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Knabel, Stephen John, Ph.D.

Iowa State University, 1989

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Effects of polyphosphates and heat on selected Gram-positive bacteria important in foods

by

Stephen John Knabel

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Co-majors: Food Technology
Microbiology

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1989

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INTRODUCTION

Preamble and Statement of Problems

Gram-positive bacteria are important in foods, because they are used to preserve foods through fermentations and because they cause spoilage, infections, or intoxications. The Gram-positive, foodborne bacteria tested in these studies were a species of *Lactobacillus* isolated from spoiled, vacuum-packaged hot dogs; *Staphylococcus aureus* and *Bacillus cereus*, toxin-producing microorganisms; and *Listeria monocytogenes*, a bacterium with the potential for causing lethal infections.

The control of Gram-positive bacteria in foods poses unique problems because they are typically more heat-, salt-, and freeze-tolerant than Gram-negative bacteria (216). In addition, *Listeria monocytogenes*, and many Gram-positive bacteria involved in food spoilage, are psychrotrophic and can grow at refrigeration temperatures (4°C). One method that has proven effective in inhibiting Gram-positive bacteria in various foods has been the use of polyphosphates (62, 341, 405, 408). Various modes of action have been proposed to explain the antimicrobial effects of polyphosphates, these include chelation of metal cations, change in pH, increased ionic strength, interactions with cell walls or cell membranes, and inhibition of transport functions. However, the true mode of antimicrobial action by polyphosphates and conditions for such action to take place remain largely unknown (385). One problem addressed by this dissertation was, therefore, what is the mechanism by which polyphosphates inhibit Gram-positive bacteria? Understanding this would allow optimization of their antimicrobial

effects in foods and might also lead to a better understanding of the Gram-positive cell.

Listeria monocytogenes is an emerging foodborne pathogen of increasing importance (292). The microorganism is widely distributed in nature (437, 438, 439) and has been incriminated in foodborne disease outbreaks involving coleslaw (363), fluid milk (128), Mexican-style cheese (214) and processed meats (25). Because *Listeria monocytogenes* is tolerant of the adverse conditions found in many foods (33), is able to grow at refrigeration temperatures (355), and is potentially lethal (266), its presence in ready-to-eat foods cannot be tolerated. Because the organism is present in milk from cows with infected udders (142), its destruction during pasteurization is critical in preventing foodborne infection. The minimum high-temperature, short-time pasteurization guidelines of the Food and Drug Administration (FDA) (131) are potentially inadequate for the destruction of *L. monocytogenes* in milk (52). However, commercial pasteurization was not considered a problem (52) because of the apparent rapid destruction of the organism during pasteurization (52) and the low levels of *L. monocytogenes* found in bulk milk tanks (178, 266).

Listeria monocytogenes ussually cannot be recovered immediately after pasteurization by using direct plating methods (52, 95, 121). Doyle et al. (95) and Fernandez Garayzabal et al. (120, 121) recovered *L. monocytogenes* however, by using various liquid enrichment techniques. Recoveries were inconsistent and did not appear to be related to the method or medium used (95). Other researchers (52, 74) suggested that the organisms recovered by those using selective enrichment techniques were not *L. monocytogenes*

cells that had survived pasteurization, but instead represented postpasteurization contaminants. The purpose of the second part of this dissertation was to determine the effect of heat shock, growth temperature, and recovery conditions on the heat resistance of *Listeria monocytogenes* in milk. It was hoped that this research would help explain the conflicting reports in the literature on the heat resistance of this organism and also lead to the establishment of safe thermal processes that would ensure the destruction of this important foodborne pathogen.

The third and last problem addressed in this dissertation is the biological function of heat shock proteins and their role in induced thermotolerance. Heat shock proteins are currently of great interest in biology because of their extremely ubiquitous and conserved nature throughout the biological world and their example as a global regulatory network (154) [for reviews see Neidhardt and VanBogelen (309) and Lindquist and Craig (257)]. Although the synthesis of heat shock proteins is very closely correlated with thermotolerance, a causative effect has not been demonstrated. The reason for lack of progress in this area is because the critical lethal lesion during thermal destruction has not been determined. Defining the specific lethal lesions would clarify the functions of heat shock proteins in conferring thermotolerance (257). The results from the heat inactivation data in the second part of this dissertation indicate that a major and critical lethal lesion in *Listeria monocytogenes* is the inactivation of the enzymes catalase and/or superoxide dismutase. The final section of this dissertation, therefore, consists of a discussion of the possible role of heat shock proteins in

stabilizing these two heat-labile enzymes, which are required for the survival of all aerobic organisms in the presence of molecular oxygen.

Explanation of Dissertation Format

The dissertation consists of three manuscripts which will be submitted for publication to professional journals and are the result of original research conducted by the candidate while working under the guidance of his co-major professors. During work on the heat resistance of *Listeria monocytogenes* it was necessary to solicit the aid of a graduate student, Aubrey Mendonca, who performed aerobic plate counts while the author performed strictly anaerobic "Hungate" roll tube techniques.

LITERATURE REVIEW

Antimicrobial Effects of Polyphosphates

Microorganisms and metals

Metal requirements The following metals are required for the growth of nearly all microorganisms: potassium, magnesium, calcium, iron, manganese, cobalt, copper, molybdenum, and zinc. Of these, potassium, magnesium, calcium, and iron are required in relatively large amounts (390). The quantitative requirements for manganese, cobalt, copper, nickel, molybdenum, and zinc are so small that it is often technically difficult to demonstrate their essentiality because they are present in adequate amounts as contaminants of the major inorganic constituents of media (390).

Microorganisms require metal ions for a range of metabolic activities and structural functions. Microorganisms cannot grow in the complete absence of magnesium (155). The bulk of bacterial magnesium is used during the assembly of ribosomes and becomes incorporated into the finished structure (400). Magnesium stabilizes spheroplasts, suggesting that it is involved in the integrity of the cellular membrane (243, 432, 453). Studies of the effects of osmotic shock (315) and EDTA (250, 251) also suggest that magnesium is important in maintaining the bacterial permeability barrier. Magnesium is required for microfiber twist development in the cell wall of *Bacillus subtilis* (295). The cytoplasmic membrane contains many enzymes that require Mg^{2+} for their activity; these include ATPase (241), peptidoglycan synthetases (140, 348) and teichoic acid synthetases (332). Magnesium is

essential for the normal cell division of yeast (102) and bacilli (425, 427, 428). Burger and Glaser (53) reported that polyglycerolphosphate (teichoic acid) synthesis decreased sharply when the Mg^{2+} concentration dropped below 0.02M in a reaction mixture containing 0.25 umoles of EDTA. The chitin and glucan synthase enzymes of fungi also require Mg^{2+} (118, 153).

The significance of metals in biological catalysis is underscored by the fact that greater than one-third of all characterized enzymes are metalloenzymes (321). Manganese can serve as an alternative cofactor to Mg^{2+} in activating many enzymes (323, 417). One key enzyme that specifically requires manganese is the superoxide dismutase found in cytoplasmic extracts of *E. coli* (289). This enzyme is responsible for converting the toxic superoxide radical ($O_2\cdot^-$) into hydrogen peroxide (H_2O_2) (133, 136), which can be converted into O_2 and H_2O by the enzyme catalase (280). Lactobacilli do not possess catalase, but contain an enzyme with a similar function, pseudocatalase, that contains manganese (15).

Iron is required in a host of important enzymes. The cytochromes and iron-sulfur proteins involved in oxidative phosphorylation require iron to fulfill their vital functions in electron transport (312). Catalase and various other oxidases are heme enzymes with an atom of iron in each heme group (312). Another form of superoxide dismutase, located in the periplasmic space of *E. coli*, requires iron as a cofactor (289). Iron is also essential for the activity of ribotide reductase, the enzyme responsible for synthesis of the deoxyribotides, which are necessary precursors for DNA (312).

Structure of microbial cell walls Gram-negative bacteria differ from Gram-positive bacteria based primarily on their wall structure. Gram-

positive bacteria possess a thick (20 - 50 um) layer of peptidoglycan immediately external to the cytoplasmic membrane (374), whereas Gram-negative bacteria contain a thin inner layer of peptidoglycan surrounded by an outer membrane composed of phospholipids, proteins, and lipopolysaccharides (34). The cell walls of molds and other fungi consist of a dense network of microfibrils embedded in an amorphous matrix. Physically fungal cell walls resemble the walls of plant cells, but cellulose is not present in the fungal cell walls. In fungal cells, the cellulose is replace by other polymers, especially chitin (poly-N-acetyl-D-glucosamine), chitosan, glucans, and galactose-containing polymers (54).

The cell walls of microorganisms perform various functions. They must permit the diffusion of nutrients into the cell and the diffusion of waste products out of the cell. Since microorganisms do not have a rigid cytoskeleton, it is the wall which forces the contour of the protoplast into the appropriate shape. Therefore, the wall can be considered as a rigid "corset" that protects the cell and prevents it from lysing under adverse conditions, such as osmotic pressure. However for a cell to grow, it must increase its volume and the cell wall must expand. This is accomplished by a process which integrates new wall material during growth without weakening the wall such that lysis occurs (35). The wall must resist physical stresses (e.g., collision with small colloidal particles and shear due to microstreaming) and withstand, to varying degrees, chemical abuses (e.g., those imposed by pH extremes, organic solvents, detergents, and denaturants). Finally, the wall acts as a chemical buffer at the cell's periphery to collect essential metal cations (e.g., Mg^{2+}, Ca^{2+}, Mn^{2+}, and Fe^{3+}) (34). Because of fundamental

differences in the composition and structure of their cell walls, Gram-positive and Gram-negative bacteria possess different mechanisms for the assimilation of metal cations (94, 122).

Binding of metals From the electrical point of view, the bacterial cell may be described as a shelled sphere. The central core appears to be an insulator at low frequencies because of the high resistance of the cytoplasmic membrane which defines its outer boundary. The shell, corresponding to the cell wall, is highly conducting by virtue of the presence of mobile counterions for the fixed charges in this region (57). Early work involving microelectrophoresis (105), established that the isoelectric points of bacteria varied between pH 2 and 4, and these could be altered by the addition of cationic surface active agents (103, 172, 293). It was concluded, from the electrokinetic data, that the low-frequency conductivities of bacteria are determined by the concentration of mobile cations in the cell wall (57). Accordingly, when a soluble polyvalent metal (e.g., Fe^{3+}) is added to growing cells, their electronegative surface property can be converted to electropositive by the bound metal (365). Gram-positive walls have a higher charge capacity than Gram-negative walls, but their isoelectric point is more variable (58). Tempest et al. (398) determined that *B. subtilis* had a greater capacity for Mg^{2+}-absorption than *Aerobacter aerogenes* but the affinity of *B. subtilis* for this ion was less. The latter difference correlated with the ability of *A. aerogenes* to outgrow *B. subtilis* rapidly in Mg^{2+}-limited chemostat cultures containing both organisms. Cutinelli and Galdiero (77) studied the cation-binding capacity of the cell walls of *S. aureus* and observed that binding of divalent cations (e.g., Ca^{2+} and Mg^{2+}) increased

dramatically with pH. Metal-wall interactions have been studied by using electron microscopy (38), electron spin resonance (123), conductivity (59), equilibrium dialysis (238), centrifugation (327), electron scattering (22), acid-base titrations (278), volume changes (276), and autoradiography (448).

In bacteria, the cell wall is a solvent-exposed organelle that may offer the first encounter between a bacterium and a molecule in its environment. The wall constitutes up to 40-50% of the cellular dry weight of bacilli, staphylococci, and streptococci (94). In *B. subtilis*, there is an approximately equal amount of peptidoglycan and teichoic acid in cell walls obtained from cultures in phosphate-deficient growth media (96). As far as is known, all walls of Gram-positive bacteria are negatively charged in media capable of supporting growth (127, 172, 213, 352). The negative charges are due mostly to phosphate groups on teichoic acids (21, 13) and the carboxyl groups on peptidoglycan (38, 283); these anionic groups can interact strongly with soluble metals in the environment (37, 325). The walls of some Gram-positive bacteria readily exchange counter ions, and an affinity displacement series for the metals has been demonstrated (277) When a series of 12 metal solutions was passed through a column containing isolated walls of *B. subtilis*, it became apparent that Mg^{2+}, Ca^{2+}, Fe^{3+}, and Ni^{2+} were strongly bound to the walls and could be detected; whereas the other metals were displaced or replaced (37). Partial lysozyme digestion of the walls greatly diminished the retention of Mg^{2+} but not that of Ca^{2+}, Fe^{3+}, or Ni^{2+}, indicating that various cations occupy different sites. The results of some experiments suggest that the walls of Gram-positive bacteria are analogous to an open, low density, ion exchange system (106, 326). Marquis et al. (277) have

pointed out that for the organism *Micrococcus lysodeikticus* the total exchange capacity of the wall may be as high as 3.5 meq per gram dry weight, which is greater than the capacity of the commercial resin Dowex A1.

The teichoic acids were discovered in 1958 by Armstrong and his colleagues (16, 17). Teichoic acids were defined as polymers of glycerol phosphate or ribitol phosphate which were substituted to various extents by ester-linked D-alanine and often also with glycosyl residues. Today the term teichoic acid has come to mean any one of a range of polymers that possess "phosphodiester groups, polyols and/or sugar residues, and usually, but not always, D-alanine ester residues" (21).

Teichoic acids probably occur in all Gram-positive bacteria (11). They are located exclusively in the outer layers (membrane, wall, and capsule) of the cell and can account for more than 10% of its dry weight (11). The results of numerous studies have indicated that teichoic acids are functionally involved in cation binding (36, 97, 113, 179, 192, 237) and in the provision of the correct cationic environment at the cytoplasmic membrane (180).

Several different structural types of teichoic acid have been described. They all contain glycerol or ribitol phosphate, and usually, a sugar or acetamido sugar and D-alanine (13, 21). These components are present in repeating structures that are held together by phosphodiester linkages to form linear polymers which usually contain between 10 and 150 units (11).

All Gram-positive bacteria so far examined contain simple 1, 3-poly(glycerol phosphate) teichoic acids which are associated with the cell membrane. Initially, teichoic acids were extracted from both bacteria and their isolated walls by prolonged treatment with cold dilute trichloroacetic

acid. However, particularly when whole organisms were examined, the results were confused by the presence in many bacteria of a closely related group of compounds which were also shown to be polymers of poly(glycerol phosphate). These could be extracted from whole bacteria although they were absent from isolated walls (76). These "intracellular" teichoic acids were subsequently shown to be located in the cytoplasmic membrane (375) and for this reason were renamed "membrane teichoic acids", or lipoteichoic acids (LTA). As the name implies, they consist of a teichoic acid chain, commonly D-alanyl-substituted 1,3-poly(glycerol phosphate), although glycosyl substituents may also be present, attached to a glycolipid. The lipid moiety serves to anchor the molecule in the cytoplasmic membrane with the hydrophilic teichoic acid chain presumably extending away from the membrane surface and, in certain organisms, through the wall (424).

Many Gram-positive bacteria also contain teichoic acids as major components of the cell wall. Such teichoic acids show greater structural diversity than the lipoteichoic acids present in association with the cytoplasmic membrane. The different structural types of teichoic acid can be classified in different ways, but perhaps the most fundamental structural difference is between those teichoic acids in which the sugar substituents form an integral part of the polymer chain and those in which the sugars are attached to a poly (alditol phosphate) "backbone" (11).

Teichoic acids, like peptidoglycan, are synthesized by enzyme systems that are located in the cytoplasmic membrane (11). Procedures that have been used for the isolation of enzymatically active preparations include the recovery of membranes from osmotically disrupted protoplasts and the

isolation, by differential centrifugation of physically disrupted bacteria, of particulate fractions rich in fragmented membrane. The method used for the isolation of particulate enzymes greatly influence their activity, but preparations obtained after disruption of cells by grinding with alumina synthesized both teichoic acid and peptidoglycan (11).

The amount of teichoic acid synthesized by the cell is controlled by environmental factors. When the uptake of Mg^{2+} is constrained by low concentrations of Mg^{2+} (294) or by the addition of high concentrations of Na^{+} (113), the bacteria respond by producing wall teichoic acid. At very low phosphate concentrations a different anionic polymer, teichuronic acid is synthesized. Teichuronic acid is composed of glucuronic acid in place of phosphoryl groups (112). Like teichoic acids, teichuronic acids are negatively charged polymers, though their charge derives from the carboxyl functions of their uronic acid components and not from phosphate ester groupings. Apparently bacteria can dispense with wall teichoic acids by synthesizing an alternative, negatively charged polymer (114). Membrane teichoic acid is still synthesized under conditions which lead to incorporation of teichuronic acid into the wall so that, although wall teichoic acids can be functionally replaced by anionic polymers, the presence of membrane teichoic acid is apparently essential for proper functioning of the cell (180).

A substantial proportion of the metabolic activity in many Gram-positive bacteria is directed toward the synthesis of teichoic acids, so it seems reasonable to assume that these polymers have some role or function that is of value to the cell. The early suggestion that teichoic acids might participate in ion-exchange reactions and influence the passage of ionic materials

through the cell surface was based largely on a consideration of its ionic nature (100). The possible importance of cell walls in the binding of Mg^{2+} was recognized by Webb (426), who observed that the magnesium requirements for Gram-positive bacteria were 10 times greater than for Gram-negative bacteria, but that the magnesium content of the cells did not differ greatly. In a later study, it was determined that the variations in Mg^{2+} requirements between Gram-positive and Gram-negative bacteria were a result of differences in their assimilatory efficiencies (429). Tempest, Dicks and Meers (398) reported that *B. subtilis* had a greater capacity for Mg^{2+} absorption than *Enterobacter aerogenes* but the affinity of *B. subtilis* for this ion was less. The use of equilibrium dialysis techniques established that isolated cell walls of *B. subtilis* possessed selective affinities for several metal cations, and that both teichoic acid and peptidoglycan contributed to the sites available for interaction with metals (97). By selectively removing teichoic acids from the cell wall, Beveridge et al. (36) determined that teichoic and teichuronic acids, not peptidoglycan, are the prime sites of metal binding in *Bacillus licheniformis* walls.

Teichoic acids appear to interact preferentially with Mg^{2+}. Lambert et al. (237) reported that one Mg^{2+} ion was found for every two phosphate groups of the teichoic acid, with an apparent association constant, $K_a = 2.7 \times 10^3 M^{-1}$. In solution, ribitol teichoic acid bound Mg^{2+} in the molar ratio $Mg^{2+}/P = 1:1$ with an apparent association constant, $K_a = 0.61 \times 10^3 M^{-1}$, and the teichuronic acid bound Mg^{2+} in the ratio $Mg^{2+}/CO_2- = 1:1$, $K_a = 0.3 \times 10^3 M^{-1}$ (179). Cell walls containing teichuronic acid exhibited binding properties similar to those containing teichoic acid; in both instances Mg^{2+} was bound in

the ratio of Mg/P or of Mg/CO_2- of 0.5:1 and with a greater affinity than was displayed by the isolated polymers in solution. The authors concluded that Mg^{2+} ions are bound bivalently between anionic centers in the walls and that incorporation of teichoic or teichuronic acid into the walls gives rise to similar ion-binding and charged properties. Membrane-bound lipoteichoic acids must also play an essential part in bacterial physiology because of their ubiquitous appearance in Gram-positive bacteria, their singular location on the membrane, and their structural homogeneity (239).

Heptinstall et al. (180) proposed that a major function of both wall and membrane teichoic acids is to maintain a high concentration of bivalent cations in the region of the membrane. This is consistent with the requirement for Mg^{2+} in maintaining the physical integrity of the membrane, as shown in studies on the survival of protoplasts (433). Hughs et al. (190, 192) provided the first direct demonstration of the function of teichoic acids in concentrating Mg^{2+} at the cytoplasmic membrane. When a membrane-bound enzyme system was used the synthesis of teichoic acid was maximal when Mg^{2+} was bound to the endogenous teichoic acid, which was in marked contrast to the absence of enzyme activity in isolated cytoplasmic membranes. They concluded that in whole cells the ordered array of anionic wall and membrane teichoic acids provides a constant reservoir of bound bivalent cations with which the membrane preferentially interacts (190). Wall and membrane teichoic acids, therefore, constitute an integrated cation exchange system between the cell exterior and the cytoplasmic membrane, where relatively high concentrations of Mg^{2+} are required for Mg^{2+}-dependent membrane systems (140, 295, 361, 433). The direct transfer of

bound Mg^{2+} from wall to membrane teichoic acid, and hence to ligand groups in the membrane, could provide a satisfactory explanation of the role of teichoic acids in the bacterial envelope (190).

Ou and Marquis (327) reported that a strain of *Staphylococcus aureus* that was deficient in cell wall teichoic acid was no more sensitive to chelating agents, high sodium chloride concentrations, divalent cation (Mg^{2+})-depletion, or low pH, than a teichoic acid-sufficient wild-type strain. Lambert et al. (239) found that this mutant still had adequate Mg^{2+}-binding capability, provided by the lipoteichoic acid and carboxyl groups of the peptidoglycan, to support growth. Matthews et al. (283) demonstrated that cell walls of *B. subtilis*, with their teichoic acids extracted, retained a high affinity for some cations although the stoichiometry was generally reduced. They concluded that the functional groups of peptidoglycan contributed to the surface binding of metals by bacteria. Beveridge and Murray (38) discovered that, in contrast to extraction of teichoic acids, alteration of carboxyl groups of the peptidoglycan severely limited metal deposition. They suggested that carboxyl groups provided the major site of metal deposition in the *B. subtilis* wall.

The cell walls of Gram-negative and Gram-positive bacteria differ fundamentally with respect to design. One of the major differences is that Gram-negative cells possess an outer membrane that consists of lipid and protein arranged in a planar, two-dimensional, bilayer matrix (35). This highly specialized structure, which resides outside a monolayer of peptidoglycan, not only physically separates the cell body from the external environment, but also serves as a selective permeability barrier that controls

the access of solutes and other external agents to the plasma membrane (170, 124).

The outer membrane of Gram-negative bacteria is essentially held together by noncovalent forces that arise from the amphiphathic nature of the constituent membrane lipids; by adopting a bilayer format in an aqueous milieu, the lipids achieve a minimum free energy conformation with their hydrophobic fatty acid chains buried in the membrane (212). The hydrophilic polar heads of the lipids, which contain anionic phosphoryl and carboxyl groups, therefore remain exposed to the external environment and effectively determine the reactivity of the cell surface (34, 124). The phosphoryl groups of LPS and phospholipid located in the outer membrane are the major sites for metallic ion interactions (123). Consequently, the outer membrane interacts efficiently with metal cations in aqueous solution (125, 189).

The outer membrane is capable of binding a wide range of metallic ions, including members of the alkali, alkaline earth, transition, and rare earth series (122, 125, 189). These metal cations are generally regarded as important accessory components that function to stabilize the outer membrane. Presumably, metallic ions bound by the outer membrane reduce charge repulsion between highly anionic constituent molecules, bridge adjacent molecules of LPS and/or protein, and help anchor the outer membrane to the underlying peptidoglycan (122, 320).

Exactly how important the metal-binding capacity of the outer membrane is to the vitality of Gram-negative bacteria is difficult to ascertain. Certainly, the structural continuity and, therefore, the discriminatory molecular sieving

properties of the outer membrane depend on the presence of bound metallic ions (122). For example, potent metal chelators, such as EDTA, can scavenge outer membrane-bound metal and cause a concomitant release of up to half of the total LPS (251). Consequently, EDTA-treated cells are typically more susceptible than untreated cells to hydrolytic enzymes and antibiotics that are normally excluded by the outer membrane (287, 370).

Based primarily on biochemical information derived from studies with metal chelators, Leive (250) proposed that calcium was the principal metal cation responsible for the stabilization of LPS in the lipid domain of the outer membrane. This hypothesis has been confirmed by data from a recent investigation which show that EDTA-modified outer membranes from *E. coli* are not only deficient in LPS but also in calcium (125). Of the other predominant metallic species normally found in the outer membrane, only the level of magnesium was slightly reduced by the chelator, whereas quantities of sodium were unaffected (125).

At least two interpretations for the more efficient extraction by EDTA of calcium from the outer membrane, compared with that of magnesium, are possible. The first is that the outer membrane-bound calcium is readily accessible to EDTA, whereas outer membrane-bound Mg^{2+} is buried within LPS-protein complexes and is not available (126). Alternatively, it is possible that the strength of magnesium binding to the outer membrane is greater that that of calcium. Because EDTA is a potent chelator of both calcium and magnesium, and extracts both of these cations from isolated LPS (71), the first possibility is favored. In this context, LPS in the calcium salt form probably represents a unique physiochemical subfraction that is more

prone to co-extraction from the outer membrane than LPS that is complexed with magnesium (125).

The action of EDTA is enhanced when used in conjunction with tris(hydroxymethyl)aminomethane (Tris) buffers (149). This large organic cation is also capable of disrupting the outer membrane alone, if used in moderately high concentrations (209). The disruptive effects of Tris are probably related to its ability to interact with LPS and displace divalent metallic ions (70). The outer membrane is also sensitive to other organic polycations (409), which compete with and displace divalent cations from the outer membrane, thereby disturbing its integrity. In each of these situations, it is the structural dependence of the outer membrane on metal cations that facilitates the perturbing effects of these polycations (122).

The binding of metallic ions by the outer membrane of Gram-negative bacteria is generally perceived as an electrostatic phenomenon mediated by interactions between the soluble metal cations and fixed anionic groups at the hydrophilic surfaces of the membrane (34, 320). However, the structural and compositional complexity of the outer membrane implies that a single metallic species would not be capable of interacting in the same way with all parts of the membrane; it would depend on the local outer membrane chemistry (125). Moreover, because different metallic ions possess distinct physical and chemical properties, there is no reason to expect that all cations interact with the outer membrane in the same way. In this context, differences in the metal binding capacity of native and EDTA-modified (LPS-deficient) outer membrane preparations provided valuable information

concerning the preferred intermolecular sites of metal coordination among the component membrane molecules (125, 126).

It can be appreciated that the outer-membrane, metal-cation requirements of different Gram-negative bacteria probably vary. Similarly, the metal-cation requirements of the outer membrane from a single species probably differ, depending on the growth conditions and the physiological status of the cells involved. Ultimately, metal requirements should depend on the native environment, and the selective pressures experienced by a microorganism. Moreover, recent evidence suggests that the physiochemical roles of metal cations extend beyond the structural level; metallic ions are known to influence outer membrane permeability profoundly (170), and may regulate autolytic enzymes (245) or signal the protoplast about events at the cell surface (12). Indeed, the ability of the outer membrane to interact strongly with metallic ions is, in the end, envisioned to facilitate cellular growth, and to couple the protoplast more effectively to its environment (122).

Enzyme treatments, combined with electron microscopy, enabled Hunsley and Burnett (196) to make proposals concerning the ultrastructural architecture of the wall components of *Neurospora crassa*. They recognized an outer, predominantly ß-glucan layer, separated by a glycoprotein reticulum from an inner region of chitin microfibrils embedded in proteinaceous material. The successive, co-axially arranged regions were not discrete but merged into each other. These findings were substantiated (195) by the immunofluorescent localization of wall antigens to antisera developed in rabbits against ß-glucan, chitin, and glycoprotein fractions.

Some 25% (w/w) of the wall in wild-type strains of *Neurospora crassa* is composed of glucans, predominantly ß-linked (54). The relative frequency of ß(1-3) and ß(1-6) linkages is unknown, as are the complete structures of the molecules. Presumably they are amorphous, rather than microfibrillar, because the region lysed by laminarinase, an enzyme specific for such glucans, appears amorphous in shadowed preparations viewed by electron microscopy (196). Chitin accounts for about 10% of the wall material as flat, ribbon-like microfibrils made of ß(1-4) 2-acetamido-2-deoxy-D-glucose units (307). A substantial amount of proteinaceous material in the cell wall is present as the glycoprotein first characterized by Mahadevan and Tatum (271). The glycoprotein can be isolated in a reasonably intact form from walls of fungi and is left structurally intact within walls treated with chitinase and glusulase (272). It is likely that there are no strong covalent links between the peptide moieties and the rest of the wall. Glucose, galactose and glucuronic acid are released from the glycoprotein polymer, which appears to be identical with that originally described in *Aspergillus parasiticus* as a polygalactosamine polymer (86) which bound polyphosphate (173). The structure of the complex carbohydrate component is not known, but it could be a branched molecule to which the peptides are covalently linked by O-glycosyl serine bonds (444). Although the analytical technique leading to the estimation of the peptides' molecular weight has been criticized (138), there is no doubt that this glycoprotein is an important wall polymer and can, in fact, be equated to a coarse, reticulated entity - the reticulum- embedded in the mature hyphal wall (196, 271).

Rothstein et al. (357) reported that metal cations bound rapidly to the anionic groups at the surface of yeast cells. In *Achyla*, about 40% of the accumulated calcium was extracellular, removable by chelates (citrate and EDTA) and bound to a low-molecular weight glycopeptide that could be removed from *Achyla* by osmotic shock (252). Both chitin and chitosan, a deacetylated derivative of chitin, are known chelators of metal cations (307). Chitin does not exhibit as high an affinity as chitosan for metal cations, because the nitrogen electrons of the acetylated amino groups are not available as they are in chitosan. However, both chitin and chitosan powders are commercially available for collecting metal cations by column chromatography (307). Indeed, whole filamentous fungi have been shown to remove considerable amounts of metals from aqueous solutions and therefore are used to detoxify waters containing heavy metals (230, 356).

Transport of Magnesium, Manganese, and Iron In *B. subtilis* there is a single transport system responsible for the uptake of Mg^{2+} (215). Co^{2+}, Mn^{2+}, and Ca^{2+} compete for transport, which is repressed by Mg^{2+} and elevated in K^{+} retention mutants (228). Co^{2+}-resistant mutants lack the Mg^{2+} uptake system and require elevated concentrations of Mg^{2+} for growth. The K_m for Mg^{2+} is 5- to 10-fold higher in *B. subtilis* (368) than in *E. coli* (379). If lower affinity is a general property of Bacilli, it may account for the inability of *B. megaterium* to grow on or take up Mg^{2+} from media that contains very low concentrations of Mg^{2+} (430, 431). Kay and Ghei (228) found that Ca^{2+} inhibited Co^{2+} uptake weakly, with a K_i of 10 mM Ca^{2+}. Scribner et al. (369) observed that at 1 mM Ca^{2+} did not inhibit Mg^{2+} uptake, but they would have found only 7% inhibition if the kinetic constants had

been the same as those reported by Kay and Ghei (228). Phosphate or, less effectively, sulfate is required for divalent cation transport by the low affinity Mg^{2+} transport system in B. subtilis (228).

Like many other species, *B. subtilis* has a high-affinity Mn^{2+} transport system with a K_m of 1.3 to 2.5 uM for Mn^{2+} (107, 228). Growth with added Mn^{2+} reduced V_{max} 3- to 6-fold (127). Overnight growth in tryptone broth low in Mn^{2+} content induced a 10-fold increase in V_{max}, however the limited supply of Mn^{2+} prevented sporulation. If the cells with the derepressed Mn^{2+} transport system were allowed to take up large amounts of Mn^{2+}, cell growth was inhibited until net efflux of Mn^{2+} reduced the intracellular Mn^{2+} concentration to nontoxic levels (127). When actinomycin or chloramphenicol were added part way into the inactivation process, rate of Mn^{2+} transport activity immediately became static. The regulation of Mn^{2+} content in *B. subtilis* is entirely by change of V_{max} of the Mn^{2+} transport system (i.e., synthesis of or inactivation of transport activity) because neither the K_m for uptake nor the rate constant for efflux changed during a 10-fold change in Mn^{2+} uptake rate and Mn^{2+} content (127).

Lactobacillus plantarum has an unusually high requirement for Mn^{2+}, and for an unusual reason. This aerotolerant microorganism lacks the enzyme superoxide dismutase (161). Instead, it relies on manganous polyphosphates to destroy superoxide ($O_2{\cdot}^-$) (15). Total intracellular Mn^{2+} is 30-35 mM, similar to the Mg^{2+} content of most bacteria, including lactobacilli, and more than enough to substitute for the micromolar levels of superoxide dismutase found in other aerobic organisms (15).

The transport of Mn^{2+} by *L. plantarum* occurs via a high-affinity (K_m of 0.2 M) transport system with the unusually high maximal velocity of 24 mol $min^{-1}g^{-1}$ (protein) (14). Cd^{2+} competes with Mn^{2+} with a K_i of 0.9 M, but Cd^{2+} transport was not saturable. Assays were performed in the presence of Mg^{2+}, so Mg^{2+} is not likely to inhibit this system. Energy is provided by pH, the proton-motive force, which is generated by the ATPase in this organism that lacks cytochromes. Thus, proton ionophores inhibit Mn^{2+} transport. Carboxylic acids or phosphate are required for Mn^{2+} uptake, which proceeds only slowly in media in which Mn^{2+} is primarily in the hexaaquo form. Although citrate was effective in stimulating Mn^{2+} uptake, essentially no [1-^{14}C] citrate was taken up (14).

Iron is usually present in aerobic environments as $Fe(OH)_3$ which is extremely insoluble (K_D = 10^{-38} M (313)). Because most microorganisms require larger amounts of Fe^{3+} than are readily available in solution, they have evolved siderophores, compounds capable of chelating Fe^{3+} and transporting it into the cell (313). Members of the genus *Bacillus* are known to synthesize two types of siderophores: schizokinen (55) is a monohydroxamate siderophore which is active in initiating cell division in *Bacillus megaterium* and 2, 3-dihydroxybenzoylglycine (396) causes the transport of Fe^{3+} into *B. subtilis* (93). Marcelis et al., 1978 (275) concluded that most of the strains of *S. aureus* that they tested synthesized siderophores, based on the ability of these isolates to grow on media containing the synthetic iron chelator ethylene diamine diorthohydroxyphenyl acetic acid (EDDA). However, the authors did not isolate and characterize the compound(s) responsible for this effect. Cowart

and Foster (71) were unable to identify any component of a high-affinity iron transport system in *L. monocytogenes*; however, the microorganism was able to remove Fe^{3+} from Fe^{3+}-transferrin by a reductive pathway. The authors proposed that this pathway was a nonspecific mechanism of iron acquisition. Archibald and Duong (14) were unable to detect a stimulatory siderophore-like chelator in *L. plantarum*.

Although wild-type *E. coli* cells display only a single K_m for Mg^{2+} transport (378), genetic studies indicate that there are actually two transport systems in *E. coli* for this metal (381). Alternative substrates Co^{2+} and Mn^{2+} are toxic, allowing the isolation of resistant mutants with altered transport properties. Nelson and Kennedy (315) found that Co^{2+}-resistant (Cor) mutants were still able to transport Mg^{2+}, but that the residual Mg^{2+}-transport activity was repressed by growth on high Mg^{2+}. Their interpretation, that there are two transport systems for Mg^{2+}, system I that also transports Co^{2+} and system II that is specific for Mg^{2+} and repressible, was confirmed and extended by Park et al. (329). Two types of system I Cor mutants were isolated, having mutations in either the *corA* or *corB* gene. Both were devoid of Co^{2+} transport activity when grown on standard low-Mg^{2+} medium. In *corA* mutant strains, growth was inhibited by Ca^{2+} but the rate of Mg^{2+} transport was not (329). Transport of Mg^{2+} was repressible. In *corB* mutants, growth on high levels of Mg^{2+} or Ca^{2+} induced transport activity for Co^{2+} and Mg^{2+}. Both mutant types were dependent on system II for the transport of Mg^{2+}. Double mutants, *corA mgt* and *corB mgt* (mutants that are Co^{2+}-resistant but defective in Mg^{2+} transport), required high concentrations of Mg^{2+} for growth (329). In the latter case, system I

was induced by growth on high levels of Mg^{2+} and 1 mM Mg^{2+} could support growth. In contrast, the *corA mgt* strain required 10 mM Mg^{2+} for growth and showed no saturable, energy-dependent Mg^{2+} transport. A *corA corB* double mutant, constructed by P1 phage transduction, was Ca^{2+} - sensitive and did not express system I after growth on high levels of Mg^{2+}. The *corA* gene product has been identified as an M_r 37,000 polypeptide (322).

While substrate specificity and regulation distinguish the two Mg^{2+} transport systems in *E. coli*, other kinetic parameters do not. Both systems have K_m values for Mg^{2+} between 15 and 60 uM and V_{max} near 10 umol min^{-1} g^{-1} (protein). In wild-type cells there is a single K_i of 0.5 mM for Mn^{2+} and a K_i of 0.4 mM for Co^{2+}, near its K_m of 0.2 mM (315, 379).

Salmonella typhimurium closely resembles *E. coli* in having two Mg^{2+} transport systems, one repressible and transporting only Mg^{2+} and the other constitutive and responsible for Co^{2+} transport as well (184). Unlinked *corA* and *corB* mutations, comparable to those in *E. coli*, affected Ca^{2+} transport. Mutations abolishing the Mg^{2+}-specific system mapped separately. Cloned *E. coli* and *S. typhimurium corA*$^+$ genes complement mutations conferring the CorA phenotype. The *corA*$^+$ plasmid caused enhanced sensitivity to Co^{2+} and increased V_{max} values for both Mg^{2+} and Co^{2+} transport, suggesting that a structural gene for Mg^{2+} transport had been cloned.

In addition to being a substrate for the Mg^{2+} transport systems, Mn^{2+} is transported by a specific, high-affinity system in *E. coli* (380). The K_m for Mn^{2+} is near 0.2 uM and its V_{max} 1-4 nmol min^{-1} per 10^{12} cells. Mg^{2+} and Ca^{2+} do not compete, even at 1 mM. Co^{2+} and Fe^{2+} are competitive inhibitors with K_i values 100-fold higher than the K_m for Mn^{2+}.

Gram-negative bacteria typically possess siderophores that have high affinity for Fe^{3+} (313). Various enteric species, such as *E. coli* and *S. typhimurium*, produce the siderophore enterobactin which has an extremely high affinity for Fe^{3+} ($K_a = 10^{52}$) (313). *Pseudomonas fluorescens* produces a fluorescent pigment called pyoverdine, which is the siderophore for this microorganism ($K_a = 10^{32}$) (296). Avirulent strains of *Pseudomonas aeruginosa* that were deficient in the synthesis of the fluorescent siderophore pyochelin could only grow around wells containing $FeCl_3$ on serum agar which contained transferrin, a strong chelator of Fe^{3+} (259). The inability of these serum-sensitive strains to grow in the absence of added iron was due to the sequestering of media-Fe^{3+} by transferrin, a strong Fe^{3+} chelator naturally present in serum.

Gram-negative bacteria possess siderophore receptors on their outer membrane that recognize siderophore-bound Fe^{3+} and enable it to be transported through the outer wall and into the cytoplasm (314). Compounds that block the receptor would prevent transport of Fe^{3+} (347) and the cell would die due to iron starvation (434).

Rothstein et al. (357) reported that mechanisms of transport of divalent cations into the yeast *Saccharomyces cerevisiae*, was somewhat similar to those described above for the Mg^{2+} transport system of *E. coli*; namely, a high affinity for Mg^{2+} but also lesser affinities for other divalent cations, including Co^{2+}, Mn^{2+}, and Ni^{2+}. There was a rapid binding to the anionic groups of the surface of the cells upon addition of cations to yeast cell suspensions. This binding exhibited little discrimination between many divalent cations (e.g., Mn^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}), and the bound cations were

exchangeable. In addition to binding, some divalent cations were transported into the cells and were then no longer exchangeable with extracellular cations. With energy-starved cells, little uptake was observed; but in the presence of, or after previous exposure to, glucose and phosphate, Mg^{2+} and Mn^{2+} were rapidly accumulated (357).

Siderophores were first discovered in the smut fungus *Ustilago sphaerogena* (311). Emery (115) demonstrated that the siderophore supplies iron to the cells via a shuttle mechanism. When thoroughly starved of iron, *U. sphaerogena* produces more deferriferrichrome A than deferriferrichrome. Although the former is inactive, the fact that its binding constant is only three times higher than that of deferriferrichrome assures a supply of the transportable chelate via iron exchange. The siderophores coprogen and ferricrocin, and possibly ferrichrome C, have been found in *Neurospora crassa* where they have been shown to serve as germination factors (188). In *Aspergillus nidulans*, a role for iron in sporulation competence has been postulated (168).

Polyphosphates

Structure and chemical properties Phosphates are salts of phosphoric acid and include the orthophosphates with a single phosphorus atom, and the polyphosphates, which are polymers of orthophosphate (PO_4) that are made up of 2 or more phosphorus atoms (416). Pyrophosphates contain 2 and tripolyphosphates contain 3 phosphorus atoms, respectively. Long-chain polyphosphates (more than 3 phosphorus atoms) are amorphous materials and are generally called glassy polyphosphates (392, 416). The average

chain lengths of different glassy polyphosphates may be 6, 12, 22 or more phosphate atoms. The most common polyphosphates are tetrametaphosphate, a ring form, and hexametaphosphate, a long-chain form. Most compounds are fully neutralized and behave as an alkali in solutions; however, acidic forms exist (e.g., sodium acid pyrophosphate) (416).

The basic chemical functions of phosphates are to control pH by acting as buffers; to sequester metal ions, and to increase the ionic strength of solutions by acting as polyvalent anions (385, 392). Orthophosphates are the best buffering agents; however, because they are calcium precipitating agents, their sequestration value by definition is zero (207). Pyrophosphate is a good buffering agent in the pH range 5.5 to 7.5; the other polyphosphates are not as effective buffering agents in this physiological pH range, and buffering capacity decreases with increasing chain length (392, 413, 415).

Polyphosphates form stable metal chelate complexes with a variety of metal cations (205, 414). Generally, polyphosphates form stronger chelates with alkaline earth metals than with alkali earth metals (207, 377). In addition, chain polyphosphates are many times more effective in sequestering Mg^{2+} ($pK_D \sim 5.5$) than Ca^{2+} ($pK_D \sim 4$) (206, 207). In contrast with their ability to sequester calcium, tetrasodium pyrophosphate (TSPP) and sodium tripolyphosphate (STPP) are more effective Mg^{2+} sequestrants than the long chain polyphosphates (207), such as sodium hexametaphosphate (SHMP), also called sodium phosphate glassy (SPG). This is because the thermodynamic stabilities of short-and long-chain polyphosphates are not much different (206), yet, the molecular weights of

the long-chain phosphates are much larger. Therefore, on a weight basis, the short-chain polyphosphates are better Mg^{2+} sequestrants (207). TSPP and STPP are also very effective chelators of Fe^{3+} (pK_D = 23) (208). Tripolyphosphate forms strong chelate complexes with the following biologically important metals: Mg^{2+} (pK_D = 5.5), Mn^{2+} (pK_D = 6.3), and Fe^{3+} (pK_D = 23) (208, 229). Manganese forms more stable complexes with the larger tetrametaphosphate (pK_D = 5.74) (222) than with the smaller trimetaphosphate (pK_D = 3.56) (223). The sequestering efficiency of polyphosphates decreases as the pH decreases, due to the competition for the sequestering anion by the hydrogen ion (207).

Effects of Polyphosphates in foods   Phosphates, through their interactions with some of the constituents of food systems, have very useful and important functions (reviewed in ref. 110). The treatment of water used in food processing is an important application of the metal chelating ability of the phosphates. Sodium tripolyphosphate (STPP) has an antioxidant effect in cooked meat and fish (344), probably because of the ability of STPP to chelate metal cations such as Fe^{3+} and Cu^{2+}, which act as catalysts in lipid oxidation. Polyphosphates increase the water-holding capacity and binding strength of meats (384, 392, 407). Other beneficial effects of phosphates in foods include stabilization of the pH, dispersion and peptization of relatively insoluble food constituents, emulsion stabilization, acidification, alkalization, and prevention of caking (110).

Inorganic polyphosphates may provide 10-20% or more of the total phosphorus ingested by Americans (450). Processed dairy products and meats, as well as soy-based animal-product analogues, often contain 0.5%

added polyphosphates (405). Consequently, these products may add 400-500 mg of polyphosphate-derived phosphorus to the daily diets of some individuals. Because of their polyanionic nature, polyphosphates have a high affinity for metals and may therefore substantially affect their utilization (450). Mahoney and Hendricks (273) reported that the addition of sodium pyrophosphate and sodium tripolyphosphate to the diets of growing rats caused decreased iron absorption, decreased hemoglobin concentrations, and depressed liver values. High dietary levels of sodium hexametaphosphate caused a 15% increase in fecal iron and a 12% decrease in liver iron; sodium orthophosphate was without effect (451). In contrast, polyphosphates increased Zn bioavailability, and orthophosphate caused a moderate decrease (449, 450). Rao and Rao (345) demonstrated that polyphosphates significantly increased iron absorption in humans fed wheat-based diets. They concluded that STPP may promote food iron absorption. In a later report, Zemel (450) studied that both TPP and HMP increased zinc absorption in 21-day-old rats. Both polyphosphates enhanced the absorption and liver accumulation of iron, but STPP depressed femur iron levels. TPP reduced calcium and Mg bioavailability.

Antimicrobial effects of polyphosphates A large portion of the research on the antimicrobial effects of polyphosphates has been performed by using artificial media. Post et al. (340) reported that most Gram-positive bacteria were prevented from growing on nutrient agar containing 0.1% SHMP. Gram-negative bacteria were capable of growing in higher concentrations, even up to 10% SHMP. Post speculated that SHMP sequestered Mg^{2+}, which resulted in the inhibition of cell wall division and loss of cell wall integrity.

A similar mechanism was also proposed by Elliott et al. (111), who discovered that commercial polyphosphates inhibited the growth of nonfluorescent pseudomonads in an artificial medium. Since the addition of Mg^{2+} and the natural competitive chelators pyoverdine and peptone reversed inhibition, Elliott et al. (111) proposed that the inhibition of nonfluorescent pseudomonads was a result of sequestration of cations by phosphate. Molins et al. (301) reported that various polyphosphates were inhibitory to Gram-positive cultures and mildly inhibitory to Gram-negative microorganisms, including *S. typhimurium* and *P. aeruginosa*. The activity of SPG was lost upon heating, as was that of STPP for all cultures except lactic acid bacteria. *S. aureus* 196E was inhibited by 0.5% levels of various polyphosphates in BHI broth (217). Supplementation of broth with Mg^{2+} was effective in overcoming inhibition by 0.5% STPP; inhibition was partially eliminated by Ca^{2+} and Fe^{2+}, but not by Zn^{2+} or Mn^{2+}. Jen and Shelef (217) speculated that the superior ability of Mg^{2+} to restore growth is related to a higher stability constant of the polyphosphate with Mg^{2+} than with other cations. Zessin and Shelef (452) reported that Gram-negative bacteria were less susceptible than Gram-positive microorganisms to polysphosphates in nutrient broth; the sensitivity to polyphosphates decreased with an increase in the metal content of the media.

Seward et al. (372) reported that 0.5% sodium tripolyphosphate in media containing 1.5% potassium sorbate inhibited cell division of *Clostridium botulinum* type E. When spores were used as the inoculum th spores germininated but the resulting vegetative cells were abnormal in shape and defective in division. Wagner and Busta (420, 421) described interactions

between pH and SAPP concentrations in the effects of SAPP against *C. botulinum*. SAPP delayed growth of various strains of *C. botulinum* in media and acted synergistically with potassium sorbate. They also found that SAPP inhibited *C. botulinum* 52A toxicity and protease activity in peptone yeast extract glucose broth. Wagner and Busta (421) speculated that toxin inhibition may be the result of SAPP chelating cations required by enzymes involved in cleavage of the protoxin. Madril and Sofos (270) showed that the inhibition of *C. sporogenes* by SAPP in meat formulations was due not only to low pH but also to the presence of the phosphate ion. The data also suggested that SAPP may be a better inhibitor at pH 6.0 than at pH values of 5.7 or 6.3.

Most of the research on the antimicrobial effects of phosphates in foods have focused on their effects in high-protein processed foods, such as processed cheeses (341) and meats (385). It is well known that adding a phosphate to sequester calcium ions can help control bacteriophage infections of lactic starter cultures (242), but this also results in injury to the starter organisms and loss of proteinase activity (244). The importance of phosphate to the microbiological stability of food is most evident in shelf-stable pasteurized process cheese products (341). These products would not be as stable if only the processing temperature, product pH, brine content or water activity are considered. The products are nutritionally adequate for microbial growth (405).

Under certain conditions of pH and NaCl and nitrite concentrations, certain polyphosphates exert antimicrobial properties in processed meat products. Interactions of polyphosphates with various antimicrobial agents

(NaCl, nitrite, pH, isoascorbate, sorbate, etc.) have been reported by various researchers (211, 315, 354, 419,). Other investigators, however, have indicated that phosphates have no effect on the antimicrobial properties aforementioned, or even enhance microbial growth (211, 354, 384). Molins et al. (300) reported bacterial were not inhibited by orthophosphate, SAPP, STPP, TSPP or SPG during refrigerated storage of cooked, vacuum-packaged bratwurst. However, SAPP significantly inhibited the growth of aerobic and anaerobic bacteria (including *C. sporogenes*) when the bratwurst was subjected to temperature abuse. TSPP and STPP were somewhate less effective than SAPP. The effects of 0.5% SAPP, STPP, and SPG on aerobic mesophilic and psychrotrophic bacteria and on the survival of *S. aureus* Z88 were investigated in uncooked bratwurst stored at 5°C for 7 days (300). Polysphosphates did not inhibit the growth of *S. aureus*, although SAPP addition resulted in consistently lower total aerobic plate counts.

Ivey and Robach (211) and Wagner and Busta (419) also observed that sodium acid pyrophosphate (SAPP) acted synergistically with sorbic acid (pH 5.84 to 6.25) in meats to delay *C. botulinum* growth and toxicity. Wagner and Busta (419) and Nelson et al. (317) observed that the maximum delay in toxin production occurred when SAPP-potassium sorbate combinations were used in beef/pork or chicken frankfurter emulsions, respectively, at various pH values ranging from 5.85 to 6.01. Molins et al. (302) reported that phosphates alone or combined with nitrite did not affect aerobic bacterial counts but resulted in reduced clostridial and anaerobic counts at 5°C. Upon temperature abuse, SAPP significantly inhibited the growth of all bacteria for up to 48 h; these effects were greatly enhanced by 100 ppm but not 50

ppm sodium nitrite. STPP was also inhibitory but lost its antimicrobial properties after 24 h. A positive correlation existed between soluble orthophosphate levels and bacterial inhibition in SAPP-treated bratwurst.

In general, there is much confusion as to the antimicrobial mechanism of phosphates (385). Some reasons for this confusion are that phosphates of different chain length were used. Other complicating factors include different polysphosphates have different affinities for essential metal cations, the influence of phosphates on pH, the presence or absence and levels of other inhibitors in the system, the microorganisms under consideration, and environmental factors involved in each study (385).

Heat Resistance of *Listeria monocytogenes*

General characteristics of *Listeria*

Description *Listeria* are Gram-positive, nonsporeforming, rod-shaped bacteria. It often occurs in pallisades in smears made from young cultures. The microorganism is facultatively anaerobic and microaerophilic. Fermentation of a variety of sugars results in the production of acid but no gas. Both aerobic and anaerobic catabolism of glucose proceeds through the Embden-Meyerhof pathway. The microorganism is catalase-positive, superoxide dismutase-positive, and oxidase-negative; all strains produce alkaline phosphatase. Some strains produce weakly ß-hemolytic reactions on blood agar. The organism is motile by a few peritrichous flagella when cultured at 20-25°C. The optimum temperature for growth is between 30° and 37°C; however, the organism can grow over a wide temperature range of

1 - 45°C (371). All strains grow best at neutral to slightly alkaline pH; *Listeria monocytogenes* grows well at pH values of 4.7 - 9.2 (336). *Listeria* grow well in the usual bacteriological media, e.g., Blood Agar Base No. 2 (Difco), tryptose Agar (Difco), or Trypticase Soy Agar (BBL) (371).

Taxonomy The taxonomic position of the genus *Listeria* with regard to other genera is still not resolved in Bergey's Manual of Systematic Bacteriology (371). On the basis of numerical taxonomic, serological, chemical and nucleic acid data, *Listeria* is a genus distinct from the coryneform bacteria; it may be more closely associated with the genera *Bacillus*, *Erysipelothrix*, *Lactobacillus* and *Streptococcus*. Data on the composition of the cell wall peptidoglycan, cytochrome and menaguinone contents, and fatty acid profiles indicate that *Listeria* are relatively closely related to the genus *Brochothrix* and to two taxa designated *Listeria grayi* and *Listeria murrayi* (371). Further DNA-DNA hybridization studies, and studies on rRNA oligonucleotide sequences, are required before the phylogenetic relationship of *Listeria* to the other genera or higher taxa can be established.

L. monocytogenes can be separated from other species of the genus based on reaction on blood agar (ß-hemolytic), fermentation pattern on various carbohydrates, hippurate hydrolysis, pathogenicity for mice, and antigenic composition (371).

Ecology Microorganisms from the genus *Listeria* are commonly found on plants and in soil. They have been isolated from old, faded, or moldy plants; top soil; river mud; and sewage and sewage sludge (439). The ability of *Listeria* to multiply at low temperatures (371), its ability to survive for

long periods in soil (437), and its presence on decaying vegetation (438) imply a saprophytic existence wherein the plant-soil environment may serve as a reservoir (435, 440). Meat, dairy products and vegetables fertilized with animal manure are important sources of contamination for humans (164, 292). A large proportion of healthy sheep and goats are latent carriers of *L. monocytogenes* and excrete the organism in feces and milk during periods of stress (163, 261). Although less frequently, cows also serve as carriers of *L. monocytogenes*, and excrete the organism into milk (142, 178, 256). Raw chickens are also frequently contaminated (60%) with *L. monocytogenes* (236, 338). Kampelmacher and Jansen (224) reported that a high percentage of healthy humans were carriers; they also isolated *L. monocytogenes* from sewage plants (225). These findings indicate that the bacteria are widespread in nature, can be present as an intestinal microorganism and can be spread to humans via consumption of contaminated foods.

Growth of *L. monocytogenes* in foods *L. monocytogenes* has been isolated from many different foods including raw milk (142, 178, 256, 266), meat (25, 220), coleslaw (363), and soft cheeses (29, 338). Because of its psychrotrophic (355) and pathogenic nature (266), considerable interest exists as to the conditions that permit the organism to grow in these foods. Donnelly and Briggs (90) found that psychrotrophic growth of *L. monocytogenes* serotype 4b was enhanced in whole milk, compared with skim milk or 11% nonfat milk solids. The stimulatory effect of whole milk was most dramatic at 40°C; cells increased from 7.9×10^0 to 5.8×10^6 CFU/ml within 48 h. Rosenow and Marth (355) observed similar growth

rates of *L. monocytogenes* in skim, whole and chocolate milk, and in whipping cream. Doubling times over all products and strains ranged from 41 min at 35°C to 45 h 33 min at 4°C. In each instance, maximum populations reached were at least 10^7 cells/ml. Highest numbers were consistently produced in chocolate milk. Ryser and Marth (359) observed that in Cheddar cheese the numbers of *L. monocytogenes* increased slightly during the first 14 days of ripening but then steadily declined. In the case of Camembert however, rapid growth of *L. monocytogenes* strain V7 occurred after 35 days of ripening, reaching levels of 1×10^7 after 56 days of storage (360). They noted that growth of strain V7 during this period paralleled increases in the pH of the cheese. The proliferation of *L. monocytogenes* on raw shredded cabbage was reported by Beuchat et al. (33). Numbers of the organism increased from 1.6×10^4 to 2.6×10^8 CFU/g when the product was stored at 5°C for 25 days. Extended storage to 64 days resulted in only a slight decrease in viable populations. No sign of growth of *L. monocytogenes* was observed in either ravioli (32) or ground beef (220) during refrigerated storage.

Detection methods *Listeria monocytogenes* is often difficult to isolate from a food because of the presence of competing flora. An early attempt to lessen this problem involved storing the food at a low temperature from one week to three months or longer. This method, known as cold enrichment (159), exploited the capability of *L. monocytogenes* to grow at 4°C. Another early method, which is still used today, involves the use of oblique lighting to differentiate colonies of *L. monocytogenes* from contaminants (157).

More recently, agents that select for *L. monocytogenes* while inhibiting other organisms have been incorporated into various media. These agents include phenyl ethanol, lithium chloride, nalidixic acid, potassium tellurite, and moxalactam. In 1960, McBride and Girard (287) developed a medium selective for *Listeria* which has been used and modified by many workers. McBride Listeria agar (MLA) is composed of phenyl ethanol agar base (Difco), lithium chloride, glycine, and blood. Phenyl ethanol and lithium chloride are inhibitory to Gram-negative microorganisms; and MLA was suitable for the isolation of *L. monocytogenes* from mixed cultures. However, Leighton (249) observed that lithium chloride had a marked inhibitory effect on *L. monocytogenes*; glycine was also inhibitory. In a recent study, Smith and Archer (383) observed that phenyl ethanol appeared to inhibit the repair of heat-injured cells of *L. monocytogenes*.

Lee and McClain (248) combined the best features of MLA and Baird-Parker medium (24) to form LiCl Phenylethanol Moxalactam agar (LPMA). LPMA is similar in composition to MLA except that there is a ten-fold increase in the lithium chloride concentration, and glycine anyhdride is substituted for glycine because the latter was found to inhibit *L. monocytogenes*. Blood was omitted from this formula; moxalactam, a ß-lactam antibiotic effective against a wide range of bacteria, was added to give a final concentration of 20 ug/ml. LPMA is very selective and inhibits contaminants commonly present in Brie cheese, cabbage, and hams (60). This medium is presently used by the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture. However, some inhibition of *L. monocytogenes* on this medium has been observed by

Swaminathan et al. (395), who reported that significantly fewer cells of certain strains of *L. monocytogenes* were recovered on LMPA than on MLA. Buchanan et al. (47) reported that LPMA and MVJ were approximately equivalent with regard to recoveries; however, MVJ allowed easier visual differentiation of *L. monocytogenes* colonies on plates contaminated with competing microorganisms (47).

Lovett et al. (266) modified MLA in 1987 by omitting the blood and adding cyclohexamide to inhibit the growth of molds. Modified McBride Listeria agar (MMLA) is presently used by the United States Food and Drug Administration in their procedure to test foods for *L. monocytogenes* (264). Buchanan et al. (47) modified MMLA by adding nalidixic acid, moxalactam, and bacitracin. This medium, known as ARS-Modified McBride agar (ARS-MMA), offered substantial improvements over selective media previously used to isolate *L. monocytogenes*. However, studies by Cassiday and Brackett (60) revealed that ARS-MMA was not satisfactory to recover *L. monocytogenes* from pasteurized milk, ice cream mix, cabbage, and Brie cheese, especially if the cells were injured. Likewise, Crawford et al. (74) concluded that the current selective FDA and USDA methods would not detect heat-injured *L. monocytogenes* that survived HTST pasteurization.

Most microorganisms undergo metabolic injury upon exposure to sublethal heating or freezing that affects their ability to recover and form colonies on selective media (reviewed in ref. 197). The methods mentioned above either lack provisions for recovering impaired cells or the suitability of the procedures to recover injured cells is unknown. Doyle and Shoeni (99) developed a selective-enrichment procedure (SEP) for detecting *L.*

monocytogenes from fecal and biologic specimens. Doyle et al. (95) used the SEP to recover *L. monocytogenes* from HTST-pasteurized milk. Other selective liquid enrichment media were developed (87) that were also capable of recovering heat-injured *L. monocytogenes* from pasteurized milk (120, 121).

A direct plating method, enabling the enumeration of *L. monocytogenes* that have survived HTST pasteurization, has yet to be realized. Golden et al. (147) determined that Modified Despierres agar (MDA) (83) and Modified McBride Listeria agar (MMLA) (266) were inferior to McBride Listeria agar (MLA) (287) and gum base-nalidixic acid-tryptone agar (GBNTSA) (279), based on their inability to recover heat injured cells. Dominguez Rodriguez isolation agar (DRIA) (87) was the medium of choice for recovering uninjured, heat-injured, and freeze-injured cells from Brie cheese. With raw cabbage, MDA was superior to Donnelly *Listeria* enrichment agar (DLEA) (89), due to the formation of small colonies on the latter medium. Van Netten et al. (412) developed a solid medium, ALPAMY, which contained acriflavine, lithium chloride, phenyl ethanol, aesculin, mannitol, and egg yolk. Solid medium repair on tryptone soya peptone yeast extract catalase agar followed by overlayering with ALPAMY tempered at 47°C an effective resuscitation procedure for very severely stressed *L. monocytogenes*.

Molecular biology techniques, including DNA probes (232) and enzyme linked immunosorbant assays (ELISA) (284), are being utilized to rapidly identify *L. monocytogenes* in foods. Recently, a rapid assay for *L. monocytogenes* by using the polymerase chain reaction was developed (30). A 606 base-pair sequence of the listeriolysin gene was amplified, and the

product was examined after either electrophoresis on an agarose gel or was dot blotted and tested with an oligonucleotide probe. Amplification, followed by either the agarose gel or dot blot techniques, resulted in the detection of as few as 10^3 cells.

Relationship to phagocytes Phagocytes play a pivotal role in immunology because they are critical for the control and physical elimination of a wide number of pathogens (231). Prior to making contact with a particle or bacterium such as *L. monocytogenes*, phagocytes exist in a so called "resting state", relying on glycolysis for their energy supply (227). Upon encountering a bacterium the cell engulfs it in a vacuole or phagosome and the cell undergoes oxidative metabolism (20). These changes, known collectively as the "respiratory burst", include the following phenomena: 1) Increased O_2 consumption by the cell; 2) Increased oxidation of glucose by the hexose monophosphate shunt (HMS); 3) Increased production of hydrogen peroxide, and 4) The production of the superoxide $O_2\cdot^-$ anion (81). The apparent purpose of these reactions is to furnish lethal oxidants for killing the engulfed microorganism (27). Besides $O_2\cdot^-$ and H_2O_2, even more toxic oxidants are produced by secondary reactions, which generate $\cdot OH$, $^1O_2$ (singlet oxygen), and ClO^- (20, 27, 66).

Listeria monocytogenes is an intracellular pathogen that survives and reproduces inside phagocytes (41, 42, 175, 78). To accomplish this feat, the microorganism must avoid the lethal effects of the respiratory bursts or fail to stimulate them. The organism is readily phagocytosed (41), triggers a burst of oxidative activity (145), and is killed by polymorphonuclear

leukocytes (PMNs) in vitro (42). However, the organism appears can survive and reproduce in macrophages (78, 175).

Welch et al. (436), using a superoxide-generating medium, found that virulent, catalase-positive *L. monocytogenes* strains were relatively resistant to killing at pH 7. They postulated that SOD might be a virulence factor for *L. monocytogenes* by eliminating $O_2^{\cdot -}$ and preventing formation of ·OH inside the phagosome. Bortolussi et al. (42) demonstrated that log-phase *L. monocytogenes* resist oxidative antibacterial agents by producing sufficient catalase to inactivate these products. After surviving the initial respiratory burst, *L. monocytogenes* would be exposed to rapidly decreasing levels of oxygen radicals (144).

Pathogenicity The first detailed description of the Gram-positive bacillus now known as *Listeria monocytogenes* was published in 1926. Murray et al. (306) described a "spontaneous" epidemic of infection among laboratory rabbits and guinea-pigs caused by a bacterium which they named *Bacterium monocytogenes* because the infection was characterized by a monocytosis. The first report of listeriosis in humans was by Nyfeldt in 1929, who isolated *L. monocytogenes* from the blood of patients with an infectious mononucleosis-like disease (158).

There are eight species of *Listeria*. In addition to *L. monocytogenes*, which is the significant pathogen for humans, there are a large number of animal species (371). In humans, meninges involvement, sometimes accompanied by septicemia, is the usual clinical manifestation. The members of the population more at risk are neonates, the aged and those compromised by pregnancy or an underlying illness such as malignancy or alcoholism or

some condition which requires immunosuppressive procedures (406). Intra-uterine infection of the fetus results in death, or an acutely ill infant with a septic disseminated form of listeriosis, Granulomatosis infantiseptica (349).

The current ability to assess the relative virulence among *L. monocytogenes* strains is imprecise. Besides catalase and superoxide dismutase activities, serotype is perhaps the best characterized virulence factor (362). Virulent *Listeria* tend to cluster in subgroups 1 through 4 (362). Recent outbreaks have been associated with serotype 4b (128, 214, 363). Serotype may not predict virulence accurately either because environmental strains of *L. monocytogenes* subgroup 4 was avirulent when injected into mice (438). When Gaillard et al. (137) inactivated ß-hemolysin production in *L. monocytogenes* by transposon mutagenesis, virulence was lost. Wexler and Oppenheim (441) described a *Listeria* endotoxin; however, the presence or absence of this endotoxin has not been studied in avirulent species, so its relevance to pathogenesis remains unclear.

Resistance on the part of the host appears to reside in the ability to mount an efficient cell-mediated immune response. This response depends on the activation of T-lymphocytes by listerial antigens and the release of lymphokines which both stimulate the bactericidal activity of the macrophages and attract more bone marrow-derived monocytes to the site of infection (274). The genetic determinant of this resistance in mice appears to reside in a single autosomal gene, termed Lr. Mice containing mutations in this gene are 100 times more susceptible than are wild-type mice to *Listeria* infections (61).

Epidemiology *Listeria monocytogenes* has recently been documented as a cause of common-source foodborne outbreaks: in the Canadian Maritime Provinces in 1981 (363), in Boston in 1983 (128), and in Southern California in 1985 (214). The outbreaks have been notable for a case-fatality rate of 30%, including stillbirths and perinatal deaths. The largest outbreak in Southern California involved at least142 cases (214). Vehicles implicated include cole slaw, pasteurized milk and Mexican-style soft cheese. It has been estimated that *L. monocytogenes* causes between 800 (45) and 1,700 (25) cases of meningitis and sepsis in the U.S. each year, with a case fatality rate of approximately 25%. Sporadic cases of listeriosis were statistically associated with the consumption of uncooked hot dogs and undercooked chicken (367). Most recently, vacuum-packaged turkey franks contaminated with *L. monocytogenes* were implicated in a fatal case of listeriosis (25). The minimum USDA thermal process for hot dogs is "borderline" for the destruction of *L. monocytogenes* (9).

Oxygen reactions

Reactivity The oxygen molecule is paramagnetic because two of its valence electrons are unpaired, a property that confers on oxygen the ability to behave as a diradical (304). This makes O_2 a powerful oxidizing agent, the two redox couples of traditional interest to the biologist being those in which the products are water and hydrogen peroxide, respectively:

$$O_2 + 4H + 4e^- = 2H_2O, \quad Eo' \text{ at pH7} = +.875v$$

$$O_2 + 2H + 2e^- = H_2O_2, \quad Eo' \text{ at pH7} = +.270v$$

In "normal" oxygen the two unpaired valence electrons are located in separate orbits and are of parallel spin ⊕⊕. This is the lowest energy state of the molecule, known as its ground or triplet state. Molecular oxygen in this triplet state may be energized to yield singlet oxygen ($^1O_2^*$) wherein the two unpaired electrons are antiparallel in spin ⊕⊖. There are thus two forms of singlet oxygen of unequal energies, that form in which the antiparallel, unpaired electrons are in identical orbit ⊕O being represented as $^1\Delta_g$, and that more highly energized form in which the antiparallel, unpaired electrons are in separate orbits ⊕ ⊖ being represented as $^1\Delta_{g+}$. Singlet oxygen can be produced in living cells by photoexcitation of triplet oxygen or the spontaneous dismutation of superoxide anion (304). However it is formed, because of its exceptional reactivity it poses a threat to the integrity of cellular components (79).

One species that has achieved particular prominence is the superoxide anion $O_2^{\cdot -}$ which, being the product of univalent reduction of the oxygen molecule, is a highly reactive free radical (135). This species is synthesized in aerated, aqueous solution by numerous procedures affecting the 1 electron reduction of molecular oxygen; e.g., pulse radiolysis, photo-illumination of suitable dyes, and reduction by ferrous ions in the presence of phosphates (304). In addition, $O_2^{\cdot -}$ is formed when O_2 reacts with various cellular constituents, including reduced flavin, flavoproteins, quinones, thiols, iron-sulfur proteins and tetrahydropteridines (283, 304, 324). Superoxide anion is also produced by the action of enzymes, including aldehyde oxidase, xanthine oxidase and numerous flavin dehydrogenases (304). It can act as a potent reducing or oxidizing agent and can serve as an initiator of free

radical chain reactions (342). In aqueous media, superoxide anions are the longest lived of all oxygen-derived free radicals, and there is ample evidence of the damage that they can do in uncontrolled reactions with numerous vital cell components (134, 135). In aqueous solutions, superoxide anions react with each other and are thus removed by dismutation reactions, $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ = H_2O_2 + O_2$, which even at pH 7 lead to destruction of superoxide anions at a rapid rate (133, 136). However this spontaneous dismutation also leads to production of singlet oxygen. Furthermore, production of hydrogen peroxide could prove additionally hazardous since, in a process known as the Haber-Weiss reaction (167), superoxide anion reacts with H_2O_2 to form another, most dangerous free radical, namely the hydroxyl radical: $O_2^{\cdot -} + H_2O_2 + H^+ = O_2 + H_2O + \cdot OH$ (169). In addition, $\cdot OH$ has been reported to be formed when H_2O_2 reacts directly with iron in a process known as the Fenton Reaction (350). Aside from the toxicity directly attributable to the hydroxyl radical which is known to account for a considerable part of the extensive cellular damage wrought by ionizing radiations (117), interaction of the hydroxyl radical with the superoxide anion could augment the yield of singlet oxygen, $O_2^{\cdot -} + \cdot OH = OH^- + {}^1O_2^*$ (304). Hence, in systems producing superoxide anion we have the additional potential of producing three more hazardous derivatives of oxygen: hydrogen peroxide, the hydroxyl radical, and singlet oxygen (134).

<u>General types of O_2 relationships</u> When the first prokaryotic life forms made their appearance some 3.5×10^9 years ago, they emerged on a planet whose surface was highly reduced and whose atmosphere was totally devoid of oxygen. Geological evidence suggests that the most abrupt increase in the

oxygen occurred only some 640 million years ago when O_2 produced by oxygenic photosynthetic organisms began to accumulate in the atmosphere (364). Because of its ready diffusibility and reactivity, O_2 supplied the requirement for an almost universally accessible electron acceptor of pleasingly high potential, the major product of whose reduction (H_2O) was innocuous and disposed of easily. Yet that very reactivity which was the basis of its effectiveness as an electron acceptor also posed a threat to organisms which did not simultaneously evolve means to deal with undesirable by-products of its consumption, or to protect or modify key "autoxidizable" cellular components (304).

Microorganisms can be classified into four broad groups based on their relationship to molecular oxygen (390). Strict aerobes are organisms that are dependent on aerobic respiration for the fulfillment of their energetic needs and for which molecular oxygen functions as a terminal electron acceptor. Facultative anaerobes are microorganisms that are able to grow either in the presence or in the absence of molecular oxygen. In metabolic terms, facultative anaerobes fall into two subgroups. Some, like the lactic acid bacteria, have an exclusively fermentative energy-yielding metabolism but are relatively insensitive to the presence of oxygen: such organisms are most accurately termed aerotolerant anaerobes. Others, (e.g., many yeasts, enteric bacteria, *Listeria*) can shift from a respiratory to a fermentative mode of metabolism. Such facultative anaerobes use O_2 as a terminal oxidizing agent when it is available but can also obtain energy in its absence by fermentative reactions. Among the strict aerobes and facultative anaerobes some organisms, such as *Listeria*, grow best at partial pressures

of oxygen considerably below that present in air. They are termed microaerophiles. It is thought that this reflects the possession of enzymes that are inactivated under strongly oxidizing conditions and can thus be maintained in a functional state only at low partial pressures of O_2 (386).

The last group of organisms is termed obligate or strict anaerobes. Morris (304) described an obligate anaerobe as an anoxybiontic, aerointolerant organism, that is, an organism which (1) is capable of generating energy and synthesizing its substance without recourse to molecular oxygen, and (2) demonstrates a singular degree of adverse sensitivity to oxygen which renders it unable to grow in air at 1 atmosphere. Obligate anaerobes are not a homogeneous group with respect to their relationships to molecular oxygen. Anaerobic organisms differ in their sensitivity to oxygen poisoning, such that a continuous spectrum of oxygen tolerance exists, from the most sensitive, strict anaerobe to the least sensitive aerotolerant anaerobe (304).

Enzyme-based theory of anaerobiosis Obligately anaerobic microorganisms are strongly inhibited or are killed by exposure to molecular oxygen (260). This phenomenon was not satisfactorily explained until McCord and Fridovich (289) reported the isolation and function of an enzyme, superoxide dismutase (SOD), which catalyzed the dismutation of superoxide radicals ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ = O_2 + H_2O_2$). When surveying the distribution of this enzyme plus catalase (CA) in various microorganisms they found that anaerobic bacteria exhibited no SOD and generally no CA activity (290). All aerobic organisms containing cytochrome systems contained both SOD and CA. Aerotolerant anaerobes, which survive exposure to air but do not utilize oxygen and do not contain cytochrome systems, were

devoid of CA activity but did exhibit SOD actively. They proposed that the prime physiological function of SOD is the protection of oxygen-metabolizing organisms against the potentially detrimental effects of the superoxide radical, a biologically produced intermediate resulting from the univalent reduction of molecular oxygen (289).

Superoxide is important in various cellular processes. When Chinese hamster ovary cells were exposed to paraquat, superoxide was toxic, but when SOD (but not CA) was introduced into the cell the toxicity of paraquat was decreased (23). The failure of exogenous SOD to protect against O_2 was also observed by Bucker and Martin (49). Gregory and Fridovich (160) showed that induction of increased levels of SOD was associated with greater tolerance to oxygen lethality mediated by $O_2{\cdot}^-$. The actual species involved in cytotoxic processes is still a matter of debate because $O_2{\cdot}^-$ can react with another $O_2{\cdot}^-$ molecule or with H_2O_2 (167) to form the very toxic hydroxyl radical ($\cdot OH$).

The syntheses of SOD and CA are induced by conditions known to increase concentrations of $O_2{\cdot}^-$ and H_2O_2, respectively. *E. coli* contains two SODs. One SOD which contains iron (FeSOD) and is located in the periplasmic space appears to be constitutive. The other SOD which contains manganese (MnSOD) is located in the cytoplasm and is made only when oxygen is present (160). The addition of paraquat to an aerobic culture of *E. coli* B resulted in a rapid and pronounced increase in the rate of biosynthesis of the MnSOD (176). Paraquat is thought to cause the production of $O_2{\cdot}^-$ which induces the synthesis of the MnSOD. Heat shock induces the synthesis of a Cu-ZnSOD in Chinese hamster ovary (CHO) cells and ovarian carcinoma (OvCa)

cells (263). The authors (263) speculated that if the observed increase in SOD activity in thermotolerant CHO + OvCa cells is the result of an increased flux of free radicals, then it is possible that cell killing by heat is associated with an increased flux of free radicals and that thermal resistance is in part due to an increased ability to metabolize free radicals.

Catalase is induced by oxygenation of cultures of *E. coli* and *Streptococcus faecalis* (177). Induction by exogenous H_2O_2 has also been reported. Glucose suppresses the synthesis of CA and peroxidase in *E. coli*, an organism which can generate two distinct catalases. One of these is constitutive and is present even in anaerobically grown or in glucose-repressed cells, whereas the other appears only when the synthesis of CA is derepressed in the absence of glucose by oxygen or nitrate (177).

Thermal inactivation of catalase and superoxide dismutase Catalase is a heat-labile enzyme in animals, plants, and bacteria. Mouse liver CA (73) and bovine milk CA (210) are inactivated rapidly above 37°C. In plants, CA is rapidly inactivated at 70°C (19). Andrews and Martin (7) demonstrated that the inactivation of CA at 52°C in *S. aureus* significantly decreased its survival. The activity of CA was further decreased when heat-injured cells were exposed to 10% NaCl (6). The heat inactivation of CA was used to differentiate species of *Mycobacterium* (285). All species demonstrated significant reduction in CA activity after 50 minutes at 53°C. Catalase activity in 4 strains of *L. monocytogenes* decreased rapidly between 55 and 60°C (80). Therefore, CA appears to be particularly heat-labile in *L. monocytogenes*.

In contrast to CA, the thermal stability of SOD varies considerably, depending on the source and the presence of specific metals. Forman and Fridovich (132) demonstrated that the presence of Cu^{+2} and Zn^{+2} greatly increased the activity and thermal stability of SOD from bovine erythrocytes. The holoenzyme that contained Cu^{+2} and Zn^{+2} was stable at 70°C, but the apoenzyme was rapidly inactivated at 50°C. The heat sensitivity of mammalian cells can be increased by the addition of diethyldithiocarbamate, a compound capable of removing Cu^{+2} from SOD in vivo (116). Superoxide dismutases from cabbage (422), *S. aureus* (48), and *L. monocytogenes* (80) were rapidly inactivated at 60°C. However, SOD in bovine milk was heat-stable at 70°C and survived the HTST pasteurization treatment given milk (182).

Anaerobic Methods Leeuwenhoek in 1680, was the first to demonstrate that "animalcules" can exist and develop without air, or at any rate, in a highly rarified atmosphere (87). Anaerobiosis was discovered 181 years later by Pasteur (331) who introduced the terms *aerobies* and *anaerobies* to denote microorganisms that live with or without oxygen, respectively. Pasteur boiled solutions to remove oxygen; however, boiling alone was not adequate to reduce the oxygen tension for surface cultivation or to allow for the growth of strict anaerobes. Various other methods were tried to further increase the recovery of the more fastidiously anaerobic bacteria; these included 1) driving O_2 from media by boiling and or sparging with O_2-free gas; 2) reducing O_2 in the environment by drawing a vacuum and or replacing air with O_2-free gas; 3) physically preventing O_2 from contacting the bacteria via agar overlays or metal or glass plates; and 4)

chemically or biologically reducing O_2 and E_h (4). Some of the other major advances in anaerobic bacteriology included the use of reducing agents, such as cysteine, and E_h indicators, such as resazurin (387).

Currently, three major methods are used routinely to enumerate of anaerobic bacteria: anaerobic jars, anaerobic glove boxes, and anaerobic roll tube ("Hungate") techniques (92). Each method has advantages and disadvantages. Anaerobic jars, although they are simple to use, pose a problem for strict anaerobes because the injured organisms are exposed to O_2 until all the molecular O_2 is removed. The exposure of anaerobic media to O_2 results in the formation of H_2O_2 and $O_2{\cdot}^-$ which results in rapid killing of strict anaerobes (376). Anaerobic glove boxes permit manipulation of all materials in an anaerobic environment (10), but are expensive and are not suitable for performing pour-plate techniques because of formation of excess humidity in the glove box. Strictly anaerobic, roll tube "Hungate" techniques (185, 186, 193, 194) permit the handling of cultures under continuous anoxic conditions such that the microorganism is never exposed to O_2. This method requires considerably less apparatus and equipment than other routine methods and less space for large scale studies than other systems (46). The main disadvantages of the Hungate method include the additional time required for preparing pre-reduced media and learning techniques which at first appear strange and arduous (4, 92). Another disadvantage is the difficulty in picking isolated colonies from inside roll tubes, compared with the ease of picking colonies on petri plates. These disadvantages are easily overcome, however, as the worker can quickly become competent with these techniques (46, 186). Most recently, the addition of stable membrane

fragments derived from *E. coli*, to liquid or solid media has been shown to produce and maintain strict anaerobic conditions (1). This has allowed manipulation of O_2-sensitive bacteria with standard aerobic techniques (2).

In reality, many food microbiology laboratories use few, if any, strict anaerobic techniques. The Compendium of Methods for the Microbiological Examination of Foods (389) contains little information on procedures for the examination of anaerobic bacteria. The three chapters on anaerobes (Chapters 20, 23, 36) mainly deal with enumeration of spores and toxin detection and do not mention the use of pre-reduced dilution blanks or media for food samples. The chapter on repair and detection of injured microorganisms (Chapter 7) makes no mention of anaerobic techniques, let alone the use of pre-reduced media.

Heat Resistance of *Listeria monocytogenes*

Factors that influence heat resistance   Numerous factors can affect the heat resistance of nonspore-forming microorganisms. These include water activity (A_w), pH, oils and fats, salts, carbohydrates, proteins, inhibitory compounds, cell concentration, age of the culture, growth temperature and other growth conditions (171). One of the first factors to be recognized to affect thermal death rates was the age of a culture. In 1923, Sherman and Albus (373) discovered that bacterial cells went through a period of physiological youth in which they were more susceptible to a variety of stresses, including heat. Six years later, Stark and Stark (391) discovered that young cultures (6 hours old) of *Streptococcus faecalis* were much less resistant than "mature" cells (24 - 40 hours old) to heat. The heat resistance

of *E. coli* was found to increase dramatically during the stationary phase, especially for cells grown above their optimum temperature (109). White (442) reported similar results for the heat resistance of *S. faecalis*. Ng et al. (319) reported that *Salmonella senftenberg* was more heat-resistant in the stationary phase of growth than when in other growth phases. Cells grown at 44°C were more heat-resistant than those grown at either 35 or 15°C. *Staphylococcus aureus* was most heat-resistant in the late logarithmic and early stationary phase (201). Cells of *S. aureus* grown at 46°C were significantly more heat resistant than cells grown at 37°C (108).

Another major factor in heat resistance is the nature of the recovery conditions after heat injury. Dubos (101) observed that the growth of various fastidious facultative pathogens is possible only when incubated a) under anaerobic conditions, b) in fresh media very recently boiled or autoclaved media, c) in fresh media reduced by means of hydrogen, or to which small amounts of cysteine or blood had been added. The favorable growth conditions obtained by these procedures may be attributed to the establishment of a proper reduction potential in the medium. Nelson (316) found that the addition of small amounts of tryptone and thioglycolic acid to a basal medium greatly increased the recovery of heat-injured facultative pathogens. This phenomenon was attributed to the ability of these compounds to reduce the oxidation/reduction (O/R) potential of the medium. In addition, Harries and Russel (174) obtained 20% higher recoveries of heated *E. coli* when pour plates techniques were used, compared with surface plating. This was attributed to the high O/R at the surface of solid media.

Gomez and Sinskey (152) determined that recovery in a minimal medium is not only dependent on heat and a nutritionally complex medium but also on air. Unlike in the presence of air, in the presence of nitrogen, heat-injured cells were able to recover their ability to grow on Trypticase soy agar. Similar recoveries in broth were observed with *E. coli* (281). Egan (104) discovered that the addition of catalase to a selective medium containing desoxycholate increased the count of heat-stressed cells by 2.3-fold, but this represented only a small percentage (13%) of the total population of injured but viable cells able to grow on nutrient agar. The sensitivity of heat-injured *S. typhimurium* to selenite and tetrathionate media was measured by viable counts in liquid and agar-solidified versions of these media and on nutrient media (267). All solid media, including the supposedly noninhibitory nutrient agar, were more inhibitory than the corresponding liquid media to injured cells. Sodium pyruvate increased counts on minimal and selenite agars, but not to the level of counts in the corresponding liquid media, implying that, with these media, factors in addition to peroxide contributed to the lower recoveries on agar plates. Mackey and Derrick (267) concluded that catalase or sodium pyruvate increased counts on nutrient agar by preventing the formation of H_2O_2.

The importance of incorporating catalase or sodium pyruvate in plating media to improve the recovery of heat-injured *S. aureus* was first suggested by Baird-Parker and Davenport (24). Subsequent work has shown that this was beneficial because of the heat inactivation of CA in *S. aureus* (50, 130, 280). Andrews and Martin (6) demonstrated that the increased sensitivity of heat-injured *S. aureus* in a selective medium containing NaCl was due to

the decrease in activity of CA in the presence of NaCl after heat-injury and prior to recovery. This suggested that the recovery mechanisms of *S. aureus* , rather than the heat-injury itself, was responsible for the decreased enumeration of heat-stressed microorganisms. The addition of CA, or other compounds that destroy H_2O_2, to solid media also aided the recovery of other facultative pathogens, including *S. senftenberg* (346), *E. coli* (291), and *L. monocytogenes* (383). Levels of pyruvate used in recovery medium vary. A level of 1.0% has been commonly used for *S. aureus* (24). Recently, Lee and Hartman (247) determined that 0.02% pyruvate was the statistically predicted optimal level to use in the Modified VRBA procedure and that 1.0% pyruvate reduced colony counts of injured cells.

Potential sites of thermal damage Moats (299) described a method for determining thermal damage by calculating k (the rate constant for thermal inactivation of individual sites within the cell), N (the number of sites per cell), and X_L (the number of sites which must be inactivated to cause death). Calculated values for N were greater than 100. The value of X_L varied depending on the recovery medium used after heating. Moats model was based on the following assumptions: 1) that inactivation of individual sites occurs at random and follows first-order kinetics, 2) that the critical sites are identical and of equal heat resistance, and 3) that the bacterial population is homogeneous in heat resistance. Moats concluded that his theory was consistent with all published experimental observations on thermal injury and death of bacteria.

Mutants of *S. aureus* that contained higher levels of wall teichoic acid than found in normal cells generally showed less injury (187). Magnesium

was lost from the cell walls during heating, and the degree of cell injury was accentuated when Mg^{2+} ions were either removed from the heating menstruum or made unavailable to the cell by chelation. These data suggest that cell-wall teichoic acids of *S. aureus* aid in survival by maintaining an accessible pool of magnesium. This hypothesis is consistent with that of Hughs and Hurst (191) who observed that Mg^{+2} loss upon heating and omission of Mg^{+2} from the repair medium diminished recovery. The outer membrane also suffers damage when Gram-negative bacteria are subjected to mild thermal stress because membrane LPS is released (358). This is not, however, considered to be a direct cause but rather a consequence of thermal injury (183).

Unless repaired, sublethal damage to the cytoplasmic membrane of both prokaryotic and eukaryotic microorganisms is associated with cell death if the cells are then exposed to deleterious chemical or physical agents. One physical agent known to cause such damage is moist heat (403, 31). Grau (156) challenged the assertion that heat injury affects the cytoplasmic membrane to any significant extent. His conclusions are based on the fact that sufficient loss of membrane integrity should drastically impair the stressed cell's ability to transport solutes into the cell and maintain a concentration gradient across the membrane. Such an effect was not observed (156). Moist heat is capable of both single- and double- strand breaks in DNA at temperatures above 50°C. (5). In thermally injured organisms, strand breakage may occur during or after heat treatment, but it is usually considered that this breakage is a consequence of the stimulation

of endonuclease activity by heating (151, 337). Repair of thermally induced DNA strand breaks has been shown to occur (335).

Iandolo and Ordal (204) implicated ribonucleic acid synthesis in the recovery of heat-stressed *S. aureus*, because actinomycin D completely repressed recovery. Additionally, they discovered that nucleic acid synthesis paralleled that of recovery and the return of salt tolerance. The lesion was the ribosomal ribonucleic acid (rRNA) (386). Sucrose-gradient analyses demonstrated that a ribosomal peak was undetectable subsequent to thermal treatment, but this peak was regenerated during the recovery period. Hurst and Hughes (198) determined that ribosomal damage is a consequence of Mg^{+2} loss and is not an effect of heat per se. They concluded that sublethal heat-injury could not be attributed to other types of ribosomal damage (199).

Tomlins and Ordal (403) stated that there was little evidence to suggest that enzyme inactivation is a significant mechanism in the thermal injury of vegetative bacteria. In addition, towards the latter stages of recovery from thermal injury, protein synthesis may occur, so the resynthesis of any heat-inactivated enzymes is possible (404). The recovery of heat-injured *S. aureus* was not inhibited by chloramphenicol. Since these early speculations, evidence has been obtained that inactivation of CA and SOD is a major factor in the loss of heat resistance (see section on thermal inactivation of CA and SOD, p. 51)

<u>Heat shock proteins</u> All organisms studied to date respond to elevations of some 5°C or more above their normal physiological temperature by the rapid and preferential synthesis of a number of highly

conserved proteins, the heat shock proteins (HSP; for a recent review see ref. 257). A heat-shock-like-response also can be induced in cells by exposure to a wide variety of other environmental insults, such as amino acid analogs, heavy metals, ethanol, H_2O_2 and glucose starvation (309). Collectively, the stress proteins appear to function in the protection and enhanced survival of the cells experiencing stress (298).

The heat shock response was first observed as an etiological phenomenon in *Drosophila* when a unique set of chromosomal puffs was induced by heat (353). Later, Tissierres et al. (399) discovered that heat shock resulted in the induction of the synthesis of a set of polypeptides. It is now known that the heat shock genes constitute a regulon, a global regulatory network under the control of a common regulatory (sigma) factor (165, 308,).

Most of what we know about heat shock in prokaryotes has been gleaned from the study of the response in *E. coli* (309). Shifts up or down in growth temperature of even a minor nature are accompanied within 1 or 2 min. by large changes in the differential rates of synthesis of many *E. coli* proteins (253, 181). Within the normal temperature range (20 - 37°C) these transient rates return to previous values for most proteins. However, after a temperature shift to the high range (37 - 46°C) the transient inductions and repressions rapidly adjust cellular content to new steady state levels (181). The HSPs are found among those that are hyperinduced upon a shift to high temperature (309). The level of synthesis of HSPs is controlled both transcriptionally (310, 446, 447) and post translationally (84) through repression of hsp70 mRNA synthesis and destabilization of hsp70 transcripts. Neidhardt and VanBogelen (308) isolated a gene, *htpR*, (*rpoH*)

which codes for a sigma factor (sigma-32) for heat-shock promoters (165). This sigma factor controls a large number of unlinked genes (operons) encoding at least 17 HSPs in *E. coli* (for a review of global regulatory networks see ref. 154). Synthesis of sigma-32 is controlled by the transient heat activation of P_{hs}, a promoter located within the protein-coding sequence for DNA primase (397). Transcription is also dependent on an activating protein factor that binds to upstream control sequences in heat shock genes (330, 445)

The heat shock response of *E. coli* was discovered by examining a mutant that was temperature-sensitive for growth (would not grow above 43°C) (69). Subsequent work led to the characterization of the mutant gene, *rpoH*, as a positive-acting regulatory gene, and its protein product, sigma-32, as a sigma-like transcription factor required for the induction of HSPs (401). Other mutations in heat shock genes also cause temperature-sensitive growth. The shift of *E. coli* Dnak null mutants to 42°C for 2 h prevented them from forming colonies at 30°C (328). Transposon mutagenesis of the *GroE* gene in *E. coli* prevented the organism from growing at 42°C (418). The genes YG100 and YG102 of *S. cerevisiae* are closely related to Hsp70 in *Drosophila*. Mutations in either of these two genes prevented *S. cerevisiae* from forming colonies at 37°C (72).

Thermotolerance has been divided into 3 phases: 1) induction, which can be triggered by treatment at all hyperthermic temperatures; 2) the development, which occurs for a period of 2 - 8 h under appropriate culture conditions, and 3) decay, which leads to the complete disappearance of thermotolerance. The induction of thermotolerance has been correlated with

the synthesis of HSPs in mammalian cells (141, 240, 255). In addition, monoclonal antibodies directed against hsp70 were lethal when injected into rat fibroblasts held at 45°C (351); cells injected with control antibodies survived a similar heat shock. The level of acquired thermal resistance in yeast and molds also are correlated with the cellular level of the HSPs (286, 339). The synthesis of HSPs is also strongly correlated with thermotolerance in prokaryotes. Yamamori and Yura (447) reported that a functional hin (*rpoH*) gene product is essential for the induction of thermotolerance in *E. coli* K-12. A typical heat shock response was reported for *S. typhimurium* when the cells were heat shocked at 48°C for 30 min., which resulted in a dramatic increase in heat resistance (268, 269). The heat shock response has been demonstrated in Gram-positive microorganisms also (393, 402). The possibility has been suggested that the heat shock response may be instrumental in the ability of bacilli such as *B. stearothermophilus* to multiply over wide temperature ranges (i.e., 37 - 65°C).

In contrast to these previous reports, VanBogelen, Acton, and Neidhardt (410) reported that no correlation existed between the development of thermotolerance and the chemical induction of HSPs in *E. coli.* In addition, Ramsay (343) discovered that, although produced in large amounts in response to thermal stress, an abnormal *Dnak* protein of *E. coli* had little or no effect on induced thermotolerance.

The mechanism by which HSPs confer thermotolerance is currently not understood. Mouse L cells transfected with a plasmid expressing high levels of hsp70 showed a more rapid recovery of nucleolar morphology following heat shock than did untransfected cells (333), suggesting that HSPs play a

role in reassembling proteins. Lewis and Pelham (254) later discovered that hsp70 was an ATP-binding protein which binds tightly to the nucleoli of mammalian cells and is released from these binding sites rapidly and specifically in vitro by as little as 1uM of ATP. Studies of hsp70 deletion mutants showed that the ability of HSPs to be released by ATP correlates with their ability to migrate to heat-shocked nucleoli and aid in their repair (255). Pelham (334) later speculated that HSPs utilized ATP hydrolysis to release HSPs from denatured proteins. The released protein would thus be able to refold into its pre-heat shock state; repeated cycles of this kind would result in full recovery of cellular functions. Minton et al. (297) proposed a model for the biological role of HSPs in which the HSPs caused a nonspecific stabilization of stress-susceptible proteins. They demonstrated experimentally that the addition of proteins that are themselves resistant to denaturation by heat or ethanol can nonspecifically stabilize other proteins that are ordinarily highly susceptible to thermal inactivation. Jinn et al. (219) demonstrated that an HSP-enriched fraction from heat shocked soybean seedlings, when added to an insoluble heat shock fraction, significantly protected the control proteins from heat denaturation.

HSPs have functions other than their proven role in growth at high temperatures (8, 72, 328) and their putative role in thermotolerance (235, 447). For example, they are involved in DNA replication (8), protein translocation into microsomes (64) and mitochondria (82), protein assembly in yeast mitochondria (63) and in *E. coli* (150) and the formation of complexes with unfolded, newly synthesized proteins in *E. coli* which may be essential for protein secretion (39). It is interesting to note that in all the

examples listed, HSPs have been shown to interact with other proteins. This idea is consistent with the proposal by Pelham (334) that HSPs bind to hydrophobic regions of unfolded proteins.

The function of one of the HSPs, the La protease, is thought to be the degradation of abnormal proteins (146, 305). The appearance of aberrant proteins may be a common signal under many adverse conditions for the induction of the La protease and other HSPs. Ananthan et al. (3) demonstrated that co-injection of various denatured, but not native proteins, along with a hsp70-ß gal hybrid gene, into frog oocytes resulted in the activation of the hsp70 gene. The fact that denatured proteins serve as a general signal for the activation of HSP synthesis would seem logical if the biological function of HSPs in thermotolerance is to prevent the denaturation of key heat-labile proteins.

Lee et al. (246) found that *S. typhimurium* and *E. coli* accumulated various adenylated nucleotides after both heat shock and oxidation stress. They proposed that these compounds were alarmones - i.e., regulatory molecules, alerting cells to the onset of oxidation stress. They suggested that oxidation stress and heat shock both result in the production of dinucleotides which signal the onset of these stresses and trigger the heat shock response. Bochner et al. (40) discovered that various oxidative stress agents, including H_2O_2, induce the synthesis of adenylated nucleotides. Hydrogen peroxide induced the synthesis of 30 proteins, nine of which were shown to be under the control of *oxyR*, a positive regulatory element. The mutant, oxyR1, overexpressed the nine oxyR-controlled proteins and was also more thermotolerant (65). Morgan et al. (303) demonstrated that, of the 30

proteins induced by H_2O_2, five were also induced by heat. The induction of one of these proteins was dependent on the positive regulator, *oxyR*. Oxidation stress is also mediated by $O_2^{\cdot -}$ production (423). Superoxide-mediated induction of the superoxide-inducible (SOI) proteins in the wild type was independent of the *oxyR*+ gene for all but the three *oxyR*-regulated proteins. Superoxide-mediated stress induced a unique group of proteins along with a small number of proteins that overlapped with other stress proteins. This suggested that the overlaps did not result from the generation of a common intracellular signal, but rather that the genes coding for the proteins that can be induced by several stresses contained cis-acting regulator sequences required for several forms of regulation. The work of VanBogelen et al. (411) showed that adenylated nucleotides were not capable of triggering the induction of all heat shock proteins, and thus were not alarmones (246). They concluded 1) the *rpoH*, SOS, and *oxyR* regulons can be separately induced, 2) some agents induce more than one regulon, 3) toxic oxidants and metals induce major responses in which the SOS, heat shock and oxidation stress regulons are minor components, and 4) adenylated nucleotides cannot be simple alarmones for the heat shock response.

Starvation for nitrogen, phosphate, or carbon when cultures enter the stationary phase all induce transcription of the bacterial DNA replication inhibitor microcin B17 and stimulate the synthesis of heat shock proteins (68). The stringent response is caused by a deficiency in amino acids. This response is clearly different from the heat shock response because the former induces HSPs in the *rpoH* 165 (defective sigma-32) mutant of *E. coli*,

whereas the latter fails to do so (166). It was concluded that the stringent response must stimulate transcription from heat shock promoters in a way that is different from stimulation during the heat shock response. Carbon-starvation of *E. coli* and *S. typhimurium* resulted in the synthesis of 30 proteins that were important in starvation survival (162). Some of these proteins were induced later in carbon-starvation and were Pex (postexponential) proteins (366). Jenkins et al. (218) demonstrated that subsets of the 30 glucose-starvation proteins were also synthesized during heat or H_2O_2 adaptation and that most of the common proteins were among the previously identified Pex proteins. Therefore, they concluded that, of the proteins common to starvation and heat shock, the eight that are Pex proteins are the most likely to be involved in conferring thermal resistance.

The induction of starvation response in *E. coli* is analogous to the induction of endospore formation in *Bacillus* sp. (262). In both instances induction: 1) is global and temporal in nature; 2) is triggered by nutrient starvation as the cells enter stationary phase; and 3) results in long term cell survival by providing resistance against various stress agents such as heat, oxidation stress, starvation stress, etc.

Heat inactivation of *Listeria monocytogenes* Conflicting results have appeared in the scientific literature concerning the heat resistance of *L. monocytogenes*. Bearns and Girard (28) reported in 1958 that *L. monocytogenes* survived the holding technique of pasteurization (61.7°C for 35 min.) whenever the initial number of cells added was 5 10^4/ml or greater. Donnelly et al. (91) compared their sealed tube method with the test tube method of Bearns and Girard (28). *L. monocytogenes* strains were

rapidly inactivated when survival was measured by using the sealed tube method. In contrast, total inactivation of *L. monocytogenes* populations measured by using the test tube method, could not be accomplished within 30 min. at 62°C. They concluded that the test tube method for measuring the thermal resistance of *L. monocytogenes* is inaccurate and that *L. monocytogenes* cells, dispersed freely in milk, will not survive pasteurization. Other authors used the sealed tube method and have reported that freely suspended *L. monocytogenes* would not survive HTST pasteurization of milk (43, 44, 51, 52) and various other dairy products (44, 29). Recently, Farber et al. (119), Lovett et al. (265), and Stroup et al. (394) determined that *L. monocytogenes* in artificially inoculated and naturally contaminated raw milk would not survive commercial HTST pasteurization. However, Doyle et al. (95) and Fernandez Garayzabal et al. (120, 121) reported that commercial HTST pasteurization did not completely destroy *L. monocytogenes* in artificial and naturally contaminated raw milk. Both Doyle et al. (95) and Fernandez Garayzabal et al. (120, 121) used liquid enrichment methods to detect viable *L. monocytogenes*. On the other hand, *Listeria monocytogenes* has not been detected immediately after pasteurization by any researchers when survival was detected by direct plating of the milk (43, 44, 51, 52, 95, 119, 120, 121, 265, 394).

Doyle et al. (95) and Fleming et al. (128) speculated that intracellular location within bovine phagocytes protected *L. monocytogenes* from heat inactivation. Bunning and co-workers demonstrated that location within mouse (51) or bovine (52) phagocytes did not significantly increase the heat resistance of *L. monocytogenes*. In the latter study, they detected no

difference in recovery between liquid or solid nonselective medium. Golden et al. (148) reported that a strain of *L. monocytogenes*, Brie -1, was consistently more heat-resistant than any strain reported; however, they concluded that even this strain would be inactivated by a standard pasteurization treatment if freely suspended in milk. Crawford et al. (74) admitted that in a previous study *L. monocytogenes* was detected in HTST milk, but only when using liquid nonselective media that were incubated at 25°C for 7 days. Most recently, Crawford et al. (75) reported a general increase in heat resistance as the time and temperature of heat shock increased; however, they concluded that the increase was not significant.

The thermal inactivation of *L. monocytogenes* has been studied in a variety of products, other than those mentioned previously. Karaioannaoglou and Xenos (226) reported that *L. monocytogenes* survived in grilled meat balls inoculated with 10^5 cells/g. Doyle et al. (98) found that a reduction of approximately 1 to 1.5 $\log_{10}$ *L. monocytogenes* occurred during spray drying of milk. The organism progressively died during storage at 25°C, a >4-$\log_{10}$ CFU/g decrease occurred within 16 weeks of storage. The rates of thermal inactivation of *L. monocytogenes* in clarified cabbage juice increased as pH was decreased from 5.6 to 4.0 (33). At 58°C (pH 5.6), 4 x 10^6 cells/ml were reduced to undetectable levels within 10 min. Glass and Doyle (143) demonstrated that *L. monocytogenes* would be inactivated in beaker sausage if the sausage was heated to an internal temperature of 62.8°C. Studies with pepperoni revealed that heating sausage at 51.7°C for 4 h after fermentation but before the drying cycle, killed most *L.. monocytogenes* however, the organism was occasionally

detected in samples during drying. Heating pepperoni at 51.7°C for 4 h after the drying cycle completely inactivated *L. monocytogenes* in all samples. Beuchat and Brackett (32) reported that initial *L. monocytogenes* populations of 3×10^5 CFU/g in ravioli were reduced to nondetectable levels after being boiled for 3, 5, and 7 min. When shrimp tails were inoculated with 10^4 - 10^5 *L. monocytogenes* cells/g and then submerged in boiling water for up to 5 min, survivors could be detected (288).

Numerous selective media have been evaluated for recovering heat-injured *L. monocytogenes*. When working with thermally processed foods, repair on a nonselective medium containing catalase was necessary prior to overlaying with ALPAMY (412). Golden et al. (147) evaluated selective, direct plating media for their suitability to recover heat-injured cells of four strains of *L. monocytogenes* from four foods. Modified Despierres agar and modified McBride agar yielded poorer recoveries of heat-injured cells than did McBride *Listeria* agar and gumbase-nalidixic acid-tryptone soya agar. They concluded that direct plating procedures can be successfully utilized for recovering healthy and injured *L. monocytogenes* from foods containing low populations of background microflora. Buchanan et al. (47) compared Vogel-Johnson Agar modified by the addition of tellurite (MVJ) with ARS-modified McBride Agar (ARS-MMA). MVJ provided a slightly higher rate of recovery of *L. monocytogenes* from food samples because the detection of *L. monocytogenes* is greatly improved in MVJ through the use of tellurite as a differentiating agent. Most recently, Crawford et al. (74) determined that any *L. monocytogenes* that survive HTST pasteurization are injured and unable to multiply either during cold storage of milk or in the FDA and USDA

recovery systems. They concluded that *L. monocytogenes* cells recovered in finished pasteurized milk products by these detection methods probably represented uninjured, environmental contaminants.

The ability of injured cells to regain pathogenicity has been reported. Sorrells et al. (388) found that mortality differences did not significantly differ between wholly uninjured and predominantly injured *Salmonella gallinarum* when they were injected into chicks. *Staphylococcus aureus* strain 36 regained its ability to synthesize normal levels of enterotoxin B after recovering from heat-injury (67). Likewise, *E. coli* fully regained its ability to produce heat-stable enterotoxin after recovery from copper-induced injury (382).

INHIBITION OF *Aspergillus flavus* AND SELECTED GRAM-POSITIVE BACTERIA BY CHELATION OF ESSENTIAL METAL CATIONS BY POLYPHOSPHATES

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ABSTRACT

A simple well-plate technique was utilized to determine the effect of various metals on the growth of microorganisms in media containing different polyphosphates. *Aspergillus flavus* and four Gram-positive bacteria were almost completely inhibited by media containing 1% of various alkaline polyphosphates, whereas four Gram-negative bacteria were not. Significant differences were observed among the type of polyphosphate added, the type of metal added, and the species of Gram-positive bacterium inhibited. The addition of Mg2+ stimulated growth of *Aspergillus flavus* and *Bacillus cereus* in the presence of tetrasodium pyrophosphate, whereas Mn2+ permitted growth of *Aspergillus flavus* and *Staphylococcus aureus* in the presence of sodium hexametaphosphate. Iron supplementation allowed the growth of *Staphylococcus aureus* and *Listeria monocytogenes* on media containing 1% tetrasodium pyrophosphate. A method for determining the amount of calcium and magnesium in water was modified to detect free Mg2+ by replacing EDTA with phosphate. The addition of free Mg2+, but not Mg2+ chelated by tetrasodium pyrophosphate, permitted the growth of *Bacillus cereus* on a medium containing tetrasodium pyrophosphate. It is speculated that polyphosphates specifically inhibit Gram-positive bacteria because they have higher affinities for essential metal cations than the cation-binding sites within Gram-positive cell walls.

INTRODUCTION

The use of polyphosphates as food additives has recently increased in the United States. This is due in part to changes in regulations permitting their use in a greater variety of meat products (42) and a need to reduce the level of NaCl used in meat processing (39). Polyphosphates are added to foods primarily because of their effects on functional properties, such as water binding, color, rancidity, texture, leavening, coagulation, emulsification, curing, and handling of foods (10). Little attention has been given to the antimicrobial effects of polyphosphates (reviewed in ref. 41). Most research that has been conducted on their antimicrobial effects has focused on optimizing botulinal protection (44) or increasing shelf life in processed cheese (34) and meats (39). Several mechanisms for the antimicrobial effects of polyphosphates have been proposed: chelation of essential cations, effect of pH, increase in ionic strength, interactions with cell walls, interactions with cell membranes, and inhibition of various transport functions (39). Elliott et al. (11) reported that microbial inhibition was due to phosphates sequestering cations. Although magnesium reversed the inhibition, Elliott et al. cautioned that the data did not warrant a conclusion as to which cations were of greatest importance. Polyphosphates have been reported to inhibit Gram-positive bacteria much more than Gram-negative bacteria (5, 30, 33, 47); the addition of Mg^{2+} overcomes the inhibitory effect (33). Supplementation of broth with Mg^{2+} was effective in overcoming inhibition of *Staphylococcus aureus* 196E by 0.5% sodium tripolyphosphate; inhibition was partly eliminated by Ca^{2+} and Fe^{2+}, but not by Zn^{2+} or Mn^{2+}

(21). The superior ability of Mg^{2+} to restore growth was believed related to a higher stability constant of the polyphosphate with Mg^{2+} than with other cations (18).

The questions addressed in the present study were: what is the antimicrobial effect of polyphosphates and why are Gram-positive bacteria more susceptible than Gram-negative bacteria? Answers to these questions would allow us to maximize the antimicrobial effect of polyphosphates and might provide some insight into the differences between these two fundamental groups of bacteria.

MATERIALS AND METHODS

Cultures

Aspergillus flavus (originally from Dr. John Tuite, Department of Plant Pathology, Purdue University, West Lafayette, Ind.), *Salmonella typhimurium* ATCC 13311, *Yersinia enterocolitica* ATCC 23715, *Aeromonas hydrophila* ATCC 7965, *Pseudomonas fluorescens* ATCC 949, *Bacillus cereus* T03, and *Staphylococcus aureus* Z88 were obtained from the culture collection in the Department of Food Technology at Iowa State University. *Listeria monocytogenes* strain Scott A was obtained from Dr. Elmer Marth, Department of Food Science, University of Wisconsin, Madison. *Lactobacillus* strain L-1 was isolated from spoiled, vacuum-packaged wieners obtained from a local supermarket.

All bacterial cultures were streaked for purity on brain heart infusion agar (BHI, Difco Laboratories, Detroit, Mich.), and their identification was confirmed by their Gram-stain reaction and appropriate biochemical tests. All Gram-negative cultures were identified by their reactions on API 20E strips (Analtab Products, Plainview, N. Y.). Fluorescent pigment production by *Pseudomonas fluorescens* was confirmed by streaking the organism on Flo Agar (BBL, Cockeysville, Md). The identities of all Gram-positive bacteria were confirmed by morphology and hemolytic reaction on blood agar, growth on *Lactobacillus* Selective (LBS) agar (BBL), color of colonies on tryptose agar (Difco Laboratories), growth in Trypticase Soy Broth (BBL) containing 10% NaCl, production of catalase, formation of spores, motility at 25°C, and growth at 4°C. Stock cultures of bacteria were maintained by

monthly subculture on BHI agar slants. Stock cultures of *A. flavus* were maintained by monthly subculture on Sabouraud dextrose agar (SDA, Difco Laboratories) slants. *Lactobacillus* L-1 was grown on MRS (Difco Laboratories) agar slants at 30° C for 24 h. All cultures were incubated at 30°C, and stock cultures were stored at 4°C. Inocula of *A. flavus* spores were prepared by suspending the surface growth of a 5-day slant culture in sterile distilled water. Bacterial inocula were prepared by growing the microorganisms in BHI (or MRS broth for *Lactobacillus* L-1) for 18 h at 30°C. The growth characteristics of *A. flavus* were observed by using the brightfield, 10x objective of an Olympus BH-2 microscope (Olympus Optical Co., Tokyo).

Media

All media and metal solutions were prepared by using water that had passed through a 2-stage resin-bed deionizer and distillation apparatus (Barnstead-Millipore Corp., Boston, Mass.). Well plates were prepared as follows: i) 10% (w/vol) solutions of the following reagent grade monophosphate and commercial grade polyphosphates (BK Ladenburg Corp., Cresskill, N. J.) were prepared in distilled water at room temperature and then sterilized by filtration through a 0.22-um membrane filter (Gelman Instrument Co., Ann Arbor, Mich.): monosodium phosphate (MSP) (Fisher Scientific Co., Fairlawn, N. J.), tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP, n = 19), and sodium acid pyrophosphate (SAPP); ii) appropriate portions of the filtrates were aseptically added to bottles of sterile, melted SDA or BHI agar

(previously tempered to 45° C) to achieve a final polyphosphate concentration (w/vol) of 1.0% (0.5% for STPP); iii) the polyphosphate solution was mixed into the agar by gently inverting the bottle several times, and the contents were then poured into sterile petri plates (ca 20 ml per plate); iv) after the plates had dried overnight, wells (1 cm in diam) were cut in the agar by using a sterile, size-6, stainless-steel cork borer. The pH of each medium was determined by using a Beckman Zeromatic SS-3 pH meter (Beckman Scientific Co., Palo Alto, Cal.) equipped with Fisher pH electrode No. 13-620-104A (Fisher Scientific Co.). Each isolate was inoculated onto the well plates by dragging one loopful of the inoculum from the outer edge of the petri plate toward the well, stopping approximately 0.5 cm from the edge of the well.

Metals

Solutions (0.1M) of the following reagent grade metals (Fisher Scientific Co.) were sterilized in an autoclave before use: $FeCl_3.6H_2O$, $MgCl_2.6H_2O$, $MnCl_2.4H_2O$, $CaCl_2.2H_2O$, $ZnCl_2$, $NiCl_2.6H_2O$, $(NH_4)_6\ Mo_7O_{24}.4H_2O$, $CuCl_2.2H_2O$, $CoCl_2.6H_2O$. Some of the Fe^{3+} formed a red precipitate upon sterilization; this was resuspended by shaking before the Fe^{3+} solution was added to the wells. A portion (0.1 ml) of the appropriate metal solution was added to the well, and the plate was incubated upright at 30°C.

In one experiment, a sterile 1M solution of $MgCl_2.6H_2O$ was serially diluted in sterile distilled water and in sterile distilled water containing 10% (w/vol) TSPP. Both diluents contained 0.0005% filter-sterilized Calmagite (1-[1-hydroxy-4 methyl-2.phenylozo]-2-naphthol-4-sulfonic acid) (Sigma

Chemical Co. St. Louis, Mo.) to detect the presence of free Mg^{2+} (26). Portions (0.1 ml) of each dilution were added to well plates containing 1% (w/vol) TSPP. Well plates had been previously inoculated by swabbing the surface of the agar with an 18-h culture of *B. cereus* T03.

Titration of Mg^{2+}

The amount of a particular phosphate required to bind all the free Mg^{2+} in solution was determined by modifying a procedure for the determination of calcium plus magnesium in water (7). In place of EDTA, a 10% solution of each phosphate (1.0% for MSP and SAPP, because of their low solubility in water) was prepared in distilled water and used to titrate 50 ml of a 0.01M solution of $MgCl_2$. The titration was carried out at pH 10; the end point was reached when the metallochromic indicator, Calmagite, changed from red to blue. The addition of masking agents proved unnecessary because the polyphosphates did not contain significant quantities of interfering ions.

RESULTS

The results of the titration of Mg^{2+} by the various phosphates are listed in Table 1. All the phosphates bound substantial quantities of Mg^{2+}. When monosodium phosphate was use to titrate Mg^{2+}, a white precipitate formed. In contrast, when polyphosphates were used, the solutions remained clear. Monosodium phosphate had little or no inhibitory effect on *A. flavus* (Fig.1) or on any of the bacteria tested (data not shown). The addition of Mg^{2+}, but not Mn^{2+}, to the well in the agar plate (Fig. 1) was effective in overcoming the inhibition of *A. flavus* on SDA + TSPP. This pattern was reversed for SDA + SHMP; Mn^{2+}, but not Mg^{2+}, permitted growth. Microscopic observation of the mold growth on SDA containing polyphosphates revealed that the spores formed short germ tubes, but subsequent mycelial development was inhibited. The addition of Mg^{2+} and Mn^{2+} allowed normal mycelial development and sporulation around the well, but the amount of growth and sporulation decreased significantly with distance from the well (Fig. 1).

The growth of the Gram-negative bacteria was only slightly inhibited by the polyphosphates (data not shown), but the growth of the Gram-positive bacteria was dramatically inhibited by all polyphosphates except SAPP (Fig. 2). The addition of Mg^{2+} permitted the growth of *B. cereus* T03 on all media containing polyphosphates, while the growth of *S. aureus* Z88 remained inhibited (Figs. 2 and 3). The combined addition of Mg^{2+} and Fe^{3+} to well plates containing TSPP and STPP enhanced the growth of *B. cereus* and also allowed the growth of *S. aureus* (Fig. 2). After dilution of Mg^{2+} in10% TSPP to a concentration of 0.04M, it was no longer effective in permitting the

growth of *B. cereus* on BHI + TSPP, in contrast to the same concentration of Mg^{2+} diluted in distilled water (Fig. 4). The separate addition of Mn^{2+} and Ca^{2+} to well plates containing BHI + TSPP did not permit growth of *B. cereus* and *S. aureus* even when high levels of these metals were added to the wells (Fig. 3). Growth of both microorganisms increased, however, when increasing levels of Fe^{3+} were added. The addition of Fe^{3+} to well plates containing BHI + SAPP resulted in better growth of *S. aureus* (Fig. 2). The addition of Fe^{3+} to well plates containing BHI + TSPP stimulated the growth of *L. monocytogenes* and inhibited the growth of *S. typhimurium* (Fig. 5), but no inhibition of *S. typhimurium* was observed when Fe^{3+} was added to BHI not containing polyphosphate (data not shown).

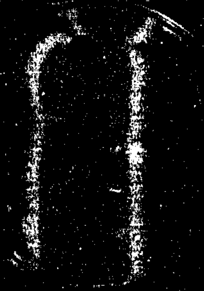
In general, for all polyphosphates and all microorganisms tested, the most inhibitory polyphosphate was STPP. Inhibition was best overcome when Mg^{2+}, Mn^{2+}, and Fe^{3+} were added in combination (Fig. 6). None of the other metals showed any ability to stimulate growth except Ca^{2+}, which was required to achieve maximum growth of *B. cereus* and *S. aureus* on BHI + STPP (data not shown). Metals that inhibited the growth of all microorganisms near the well when 0.1 ml of a 0.1M solution was added were Cu^{2+}, Co^{2+}, Ni^{2+}, Mo^{2+}, and Zn^{2+}. When BHI and SDA plates containing 1% EDTA were used in place of polyphosphate-containing plates, similar tapered-growth patterns of *A. flavus* and *S. aureus* were observed around wells containing a combination of Mg^{2+}, Mn^{2+}, and Fe^{3+} (data not shown). Media containing EDTA were much more inhibitory than media containing polyphosphates.

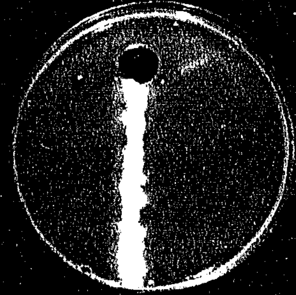
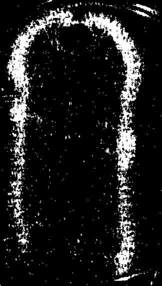
TABLE 1. Quantity of phosphate required to titrate 5 mmoles of $MgCl_2$

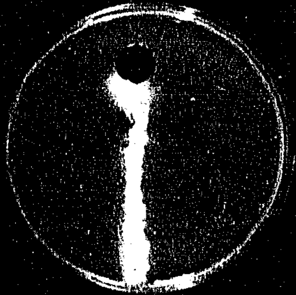
Type of phosphate	ml of phosphate[a]
Monosodium phosphate (MSP)	6.7
Tetrasodium pyrophosphate (TSPP)	4.5
Sodium acid pyrophosphate (SAPP)	5.0
Sodium tripolyphosphate (STPP)	4.8
Sodium hexametaphosphate (SHMP)	10.0

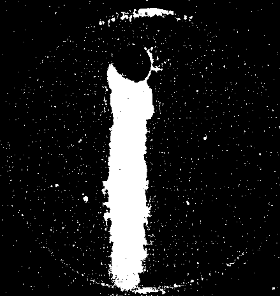
[a] ml of a 10% solution of phosphate in distilled water, determined by the method reported in (6), modified (see text). Results for 1.0% solutions of MSP and SAPP were converted to a 10% basis by dividing the volume of 1% phosphate by 10. Each value is the average of three titrations.

FIG. 1. Effects of Mg^{2+} and Mn^{2+} on the growth of *Aspergillus flavus* on Sabouraud dextrose agar containing monosodium phosphate (MSP), tetrasodium pyrophosphate (TSPP), and sodium hexametaphosphate (SHMP). Each well contained 0.1 ml. of a 0.1M solution of metal. Control, SDA + H_2O







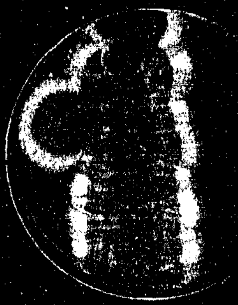

MSP
TSPP
SHMP
H_2O
Mg
Mn
CONTROL

FIG. 2. Effects of various combinations of Mg^{2+}, Mn^{2+}, and Fe^{3+} on the growth of *Bacillus cereus* and *Staphylococcus aureus* on brain heart infusion agar containing tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium acid pyrophosphate (SAPP), and sodium hexametaphosphate (SHMP). On the left side of the plate, *Bacillus cereus* T03; on the right, *Staphylococcus aureus* Z88. A and B, Mg^{2+} + Fe^{3+}; C, Fe^{3+}; D, Mg^{2+} + Mn^{2+}; each well contained a total volume of 0.2 ml. Control, BHI + H_2O

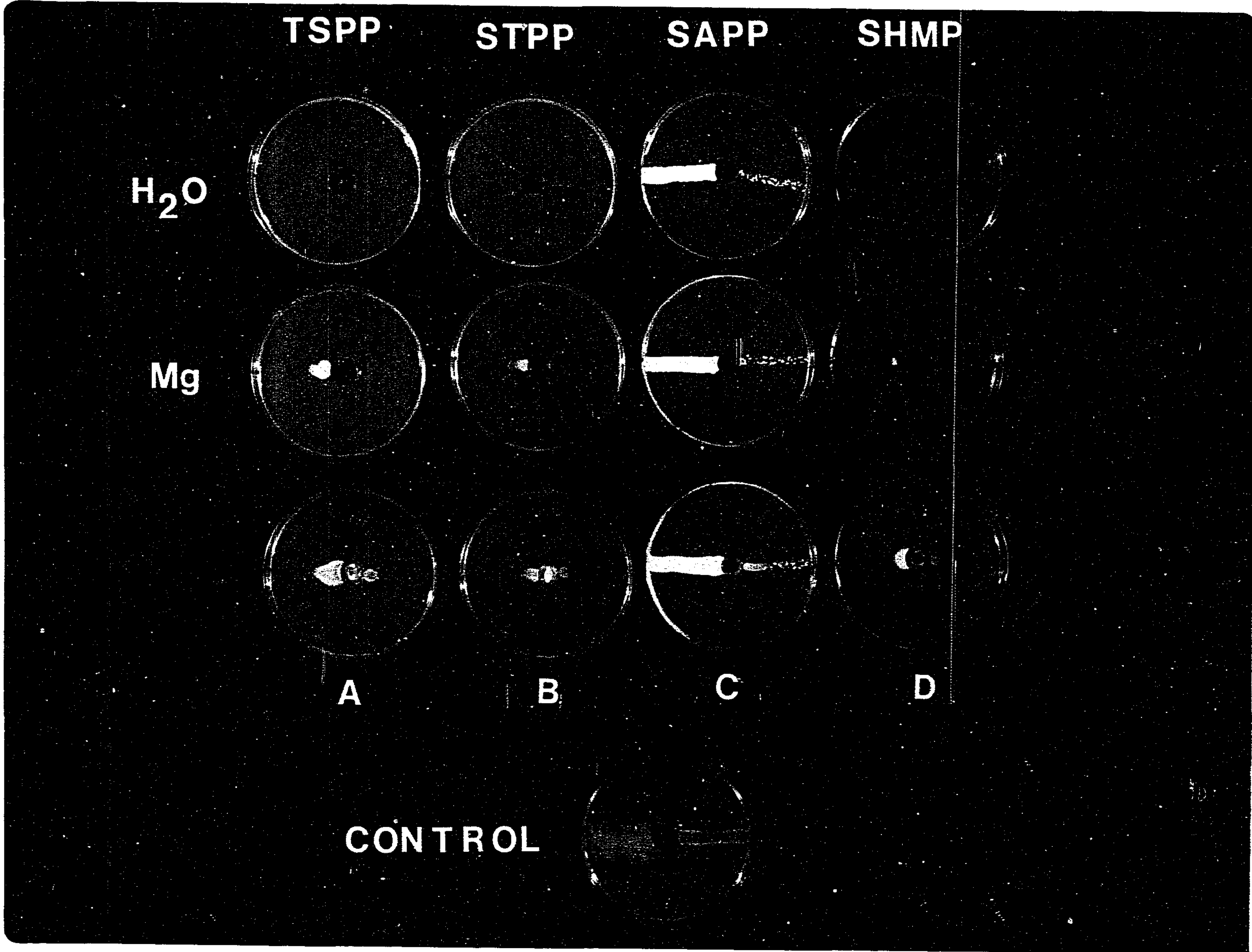
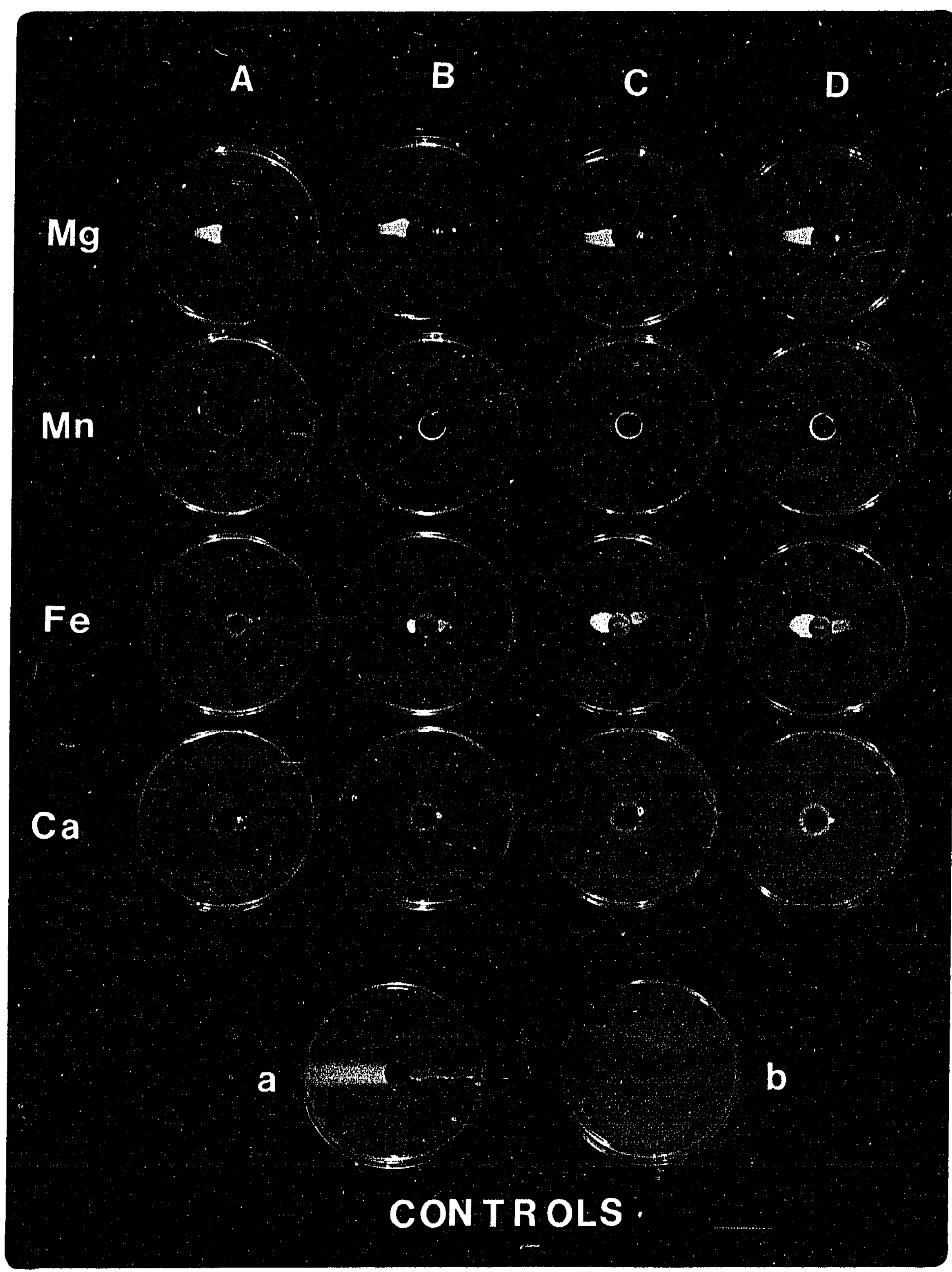

TSPP
STPP
SAPP
SHMP
H_2O
Mg
A
B
C
D
CONTROL

FIG. 3. Effects of increasing levels of Mg^{2+}, Mn^{2+}, Fe^{3+}, and Ca^{2+} on the growth of *Bacillus cereus* and *Staphylococcus aureus* on brain heart infusion agar containing 1% tetrasodium pyrophosphate (TSPP). On the left side of the plate, *Bacillus cereus* T03; on the right, *Staphylococcus aureus* Z88. Volume of 0.1M metal: A, 0.1 ml; B, 0.2 ml.; C, 0.3 ml; D, 0.4 ml. Controls; a, BHI + H_2O; b, BHI + 1% TSPP



A
B
C
D
Mg
Mn
Fe
Ca
a
b
CONTROLS

FIG. 4. The effects of free and chelated Mg^{2+} on the growth of *Bacillus cereus* on brain heart infusion agar containing 1% tetrasodium pyrophosphate (TSPP). Concentration of Mg^{2+}: 1, 1M; 2, 0.2M; 3, 0.04M; 4, 0.008M. A, Mg^{2+} diluted in sterile distilled water; B, Mg^{2+} diluted in 10% TSPP. Color of Calmagite (metallochromic indicator) in diluent: 1A - 4A, all pink (free Mg^{2+} present); 1B and 2B, pink (free Mg^{2+} present); 3B and 4B, blue (free Mg^{2+} not present). Controls; a, BHI + H_2O; b, BHI + 1% TSPP + H_2O

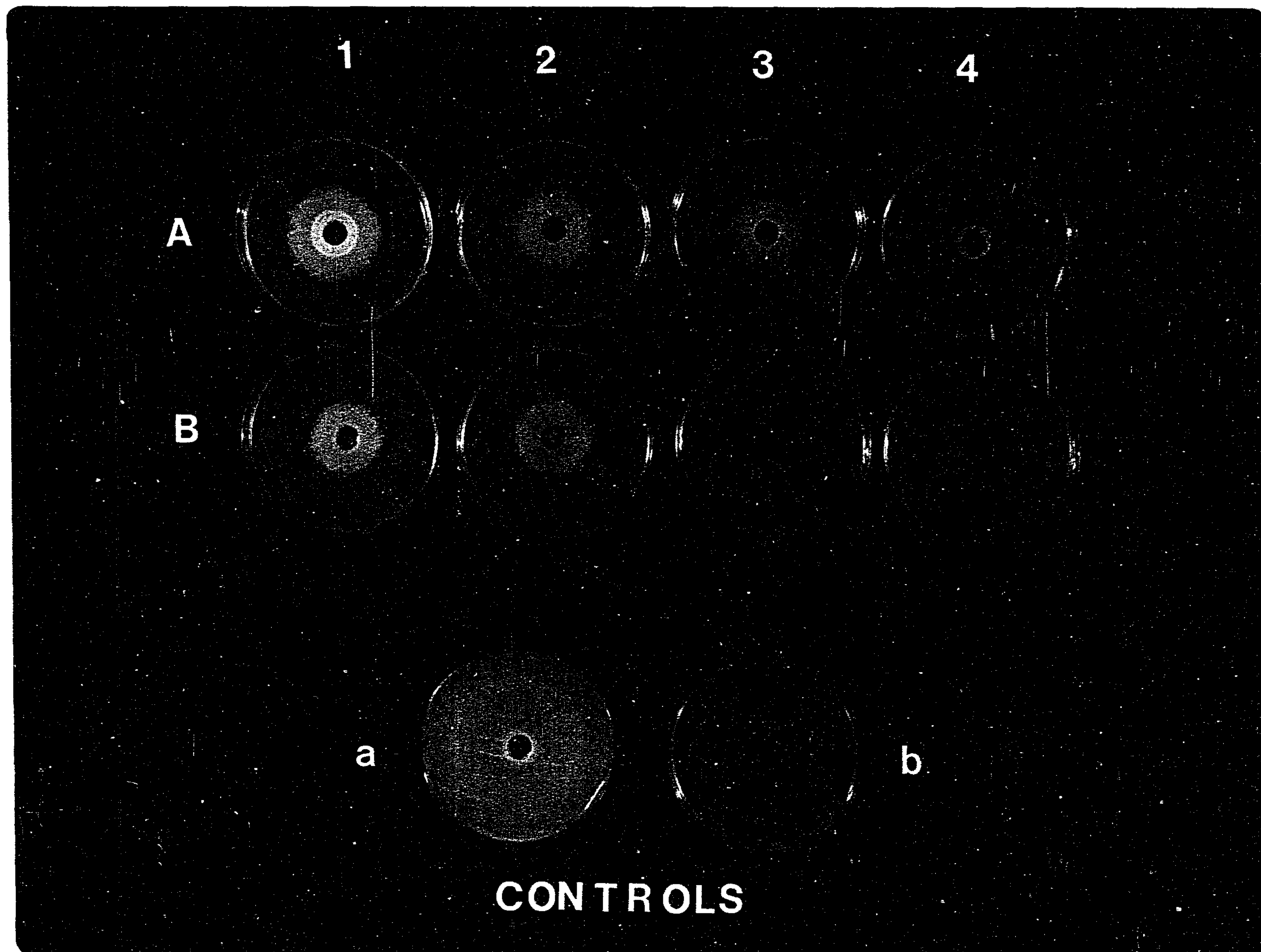
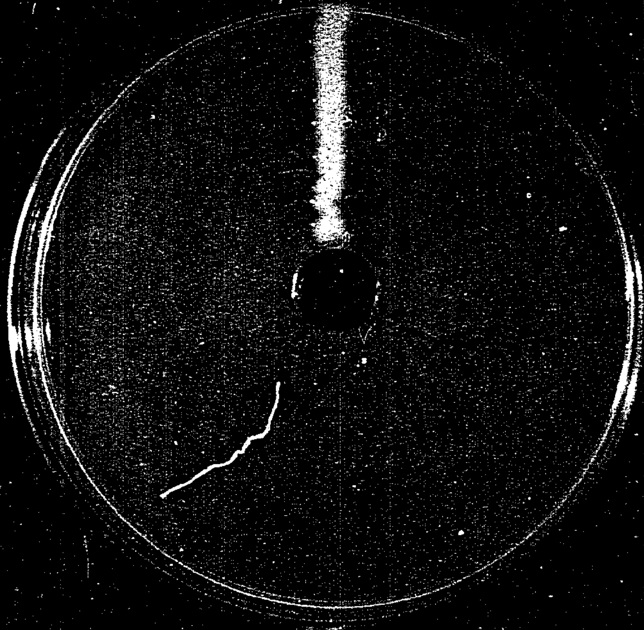

1
2
3
4
A
B
a
b
CONTROLS

FIG. 5. Effects of Fe^{3+} on the growth of *Salmonella typhimurium* ATCC 13311 and *Listeria monocytogenes* strain Scott A on brain heart infusion agar (BHI) containing 1% tetrasodium pyrophosphate (TSPP). On left BHI + 1% TSPP + 0.1 ml H_2O; on right, BHI + 1% TSPP + 0.1 ml of 0.1M Fe^{3+}. Vertical streak, *Salmonella typhimurium*; horizontal streak, *Listeria monocytogenes*

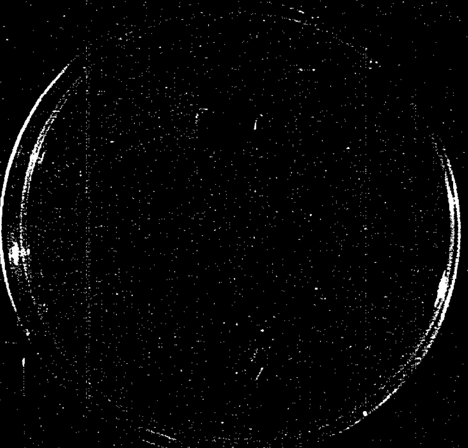
H_2O



Fe

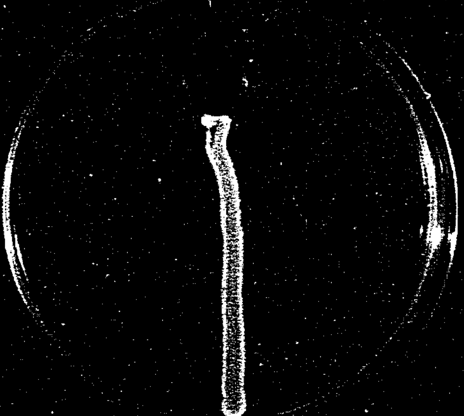
FIG. 6. Effect of Mg^{2+}, Mn^{2+}, and Fe^{3+} on the growth of *Lactobacillus* L-1 on MRS agar. A, + 0.5% STPP + H_2O; B, + 0.5% STPP + Mg^{2+}, Mn^{2+}, Fe^{3+} (0.1 ml of 0.1M solution of each metal); C, No STPP + H_2O

A



B

C



DISCUSSION

The titration results (Table 1) demonstrated that polyphosphates chelated free Mg^{2+} and removed it from the metallochromic indicator. The titrations were not stoichiometric, probably because the affinity of most polyphosphates for Mg^{2+} does not greatly exceed that of Calmagite (pK_D = 5.69 at pH 10.0) (26). Therefore, polyphosphates must be in excess to completely remove Mg^{2+} from the indicator. Also, the actual molecular weight of commercial polyphosphates varies significantly, so the actual number of reactive sites per molecule cannot be determined. The results indicate, however, that TSPP and STPP can chelate more than twice the amount of Mg^{2+} as SHMP, on a weight basis, which agrees with the conclusions of Irani and Callis (18). Calmagite was useful for indicating the absence of free Mg^{2+} when Mg^{2+} was serially diluted in solutions of TSPP (Fig. 4).

In the present study, the use of well plates permitted the manipulation of three important variables: type of polyphosphate, type of metal cation, and type of micoorganism. Thus, numerous combinations of variables could be tested rapidly for their effects on microbial growth.

The inability of orthophophates to inhibit any of the microorganisms tested in the present study should be of concern to any food industry because foods may contain phosphatases (2) capable of hydrolyzing polyphosphates into ineffective orthophosphates (19). The lack of significant inhibition of *S. aureus*, and especially *B. cereus*, on BHI + SAPP (Fig. 2) probably is caused by protonation of the O^--binding sites in this acidic

polyphosphate at low pH (6.0), which would have decreased the ability of SAPP to chelate metal ions such as Mg^{2+} (18). This contrasts with SAPP's ability to chelate significant quantities of Mg^{2+} at pH 10 (Table 1). Media containing alkaline polyphosphates had a higher pH (7.8) and greater antimicrobial effectiveness (Fig. 2). This agrees with previous research which demonstrated that 0.5% STPP did not inhibit growth of *S. aureus* 196E in BHI broth adjusted to pH values below 6.0, but growth was inhibited at pH 7.0, and cell numbers declined at pH 7.4 and above (21).

The tapered pattern of growth around wells containing various metals (all figures) is consistent with the hypothesis that polyphosphates inhibit microorganisms by chelating metal cations essential for their growth (21). Liu and Shokrani (27) observed a similar tapering-growth pattern with serum-sensitive strains of *Pseudomonas aeruginosa* on rabbit serum agar around a well containing a 0.01% solution of $FeCl_3$. They attributed this growth pattern to the inability of serum-sensitive (siderophore-deficient) strains to acquire Fe^{3+} that had been chelated by the transferrin present in the serum agar. Siderophores specifically chelate Fe^{3+} with great affinity ($pK_D = 32$) (32), but polyphosphates are less efficient chelators of a wider variety of metal cations (pK_Ds ranging from 2 to 23), depending on the type of metal and its valence state (38). This explains the different reactions of the various microorganisms when Mg^{2+} and Mn^{2+} were added to media containing different polyphosphates (Figs. 1 and 2). The low solubility of Fe^{3+} in water, in contrast to Mg^{2+}, limited the amount of Fe^{3+} that diffused into the medium surrounding the wells (Figs. 2 and 3), which tended to

underestimate the effect of Fe^{3+} on the growth of various microorganisms in media containing polyphosphates.

Sofos (39) stated that heavy-metal cations are sequestered best by shorter-chain phosphates. Our data indicate that, although this seems to be true for the chelation of Fe^{3+} by TSPP (Fig. 2), SHMP ($n = 19$) seemed to be a superior chelator of Mn^{2+} (Figs. 1 and 2). Jones et al. (23) demonstrated that tetrametaphosphate possessed a greater affinity for Mn^{2+} ($pK_D = 5.74$) than the smaller trimetaphosphate ($pK_D = 3.57$). In addition, Jones and Monk (22) found that tetrametaphosphate has a greater affinity for Mn^{2+} than for Mg^{2+}. Therefore, the addition of Mn^{2+}, an essential metal cation (43), to media containing a long-chain polyphosphate (i.e., SHMP), may have resulted in the release of additional essential metal ions, such as Mg^{2+} and possibly Fe^{3+}, which explains the growth of *A. flavus*, *S. aureus*, and *B. cereus* on SHMP well plates containing Mn^{2+} (Figs. 1 and 2).

Long-chain polyphosphates have been descibed as the best sequestering agents for light-metal cations such as Ca^{2+} and Mg^{2+} (39). However, Irani and Callis (18) stated that, on a weight basis, short-chain polyphosphates (TSPP and STPP) were more effective because the thermodynamic stabilities of short- and long-chain polyphosphates are not much different (17), whereas the molecular weights of TSPP and STPP are smaller. This is not a trivial distinction, because current legal limits for the addition of polyphosphates to foods are on a percentage (weight) basis (42).

Jen and Shelef (21) reported that *Staphylococcus aureus* 196E was not inhibited in BHI broth containing 0.5% TSPP, but in our study, inhibition was observed in BHI agar + 1% TSPP. This difference may be due to the use of a

higher level of polyphosphate (1%), or perhaps *S. aureus* 196E has a stronger affinity for Mg^{2+} or Fe^{3+} than either 0.5% TSPP or *S. aureus* Z88. Marcelis et al. (28) observed that not all strains of *S. aureus* were able to remove Fe^{3+} from ethylene diamine diorthohydroxylphenyl acetate (EDDA), a synthetic iron chelator.

The results of our study indicate that STPP is very inhibitory toward Gram-positive bacteria in general, not just *S. aureus* 196E (21). Because STPP chelates a wide variety of metals with high affinity (18, 19, 24), Gram-positive bacteria required the addition of Mg^{2+}, Mn^{2+}, and Fe^{3+} before significant growth occurred on BHI + STPP (data not shown and Fig. 5). This agrees with the high association constants for the interaction of STPP with a variety of metal cations (24). Because lactobacilli are one of the few groups of microorganisms that do not show a requirement for Fe (32), the increased growth seen upon the addition of this metal probably was a result of the ability of Fe^{3+} to release essential metal cations from STPP ($pK_a = 23$) (19) rather than its ability to provide a direct benefit to the organism.

Magnesium is an essential metal cation responsible for membrane integrity (16), cell division (45), and many membrane-bound enzyme activities (15). *Bacillus cereus* demonstrated good growth around wells containing Mg^{2+} on all media containing polyphosphates (Figs. 2, 3 and 4). The addition of free Mg^{2+}, but not Mg^{2+} chelated by TSPP, permitted the growth of *B. cereus* on BHI + TSPP (Fig. 4). This finding is further proof that polyphosphates inhibit Gram-positive bacteria by the chelation of metal cations. Aerobic microorganisms, such as *Bacillus* spp, have a high requirement for Fe^{3+} (32), which is sequestered much more efficiently (pK_D =

23) (19) by TSPP than Mg^{2+} (pK_D = 5.4) (17). Therefore, why did *B. cereus* grow on TSPP well plates containing Mg^{2+}? This phenomenon may be explained by the ability of *B. cereus* to synthesize schizokinen (4) or 2, 3-dihydroxybenzoate (8), hydroxamate siderophores produced by members of the genus *Bacillus*, which are capable of binding Fe^{3+} with great affinity and transporting it into the cell (32). Because siderophores are specific for the acquisition of Fe^{3+} (32), the addition of Mg^{2+} was still required to allow the growth of *B. cereus* on a Mg^{2+}-deficient medium containing TSPP (Figs. 1 and 2). The failure of *S. aureus* Z88 to grow around wells containing only Mg^{2+} may reflect its inability to acquire iron via a siderophore (28). In as much as TSPP has a higher affinity for Fe^{3+} (19) than for Mg^{2+} (17), the addition of high levels of Fe^{3+} would have released free Mg^{2+}. This explains the growth-stimulating effect of of Fe^{3+} on *S. aureus* inoculated onto BHI + SAPP (Fig. 2).

Wagner and Busta (44) reported that SAPP inhibits the activity of a protease responsible for toxin production in *Clostridium botulinum* 52A, resulting in delayed toxin production. They suggested that this might be caused by the chelation by SAPP of a metal cation required for protease activity. The results in Fig. 2 suggest that the addition of Fe^{3+} might release this putative cation; further research is needed in this area. The release of essential metals by the addition of Fe^{3+} also explains the growth of both *B. cereus* and *S. aureus* around TSPP well plates containing high levels of Fe^{3+} (Fig. 3). These observations are consistent with the hypothesis of Jen and Shelef (21) that a metal cation with a high stability constant for a particular phosphate may cause the release of essential chelated cations with lower

stability constants so that they are available. However, Mg^{2+} does not have superior affinity for most polyphosphates (38) as they previously suggested (21) and therefore would not release some essential metals. This is supported by the ability of Mn^{2+}, but not Mg^{2+}, to stimulate the growth of *A. flavus* on SDA + 1% SHMP (Fig. 1) and the ability of Fe^{3+}, but not Mg^{2+}, to stimulate the growth of both *S. aureus* and *B. cereus* on BHI + 1% TSPP (Fig. 3). Jen and Shelef (21) reported that the addition of Fe^{2+}, while effective at higher concentrations, was not as effective as Mg^{2+} at lower concentrations in stimulating the growth of *S. aureus* 196E in BHI + 0.5% STPP. This can be attributed to their use of Fe^{2+}, which has a much lower affinity for STPP than Fe^{3+} (19), the form of iron typically present in aerobic environments (32) and that used in the present study (Figs. 3, 5, and 6).

Listeria monocytogenes strain Scott A grew better than any of the other Gram-positive bacteria tested on media containing polyphosphates. This bacterium produces high levels of alkaline phosphatase (37), which might have hydrolyzed the polyphosphates into ineffective orthophosphates (18) (Fig. 1). Cowart and Foster (6) were unable to detect siderophore-mediated Fe^{3+} transport in *L. monocytogenes*; however, they discovered that the organism can acquire Fe^{3+} by secreting a soluble reductant that effectively removes iron from transferrin. This mechanism, if operative, was not effective in our study because *L. monocytogenes* grew only on media containing polyphosphates with added Fe^{3+} (Fig. 6). The stimulatory effect of Fe^{3+} on the growth of *L. monocytogenes* contrasted with its inhibitory effect on *S. typhimurium* in BHI + TSPP (Fig. 6). Interestingly, *S. typhimurium* was not inhibited by Fe^{3+} on BHI agar that did not contain

polyphosphates. Perhaps the polyphosphate-Fe^{3+} chelate complex inhibited *S. typhimurium* by blocking siderophore receptors on the surface of the cell, which prevented Fe^{3+} transport (35).

Although a hypothesis was proposed to explain how Mg^{2+} can release other metals and make them available for growth (21), no satisfactory model has yet been proposed to explain why Gram-positive bacteria are inhibited to a greater extent than Gram-negative bacteria by polyphosphates. We believe that polyphosphates inhibit Gram-positive bacteria by removing essential metal cations from unique binding sites within their cell walls (see ref. 9 for a current review).

The cell walls of most Gram-positive bacteria (9), but not Gram-negative bacteria (13), consist of a thick layer of peptidoglycan and usually large amounts of teichoic and/or teichuronic acids, which serve as major sites for binding metals, such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{3+} (2). The major function of these anionic polymers in the cell wall is to maintain a high concentration of bivalent cations in the region of the membrane (15), which provides the correct ionic environment for cation-dependent membrane systems (16). The requirement for Mg^{2+} seems to be especially rigid because Gram-positive bacteria synthesize more teichoic acids when in an environment containing low levels of Mg^{2+} and shift to producing Mg^{2+}-binding teichuronic acids when phosphate (a necessary precursor for teichoic acid synthesis) is in short supply (12). Because polyphosphates possess higher affinities (17, 18, 19, 24) than either peptidoglycan (29) or teichoic and teichuronic acids (13, 25) for metal cations, they can remove these essential metals from the cell walls of Gram-positive bacteria and inhibit their growth

(all figures). In contrast, Gram-negative bacteria can grow in the presence of polyphosphates because they possess higher affinity systems for binding (13, 40, 46) and transporting (20) essential metal cations. Fungal cell walls also possess anionic polymers such as chitin, chitosan, and glycoproteins (3), which are involved in the passive uptake of metals by fungi (36). Chitin and chitosan are known metal chelators, the latter polymer being used in column chromatography to extract metal cations from aqueous solutions (31). Therefore, the inhibition of *A. flavus* observed in the present study may also be due to the ability of polyphosphates to remove metal cations from unique cation-binding sites in the fungal cell wall.

ACKNOWLEDGMENTS

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EFFECT OF GROWTH TEMPERATURE AND STRICTLY ANAEROBIC RECOVERY ON THE SURVIVAL OF *Listeria monocytogenes* DURING PASTEURIZATION

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ABSTRACT

Listeria monocytogenes F5069 was suspended in either Trypticase soy-0.6% yeast extract broth (TSBYE) or sterile, whole milk and heated at 62.8°C in sealed thermal death time tubes. Severely heat-injured cells were recovered in TSBYE within sealed thermal death time tubes because of the formation of reduced conditions in the depths of the TSBYE. The use of anaerobic "Hungate" techniques resulted in efficient enumeration of injured cells in Trypticase soy yeast extract broth containing 1.5% (w/v) agar (TSBYEA), compared with aerobically incubated controls. The exogenous addition of catalase, but not superoxide dismutase, slightly increased the recovery of heat-injured cells in TSBYEA incubated aerobically. Growth of cells at 43°C caused a greater increase in heat resistance as compared with cells heat shocked at 43°C or cells grown at lower temperatures. Growth of *L. monocytogenes* at 43°C and enumeration by the use of strictly anaerobic "Hungate" techniques, resulted in D62.8°C values that were approximately 6 times greater than those reported recently which were obtained by using cells grown at 37°C and aerobic plating. Results indicate that, under the conditions of the present study, low levels of *L. monocytogenes* would survive the minimum high-temperature, short-time treatment required by the U.S. Food and Drug Administration for pasteurizing milk. The direct enumeration of other injured facultative pathogens by recovery in selective media under strictly anaerobic conditions is discussed.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, catalase-positive, aerobic-to-facultatively anaerobic bacterium that has recently been implicated in several fatal foodborne disease outbreaks (26, 39, 58). Because the organism is present in raw milk (6, 26, 29, 46), can grow well in foods at refrigeration temperatures (8, 18, 57), and is a potentially lethal pathogen (46), considerable emphasis has been placed on ensuring its complete destruction during pasteurization.

Conflicting reports have appeared on the ability of *L. monocytogenes* to survive the minimum High-Temperature Short-Time (HTST) pasteurization processing guidelines (71.7°C for 15 s) of the Food and Drug Administration (27). Bearns and Girard (5) were the first to report that *L. monocytogenes* was able to survive pasteurization; however, Donnelly et al. (19) determined that the method used by the previous authors could have resulted in an overestimation of the heat resistance of the organism. Numerous studies have indicated that *L. monocytogenes* would not survive the minimum HTST treatment given fluid milk (6, 10, 11, 13, 14, 18, 19, 31). However, a few workers have reported that *L. monocytogenes* in naturally and artificially contaminated raw milk can survive the minimum HTST process (20, 24, 25). Although viable *L. monocytogenes* could not be detected by direct plating immediately after pasteurization, they were detected after a period of liquid enrichment. Fleming et al. (26) and Doyle et al. (20) postulated that the ability of *L. monocytogenes* to survive pasteurization was due to a thermal protective effect provided by an intracellular location within

polymorphonuclear leukocytes (PMNL). Bunning et al. (13, 14) determined that intracellular location within bovine phagocytes did not significantly increase heat resistance. In addition, they did not obtain a significant difference between the direct plate and liquid enrichment methods in detecting positive samples (14). Incubation at 25°C resulted in the detection of more organisms at higher heating times, and a potentially unsafe HTST process of 3.7 D was indicated (14). Bunning et al. (14) concluded, however, that commercial pasteurization would completely inactivate the low levels of *L. monocytogenes* found in raw milk. Golden et al. (31) reported that different strains of *L. monocytogenes* differed in heat resistance. One strain, Brie-1, was significantly more heat resistant than previously reported, but the authors still believed that it was unlikely that this organism would survive pasteurization if freely suspended in milk. Most recently, in joint studies between the FDA and USDA, viable cells were not detected in milk that had been contaminated both in vitro and in vivo with *L. monocytogenes* and then subjected to the minimum HTST pasteurization process (32).

Many factors influence the heat resistance of nonspore-forming microorganisms (34). Time and temperature of incubation dramatically affect the heat resistance of both Gram-negative (22) and Gram-positive bacteria (38, 63). Beuchat (7) speculated that growth at high temperatures results in the production of thermostable membranes, which results in increased thermotolerance. A factor that has received considerable attention recently is sublethal heat shock (48, 65), which induces the rapid synthesis of heat shock proteins (see reference 44 for a current review). Factors other than heat, such as hydrogen peroxide (52) and glucose starvation (40), also

induce the synthesis of heat shock proteins. Evidence is accumulating that heat shock proteins are a major determinant of bacterial thermotolerance (40, 65). An important factor in thermotolerance may be the effect of superoxide ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) on the recovery of bacteria injured by heat. Recoveries of heat-stressed facultative pathogens were greatly increased in media containing compounds such as catalase (CA) (49) and pyruvate (61) that degrade hydrogen peroxide; the addition of exogenous superoxide dismutase (SOD) had no effect on the recovery of heat injured *S. aureus* (12). Gregory and Fridovich (32) reported that the induction of superoxide dismutase in *E. coli* dramatically increased its resistance to hyperbaric oxygen, presumably by eliminating intracellular superoxide. Dallmier and Martin (17) observed no discernible correlation between intracellular levels of either CA or SOD and the resistance of *L. monocytogenes* to sublethal heat (17). However, a correlation was noted in their data between the rate of inactivation of SOD and resistance to sublethal heat injury.

The purpose of the present study was to determine if the thermal resistance of *L. monocytogenes* could be enhanced by growth or sublethal heat shock at 43°C. In addition, an attempt was made to determine if and why liquid medium results in superior recoveries of heat injured *L. monocytogenes* compared with direct aerobic plating in a solid medium.

MATERIALS AND METHODS

Bacterial Culture Conditions and Media Preparation

L. monocytogenes F5069, serotype 4b, was obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. This strain was isolated from raw milk obtained from a farm that supplied dairy products incriminated in an outbreak of listeriosis (26). The identity of the isolate was established by Gram stain, presence of catalase, hemolysis on sheep blood agar, tumbling motility at 25°C, formation of umbrella-shaped growth in motility medium, and growth at 4° and 45°C. Stock cultures were grown on Trypticase soy agar-0.6% yeast extract (TSAYE) (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h and were maintained at 4°C with monthly transfer.

Prereduced Trypticase soy broth-0.6% yeast extract (TSBYE) (BBL Microbiology Systems) and TSBYE containing 15 g/liter (TSBYEA) were prepared as described previously (36, 37). Resazurin (0.25 g/liter) was added as an E_h indicator, and cysteine $HCl \cdot H_2O$ (0.5 g/liter) was added after boiling to reduce the medium. Oxygen-free N_2 was obtained by passing N_2 gas through a hot copper column (36, 37). Extreme care was taken to avoid the introduction of even slight amounts of O_2 into any of the prereduced media. Any tubes containing oxidized medium (as detected by the presence of a pink color at the surface of the medium) were discarded.

Preparation of Bacteria

L. monocytogenes F5069 was grown in TSBYE at 37, 39, 41, 43, and 45°C for 18 h. In addition, the organism was grown in TSBYE at 37°C and then subjected to a sublethal heat shock at 43°C for 5, 30, and 60 min. Cultures of *L. monocytogenes* subjected to the various incubation and incubation/heat-shock treatments were inoculated into blender jars containing 200 ml of either TSBYE or sterile whole milk to yield approximately 10^6 cells/ml. The heating menstruum and inoculum were mixed by using five 1-s high-speed bursts on a Waring Blendor; 2.5 ml portions of this mixture were dispensed into Pyrex thermal death time (TDT) tubes (9mm OD, 7mm ID x 150mm length). The TDT tubes were sealed by using a Type 3A Blowpipe (Veriflo Corp., Richmond, Calif.) and then were placed in a water bath at 10°C for 3 min to allow temperature equilibration before thermal inactivation.

Thermal Inactivation and Enumeration

Thermal inactivation was accomplished by completely submerging TDT tubes in a model 11-V-8 circulating water bath (Precision Scientific, Chicago) equipped with a thermoregulator accurate to within 0.05°C. Thermal Death Time tubes were heated at 60°C (TDT tubes pulled 0, 5, 10, 15, 30, and 60 min) or 62.8°C (TDT tubes pulled 0, 2, 5, 10, 15, and 30 min). The time for the TDT tube contents to reach water-bath temperature (0 time) was measured by using a TDT tube equipped with a Thermal Death Time Thermocouple (O. F. Ecklund Inc., Cape Coral, Fla.). Six TDT tubes containing inoculated milk from each treatment/time combination were removed at appropriate times. Four tubes were left sealed, one was plated in duplicate

in TSBYEA and incubated aerobically, and one was enumerated in duplicate in prereduced TSBYEA by using the "Hungate" roll tube method (36, 37). In the latter procedure, 1:10 dilutions were made in tubes of prereduced TSBYE while the tubes were being flushed with oxygen-free N_2. Roll tubes containing 12 ml of melted, prereduced TSBYEA equilibrated to 45°C were inoculated with 0.1-ml portions of the appropriate dilutions. The roll tubes were purged of O_2 by using O_2-free N_2 gas and stoppered as described previously (36, 37). An Anoxy Tube Roller Model C (The McBee Laboratory, Bozeman, Mont.) containing ice water was used to solidify the agar evenly against the inside of the tubes. Any tubes showing signs of oxidation (pink color) were labelled as such, and the procedure was repeated, if necessary, to obtain roll tubes that were completely anaerobic (showing no sign of pink color). Sealed TDT tubes, aerobic plates, and roll tubes were incubated for 3 weeks at 25°C, a temperature found to be superior for the recovery of heat injured *L. monocytogenes* (14). Isolates from the plates and roll tubes were then confirmed as *L. monocytogenes* as already described.

Effects of Catalase and Superoxide Dismutase

Bovine liver catalase (2,600 U/mg) and iron-superoxide dismutase (FE-SOD) from *E. coli* (2,940 U/mg) were obtained from Sigma Chemical Co., St. Louis, Mo. The enzymes were rehydrated with distilled water and sterilized by filtration to obtain stock solutions with enzyme activities of 10,000 U/ml. *L. monocytogenes* F5069 was grown at 43°C for 18 h, diluted in TSBYE to approximately 10^6 cells/ml, and heated for 10 min at 62.8°C by using the sealed tube method. Enzyme(s) and heat-injured cells were added (0.1 ml

each) to tubes containing 20 ml of melted, prereduced TSAYE that had been equilibrated to 45°C. Inactive enzymes (heated at 100°C for 5 min) were added to other tubes as controls. The contents of the tubes were inverted several times to distribute the cells and achieve a uniform enzyme concentration (50 U enzyme activity/ml) before their contents were poured into sterile petri plates. The plates were incubated aerobically at 25°C, and CFUs were counted after 14 days. Anaerobic roll tubes containing cells but no enzyme were prepared as already described.

RESULTS

Recovery in Sealed Thermal Death Time Tubes

Growth temperature and type of recovery method had significant effects on the detection of heat-injured *L. monocytogenes.* Cells grown at elevated temperatures and both heated and recovered in TSBYE within sealed TDT tubes survived severe heat treatments (Tables 1 and 2), whereas cells plated aerobically in TSBYEA did not (Table 1). Growth of *L. monocytogenes* in sealed TSBYE within TDT tubes was accompanied by a drop in pH from 7.2 to 5.5. Elevated growth temperatures increased the organism's thermal resistance, as measured by its ability to recover in TSBYE within sealed TDT tubes (Table 2). Recovery was not observed in milk that had been heated at 62.8°C for 5 min or longer in sealed TDT tubes and then incubated at 25°C (data not shown). Reduction of resazurin, an E_h indicator, occurred in the bottom of sealed TDT tubes containing TSBYE immediately after heating, but not in TDT tubes containing sterile whole milk treated similarly. The depth of reduction in TDT tubes containing TSBYE became progressively greater as heating time increased. The time required to detect growth in TSBYE within sealed TDT tubes increased as the heating time increased; TDT tubes heated at 62.8°C for 30 min required 12 days at 25°C before growth became visible.

TABLE 1. Effects of growth temperature and recovery method on survival of *L. monocytogenes* F5069 heated at 60°C in Trypticase soy yeast extract broth in sealed thermal death time tubes

Growth Temperature	Recovery Method[a]	Time(min) at 60°C 0	5	10	15	30	60
37°C	APC[b]	6.7	6.1	2.2	<0	<0	<0
	TDT tube[c]	3	3	3	0	0	0
45°C	APC	6.1	5.8	5.5	2.9	<0	<0
	TDT tube	3	3	3	3	3	3

[a] Pour plates and TDT tubes incubated at 25°C for 12 days.

[b] Aerobic plate count (Log_{10} CFU/ml in TSAYE) immediately after heat treatment.

[c] Number of sealed thermal death time tubes, of 3 possible, showing growth after 12 days at 25°C.

TABLE 2. Effects of growth temperature on survival of *Listeria monocytogenes* F5069 heated at 62.8°C in Trypticase soy yeast extract broth and recovered in sealed thermal death time tubes

Time at 62.8°C (min)	Growth Temperature (°C)[a] 37°	39°	42°	43°
0[b]	3[c]	3	3	3
10	3	3	3	3
15	3	3	3	3
20	0	1	3	3
25	0	0	1	3
30	0	0	0	1

[a] 18 h cultures.

[b] Approximately 10^6 *L. monocytogenes* cells/ml.

[c] Number of sealed thermal death time tubes, of 3 possible, showing growth after 12 days at 25°C.

Aerobic and Anaerobic Plate Counts

Aerobic plating of heat-injured *L. monocytogenes* yielded nonlinear thermal destruction curves (Table 1 and Fig. 1). The rate of destruction increased after 5 min with cells grown at 37°C and after 10 min with cells grown at 43°C or 45°C (Table 1 and Fig. 1). Heat resistance increased as the heat shock time increased, but cells grown at 43°C were the most heat resistant (Fig. 1). Incubation of TSBYEA pour plates in anaerobic jars did not result in a significant increase in CFUs compared with aerobically incubated controls (data not shown).

Strictly Anaerobic Roll Tube Counts

Strictly anaerobic incubation greatly increased the apparent heat resistance of *L. monocytogenes* heated in sterile whole milk when compared with the aerobic plate count method (Figs. 2 and 3). Heat inactivation of *L. monocytogenes* cells grown at 43°C and inoculated into sterile, whole, homogenized milk resulted in a $D_{62.8^\circ C}$ value of 243 s (Fig. 2). In contrast, cells grown at 37°C, which were treated the same but plated aerobically, yielded a $D_{62.8^\circ C}$ value of 36 s (Fig. 2). Cells grown at 43°C, inactivated in sterile, whole, nonhomogenized milk, and enumerated by using strictly anaerobic "Hungate" techniques had a $D_{62.8^\circ C}$ value of 441 s compared to 72 s for cells grown at 37° and plated aerobically (Fig. 3). Cells grown at 43°C and enumerated by using anaerobic "Hungate" techniques resulted in thermal inactivation data with little deviation from a straight line.

Effects of Catalase and Superoxide Dismutase

The addition of both CA and SOD to TSBYEA increased the recovery of heat-injured *L. monocytogenes* upon aerobic incubation; CA was more effective than SOD (Table 3). Neither of these enzymes alone or in combination, increased the number of CFUs as much as the use of anaerobic "Hungate" techniques (Table 3). The addition of heat-inactivated enzymes had no effect on the number of CFUs compared with the aerobic control (Table 3).

FIG. 1. Effects of heat shock time (min) at 43°C versus growth at 43°C for 18 h on the heat resistance of *L. monocytogenes* F5069. Cells grown at 37°C for 18 h: not heat shocked (●——●); heat shocked for 5 min (o——o), 30 min (▲—▲), and 60 min (△—△). Cells grown at 43°C for 18 h (□——□). All cells plated and incubated aerobically

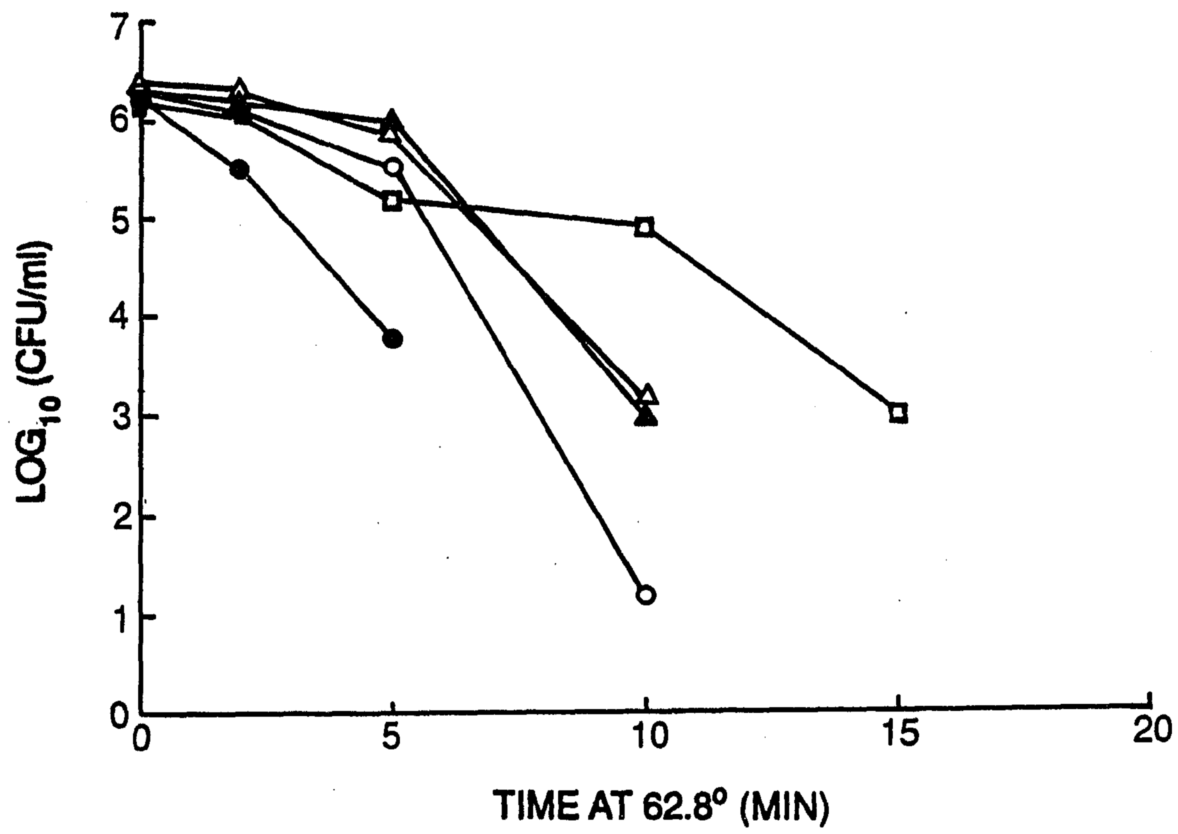

LOG10 (CFU/ml)
0
1
2
3
4
5
6
7
0
5
10
15
20
TIME AT 62.8° (MIN)

FIG. 2. Effects of growth temperature and strictly anaerobic recovery on the heat resistance of *L. monocytogenes* F5069 suspended in sterile, whole, homogenized milk. Cells grown at 37°C for 18 h; aerobic plate count (o——o), anaerobic roll tubes (●——●). Cells grown at 43°C for 18 h; aerobic plate count (□——□), anaerobic roll tubes (■——■)

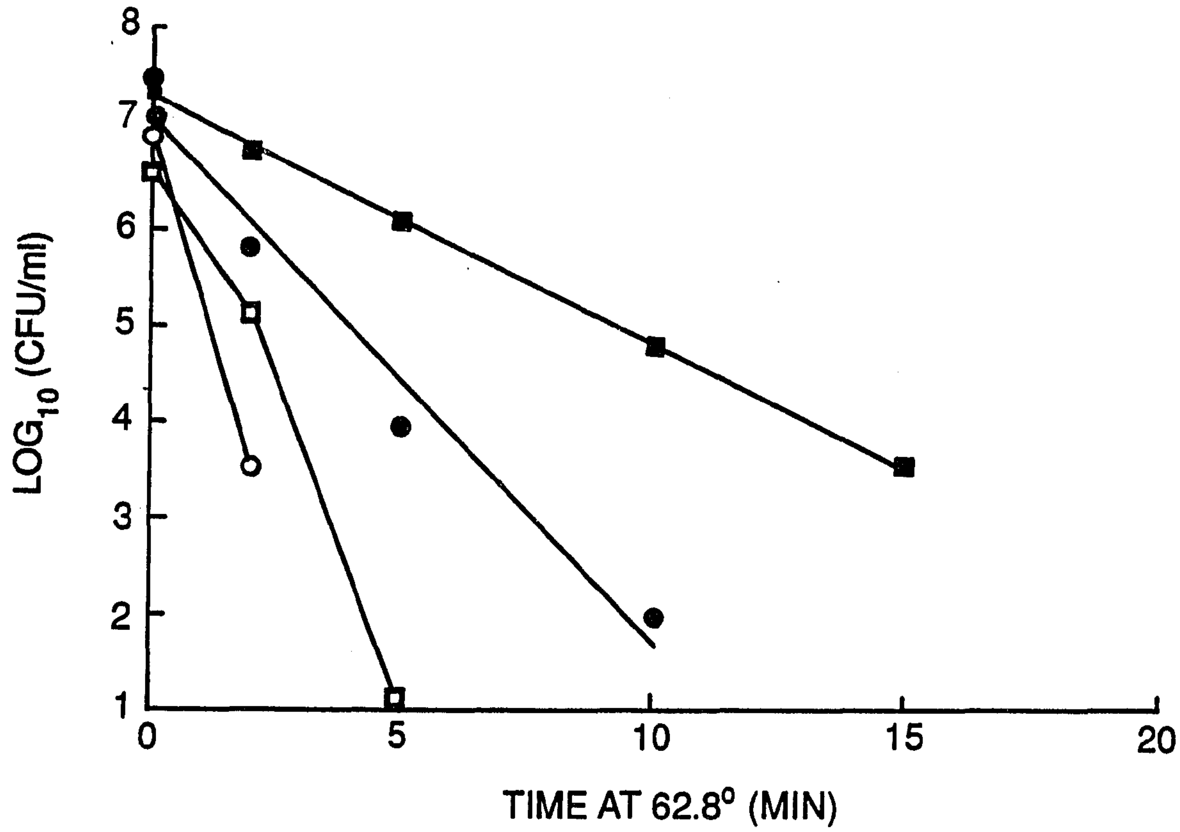
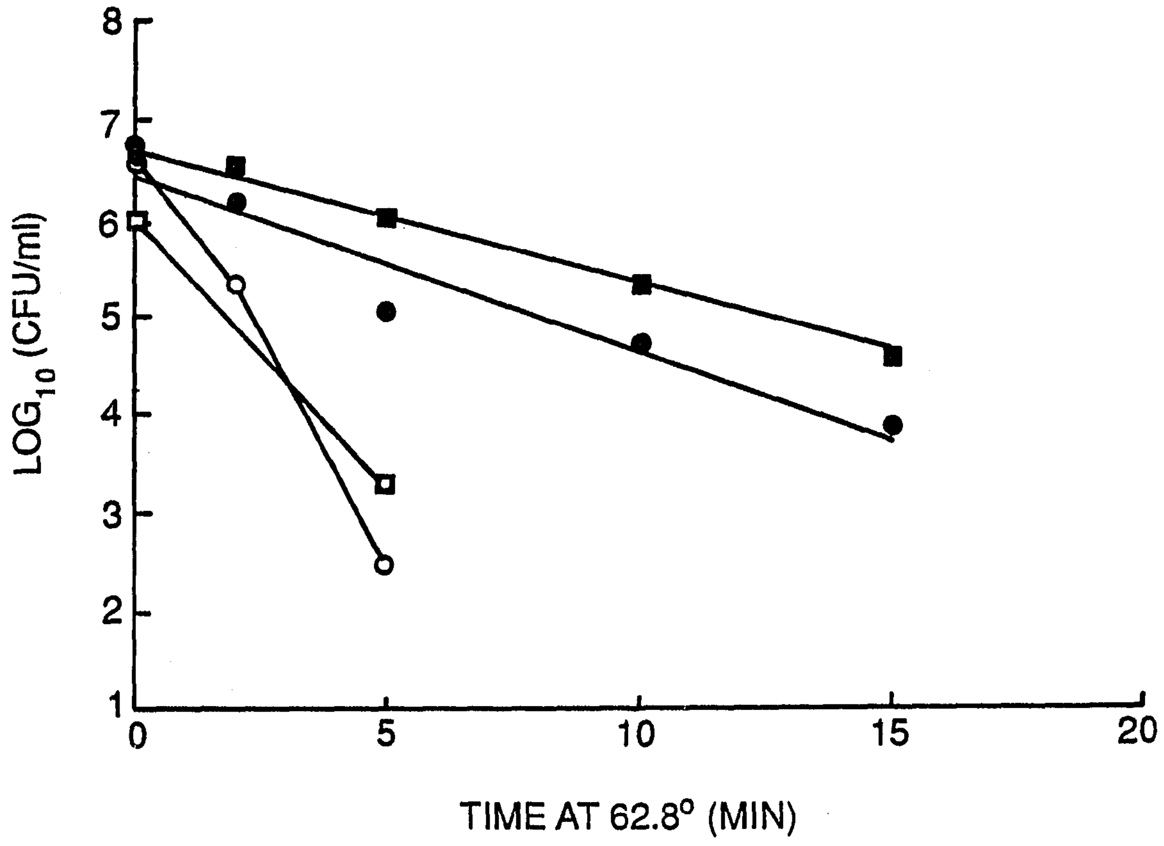

LOG10 (CFU/ml)
8
7
6
5
4
3
2
1
0
5
10
15
20
TIME AT 62.8° (MIN)

FIG. 3. Effects of growth temperature and strictly anaerobic recovery on the heat resistance of *L. monocytogenes* F5069 suspended in sterile, whole, nonhomogenized milk. Cells grown at 37°C for 18 h; aerobic plate count (o——o), anaerobic roll tubes (●——●). Cells grown at 43°C for 18 h; aerobic plate count (□——□), anaerobic roll tubes (■——■)



8
7
6
5
4
3
2
1
0
5
10
15
20
LOG10 (CFU/ml)
TIME AT 62.8° (MIN)

TABLE 3. Effects of catalase, superoxide dismutase, and strictly anaerobic recovery on the enumeration of heat-injured *Listeria monocytogenes* F5069

Treatment[a]	Log_{10} cells/ml[b]
APC[c]	2.0
APC + Active CA[d]	2.8
APC + Active SOD[e]	2.2
APC + Active SOD + Active CA	2.8
Strictly anaerobic roll tube	4.0
APC + Inactive CA[f] + Inactive SOD[f]	2.1
APC + Active CA + Active SOD - cells	<1.0

[a] *L. monocytogenes* was grown at 43°C for 18 h, diluted to approximately 10^6 cells/ml in TSBYE, and then heated in sealed TDT tubes at 62.8°C for 10 min. before enumeration.

[b] Average of two replicate experiments

[c] Aerobic plate count; plates were poured with TSBYEA and then incubated aerobically for 14 days at 25°C.

[d] Catalase (50 U/ml of TSBYEA).

[e] Superoxide dismutase (50 U/ml of TSBYEA).

[f] Heated at 100°C for 5 min.

DISCUSSION

The observation that heat-injured *L. monocytogenes* grew in sealed TDT tubes containing TSBYE but not in aerobic TSBYEA plates (Table 1) was unexpected. The addition of resazurin, an E_h indicator, to the TSBYE demonstrated that the difference between the two recovery methods might be caused by molecular O_2. Resazurin was reduced at the bottom of TDT tubes containing TSBYE after heating, probably because of a combination of low solubility of O_2 in water at high temperatures and the creation of a vacuum in the headspace of the TDT tube during sealing. Upon heating, the O_2 moved from the broth into the headspace, which left the bottom of the tube in a reduced state suitable for the repair and multiplication of heat-injured *Listeria*. In contrast to TDT tubes containing TSBYE, TDT tubes containing milk did not become reduced, as evidenced by the uniform pink color of the milk throughout the TDT tube after heating. Viable *Listeria monocytogenes* could be recovered from milk heated for 15 min at 62.8°C if the milk was sampled immediately after heating by using strictly anaerobic techniques (Figs. 2 and 3), but, unlike TSBYE in TDT tubes, could not be recovered from milk in TDT tubes that had been heated for 5 min or longer and then incubated for 2 weeks at 25°C before aerobic sampling (data not shown). The exposure of heat-injured *Listeria* to the oxidized environment of the milk probably led to inactivation of the cells after prolonged incubation. Growth of heat-injured cells in anaerobic roll tubes, but not in aerobic plates containing the same medium (Table 3, Figs. 2 and 3),

confirmed the hypothesis that the absence of O_2 significantly increased the recoveries of heat-injured *L. monocytogenes* F5069.

The O_2-sensitivity of heat-injured *L. monocytogenes* F5069 observed in this study probably was caused by the inactivation of CA and SOD. Dallmier and Martin (17) reported that these two enzymes were rapidly inactivated when *L. monocytogenes* was heated at temperatures of 55 to 60°C; SOD was slightly more heat labile than CA. A review of their data revealed that CA and SOD activities in *L. monocytogenes* would be almost totally inactivated during pasteurization. Complete inactivation of these enzymes would convert *L. monocytogenes* into a strict anaerobe because lethal levels of toxic O_2 products, such as $O_2{\cdot}^-$ (28) and H_2O_2 (49), would accumulate under aerobic conditions. Hydrogen peroxide can undergo a secondary reaction with iron (56) or $O_2{\cdot}^-$ (33) to form the extremely toxic hydroxyl ($\cdot$OH) radical. This hypothesis agrees with the enzyme-based theory of obligate anaerobiosis proposed by McCord et al. (50) and accounts for the large differences in thermotolerance observed when heated cells were incubated under aerobic and strictly anaerobic conditions (Figs. 2 and 3).

It is difficult to assess which enzyme, CA or SOD, is more critical in preventing cell death (28). In our study, added CA and SOD failed to effectively protect heat-injured *Listeria* plated aerobically (Table 3). Martin et al. (49) determined that the inhibition of *S. aureus* observed after thermal inactivation of CA was due to the production of H_2O_2. The addition of SOD to an $O_2{\cdot}^-$-generating system resulted in protection of thermally stressed cells of *S. aureus*; however, incorporation of $O_2{\cdot}^-$-scavengers into the medium did not increase enumeration upon aerobic incubation, which

was explained by the failure of SOD to penetrate the cell membrane and react with $O_2^{\cdot -}$ formed intracellularly (12). Bagley et al. (3) treated Chinese hamster cells with paraquat, a chemical that mediates the production of intracellular $O_2^{\cdot -}$, and observed that when added to the growth medium neither CA nor SOD enhanced the formation of colonies. When introduced into the cells, SOD but not CA inhibited the toxicity of paraquat, indicating that the cytocidal action of paraquat was caused by the intracellular production of $O_2^{\cdot -}$. Therefore, the thermal inactivation of SOD and subsequent intracellular accumulation of $O_2^{\cdot -}$ may play a large role in cell killing. This hypothesis is supported by the observation that strains of *E. coli* low in SOD activity were more susceptible to heat injury than cells with high SOD activity (51).

Failure to detect an increase in heat resistance in the present study by incubation of pour plates in anaerobic jars (data not shown) has been reported previously (16, 25) and can be attributed to the "strict anaerobe" nature (50) of severely heat-injured *L. monocytogenes* (Table 3, Figs. 2 and 3). Toxic O_2 products would have accumulated inside the cells during aerobic diluting and plating operations until all O_2 had been expended inside the anaerobic jars. This would explain why no significant differences were observed between the numbers of colonies formed in the TSBYEA plates incubated aerobically and those incubated in anaerobic jars when the samples were processed aerobically before incubation (data not shown).

Doyle et al. (20) and Fernandez Garayzabal and coworkers (24, 25), using naturally and artificially contaminated raw milk, did not detect viable *L. monocytogenes* by direct aerobic plating of milk immediately after

pasteurization, but they were able to detect viable cells when liquid enrichment techniques were used. Doyle et al. (20) recovered viable *L. monocytogenes* in three different enrichment media, but they were unable to obtain consistent recovery with any one medium. Recently, Smith and Archer (61) and Crawford et al. (15) determined that the selective media currently in use to detect *L. monocytogenes* in foods are not satisfactory for the recovery of heat-injured cells. Crawford et al. (15) therefore suggested that the presence of viable *L. monocytogenes* in HTST pasteurized milk, as detected by using selective enrichment media (20, 24, 25), was the result of postpasteurization contamination with uninjured cells. Dubos (21) observed that only a few cells of various facultative pathogens were required to initiate growth in a plain meat broth if the broth had been recently boiled, but after cooling for 24 h that same broth would not allow the growth of large numbers of the same microorganisms unless it was first reduced by the addition of cysteine. The addition of thioglycolic acid to a liquid enrichment medum greatly increased its ability to support the growth of heat-treated facultative bacteria (55). Dubos (21) and Nelson (55) both concluded that low oxidation-reduction potential was the main factor in allowing growth of facultative microorganisms in their studies. Mackey and Derrick (47) discovered that the solid form of selective and nonselective media were significantly more inhibitory to heat-injured *Salmonella typhimurium* than the corresponding liquid media. The authors hypothesized that aerobic incubation of solid media exposed the cells to higher levels of O_2 which resulted in the formation of toxic H_2O_2, because the addition of catalase increased the counts on nutrient agar to the level obtained in nutrient broth.

Therefore, the negative recovery of heat-injured *L. monocytogenes* by using direct aerobic plate counts (10, 11, 13, 14, 18-20, 25) and the negative (15, 45) and inconsistent (20, 25) recovery in liquid media may have depended more on the concentration of O_2 in the individual media rather than on the composition of the media per se. In the present study, recovery of severely heat-injured cells occurred only in reduced TSBYE inside sealed, undisturbed TDT tubes (Tables 1 and 2) or in roll tubes containing pre-reduced TSBYEA (Figs. 2 and 3). After a recovery period in an anaerobic environment, *Listeria* would regain CA and SOD activities and again become tolerant of oxygen (47). This hypothesis was supported by the finding that heat-injured cells that initially grew only in sealed TDT tubes or strict anaerobic roll tubes, later demonstrated normal colony formation when transferred to the surface of TSBYEA incubated aerobically (data not shown).

The answer to how heat-injured *L. monocytogenes* can survive and reproduce in oxygenated milk after pasteurization may lie in its special intracellular relationship with phagocytes. Resting phagocytes consume little oxygen but obtain most of their energy from glycolysis (43). Oxygen consumption occurs only during the respiratory burst, which results in the introduction of $O_2{\cdot}^-$ into the phagosome (2). *L. monocytogenes* is an intracellular pathogen that can survive and grow inside phagocytes because it may fail to initiate the respiratory burst (30) and possesses significant CA and SOD activities (9) that enable it to remove toxic O_2 metabolites. Therefore, *L. monocytogenes* might not be exposed to significant levels of O_2 or its toxic by-products when inside heat-killed phagocytes. Besides the possibility of providing an anaerobic environment, an additional protective

mechanism might exist for *L. monocytogenes* because of its location within phagocytes. Bovine CA and SOD, located within the cytoplasm of phagocytes (41), are more thermostable than the corresponding enzymes in *L. monocytogenes* (compare reference 17 with reference 35). Therefore, the presence of these enzyme activities in the cytoplasm of the phagocyte after HTST pasteurization might protect heat-injured, intracellular *L. monocytogenes* from $O_2^{\cdot -}$ and/or H_2O_2, thereby allowing it to resuscitate. Localization of *L. monocytogenes* inside PMNL was originally thought to serve as a "heat shield" and provide direct thermal protection (20, 26), but Bunning et al. (14) and Crawford and coworkers (15) determined that localization inside bovine phagocytes did not provide significant protection against heat. In the latter studies, heat-injured *L. monocytogenes* were released from the phagocytes by sonication immediately after heating, followed by aerobic plating on a solid medium. This procedure would have subjected the heat-injured, oxygen-sensitive bacteria to a toxic, oxygenated environment. This could explain why Bunning et al. (14) were unable to detect viable *Listeria* by the direct plating of severely heat-injured cells. We believe that *L. monocytogenes* may survive pasteurization of milk when allowed to resuscitate within heat-killed phagocytes, protected from contact with O_2 and toxic O_2 radicals.

An increase in the time that cells were heat shocked at 43°C resulted in increased heat resistance (Fig. 1). This has been reported previously for *Salmonella typhimurium* (48) and it may reflect a quantitative increase in the amounts of heat shock proteins synthesized with time (40, 44). Crawford et al. (16) observed a general trend of increased thermotolerance with an

increase in time and temperature of heat shock for *L. monocytogenes* F5069; however, they concluded that the increased thermotolerance was not statistically significant. Most thermotolerance studies of *L. monocytogenes* have utilized cells grown at, or below, 37°C (6, 10, 11, 13, 14, 18, 19, 31). The normal body temperature of a cow is approximately 39°C, but temperatures can reach as high as 42.8°C when the cow is infected with *L. monocytogenes* (20). The large increase in heat resistance observed when cells were grown at 43°C, compared to cells that were heat shocked at 43°C (Fig. 1), may be due to the accumulation of large amounts of heat shock proteins during the stationary phase of growth. Jenkins et al. (40) concluded that the increased heat resistance of stationary phase cells (22, 38, 63) could be a result of the synthesis of Pex (post exponential heat shock proteins) that are induced during glucose starvation. The presence of large amounts of Pex proteins in stationary phase cells grown at 43°C would explain why these cells were more heat resistant than either those grown at lower temperatures (Tables 1 and 2) or those heat shocked at 43°C (Fig. 1). Heat resistance can also be induced by exposure to H_2O_2 (52), a condition known to occur within the phagosome (41). Therefore, cells of *L. monocytogenes* growing inside bovine phagocytes within an infected cow could be in a heat-resistant state that might persist under refrigerated storage prior to pasteurization. Recently, heat-shocked cells of *L. monocytogenes* have been reported to remain heat-resistant after being held for 24 h at 4°C (23).

The increased heat resistance observed in nonhomogenized milk (Fig. 3) was probably due to the protective effect of milk fat. The heat resistance of *L. monocytogenes* has been shown to increase in ice cream (11). If *L.*

monocytogenes becomes embedded or coated in fat during homogenization it may be protected from the rapidly lethal effects of moist heat. Therefore, the performance of heat resistance studies by the addition of *L. monocytogenes* to previously homogenized milk (14, 18, 19) might have resulted in an underestimation of the microorganism's actual heat resistance under normal processing conditions.

Recently, Bunning et al. (14) used cells grown at 37°C, aerobic plating, and sterile, homogenized, whole milk to obtain a $D_{62.8^{\circ}C}$ value of 38.3 s for freely suspended *L. monocytogenes*. Based on their results and the use of a detailed risk analysis model (53), Bunning et al. (14) concluded that commercial HTST pasteurization of milk did not appear to be a problem. In an earlier paper Mossel and Van Netten (54) cautioned that if careful attention is not paid to the recovery of stressed microorganisms, substantial overestimation of the microbial kill attained in processing may result. In the present study, $D_{62.8^{\circ}C}$ values were approximately 6.3 times greater than the $D_{62.8^{\circ}C}$ value of 38.3 s just mentioned. Extrapolation of our results to the HTST range is scientifically inappropriate; however, given a z_D value of 5.6°C (14), we believe that low levels of *L. monocytogenes* (i.e., 10 per ml of raw milk) might survive the HTST process under the conditions described in the present study.

The heat resistance of *L. monocytogenes* in foods other than dairy products is also of current interest (1, 8). The development of anaerobic environments in foods after thermal processing might permit the growth of heat-injured *L. monocytogenes*. Such conditions might exist in cooked meats (42) and other pasteurized foods that are either canned, vacuum packaged,

or held in modified atmosphere packages that contain low levels of O_2. Potentially inadequate thermal processes currently exist for hot dogs (1), the consumption of which has been associated with sporadic listeriosis (59). Recently, a fatal case of listeriosis was associated with the consumption of vacuum-packaged turkey franks (4). Although it has not been shown that *L. monocytogenes* can survive the thermal process for hot dogs, the results of the present study should give cause for concern.

Many attempts have been made to develop an improved medium for the enumeration of injured *L. monocytogenes*, but such a medium is still to be realized (15, 61). The lethal effects of selective agents on injured cells is the main impediment to developing a selective yet efficient recovery method. Selective agents might increase lethality by enhancing the oxidative stresses that injured cells experience under aerobic incubation. Removal of this oxidative stress might allow efficient recovery of injured cells in selective media. This hypothesis is supported by previous work with *L. monocytogenes* (61), *S. aureus* (49), and *S. typhimurium* (47), where the toxic effects of various selective agents were partially overcome by the addition of CA. The use of a strictly anaerobic recovery period would eliminate oxidation stress by excluding O_2, the necessary precursor of toxic O_2 radicals. The recovery of injured cells by direct plating on selective media under strictly anaerobic conditions might permit more rapid and accurate enumeration of stressed cells than is currently possible. This method may be applicable to the enumeration of other injured, facultatively anaerobic, foodborne pathogens such as *S. aureus*, *Salmonella* spp., *Shigella* spp., *E. coli*, *Yersinia enterocolitica*, *Vibrio* spp., etc. These direct enumeration

methods would be of significant benefit to the food industry as it attempts to identify both the sources of contamination and the levels of various pathogens present in different foods.

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HEAT SHOCK PROTEINS MAY CAUSE THERMOTOLERANCE IN *Listeria monocytogenes* BY PREVENTING THE DENATURATION OF CATALASE AND SUPEROXIDE DISMUTASE

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ABSTRACT

Listeria monocytogenes F5069 was suspended in Trypticase soy-0.6% yeast extract broth (TSBYE) and heated at 60°C in sealed thermal death time tubes. Heat resistance increased with the time cells were subjected to a prior sublethal heat shock, reaching maximum heat resistance after 30 min at 43°C. Cells grown at 43°C for 18 hours were more heat resistant than cells that had been heat shocked at 43°C; cells grown at 37°C were the least heat resistant. Thermal inactivation at 60°C of cells grown at 43°C resulted in linear inactivation kinetics, whereas at 62.8°C the rate of inactivation increased with heating time (nonlinear kinetics). Anaerobic roll tube ("Hungate") techniques resulted in an approximately 6-fold increase in heat resistance and linear inactivation kinetics. The possibility that heat shock proteins cause thermotolerance by preventing the denaturation of essential, heat-labile proteins, such as catalase and superoxide dismutase, is discussed.

INTRODUCTION

A sudden shift-up in temperature results in the synthesis of a group of proteins known as heat shock proteins (HSPs); concomitantly, the synthesis of most other proteins is repressed. The heat shock response is the most highly conserved genetic system known, existing in every organism in which it has been sought, including archaeobacteria and eubacteria, plants and animals (for a recent review see ref. 20). The fact that this very conserved group of proteins is synthesized during a marginal upshift in temperature indicates that the HSPs must serve essential biological functions (23). Results from studies on both eukaryotes (16, 19, 34) and prokaryotes (14, 42) indicate that a major function of HSPs is the development of thermotolerance. However, VanBogelen et al. (37) recently reported that artificial induction of the heat shock regulon did not produce thermotolerance. A variety of stress agents, other than heat shock, are believed to induce thermotolerance. These include hydrogen peroxide (H_2O_2) (27), ethanol (18), and glucose starvation (14). The growth of an organism near its maximum temperature and the use of stationary-phase cells greatly increases the heat resistance of *E. coli* (9), *Streptococcus faecalis* (41), and *Staphylococcus aureus* (13). Recently, Jenkins *et al.* (14) correlated the increased heat resistance of stationary-phase cells to the presence of Pex (post-exponential) proteins, which are synthesized during both heat shock and glucose starvation. Cells grown at 37°C and then glucose-starved for 4 h were much more heat resistant than cells that had been heat shocked at 42°C for 30 min.

The exact role of HSPs in the development of thermotolerance is not known (20). It has been postulated that they stabilize heat-labile proteins by nonspecific interactions (26) or catalyze the refolding of denatured proteins (31). Munro and Pelham (29) observed that most, if not all, of the stress-inducing agents have a common property of causing an accumulation of denatured proteins, which may be a common signal for the induction of HSPs (11). This view is in agreement with the work of Ananthan et al. (1), who discovered that denatured proteins, but not native proteins, triggered the activation of heat shock genes injected into frog oocytes. In addition, Goff and Goldberg (11) reported that the production of abnormal proteins in *E. coli* stimulated the transcription of *lon* and other heat shock genes. It is not known which, if any, of these denatured proteins is the most critical lesion in terms of viability for vegetative bacteria (35). Identification of the most critical and heat-labile enzymes in the cell might allow one to test the hypothesis that thermotolerance can be induced because HSPs either prevent the denaturation or affect the renaturation of enzymes that are critical to survival.

Catalase (CA) and superoxide dismutase (SOD) are enzymes found in almost all aerobic cells where they remove the toxic by-products of O_2 metabolism, H_2O_2 (22) and $O_2\cdot^-$ (10), respectively. Strictly anaerobic bacteria lack these enzymes and can only grow anaerobically because they are rapidly killed upon exposure to molecular oxygen because of the formation of $O_2\cdot^-$ and H_2O_2 (24, 28). Dallmier and Martin (7) reported that CA and SOD were rapidly inactivated when *Listeria monocytogenes* cells were heated to temperatures above 55°C. Examination of their data revealed that CA and

SOD would be almost completely inactivated during pasteurization. Subsequent exposure to O_2 would then place *L. monocytogenes* under severe oxidation stress (24, 28).

Lee et al. (17) hypothesized that adenylated dinucleotides produced during both heat shock and oxidation stress are alarmones-i.e., regulatory molecules that signal the onset of oxidation stress. However, VanBogelen et al. (38) reported that the response to heat shock is not regulated by alarmones, but is primarily an independent response of the *rpoH* regulon.

Listeria monocytogenes is a Gram-positive, thermotolerant, facultatively anaerobic bacterium (36) that has been reported to survive pasteurization (8). The organism produces a typical heat shock response upon exposure to sublethal heat treatments (6). Previous work with *E. coli* (25), *Salmonella senftenberg* (33), and *Staphylococcus aureus* (2, 4) indicated that the thermal inactivation of CA, and possibly SOD, greatly decreased the recovery of these facultative microorganisms upon aerobic plating. The dramatic increase in thermotolerance observed when cells were incubated under anaerobic, compared to aerobic conditions (see preceding manuscript), indicated that the most critical thermal lesion in *L. monocytogenes* was the inactivation of CA and/or SOD. The facultatively anaerobic and heat-resistant nature of *L. monocytogenes* make this microorganism an excellent tool to test the hypothesis that HSPs play a significant role in thermotolerance by preventing the inactivation of these two critical, heat-labile enzymes.

MATERIALS AND METHODS

Bacterial Culture Conditions and Media Preparation

L. monocytogenes F5069, serotype 4b, was obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. The identity of the microorganism was established as described previously (36). Stock cultures were grown on Trypticase soy agar-0.6% yeast extract (TSAYE) (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h and were maintained at 4° C with monthly transfer.

Pre-reduced Trypticase soy broth-0.6% yeast extract (TSBYE) (BBL Microbiology Systems) and TSAYE were prepared as described previously (12). Resazurin (0.25 g/l) was added as an E_h indicator, and cysteine $HCl{\cdot}H_2O$ (0.5 g/l) was added after boiling to reduce the medium. Oxygen-free N_2 was obtained by passing N_2 gas through a hot copper column (12). Any tubes containing oxidized medium (as detected by the presence of a pink color at the surface of the medium) were discarded.

Preparation of Bacteria

L. monocytogenes F5069 was grown in TSBYE at 37, 39, 41, 43, and 45°C for 18 h. In addition, the organism was grown in TSBYE at 37°C and then subjected to a sublethal heat shock at 43°C for 5, 30, and 60 min. Cultures of *L. monocytogenes* subjected to the various incubation and incubation/heat-shock treatments were inoculated into blender jars containing 200 ml of either TSBYE or sterile whole milk to yield approximately 10^6 cells/ml. The heating menstruum and inoculum were mixed by using five 1-second high-

speed bursts on a Waring Blendor; 2.5 ml of this mixture were dispensed into Pyrex thermal death time (TDT) tubes (9mm OD, 7mm ID x 150mm length). The TDT tubes were sealed immediately by using a Type 3A Blowpipe (Veriflo Corp., Richmond, Calif.).

Thermal Inactivation and Enumeration

Thermal inactivation was accomplished at 60°C or 62.8°C. The time for the tube contents to reach water bath temperature was measured by using a TDT tube equipped with a Thermal Death Time Thermocouple (O. F. Ecklund Inc., Cape Coral, Fla.). Six TDT tubes containing inoculated milk from each treatment/time combination were removed at appropriate times. Four tubes were left sealed, one was plated in duplicate in TSAYE and incubated aerobically, and one was enumerated in duplicate in pre-reduced TSAYE by using the "Hungate" roll tube method (12). In the latter procedure, 1:10 dilutions were made in tubes of pre-reduced TSBYE while the tubes were being flushed with oxygen-free N_2. Roll tubes containing 12 ml of melted, pre-reduced TSAYE equilibrated to 45°C were inoculated with 0.1-ml portions of the appropriate dilutions. The roll tubes were purged of O_2 by using O_2-free N_2 gas and stoppered as described previously (12). An Anoxy Tube Roller Model C (The McBee Laboratory, Bozeman, Mont.) containing ice water was used to solidify the agar evenly against the inside of the tubes. Any tubes showing signs of oxidation (pink color) were labelled as such, and the procedure was repeated, if necessary, to obtain roll tubes that were completely anaerobic (showed no sign of pink color). Sealed TDT tubes, aerobic plates, and roll tubes were incubated at 25°C for 3 weeks to give

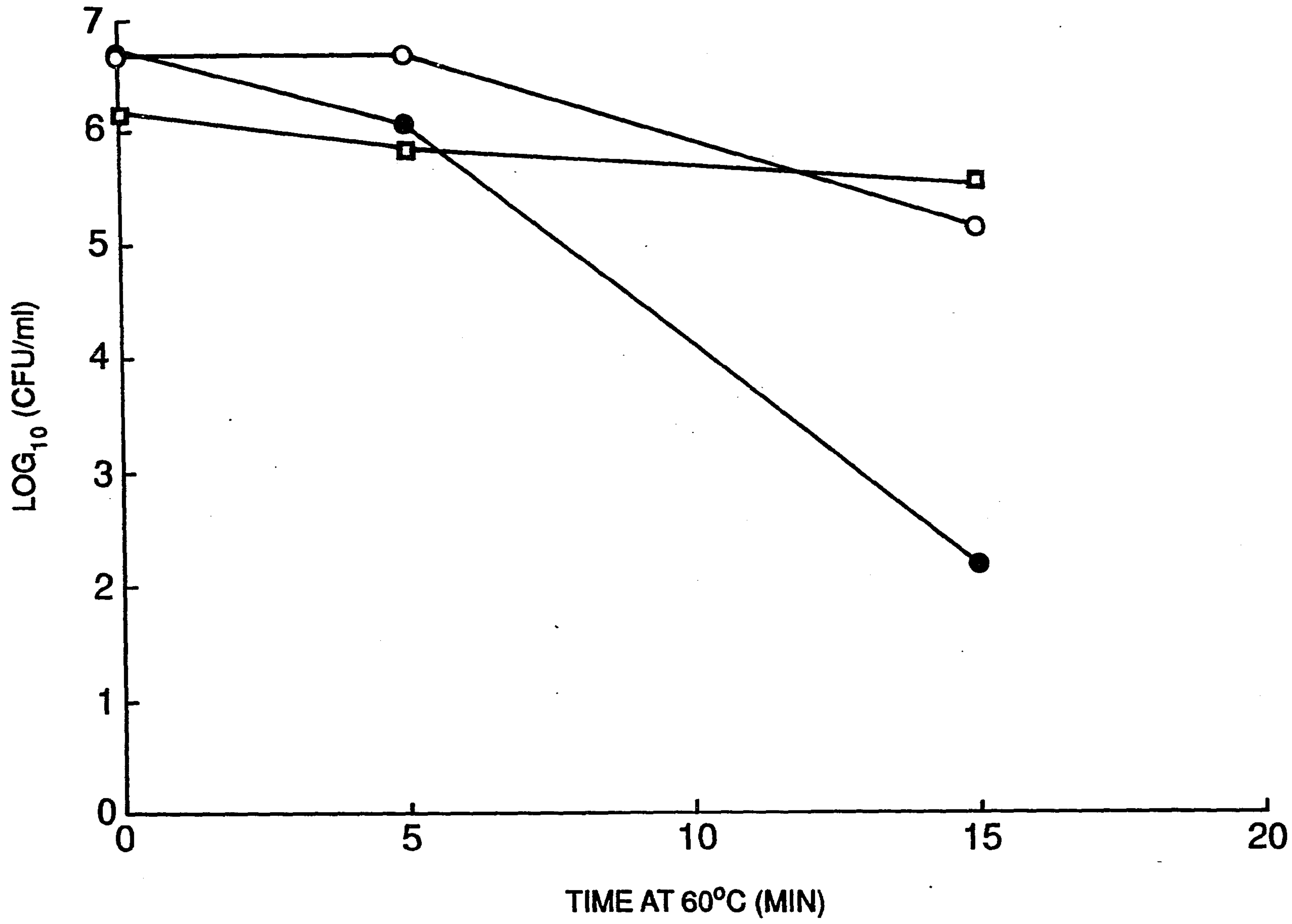
severely injured cells time to resuscitate. Isolates from the plates and roll tubes were then confirmed as *L. monocytogenes* (36).

RESULTS AND DISCUSSION

Heat resistance increased with the time cells were heat shocked at 43°C, reaching a maximum at 30 min (data not shown). Cells grown at 43°C were much more thermotolerant than cells that were heat shocked at 43°C for 30 min (Fig. 1). These results agree with those of Jenkins et al. (14), who found that glucose starvation for 4 h resulted in much greater thermotolerance than heat shock at 43°C for 30 min. They concluded that longer incubation times were needed before pertinent Pex proteins reached levels required for maximal thermotolerance. The Pex proteins include HSPs thought to be involved in thermotolerance; however, it is not known whether or not these proteins are encoded by the *rpoH*-controlled heat shock regulon (14). If they are *rpoH*-controlled, it is possible that their regulation might involve multiple promoters or individual promoters that respond to different stress stimuli.

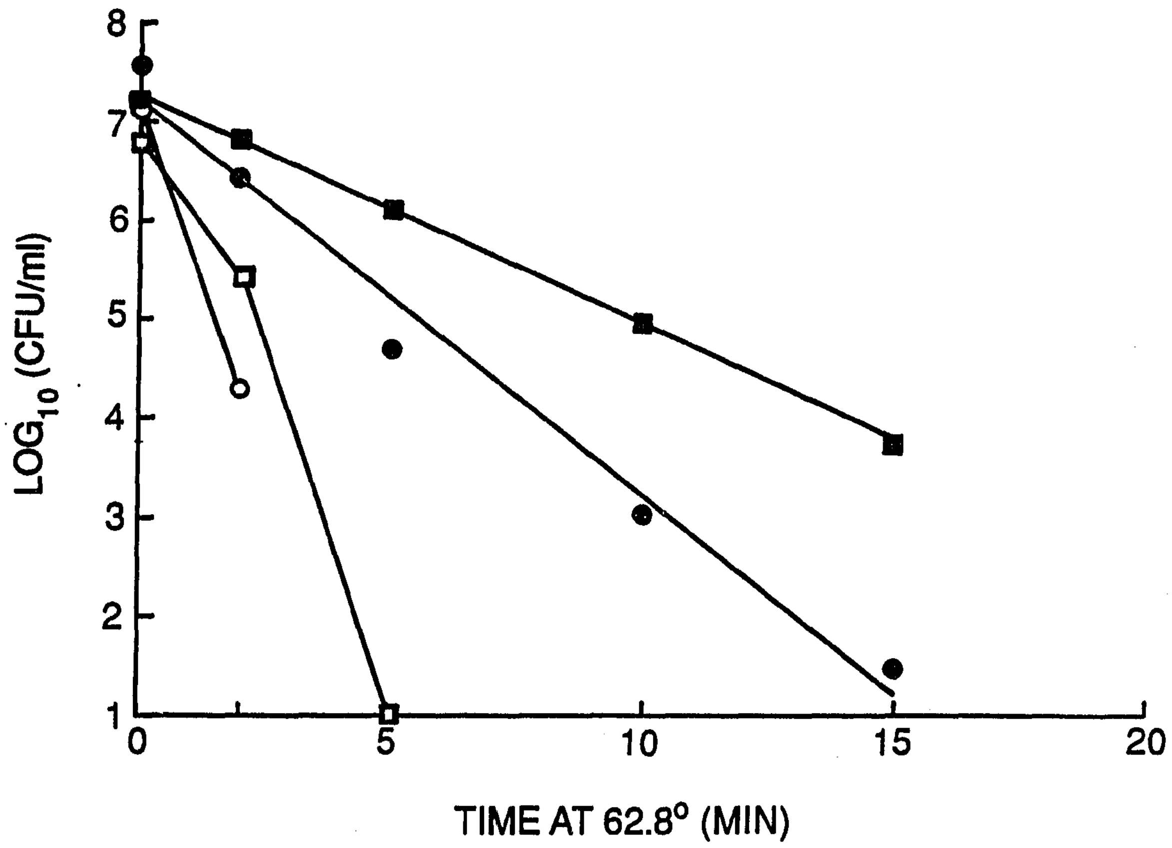
Growth of *L. monocytogenes* cells at 43°C had a definite effect on the recovery of heat-injured cells under both aerobic and anaerobic conditions (Fig. 2). The dramatic decrease in thermotolerance upon aerobic incubation, compared to anaerobic incubation, is probably a result of the production of $O_2\cdot^-$ and H_2O_2 by *L. monocytogenes* when exposed to O_2 (28). The rapid inactivation of SOD (4, 7) and CA (2, 7) above 55°C would have converted *L. monocytogenes* into an obligate anaerobe (24), unable to grow under aerobic conditions (Fig. 2).

FIG. 1. Effects of heat shock at 43°C versus growth at 43°C for 18 h on the heat resistance of *L. monocytogenes* F5069 suspended in Trypticase soy - 0.6% yeast extract broth. Cells grown at 37°C for 18 h; not heat shocked (o——o), heat shocked at 43°C for 30 min (●——●); cells grown at 43°C for 18 h (□——□). All cells plated and incubated aerobically



LOG10 (CFU/ml)
7
6
5
4
3
2
1
0
0
5
10
15
20
TIME AT 60°C (MIN)

FIG. 2. Effects of growth temperature and strictly anaerobic recovery on the heat resistance of *L. monocytogenes* F5069 suspended in sterile, whole, homogenized milk. Cells grown at 37°C for 18 h; aerobic plate count (o——o), anaerobic roll tubes (●——●). Cells grown at 43°C for 18 h; aerobic plate count (□——□), anaerobic roll tubes (■——■)



8
7
6
5
4
3
2
1
LOG10 (CFU/ml)
0
5
10
15
20
TIME AT 62.8° (MIN)

Lee et al. (17) reported that adenylated nucleotides accumulated in *Salmonella typhimurium* during both oxidation stress and heat shock, and therefore postulated that these alarmones were responsible for regulating the synthesis of both oxidation stress and HSPs. They suggested that oxidation stress and heat shock have a common physiological effect (oxidation stress) on cells, with heat mediating oxidation stress through damage to cell membranes. Based on our results (Fig. 2) and those of Dallmier and Martin (7), we believe that heat shock can cause oxidation stress by the inactivation of SOD and CA, which results in the accumulation of $O_2 \cdot^-$ and H_2O_2. Therefore, although the heat shock (*rpoH*-controlled) and the oxidation stress (*OxyR*-controlled) regulons might not utilize a common signal, both heat shock and oxidation stress would expose the cell to agents of oxidation stress, i.e., $O_2 \cdot^-$ and H_2O_2. This would explain why proteins induced by heat shock overlap with those induced by exposure to H_2O_2 (27) or $O_2 \cdot^-$ (40). Thermal inactivation of these enzymes would also explain why SOD (32) and CA (27) synthesis is induced by heat shock.

The results in Fig. 2 indicate that thermal inactivation of SOD and/or CA may be the primary lethal lesion that occurs during heat inactivation of *L. monocytogenes*. Therefore, one logical function for HSPs in the production of thermotolerance in *L. monocytogenes* is to prevent the inactivation of these two critical, heat-labile enzymes. This may represent a general biological phenomenon because CA and SOD from other prokaryotes (2, 4), plants (3, 39) and animals (5) are also heat-labile.

Evidence from various sources indicates that protein stabilization is a general function of HSPs. Jinn et al. (15) reported that extracts of heat

shocked, but not nonheat shocked, soybean seedlings prevented the in vitro denaturation of soluble soy proteins; the degree of protection was proportional to the amount of heat shock protein fraction added. Pelham (30) suggested that an important function of hsp70 in thermotolerance is to catalyze the reassembly of damaged pre-ribosomes and other ribonuleoproteins after heat shock. In the present study, cells grown at 43°C had increased thermotolerance under anaerobic conditions, where CA and SOD inactivation would not be expected to influence recovery (Fig. 2). The discovery that HSPs are induced by a wide variety of denatured proteins, but not native proteins (1), also is consistent with the hypothesis that HSPs have the general function of maintaining proteins in their native or "active" state. HSPs might prevent the inactivation of different proteins by nonspecific stabilization (26) and/or by an active process in which heat shock proteins catalyze the refolding of denatured proteins (31).

The pattern of thermal inactivation of *L. monocytogenes* differed, depending on prior growth/heat shock treatment, heating temperature, and aerobic/anaerobic incubation conditions (Figs. 1 and 2). The rate of inactivation of cells grown at 43°C and plated aerobically was linear (first-order inactivation kinetics) for the first 15 min at 60°C (Fig. 1), and then decreased sharply after 2 min at 62.8°C (Fig. 2). This same phenomenon was reported by Mackey and Derrick (21), who observed that a linear (first-order) inactivation curve was obtained for heat-shocked *Salmonella typhimurium* at 50°C but not at 55°C. The high rate of inactivation at high heating temperatures may be caused by the denaturation of HSPs themselves. Left unprotected, SOD and CA would rapidly denature, resulting

in the death of the cell upon exposure to heat and O_2 (multiple-hit kinetics) (21, Figs. 1 and 2). In contrast, HSPs may be stable at the lower heating temperatures and thus able to stabilize SOD and CA, resulting in linear (first-order) inactivation kinetics (21, Fig. 1, 43°C-grown cells). Linear inactivation of 43°C-grown cells, when heated at 62.8°C and recovered anaerobically (Fig. 2), may indicate that severely heat-injured cells, given sufficient time, could recover in the absence of toxic O_2 metabolites. In the present study, numerous colonies continued to appear, even after 10 days at 25°C in anaerobic "Hungate" tubes, but not in aerobic plates (data not shown). The recovery of heat-injured cells after long incubation times is consistent with the catalytic model for HSP function proposed by Pelham (31), who postulated that hsp70 utilizes an ATPase activity to refold denatured proteins into their native state. If HSPs are denatured at high temperatures, a few residual, undenatured HSPs might mediate the refolding of denatured HSPs. This putative autoreactivation process would lead to a HSP-activation cascade, followed by the reactivation of other denatured enzymes and eventual cell recovery. Thermal inactivation studies are currently being conducted in conjunction with enzyme assays for CA and SOD in attempts to show conclusively that HSPs cause thermotolerance in *L. monocytogenes* by preventing the inactivation of these two critical enzymes.

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GENERAL SUMMARY AND DISCUSSION

Gram-positive bacteria are important in foods because some produce beneficial effects through fermentations while others produce harmful effects through spoilage, foodborne diseases, and intoxications. Because they are typically more resistant than Gram-negative bacteria to adverse conditions in foods (216), special measures are required to control their presence and/or growth in foods. Two measures of control are the use of polyphosphates and heat. This dissertation is comprised of two separate series of studies, each based on a separate question. One question was: What is the mechanism by which polyphosphates specifically inhibit Gram-positive bacteria. The second question was: Does the Gram-positive bacterium *Listeria monocytogenes* survive the minimum low-temperature, long-time pasteurization process? A third question, which associated with the second question but has more general biological implications was: What is the role of heat shock proteins in thermotolerance? This dissertation attempted to address these important questions.

The use of the well-plate technique in Part I permitted rapid manipulation and assessment of three important variables: type of polyphosphate, type of metal, and species of microorganism. The formation of tapered-growth patterns on well-plates containing added Mg^{2+}, Mn^{2+}, and Fe^{3+} indicated that inhibition was caused by the chelation of essential metal cations (259).

The procedure for the determination of Ca and Mg (85) was modified by substituting polyphosphate for EDTA. The change from red to blue of the

metallochromic indicator, Calmagite (258), indicated that all free Mg^{2+} had been chelated. The addition of free Mg^{2+}, but not chelated-Mg^{2+}, permitted the growth of *B. cereus* on media containing polyphosphates. These results were in agreement with the work of various authors who demonstrated the importance of Mg^{2+} in cell division (425-428), membrane (432) and ribosome (129) stability, and peptidoglycan (140, 348) and teichoic acid (332) synthesis.

Various authors observed that polyphosphates inhibit Gram-positive bacteria much more than Gram-negative bacteria (62, 282, 340, 452). The results of this study agreed with these previous reports. One of the main differences between these two groups of bacteria is the metal-binding structures in their outer walls (34, 35, 94, 122). The Gram-positive cell wall relies on teichoic and/or teichuronic acids and peptidoglycan to retain metals scavenged from the environment (36, 37, 38), while the Gram-negative cell uses anionic groups in the LPS layer (122). The hypothesis was proposed that polyphosphates can inhibit Gram-positive bacteria because the polyphosphates remove metals from the Gram-positive cell wall. This is consistent with the fact that polyphosphates have a greater affinity for essential metal cations, such as Mg^{2+} (207, 229, 377), than unique cation-binding sites in the Gram-positive wall, such as teichoic or teichuronic acids (179, 237) or peptidoglycan (37, 38). The failure of orthophosphates to inhibit Gram-positive bacteria can be explained by their inability to form chelate complexes with essential metal ions. Thus, phosphatases naturally present in meats (18) would decrease the antimicrobial effects of polyphosphates by hydrolyzing them to relatively noninhibitory

orthophosphates. Research is needed to determine the rate of hydrolysis of various polyphosphates in meats and the effect this has on microbial inhibition. Research is also needed to prove that polyphosphates remove metal cations from isolated cell walls and that it is the removal of these metal cations in vivo which results in either loss of viability or inhibition of growth. In addition, it would be important to determine which of the metals are removed most efficiently by polyphosphates and which of those that are removed are most important to viability.

Conflicting reports have appeared in the literature concerning the heat resistance of *L. monocytogenes*. Most research has indicated that the microorganism would not survive the HTST pasteurization process given milk. These results led the World Health Organization to recently declare that the presence of *L. monocytogenes* in pasteurized milk was a postpasteurization contamination problem (443). However, a few workers previously recovered *L. monocytogenes* from pasteurized milk by using liquid enrichment techniques (95, 120, 121). The use of strictly anaerobic recovery conditions in the present study resulted in a 6-fold increase in the $D_{62.8^{\circ}C}$ value. *L. monocytogenes* probably did not grow under aerobic conditions because catalase and superoxide dismutase were inactivated during pasteurization (80) resulting in the accumulation of lethal levels of $O_2\cdot^-$, H_2O_2, and possibly the production of $\cdot OH$ (290, 304). The addition of catalase had a slight effect on recovery of heat-injured cells, but recoveries were significantly greater when strictly anaerobic techniques were used to enumerate viable cells. The failure of exogenously-added superoxide dismutase to enhance recovery may be explained by its failure to penetrate

the cell membrane and react with superoxide formed inside the cell (49). No difference was detected between the recovery of heat-injured *L. monocytogenes* incubated in air and in anaerobic jars. This can be explained by the strictly anaerobic nature (290) of *L. monocytogenes* after thermal treatment which resulted in the inactivation of CA and SOD activities. A definitive experiment is still required to demonstrate that the inactivation of catalase and/or superoxide dismutase is solely responsible for the O_2-sensitivity observed in the present study. It may be possible to induce high levels of CA and/or SOD activities prior to heat treatment and observe the effect on survival under aerobic conditions, compared with control (non-induced cells). Increased survival for those cells having higher CA and/or SOD activities would provide further support for this theory. In addition, it remains to be seen if one of these enzymes is more important to cell viability. This question might be resolved by selectively inducing the synthesis of CA or SOD or by the use of mutants that are defective in the synthesis of one of the enzymes.

Other factors besides strictly anaerobic recovery also increased the heat resistance of *L. monocytogenes*. Growth of the microorganism at high temperatures (43°C) caused a 2-fold increase in the $D_{62.8}$°C value, compared with growth at 37°C. These results agree with the work of White (442), El-Banna and Hurst (108), and Hurst et al. (201), who demonstrated that the heat resistance of other Gram-positive bacteria increased with time and increased temperature of incubation. The high heat resistance observed with 18-h (stationary phase) cells is consistent with the finding that glucose-starved cells were more heat resistant than cells that were not starved, but

had been heat-shocked at 42°C (218). Jenkins et al. (218) attributed this effect to the efficient synthesis of postexponential proteins during glucose starvation in the stationary phase of growth. The presence of stationary phase cells in the udder that had been growing at high temperatures is a possible because cows infected with *Listeria monocytogenes* have been reported to have fevers as high as 42.8°C (95). These infected cows also shed large numbers of *L. monocytogenes* in their milk (142). Further research should be conducted to determine which combination of incubation time and temperature would yield the greatest heat resistance. These cells could then be used in laboratory experiments to determine the minimum process required to ensure complete destruction of maximally heat-resistant cells of *L. monocytogenes*.

The increased heat resistance observed with nonhomogenized milk may be caused by the thermal protective effect of milk fat (44). Future research should involve adding cells to nonhomogenized milk to simulate natural contamination of milk in the infected udder of a cow before the milk is homogenized and then pasteurized. Increased heat resistance would also be expected in such high-fat products as ice cream and whipping cream.

The combined effects of strictly anaerobic recovery, high growth temperature, and the use of nonhomogenized milk would probably allow initially low levels of *L monocytogenes* to survive the current minimum HTST pasteurization process. Additional research is needed to determine the effects of these three variables on the heat resistance of *L. monocytogenes* at different temperatures, especially the minimum HTST temperature (71.7°C). This information is required to determine the correct z value for *L.*

monocytogenes in milk. This z value may be quite different from the current z value calculated by the FDA (52), because the current one was derived from the results of aerobic plate counts and thus would be based more on enzyme inactivation than on thermal destruction per se.

In addition to the effects of strictly anaerobic recovery, growth temperature, and nonhomogenized milk, the results of the present study point to other areas that need further research efforts. An effort should be made to determine if the location inside bovine milk phagocytes actually prevents heat-injured *L. monocytogenes* from contacting O_2 or toxic O_2-intermediates. The use of strictly anaerobic techniques may allow direct enumeration of other injured, facultatively anaerobic, foodborne pathogens on selective media. Finally, determining the second most critical thermal lesion in the cell may now be possible by using strictly anaerobic techniques.

Although the research on polyphosphates and the heat resistance of *L. monocytogenes* was conducted separately, the two projects are related. Garibaldi et al. (139) reported that the supplementation of egg white with EDTA at 7 mg/ml or with Kena, a commercial blend of polyphosphates containing 75% $Na_5P_3O_{10}$ and 25% $Na_4P_2O_7$ at 10 mg/ml, affected the heat resistance of *Salmonella typhimurium* Tm-1 and *Salmonella senftenberg* 775W at 52.5°C, reducing the time required to kill 90% of the population by a factor of 2 to 6. In contrast to other foods in which polyphosphates have been studied, egg whites have an alkaline pH which explains the effectiveness of polyphosphates in this product. Kohl et al. (234) and Kohl (233) patented a pasteurization process for egg whites in which 0.5%-0.75% sodium hexametophosphate (average chain length of 10-12 phosphorous

atoms) and pH adjust between 9.0 to 9.5 were claimed to destroy salmonellae at lower temperatures (52.5 - 55°C). Use of this process allowed the functional properties of egg whites to be retained while preventing subsequent microbial spoilage. The prevention of spoilage was attributed to the phosphate rather than the elevated pH.

The mechanism responsible for lowered heat resistance in the presence of phosphate may be the rapid loss of Mg^{2+} from cells upon heating (203). Hughes and Hurst (191) reported that EDTA prevented the repair of heat-injured *S. aureus* by chelating Mg^{2+} and thus suggested that heat damage is associated with two separate primary targets in *S. aureus*: the membrane, which is modified by loss of salt tolerance (200), and teichoic acids, manifested by the loss of D-ala and Mg^{2+} (203). Hoover and Gray (187) reported that cells that contained high levels of wall teichoic acid generally showed less injury than normal cells. Cells with the weaker cation-binding polymer, teichuronic acid, in the cell wall generally were generally more susceptible to heat injury. Sogin and Ordal (386) demonstrated that rRNA synthesis was required for the repair of *S. aureus* after sublethal heat injury. Hurst and Hughes (198) then demonstrated that cells of *S. aureus* heated at 52°C in Mg^{2+}-chelating buffers (pH 7.2, 50mM potassium phosphate) released 260-nm absorbing material shown to be RNA. Their ribosomes were also destroyed, and the cells did not regain salt tolerance in the presence of actinomycin D. Cells similarly heated in Mg^{2+}-conserving buffers did not leak RNA, suffered no ribosomal damage, and recovered in the presence of actinomycin D. Therefore, polyphosphates may cause sublethal heat injury by removing Mg^{2+} from the cell, which would

destabilize ribosomes (198). The addition of polyphosphates to foods might permit utilization of less-destructive heat treatments by decreasing the heat resistance of Gram-positive bacteria.

Part three of this dissertation speculated on the role of heat shock proteins (HSPs) in thermotolerance. Various authors have speculated that the function of HSPs is to prevent denaturation of (297) or actively cause renaturation of (334) critical, HSPs in the cell. The main impediment to defining the role of HSPs in thermotolerance has been the inability to define the critical, lethal, thermal lesion (258). The results of the present study indicate that, in the case of *L. monocytogenes*, that lesion is probably the inactivation of catalase and/or superoxide dismutase. Because of the heat-labile nature of catalase and superoxide dismutase (80), and the ability of these enzymes to detoxify lethal O_2-intermediates, one of the prime functions for HSPs in cells may be to prevent the inactivation of these enzymes.

Listeria monocytogenes could be a model organism for research on the biological function of HSPs. The microorganism is heat resistant and shows a heat shock response (present study), is facultatively anaerobic (371), and possesses significant catalase and superoxide dismutase activities (80). To test the hypothesis that HSPs induce thermotolerance, one can heat shock cells of *L. monocytogenes* and compare the loss of enzyme activities and viability during heating with nonheat shocked control cells. The effects of HSPs on pure enzymes could also be determined in vitro by adding HSP-enriched cell extracts or isolated heat shock proteins to solutions of pure enzymes and then assaying for enzyme activities during heating. The

combined results of these in vivo and in vitro tests might provide evidence that one of the roles of HSPs in thermotolerance is the stabilization of important enzyme activities.

HSPs undoubtedly evolved first in bacteria, but have remained very conserved throughout nature because of some universally important function. The use of *L. monocytogenes* to determine the role of HSPs in thermotolerance may lead to an understanding of their primary function in nature.

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