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Adaptation of Microorganisms to Cold Temperatures, Weak Acid Preservatives, Low pH, and Osmotic Stress: A Review

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ABSTRACT: The application of physical stress to microorganisms is the most widely used method to induce cell inactivation and promote food stability. To survive, microorganisms have evolved both physiological and genetic mechanisms to tolerate some extreme physical conditions. This is clearly of significance to the food industry in relation to survival of pathogens or spoilage organisms in food. In some microorganisms, the "cold shock response" has been observed in response to abrupt changes to lower temperatures. This results in the production of specific sets of proteins (cold shock proteins), the continued synthesis of proteins involved in transcription and translation, and the repression of heat shock proteins. The addition of weak acid preservatives (for example, sorbates, benzoates) also induces a specific pattern of gene expression (for example, 'Acid Tolerance Response'), which is likely to be required for optimal adaptation of bacteria to weak acid preservatives and low pH. The primary mode of the antimicrobial action of low pH is to reduce the internal cell pH (pHi) below the normal physiological range tolerated by the cell, leading to growth inhibition. Survival mechanisms involve maintaining pH homeostasis, and this is achieved by a combination of passive and active mechanisms. Microorganisms adapt to osmotic stress by accumulating non-ionic or compatible solutes such as trehalose, glycerol, sucrose, and mannitol. These compatible solutes help balance the osmotic pressure and help preserve protein function inside the cells. By understanding and controlling such mechanisms of adaptation, it may be possible to prevent growth of key microorganisms in food products.

Introduction

Microorganisms are adapted for optimum functioning in their normal physiological environments. Any extreme change in environmental conditions from the optimum inflicts a stress on an organism. The extent of the change will determine whether the organism is killed, ceases growth, or has an increased lag time and reduced growth rate (Ray 1986; Russell and others 1995). Most bacteria are able to tolerate small changes in an environmental parameter and can adapt over the time scale of minutes, hours, or days (Hill and others 1995). Microorganisms do this by both yielding to the stress conditions and making suitable provisions for survival or attempting to resist the stress (Herbert 1989). For most organisms, this tolerance can be pushed to maximum limits if the cell is provided with sufficient opportunity to sense and adapt to the deteriorating environment. Entire groups of microorganisms such as psychrophiles, acidophiles, and halophiles have adapted their lifestyles to prefer these extreme environments (Herbert 1986; Bower and Daeschel 1999). Psychrophiles are microorganisms that can grow at 0 °C and have an optimum growth temperature of 15 °C or less and a temperature maximum of around 20 °C (Prescott and others 1990). Acidophiles are microorganisms that have their growth optimum between about pH 1.0 and 5.5, and halophiles are microorganisms that require high levels of sodium chloride for growth such as 2.8 molal and up to 6.2 molal for extreme halophiles (Prescott and others 1990).

Changes in environmental conditions away from the optimal value can cause the induction of many elaborate stress responses. These strategies are generally directed at survival rather than growth.

The control of microorganisms is one of the most important aspects of food preservation. Bacterial destruction ensures food safety, but it also involves the application of more intense treatments that may cause additional food quality losses (Ray 1986). In many cases, bacterial destruction is not necessary for food preservation and controlling the environmental factors that affect viability can be sufficient to inhibit bacterial growth. In this case, the microorganisms will not be destroyed, but they will not be able to grow, and the preservation techniques used, which are much less intense, will affect food quality to a lesser extent. However, micro-

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organisms will still be metabolically active and viable if transferred to favorable conditions (Busta 1978; Abbiss 1983; Ray 1986). Preservation techniques are designed to prevent microbial growth in processed foods, by disrupting the internal environment of the cell such that growth is no longer possible.

Temperature, water activity, addition of preservatives, and pH are all parameters used to inhibit or destroy microorganisms and their spores and are also used as an aid for food preservation (Marechal and others 1999). Other parameters such as heat and bacteriostatic and bactericidal agents are also used in food preservation; they are not included in this review, but have been reviewed extensively (Lindquist 1986; Hendrick and Hartl 1993; Abee and Wouters 1999; Brul and Coote 1999). Some microorganisms have evolved and are capable of rapidly adapting to a continuously changing environment, thus developing tolerance or resistance to increased "doses" of particular stresses. Different types of organism possess different inherent resistances and susceptibilities to stress. For instance, Gram-negative bacteria are regarded as being more sensitive to cold shock, chilling, and freezing than Gram-positive bacteria (Straka and Stokes 1959). The microbial stress responses that have been selected during evolution include reactions to many of these preservation techniques and result principally in increased resistance to the applied stress. A result of common elements of the global stress and stationary phase responses that underlie many stress reactions is an increase in tolerance to other unrelated stresses (Gould 1999). Acid-adapted *Salmonella* Typhimurium, for example, have been found to exhibit increased resistance to heat and salt and thus have an increased tolerance to the parameters that are used to inhibit microbial growth in foods (Leyer and Johnson 1993). After exposure to physical stresses, it is necessary for the microorganism to maintain the physiology and operation of the cell. This is done by maintaining structural integrity of cell components such as proteins, membranes, and ribosomes and to keep important systems in the cell, such as protein synthesis, functioning (Berry and Foegeding 1997). Some microorganisms are able to alter their cell membrane fatty acid composition after a reduction in temperature, pH, or water activity (a_w) or in the presence of weak acid preservatives. This results in increased or decreased membrane fluidity, and allows the membrane to carry on its normal function under these stressful conditions (Russell and others 1995). Many organisms have also been shown to induce a specific pattern of gene expression, which is likely to be required for optimal adaptation to a particular stress (Berry and Foegeding 1997). The resistance or adaptation of microorganisms to such conditions will permit the growth of both spoilage and pathogenic microorganisms and thus have implications for microbial spoilage and safety of food products.

Low Temperature

Introduction

Storage temperature is one of the most important parameters regulating the activities of microorganisms in food systems. Because of the impact of temperature on all reactions of the cell, adaptation to fluctuations in temperature is possibly the most common response researched (Palumbo 1986; Gounot 1991; Berry and Foegeding 1997). However, the sensitivity of cells to cold stress is dependant on several factors including temperature, rate of cooling/freezing, culture medium, microbial strain, and duration of storage.

Growth of microorganisms at temperatures below the optimum for growth can cause a number of physiological and morphological changes as described in several reviews including Herbert (1986); Russell and others (1995); Berry and Foegeding (1997); Montville (1997), and Russell (2002). Changes to metabolic products can occur as a result of delays in enzyme activity (Witter and others 1966; Olson and Nottingham 1980). Reduced temperature during growth can also lead to metabolic imbalance and growth cessation due to the sensitivity of some metabolic regulatory processes (Busta 1978; Herbert 1986; Ray 1986; Abbiss 1983).

Incubation at low temperatures can also change the lipid composition of microbial cells. Both bacteria and yeasts have been reported to contain an increasing proportion of unsaturated fatty acids as the growth temperature decreases (Russell and others 1995; Berry and Foegeding 1997). This increase in the proportion of unsaturated fatty acids with decreasing temperature is believed to be essential for membrane function at low temperatures. When the temperature is lowered, some of the normally fluid components become gel-like, which prevents the proteins from functioning correctly, resulting in bacterial membrane leakage. However, if the membrane components change as described previously, allowing the membrane to retain fluidity as temperature decreases, then gel formation is prevented and the bacteria are still able to grow. Other responses to an abrupt reduction in temperature involve a pattern of gene expression, termed the "cold shock response." This involves the induction of cold shock proteins and cold acclimatization proteins, and the repression of heat shock proteins (Jones and others 1987; Berry and Foegeding 1997).

Modes of action of low-temperature stress

Temperature can influence the response of microorganisms either directly, by its effects on growth rate, enzyme activity, cell composition, and nutritional requirements, or indirectly by its effects on the solubility of solute molecules, ion transport and diffusion, osmotic effects on membranes, surface tension, and density (Herbert 1986).

As the temperature decreases, the lag phase before growth extends, the growth rate decreases, and the final cell numbers may decrease. During the lag phase, many physiological changes occur, including a decrease in the saturation of fatty acids and inhibition of DNA, RNA, and protein synthesis (Russell and others 1995). Growth of microorganisms at temperatures below their growth optimum can also cause a number of structural changes as shown in Table 1 (McCarthy 1991; Flanders and Donnelly 1994; Smith 1996).

An increase in production of pigments and other enzymatic activities are enhanced at low temperatures; for example, lipase and proteinase production by *Pseudomonas* and certain other genera occurs preferentially at low temperatures (Witter and others 1966; Olson and Nottingham 1980). Also, *Serratia marcescens* produces red pigments at a lower temperature (25 °C) compared with no red pigment produced at 37 °C.

Tolerance to reduced temperatures can also be observed after pretreatment of organisms to sublethal temperatures, which results in greater survival. For example, a pretreatment at 20 °C of *Streptococcus thermophilus*, resulted in a 1000-fold increase in survival after 4 freeze–thaw cycles compared with cells that were not pretreated (Wouters and others 1999). The prior temperature history of the cell has been found to be an important determinant of the survival and growth of organisms because of its effect on the length of lag phase before growth is initiated (Dufrenne and others 1997; Gay and Cerf 1997). Such results have consequences for predictive microbiology in that the length of the lag time of an organism within a food will depend on many other factors, including the temperature at which the organism was previously incubated. Bacteria are also likely to encounter a number of temperature shifts in a food-processing environment, and this will affect the lag time of the microorganism for the given conditions. For example, if a microorganism within a refrigerated food was derived from mammalian sources, it is likely that the lag time will be the maximum for the given conditions; however, if the organism was

Table 1—Examples of structural changes during growth at low temperature

Structural change	Examples	References
Increase in cell size	*Candida utlis*	Rose (1968); Olson and Nottingham (1980); Herbert (1986)
Filament formation	*Escherichia coli*; *Pseudomonas putida;* *Salmonella enteritidis* PT4 strain E isolate	Shaw (1968), Jensen and Woolfolk (1985), Phillips and others (1998)
Mesosome deterioration	*Bacillus subtilis*	Neale and Chapman 1970
Formation of a double cell wall	*Bacillus subtilis*	Neale and Chapman 1970

derived from a chilled environment, then the lag time is likely to be the minimum for the given conditions. Thus the source and the physiological state of the organism will need to be estimated before predictions on lag time can be given.

Cell membrane response to low temperature

Cell membranes are complex heterogeneous systems whose properties are to a large extent determined by their composition and spatial organization as well as by external influences, of which temperature is one of the most important (Alberts and others 1994).

Effect of temperature on solute uptake. Evidence over the years has shown that in order for microorganisms such as psychrotrophs to grow rapidly at reduced temperatures, they must be able to effectively transport soluble molecules across the cytoplasmic membrane (Herbert 1989). Wilkins and others (1972) and Wilkins (1973) found that a cold-resistant sugar transport system was present that provided high concentrations of intracellular substrates to stimulate growth of psychrotrophic *Listeria monocytogenes*.

Herbert (1986, 1989) describes the findings of Baxter and Gibbons (1962) who compared the metabolic activity of a psychrotrophic *Candida* species and a mesophilic strain of *Candida lipolytica* at different temperatures. The psychrotroph was able to oxidize exogenous glucose at 0 °C, whereas the mesophile showed no metabolic activity at temperatures less than 5 °C. However, the mesophile was able to metabolize endogenously at temperatures less than 5 °C, suggesting that the limiting factor was the ability to transport sugars into the cell at temperatures near 0 °C. In contrast, other investigations have shown substrate uptake as being largely independent of temperature (Herbert and Bell 1977; Herbert 1989). Several theories suggested by Farrell and Rose (1967) to explain differences between the abilities of psychrophiles/psychrotrophs and mesophiles to transport solutes into the cell at reduced temperatures have been reviewed by Herbert (1986, 1989) and include the following: membrane permeases of psychrophiles are less sensitive to low-temperature inactivation than those of mesophiles; permeases in mesophiles are not cold-sensitive, but membrane lipid bilayer changes render them unable to bind to substrate; and at low temperatures, there is insufficient energy in mesophiles to allow active transport across the membranes (Herbert 1986, 1989).

Temperature-induced changes in fatty acid composition. Within the cell membrane, the phospholipid molecules are arranged in the form of a bilayer with the polar head groups at the intracellular and extracellular surfaces. These groups are thus able to interact with the aqueous phases on the inside and outside of the cell (Neidhart and others 1990; Alberts and others 1994). The fatty acid acyl chains, in contrast, are stacked in parallel at right angles to the plane of the membrane, with the terminal methyl groups situated in the interior of the bilayer (Alberts and others 1994). It is well documented that microorganisms adjust their membrane lipid composition in response to changes in growth temperature to ensure membrane function such as enzyme activity and solute transport (Brown and Minnikin 1973; Russell 1984; Tsuchiya and others 1987; Russell and others 1995; Mastronicolis and others 1998). Generally during low-temperature growth, the fatty acid composition of phospholipids and glycolipids are changed most commonly because the fluidity of the membrane is altered much more effectively by changes in fatty acid structure than in the head group (Russell and others 1995).

For the cell to function normally, the membrane lipid bilayers need to be largely fluid so that the membrane proteins can continue to pump ions, take up nutrients, and perform respiration (Berry and Foegeding 1997). Therefore, it is essential that the membrane lipids are in the liquid crystalline state. When the growth temperature of a microorganism is reduced, some of the normally fluid components become gel-like, which prevents the proteins functioning normally; therefore, for these components to remain fluid, a number of changes in the pattern of fatty acids must occur.

Unsaturation of fatty acid chains is the most commonly found change that occurs when the temperature is reduced; this increases the fluidity of the membrane because unsaturated fatty acid groups create more disturbance to the membrane than saturated chains and is achieved by desaturases situated in the membrane itself and thus are able to react quickly. For example, *Clostridium botulinum* has an increased level of unsaturation, from 27% to 40%, after a reduction in temperature from 37 °C to 8 °C (Russell and others 1995). Increased fatty acid unsaturation in *Aspergillus niger, Neurospora crassa, Penicillium chrysogenum*, and *Trichoderma reesei* is also observed at reduced temperature (Suutari 1995). There are, however, a number of other alterations that can occur after a fall in temperature (Russell 1984). The average fatty acid chain length may be shortened, which would have the effect of increasing the fluidity of the cell membrane because there are fewer carbon-carbon interactions between neighboring chains (Russell 1990). A psychrophilic organism, *Micrococcus cryophilus* for example, which contains high proportions of unsaturated fatty acids under all growth conditions, responds to a decrease in temperature, from 20 °C to 0 °C by a reduction in the average chain length of the fatty acids (McGibbon and Russell 1983). The fatty acid mean chain length of the yeast *Zygosaccharomyces bailii* also decreases at low temperature (Baleiras-Couto and Huisin't-Veld 1995). After a fall in temperature, an increase in the amount and/or kind of branched fatty acids may also occur. There may be a reduction in the proportion of cyclic fatty acids and thus an increase in mono-unsaturated straight chain fatty acids as shown with *Salmonella* spp. (Russell 1984) and *C. botulinum* (Russell and others 1995; Evans and others 1998). This again increases the fluidity of the membrane because a double bond creates more disturbances to the packing of the fatty acid chains in the bilayer than does a cyclopropane ring (Russell and others 1995). All these changes, as summarized in Table 2, result in the "membrane maintaining its fluidity, by producing lipids with a lower gel to liquid crystalline transition temperature, by incorporating proportionally more low melting point fatty acids into membrane lipids" (Russell 1984). This allows the membrane to function normally and therefore retain its ability to regulate the activity of solute transport systems and the function of essential membrane bound enzymes (Berry and Foegeding 1997) and also com-

Table 2—Changes in fatty acid composition at low temperatures

Changes in fatty acid composition	Aim	References
Unsaturation of fatty acids Modifications to ante-iso/iso branching patterns Shortening in the fatty acid chain length	Maintain membrane fluidity	Russell and others (1995), Russell 2002

pensates for the decreased growth temperature.

The effects of reduced temperature on the membrane fatty acid composition of *L. monocytogenes* has been studied extensively (Püettman and others 1993; Russell and others 1995; Annous and others 1997; Mastronicolis and others 1998). The main change observed when the temperature is reduced to below optimum (for example, 7 °C) is an increase in the proportion of $C_{15:0}$ at the expense of $C_{17:0}$ (Russell and others 1995). Such a shortening in the fatty acid chain length will lower their melting temperature and hence help to maintain membrane fluidity at a lower temperature (Russell and others 1995). There is also a small increase in $C_{18:1}$, and this increase in unsaturation will contribute to fluidizing the membrane at lower temperature (Russell and others 1995). Six species of *Listeria,* including *L. monocytogenes*, also responded to low temperature by increasing the proportion of $C_{15:0}$ (Püettman and others 1993). Annous and others (1997) also found that $C_{15:0}$ has a critical role in the growth of *L. monocytogenes* at cold temperatures, presumably through its physical properties and its effects in maintaining a fluid, liquid-crystalline state of the membrane lipids.

Other microorganisms with similar responses include *Pseudomonas fluorescens* (Gill 1975), *Escherichia coli* ML30 (Marr and Ingraham 1962), *Lactobacillus plantarum* (Russell and others 1995), and *Yersinia enterocolitica* (Abbas and Card 1980; Tsuchiya and others 1987; Goverde and others 1994) that respond to low temperatures by synthesizing increased proportions of unsaturated fatty acids at the expense of saturated fatty acids. Fatty acid composition has also been studied in *Serratia* species (Kates and Hagen 1964), *E. coli* (Marr and Ingraham 1962), marine pseudomonads (Brown and Minnikin 1973), and yeasts such as *Candida utilis* (Brown and Rose 1969) and *Saccharomyces cerevisiae* (Hunter and Rose 1972).

The mechanisms by which changes in fatty acid composition are brought about in response to low temperature and the different levels at which regulation of membrane lipid modifications occur, has been studied in some detail; however, such mechanisms are complex (Fulco 1970; Clarke 1981; Russell 1984) and cannot be described in detail in this review. Mechanisms are reviewed in Herbert (1986, 1989) and Russell (1990).

Klein and others (1999) studied the response of *Bacillus subtilis* to reduction in temperature from 37 °C to 15 °C. During cold shock, *B. subtilis* changed its membrane composition by increasing the anteiso branched fatty acid content and decreasing the iso-branched content. However, a strain that was isoleucine-deficient was unable to synthesize more anteiso branched fatty acids after a reduction in temperature, suggesting the importance of isoleucine for the survival of *B. subtilis* to reductions in temperature from 37 °C to 15 °C.

Other effects of reduced temperature. To compensate for reduced metabolic activity at low temperatures, it has been suggested that psychrophiles also synthesize elevated levels of enzymes (Herbert 1989). At low temperatures, the high specific enzyme activity of psychrophilic microorganisms has been thought to be due to a looser, more flexible conformational structure of the protein (Jaenicke 1990; Davail and others 1994; Berry and Foegeding 1997). Because of the ability of psychrophiles to produce cold-adapted enzymes such as β-galactosidase (Nakagawa and others 2003), which exhibit high catalytic activities at low temperature, much interest has been received of such cold-adapted enzymes for use in food processing, such as for processing fruit juices and milk, under low-temperature conditions rather than at ambient temperatures. This will help prevent spoilage, which may occur if produced at ambient temperatures.

Effects of low-temperature stress on gene expression: the cold shock response

Jones and others (1987) made the initial discovery of the cold shock response in *E. coli* and have described the response as a specific pattern of gene expression in response to abrupt shifts to lower temperature. This pattern is now known to include the induction of cold shock proteins, continued synthesis of proteins involved in transcription and translation (Jones and others 1992), and the repression of heat shock proteins (Jones and others 1987; Berry and Foegeding 1997). Transcription is the process in which single-stranded messenger RNA (mRNA) with a base sequence complementary to the template strand of DNA or RNA is synthesized. Translation is the process whereby the genetic message carried by the mRNA directs the synthesis of polypeptides with the aid of ribosomes and other cell components (protein synthesis).

The function of the cold shock response is not known, although Jones and Inouye (1994) suggested that the function may be to overcome the partial block in protein synthesis, the process whereby the genetic message carried by the mRNA directs the synthesis of polypeptides with the aid of ribosomes and other cell components (translation), thereby increasing the translational capacity of the cell and vice versa. The cold shock proteins are thought to do this by binding to the RNA during transcription and facilitating initiation of translation. Yamanaka (1999) reviewed how *E. coli* responds to cold shock and the function of the cold shock proteins. Mihoub and others (2003) have also looked at cold adaptation of *E. coli* using proteomic approaches, which showed changes in the expressions of proteins resulting from the cold adaptation.

Within the normal temperature range, 20 °C to 37 °C, the levels of proteins did not vary for *E. coli*, and any temperature shifts within this range resulted in the same characteristic growth rate (Jones and Inouye 1994). This suggests that cellular composition is similar within this growth range for *E. coli*. When a temperature shift from the normal range to a temperature below 20 °C occurred, changes in cell physiology were observed. Jones and Inouye (1994) suggested that the biochemical composition of the cells at the lower temperature range was probably very different from that at the normal temperature range. This cold shock response occurs when *E. coli* growing at 37 °C is transferred to 10 °C, and reaches its maximum induction after 3 h; but after 4 h, protein synthesis and growth resumed resulting in a 4-h growth lag phase (Jones and others 1987). Within this study, the cold shock response occurred with every 13 °C decrease in temperature; however, the greater the magnitude of the drop in temperature, the greater was the effect (Jones and Inouye 1994).

During the lag period, called the acclimation phase, many physiological changes occur, including a decrease in saturation of fatty acids, the induction of some 16 proteins called the cold shock proteins (Csp's) and the specific repression of heat shock

Table 3—Proteins involved in the cold shock response of *Escherichia coli*

Cold shock protein	Comments	Reference
Protein RecA	Role in recombination and the induction of the SOS response.	Berry and Foegeding (1997)
Hsc66 (70-kDa heat shock protein)	Thought to act as a molecular chaperone in the cold shock response. To ensure the conformation of proteins and refolding of denatured proteins occurs correctly.	Lelivelt and Kawula (1995)
CspA (70 amino acid protein encoded by the *cspA* gene)	Designated the major cold shock protein. It has a high induction level, increasing 200-fold after a reduction from 37 °C to 10 °C, and reaches 13% of total protein synthesis within 1.5 h after the shift in temperature. CspA is a transcriptional regulator, which recognizes gene promoters and switches them on, thus producing cold shock proteins (Csp's)	Jones and others 1987, Inouye 1994
CspB, CspG	Cold shock inducible. The temperature dependence of CspB and CspG induction is restricted to low temperature ranges, compared with that of CspA. and that, as for CspA, CspB,CspG, induction, is transient during the lag phase upon cold shock.	Etchegaray and Inouye (1999)
CspI	CspI belongs to the same group as CspA, CspB, and CspG, all of which are cold shock inducible. As with CspA, it is suggested that CspI may also bind to RNA and single-stranded (ss)DNA.	Wang and others (1999)

proteins. The identified cold shock proteins are a set of proteins involved in various cellular processes that are made at differential rates 2 to 10 times greater following a shift to 10 °C than at 37 °C (Jones and others 1987; Jones and Inouye 1994).

A wide range of proteins involved in cellular processes are included in the cold shock response of *E. coli* and are reviewed in Jones and others (1987); Goldstein and others (1990); Tanabe and others (1992); Jones and Inouye (1994); and Berry and Foegeding (1997), and some are described in Table 3.

The cold shock protein of *E. coli* (CspA) has a sequence similarity with proteins from other organisms such as *B. subtilis* and *Streptomyces clavuligerus. B. subtilis* cold shock protein CspB has 61% homology with *E. coli* CspA (Willimsky and others 1992; Graumann and Marahiel 1999a, 1999b) and a 7.0 kDa protein from *S. clavuligerus* has a 56% identity to *E. coli* CspA (Jones and Inouye 1994).

In addition to a reduction in temperature resulting in the expression of cold shock proteins, continued synthesis of many of the components of the transcriptional and translational machinery also occur (Jones and others 1992). Normally when growth is prevented, proteins that are involved in transcription and translation are repressed. Jones and others (1992) found that when *E. coli* was subjected to temperatures of 10 °C during lag phase, these proteins were still synthesized. The synthesis of cold shock proteins is affected by regulators including guanosine 5'triphosphate-3'diphosphate [(p)ppGpp] and guanosine 5'diphosphate-3'diphosphate (ppGpp) and variations in the levels of these regulators have been observed at reduced temperatures. In *E. coli*, a reduction in temperature results in a decrease in the (p)ppGpp level. This decrease is proportional to the range of temperature shift: the larger the fall in temperature, the larger the decrease in (p)ppGpp. Jones and others (1992) found that increasing the level of (p)ppGpp level before a downshift in temperature, by overproduction of the enzyme that catalyses the synthesis of (p)ppGpp or by subjecting the bacteria to a nutritional downshift, resulted in a decreased induction of many cold shock proteins and decreased synthesis of transcriptional and translational proteins. They also found that moving a mutant that did not contain a detectable level of (p)ppGpp to 10 °C resulted in a higher induction of many cold shock proteins and increased synthesis of transcriptional and translational proteins. Jones and others (1992) therefore suggested that the decrease in (p)ppGpp level positively affects the synthesis of both transcriptional and translational proteins and cold shock proteins and thus is part of an adaptive response. This suggests that, following a reduction in temperature, the decreased translational capacity of the cell triggers the decrease in the (p)ppGpp level with the corresponding changes in gene expression (Jones and others 1992).

It is known that chemicals such as chloramphenicol and tetracycline, which affect bacterial ribosomes, also result in the induction of cold shock response (Van Bogelen and Neidhart 1990; Jones and Inouye 1994). These authors also showed that a decrease in (p)ppGpp level occurs in the presence of many of the inhibitors of translation that induce the cold shock response. This observation shows consistency with the proposed involvement of the (p)ppGpp level in the cold shock response. This observation, combined with the observation that moving cells to a lower temperature causes inhibition of protein synthesis, led to the proposal that the state of the ribosome is the physiological sensor for the induction of the response (Van Bogelen and Neidhart 1990).

Cold shock responses have been found in other microorganisms including *S. cerevisiae* (Kondo and Inouye 1991), *Bacillus psychrophilus* (Whyte and Inniss 1992), *Pseudomonas fragi* (Berry and Foegeding 1997), *Bacillus cereus* (Berry 1996; Mayr and others 1996), *Trichosporon pullulans* (Julseth and Inniss 1990), *Listeria* sp. (Phan-Thanh and Gormon 1995; Bayles and others 1996), *Lactococcus lactis* subsp. *lactis* (Panoff and others 1994), *B. subtilis* (Graumann and Marahiel 1994, 1999a, 1999b; Lottering 1994; Graumann and others 1996), and *Vibrio vulnificus* (McGovern and Oliver 1995).

The cold shock response can involve the expression of up to 50 different cold shock proteins depending on the species, as well as the rate and extent of temperature drop. *P. fragi*, for example, produces 15 cold shock proteins on decreasing from 20 °C to 5 °C and 24 Csps when reduced from 30 °C to 5 °C (Russell 2002). The reason for the increase in cold shock proteins produced is not clear but may be to help to overcome the added stress.

Hebraud and Potier (1999) describe the ability of psychrotrophic bacteria to grow at low temperatures close to freezing. The cold shock response described in several psychrotrophic bacteria is described as being different from that of mesophilic bacteria. The synthesis of the housekeeping proteins is not repressed after a reduction in temperature, and thus are expressed at both optimal and reduced temperatures. Cold shock proteins are also synthesized and the larger the severity of the shock, the more Csps are produced. A second group of cold-induced proteins, the cold acclimation proteins (caps) has also been described that are compa-

rable to Csps and are continuously synthesized during prolonged growth at low temperatures, and differentiate psychrotrophs from mesophiles.

Mizushima and others (1997) found that subjecting *E. coli* to cold shock increased negative supercoiling in the cells. DNA in cells is negatively supercoiled, that is, the coiling of the double-stranded DNA helix around itself. The extent of DNA supercoiling has been observed to change in response to various environmental factors such as changes in osmolarity (Higgins and others 1988) and heat shock (Mizushima and others 1997).

In summary, the cold shock response is a specific pattern of gene expression in response to abrupt changes to lower temperatures. One effect of reducing temperature is to block initiation of protein synthesis. Cold shock proteins can stabilize mRNA and re-initiate protein production. Others are also linked to maintaining the fluidity of the membrane such as inducible desaturases.

Conclusions

Modification of membrane lipid composition with temperature reduction is clearly an important adaptation in some microorganisms, which allows them to grow at low temperatures. Other microorganisms show no need to alter membrane fatty acid composition, suggesting that it is not a primary factor in determining minimum growth temperature, but it does serve as an important adaptive function, which offers a selective advantage. Upon an abrupt reduction in temperature, *E. coli* and other organisms have been shown to induce a specific pattern of gene expression, which is likely to be required for optimal adaptation to low temperature. In *E. coli*, temperature reduction appears to influence ribosomal activity and other physiological changes, which lead to preferential synthesis of proteins involved in various cellular functions from supercoiling of DNA to initiation of translation—the cold shock response. Such stress responses can play an important role as to the organisms survival in a particular product and thus can affect the shelf-life of a product.

Information on the stress response after temperature reduction will help the food industry to understand how organisms can behave in their products and how the extent of the stress responses can be affected by pre-storage temperatures. Different cold shock treatments before freezing or chilling can result in differences in microbial survival and growth. This might result in higher survival and growth rate of microorganisms in low-temperature chilled and frozen products resulting in shorter lag times and thus shorter shelf-life. A greater understanding of the mechanisms of cold adaptation may offer insight into methods for controlling growth of psychotropic microorganisms in chilled and frozen foods.

Weak Acid Preservatives

Introduction

Weak lipophilic acids can occur naturally in many fruits and vegetables and have been widely used to maintain microbial stability in low pH foods including fruit juices, beverages, wines, pickled vegetables, mayonnaise, and salad dressings (Sofos and Busta 1981; Restaino and others 1982; Pilkington and Rose 1988; Seymour 1998). Spoilage of such foods is most often caused by yeasts, molds, and lactic acid bacteria, since environmental conditions in these foods generally inhibit the growth of bacteria (Beuchat 1982). Weak acid preservatives affect the cells' ability to maintain pH homeostasis, disrupting substrate transport and inhibiting metabolic pathways. Most of these acids behave primarily as fungistatic agents, whereas others are more effective at inhibiting bacterial growth. The addition of weak acid preservatives to low pH foods provides microorganisms with a further hurdle to overcome in order to grow. However, such environments may encourage osmophilic yeasts such as *Zygosaccharomyces rouxii* and *Z. bailii* to grow, which will cause food spoilage despite the maximum permitted level of preservative and good manufacturing practice (Pitt 1974; Thomas and Davenport 1985; Golden and Beuchat 1992a, 1992b). However, in some instances, *Z. bailii* can tolerate preservative concentrations in excess of those legally permitted (Splittstoesser and others 1978; Neves and others 1994).

Lowering the pH value of the suspending medium increases the antimicrobial effect of weak acid preservatives. When exposed to a mild concentration of a weak acid preservative, microorganisms may adapt to the stress, thus developing tolerance or resistance to stronger doses of the weak acid stress (Bills and others 1982; Warth 1985, 1988; Goodson and Rowbury 1989a, 1989b). Some organisms with resistance to one preservative such as benzoate, were also resistant to others such as sorbate and acetate (Fleet 1992).

Phenotypically acquired resistance to lipophilic acid preservatives is well documented for yeasts, and this results from the enhanced ability of adapted cells to catalyze energy-dependent extrusion of the acids (Warth 1977, 1985). No such mechanism has been claimed with bacteria. Unlike many of the yeasts, stress response mechanisms in bacteria produced in the presence of weak acid preservatives allow the bacteria to be able to survive in the harsh environment rather than grow (Nikaido and Varra 1985).

Mode of action of weak acid preservatives

Minimum inhibitory concentrations reported to inactivate various microorganisms may vary considerably (Eklund 1989; Kabara and Eklund 1991). In principle, growth inhibition can be caused by inactivation of, or interference with, the cell membrane, cell wall, metabolic enzymes, protein synthesis system, or genetic material (Eklund 1989). The antimicrobial action of sorbate, for example, is based on the inhibition of metabolic enzymes involved with the metabolism of carbohydrates such as fumerase and aspartase. The sorbic acid binds covalently with the sulphydryl groups of the enzymes and inactivates this part of the enzyme (Russell 1982; Eklund 1989). It may also be that more than one target is involved and that growth inhibition may be a result of the combined load on the cell (Eklund 1989). Warth (1985) reported that weak acid preservatives may also affect the cell yield, ATP levels, and the cells' ability to maintain pH homeostasis, therefore disrupting substrate transport and oxidative phosphorylation. This may account for their effectiveness in preventing the growth of many sensitive bacteria and yeasts under some conditions (Seymour 1998).

Generally, Gram-negative bacteria are more resistant to weak acid preservatives than are Gram-positive bacteria (Russell 1991). One reason for this is to be found in the different structural and chemical composition of the outer layers of the cells (Nikaido and Varra 1985).

Effect of pH on the antimicrobial action of weak acid preservatives. The antimicrobial effect of weak acids is both pH-dependent and non–pH dependent, although this has been long established to be more active in an acid than in a neutral environment (Macris 1975; Eklund 1983). Acids generally inhibit molecular reactions essential to the microorganisms by increasing the hydrogen ion concentration, which results in a decrease in internal pH (pH_i) (Brown and Booth 1991). This fall in pH_i is a major cause of growth inhibition by weak acids (Seymour 1998). The pH of the environment and the dissociation constant (pKa) of the weak acid determine the proportion of the hydrophobic (undissociated) form in the medium and thus the effectiveness of the weak acid (Brown and Booth 1989; Vasseur and others 1999). The strength of an acid is defined by its dissociation constant (pKa). This is the pH value when the dissociated and undissociated forms of the

acid are in equal amounts. Strong acids such as hydrochloric acid have a much lower pKa value than weak acids. Thus at a pH of between 3 and 6, the pH range for normal food, strong acids will be dissociated whereas weak acids will be undissociated. This latter form is membrane-permeable and thus allows the weak acid to enter the microbial cell (Booth and Kroll 1989). Once inside the cell, weak acids generally encounter a higher pH due to the cell buffers, dissociate, and become toxic, which ultimately inhibits cell growth due to the acidification of the cell interior (Krebs and others 1983; Warth 1985). Therefore the lower the pH value, the greater the proportion of the acid in the undissociated form and thus the greater the antimicrobial effect (Beuchat 1981; Sofos and Busta 1981).

Recent studies with sorbic, benzoic, and propionic acids investigated the antimicrobial effects of dissociated and undissociated forms (Eklund 1983, 1985a, 1985b). Here the undissociated form of sorbate ($pK_a = 4.74$) was 10 to 600 times greater at inhibiting microbial growth than was the dissociated acid, but dissociated acids also possessed antibacterial activity, which was a major factor in the growth inhibition at pH 6 and above (Eklund 1983).

Salmond and others (1984) also showed that growth inhibition is predominantly due to the undissociated form of the acid, even though both the undissociated and dissociated forms result in a drop in internal pH (pH_i). In addition, they suggested that sorbic acid affects the proton-motive force in *E. coli* and accelerates the movement of hydrogen ions from low pH media into the cytoplasm and indicated that for *E. coli*, acidification of the cytoplasm to pH 6 is sufficient to prevent growth.

Bacteria and yeasts usually maintain their pH_i around neutrality (Salmond and others 1984; Serrano 1991), which is essential for optimal activity of many important cellular processes including the activity of a number of enzymes (Sofos and Busta 1981), the efficiency of contractile elements, and the conductivity of ion channels (Madshus 1988). Changes in pH_i also seem to be important in controlling the cell cycle (Anand and Prasad 1989), and rates of DNA and RNA synthesis appear to increase with higher pH_i within the normal physiological range (Madshus 1988).

Effects of weak acid stress on gene expression

As discussed earlier, the primary mode of action of weak acid preservatives is to reduce pH_i below the normal physiological range, which results in an extension to the lag phase and inhibition of growth (Restaino and others 1982). Fungi have developed mechanisms to counteract the effect of weak acids and maintain pH_i homeostasis (Figure 1).

Weak acid stress response in yeasts. The resistance of *Z. bailii* to weak acids is believed to be due to the induction of an energy-requiring system for transport of the preservative anion from the cell (Warth 1977). It is now known that to counteract the effect of weak acids and maintain pH_i homeostasis, yeasts have developed systems that are dependent on the plasma membrane H^+-adenosine triphosphatase (ATPase) (Serrano and others 1986; Serrano 1988; Warth 1989; Verduyn and others 1992), a member of a family of cation-translocating ATPase's found in a number of yeast and fungal species (Serrano 1991; Sigler and Höfer 1991). An extensive review of this area is found in Seymour (1998), who reported that the activity of the H^+-ATPase results in ATP hydrolysis extruding protons and therefore reducing the intracellular pH, thus retaining favorable internal conditions in the cell.

Plasma-membrane H^+-ATPase in yeasts. The H^+-translocating ATPase of the yeast plasma membrane is an important system involved in pH homeostasis, which mediates growth responses through the regulation of pH_i (Serrano and others 1986; Seymour 1998). Thus, if the principle inhibitory effect of weak acids is to reduce pH_i, a component of any potential mechanism of resistance to weak acid preservatives could involve the proton-translocating H^+-ATPase.

Holyoak and others (1996) report results that support this hypothesis that H^+-ATPase plays a critical and energy-demanding role in adaptation to weak acid stress. Results suggested that in the presence of sorbate, the membrane H^+-ATPase activity increased. This is due to activation of H^+-ATPase in response to reduced pH_i, which is known to occur in the presence of sorbic acid. It was further reported that H^+-ATPase activity is required for optimal adaptation to sorbic acid stress when a strain with reduced levels of H^+-ATPase displayed significantly extended lag phases in response to sorbic acid stress (Holyoak and others 1996).

The antimicrobial effect of sorbic acid was synergistically increased by the presence of compounds that inhibited the plasma membrane H^+-ATPase (Kubo and Lee 1998). This again supports the hypothesis that H^+-ATPase plays a critical role in adaptation to weak acid stress.

Several stresses have been studied and found to stimulate H^+-ATPase activity. These include heat shock, ethanol, low pH, and weak lipophilic acids that act to cause a reduction in pH_i by depleting the proton motive force across the plasma membrane (Eraso and others 1987; Coote and others 1991; Fernanda Rosa and Sá-Correia 1991; Viegas and Sá-Correia 1991; Eraso and Portillo 1994). Such a reduction in internal pH requires an increased ATPase activity to restore homeostasis, which exerts a heavy energy load on the cell. During normal growth, the H^+-ATPase is estimated to use 10% to 15% of the total ATP produced. However, during the increased ATPase activity required to restore pH homeostasis, 40% to 60% of the total cellular ATP can be consumed (Serrano 1991; Holyoak and others 1996). Therefore, in the pres-

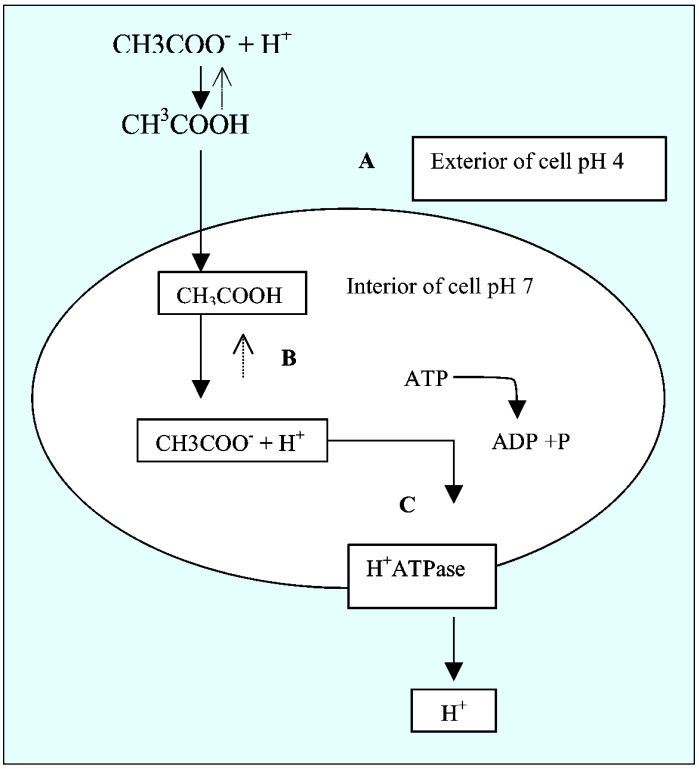

Figure 1—Interaction of weak acids on the microbial cell. (a), Exterior of the cell favors undissociated weak acid; (b), on entering the cell, the interior of the cell favors dissociated molecular and the acid dissociates; (c), proton pumps remove excess H+ ions and use energy.

ence of weak acid preservatives, cellular ATP levels may be reduced to such an extent that growth rate is reduced (Viegas and Sá-Correia 1991; Holyoak and others 1996; Piper and others 1997; Seymour 1998).

Piper and others (1997, 1998) showed that 2 plasma membrane proteins, Hsp30 and Pdr12, were also involved in the long-term stress response of yeasts to weak acid preservatives at low culture pH.

Hsp30 is a highly hydrophobic integral membrane heat shock protein that has been shown to be induced by heat shock, ethanol, and weak acid preservatives (Panaretou and Piper 1990; 1992; Piper and others 1994, 1997). Detailed studies on an *hsp30* gene in a mutant yeast and its wild-type parent (Piper and others 1997) revealed that Hsp30 was acting as a "molecular switch." This led to down-regulation of the stress stimulation of the H^+-ATPase and suggested that the role of Hsp30 may be to conserve the cellular ATP levels under long-term stress, which would have been consumed by the enzyme while attempting to restore homeostasis. Thus, the cell must be able to balance the need for H^+-ATPase activity for maintaining homeostasis and the need for appropriate ATP levels for growth in order to adapt to growth under weak acid stress (Braley and Piper 1997).

Henriques and others (1997) showed evidence of an efflux system that removes accumulated anions from inside the cell. This has been further supported by Piper and others (1998), who demonstrated the existence of a large membrane protein of approximately 170 kDa in sorbate-adapted cells of *S. cerevisiae*. This has been identified as a multidrug resistance pump, the ATP-binding cassette (ABC) transporter Pdr12, which is believed to confer resistance by actively extruding preservative anions from the cell. In the absence of weak acid stress, yeast cells grown at pH 7 express low levels of Pdr12. However, sorbate treatment caused a dramatic increase in Pdr12. Pdr12 is believed to be essential for the adaptation of yeasts to growth under weak acid stress (Piper and others 1998). Brul and Coote (1999) describe how such a mechanism of pumping preservative anions could be a futile process because the anions will reassociate and reenter the cell. However, they go on further to show that adapted yeasts actually reduce the diffusion coefficient of preservatives across the membrane, resulting in a reduced ability of weak acids to enter the cell. This suggests that if there was a reduction in the ability of compounds to diffuse across the membrane, efflux of protons and anions by H^+-ATPase and Pdr12 would not be futile.

The weak acid stress response in bacteria. Unlike yeasts, very little information is available concerning the resistance of bacteria to organic acids. However, several bacteria such as *S.* Typhimurium and *E. coli* often encounter acidic pH environments that also contain organic acids and can survive these potentially lethal situations (Goodson and Rowbury 1989a; Baik and others 1996; Greenway and England 1999). Studies using acetic, citric, and lactic acid at concentrations of up to 1.5% as organic sprays on beef revealed that *E. coli* O157:H7 populations were not appreciably affected by the treatments and thus were tolerant to acidic environments. The mechanism of acid tolerance has not been fully elucidated but appears to be associated with a protein or proteins that can be induced by pre-exposing the bacteria to acidic conditions (Brackett and others 1994).

Escherichia coli possesses several systems of acid resistance that can be classified into 2 general categories on the basis of whether a system can be induced and function in an unsupplemented minimal medium (acid-tolerance response [ATR] systems) or requires some form of supplementation (acid-resistance [AR] mechanisms). Three distinct AR systems are identified for *E. coli* and *Shigella flexneri* (Lin and others 1995). One system is oxidative-dependent AR, expressed in oxidatively metabolizing bacteria grown in a complex medium but which also protects the cells in minimal medium to pH 2.5. This system, however, is not apparent in cells grown in complex medium containing glucose. The other 2 systems of acid survival become evident under these conditions and will also protect against pH 2.5 in minimal medium but only if supplemented with arginine (arginine-dependant AR) or glutamate (glutamate-dependant AR). The mechanisms by which these amino acids protect *E. coli* and *S. flexneri* from acid could involve precursors of some molecule, such as an amine, which because of its alkaline nature would counteract the effect on intracellular pH and protect the interior of the cell from acid stress (Lin and others 1995).

The presence of weak organic acids hastens the death of organisms during an acid challenge. However, all strains of *E. coli* tested, including enterohaemorrhagic strains, could effectively use the glutamate-dependent AR system to ward off the lethal effects of weak acids (Lin and others 1995); however, the oxidative-dependent and arginine-dependent AR systems were not very effective at combating benzoate stress.

Salmonella Typhimurium does not appear to possess these complex medium-dependent AR systems. However, ATR system could potentially combat organic-acid stressed *S.* Typhimurium and is discussed in Lin and others (1995). The ATR is a complex stress-response system that enables *S.* Typhimurium to survive brief encounters with extreme acid environments as low as pH 3 (Foster and Spector 1995; Baik and others 1996). There are ATR systems induced in exponential and stationary-phase cells. The exponential-phase ATR involves the induction of at least 50 proteins called acid shock proteins (ASPs). Synthesis of 8 of these proteins requires an alternative sigma factor called σ^s, with a different promoter specificity from that used under normal growth conditions. The σ^s protein, encoded by *rpoS,* is important as a regulator of stationary-phase physiology and during osmotic shock. Several other studies have indicated that during the stationary-growth phase, the *rpoS* regulon induced several genes that contribute to the organisms survival during acid challenge (Lee and others 1994, 1995; Lin and others 1995; Lambert and others 1997).

In *S.* Typhimurium, σ^s levels increase upon an acid shock of exponential-phase cells, thereby partially explaining the acid shock induction of this subset of ASPs (Baik and others 1996). Eight σ^s-dependent proteins were required for sustained induction of acid tolerance. Without them, acid tolerance was only transiently induced via acid shock for about 20 min. In addition to a general stress resistance induced by the stationary-phase itself, stationary-phase cells have an acid pH–inducible system of acid tolerance that is independent of σ^s. Only 15 stationary-phase ASPs are produced, 4 of which are also induced during the exponential growth phase (Baik and others 1996).

In addition to its importance for surviving strong acid environments, the inducible ATR is an important means of surviving exposures to weak acids (Baik and others 1996). Thus, the ATR will protect *S.* Typhimurium against both types of acid stress that the microorganism will encounter. Organisms with such inducible mechanisms of acid tolerance will be better equipped to survive in the presence of weak acid preservatives and cause disease.

Effect of weak acid preservatives on the fatty acid composition of the membrane

The effect of many weak acid preservatives is dependent on the permeability of the cytoplasmic membrane. The fatty acid composition of *Z. rouxii* cells is altered in the presence of sorbate (Golden and others 1994). Such changes in the fatty acid profile of the plasma membrane may alter membrane permeability and fluidity, which may in turn contribute to sorbate tolerance. When *L. monocytogenes* cells were grown in the presence of exogenous fatty acids ($C_{14:0}$, $C_{18:0}$), cells showed increased resistance to me-

thyl parabens and propyl parabens, whereas decreased resistance was observed in cells grown in the presence of $C_{18:1}$ (Juneja and Davidson 1993). Low-melting-point fatty acids (unsaturated and branched chain fatty acids) increase membrane fluidity, whereas high-melting-point fatty acids (saturated long and straight chain fatty acids) decrease membrane fluidity. In the resistant lipid-adapted *L. monocytogenes* cells, the physical state of the fatty acids was less fluid because of the increased levels of saturated fatty acids ($C_{14:0}$ or $C_{18:0}$), which may have acted as a defense mechanism, decreasing the ability of the weak acid preservatives to pass through the membrane and thus conferring protection. Consequently, any food-preservation system that alters the membrane lipid composition of certain microorganisms could result in changes in weak acid preservative susceptibility (Juneja and Davidson 1993).

Other effects of weak acid preservatives on the cell

Microbial resistance and tolerance to weak acid preservatives and the mechanisms of weak AR in yeasts and bacteria are, as yet, only partly resolved. Significant knowledge, however, is available on intrinsic resistance mechanisms to weak acid preservatives in bacteria and are described in detail in Russell (1991). Resistance mechanisms are more complicated in Gram-negative cells than in Gram-positive cells. Most weak acid preservatives must enter the Gram-negative cell to bring about an inhibitory or lethal effect. They must cross the outer membrane and in some instances the inner membrane before reaching their site of action within the cell. Thus, penetration of a preservative into the Gram-negative cell may be limited by these membranes, and so the membrane has a role in controlling the accessibility of the interior of a cell to preservatives (Nikaido and Varra 1985; Russell and Gould 1988).

Other effects of weak acids on the cell have been shown with *Z. bailii* (Cole and Keenan 1987) and *Z. rouxii* (Golden and Beuchat 1992a, 1992b) in which a decrease in protoplast volume, cell size, and alterations in cellular morphology were observed during exposure to sorbate. It has also been suggested by Warth (1991a, 1991b) that the variability in the resistance to weak acid preservatives is due to physiological differences between species, such as cell permeability, enzyme structure, and induction or suppression of genes that affect susceptibility to weak acids (Warth 1991a, 1991b).

Conclusion

An acid pH and the presence of organic acids in certain foods play important roles in both conferring flavor and preventing the growth of contaminating microorganisms. Among the many food preservatives, weak acid preservatives have several mechanisms of action that adversely affect several systems in an organism and thus exhaust the microbial cells, limiting their ability to grow. However, in the presence of weak acid preservatives, many organisms have been shown to have stress adaptations, which aid microbial survival by inducing a specific pattern of gene expression, which appears to be required for optimal adaptation to weak acid conditions. Most of these stress protection systems include a mechanism for sustaining cytoplasmic pH.

The resistance or adaptation of microorganisms to such conditions may result in microbial spoilage of products and affect food safety and so is clearly of significance to the food industry in relation to survival of pathogens and growth of spoilage organisms in food. It is therefore important for the food industry to understand more comprehensively the ways and conditions under which cells can become adapted to low pH environments to investigate how this can be prevented. Prevention of acid adaption may be achieved by using different acid types such as lactic and acetic acids, which have been shown to be more antimicrobial than other acids. Storage temperature has also been seen to be important; ambient storage temperature, for example, decreases acid survival more than storage at chill temperatures (Skandamis and Nychas 2000).

Low pH

Introduction

Microorganisms have defined optimum ranges of external pH required for growth and survival, and so acidification is often quite effective in controlling microbial growth. Organisms are in general more sensitive to changes in the pH_i than to changes in external pH, although significant changes in either will lead to loss of viability.

Because strong nonpermeant acids do not affect the pH of the cytoplasm to the same extent as weak permeable acids, relatively large changes in external pH are required for effective preservation, which may be detrimental to the foods' sensory properties. Therefore, in many cases, acidification alone is too detrimental to the sensory quality of foods to be acceptable as the only means of controlling microbial growth (Brown and Booth 1991).

In general, for growth and survival, bacteria require pH values that are between 4 and 8, whereas the yeasts and molds are able to grow and survive at a wider range of pH values; in some instances, the pH range can be between 2 and 11 (Wheeler and others 1991). However, microorganisms may survive in conditions of low pH, and although growth may have stopped, the cells may still be metabolically active. The energy requirements of a microorganism in a low pH environment are greater than the energy required at optimal pH values. This is because an energy-requiring proton pump is in use, with protons being pumped out of the cell. In high pH environments, protons may be pumped into the cell. If the pH is not balanced, the cell is unable to synthesize normal cellular components and is unable to divide and grow (Booth and Kroll 1989; Brown and Booth 1991).

Modes of action

Unlike undissociated lipid-permeable weak acids, which can diffuse freely through the cell membrane and ionize in the cell to yield protons that acidify the interior of the cell (Krebs and others 1983; Warth 1985), strong acids lower the external pH (pH_o) but are not able to permeate through the cell membrane. These acids exert their antimicrobial effect by denaturing enzymes present on the cell surface and by lowering the cytoplasmic pH due to increased proton permeability when the pH gradient is very large. This can result in reduced growth rate and cause an extension to the lag phase (Cheroutre-Vialette and others 1998). Growth may be limited by a reduction in the activity of ion transport systems in which case essential ions and nutrients will not be taken up (Booth and Kroll 1989).

Yeast and molds are able to tolerate lower pH values than bacteria and as a consequence are associated with spoilage of pickled, acidified, and fruit products. There are several factors that can influence the pH tolerance of a microorganism. These factors include changes brought about by the competing microflora, damage by prior treatments such as heat, or lowered a_w. For many microorganisms in environments where the pH is lower than optimal, the minimum temperature of growth may also be raised, and in this respect there is also a very strong interaction with reduced a_w. In laboratory media at an a_w of 0.97, *P. fluorescens* has a minimum growth temperature of 15 °C to 20 °C at pH 7, which is raised to 20 °C to 25 °C at pH 5.4 (Brown and Booth 1991).

Several investigations have focused on the adaptive responses of microorganisms to assorted pH conditions. The microorganisms studied include several Enterobacteriaceae, *Streptococcus*, *Enterococcus*, *Actinomycetes*, *Lactobacillus*, *Rhizobium*, *Thioba-*

cillus, *Sarcina*, *Staphylococcus*, *Yersinia*, and *Listeria* species. The adaptive mechanisms observed during growth at low pH range from AR to specific inducible systems, but are all based on either maintaining the pH homeostasis or restoring the internal pH to neutrality (Hall and others 1995).

In low pH environments, populations of cells may consist of 2 types. First, cells whose cytoplasmic pH has not been lowered enough to degrade proteins and so can completely recover with no protein synthesis. Second, a group of cells that require protein synthesis for recovery because they have experienced a lowering of their cytoplasmic pH, which has caused protein degradation (Brown and Booth 1991).

Table 4—pH tolerance and internal pH values for various microorganisms

Organism	pH for growth	Internal pH (pH_i)
Alicyclobacillus	2.0 to 5.0	5.9 to 6.1
Acetobacterium	2.8 to 4.3	4.0 to 6.0
C. thermoacetium	5.0 to 8.0	5.7 to 7.3
Saccharomyces cerevisiae	2.35 to 8.6	6.0 to 7.3
Enterococcus faecalis	4.4 to 9.1	7.2 to 7.4
Escherichia coli	4.4 to 8.7	7.5 to 8.2

Taken from Booth and Kroll (1989)

Maintaining pH homeostasis

Bacteria can only survive in acidic environments because of their ability to regulate their pH_i, a process primarily driven by the controlled movement of cations across the membrane. However, this ability to maintain pHi close to neutrality (pH homeostasis) can be overwhelmed at low extracellular pH values, leading to the death of the cell (Booth and Kroll 1989). Examples of growth pH values and internal pH for various organisms are shown in Table 4.

Escherichia coli controls its internal pH very well, and as the external pH drops, the internal pH remains constant; other microorganisms allow the internal pH to drop. In general, yeasts and filamentous fungi have been shown to be able to withstand rapid acidification of the medium without excessive lowering of the cytoplasmic pH (Sanders and Slayman 1982). The ability of a microorganism to maintain its pH_i at a value close to neutral is achieved by a combination of passive and active mechanisms as reviewed by Brown and Booth (1991), Booth and Kroll (1989), and Hill and others (1995).

Passive homeostasis. In the prevention of large changes in pH_i as the pH of the environment varies, it is thought that the permeability of the cell membrane to protons plays an important role, with protons that are present in the environment being prevented from crossing the membrane and reducing the internal pH. However, undissociated lipid-permeable weak acids can overcome this by passing the membrane in an undissociated form, as can treating the cell with an ionophore, which results in the membrane being permeable to protons (Hill and others 1995).

A cell exposed to low pH environments can also increase the cytoplasmic levels of proteins and glutamates and increase buffering capacity, which may prevent internal pH disruption (Booth and Kroll 1989).

Active homeostasis. Active pH homeostasis depends primarily on the potassium ion and proton movement (Hill and others 1995). At low external pH, a cell must force out ions entering the cell that are associated with weak acids to prevent acidification of the interior of the cell. However, the movement of protons across the membrane generates a membrane potential, preventing further proton removal. This membrane potential is, however, broken up by the movement of cations into the cell such as potassium ions, which generates a transmembrane pH gradient and helps maintain the internal pH (Booth and Kroll 1989).

In general, pH is important in microbial control, but acid type and concentration are of equal importance. The species and strains differ in pHi and their physiological and genetic response to pH and acidity. There are short-, medium-, and long-term adaptations, which can aid microbial survival in acid conditions, and the rate of acidification affects the acid tolerance observed.

Membrane response to low pH stress

Effect of low pH on the structure of the outer membrane. At very acidic pH values, it has been proposed that alterations in the structure of the outer membrane may be a mechanism for enhancing microbial survival (Brown and Booth 1991). Some adaptations to low pH have also been noted in the lipid composition of the inner membrane, which interacts with the periplasm (Roth and Keenan 1971; Coleman and Leive 1979; Booth and Kroll 1989).

Effect of low pH on membrane fatty acid composition. As with temperature, membrane lipid composition may be determined by the pH of the growth medium and thus possibly represent an adaptation process to low pH (Booth and Kroll 1989). When *Clostridium acetobutylicum* is grown at a low pH, a decrease in the ratio of unsaturated to saturated fatty acids coupled with an increase in the amount of cyclopropane fatty acids is observed (LePage and others 1987); this results in a decrease in the fluidity of the membrane and so protects the cell from the low pH. The membrane composition of acid-adapted *E. coli* has more cyclopropane derivatives and fatty acids present, compared with strains that have not been exposed to low pH conditions (Brown and others 1997).

During incubation at low pH, an increase in synthesis of aminophospholipids (such as alanyl- and lysyl-phosphatidylglycerol) has been observed in *Staphylococcus aureus*. It has been proposed that such changes give the membrane surface a net positive charge, which may act as a barrier to protons (Brown and Booth 1991).

Yersinia enterocolitica also produces increased proportions of cyclopropane and saturated fatty acids (increases of saturated fatty acids from 47% to 72% of total fatty acids to 73% to 80%) and a decreased level of unsaturated fatty acids when grown at pH 5 compared with pH 9 (Bodnauk and Golden 1996).

Brown and others (1997) also revealed that, during acid habituation of *E. coli,* a proportion of the mono-unsaturated fatty acids were either converted to cyclopropane fatty acids or replaced by saturated fatty acids. They also found that cells with high levels of intrinsic acid tolerance had high levels of cyclopropane fatty acids compared with strains with low level intrinsic tolerance, and suggested that this may play a role in protection of cells from low pH.

Other effects of low pH stress

Studies with *E. coli* have shown that cells will move away from acidic environments (chemotaxis) by sensing the decrease in pH via the transmembrane transducer, a single protein, one part of which is exposed to the cytoplasm and the other to the periplasm. The cells tumble, which aids overall movement in the direction of a more favorable environment (Brown and Booth 1991). This protein exists in 2 forms, depending on methylation, and is induced under low pH conditions and allows detection of lowered pH. Such a transducer system could be sensitive to the transmembrane difference in pH since the 2 parts would allow the protein to respond to changes in both external and internal pH (Brown and Booth 1991; Rowbury 1997).

ATPase synthesis is important for maintaining the internal pH gradient at low pH conditions for *Enterococcus faecalis*. As the in-

ternal pH of the cell decreased, both the proton translocating F_1F_0-ATPase activity and the removal of protons from the cell increased, restoring the internal pH to neutrality. Protein synthesis was also necessary to return the cytoplasmic pH to normal (Hall and others 1995).

Morphological changes such as cell elongation in *Bacillus polymyxa* and *Clostridium tyrobutyricum* as a stress response to suboptimal pH conditions has been observed (Everis and Betts 2001). Once the cells are put back into favorable conditions, the cells reverted and gave a rapid apparent increase in cell numbers. Other stress responses, such as chilled temperatures for *Salmonella enteritidis* (Phillips and others 1998) and high salt concentrations for *L. monocytogenes* (Isam and others 1995) show similar results.

Samelis and others (2001) has also shown that the predominant natural flora (Gram-negative aerobic bacteria) of a fresh meat factory may sensitize bacterial pathogens such as *L. monocytogenes* to acid.

Effects of low pH stress on gene expression

As with many other physical stresses, alterations in pH can cause a number of genes to be switched on. A number of inducible responses and their general characteristics in *E. coli* are reviewed in detail in Rowbury (1997), Gahan and Hill (1999), and Masuda and Church (2003). A review of the genes regulated by pH are also detailed in Hall and others (1995). During growth at acid pH, the porin gene, *ompC* is induced as described in detail by Heyde and Portalier (1987) and Rowbury (1997). As well as pH stress, efficient regulation of this gene also affects responses to osmotic stress, nutrient limitation, and temperature shifts.

To adapt to and tolerate low pH environments, cells try to alter the external pH value. For example, *E. coli* expresses carboxylase enzymes preferentially at low pH (Brown and Booth 1991). The function of such enzymes is to raise the external pH value and contribute to induced acid tolerance in some situations (Rowbury 1997). Lysine decarboxylase, encoded by *cadA*, is a 715 amino acid protein that contributes to pH homeostasis by converting exogenously supplied lysine to cadaverine, an alkaline product that, when secreted from the cell, neutralizes the acidic environment (Hall and others 1995; Rowbury 1997). For example, the low pH–inducible lysine decarboxylase contributes significantly to pH homeostasis in environments as low as pH 3 for *S.* Typhimurium (Park and others 1996). For this response to occur, both lysine decarboxylase and σ^s-dependent ASPs are required. However, only the lysine decarboxylase contributed to the homeostasis.

Arginine decarboxylase (*adi*) of *E. coli* has several properties in common with lysine decarboxylase in that they can both increase the surrounding pH value by removing acidic carboxyl groups and releasing CO_2 from their substrates (Hall and others 1995; Rowbury 1997).

Two systems for acid stress protection are the inducible pH homeostasis systems of ATR and acid shock response, which both require protein synthesis. These acid stress responses of *Salmonella* and *E. coli* are described in detail in Foster (2001). *S.* Typhimurium possesses different survival mechanisms, depending on whether the cells are in exponential or stationary phase (Bearson and others 1997). Omp R is an acid–induced response regulator critical to stationary-phase ATR but not to log-phase ATR (Iel and others 2002).

During exposure to mildly acidic conditions, bacteria acquire the ability to survive lethal acid concentrations. This inducible mechanism, the ATR system, is a complex phenomenon involving a number of changes in the levels of different proteins and events at the level of gene regulation. For example, the ATR of *Salmonella* spp. involves the transient synthesis of key ASPs (Foster 1991, 1993). Using a number of molecular techniques, numerous loci have been located that are involved both in sensing and responding to such a stress (Gahan and Hill 1999). Foster and Hall (1991) found that acid tolerance correlated with improved pH homeostasis at low external pH, indicating that acid induction of extreme acid tolerance may include inducible pH homeostasis.

Among several factors that influence the ability of cells to withstand acid challenge, the role of the stationary-phase σ–transcription factor (encoded by *rpoS*) is well documented and regulates the expression of a number of acid-induced genes (Fang and others 1992; Lee and others 1994, 1995; Foster and Spector 1995; Bearson and others 1997). This ability to survive acid challenge has also been shown to be severely affected in strains of *S.* Typhimurium defective in *rpoS* (Lee and others 1994; Gahan and Hill 1999).

Acid tolerance is induced at low pH in several microorganisms including *L. plantarum, Leuconostoc mesenteroides* (McDonald and others 1990), *L. lactis* (O'Sullivan and Condon 1997*), Lactobacillus acidophilus* (Lorca and others 2001), *L. monocytogenes* (Kroll and Patchett 1992; O'Driscoll and others 1996a, 1996b, 1997; Davis and others 1996; Cotter and others 2001), *S.* Typhimurium (Foster and Hall 1990; Foster 2001), *E. coli* O157:H7 (Leyer and others 1995; Brudzinski and Harrison 1998), and *E. coli* (Rowbury 1995; Brown and others 1997; Rowbury and Goodson 1999; Foster 2001). Acid stress has also been studied in *B. cereus* (Browne and Dowds 2002).

Salmonella spp. and *E. coli* can adapt and grow at low pH values if sequential acid adaptation is performed (Foster and Hall 1990; Brown and others 1997). Goodson and Rowbury (1989b) found that *E. coli* strains grown at pH 7 failed to grow after a short exposure to pH 3 or 3.5. However, *E. coli* cells that had previously grown at pH 5.0 were unaffected by exposure to acid pH values of 3.0 or 3.5. Therefore, to control such acid tolerance of organisms in foods, it is necessary to prevent organisms from becoming acid-adapted. For example, if the acidification process of a product is not performed quickly, organisms present in the product will become adapted to the gradual reduction in pH and thus be unaffected by the final pH value of the product and survive longer in the acidic foods. For *L. monocytogenes*, prior growth at a mild pH value of pH 5 to 6 protected cells from severe acid stress of pH 3.5 (O'Driscoll and others 1996a; Koutsoumanis and others 2003), and such acid-adapted cells were shown to survive longer in acidic foods than unadapted cells (Gahan and others 1996). The increased protection of acid-adapted *E. coli* O157 cells also increased protection to lower pH values than nonadapted cells (Leyer and others 1995). *Shigella flexneri* also had increased resistance to extreme acid after pre-exposure to an acidic environment (Tetteh and Beuchat 2003). Therefore, the acidification process of low acidic foods must be performed quickly.

In *Salmonella* spp., 3 stages of pH homeostasis have been identified. At pH values greater than 6, proton pumps are regulated. When exponentially growing cells (at pH 7.6) are shifted to mildly acidic conditions (pH 5.5 to 6.0), an ATR is triggered (Foster and Hall 1990). This acid adaptation is referred to as pre-shock and involves the induction of 12 proteins and the repression of 6 proteins. Prolonged time at this pH range allowed cells to grow at pH values as low as 4. A pH of between 3.0 and 5.0 caused an acid shock response. During acid shock, ASPs were synthesized that were different from the acid-tolerance proteins (Foster 1991). For *S.* Typhimurium to adapt to very low pH conditions, sequential acid adaptation is required, so as to protect against the more severe acid stress. If a cell is transferred directly from pH 7.0 to pH 3.3, these ASPs will not be synthesized and the cell will be killed (Brown and others 1997).

Most of the ASP remain poorly characterized, and their role in acid response and survival is unknown. Recently an *Escherichia coli* gene *asr*, which encodes a protein of unknown function (Sep-

utiene and others 2003), has been shown to be strongly induced by increased environmental acidity of less than pH 5.

Lin and others (1995) compared acid survival systems in several microorganisms. Both *S.* Typhimurium and *E. coli* but not *Shigella flexneri* expressed low pH–inducible log-phase and stationary-phase ATR systems that function in both minimal or complex medium to protect cells at pH 3.0. All 3 microorganisms also expressed a pH-independent general stress resistance system that contributed to acid survival during stationary-phase. *E. coli* and *Shigella flexneri* possessed several acid-survival systems including AR systems that were not demonstrable in *S.* Typhimurium. These additional AR systems protected cells to pH 2.5 and below but required supplementation of minimal medium with glutamate or arginine for either induction or function during acid challenge. Another acid-inducible AR system required oxidative growth in complex medium for expression but protected cells to pH values of 2.5 in unsupplemented minimal medium (Lin and others 1995).

The survival of stationary-phase cells at extreme low pH is termed *acid resistance*. As *E. coli* enters the stationary phase, slowing and finally ceasing to grow, genetic changes occur to prolong survival and increase resistance to a variety of stress conditions (Lange and Hengge-Aronis 1991a, 1991b; Siegele and Kolter 1992; Small and others 1994). Stationary-phase cultures of *S.* Typhimurium and *E. coli* O157, for example, are able to survive a challenge for 2 h at pH 2.5 (de Jonge and others 2003a); such survival at extreme low pH environments found also in the human stomach is a serious concern to public health.

Generally for Enterobacteriaceae, several survival responses to extreme low pH environments have been described (Bearson and others 1997) and consist of (1) the induction of enzymes capable of raising the pH_i, and (2) induction of enzymes involved in the protection of repair of proteins and DNA

Conclusion

Although pH is important in microbial control, the acid type and concentration of the acid are both important. Increasingly acidic conditions can affect the growth of microorganisms by interfering with the synthesis of cellular components and inducing cell death as a result of damage to the outer membrane, disruption of the cytoplasmic pH homeostasis, and subsequent damage to DNA and enzymes (Brown and others 1997).

In the presence of low pH, many organisms have been shown to have short-, medium-, and long-term stress adaptations that aid microbial survival by inducing a specific pattern of gene expression, which appears to be required for optimal adaptation to low pH conditions. The resistance or adaptation of microorganisms to such conditions may result in microbial spoilage of products and/or affect food safety. Most pH-stress protection systems include a mechanism for sustaining cytoplasmic pH, and many pH stress-inducible systems offer cross protection to other stresses such as increasing heat and salt tolerances (Hall and others 1995).

Acid-habituation of pathogens may enhance survival in an acidic food or in the stomach and subsequently cause infection after ingestion (Goodson and Rowbury 1989b). In an environment with changing pH, acid-sensitive *E. coli* O157 cultures can become acid-resistant within 17 min (de Jonge and others 2003b). Acid resistance and survival have significant implications for food safety and the virulence of pathogenic microorganisms, and the ability of non–acid-adapted *E. coli* O157 to adapt within a very short period under extreme conditions further contributes to their virulence.

Osmotic Stress

Introduction

Increasing the osmotic pressure (lowering a_w) is among one of the most widely used methods to preserve food products. This results in the reduction of the amount of available water to a microorganism and is reviewed in detail in Csonka (1989); Gallinski and TrÜper (1994); Gutierrez and others (1995); Miller and Wood (1996); and Kempf and Bremer (1998). The a_w of a product is very important with respect to bacterial growth, and microorganisms have minimum a_w limits, below which growth cannot occur. This limiting a_w value will depend on the solute used such as salts (for example, NaCl and KCl), sugars (for example, glucose and sucrose), or glycerol (Troller 1980; Sperber 1983). For example, the minimum growth a_w for *P. fragi* is 0.957 using NaCl. When glycerol is used, the minimum a_w for growth is 0.94 (Sperber 1983). Below an a_w value of 0.80, it is assumed that bacteria will be unable to grow; however, fungal growth can occur to levels as low as 0.60 (Gould 1989).

A well-established response to the temporary loss of turgor pressure after a hyperosmotic shock (for example, a reduction in a_w surrounding the cell) is osmoregulation. If the a_w of the environment is reduced because of an increase in solutes unable to be transported across the cell membrane, then the bacteria raise their internal solute levels (compatible solutes) (Gould and Christian 1988; Gutierrez and others 1995). This results in an increase in internal osmotic pressure and restores turgor pressure. Besides the accumulation of compatible solutes, changes in the membrane lipid composition, including phospholipid and fatty acid changes, have been observed (Russell 1989; Russell and others 1995).

Mode of action

The mode of action of food preservation procedures that involve reduction of a_w by drying, salting, or sugaring is based on the effectiveness of the procedure for exceeding the osmoregulatory capacity of the cell, or in some way reducing the amount of energy available for osmoregulation. This may be achieved by limiting the amount of available oxygen for facultative microorganisms (Csonka 1989; Gutierrez and others 1995).

Most microorganisms have evolved to function only within certain ranges of water activities. Exposure to a_w values outside of this range may result in essential cellular functions becoming impaired and in inhibition of a variety of physiological processes such as nutrient uptake (Roth and others 1985b). For example, in *E. coli*, it has been observed that osmotic stress drastically inhibits active transport of carbohydrates (Roth and others 1985a) and deoxyribonucleic acid (DNA) replication (Meury 1988).

Reduction in a_w has a dramatic effect on bacterial growth, for which an increased lag phase and decreased growth rate and cell yield have been reported (Troller 1980; Sperber 1983; Cheroutre-Vialette and others 1998). Many bacteria have maximum growth rates between a_w values of 0.990 and 0.995. As the a_w is decreased from the maximum point to the minimum a_w value required for growth, the growth rate decreases. When *S. aureus* is grown at an a_w value of 0.90, its growth rate is only 10% of its maximum growth rate (Scott 1957). However, *L. monocytogenes* can grow in the presence of salt concentrations of 10% (w/v) in a rich medium such as brain heart infusion (BHI) broth, which contains osmoprotectants such as betaine, carnitine, and peptides (Beumer and others 1994; Amezega and others 1995). Pusey (2001) describes work carried out at Campden & Chorleywood Food Research Association, U.K., which found that *E. coli* O157 and other verocytotoxigenic strains are able to survive in low a_w foods such as chocolate, ambient stable cakes, and biscuits. Initially these organisms experience a period of rapid die off, which is then followed by an extended die-off period, which leads to prolonged survival of the organisms. Such factors that can affect microbial response to low a_w is described subsequently and summarized in Table 5.

Exposure of microorganisms to salt and other solutes causes an instantaneous loss of water, which is accompanied by a decrease in the cytoplasmic volume. Hypo-osmotic shock generally results in minor changes in cell volume. On the other hand, hyperosmotic shock causes considerable shrinkage of the cytoplasmic volume. If the osmotic shock is not too severe, after extended lag phase, the cytoplasmic volume will increase as a result of osmotic adjustments made by the cells (Csonka 1989).

Table 5—Factors affecting microbial response to low water activity

Factors affecting microbial response to low water activity	
A	Microbial type and sporulation activity
B	Solute type and associated osmotic effect
C	Rate of dehydration
D	Alteration of membrane composition
E	Compatible solute accumulation

Osmoregulation

Bacteria are constantly in contact with their surroundings, and the barriers surrounding the cell are water permeable and thus ensure that bacteria come into rapid equilibrium with their environment (Gutierrez and others 1995). Generally, the internal osmotic pressure in bacterial cells is greater than that of the surrounding environment and so pressure is exerted outward on the cell wall; this is called *turgor pressure* (Gutierrez and others 1995). Therefore, to survive variations in osmotic pressure of the surrounding medium, the bacterial cells must maintain turgor. If the a_w of the environment is reduced, the cells would lose water to ensure that restoration of the osmotic equilibrium across the cell occurred. If the reduced a_w is due to the increase in solutes unable to be transported across the cell membrane, then a temporary loss in turgor pressure would occur. The bacteria then raise their internal solute levels (compatible solutes), resulting in an increase in internal osmotic pressure and restoration of turgor pressure (Figure 2). This is termed *osmoregulation* (Gutierrez and others 1995). Therefore, osmoregulation is initiated after the reduction in turgor of the cell following loss of cell water or an increase in the external solute concentration that cannot cross the membrane.

At high osmolarities, bacteria have evolved to accumulate high cytoplasmic levels of a certain class of solutes (compatible solutes) that do not interfere too seriously with the functioning of cytoplasmic enzymes (Gutierrez and others 1995). The internally accumulated solutes allow continued activity of cytoplasmic enzymes at lower water activities (Booth and others 1994). Osmoregulation also regulates the activity of porins, transport proteins, and potassium in the cell membrane (Gould 1989). Bacterial compatible solutes are accumulated either by de novo biosynthesis (endogenous osmolytes, such as glutamate, proline, ectoine, trehalose, and sucrose) or by uptake from the environment (exogenous osmolytes such as glycine betaine) (Csonka and Hanson 1991).

The compatible solutes produced internally are highly soluble, pH neutral, and are usually end product metabolites. They can include sugars from the breakdown of carbohydrates, amino acids from protein degradation, and cations such as K^+. Examples include betaine, trehalose, glycerol, sucrose, proline, choline, carnitine, mannitol, glucitol, and ectoine. The cell membrane is selectively permeable to them, allowing the cytoplasmic pool to be determined by the external osmotic pressure (Galinski 1995; Gutierrez and others 1995). The preferred exogenous bacterial-compatible solutes are glycine betaine, which is found in higher plants, and the amino acid proline. In many foods, however, peptides are more readily available than free amino acids and hence peptides have become an important source of both nutrients and compatible solutes (Amezega and others 1995). Peptides have also been shown to play an important role in the adaptation of *L. monocytogenes* to osmotic stress (Amezega and others 1995).

Cell membrane composition response to osmotic stress

Growth at low a_w values also leads to changes in membrane lipid composition, and it has been suggested that this change is part of the osmoregulation sensing mechanism (Russell and Kogut 1985). In contrast to the effect of temperature on membrane composition, the major change in response to salt is in the head group of the lipids. The most common alteration is the increase in the proportion of anionic phospholipids and/or glycolipids when external a_w is lowered by means of preservative solutes (Russell and others 1995). The reasons for these changes are to preserve the membrane lipids in the proper bilayer phase because lowered a_w increases the likelihood of the membrane lipids adopting a nonbilayer phase in the membrane, which would disrupt the membrane function. These changes have been observed in a number of microorganisms including those that cause food spoilage (Bygraves and Russell 1988; Russell 1993).

Osmotic stress–induced changes in phospholipids. The major change in composition after growth at low a_w is that the levels of anionic lipids such as diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) increase relative to the levels of neutral, zwitterionic lipids such as phosphatidylethanolamine (PE). This has been found to be true for *E. coli* (McGarrity and Armstrong 1975); *Pseudomonas halosaccharolytica* (Ohno and others 1976), *Vibrio costicola* (Kogut and Russell 1984; Russell and others 1985), and *Z. rouxii* (Hosono 1992).

When 2% (w/v) sodium chloride was added to the growth medium, *L. monocytogenes* responded by increasing the ratio of DPG/PG compared with control samples (Russell and others 1995). Therefore, the presence of the salt triggered changes in the membrane lipid composition.

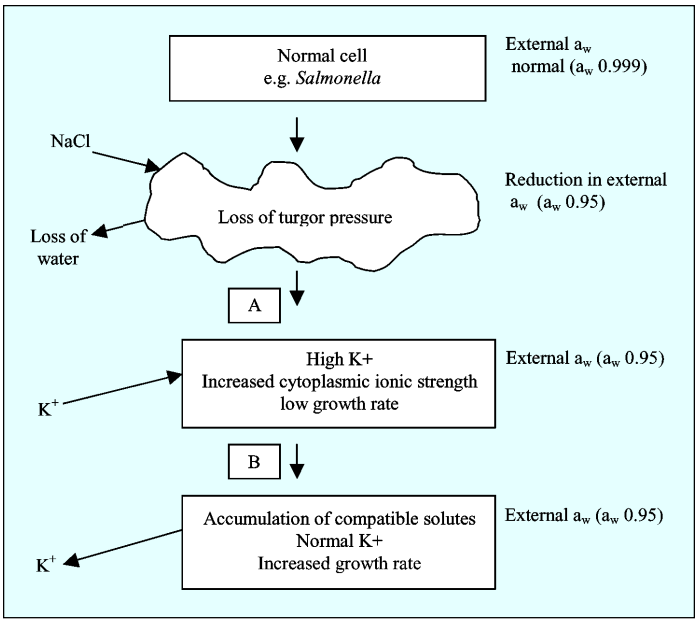

Figure 2—Osmoregulation. 'A' as the bacterium loses water, cytoplasmic level of K+ increases 'B'. This triggers enzymes such as glutamate dehydrogenase to form glutamate from a ketoglutamate. As the glutamate levels increase, water starts to re-enter the cell and growth resumes.

Lactobacillus plantarum has also been found to change its phospholipid composition in the presence of solutes (Russell and others 1995). With this organism, addition of glycine betaine and a solute to the growth medium resulted in the synthesis of membranes with a much higher proportion of lysyl-phosphatidylglycerol (LPG) at the expense of both PG and DPG. Therefore, the compatible solute stabilized the intracellular enzymes and other proteins and enabled the organism to continue functioning at reduced a_w (Russell and others 1995). *Bacillus subtilis* grown in hypertonic LBN medium, changed its phospholipid composition with PG content decreasing from 40% to 31% and the cardiolipin (CL) content increasing from 24% to about 31% (Lopez and others 2000). This increase in CL has been suggested to behave as a barrier against the high ionic level.

Lactococcus lactis has been shown to make significant modifications to the fatty acid composition of its membrane when cells are subjected to osmotic stress. The main modification is an increase in cyclopropane fatty acid $C_{19:0}$, whereas the unsaturated-to-saturated ratio remains unchanged (Guillot and others 2000).

Osmotic stress–induced changes in fatty acid composition. Salts and other solutes can also alter the fatty acid composition of the membrane lipids (Russell 1989, 1993). Sutton and others (1990) found changes in fatty acid composition of *V. costicola* when grown in 3 molal sodium chloride compared with 1 molal. It was found that extremes of salt levels had more of an effect on fatty acyl constituents of PG than PE. The fatty acid composition of PG at 3 molal NaCl was more unsaturated with a different double bond isomeric distribution and a shorter chain length compared with 1 molal. However, the PE in cells had the same unsaturation index and average chain length at both concentrations but had a different double bond isomeric distribution.

The effect of osmotic stress on gene expression

Analysis of the genetic response to a reduction in a_w has shown that as many as 12 genes are involved in the osmoregulation of Gram-negative bacteria (Csonka 1989; Booth and others 1994; Gutierrez and others 1995). Under conditions of low osmolarity, the potassium transport systems are operating at relatively low rates, and the betaine transport systems are almost completely inactive. An immediate increase in the osmolarity of the medium causes immediate loss of cell turgor accompanied by a loss of water and cell shrinkage (Gutierrez and others 1995).

In *E. coli,* the primary response to restore turgor pressure is the controlled accumulation of potassium and glutamate. The loss of turgor activates the major potassium transport systems: TrkG, TrkH, and Kup, and induces the expression of the *kdp* gene. This gene codes for the high-affinity potassium uptake system Kdp. This results in an uptake of potassium, which can last until the turgor is restored and can accumulate to levels as high as 0.7 to 0.8 *M*, which is sufficient to inhibit enzyme function (Booth and others 1994; Gutierrez and others 1995). Therefore, to enhance survival, a secondary response is required: the accumulation of compatible solutes.

When *E. coli* growing in a minimal medium is subjected to an osmotic shock, there is an uptake of potassium glutamate, which is followed by an accumulation of trehalose. The accumulation of trehalose allows potassium glutamate to be released at steady rate until there is a balance between potassium glutamate and trehalose. Once this occurs, growth rate is improved (Dinnbier and others 1988, Gutierrez and others 1995). This involvement of trehalose was also observed in mutants that were unable to synthesis trehalose, where slow growth rates at high osmolarity were seen (Giaever and others 1988).

The rate at which trehalose is produced is dependent on the stresses to which the microorganism has already been subjected (Gutierrez and others 1995). If the microorganisms had already been subjected to a stress, the enzymes involved in the synthesis of trehalose may already be present and are thus activated. However, microorganisms from stress-free environments will need to express the genes for trehalose biosynthesis, which is under the control of RpoS stationary phase sigma factor (σ^{38}), which will not be active unless the cell has undergone stress.

Gutierrez and others (1995) also discuss how adaptive mechanisms can alter if other compatible solutes such as betaine or proline are present. Accumulation of betaine or proline from the environment takes place shortly after the onset of potassium glutamate accumulation. In *S.* Typhimurium and *E. coli*, the glycine betaine transport is affected through the two general ProP and ProU transport systems in response to osmotic changes. However, ProU is not present in nonstressed cells, but will be induced after osmotic stress (May and others 1986; Gutierrez and others 1995). ProU belongs to the family of binding protein–dependent ABC transporters, and ProP is an integral membrane protein.

The *S.* Typhimurium ProP system is a proton-driven transport system. It is active in membrane vesicles, and it is constitutively expressed. The ProU system is only expressed at significant levels after exposure to osmotic stress (Csonka 1989). However, ProU expression is reduced if ProP is active and glycine betaine is present (Cairney and others 1985). This suggests that during adaptation to osmotic shock, ProP is the major route for glycine betaine accumulation.

The regulation of glycine betaine accumulation has been investigated by Koo and others (1991) who found that at high osmolarity, another transport component controls the glycine betaine pool and is believed to be independent of the activation of ProU and ProP. Strom and others (1986) also found that although ProP and ProU systems are involved in the uptake of glycine betaine and proline, the synthesis of glycine betaine occurred only in the presence of choline.

Other genes that are osmotically inducible and are involved in the synthesis or uptake of compatible solutes include *bet* required for choline uptake and endogenous glycine betaine uptake (Anderson and others 1988), *otsA* and *otsB* required for cytoplasmic trehalose synthesis (Giaever and others 1988), and *treA*, which encodes a periplasmic trehalase involved in the catabolism of trehalose (Gutierrez and others 1989).

Bacillus subtilis contains 5 osmoprotectant uptake systems, named OpuA to OpuE. Unlike the osmoporters in *E. coli,* each system can accept only a few molecules. OpuA and OpuD appear to be very specific to glycine betaine, and OpuB and OpuE are specific to choline and proline, respectively (Pichereau and others 2000).

Unlike Gram-negative bacteria and their osmotic stress adaptation, the Gram-positive microorganisms have received less attention. Killham and Firestone (1984) studied *Streptomyces* species and showed that in the presence of increased salt concentrations, increased synthesis of proline, glutamine, and alanine occurred. Other work has been carried out with *S. aureus*, and *L. acidophilus*, in which betaine and proline were shown to play a role in osmoregulation (Bae and Miller 1992; Graham and Wilkinson 1992).

Work has also been carried out with *L. monocytogenes*; as with many other microorganisms, it is dependent on its ability to accumulate betaine and amino acids to adapt to osmotic stress conditions (Amezega and others 1995). *Listeria monocytogenes* cannot synthesize glycine betaine and it must be provided in the growth medium and transported into the cell. The accumulation of glycine betaine has also been observed after low-temperature storage (Smith 1996). Beumer and others (1994) also found that exogenously supplied carnitine contributed to growth of *L. monocytogenes* at low a_w, as for *E. coli* (Verheul and others 1998). The up-

take of betaine and carnitine in *L. monocytogenes* is mediated by transport systems called glycine betaine porter I, glycine betaine porter II, and a carnitine transporter OpuC (Gutierrez and others 1995; Angelidis and Smith 2003a). The stress-inducible sigma factor sigmaB has also been shown to play a major role in the regulation of carnitine utilization by *L. monocytogenes*, but was not essential for betaine utilization (Fraser and others 2003).

Proline confers osmoprotection in *L. monocytogenes* when present at high concentrations (Amezega and others 1995). When supplied as a peptide, significant osmoprotection is provided at low concentrations. They also found that peptone plays 2 important roles when *L. monocytogenes* is grown in peptone-containing medium. It serves as a nutrient supplement for protein synthesis, and it functions as a source of amino acids and peptides that serve in addition to betaine as a mechanism for maintaining turgor of the cell (Gutierrez and others 1995). Transport of proline has also found to be osmotically regulated by *L. acidophilus* (Jewell and Kashket 1991). Peptone has also been found to protect *E. coli* and *S. aureus* from growth inhibition caused by osmotic stress, although these microorganisms, unlike *L. monocytogenes,* do not accumulate peptides. *Escherichia coli* cells benefit from peptone via the osmotically regulated accumulation of free proline present in peptone, and the peptides have a nutritional role (Amezega and others 1995; Gutierrez and others 1995).

The osmoprotectants of *S. aureus* include taurine, proline, choline, and betaine when exogenously supplied (Graham and Wilkinson 1992; Gutierrez and others 1995). In response to osmotic stress, the uptake of choline has been found to occur via an inducible transport system, which is energy- and sodium-specific (Kaenjak and others 1993; Gutierrez and others 1995). For uptake of taurine, proline, and betaine, different sodium-dependent transport systems have been characterized as discussed in Booth and others (1994), Pourkomailian and Booth (1994), Stimeling and others (1994), and Gutierrez and others (1995).

The presence of a glycine betaine transport system is also required for *L. lactis* strains to tolerate high salt concentrations (O'Callaghan and London 2000). Strains that were sensitive to salt did not accumulate glycine betaine or display any sign of glycine betaine transport. *Lactobacillus casei* can use several osmocompatible solutes to overcome an osmotic upshift. Glycine-betaine and carnithine were efficient osmoprotectants. A contribution of the proteolytic system peptide supply in adaption to osmotic conditions has also been suggested (Piuri and other 2003).

Some of the compatible solutes may also be produced during exposure to reduced storage temperature conditions. *Lactococcus lactis*, for example, has been shown to accumulate betaine and carnitine at refrigeration temperatures, where there is no osmotic stress. The reasons for this is not clear, but clearly shows how closely linked the various mechanisms are for the various environmental stresses (O'Byrne and Booth 2002; Angelidis and Smith 2003b).

The osmoresistance of spores of *B. subtilis* has also been studied, and such resistance appears as a result of 3 major factors as detailed by Tovar-Rojo and others (2003):

(1) Specific characteristics of spores and cells of individual species

(2) The precise sporulation conditions that produce the spores

(3) Sufficient energy by the germination and outgrowing spore to adapt to conditions of osmotic strength

Induced changes in yeast gene expression have also been observed after osmotic stress. Salt shocked cells of *S. cerevisiae* showed an elevated synthesis of 9 proteins (Varela and others 1992), of which 2 were the heat shock proteins *hsp12* and *hsp26.* The induction of glycerol-3-phosphate dehydrogenase gene expression was also demonstrated.

Conclusions

The use of a_w as a determinant of microbial growth and metabolic activity has been of great practical value as a preservation system for foods. However, a number of microorganisms are able to tolerate osmotic stress. There are 2 important components of osmotic tolerance:

(1) Restoration and stabilization of the membrane lipid bilayer phase, which is achieved by an increase in the proportion of anionic membrane lipids and a decrease in the proportion of zwitterionic membrane lipids, which helps restore the lipid bilayer phase of the membrane

(2) The production of solutes that do not interfere with cell components and may reduce undesired chemical or enzymatic reactions by forming viscous states (Leistner and Russell 1991)

Many microorganisms are able to accumulate a wide range of compatible solutes, most of which are present in foods, allowing growth in reduced a_w foods. Knowledge of osmoregulation, including information about the regulation of the synthesis and activity of the systems, may help apply osmotic stress as an additional hurdle to preserve foods.

Combination of preservation factors

Each of the preservatives reduced temperature, pH, a_w, and weak acid preservatives have been discussed in isolation. However, it is usual to combine sublethal factors, to target different groups of organisms within a food product termed the *hurdle concept*. The hurdle effect is of fundamental importance for the preservation of foods because the hurdles in a stable food control the growth of microorganisms. If the microorganisms present in the food are not able to overcome the hurdles present, they will cause the food to spoil or even cause food poisoning, dependent on the microorganisms present.

A limitation to the success of hurdle technology foods could be the stress reactions of microorganisms, since exposure of a cell to multiple stresses can give some microbial cells an enhanced stress response system. For example, a combination of 4% NaCl at a pH value of 4.2 (adjusted with lactic acid) resulted in 10^3-fold more survivors of *E. coli* O157:H45 (1-log reduction) than lactic acid alone (4-log reduction). However, if the salt was added 45 min into the experiment, no cross-protective effects were observed. The pHi of the cell was 5.23 with no salt, whereas in the presence of 4% salt, the pHi was 5.79, suggesting that *E. coli* O157:H45 can use NaCl to counteract acidification of its cytoplasm by organic acids (Casey and Codon 2002). However, when the salt was added 45 min after acid stress, the pH of the cytoplasm was already reduced, and thus the cell was unable to counteract the effects.

O'Byrne and Booth (2002) also describes how if *L. monocytogenes* were pre-adapted to chill temperatures and then placed into a low a_w product, it would be able to respond more quickly to restore is osmotic balance, since at low temperatures, compatible solutes such as betaine and carnitine are produced that are also response mechanisms to osmotic stress.

Cross-protection against salt, heat, and hydrogen peroxide, for example, has been established in *B. subtilis*. Browne and Dowds (2001) describe a hierarchy of resistance in *B. cereus* with salt protecting against hydrogen peroxide, which protects against ethanol and oxidative stress. Garren and others (1998) also describe how acid tolerance and acid shock responses provide cross-protection to sodium lactate and sodium chloride in *E. coli* O157:H7 and non-O157:H7 strains.

Therefore, combinations of preservative effects may not always be relied on to achieve an additive effect on the inhibition of microorganisms in food if such stress responses can be produced and thus allow the microorganisms to grow. However, simulta-

neous exposure to different stresses will require energy-consuming synthesis of several protective stress shock proteins, which may cause the microorganisms to be metabolically exhausted.

Overall Conclusions

The study of stress adaptation in microorganisms has shown that complex changes in cell composition and regulation take place as a result of exposure to stressful environments. Such changes enable microorganisms to maintain the physiology of the cell and thus potentially survive and grow after exposure to stress conditions in food products. It has also been shown that there are many molecular mechanisms that microorganisms use to adapt and survive. Adaptive changes to environmental stresses require large amounts of energy, and during the adaptation stages, all normal cell division is stopped. This has several important consequences for microbial food poisoning or spoilage; as the lag phase before growth extends, the growth rate decreases and the final cell numbers decrease. The nutritional requirements and the enzymatic and chemical composition of cells are also affected (Herbert 1986, 1989). Microorganisms that have been stressed may therefore be more susceptible to food preservatives (such as organic acids) than nonstressed cells, resulting in changes in product shelf-life. This can be seen for a range of bacteria and yeasts in which the lag time at chill temperature is increased dramatically when there is an additional stress added, such as pH or salt. Betts and others (2000) showed that under conditions that included high levels of environmental stress, the duration of the lag phase of a cocktail of food spoilage yeasts was extremely long, and under the most extreme conditions tested (1 °C, pH 5.8, and 6% NaCl), the lag phase was more than 1000 h. Once bacteria have adjusted to a stressful environment, however, the lag phase may be shorter. Similar results have also been shown in Beales and others (2001), where shorter lag phases were observed with both *L. monocytogenes* and *P. fragi* when subjected to 8 °C, following initial adjustment to 8 °C compared with organisms grown optimally and then subjected to lower temperatures.

Stress responses toward a variety of extreme or changing conditions including temperature, acidity, osmolarity, and presence of preservatives have been demonstrated in a number of bacteria. Although the physiology, biochemistry, and genetic mechanisms have not been outlined for all stress responses (Berry and Foegeding 1997), in most cases when exposed to a mild stress, microorganisms may adapt to the stress, thus developing tolerance or resistance to greater amounts of that stress (Leyer and Johnson 1992). It is also clear that exposure to a low level of stress protects microorganisms against the otherwise lethal effect of a high level of the same stress or to other different stresses (Leyer and Johnson 1992).

Examinations of *E. coli* O157:H7 grown in liquid medium, for example, showed that the presence of organic acids favorably affected the survival of the bacterium at 4 °C (Conner and Kotrola 1995). Other studies have shown that acid shock and cold shock may lead to cross-protection to heat or high-pressure treatments (Berry and Foegeding 1997). In light of this, the current trends toward the adoption of milder minimal preservation techniques that leave more microorganisms viable, though inhibited in foods, may lead to more stress responses in foodborne microorganisms and therefore increased problems in the control of food spoilage and food poisoning. Thus, microorganisms that contaminate food products and which have adapted to stressful environmental conditions may have already developed the defenses to survive and may possibly grow in the foods. This cross-protection should be remembered when investigating the ability of microorganisms to grow or survive in foods. Therefore, despite the presence of preservation barriers such as low temperature, preservatives, and low pH, such adapted microorganisms may exhibit shorter lag phases, which will be of significance in terms of quality and safety of foods. Organisms used for bacterial challenge testing experiments should be considered carefully because organisms that have been previously adapted to the new situation will have a much shorter lag phase than organisms that have not been previously adapted and grown at optimal conditions. For example, Duffy and others (2000) recommended that pre-acid adapted *E. coli* O157 cultures should be used for challenging the microbiological safety of acidified foods. They state that the industry should characterize the range of ATRs and mechanisms, identify the most resistant strains, and ensure that these strains are easily available in the adapted state. They also concluded that the acid type, how the acidic environment is achieved, previous adaption, and different strains' responses should all be considered because these can all have an effect on the survival of *E. coli* O157. This is also true for the other parameters discussed in this review; therefore, careful consideration should be made when investigating the effects of microorganisms in foods.

Understanding microbial stress response mechanisms will improve the effective use of preservative factors, help manipulate the survival and growth of foodborne microorganisms, and exert microbial control at these points. It has been shown that the primary reaction of a microorganism to a stressful environment is to shut down replication processes and switch on adaptive mechanisms; while this reaction is in progress, no further growth will occur. Therefore, if these adaptive mechanisms can be disrupted, it is possible that the stress responses to environmental conditions will be incomplete and the microbial lag phase will be increased. For example, it has been stated that important responses to temperature are to (1) change membrane fluidity, (2) switch enzyme systems, and (3) use different metabolic pathways. If the storage conditions of the food or substrate availability was altered such that the microbes never attain the correct balance of these factors, then growth could be suspended. If this could be achieved, it would be of particular interest to the development of food-preservation technologies whose primary goal is to prevent food poisoning and spoilage due to microbial growth.

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