ELSEVIER

International Journal of Food Microbiology 50 (1999) 65–91

International Journal of Food Microbiology

# Microbial stress response in minimal processing

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**Abstract**

''Bacteria have evolved adaptive networks to face the challenges of changing environments and to survive under conditions of stress. Therefore, the efficiencies of inactivation and preservation methods need to be assessed, especially with regard to the enormous potential of food pathogens to adapt to a wide variety of stress conditions. All adaptive responses, whether to changing nutrients or to various stresses encountered in minimal processing, involve a series of genetic switches that control the metabolic changes taking place. A common regulatory mechanism involves the modification of sigma ($\sigma$) factors whose primary role is to bind to core RNA polymerase conferring promoter specificity directing expression of specialty regulons involved in heat-shock response, the chemotactic response, sporulation, and general stress response. Examples of the latter regulon in Gram-positive bacteria (the $\sigma^B$ regulon) and in Gram-negative bacteria (the RpoS regulon) will be discussed in more detail. Cellular adaptive mechanisms to starvation, cold shock, heat shock, (weak) acids, high osmolarity and high hydrostatic pressure will be described and their significance in food preservation and safety will be discussed.'' 

*Keywords:* Stress response; Sigma factors; Pathogens; Minimal processing

## 1. Introduction

Traditional ways to control microbial spoilage and safety hazards in foods, such as freezing, blanching, sterilization, curing and use of preservatives are being replaced by new, innovative techniques including mild heating, modified atmosphere and vacuum packaging, and the employment of natural antimicrobial systems. Additionally, the food industry has renewed interest in the use of high hydrostatic pressure as a food processing method because of reported quality improvements in specific food products subjected to high pressure treatments after packaging. Other new physical inactivation techniques such as pulsed electric field and high intensity laser are also gaining more attention and their possible application is being explored.

Preservation techniques are becoming milder in response to consumers' demands for higher quality, more convenient foods, that are less heavily processed (REPFEDs and 'Sous Vide' foods that are mildly heated), less heavily preserved (e.g. less acid,

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0168-1605/99/$ – see front matter 
PII: S0168-1605(99)00078-1

salt and sugar), and less reliant on preservatives such as, e.g. sulphite and nitrite. These products commonly rely on refrigerated storage and distribution for their preservation, both from microbial and quality retention standpoints. The major microbiological concerns associated with minimal processed foods are psychrotrophic and mesophilic microorganisms. Psychrotrophic microorganisms can grow at refrigeration temperatures, mesophilic pathogens can survive under refrigeration and may grow during temperature abuse. The types of microorganisms that cause the majority of outbreaks and sporadic cases of food poisoning, and which are of major concern in minimally processed foods are listed in Table 1 (Gould, 1998). Their temperature relationships are indicated to highlight firstly the wide range of minimum temperatures at which they may grow, and secondly the range of their tolerances to thermal inactivation.

Microorganisms have evolved signal transduction systems, which in response to environmental stresses, control the coordinated expression of genes involved in cellular defence mechanisms (Huisman and Kolter, 1994; Rees et al., 1995; Salmond et al., 1995; Hengge-Aronis, 1996a; Kennelly and Potts, 1996; Kleerebezem et al., 1997). Therefore, the efficiencies of these inactivation and preservation methods need to be assessed, especially with regard to the enormous potential of food pathogens to adapt to a wide variety of stress conditions. Cellular adaptive mechanisms to starvation, cold shock, heat shock, (weak) acids, high osmolarity and high hydrostatic pressure will be described and their significance in food preservation and safety discussed.

## 2. Regulation of gene expression: adaptive responses to environmental stresses

Bacteria have evolved adaptive networks to face the challenges of changing environments and to survive under conditions of stress. In some gram-positive organisms such as *Bacillus subtilis* the survival strategy involves differentiation to spores, whereas other organisms such as *Escherichia coli* enter what is normally referred to as stationary phase. In this apparently, non-differentiated state, significant physiological changes have taken place that allow the cells to survive a wide variety of environmental stresses including starvation, near UV-radiation, hydrogen peroxide, heat and high salt (Matin et al., 1989; Kolter et al., 1993; Nyström, 1995; Hengge-Aronis, 1996b). All adaptive responses, whether to changing nutrients or to various

Table 1
Food-poisoning microorganisms of concern in minimally processed foods (adapted from Gould, 1998)

| Minimum growth temperature | Heat resistance | |
|---|---|---|
| | Low[a] | High[b] |
| Approx. 0–5°C | *Listeria monocytogenes* (INF[c]) | *Clostridium botulinum* E and non-proteolytic B (TOX[d]) |
| | *Yersinia enterocolitica* (INF) | *Bacillus cereus* (INF and TOX) |
| | *Aeromonas hydrophila* (INF) | *Bacillus subtilis* (TOX) |
| | | *Bacillus licheniformis* (TOX) |
| Approx. 5–10°C | *Salmonella* species (INF) | |
| | *Vibrio parahaemolyticus* (INF) | |
| | *Escherichia coli* enteropathogenic and verocytotoxigenic strains (INF) | |
| | *Staphylococcus aureus* (TOX) | |
| Approx. 10–15°C | | *Clostridium botulinum* A and proteolytic B (TOX) |
| | | *Clostridium perfringens* (INF) |

[a] In excess of a 6 log inactivation of vegetative microorganisms by pasteurization, e.g. at a temperature of about 70°C for 2 min.
[b] In excess of a 6 log inactivation of spores at temperatures ranging from about 90°C for most heat-sensitive types to about 120°C for 10 min for the most heat-tolerant types.
[c] INF, organisms that may contaminate foods, and may multiply in them, and which cause food poisoning by infection.
[d] TOX, organisms that may contaminate food and multiply in them to form toxins that then cause food poisoning by intoxication.

stresses, involve a series of genetic switches that control the metabolic changes taking place. A common regulatory mechanism involves the modification of sigma (σ) factors whose primary role is to bind to core RNA polymerase conferring promoter specificity (Haldenwang, 1995). Sigma factors of *B. subtilis* and *E. coli* have been studied most extensively, and Table 2 provides an overview of their characteristics and possible functions. The main, or housekeeping σ factor ($\sigma^{70}$ in *E. coli* and $\sigma^{A}$ in *B. subtilis*) is responsible for transcription from a majority of the promoters. Alternate σ factors have different promoter specificities, directing expression of specialty regulons involved in heat-shock response, the chemotactic response, sporulation, and general stress response. Examples of the latter regulon in gram-positive bacteria (the $\sigma^{B}$ regulon) and in gram-negative bacteria (the RpoS regulon) will be discussed in more detail. For information about the other responses, the reader is referred to reviews by Haldenwang (1995) and Helmann and Chamberlain (1988).

Table 2
Sigma factors[a] of *B. subtilis* (upper panel) and *E. coli* lower panel; adapted from Helmann and Chamberlain (1988); Haldenwang (1995); Raina et al. (1995); Hengge-Aronis (1996a)

*B. subtilis*

| Sigma factor (alternative designation) | Gene(s) | Function | Promoter sequence[b] | | |
|---|---|---|---|---|---|
| | | | −35 | Spacer (bp) | −10 |
| Vegetative-cell factors | | | | | |
| $\sigma^{A}$ ($\sigma^{43}$, $\sigma^{55}$) | *sigA, rpoD* | Housekeeping/early sporulation | TTGACA | 17 | TATAAT |
| $\sigma^{B}$ ($\sigma^{37}$) | *sigB* | General stress response | RGGXTTRA | 14 | GGGTAT |
| $\sigma^{C}$ ($\sigma^{32}$) | Unknown | Postexponential gene expression | AAATC | 15 | TAXTGYTTZTA |
| $\sigma^{D}$ ($\sigma^{30}$) | *sigD, flaB* | Chemotaxis/autolysin/flagellar gene expression | TAAA | 15 | GCCGATAT |
| $\sigma^{H}$ ($\sigma^{30}$) | *sigH, spoOH* | Postexponential gene expression; competence and early sporulation genes | RWAGGAXXT | 14 | HGAAT |
| $\sigma^{L}$ | *sigL* | Degradative enzyme gene expression | TGGCAC | 5 | TTGCANNN |
| Sporulation-specific factors | | | | | |
| $\sigma^{E}$ ($\sigma^{29}$) | *sigE, spoIIGB* | Early mother cell gene expression | ZHATAXX | 14 | CATACAHT |
| $\sigma^{F}$ ($\sigma^{spoIIAC}$) | *sigF, spoIIAC* | Early forespore gene expression | GCATR | 15 | CGHRARHTX |
| $\sigma^{G}$ | *sigG, spoIIIG* | Late forespore gene expression | GHATR | 18 | CATXHTA |
| $\sigma^{K}$ ($\sigma^{27}$) | *sigK, spoIVCB:spoIIIC* | Late mother cell gene expression | AC | 17 | CATANNNTA |

*E. coli*

| Sigma factor (alternative designation) | Gene(s) | Function | Promoter sequence | |
|---|---|---|---|---|
| | | | −35 | −10 |
| $\sigma^{70}$ | RpoD | Housekeeping functions | TTGACA | TATAAT |
| $\sigma^{54}$ | GlnF, nrtA, rpoN | Nitrogen-regulated genes | CTGGCAC | TTGCA |
| $\sigma^{32}$ | HtpR, rpoH | Heat-shock genes | CTTGAA | CCCCAT-TA |
| $\sigma^{24}$ | rpoE | Heat-shock genes | GAACTT | TCTGA |
| $\sigma^{28}$ | FlbB+flaI, rpoF | Flagellar synthesis/chemotaxis | TAAA | GCCGATAA |
| $\sigma^{38}$ | RpoS, katF | Starvation/general stress response[c] | | |

[a] The designations for the sigma proteins and their structural genes as well as likely functions of their regulations are listed. The probable consensus sequences for the holoenzyme forms are aligned at their −10 positions (underlined). The spacer region (*B. subtilis*) represents the number of bases between the upstream-most −10 region base that is given and the downstream-most base of the −35 region.

[b] H, A or C; N, A, G, C or T; R, A or G; W, A, G or C; X, A or T; Y, C or T; Z, T or G.

[c] No consensus promoter sequence supplied. See text for details.

*B. subtilis* responds to signals of environmental and metabolic stress by inducing over 40 general stress genes under the control of the $\sigma^B$ transcription factor (Boylan et al., 1993; Haldenwang, 1995; Akbar et al., 1997). The $\sigma^B$ regulon includes the *katE* gene, encoding a catalase (Engelmann and Hecker, 1996), the *opuE* gene, encoding an osmoregulated proline transporter (Von Blohn et al., 1997), the *clpC* gene, which is similar to ATPase subunits of ClpP-type proteases (Kruger et al., 1996; Gerth et al., 1998), the *gtaB* gene, encoding a UDP-glucose pyrophosphorylase involved in trehalose synthesis (Varon et al., 1993), and several genes whose functions cannot be inferred from their sequences (Haldenwang, 1995).

The $\sigma^B$-dependent promoter of the *sigB* operon is principally regulated by the activity of $\sigma^B$ itself (Haldenwang, 1995). Sigma$^B$ activity is regulated post-translationally by a multi-component network composed of two partner-switching modules, RsbX-RsbS-RsbT and RsbU-RsbV-RsbW, each containing a serine phosphatase (X or U), an antagonist protein (S or V), and a switch protein/serine kinase (T or W; Benson and Haldenwang, 1993a; Alper et al., 1994; Kang et al., 1996; Akbar et al., 1997). As a result, $\sigma^B$ partitions between inactive complexes with an anti-sigma factor protein, RsbW (for regulation of $\sigma^B$), and free $\sigma^B$, which is capable of forming holoenzyme complexes with core RNA polymerase. At least two independent pathways exist which can alter the affinity of RsbW for its antagonist RsbV, or $\sigma^B$. First, it has been proposed that ATP stimulates the formation of RsbW-$\sigma^B$ complexes and activates a serine kinase activity in RsbW that phosphorylates its antagonist, RsbV, into an inactive state. Physiological signals such as temperature, pH, ethanol, and osmolarity can also invoke $\sigma^B$ activity (Völker et al., 1995), by stimulating the function of the RsbV-phosphate phosphatase RsbU (Kang et al., 1996). At least four other Rsb proteins (RsbR, RsbS, RsbT, and RsbX) modulate the activity of RsbU and serve as distinct points of signal input into the system (Kang et al., 1996; Akbar et al., 1997). While disruption of *sigB* in *B. subtilis* has no apparent effect on the organism's ability to grow under many conditions, $\sigma^B$ mutants have been shown to be sensitive to oxidative stress (Engelmann and Hecker, 1996; Antelmann et al., 1996). In cases where disruption of $\sigma^B$ did not effect stress resistance it appeared that other $\sigma$ factors, such as the house keeping transcription factor $\sigma^A$, can take over the stress-activated transcription of $\sigma^B$-dependent genes (Haldenwang, 1995; Kruger et al., 1996).

Recently, chromosomal gene clusters have been identified in *Staphylococcus aureus* (Wu et al., 1996; Kullik and Giachino, 1997) and in *Listeria monocytogenes* (Becker et al., 1998; Wiedmann et al., 1998), the gene products of which are highly homologous to the $\sigma^B$ operon in *B. subtilis*. The *L. monocytogenes* open reading frames (ORFs) showed significant predicted amino acid identities to RsbU, RsbV, RsbW, $\sigma^B$, and RsbX in *B. subtilis* and RsbU, RsbV, RsbW, and $\sigma^B$ in *S. aureus*. *L. monocytogenes* $\sigma^B$ mutants were shown to have reduced resistance to acid stress (Wiedmann et al., 1998) and to osmotic stress (Becker et al., 1998; see Section 3). Details about the effect of null mutations in the *sigB* gene on resistance to other stress conditions is currently lacking. It should be noted, however, that *sigB* transcription was stimulated in *L. monocytogenes* under a wide range of stress conditions including exposure to high osmolarity, low and high temperature, ethanol, EDTA, and entrance into stationary phase (Becker et al., 1998). In *S. aureus* stress resistance studies, $\sigma^B$ was shown to be involved in recovery from heat shock at 54°C and in acid and hydrogen peroxide resistance, but not in resistance to ethanol or osmotic shock (Kullik and Giachino, 1997; Chan et al., 1998). Transcription of *sigB* was shown to be effected by SarA, inferring a putative role for SarA in the *S. aureus* stress response. SarA is a DNA-binding protein, which binds to the *agr* regulatory region and mediates changes in virulence determinant production (Chan et al., 1998). The interaction between SarA and $\sigma^B$ determines the efficient transduction of environmental signals so as to allow bacterial adaptation and survival.

The $\sigma^s$ subunit of RNA polymerase (RpoS) is the master regulator of the general stress response in *E. coli* and other enteric bacteria including *Shigella flexneri* and *Salmonella typhimurium* (Small et al., 1994; Hengge-Aronis, 1996b). Regulation of *rpoS* transcription and translation, and RpoS activity, has been most extensively studied in *E. coli*. While levels of RpoS are low in rapidly growing cells not exposed to any particular stress, $\sigma^S$ is induced in response to a variety of rather diverse environmental stresses that

include starvation for various nutrients, stationary phase in general, high osmolarity, diauxic shift from glucose to lactose, and high or low temperature (Hengge-Aronis, 1993,1996a,b; Jishage et al., 1996; Fischer et al., 1998). These stresses differentially effect *rpoS* transcription and translation, as well as the rate of proteolysis of $\sigma^S$, which under non-stress conditions is a highly unstable protein.

Transcriptional control of *rpoS* is observed during entrance into the stationary phase, whereas it is not observed under osmotic stress conditions. For both conditions, it was found that highest levels of control of $\sigma^S$ activity are exerted during translation and by regulation of $\sigma^S$ turnover. *rpoS* mRNA is predicted to form a stable secondary structure resulting in poor translation. Under inducing conditions, an increased frequency of translational initiation may be obtained by alterations in this secondary structure. The RNA-binding protein HF-1 has been found to be essential for *rpoS* translation (Muffler et al., 1996), presumably by binding to *rpoS* mRNA, thus inducing conformational changes allowing translational initiation. The DNA-binding protein H-NS has also been implicated to be a component involved in the transduction of osmotic and growth-phase-related signals that cause induction of $\sigma^S$ (Yamashino et al., 1995). The effects of H-NS in the control of *rpoS* are post-transcriptional, with *hns* mutants showing enhanced *rpoS* translation as well as reduced $\sigma^S$ turnover. H-NS has been found to tightly associate in vitro with the HF-1 protein and it was speculated that such a complex may also be formed in vivo, thereby inhibiting the HF-1 activity resulting in low $\sigma^s$ levels (Hengge-Aronis, 1996b). Additionally, evidence has been presented that guanosine-3′,5′-bispyrophosphate (ppGpp; Lange et al., 1995) and a homoserine lactone (HSL; Huisman and Kolter, 1994) act as positive signals for *rpoS* transcription. When enterobacteria such as *E. coli* are starved for amino acids, they elicit the stringent response, characterized by the accumulation of the nucleotide ppGpp. Other metabolic processes such as nutrient limitation also cause accumulation of this compound. Two different enzymes, the ribosome-associated RelA (ppGpp synthetase I) and SpoT (ppGpp synthetase II) synthesize ppGpp. The latter enzyme can either act as a synthetase or as a hydrolase, depending on the growth conditions (for a review see Cashel et al., 1996). Alterations at the intracellular level of ppGpp have pleiotropic effects on metabolism, e.g. the nucleotide binds to the β subunit of RNA polymerase thereby modulating polymerase specificity, inhibits the accumulation of ribosomal RNA (rRNA) and protein synthesis, and stimulates metabolism of certain amino acids. The altered specificity of the RNA polymerase may result in enhanced transcription of *rpoS* (Hengge-Aronis, 1996b). It has been proposed that the synthesis of HSL arises as a natural response to starvation/stationary phase in *E. coli* (Huisman and Kolter, 1994). However, the relationship between starvation, HSL production and RpoS activity is still unclear.

The levels of $\sigma^S$ in growing non-stressed cells are low, due to rapid turnover mediated by ClpXP protease (Schweder et al., 1996). ClpXP degradation of $\sigma^S$ absolutely requires the two-component regulator RssB, also called SprE (Pratt and Silhavy, 1996) or, in *S. typhimurium*, MviA (Bearson et al., 1996). The activity of response regulators is mediated by a C-terminal 'output' domain and controlled by an N-terminal 'receiver' domain. The receiver domain may be phosphorylated or dephosphorylated in response to signals transmitted by sensory histidine kinases. Conceivably, the state of phosphorylation of RssB and therefore its activity may be altered in response to osmotic and starvation signals (Hengge-Aronis, 1996a). Recently, the phosphorylation of RssB at the conserved aspartic acid was demonstrated in vitro with acetyl phosphate, and a mutant unable to synthesize this compound showed significantly decreased RssB-promoted proteolytic degradation (Bouche et al., 1998). RssB may effect the activity of the ClpXP protease in general or its recognition of $\sigma^S$ as a substrate. Gibson and Silhavy (1999) provided evidence that the activity of RssB is modulated by LrhA. Since LrhA is a putative DNA-binding transcription factor, it is likely to regulate RssB activity indirectly.

RpoS controls the expression of more than 35 genes involved in general stress response in *E. coli*. Inactivation of *rpoS* renders cells sensitive to heat shock, oxidative stress, starvation, acid, ethanol, and near UV light (Hengge-Aronis, 1996a; Farewell et al., 1998). Among these stress-induced genes are *bolA*, involved in modulating cell morphology; *cfa*, involved in cyclopropane fatty acid synthesis; *uspB*, involved in ethanol resistance; *katG* and *katE*, two catalases which degrade hydrogen peroxide; *otsA*,

*otsB* and *treA*, involved in trehalose synthesis; *ftsQAZ*, involved in cell division; *glgS*, involved in glycogen synthesis; *csgBA* and *gscCDEF* operons, involved in the synthesis of curli fimbriae. Derivation of a consensus sequence for RpoS-dependent promoters proved to be very difficult because of the similarity to $\sigma^{70}$ promoters and because of sequence variations arising from the involvement of additional regulatory factors (Table 2; Hengge-Aronis, 1996a). The direct relevance of RpoS to food microbiology has been the discovery that bacteria defective in *rpoS*, are highly sensitive to food processing conditions (Rees et al., 1995), including stress conditions imposed on bacteria during minimal processing (see below).

## 3. Osmotic stress

Among the many ways used to preserve food products, increased osmotic pressure, i.e. lowering of water activity ($a_w$), is one of the most widely used. Desiccation or addition of high amounts of osmotically active compounds such as salts or sugars lowers the water activity of the food. Therefore, understanding the processes underlying osmotic adaptation of pathogenic microorganisms is of crucial importance in trying to design new ways for controlling growth of food spoilage and pathogenic bacteria in low and medium water activity foods. The internal osmotic pressure in bacterial cells is higher than that of the surrounding medium. This results in a pressure exerted outwards on the cell wall, called the turgor pressure, which is thought to provide the mechanical force necessary for cell elongation (Csonka, 1989). Therefore, bacterial cells must be able to maintain turgor despite variations in the osmotic pressure of the surrounding medium.

A universal response to the temporary loss of turgor following a hyperosmotic shock is the cytoplasmic accumulation of a certain class of solutes that do not interfere too seriously with the functioning of cytoplasmic enzymes, the so-called 'compatible solutes' (Csonka, 1989; Booth et al., 1994; Csonka and Epstein, 1996). These compounds are small organic molecules, which share a number of common properties: they are soluble to high concentrations and can be accumulated to very high levels in the cytoplasm of osmotically-stressed cells; they are usually either neutral or zwitterionic molecules; specific transport systems are present in the cytoplasmic membrane allowing the controlled accumulation of these compounds, and, they do not alter enzyme activity and may even protect enzymes from denaturation by salts or protect them against freezing and drying. There is great variation in the compatible solutes accumulated by microorganisms: betaine, carnitine, trehalose, glycerol, sucrose, proline, mannitol, glucitol, ectoine, and small peptides.

The adaptability of food spoilage microorganisms and food pathogens, including *E. coli* O157:H7, *S. typhimurium*, *B. subtilis*, *L. monocytogenes* and *S. aureus*, to osmotic stress is most efficiently mediated by the accumulation of betaine (*N*,*N*,*N*-trimethylglycine) via specific transporters (Booth et al., 1994; Patchett et al., 1994; Csonka and Epstein, 1996; Verheul et al., 1997). *E. coli*, *B. subtilis* and *S. aureus* can also synthesize betaine from exogenously provided choline. This pathway, which is under osmotic control, involves a choline transporter, an alcohol dehydrogenase and a betaine aldehyde dehydrogenase, which after uptake and enzymatic conversion results in the accumulation of betaine (Landfald and Strom, 1986; Kaenjak et al., 1993; Bloch et al., 1994, 1996). The known transport systems for betaine and proline can be subdivided into two groups: (i) the binding protein-dependent, ATP-driven systems (Ames et al., 1990) that include ProU of *E. coli* and *S. typhimurium*, and OpuA and OpuC of *B. subtilis*; (ii) the ion-motive force-driven transporters examplified by ProP of *E. coli*, OpuD and OpuE of *B. subtilis*, BetL of *L. monocytogenes,* and the *S. aureus* betaine and proline transporters (see below).

Betaine is present at high concentrations in sugar beets and other foods of plant origin (Rhodes and Hanson, 1993). Betaine transport has been most extensively characterized in *E. coli* and *S. typhimurium*, and was shown to proceed via the constitutive, $Na^+$-dependent secondary transporter ProP (Csonka, 1989; Culham et al., 1993). The high affinity, ATP-driven, binding protein-dependent transport system ProU is induced under conditions of osmotic stress either in the absence or in the presence of low (micromolar) levels of betaine (Lucht and Bremer, 1994). Additionally, the activity of the ProP and ProU systems is significantly increased upon exposure of cells to an osmotic

upshift. The ProP transport protein contains 12 transmembrane regions, typical for secondary transporters, but is characterized additionally by the presence of an extended central hydrophilic loop and a carboxy-terminal extension that is predicted to form an alpha-helical coiled coil; both structural elements are located internally (Culham et al., 1993). It was speculated that the carboxy-terminal extension is relevant for the osmoregulation of activity. Clear evidence that the carboxy-terminal extension has a role in osmosensing comes from studies on the betaine transporter BetP of *Corynebacterium glutamicum* (Peter et al., 1998). The carboxy-terminal extension is 55 amino acids long, and has a large excess of positively charged residues. Deletions in this domain result in complete loss of regulation. A similar carboxy-terminal extension has been observed for other osmotically-activated, secondary trimethylammonium compound transporters including BetT of *E. coli* and BetT-like of *Haemophilus influenzae* (Lamark et al., 1991; Poolman and Glaasker, 1998). However, the exact role of the carboxy-terminal extension in osmoregulation of transporter activity, other than BetP, remains to be elucidated. As noted by Poolman and Glaasker (1998), with the exception of ProP in enteric bacteria, BetP in *C. glutamicum* and the transport systems for compatible solutes in *L. monocytogenes* (and *Lactobacillus plantarum*), the hyperosmotic activation is often poorly described (see below).

It is assumed that the ProP system is the main osmolyte transporter in *E. coli* and *S. typhimurium* cells in foods, since synthesis of the ProU system is most likely prevented due to the relatively high (mM) levels of betaine, or alternatively carnitine (see below), encountered in these environments. It can, however, not be excluded that starved cells, which have synthesised ProU, are introduced into the food. Whether such cells, containing both active ProP and ProU, have an advantage over the ProP containing cells is not known. Koo and Booth (1994) have shown, using betaine transport mutants of *S. typhimurium*, that ProU in particular can provide the cell with sufficient betaine when this compound is supplied at only very low levels in the environment.

Multiple transport systems are involved in the uptake of betaine in *B. subtilis*. Two systems, OpuA (osmoprotectant uptake) and OpuC (ProU) have been identified (Kempf and Bremer, 1995; Lin and Hansen, 1995) as members of the ATP-driven, binding protein-dependent transport systems. The OpuA system comprises three components: OpuAA, an ATPase; OpuAB, an integral protein of the cytoplasmic membrane; and OpuAC, an extracellular substrate binding protein that is anchored in the cytoplasmic membrane via a lipid modification. The OpuC betaine uptake system is related to OpuA but contains an additional integral membrane component. Both OpuA and OpuC exhibit structural and functional similarities to the ProU system from *E. coli* (Gowrishankar, 1989; Lucht and Bremer, 1994). The third betaine uptake system, OpuD, consists of only one component. OpuD (512 amino acid residues, 56.1 kDa) is a member of a small family of transport proteins involved in the accumulation of trimethylammonium compounds. The OpuD-mediated betaine uptake is controlled by the environmental osmolarity. High osmolarity stimulates de novo synthesis of OpuD and activates pre-existing OpuD proteins to achieve maximal betaine uptake activity (Kappes et al., 1996). Each of the three betaine transport systems in *B. subtilis* shows high substrate affinity, with $K_m$ values in the low micromolar range, allowing uptake of betaine from the environment even when this osmoprotectant is present at a very low concentration. The OpuA system appears to be the predominant betaine transporter in *B. subtilis*. Under low osmolarity conditions, the contribution of the OpuA system to betaine uptake exceeds by far that made by the OpuC and OpuD transporters. The high basal level in OpuA activity is a reflection of the dual transcriptional regulation of the *opuA* operon. It is expressed both from an osmotically inducible promoter and from transcription initiation signals that mediate constitutive expression of *opuA* in log-phase cells (Kempf and Bremer, 1995). The accumulation of this osmoprotectant at low osmolarity in gram-positive bacteria appears to be of general physiological importance, and it is probably connected with the high turgor maintained by these organisms (Poolman and Glaasker, 1998).

*B. subtilis sigB* mutants do not show obvious defects in osmotolerance (Von Blohn et al., 1997), likely due to the existence of both multiple regulatory systems and redundant transporters in this organism (see above). Moreover, in the case of the *opuE* gene, encoding a proline transporter, its $\sigma^B$-dependent promoter OpuE-p2, is one of the two

osmotically inducible promoters, the other one, OpuE-p1, being dependent on the house-keeping transcription factor $\sigma^A$. An elevation of the osmolarity of the medium by either ionic or non-ionic compounds resulted in a strong increase in the OpuE-mediated proline uptake, which appeared to be entirely dependent on de novo protein synthesis, suggesting a transcriptional control mechanism. Such a dual control has already been described for a number of $\sigma^B$-responsive genes (Kruger et al., 1996; Hecker et al., 1996), indicating that many members of the *B. subtilis* general stress regulon are part of a redundantly regulated system. The $\sigma^B$-dependent control of *opuE-p2* activity links the OpuE-mediated uptake of proline to the general stress regulon of *B. subtilis*. Although the addition of salt triggers enhanced expression of many genes from the $\sigma^B$ regulon (see above), no clear function in osmoadaptation has emerged for any of its members (Varon et al., 1993). However, $\sigma^B$ is dispensable for the induction of the OpuE system under high osmolarity conditions, indicating that the activity of the $\sigma^A$-dependent *opuE*-p1 promoter is sufficient for the overall osmotic control of *opuE* (Von Blohn et al., 1997). Apparently, two independent signal transduction pathways operate in *B. subtilis* to control the level of *opuE* expression osmotically.

*S. aureus* is the most halotolerant, non-halophilic eubacterium, and the organism can grow at $a_w$-values as low as 0.86 (equivalent to 3.5 M NaCl; Graham and Wilkinson, 1992; Townsend and Wilkinson, 1992). *S. aureus* is a common cause of food poisoning outbreaks in western countries. Growth studies have shown that exogenously supplied taurine, proline, choline, and betaine are osmoprotectants for *S. aureus* (Graham and Wilkinson, 1992). Furthermore, different sodium-dependent transport systems for the uptake of taurine, proline and betaine have been characterized (Graham and Wilkinson, 1992; Pourkomailian and Booth, 1992; Townsend and Wilkinson, 1992; Kaenjak et al., 1993; Stimeling et al., 1994). The proline content of *S. aureus* increases upon osmotic stress with sodium chloride. Studies of the kinetics of proline transport revealed high-affinity ($K_m$ is 1.7 μM) and low-affinity ($K_m$ is 132 μM) transport systems (Townsend and Wilkinson, 1992). The proline transport activity of the low-affinity system was stimulated by increased osmotic strength. It was suggested that the low-affinity system is involved in adjusting to increased environmental osmolarity and that the high-affinity system is involved in scavenging low concentrations of proline (Townsend and Wilkinson, 1992).

Betaine is the most efficient osmoprotectant in *S. aureus* (Graham and Wilkinson, 1992). Betaine accumulation to high levels is achieved by the action of two sodium-dependent transport systems, which could be differentiated on the basis of their affinity for betaine and their activation by osmotic pressure (Pourkomailian and Booth, 1992, 1994; Stimeling et al., 1994). The high-affinity system ($K_m$ is 3 μM) was highly specific for betaine, and was independent of osmotic pressure. The low-affinity system was suggested to be identical to the osmotically-activated, low-affinity proline transport system (see above). Recently, evidence was presented that feedback regulation of the activity of the uptake systems was the major mechanism for controlling the level of compatible solute accumulation (Pourkomailian and Booth, 1994; Stimeling et al., 1994). Furthermore, in cells that had been preloaded with betaine, the high-affinity proline transport system was undetectable and the $V_{max}$ of the low-affinity system was reduced almost 30-fold. It was suggested that the high-affinity proline and betaine transport systems, though separate proteins, are regulated in the same manner by compatible solute accumulation, i.e. the intracellular osmolarity (Pourkomailian and Booth, 1994).

Betaine transport in *L. monocytogenes* has been shown to be mediated by a highly specific, constitutive, energy-dependent secondary transport system (Patchett et al., 1994; Verheul et al., 1997). Gerhardt et al. (1996) have shown that betaine is transported in symport with sodium ions in membrane vesicles of *L. monocytogenes*. Recently, Sleator et al. (1999) cloned and characterized *betL*, a gene encoding a 507 amino acid residue (55 kDa) betaine transporter from *L. monocytogenes*. BetL is highly specific for betaine ($K_m$ value of 7.8 μM) and fails to transport other trimethylammonium compounds such as carnitine. The transporter exhibits significant sequence homologies to OpuD from *B. subtilis* (57% identity) and BetP from *C. glutamicum* (41% identity; Kappes et al., 1996; Peter et al., 1998). In addition, a *betL* knockout mutant was constructed in *L. monocytogenes*, which was significantly affected in its ability to accumulate betaine in the presence or absence of NaCl. This mutant is also unable to tolerate the same

salt concentrations as the *betL*+ parent, signifying its role in osmotolerance of *L. monocytogenes* LO28 (Sleator et al., 1999). Interestingly, the *betL* -10 and -35 promoter binding sites (Sleator et al., 1999) show similarity to the recently characterized $\sigma^B$-dependent promoters (Becker et al., 1998). The activity of $\sigma^B$ was shown to be highly responsive to an osmotic upshift, suggesting a role in coordinating osmotic responses in *L. monocytogenes*. Indeed, a null mutation in $\sigma^B$ reduced the ability of *L. monocytogenes* to accumulate betaine, i.e. stimulation of betaine uptake after osmotic upshock was not observed (Becker et al., 1998). BetL thus may represent this predicted $\sigma^B$-mediated sodium or osmotically inducible component of betaine transport in *L. monocytogenes*. Recently, an operon encoding an ABC transporter, homologous to the *opuC* operon encoding a betaine transporter in *B. subtilis*, was identified in *L. monocytogenes* (C.P. O'Byrne, personal communication). The role of this putative ATP-dependent betaine transporter remains to be elucidated.

Beumer et al. (1994) have shown that exogenously supplied carnitine (β-hydroxy-L-τ-*N*-trimethyl aminobutyrate) can contribute significantly to growth of *L. monocytogenes* at high osmolarity. L-Carnitine occurs ubiquitously in biological material because of its crucial function in the oxidation of fatty acids in mitochondria (Bieber, 1988). An ATP-dependent, high-affinity L-carnitine transport system in *L. monocytogenes* enables the bacterium to scavenge L-carnitine when it is available at trace levels in foods (Verheul et al., 1995; Fig. 1). Strikingly, the transport capacity of the L-carnitine permease was very high over a wide range of temperatures and this activity could not be stimulated upon imposition of an osmotic stress. This implicates that carnitine accumulation in *L. monocytogenes* can occur even under conditions of low osmolarity. Smith (1996) reported the accumulation of betaine and carnitine by *L. monocytogenes* grown on processed meat surfaces. Uptake of these osmoprotectants was increased at low temperature and at high osmolarities, signifying the importance of these transporters in growth of this pathogen in meat products. As described above, the structural analogues betaine and carnitine are taken up via separate systems that respond to an osmotic upshock only when *L. monocytogenes* ScottA cells have been pregrown in the presence of betaine or carnitine. These systems, although highly specific for their substrates, are inhibited by both betaine and carnitine at the cytoplasmic face (*trans* site) of the membrane. Without intracellular betaine and/or carnitine, the activity is maximal and not affected by medium osmolarity. The inhibition by intracellular betaine and carnitine is relieved upon osmotic upshock, which allows these cells to accumulate these compatible solutes further and restore turgor more rapidly (Verheul et al., 1997). In kinetic terms, the activation of the betaine and carnitine uptake systems of *L. monocytogenes* upon osmotic upshock is thought to reflect an increase in the apparent $K_i$ (apparent inhibition constant) for the compatible solutes at the inner surface of the membrane. Apparently, a decrease in turgor alters the internal binding site for betaine and carnitine, a phenomenon that is observed for both the ion-motive force driven betaine uptake system and the ATP-driven carnitine system.

Carnitine can also function as an osmoprotectant in *E. coli*, including the enterohaemorrhagic *E. coli* O157:H7, and it is taken up via the ProP and ProU transporters both under aerobic and anaerobic conditions (Verheul et al., 1998). This finding may be important for the microbiological safety of meat products. Apparently, microorganisms can accumulate a wide spectrum of compatible solutes, most of which are present in significant amounts in foods, thereby allowing growth at reduced water activities. Knowledge about the osmoregulation, including information about the regulation of the synthesis and activity of the various transport systems for compatible solutes, and their role in cryoprotection, may provide clues to impose osmotic stress and low temperature optimally as hurdles in the preservation of foods.

## 4. pH stress

Sudden as well as gradual exposures to acid stress occur in a variety of ecological niches occupied by food pathogens. The acidification of foods is an age-old means of food preservation, and is still used as the principle barrier to the outgrowth of pathogens and spoilage bacteria. *S. typhimurium*, *E. coli* O157:H7, *Shigella flexneri* and *L. monocytogenes* are neutrophiles; they grow best at neutral pH. These

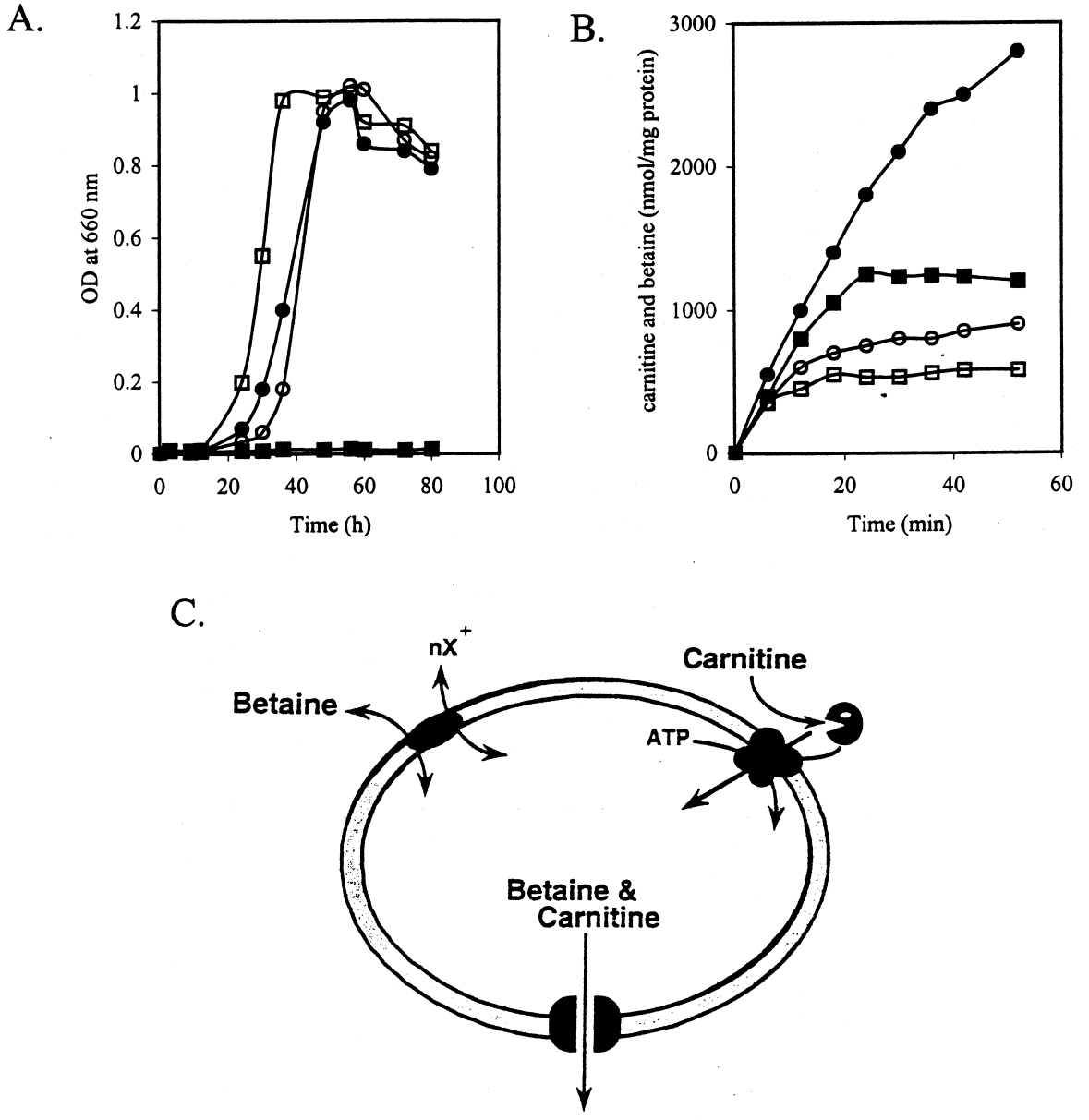


Fig. 1. Growth, transport and overview of uptake systems involved in osmoregulation of *L. monocytogenes*. Growth of *L. monocytogenes* ScottA in Defined Medium (DM, open squares), in DM supplemented with 3% NaCl (closed squares) and in DM supplemented with 3% NaCl and 1 mM betaine (closed circles) or 1 mM carnitine (open circles) at 37°C as determined by $OD_{660}$ (A). Simultaneous uptake of [$^{3}$H]betaine (circles) and L-[$^{14}$C]carnitine (squares) under low (open symbols) and high (closed symbols) osmolarity conditions (B). Overview of uptake and efflux systems for betaine and L-carnitine in *L. monocytogenes*. Betaine and L-carnitine enter the cytoplasm of *L. monocytogenes* via distinct transport systems. Upon an osmotic downshock, betaine and L-carnitine are rapidly released to the external medium through activation of protein channels in the cytoplasmic membrane (C). After Beumer et al. (1994) and Verheul et al. (1997). See text for details.

microorganisms may be exposed to dramatic pH fluctuations in nature, for example in foods, and during pathogenesis. While travelling through the gastrointestinal tract they must endure extreme low pH in the stomach, as well as volatile fatty acids present in the intestine. Facultative intracellular pathogens such as *Salmonella* and *Listeria* also tolerate episodes of low pH within macrophage phagolysosomes. Research on acid tolerance response (ATR) in *L. monocytogenes* was recently initiated and will be discussed by Hill et al. in this issue.

Acid stress can be described as the combined biological effect of low pH and weak (organic) acids, such as acetate, propionate and lactate present in the environment (food) as a result of fermentation, or alternatively, when added as preservatives (Zhao et al. 1993; Garlant Miller and Kaspar, 1994; Bearso et al., 1997). Weak acids in their unprotonated form can diffuse into the cell and dissociate, thereby lowering the intracellular pH ($pH_{in}$) resulting in the inhibition of various essential metabolic and anabolic processes. In response to encounters with acids these organisms have evolved complex, inducible acid survival strategies (Fig. 2). Regulatory features include an alternative σ factor $\sigma^s$, two-component signal transduction systems (e.g. PhoP and PhoQ), and the major iron regulatory protein Fur (Foster, 1995; Waterman and Small, 1996; Maham et al., 1996; Bearso et al., 1997). The alternative sigma factor $\sigma^s$, encoded by *rpoS*, was shown to be an acid shock protein (ASP) that controls the expression of at least eight other ASPs in *S. typhimurium* (Lee et al., 1995). The acid shock induction of RpoS appears to be controlled by a 38-kDa protein, encoded by the mouse virulence gene *mviA* (Bearson et al., 1996). MviA controls the accumulation of RpoS and of RpoS-dependent ASPs by regulating the proteolytic turnover of RpoS; MviA stimulates turnover in the absence of stress while allowing RpoS to accumulate in the presence of stress. MviA is probably a sensor of perturbation of cellular physiology, and somehow can activate the ClpXP protease (Gottesman et al., 1997), which degrades RpoS (Schweder et al., 1996). Mutations in either *rpoS* or *mviA* render *Salmonella* avirulent, which suggests that either under or overproducing RpoS is detrimental to the pathogenic process (Benjamin et al., 1991; Fang et al., 1992). Interestingly, the presently used live oral typhoid vaccine, attenuated *S. typh* Ty21a, is, in fact, an *rpoS* mutant which is highly susceptible to various environmental stresses (Robbe-Saule et al., 1995).

Other subsets of ASPs produced by *S.*

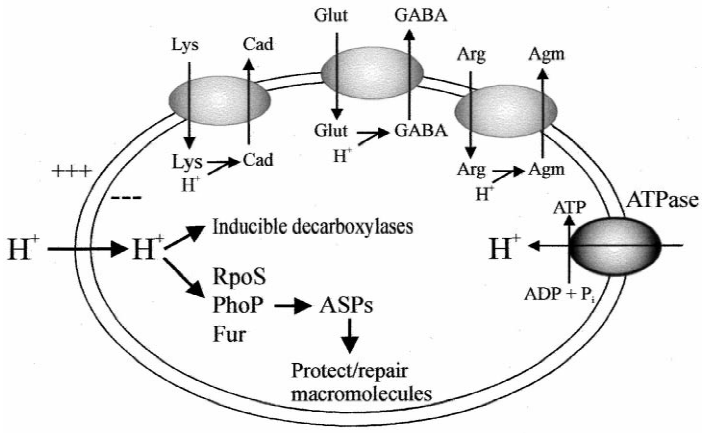


Fig. 2. Acid survival response in enteric microorganisms. The figure represents a composite cell containing all known components of inducible acid tolerance and acid resistance. Low $pH_{in}$ will induce several amino acid decarboxylases (Glut, glutamate; Lys, lysine; Arg, arginine; GABA, gamma-amino-isobutyrate; Cad, cadaverine; Agm, agmatine). Furthermore, low pH will increase accumulation of RpoS, PhoP and Fur that control distinct sets of ASPs defining partially redundant systems of acid tolerance. The function of ASPs is presumed to include the prevention and/or repair of acid-induced damage to macromolecules. The membrane potential (negative inside) generated by substrate product exchange, and the pH gradient generated by decarboxylase activity can drive ATP synthesis by the $F_0F_1$ ATPase (adapted from Bearso et al., 1997). See text for details.

*typhimurium* are dependent on Fur and PhoP and a total of 51 for log-phase ATR and 15 for stationary phase ATR have been described (Bearso et al., 1997). Effective acid tolerance involves RecA-independent DNA repair systems, iron, and facets of fatty acid metabolism (Foster, 1995). The ATR described for *S. typhimurium* differs from the acid response (AR) described for *E. coli* and *S. flexneri*. An important feature of the ATR is that it can function within the confines of a minimal medium, whereas AR requires components of complex medium for induction and/or function (Lin et al., 1995). Furthermore, AR enables survival at pH 2, whereas the maximum limit of ATR is pH 3. It has become clear that several inducible amino acid decarboxylases play an important role in $pH_{in}$ maintenance in enterobacteria (Bearso et al., 1997). The low pH-inducible lysine decarboxylase was shown to contribute significantly to pH homeostasis in *S. typhimurium* in environments as low as pH 3. Under these conditions, both lysine decarboxylase and RpoS-dependent ASPs were required for acid tolerance but only lysine decarboxylase contributed to pH homeostasis (Park et al., 1996). The lysine decarboxylase (CadA) works in cooperation with a lysine-cadaverine antiporter (CadB; Fig. 2). CadA decarboxylates intracellular lysine to cadaverine consuming a proton in the process. Cadaverine (charge $+2$) is then exchanged for extracellular lysine (charge $+1$) from the medium via the CadB antiporter. In addition to the rise in intracellular pH due to proton consumption, the net efflux of positive charge results in the generation of a membrane potential (negative inside), such that the generation of both components of the proton motive force, the pH gradient (alkaline inside) and the membrane potential (negative inside), can drive ATP synthesis via the $F_0F_1$ $H^+$-ATPase. *S. typhimurium* also contains inducible ornithine and arginine decarboxylases and the corresponding antiporters, which suggests that this organism can survive many extreme acid pH situations depending on which amino acids are present in the surrounding food environment.

For *E. coli*, three complex medium-dependent AR systems not present in *S. typhimurium* have been described (Lin et al., 1995). Two of these systems are also present in *S. flexneri*. The activity of each system depends in part on whether cells have undergone fermentative or oxidative metabolism. Two fermentative AR systems involve arginine decarboxylase and glutamate decarboxylase with the corresponding antiporters mediating arginine/agmatine and glutamate/gamma-amino-isobutyrate exchange, respectively (Park et al., 1996, Bearso et al., 1997; Fig. 2). The arginine and glutamate systems are encoded for by the *adi* and the *gadB*/*gadC* operons, respectively. The *S. flexneri* glutamate system is very dependent on RpoS due to the role of this sigma factor in *gadC* expression (Park et al., 1996). GadBC was also shown to provide acid resistance in the lactic acid bacterium *Lactococcus lactis* (Sanders, 1997). Promoter studies revealed that the *gadBC* operon is induced by chloride. Remarkably, glutamate-dependent acid resistance in *S. flexneri* is enhanced in the presence of NaCl; an effect not caused by the higher osmolarity of the medium (Waterman and Small, 1996). Sanders (1997) suggested that it might well be that *gadC* expression in *S. flexneri* is also stimulated by chloride. This is especially important with regard to the exposure of these microorganisms to highly acidic and chloride rich conditions in the stomach. Clearly, the exact regulatory mechanisms involved in the expression of antiporter/decarboxylase systems remain to be elucidated. The third AR system is the so-called oxidative system, found both in *E. coli* and *S. flexneri*. It is induced by growth to stationary phase in LB, is repressed by glucose and, once induced does not require the presence of amino acids in the medium during a subsequent challenge at pH 2.5. The oxidative system is dependent on RpoS in both organisms, whereas the regulation for the other AR and ATR systems in enterobacteria may be regulated differently (Bearso et al., 1997).

An important additional aspect of the ATR is the induction of cross-protection to a variety of stresses (heat, osmolarity, oxidation, membrane active compounds) in exponentially grown (log phase) cells. Cross protection of acid-shocked and acid-adapted *S. typhimurium* and *E. coli* O157:H7 against thermal and osmotic stresses has been reported (Leyer and Johnson, 1993; Ryu and Beuchat, 1998; Garren et al., 1998). Acid-adapted cells are those that have been exposed to a gradual decrease in environmental pH, whereas acid-shocked cells are those which have been exposed to an abrupt shift from high pH to low pH. As suggested by Ryu and Beuchat (1998), these

processes may result in cells exhibiting different physiological states and, thus, potentially different tolerances to secondary stresses imposed by food environments. Acid-adapted, but not acid-shocked *E. coli* O157:H7 cells in acidified TSB and low pH fruit juices, were shown to have enhanced heat tolerance in TSB at 52°C and 54°C, and in apple cider and in orange juice at 52°C (Ryu and Beuchat, 1998). Acid-induced general stress resistance may reduce the efficiency of hurdle technologies, which rely upon the imposition of multiple stresses, i.e. 'combination preservation' techniques (Gould, 1998).

## 5. Heat-shock response

Food preservation by elevation of temperature (i.e. blanching, pasteurisation, sterilisation) is a common process of food preservation in use today. Reduction or inactivation of microbial populations can be achieved by thermal processes using different means such as water, steam, hot air, electrical, light, ultrasound or microwave energy (Heldman and Lund, 1992). However, in the majority of cases, the process variables and controls have been derived by empirical investigation of the effect of temperature and time of exposure on microbial survival kinetics. There are major differences in the heat resistance of food poisoning bacteria. These are most clearly demonstrated by the ability of some counterparts of this group to form spores (Gould, 1998; Table 1).

Cells contain several targets for the action of heat, and so it can be proposed that the basal heat resistance of microorganisms may be due to the intrinsic stability of macromolecules, i.e. ribosomes, nucleic acids, enzymes and proteins inside the cell and the membrane. Ribosomal subunits may lose their specific secondary and tertiary structure whereas proteins may coagulate upon heating. Single-stranded RNA, as well as single stranded DNA are susceptible to heat damage, however the latter is ~30 times less sensitive (Earnshaw et al., 1995). Membrane damage is mentioned as another target for the heat inactivation of bacterial cells. Mild heat treatments can lead to adaptation of the cell membrane by increasing the saturation and the length of the fatty acids in order to maintain optimal fluidity of the membrane and activity of intrinsic proteins (Russell and Fukanaga, 1990). Vossenberg et al. (1995) reported that the proton permeability of the cytoplasmic membrane probably determines the maximum growth temperature. The exact prime cause for cell death upon heat exposure is still not clearly understood (Earnshaw et al., 1995).

It has been reported that bacterial heat tolerance is affected by a wide variety of factors. Bacterial thermotolerance was shown to increase upon exposure to sublethal heating temperatures, viral infections and chemical compounds such as ethanol, methylating agents, antibiotics (such as kanamycin, streptomycin) and amino acid restrictors (Neidhardt et al., 1984). Protection against heat may be achieved by the accumulation of osmolytes that may enhance protein stability and protect enzymes against heat activation (Taneja and Ahmed, 1994; Earnshaw et al., 1995). Another successful adaptation of certain microorganisms, like the members of the genera *Bacillus* and *Clostridium*, to resist exposure to heat is their ability to produce spores (Gould et al., 1995). Evidence also exists for a connection between the synthesis of heat-shock proteins (HSPs) and the development of thermotolerance (Georgopoulos and Welch, 1993; Hecker et al., 1996).

When bacterial cells are exposed to higher temperatures, a set of HSPs is rapidly induced. The primary structure of most HSPs appears to be highly conserved in a wide variety of microorganisms. HSPs involve both chaperones and proteases which act together to maintain quality control of cellular proteins. Both types of enzymes have as their substrates a variety of misfolded and partially-folded proteins that arise from slow rates of folding or assembly, chemical or thermal stress, intrinsic structural instability, and biosynthetic errors (Gottesman et al., 1997; Fig. 3). The primary function of classical chaperones, such as the *E. coli* DnaK (Hsp70) and its co-chaperones, DnaJ and GrpE, and GroEL (Hsp60) and its co-chaperone, GroES, is to modulate protein folding pathways, thereby preventing misfolding and aggregation, and promoting refolding and proper assembly (Georgopoulos and Welch, 1993). HSPs are induced by several stress situations, e.g. heat, acid, oxidative stress and macrophage survival, which suggests that HSPs contribute to bacterial survival during infection. In addition, HSPs may enhance the survival of (pathogenic) microorganisms in foods during exposure to high temperatures.

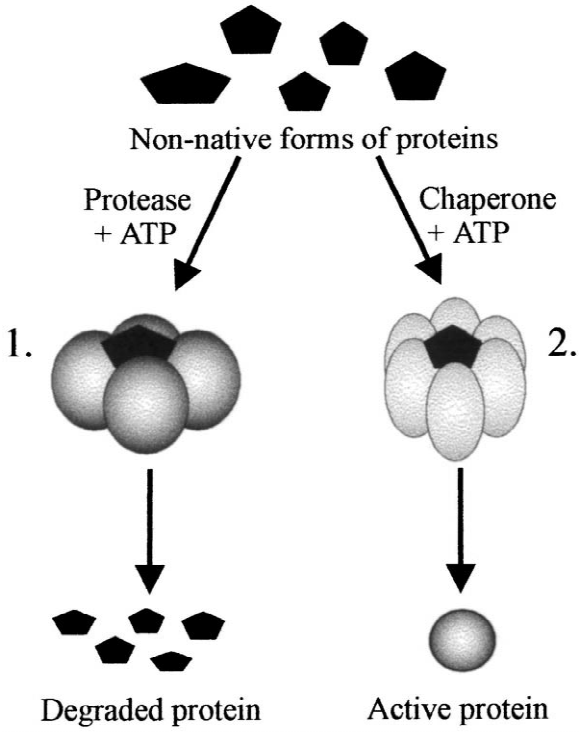


Fig. 3. Mode of action of HSPs. Inactive, non-native or unstable proteins are recognized by either proteases (1) or chaperone complexes (2) which leads to degradation of the proteins to smaller peptides or formation of active, native and/or stable proteins, respectively, at the expense of ATP (after Gottesman et al., 1997).

Recently, evidence was obtained that ATP-dependent proteases have intrinsic chaperone activity, suggesting that initial steps in energy-dependent protein degradation may be similar to those of chaperone-dependent protein folding. Chaperone activities have been demonstrated for three different families of ATP-dependent proteases: the Clp family, the AAA proteases such as FtsH, and Lon proteases, which are found universally throughout both prokaryotes and eukaryotes (Gottesman et al., 1997). Recently, several energy-dependent ATPases have been implicated to play a significant role in stress resistance. The FtsH family of proteases is anchored to membranes but contains cytoplasmic domains with ATPase activity and $Zn^{2+}$ metalloprotease active sites. In *E. coli*, and probably also in other organisms, some aspects of membrane assembly and protein localisation depend on FtsH activity (Tomoyasu et al., 1993; Tomoyasu et al., 1995). Furthermore, synthesis of FtsH appeared to be acid-inducible which, combined with the information above, indicates that this ATP-dependent protease may play a general role in maintaining protein quality in stressed cells. Members of the ClpC subfamily of stress-response-related Clp ATPases constitute a family of proteins which are highly conserved and universal, and are found for example in *B. subtilis*, *E. coli* and *Streptococcus pneumoniae* (Gottesman et al., 1997). The ClpC ATPase from *L. monocytogenes* was cloned and sequenced (Rouquette et al., 1996). This locus displays the same organisation as that of the *clpC*/*MecB* locus of *B. subtilis* and consists of an operon of four genes whose transcription is thermoregulated. *L. monocytogenes clpC* mutants exhibited highly sensitive phenotypes to several stresses and in addition their virulence capabilities were clearly impaired in comparison to the wild type strain both in murine models and macrophage cell lines (Rouquette et al., 1996). Recently, the *clpP* gene, encoding the Clp or Ti protease of *B. subtilis*, was cloned and sequenced and appeared to be induced upon heat shock, salt and ethanol stress. Initiation of transcription appeared to take place at both a $\sigma^A$-, as well as a $\sigma^B$-dependent promoter, but the $\sigma^A$-dependent promoter appeared to be essential for heat induction. In a *sigB* mutant strain, *clpP* remained heat-and stress-inducible at the $\sigma^A$-dependent promoter. Mutants lacking either the proteolytic component ClpP or the proteolytic component ClpX produced chains of elongated cells and exhibited severely impaired growth under stress conditions (Gerth et al., 1998).

Most HSPs are synthesised at low levels under non-stress conditions but are induced rapidly and transiently upon exposure to high temperature. In *E. coli* and *B. subtilis*, the induction of HSPs is obtained through alternative sigma factors ($\sigma^{32}$, $\sigma^E$, $\sigma^N$ or $\sigma^B$) which modify the promoter recognition specificity of the RNA polymerase to enable the expression of heat shock genes. The increase in $\sigma^{32}$ in *E. coli* results from both increased synthesis and stabilisation of $\sigma^{32}$, which is ordinarily very unstable. Transcription of some 30 genes, constituting the $\sigma^{32}$ regulon, is transiently increased as a consequence of elevated cellular level of $\sigma^{32}$. This response is feedback controlled by the DnaK machinery that sequesters $\sigma^{32}$ under physiological conditions and may also deliver it to FtsH, a protease

that degrades $\sigma^{32}$. In *E. coli*, a second set of heat-inducible genes is controlled by $\sigma^{E}$. This sigma factor belongs to a class of sigma factors that respond to extracytoplasmic stimuli, such as unfolded proteins in the periplasm. A third heat-shock regulon in *E. coli* is controlled by $\sigma^{N}$, an alternative sigma factor that requires an activator (PspF) for transcriptional activation (Model et al., 1997).

In *B. subtilis*, three classes of heat-inducible genes can be defined by their common regulatory characteristics. Class I gene expression involves a vegetative promoter and a short inverted repeat element upstream from the coding region (a so-called CIRCE element). This inverted repeat plays a role in repression under normal growth and has also been found in other heat shock genes of gram-positive bacteria, including *L. lactis* (Van Asseldonk et al., 1993). Class I genes are particularly induced by heat stress. Class II genes, the majority of general stress genes, are induced at $\sigma^{B}$-dependent promoters and can be induced by different stressors. The crucial event in the expression of these $\sigma^{B}$-dependent genes is the regulation of $\sigma^{B}$ itself, which is dependent on RsbW (see paragraph on sigma factors) and is induced by heat shock (Benson and Haldenwang, 1993b). A few genes (class III) can respond to various stress factors independently of $\sigma^{B}$ or CIRCE elements. This subgroup is characterised by a rather complex regulation pattern that may be exerted by additional regulatory proteins. This global control allows the cell to fine-tune the expression of each single gene that is under epistatic control of $\sigma^{B}$ (Hecker et al., 1996; Narberhaus, 1999).

Bacterial ribosomes seem to play a central role in the heat-shock regulatory pathways. Upon an increase in temperature, the translation proceeds faster than the instantaneous supply of charged tRNA. This results in an empty A-site of the ribosome. This might signal the ribosome to induce the heat-shock response and leads to an increased (p)ppGpp level. This means that the physical state of the ribosome or the nature of some product produced by the ribosome is involved as a signal linking the environmental stimulus (temperature) and the increased expression of heat-shock genes. One of the functions of the cellular heat-shock response may be to correct a temperature-imposed dysfunction of translation (Van Bogelen and Neidhardt, 1990). However, it has also been reported that RNA modification has been associated with the early events of heat damage in a cell. Heating causes membrane damage by which $Mg^{2+}$ leaks out of the cell. $Mg^{2+}$ has been mentioned to have a stabilising effect on ribosomes. Early studies revealed degradation of rRNA and ribosomal subunits during heat injury at 47°C (Miller and Ordal, 1972).

It is important to note that microorganisms develop a complicated, tightly regulated response upon an upshift in temperature. Different stressors can activate (parts of) this stress regulon by which they can induce an increased heat-tolerance. The process of adaptation and initiation of defence against elevated temperature is clearly an important target when considering food preservation and the use of hurdle technology.

## 6. Low temperature response

The extended use of frozen and chilled (convenience) foods and the increased popularity of fresh or minimally processed food, often preservative-free, greatly increased the interest in cold adaptation behaviour of microorganisms and food pathogens, in particular. Due to the longer time intervals between production and consumption of food products and the extended use of refrigerators, the importance of foodborne psychrotrophic pathogens, such as *L. monocytogenes*, *Y. enterocolitica*, *B. cereus* and *C. botulinum*, increased. Microorganisms that are a major concern for food poisoning show a wide variety in minimum growth temperatures as is indicated in Table 1 (Gould, 1998). The increased use of freezing as preservation method has led, in recent years, to a keen interest in microbial adaptation to freeze-conditions. Because of these important economical factors, research on cold adaptation of microorganisms has gained considerable interest in recent years (Berry and Foegeding, 1997; Yamanaka et al., 1998; Graumann and Marahiel, 1998).

Mechanisms that permit low-temperature growth involve membrane modifications maintaining membrane fluidity (involving nutrient uptake; Russell, 1990) and the maintenance of the structural integrity of macromolecules and macromolecule assemblies such as proteins and ribosomes (Jaenicke, 1991; Berry and Foegeding, 1997). One of the best studied reactions to low temperature is the synthesis of

cold-shock proteins, which are reported to be involved in protein synthesis and mRNA folding (Graumann and Marahiel, 1998). Furthermore, DNA supercoiling plays a role in the transduction of environmental signals to the bacterial nucleoid with important consequences for the regulation of transcription. Changes in growth temperature modify DNA supercoiling in which DNA topoisomerase and DNA gyrase activity play an essential role for the transduction of the environmental signal (Drlica, 1992). It was reported that the negative supercoiling of plasmid DNA in *E. coli* increases upon a cold-shock treatment and that DNA gyrase and the HU protein have an important role in this process (Mizushima et al., 1997).

Microorganisms have developed a number of strategies to maintain their membrane lipids fluid and functional at low growth temperature. In general, as the growth temperature is decreased, an increase in the proportion of shorter and/or unsaturated fatty acids in the lipids is observed. One of the most important consequences of membrane lipid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (Russell, 1990). The membrane adaptation response has been most extensively studied for *E. coli* and it has been shown that the proportion of *cis*-vaccenic acid (C18:1) increases at low temperature at the expense of palmitic acid (C16:0). The increase in average chain length has the opposite effect on membrane fluidity, but is outweighed by the greater fluidity effect of increased unsaturation. The increase in C18:1 is catalysed by the constitutive enzyme β-keto-acyl-acyl-carrier protein synthase I, that is active only at low temperature (Garwin and Cronan, 1980; De Mendoza and Cronan, 1983; De Mendoza et al., 1983). In *L. monocytogenes*, the fatty acid composition was altered in response to low temperature by an increase of a-C15:0 and a decrease of a-C17:0 (Püttman et al., 1993; Russell et al., 1995; Annous et al., 1997). Transposon-induced cold-sensitive mutants incapable of growth at low temperature have dramatically altered fatty acid compositions with low levels of i-C15:0, a-C15:0 and a-C17:0. The levels of a-C15:0 and a-C17:0 and the growth at low temperature could be restored by the addition to the medium of 2-methylbutyric acid, a precursor of anteiso odd-numbered fatty acids. A-C15:0 appears to be critical for the ability of this strain to grow at low temperature (Annous et al., 1997). For *B. subtilis*, the synthesis of C16:1 is induced when a culture is transferred from 37 to 20°C, whereas unsaturated fatty acids are absent in 37°C grown cultures (Grau and De Mendoza, 1993). Follow-up studies suggested that an increase in DNA supercoiling associated with a temperature downshift could regulate the unsaturated fatty acid synthesis in *B. subtilis*. It is likely that the transcription of the gene(s) involved in unsaturated fatty acid synthesis is regulated by a promoter(s) that is activated upon an increase in negative supercoiling. However, the molecular mechanism that links these two events remains to be elucidated (Grau et al., 1994).

Compatible solutes (such as betaine, proline and carnitine) may play a role in osmoprotection and in cold adaptation. For *L. monocytogenes*, growth at 7°C was stimulated in the presence of betaine and cells transported betaine 15-fold faster at 7°C than at 30°C (Ko et al., 1994). For different compatible solutes, such as betaine, ectoine and mannitol, a protective effect during freeze drying, has been reported. The mechanisms behind this effect remain to be elucidated but increased levels of compatible solutes have positive effects on cell survival and activity of enzymes (Louis et al., 1994).

A considerable amount of research is directed to the response of bacteria to an abrupt decrease in growth temperature (cold shock). It has been observed that many bacteria synthesise increased amounts of small (7 kDa) proteins upon a sudden decrease in temperature, the so-called cold-shock proteins (CSPs). These proteins share a high degree of similarity (>45%) in a variety of gram-positive and gram-negative bacteria, including food-related microorganisms, like *E. coli* (Goldstein et al., 1990), *B. subtilis* (Willimsky et al., 1992), *Bacillus cereus* (Mayr et al., 1996), *Salmonella enteriditis* (Jeffreys et al., 1998), *S. typhimurium* (Craig et al., 1998), *L. lactis* (Wouters et al., 1998), and *Lactobacillus plantarum* (Mayo et al., 1997). However, CSPs are not observed in all bacteria, for example, *Helicobacter pylori* (Tomb et al., 1997) and *Campylobacter jejuni* (Hazeleger et al., 1998). The most extensively studied CSPs are CspA of *E. coli* ($CspA^E$) and CspB of *B. subtilis* ($CspB^B$). For *B. subtilis* (Graumann et al., 1997), *L. lactis* (Wouters et al., 1998), *B. cereus* (Mayr et al., 1996) and *E. coli* (Yamanaka et al.,

1998) families of three, five, six and nine members, respectively, have been found. For *L. lactis*, a clustered organisation of two tandems of two *csp* genes was observed (Wouters et al., 1998), whereas for *E. coli* a clustered organization of the *csp* genes on the chromosome was observed (Yamanaka et al., 1998). Not all members of CSP families appeared to be cold induced (Lee et al., 1994; Wouters et al., 1998; Yamanaka et al., 1998). CspD of *E. coli* and CspB and CspC of *B. subtilis* were shown to be induced at stationary phase (Yamanaka and Inouye, 1997; Graumann and Marahiel, 1999). CspC and CspE, two non-cold-induced members of the CspA family of *E. coli*, have been implicated in chromosomal condensation and/or cell division (Yamanaka et al., 1994, 1998). Strikingly, CspA of *E. coli* also appeared to be induced upon exposure to ultra-high pressure treatments which was correlated to the inactivation of ribosomes (see section on ultra high pressure, Welch et al., 1993).

The determination of the crystal structures of $CspA^E$ and $CspB^B$ revealed that both proteins consist of five antiparallel β-strands which together form a β-barrel structure (Schindelin et al., 1993; Newkirk et al., 1994). Both proteins have been characterised as single-stranded DNA binding proteins (Jones et al., 1992b; Graumann and Marahiel, 1994). $CspA^E$ acts as a transcriptional activator of at least two other genes encoding cold-induced proteins, GyrA (Jones et al., 1992b) and H-NS (LaTeana et al., 1991), both involved in DNA supercoiling. CSPs contain regions highly homologous to the cold-shock domain of eucaryotic DNA-binding proteins, like YB1 and FRGY2. These proteins are known to act as transcription factors with the ability to bind to the Y-box motif (ATTGG), thereby regulating gene expression (Jones and Inouye, 1994). Both $CspA^E$ and $CspB^B$ are able to bind specifically to single-stranded DNA containing this motif or its complementary sequence (Newkirk et al., 1994; Graumann and Marahiel, 1994). $CspA^E$ and $CspB^B$ are also considered RNA-binding proteins because they both possess highly conserved RNA-binding motifs, i.e. RNP1 and a rudimentary RNP2 motif (Schindelin et al., 1993; Jones and Inouye, 1994) and recently Jiang et al. (1997) showed that $CspA^E$ acts as an RNA chaperone. For $CspB^B$, a function as an anti-freeze protein has been suggested because a lower survival has been observed after freezing of *B. subtilis* cells in which the *cspB* gene, encoding $CspB^B$, was disrupted (Willimsky et al., 1992). A deletion of $CspA^E$ did not reveal a distinct phenotype in relation to cold adaptation (Mitta et al., 1997). Multiple deletion analysis of the CSP family of *B. subtilis* revealed a lethal phenotype upon deletion of all three counterparts and severe growth inhibition at high as well as at low temperatures whenever two *csp* genes were deleted. It appeared that loss of one or two of the CSPs led to an increase in the synthesis of the remaining CSP(s) at 37°C and at low temperature. CSPs are suggested to function as RNA chaperones facilitating the initiation of translation under optimal and low temperatures (Graumann et al., 1997; Fig. 4). Recently, a method was described for the discrimination of psychrotrophic and mesophilic strains of the *B. cereus* group based on differences in the *cspA* sequence. It appeared that by use of a specific set of primers, PCR products were only obtained for species falling in the psychrotrophic *B. cereus* group (Francis et al., 1998). Whether the differences in the observed minimum growth temperature for psychrotrophic and non-psychrotophic strains are solely linked to differences in the *cspA* sequence remains to be elucidated.

The regulation of $CspA^E$ after cold shock takes place at the level of transcription (Jiang et al., 1993; Mitta et al., 1997) as well as at the level of mRNA stability (Brandi et al., 1996; Goldenberg et al., 1996). An AT-rich sequence (UP-element) upstream from the −35 region of the *cspA* promoter enhances *cspA* transcription at low temperature (Mitta et al., 1997; Goldenberg et al., 1997). The *cspA* mRNA is highly unstable at 37°C and stabilized after cold shock. The increased stability upon cold shock appeared to be dependent on the unusually long, untranslated *cspA*-mRNA leader region (5′UTR). It has been suggested that under conditions during which protein synthesis is blocked as a result of ribosomal malfunctioning, the mRNAs of CSPs are still translatable during these conditions because of the presence of a downstream box (DB). This DB, located in the coding region of *E. coli* CspA, is complementary to a sequence proximal to the RBS-decoding region in 16S rRNA and was shown to be required for efficient translation under cold-shock conditions (Mitta et al., 1997). The mRNA stabilisation of cold-shock genes upon cold shock appeared to be transient and is lost once cells have adapted to

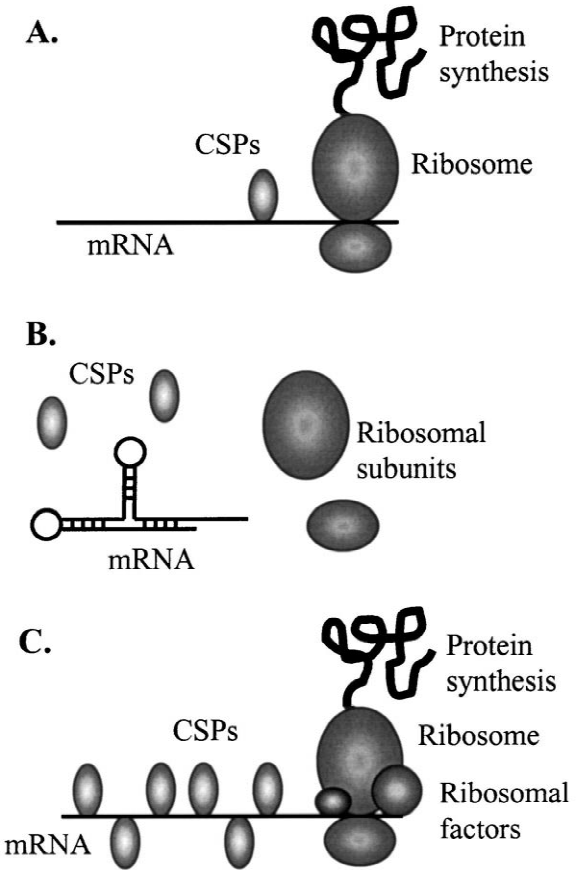


Fig. 4. Model of the mode of action of CSPs during the initiation process of translation. At high temperature the mRNA molecules are hardly folded and translation takes place at a maximum rate (A). Upon cold shock the ribosomal structure is disrupted and the secondary structure of mRNA molecules is drastically increased (B). Easy-translatable mRNAs of cold-shock genes are translated by partially intact or by a minority of intact ribosomal structures. In response to cold shock, ribosomal structure is restored by ribosomal binding factors and mRNA secondary folding is reduced by the increased number of CSPs by which translation can proceed at low temperature (C). See text for details.

low temperature (Goldenberg et al., 1996). It appeared that the CSPs of *B. subtilis* have high sequence affinity to bind to the first 25 bases of their 5′UTRs. In this way, CSPs could downregulate translation of their messengers by which they limit their cellular concentrations. This may be an important regulatory mechanism since levels of CSPs which are too high have a growth inhibitory effect as was shown using artificial overexpression (Graumann and Marahiel, 1997, 1998).

The response to cold shock of *E. coli* showed induction of an additional, specific set of ~15 proteins. These proteins play a role in various cellular processes and include, among others: NusA (involved in both termination and anti-termination of transcription), RecA (dual roles in recombination and in the SOS response), H-NS and GyrA (both involved in DNA supercoiling), polynucleotide phosphorylase (involved in mRNA degradation; Jones et al., 1987; Jones and Inouye, 1994). Furthermore, cold-induction is observed for CsdA (a ribosome associated helicase; Jones et al., 1996) and RbfA (ribosome binding factor A; Jones and Inouye, 1996) which are both important for ribosomal structure. It is believed that upon cold shock the ribosomal functioning is blocked and that for translation of non-cold-shock gene mRNAs the presence of cold-shock-specific ribosomal factors, such as CsdA and RbfA, is required for the formation of the translation initiation complex (Mitta et al., 1997; Fig. 4). An important role in the regulation of the temperature stress response has been elucidated for CsdA, which is able to unwind double-stranded RNA. Disruption of this gene resulted in a significant growth defect and in expression of certain genes including heat-shock proteins only at low temperature. It is proposed that at low temperature, CsdA is essential for ribosomal functioning to increase translational efficiency of mRNAs by unwinding stable secondary structures formed at low temperature (Jones et al., 1996).

For *B. subtilis*, a set of specific proteins (~37 proteins) is induced upon cold shock, which are involved in a variety of cellular processes, such as chemotaxis, sugar uptake, translation, protein folding and general metabolism (Graumann et al., 1996). *L. monocytogenes* cells induce a set of 12 unidentified proteins upon cold shock from 37°C to 5°C as was observed using two-dimensional gel electrophoresis (Bayles et al., 1996). Among this group no homologues to the CspA family could be observed, probably due to the separating conditions used, which did not allow identification of proteins smaller than 14 kDa. Using a PCR strategy one *csp* gene was identified in *L. monocytogenes* (Francis and Stewart, 1997).

The functioning of the ribosomes seems to play a central role in cold adaptation process. A downshift in temperature causes a cold-sensitive block in

initiation of translation, resulting in a decrease in polysomes and an increase in 70S monosomes and ribosomal subunits. Upon cold shock, mRNAs of cold-induced genes can be translated because of the presence of DB elements. The induction of these cold-shock proteins leads to restoration of the ribosomal structure to form intact translation initiation complexes (Jones and Inouye, 1996; Fig. 4). Van Bogelen and Neidhardt (1990) stated that the ribosome might be the temperature sensor in bacteria. During a cold-shock treatment the translational capacity becomes low which would lead to the concentration of charged tRNA becoming too high, thus blocking the A-site of the ribosome. This in turn lowers the (p)ppGpp concentration by the diminished synthesis of (p)ppGpp by RelA (which in turn controls the stringent response). In *E. coli*, artificially low concentrations of (p)ppGpp increase the synthesis of cold-induced proteins (Van Bogelen and Neidhardt, 1990; Jones et al., 1992a; Graumann and Marahiel, 1996).

The significance of psychrotrophic organisms is well understood, however the significance of the cold shock response of these organisms is still uncertain. Different cold-shock treatments prior to freezing results in clear differences in microbial survival after freezing. (Goldstein et al., 1990; Willimsky et al., 1992). This might result in a high survival rate of bacteria in frozen food products. Furthermore, low temperature adapted bacteria show shorter lag times at cold temperature and their higher growth rate at low temperature may be relevant to food quality and safety. Greater understanding of the mechanisms of cold adaptation may offer insight into methods to control the growth of psychrotrophic microorganisms, which continue to challenge the shelf-life and safety of refrigerated foods.

## 7. High hydrostatic pressure

Because of its inactivating effect on microorganisms and enzymes, the potential use of pressure technology as a novel food preservation method is currently being investigated (Knorr, 1993). Elevated pressure can exert detrimental effects on microbial physiology and viability. Growth of microorganisms is generally inhibited at pressures in the range of 20 to 130 MPa, while higher pressures of between 130 and 800 MPa may result in cell death; the maximum pressure allowing for growth or survival depends on the species and medium composition. The exact nature of cellular damage responsible for pressure killing remains to be elucidated, but membranes and ribosomes have been suggested as important determinants in pressure sensitivity (Welch et al., 1993; Earnshaw et al., 1995; Patterson et al., 1995). Additionally, Welch et al. (1993) have shown that exposure of *E. coli* to high hydrostatic pressure induces a unique stress response which results in higher levels of both cold-shock proteins CSPs and heat shock proteins HSPs, as well as other proteins which appear only in response to high pressure. Recently, differential scanning calorimetry allowed the detection of in vivo changes in ribosome conformation (Niven et al., 1999). Indeed, cell death upon exposure to ultra high pressure (UHP) was associated with ribosome damage. Since ribosomes have been implicated as temperature sensors, CSPs (and HSPs) may also play a role in stress response under inactivation conditions such as UHP. Attention has also been paid to the kinetics and mechanisms of pressure inactivation of microorganisms, with a special focus on the occurrence of mutants resistant to inactivation by high hydrostatic pressure (Lechowich, 1993; Hauben et al., 1997).

Hauben et al. (1997) used alternating cycles of exposure to high pressure and outgrowth of surviving populations to select for highly pressure-resistant mutants of *E. coli* MG1655. Three barotolerant mutants (LMM1010, LMM1020 and LMM1030) were isolated independently by using outgrowth temperatures of 30, 37 and 42°C. Survival of these mutants after pressure treatment for 15 min at ambient temperature was 40 to 85% at 220 MPa and 0.5 to 1.5% at 800 MPa, while survival of the parent strain decreased from 15% at 220 MPa to $2\times10^{-8}$% at 700 MPa. Two of the three mutants, LMM1020 and LMM1030, also showed a different heat resistance as evident from higher $D$ values at 58 and 60°C and reduced $Z$ values compared to those for the parent strain. Surprisingly, the ability of the mutants to grow at moderately elevated pressure (50 MPa) was reduced at temperatures above 37°C, indicating that resistance to pressure inactivation in these mutants is unrelated to barotolerant growth. Since membranes have been implicated as targets for pressure damage, the fatty acid composition of the

mutants was compared to that of the parent strain, but no significant differences could be found (Hauben et al., 1997).

Subsequently, Garcia-Graells et al. (1998) performed a follow-up study using the high-pressure-resistant mutants of *E. coli* described above, to analyse their survival capacity during high pressure pasteurization in fruit juices and in low pH. The barotolerant mutants also showed enhanced pressure resistance in juices when compared to the parent strain. However, a considerable decrease in the number of survivors was found upon storage of the pressure-treated juices at 8°C, in some cases resulting in undetectable levels after 5 days. This phenomenon illustrated that the pressure treatment (300–400 MPa) caused sublethal injury to a large proportion of the cells, resulting in reduced resistance to low pH. Since enterohemorrhagic *E. coli* strains have a low infectious dose, in some cases the reduction in cell numbers may not be sufficient to provide the desired level of safety, therefore the authors recommend a higher pressure. As an alternative, they suggest to include a 2-day quarantine period (low pH fruit juice stored at low temperature) between pressure treatment and consumption, which may significantly enhance the safety of pressure-pasteurized fruit juices (Garcia-Graells et al., 1998).

The development of high levels of barotolerance may have important implications for the practical application of pressure technology in food preservation. If barotolerant strains can survive in commercial pressurisation equipment they can form a serious threat to the safety and stability of pressure-processed foods. One approach to increase microbial inactivation is to combine pressure with another processing technology such as exposure to mild heat. This strategy is likely to be successful, as there is evidence that microbial injury (see also above) can occur at significantly lower pressures than are required for inactivation (Patterson et al., 1995). Indeed, Patterson and Kilpatrick (1998) found that inactivation of *E. coli* O157:H7 and *S. aureus* in poultry meat and in ultra-high-temperature-treated (UHT) milk was far more efficient when pressure (400–500 MPa) was combined with high temperature (50°C). The use of pressure with heat has also been found to be more effective for inactivating *Bacillus* and *Clostridium* spores (Gould, 1973; Mills et al., 1998). Alternatively, pressure cycling has been proposed to efficiently inactivate bacterial spores. Here, spore germination is induced at low pressure (100–250 MPa) followed by inactivation at high pressure. Most of the studies have been performed with *Bacillus* species, including *B. subtilis*, *B. cereus* and *B. stearothermophilus*, and these show great variability in ultra high pressure resistance. At present, there is still only limited knowledge on the factors affecting pressure-induced germination and on its kinetics and mechanism (Holters et al., 1997; Wuytack et al., 1997; Raso et al., 1998).

Recently, Wuytack et al. (1998) studied pressure-induced germination of *B. subtilis* spores at moderate (100 MPa) and high (500 to 600 MPa) pressures. At 40°C, the degree of pressure-induced germination, using heat sensitivity as a criterion, was roughly constant between 100 and 600 MPa, while inactivation was maximal at 200 MPa and almost non-existent at 600 MPa. Spores germinated at 100 MPa were more sensitive to pressure ($>$200 MPa), UV light, and hydrogen peroxide than those germinated at 600 MPa. In the latter type of spores no degradation of small acid soluble proteins was observed. These proteins are known to be involved in spore resistance to UV light and hydrogen peroxide, thereby offering an explanation for the resistance of these spores against these stresses. Furthermore, germination at 100 MPa was accompanied by rapid ATP generation, as is the case with nutrient-induced germination, however, no ATP was formed during germination at 600 MPa. The authors concluded that germination can be initiated by low and high pressure treatments but is arrested at an early stage in the latter case. The results also indicate that there is not necessarily a link between heat and pressure resistance, and this may have important consequences for the use of high pressure as a non-thermal preservation process.

Most studies using UHP have been performed with *Bacillus* species, whereas information about the efficiency of inactivation of *Clostridium* spores by this technique is scarce. Although it could be assumed that *Clostridium* spores will respond to ultra high pressure in a similar manner to *Bacillus* spores, there is little evidence to support this assumption. A recent study by Mills et al. (1998) showed that *Clostridium sporogenes* spores could not be inactivated by pressure alone. Combination treatments including heat and pressure applied simultaneously

(400 MPa at 60°C for 30 min) or sequentially (80°C for 10 min followed by 400 MPa for 30 min) proved more effective at inactivating spores than treatments at 600 MPa (30 min at 20°C). Pressure cycling (60 MPa followed by 600 MPa at 60°C) also reduced spore numbers. However, all these treatments resulted only in less than a 3-log reduction. The combination of pressure inactivation with other preservation methods or application of multiple low pressure–high pressure cycles may result in a treatment that is acceptable to the food industry i.e. an effective inactivation of *Clostridium* spores.

## 8. Perspectives

Recently, the whole genome sequences of *B. subtilis* and *E. coli* have been published (Kunst et al., 1997; Blattner et al., 1997). The 4,214,810 and 4,639,221-base pair sequences of *B. subtilis* and *E. coli* comprise 4100 and 4288 protein-coding genes, respectively, of which ~40% have no attributed function. Large families of transcription regulatory factors, two-component signal-transduction pathways, solute transport systems, including 77 and 80 ABC transporters (Ames et al., 1990) in *B. subtilis* and *E. coli*, respectively, could be identified. Further research will be required to determine the precise functions for all the genes by global transcription analysis of mutants, and analysis of biochemical and catalytic properties of the expressed proteins. Another important area for exploration will lie in whole sequence comparisons, both with related pathogens to identify those genes that confer detrimental or beneficial properties, and with other microbial genomes to ascertain evolutionary relations (Blattner et al., 1997).

Stress response and cross protection in vegetative cells of spoilage and pathogenic microorganisms to high pressure and other inactivation techniques including high-voltage pulsed electric fields is largely unexplored. Furthermore, substantial research is needed to identify factors affecting pressure-induced germination and its mechanism and kinetics. All this information is essential for increasing the efficiency and application of pressure cycling and combination treatments for inactivation of spores, including that of pathogenic *Clostridia* in minimally processed foods. In this respect, information available from genome sequences may be used to identify target genes or encoding proteins playing an important role in stress response, with the subsequent aim being to prevent the adaptation response, thereby enhancing the efficiency of (combination) preservation and inactivation techniques.

Evidence has accumulated that environmental stresses may modulate bacterial virulence (Benjamin et al., 1991; Fang et al., 1992; Garcia-del Portillo et al., 1993; Robbe-Saule et al., 1995; Archer, 1996; Bearson et al., 1996; Rouquette et al., 1998). The ability to proliferate in fluids or within cells of a living host is the key attribute of pathogenic bacteria that distinguishes them from commensal species. Any product that a bacterium synthesises which enhances the growth or survival of a bacterium during its interaction with the host, can be thought of as a virulence factor and its corresponding coding sequence as a 'virulence gene'. Included in this broad category of virulence properties are some adaptive responses to environmental stresses, such as pH extremes, oxidative stress, lack of iron, heat and cold stress, osmolarity extremes and starvation. Similar stresses may therefore be imposed on a bacterium in foods and by host defenses. Cellular adaptive mechanisms to environmental or food-processing and food-preservation-related stresses including starvation, temperature extremes and (weak) acids, may affect virulence of food pathogens (see also Hill, this issue). Alternative σ factors, including RpoS (e.g. *E. coli* and other enteric bacteria) and $\sigma^B$ (e.g. *B. subtilis*, *L. monocytogenes*, *S. aureus*) may play key roles in the pathogenicity of foodborne pathogens, thereby highlighting the stress response as an important factor in potentiating virulence gene expression. Therefore, the effects of preservation combination and inactivation techniques such as UHP and pulsed electric field, on the virulence of foodborne pathogens and on the possible selection of stress-resistant, virulent sub-populations should be studied.

In this respect, the application of flow cytometry (FCM) may provide a powerful tool for the quantitative analysis of microbial populations. FCM measures physical or chemical characteristics of individual cells as they move in a fluid stream past optical or electronic sensors, typically at a rate of some 1000 cells per second (for reviews, Shapiro, 1995; Davey and Kell, 1996; Nebe-Von Caron et al.,

1998). The non-fluorescent sideways and forward scatter parameters provide information about particle granularity, i.e. surface characteristics and particle size, respectively. A wider range of cellular parameters is accessible via fluorescence measurements. Some common cellular constituents such as pyridine and flavin nucleotides, photosynthetic pigments, and fluorescence can be determined by FCM of unstained cells. However, most measurements rely upon the use of fluorescent reagents or probes, and require staining procedures (Shapiro, 1995). An additional advantage is provided by the ability to sort cells with selected properties, i.e. allowing selection and subsequent characterization of subpopulations, for example, for their outgrowth potential. Application of FCM in food microbiology has also become more feasible due to improved instrumentation and labelling of cells.

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