**Review**

**Cold Stress Tolerance of *Listeria monocytogenes*: A Review of Molecular Adaptive Mechanisms and Food Safety Implications**

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**ABSTRACT**

The foodborne pathogen *Listeria monocytogenes* has many physiological adaptations that enable survival under a wide range of environmental conditions. The microbes overcome various types of stress, including the cold stress associated with low temperatures in food-production and storage environments. Cold stress adaptation mechanisms are therefore an important attribute of *L. monocytogenes*, enabling these food pathogens to survive and proliferate to reach minimal infectious levels on refrigerated foods. This phenomenon is a function of many molecular adaptation mechanisms. Therefore, an improved understanding of how cold stress is sensed and adaptation measures implemented by *L. monocytogenes* may facilitate the development of better ways of controlling these pathogens in food and related environments. Research over the past few years has highlighted some of the molecular aspects of cellular mechanisms behind cold stress adaptation in *L. monocytogenes*. This review provides an overview of the molecular and physiological constraints of cold stress and discusses the various cellular cold stress response mechanisms in *L. monocytogenes*, as well as their implications for food safety.

*Listeria monocytogenes* are gram-positive, foodborne pathogens that are found in a wide variety of environments as a result of their robust physiological adaptation capacity, which enables colonization and persistence in various locations. Human infection with this bacterium may lead to listeriosis, a serious and potentially life-threatening illness (67). Clinical features of systemic listeriosis include abortion, prenat al infection, meningitis, septicemia, and gastroenteritis. The susceptible group includes pregnant women, neonates, elderly people, and immunocompromised individuals (67). Some of the recent epidemiological reports have shown that *L. monocytogenes* infections are responsible for the highest hospitalization rates (91%) among known foodborne pathogens in the United States (56). Various cases of both sporadic and large outbreaks of human illness have also been linked to *L. monocytogenes* infection in various parts of the world (24). A notable observation from the available epidemiological data is that the majority of human outbreaks are primarily associated with only 3 *L. monocytogenes* serovars (1/2a, 1/2b, and 4b), even though 13 *L. monocytogenes* serovars capable of human infection are known to exist (24, 72, 76, 88, 93, 104).

The current transmission models assume contaminated food products to be the main routes for human infection (71). In this context, it is therefore plausible to assume as one hypothesis that the epidemiological trends observed so far are reflective of better adaptation by some *L. monocytogenes* subtypes to food environments and subsequent human infection (37, 42, 46, 62, 63). In this regard, the cold stress and other food-environment-associated stresses may actually be selecting for *L. monocytogenes* subtypes possessing the appropriate adaptive physiological attributes that promote efficient survival and proliferation during food processing and storage.

In general, the severity of the human clinical disease, coupled with the high case fatality rates associated with *L. monocytogenes* infections (24, 56, 76), emphasize the critical importance of effective control measures against these pathogens in food. But the ubiquity of these organisms in food-processing, distribution, and storage environments and their efficient stress adaptation capabilities make their control in food a great challenge. Therefore, it is widely presumed that food products are contaminated by *L. monocytogenes* organisms that occasionally pass from food processing plant environments, including work-contact surfaces and equipment (54). Consequently *L. monocytogenes* contamination occurs widely in foods, with the highest prevalences documented in meat, poultry, and seafood products (24, 29, 74, 77, 90).

In view of food safety, it is important to understand how these organisms are able to adapt their cellular physiology and efficiently overcome the various forms of food- and food-processing–related stresses, as well as resist current control measures. Such information may be relevant for developing better ways of contamination prevention and control in food. Cold stress tolerance is one of the fundamental attributes of *L. monocytogenes* that markedly contributes to the microbes’ dissemination through refrigerated.

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food products. This phenomenon renders the use of low temperatures during food processing and subsequent long-term storage under refrigeration ineffective against *L. monocytogenes* proliferation, although cooling can effectively control most other foodborne pathogens. Consequently, cold-stored contaminated foods provide rich environments for these organisms (45, 98). As in other psychrotolerant bacteria, cold stress resistance in *L. monocytogenes* is a biological property mediated through many molecular response mechanisms at the microbes’ disposal. The nature of such molecular cold-adaptation responses remains largely elusive, although some aspects of this phenomenon have been illuminated in model microorganisms (23, 106). Over the past few years, some aspects of cold stress adaptation mechanisms in *L. monocytogenes* have come to light, and here, we discuss these different molecular aspects and their implications for food safety.

**FOOD-ENVIRONMENT-RELATED COLD STRESS MOLECULAR CHALLENGES AND GENERAL MICROBIAL RESPONSE STRATEGIES**

*L. monocytogenes* faces various forms of cold stress challenge at various stages in food-processing and storage environments, and the organisms must efficiently adapt their molecular responses in order to overcome the challenges, as well as proliferate in food products (73). If the transfer to low temperature is sudden, it will be accompanied by cold shock stress on the bacterium. This requires the microbes to induce appropriate cold shock adaptation molecular response mechanisms. Once at low temperature, the microbes must acclimatize to the cold stress environment, then eventually resume growth at low temperatures. This process requires induction and implementation of various cold-acclimation molecular responses. In foods, the *L. monocytogenes* organisms might also face additional cold stress associated with freezing and freeze-thawing. Furthermore, these cold stress challenges in food environments are often coupled with exposure to other food-environment–related stresses such as high osmolarities, low pH, starvation, and dehydration (24, 53).

Cold stress has profound effects on several cellular events. Temperature reduction immediately leads to a general slowdown of reaction rates in various cellular processes. At the cell-membrane level, there is decreased fluidity in the lipid bilayer, which compromises membrane structural integrity and various other cell-membrane–associated functions. An increased stability of RNA and DNA secondary structures, as well as destabilization of cellular macromolecules, in particular the ribosomes, are also presumably associated with cold stress exposure. A differential gene expression–based study showed that cold stress acclimation process in *L. monocytogenes* is accompanied by oxidative and amino acid starvation stresses in the cells, as well as alterations in other metabolic pathways (52). Typical molecular cold stress adaptation mechanisms documented in microbes include: (i) modulation of nucleic acid structures (DNA and RNA) in order to relieve constraints associated with increased stability, (ii) maintenance of structural integrity in cell membranes, (iii) uptake of compatible solutes, (iv) nonspecific stress response mechanisms, and (v) production of various cold stress proteins, popularly known as cold shock proteins (Csps) and cold acclimation proteins (Caps).

In view of *L. monocytogenes*, the response mechanisms described so far involve maintenance cell-membrane lipid fluidity, intracellular uptake of compatible solutes, and production of several cold stress proteins (Csps and Caps). A comparative relative gene expression study of cold-acclimated *L. monocytogenes* cells also implicates the potential involvement of several other molecular factors (52). A simplified schematic overview on some of the molecular events that might be envisaged to be part of the cold stress adaptation process in *L. monocytogenes* is shown in Figure 1. On the basis of this scheme, the external cold stress is sensed and the stress signals conveyed through as yet unknown molecular transduction pathway or pathways into the cell. These signaling pathways eventually lead to the induction of required cellular response proteins that subsequently implement the appropriate molecular response mechanisms. The overview in Figure 1 also incorporates presently known aspects of molecular cold stress adaptation in *L. monocytogenes*. However, on the basis of the available literature, many other genetic factors are probably involved in cold stress adaptation of *L. monocytogenes*, and these are presented in Table 1. For discussion purposes, these have been grouped into following categories: (i) cell-membrane–based responses, (ii) Csps, (iii) adaptive regulatory proteins, (iv) general stress response proteins, and (v) other proteins. It must be emphasized that for most of these genes, the available information is currently putative. Future validation and molecular investigations are essential to understanding their possible roles in *L. monocytogenes* cold adaptation.

**MOLECULAR COLD-ADAPTATION RESPONSES INVOLVING CELL-MEMBRANE–BASED FUNCTIONS**

Maintenance of lipid fluidity in cell membranes. The correct physical state of the membrane lipids is crucial to optimal structural and functional integrity of cell membranes. Low temperatures lead to reduced membrane lipid fluidity. The microbes principally respond by altering the fatty acid composition of their cell-membrane lipids to correspondingly lower the liquid to solid phase transition temperatures. Therefore, the molecular adaptation measures adopted in cell-membrane lipids include (i) a change in the fatty acid chain lengths, (ii) an alteration in the degree of fatty acid unsaturation, and (iii) a change in the type of branching at the methyl end of the fatty acids (reviewed in reference (86)). Numerous studies to date have examined cold stress–associated changes in the cell-membrane fatty acid composition of *L. monocytogenes* organisms (5, 43, 44). Puttmann et al. (69) discovered that the proportion of a-C$_{17:0}$ decreased and that of a-C$_{15:0}$ increased in cell membranes of *L. monocytogenes* grown at 4°C compared with 37°C.

Similarly, Jones et al. (43) described lower a-C$_{17:0}$ and increased a-C$_{15:0}$ and short chain fatty acids proportions in
FIGURE 1. Schematic outline of sequence of molecular events envisaged during the cold stress adaptation process in L. monocytogenes. The cold stress is sensed and signals are relayed through presently unknown molecular mechanisms. This might in turn lead to induction of adaptive regulatory protein networks. These will eventually lead to the coordinated activation of many response proteins involved in the required molecular responses to alleviate the constraints of cold stress on the microbe, which in turn leads to its survival and proliferation in low-temperature environments. Broken arrows highlight the fact that several steps of molecular events are probably involved in the implementation of the cold stress response mechanisms. The established cold stress adaptation responses of L. monocytogenes that involve increased membrane lipid fluidity and intracellular accumulation of osmolytes and oligopeptides are highlighted.

*L. monocytogenes* cell membranes at 10°C compared with 30°C. Juneja et al. (44) also documented an increase in branched C\textsubscript{15:0} and a decrease in C\textsubscript{17:0} proportions after lowering the temperature of *L. monocytogenes* cells from 37°C to 10°C. Annous et al. (5) described two modes of cell-membrane-based cold stress adaptation in *L. monocytogenes* 10403S and SLCC 53 strains that involve shortening of fatty acid chains and an alteration in branching of the fatty acids from iso to anteiso. This study also described two transposon-induced cold-sensitive mutants that grew at 37°C but failed to grow at 10°C. These mutants were found to be deficient in odd-numbered branched chain fatty acids, particularly a-C\textsubscript{15:0} and a-C\textsubscript{17:0} in their cell membranes. Growth at 10°C could be restored in the mutants upon supplementation of the growth media with 2-methylbutyric acid. Interestingly, this also restored the normal a-C\textsubscript{15:0} and a-C\textsubscript{17:0} composition in the mutant cells’ membranes (5). It has previously been shown that cold stress leads to induction of fatty acid desaturase enzyme systems in *Bacillus* species (20), but the study by Annous et al. (5) could not find any evidence to support the role of such a system during cell-membrane-based cold stress adaptation responses of *L. monocytogenes*.

**Uptake of cryoprotective osmolytes and peptides.** Two other cell-membrane–based functions that have significant cold stress adaptation roles are also reported in *L. monocytogenes*. They involve intracellular accumulation of compatible solutes and short peptides as a response to cold stress. Thus the primary osmoadaptation mechanisms involving intracellular accumulation of osmolytes are also an important cold stress adaptation strategy of *L. monocytogenes* organisms (2–4, 9, 25, 28, 56, 78–80, 83). Osmolytes are low-molecular-weight organic compounds that can accumulate to high intracellular concentration with minimal effects on normal functions in microbes (reviewed in reference (82)). It is not clear how their intracellular accumulation leads to cold stress alleviation, although there are suggestions they may act through stabilization of enzymatic functions and the cell-membrane lipid bilayer at low temperatures (51). Interestingly, *L. monocytogenes* does not appear to possess the molecular ability to synthesize the principal cryoprotective osmolytes, glycine betaine and carnitine. But on the other hand, these are found at high levels in foods of plant and animal origin, respectively (70). Therefore, osmolyte-based cold-adaptation mechanisms in *L. monocytogenes* are primarily based on their uptake directly from extracellular environments.

A proposal supported by several studies showing that inclusion of osmolytes such as glycine betaine and carnitine in growth media enhances *L. monocytogenes* growth at low temperatures (8, 11, 49, 84). Furthermore, cold stress exposure directly induces an increased uptake of cryoprotective osmolytes by *L. monocytogenes* (2, 4, 57, 101). The uptake of cryoprotective osmolytes glycine betaine and car-
TABLE 1. L. monocytogenes genes with defined or putative involvement in cold stress adaptation mechanisms

<table>
<thead>
<tr>
<th>Gene type</th>
<th>Reference(s)</th>
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<tr>
<td>Cell-membrane-associated protein genes</td>
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<tr>
<td>oppA</td>
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</tr>
<tr>
<td>gbuABC</td>
<td>2, 48, 101</td>
</tr>
<tr>
<td>betL</td>
<td>57, 101</td>
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<tr>
<td>opuC</td>
<td>2, 25, 101</td>
</tr>
<tr>
<td>flaA</td>
<td>52</td>
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<tr>
<td>fbp</td>
<td>52</td>
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<tr>
<td>Cold stress adaptive regulatory protein genes</td>
<td></td>
</tr>
<tr>
<td>orfX</td>
<td>14</td>
</tr>
<tr>
<td>sigB</td>
<td>9, 10, 60</td>
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<tr>
<td>rpoN</td>
<td>52</td>
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<tr>
<td>bggA</td>
<td>52</td>
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<tr>
<td>degU</td>
<td>47</td>
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<tr>
<td>yycJ</td>
<td>52</td>
</tr>
<tr>
<td>lbkA</td>
<td>52</td>
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<tr>
<td>psr</td>
<td>52</td>
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<tr>
<td>Cold shock phase protein genes</td>
<td></td>
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<tr>
<td>cspLA</td>
<td>100</td>
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<tr>
<td>fri (flip)</td>
<td>40, 52</td>
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<tr>
<td>Other cold stress protein genes</td>
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<tr>
<td>trxB</td>
<td>52</td>
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<td>aroA</td>
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<td>cysS</td>
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<td>trpG</td>
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<td>lirA</td>
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<td>lirB</td>
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<tr>
<td>lirC</td>
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<tr>
<td>General stress response protein genes</td>
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<tr>
<td>groEL</td>
<td>52</td>
</tr>
<tr>
<td>clpP</td>
<td>52</td>
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<tr>
<td>clpB</td>
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Carnosine is mediated by means of three membrane transporter systems. Glycine betaine uptake is mainly through BetL (betaine porter I) and GbuABC (betaine porter II) transporter systems (48, 79, 81). BetL is a secondary uptake system coupling glycine betaine accumulation to Na\(^+\)-motive force, whereas GbuABC is an ATP-driven system (48, 79). Carnosine uptake, on the other hand, is mainly via OpuC, which is also an ATP-driven transporter system (25, 96). In the context of cold stress adaptation, GbuABC is the major betaine transporter, although small cryoprotective amounts are also accumulated via the BetL and OpuC systems (4, 57). Under cold stress, major carnosine uptake is mediated by OpuC, and small cryoprotective amounts are similarly accumulated through GbuABC and BetL systems (4). In fact, the physiological characterization of single and multiple osmolyte transporter deletion mutants revealed the existence of a hierarchy in osmolyte transporter importance. This hierarchy differs depending on the growth media, food, or animal host environment backgrounds (78, 101).

Wemekamp-Kamphuis et al. (101) showed that during growth in brain heart infusion broth at 7°C, GbuABC and OpuC have contributions to cryoprotection, whereas the BetL system does not seem to be significant. However, in a defined minimal medium, the addition of betaine results in a shift in the hierarchy of transporters. In this case, GbuABC followed by BetL becomes more important in cryoprotection compared with OpuC. While under carnitine supplementation, only the OpuC system had important contributions to cryotolerance (101). The study by Sleator et al. (78) found that the contribution of each osmolyte transporter system depended on the growth environment. GbuABC seems to be the predominant osmolyte transporter for L. monocytogenes survival in foods, and the OpuC system appears to be more important during infection (78).

The upregulation of osmolyte uptake in face of cold stress includes the induction of all the three transport systems at transcription level (101). This feature probably also involves transcription components that are directly upregulated via a σ\(^B\)-dependent system, a proposal supported by the presence of σ\(^B\)-dependent promoter sites upstream to the gbuABC and opuC operons in L. monocytogenes (15, 26). In fact, transcription from the opuC operon seems to be primarily σ\(^B\)-dependent (26). The gbuABC operon, on the other hand, is under a dual-promoter system control that includes a σ\(^B\)-dependent promoter and another promoter controlled by an as yet unknown factor (15). The transcription regulation mechanisms involved in betL induction are presently not clear, although putative σ\(^H\)- and σ\(^A\)-dependent promoter binding sites upstream to the betL operon have been proposed (79). Cetin et al. (15) were not able to detect any primer extension activity from a putative σ\(^B\)-dependent promoter by using L. monocytogenes 10430S strain cells.

Another important cold stress adaptation strategy is linked to the accumulation of short peptides from the growth medium by L. monocytogenes. Two-cell membrane-based transport systems mediate peptide accumulation in L. monocytogenes: a proton motive force-dependent system that mediates the accumulation of di- and tripeptides, and a system that mediates oligopeptides accumulation (94, 95). The first report suggesting a role of oligopeptide permease (Opp) transport system in cold stress adaptation by L. monocytogenes was reported by Borezee and coworkers (12). In this study, they showed that the oligopeptide binding protein (OppA) of L. monocytogenes strain LO28 is essential for microbial growth at low temperatures. They generated an oppA gene deletion mutant that was unable to grow at 5°C during 20 days of incubation, whereas under the same conditions, its isogenic wild-type strain was able to reach maximal growth within 15 days (12). The deletion mutant phenotype was subsequently rescued through oppA gene reintroduction on a plasmid.

A follow-up of oppA gene transcripts by Northern blot test showed that its transcription was markedly higher at 5°C compared with 37°C. Interestingly, the predominant oppA transcripts detected at 5°C were shorter (0.8 kb versus 2 kb) compared with those detected at 37°C, suggesting activation of a second promoter that drives production of shorter oppA transcripts upon cold stress exposure (12). The molecular functions of the short peptides accumulated via the OppA transport system in view cold stress alleviation.
is not known. Suggestions include the notion that the accumulated short peptides might be involved in activation of certain signal transduction pathways that promote other L. monocytogenes cold-adaptation and growth mechanisms at low temperatures. Another suggested alternative is that the accumulated peptides may provide substrates that are hydrolyzed providing amino acids or peptide derivatives—for example, proline—that have cryoprotective functions, and these confer cold stress tolerance to microbial cells (12).

**Alterations in cell-surface protein expression.** Temperature-dependent changes in protein expression at the cell-membrane surface are another potential microbial mechanism for adaptation to a cold stress environment. L. monocytogenes strains are highly flagellated and motile at temperatures of 30°C and below, but are typically not motile at temperatures above 37°C (64, 99). Temperature-dependent induction of flaA transcription has also been documented (21, 38, 52). Although it is not clear why cold stress induces flagellation of L. monocytogenes organisms, motility has been associated with biofilm formation in other microbes. One possibility is that cold stress might serve as a signal that prompts biofilm formation as an adaptation strategy of microbes against cold stress environments. Motility might also be essential to allow translocation of the microbes to suitable environmental locations in terms of nutrition or other requirements compatible with growth at reduced temperatures. The mRNA encoding the fibronectin-binding protein (Fbp) is also upregulated in response to cold stress of L. monocytogenes (52). Therefore, it appears that a number of cell-surface modifications are induced under cold stress, highlighting potential but as yet unknown contributions of cell-membrane-associated molecular factors in cold-adaptation responses.

**RIBOSOME FUNCTIONS AND COLD STRESS ADAPTATION**

Because of their central role in protein synthesis, ribosomes are considered one of the key cellular structures involved in microbial cold stress adaptation. It is presumed that one of the effects of cold stress exposure is to severely compromise ribosome structural stability and function, and this manifests through a general reduction in microbes’ protein synthesis capacity in the immediate aftermath of cold shock. In fact, VanBogelen and Neidhardt (91) proposed ribosomes to serve as early sensors of cold shock as well as heat shock stresses in *Escherichia coli*. This role is supported by observations from *Bacillus subtilis* species, where a study by Zhang et al. (108) found that the activation of general stress response modulator σB was blocked by deletion of the L11 ribosomal protein. But for *L. monocytogenes*, the molecular cold-adaptation mechanisms that involve the ribosomes remain largely unexplored.

To date, only a single study has reported on the instability of ribosome structures after cold shock stress of *L. monocytogenes* Scott A strain cells (7). This study detected reduced thermal tolerance in *L. monocytogenes* cells cold-shocked before thermal inactivation at 60°C. The authors used differential scanning calorimetry and found that cold shock led to a decrease in maximum denaturation temperatures (from 73.4 ± 0.1°C to 72.1 ± 0.5°C [mean ± SD]) of the 50S subunit and 70S ribosomal particle peak (7). They suggested that decreased thermal tolerance of cold-shocked *L. monocytogenes* cells was possibly a result of cold shock stress–associated ribosomal damage that led to decreased thermal stability of the ribosomal structures (7). A further support for this phenomenon was provided in the same study when treatment of *L. monocytogenes* with antibiotics (kanamycin and tetracycline) known to affect ribosomal structural stability similarly resulted in reduction of thermal tolerance of the microbial cells, as well as ribosomal structures characterized by reduced melting peaks (7).

Studies conducted in enteric bacteria species suggest that some of the ribosome-associated cold-adaptation responses might include increased expression of the accessory ribosomal factors to promote normal protein production after cold shock (reviewed in reference (106)). Another aspect of cold stress adaptations at the ribosome level might be modulated via the structure and number of *rrn* operons in the microbes (19, 68). Thus it appears that there are different ribosomal aspects of cold adaptation that remain unclear and warrant further exploration for *L. monocytogenes* as well as other microbes.

**Csps AND THEIR INVOLVEMENT IN COLD STRESS ADAPTATION**

Many cold-adaptation studies to date have explored the induction of cold stress proteins in response to abrupt cooling. One group of these proteins has been the Csps (see reviews in (65, 106)). Most Csps have been detected by means of two-dimensional gel electrophoresis approaches to visualize proteins whose production is increased in response to cold shock stress. Several Csps that vary in size have been demonstrated in different *L. monocytogenes* strains (6, 41, 66), although the total number of Csps detected has also varied among the strains investigated. This fact has been attributed to strain differences, variation in the experimental conditions applied, and possibly differences in criteria used for defining the Csps.

The temperatures used for induction of Csps generally range between 4 and 10°C, and most of the studies detected between 10 to 13 Csps (6, 40). But the earliest study published by Phan-Thanh and Gorman (66) documented the induction of up to 38 Csps in *L. monocytogenes* EGD-e strain. On the basis of these studies, the major Csp induced in *L. monocytogenes* seems to be an 18-kDa polypeptide (40). This protein was identified by N-terminal amino acid sequencing to share 100% identity with the ferritin protein of *Listeria innocua* (40). Northern blot analysis also shows that this protein is translated from monocistronic 0.8-kb mRNA transcripts. Even though *fbp* mRNA transcripts are found in normal exponentially growing cells, their production is greatly upregulated after both cold and heat shock stress (40). Therefore, part of this protein’s induction is directly regulated at the transcription level and seems to be required for alleviation of both cold and heat shock stress–associated molecular constraints in the organism (40). Pu-
rified ferritin-like proteins (Flps) of \textit{L. innocua} assemble into a 240-kDa multimeric complex that functions in oxidation and chelating of inorganic iron (13). Although purified Flp of \textit{L. monocytogenes} also assemble into multimeric complex made up of six 18-kDa subunits, their molecular functions are not yet clear (40).

Elevated Flp transcription is also detected during balanced growth of cold-acclimated \textit{L. monocytogenes} cells (52). Possible functions of \textit{L. monocytogenes} Flp proteins in metabolic molecular adaptations designed to alleviate oxidative stress—associated with the cold-adaptation processes have been suggested (52). Another observation was that Csp induction profiles differ among \textit{L. monocytogenes} strains. As a notable example, Bayles et al. (6) found that despite similar cold stress exposure conditions, there were differences in cold shock induction profiles between \textit{L. monocytogenes} SLC53 and 10430S strains. For example, the SLC53 strain cells had three Csp spots of 23.7-, 22-, and 21.4-kDa polypeptides induced that were not observed in 10430S strain cells, although this strain lacked two Csps (19.4-kDa and 15.5-kDa polypeptides) that were induced in the 10430S strain cells (6).

Any implications of this kind of genetic or gene expression variation may have in view of cold stress adaptation of different \textit{L. monocytogenes} strains remain to be elucidated. However, the bulk of research on Csps in microorganisms has to date focused on a family of low-molecular-weight structurally related CspS, which are characterized by the presence of a conserved domain that is highly homologous to the "cold shock domain" of eukaryotic Y-box proteins (reviewed in references (34, 85)). This group, also generally referred to as CspA family proteins, are widely distributed in various prokaryotes (34, 65, 106), although most of our current understanding of these CspA family proteins comes from studies on \textit{E. coli}, \textit{Bacillus}, and \textit{Lactobacillus} organisms (reviewed in references (89, 106, 107)). In \textit{E. coli}, CspA is the main protein that is indirectly induced in response to cold shock (31), although these organisms also harbor eight other CspA homologs that are designated CspB to CspI (89, 107). Interestingly, it is only CspA, CspB, CspG, and CspI that are cold stress inducible in \textit{E. coli}; the other remaining CspA family members in this organism are not induced by cold stress. In fact, some CspA family members have been implicated in other cellular processes in this organism (23, 65, 89, 107).

Three proteins also belonging to the CspA family and designated CspB to CspD have been described in \textit{Bacillus subtilis} (36). They appear to be required under a variety of physiological conditions (33, 35, 36). CspB and CspC are important for cold shock responses and for adaptation to stationary phase (33, 36). But despite the wide distribution of CspA homologs among prokaryotes, in microbes, their functions are not known. Moreover, some organisms completely lack CspA homologs, such as \textit{Helicobacter pylori} and \textit{Mycoplasma genitalium} (32). In context of cold stress adaptation, one of the current assumptions is that some of the CspA homologs (e.g., \textit{E. coli} CspA) may help cells adapt to growth at low temperature through RNA-chaperone activities. This presumably promotes transcription and translation functions that may otherwise be hindered under cold stress. The contributions of CspA family protein to cold adaptation of \textit{L. monocytogenes} have not yet been extensively explored. The completed genome sequences of \textit{L. monocytogenes} strains EGD-e and 4b F2365 show that these organisms harbor three CspA family proteins (CspLA, CspLB, and CspD) (30, 61).

To date, only one study has explored possible involvement of CspA family proteins in cold stress adaptation of \textit{L. monocytogenes} with a two-dimensional gel-based approach (100). This study, by Wemekamp-Kamphuis et al. (100), describes four polypeptides (Csp 1 to Csp 4) in \textit{L. monocytogenes} LO28 strain. These four proteins displayed varying degrees of reactivity to anti-\textit{Bacillus subtilis} CspB antibodies in Western blot tests. Csp 1 and Csp 2 polypeptides in this study are described, although not confirmed, as products encoded by the \textit{L. monocytogenes} cspLA and cspLB genes, respectively (100). All four Csps are constitutively expressed during normal growth at 37°C, but Csp 1 and Csp 3 synthesis is greatly increased in response to cold shock stress induced by lowering the temperature from 37°C to 10°C. Maximal induction of these two Csps was observed in cold-adapted cells after a 20-h incubation at 10°C. The expression of Csp 2 and Csp 4 remained unchanged in response to cold stress. Interestingly—and similar to the \textit{E. coli} CspA protein—Csp 1 and Csp 4 in the \textit{L. monocytogenes} LO28 strain were also induced after exposure to hydrostatic pressure stress (100), although similar induction of these CspA family proteins by hydrostatic pressure was also confirmed in \textit{L. monocytogenes} EGD-e strain cells (102). In addition, preexposure to cold stress was also found to induce a cross-protective effect against hydrostatic pressure or freezing stress (100). Therefore, these studies indicate that although CspA family proteins of \textit{L. monocytogenes} are induced in response to cold shock stress and hydrostatic pressure, their molecular functions and possible contributions to cold adaptation are still unexplored.

**ADAPTIVE REGULATORY PROTEINS INVOLVED IN COLD STRESS ADAPTATION**

Many cellular regulatory protein networks are essential in implementation and coordination of the various molecular events at the interface of efficient cold stress adaptation responses. Therefore, regulatory protein networks are crucial in the tight coordination of the many cold stress adaptation responses through various molecular signal transduction networks at the microbe’s disposal. Present knowledge about the key molecular mechanisms behind cold stress sensing and signal transduction in most organisms, including \textit{L. monocytogenes}, remains elusive. One proposal is that ribosomes may be the early sensors of cold stress in microbes (91); this has been supported by an observation that loss of ribosomal protein L11 blocks stress activation of the pB transcription factor in \textit{B. subtilis} (108).

Another possibility involving cell-membrane-based component sensors of environmental stresses, including cold stress, has been suggested (97); that environmental...
changes detected at the membrane level lead to transmission of signals that eventually culminate in the activation of response gene transcription in the cell (97). Many studies have also focused on the roles of the general stress response modulator σB in cold stress adaptation (reviewed in (92)). Present data show that σB is activated in response to cold stress in *L. monocytogenes*, although the initial stress sensing and signaling pathways leading to its activation are unknown (9). σB is a dissociable initiation factor for the multisubunit RNA polymerase holoenzyme, targeting the transcription complex to σB-dependent promoters, thereby activating transcription from operons preceded by σB recognition sequences in their promoter region. Phenotypic characterization of the σB deletion mutants of *L. monocytogenes* shows that σB functions are essential for efficient cold adaptation and proliferation under certain experimental conditions (10, 60).

Becker et al. (10) found that σB is needed for efficient cold adaptation in stationary-phase cells, but its deletion has no influence in log-phase cells. One of the conclusions they drew is that independent cold stress adaptation pathways may be present in *L. monocytogenes*. Some of the pathways entered in stationary-phase cells needed a component contributed by σB functions, whereas alternative cold-adaptation pathways independent of σB functions were used in log-phase cells. The influence of σB deletion in cold adaptation may in part be attributed to defects in accumulation of cryoprotective solutes such as glycine betaine and carnitine as a result of a loss of σB-dependent induction on the GbuABC and OpuC osmolyte transporter operons (10, 15, 26). Another potential σB downstream target that might be involved in cold stress adaptation is the *L. monocytogenes* hfq gene, which encodes a putative RNA-binding regulatory protein Hfq and various transcription regulatory functions that are essential for growth and survival under harsh environmental stress conditions such as osmotic and ethanol stresses (18). Christiansen et al. (18) showed that hfq transcription induction is lost in σB deletion mutants of *L. monocytogenes* strain 10403S. Interestingly, the hfq deletion mutant described in this work also displays reduced cold stress adaptation ability, which was characterized by a prolonged lag phase during growth at 4°C.

Moorhead and Dykes (60) published a study further supporting σB roles in cold stress adaptation. They described reduced cold stress survival and recovery in σB deletion mutants in *L. monocytogenes* strains of serotypes 1/2a (meat isolate) and 4c (clinical isolate) that had been inoculated on beefsteaks or in phosphate-buffered saline and incubated at 4°C (60). Regulation of σB under various stress conditions is complex and probably involves several molecular mechanisms acting at both the transcription and posttranscription levels. For the various stress responses of *L. monocytogenes*, only a few studies have investigated potential roles of other adaptive regulatory proteins that act upstream to σB during its activation (14, 17). In one such study, Brondsted et al. (14) investigated the contributions of genes within the kdp locus in the growth of *L. monocytogenes* strain EGD under osmotic and cold stress. In particular, they focused on the kdpE and orfX genes within this locus with regard to cold stress adaptation. They found that deletion of the orfX gene resulted in mutants displaying similar growth behavior to that of σB-deletion mutants in an *L. monocytogenes* strain LO28 background. In this case, deletion of either orfX or sigB gave rise to mutant cells that displayed a shortened growth lag phase compared with their wild-type isogenic counterparts after a temperature reduction from 37°C to 3°C. The orfX gene product is putative homologue of the RsbQ protein. The RsbQ protein in *B. subtilis* is a known modulator of σB activity, and thus if a similar model is assumed, then orfX presumably serves a similar function in *L. monocytogenes*. Furthermore, because in this study orfX deletion had an effect similar to sigB deletion, it was suggested that the loss of orfX activity indirectly abolished downstream σB activation. Although in contrast to observations in other sigB deletion mutants (see references (10, 60)), the loss of σB functions through sigB or orfX gene deletions resulted in cells that actually needed short cold-adaptation periods compared with the wild-type strain when the temperature was decreased from 37°C to a low of 3°C. The authors attributed these contrasting phenotypic behaviors of the σB deletion mutants to possible strain- or experimental condition–associated differences.

Similarly, variations in the general stress response by different *L. monocytogenes* serotypes have also been attributed to strain-related differences in the magnitude of σB activation (59). Despite being a key modulator of various stress responses, σB deletion has so far only resulted in minimal observable phenotypic defects in view of the cold stress adaptation using the different *L. monocytogenes* strain backgrounds tested to date. This possibly reflects redundancy in the pathways that connect the cold-adaptation regulatory protein networks in the organism, a phenomenon that might not be surprising in view of the crucial importance of various stress adaptation mechanisms on microbial survival and dissemination in different environments. This also emphasizes the need to elucidate the role of other regulatory protein networks in view of cold stress adaptation regulation in *L. monocytogenes*.

Some potential alternative cold stress adaptation regulators were recently highlighted by using differential gene expression studies of cold-acclimated *L. monocytogenes* cells (52). Notably, rpoN gene transcripts were found to be significantly increased during growth at 10°C compared with 37°C in *L. monocytogenes* EGD-e cells. This implies potential roles of the alternative sigma factor 54 (σ54), the rpoN gene product in regulation of gene expressions during cold adaptation of *L. monocytogenes* (52). Alternatively, σ54 might regulate several cold-adaptation proteins, and it might act redundantly with σB, thus providing one possible explanation for the minimal phenotypic cold-adaptation defects observed in σB deletion mutants so far. The adaptive proteins of the bacterial two-component signal transduction systems are also another group of regulatory protein networks that may mediate gene expression regulation during cold adaptation. These adaptive regulatory protein systems typically consist of histidine kinase sensors and their cognate DNA binding response regulators, and they enable mi-
crobases to respond to diverse environmental stimuli (reviewed in reference (103)).

Sensor kinases are usually integral membrane proteins capable of responding to environmental signals and thus are ideally positioned for cell-membrane-based cold stress sensing and signal transduction (22). L. monocytogenes harbors 16 two-component transcription regulatory systems, although little is known about their possible involvement in cold stress adaptation (30, 105). The study of Liu et al. (52) reported enhanced transcription of the yycJ gene in response to cold stress. This gene encodes a putative member of the two-component signal transduction system in L. monocytogenes. Similarly, the lhkA gene, which encodes a histidine kinase sensor, was also cold stress induced. Therefore, this might act as a kinase sensor for a two-component regulatory system required in the cold adaptation of L. monocytogenes (52). In their primary characterization of L. monocytogenes degU gene encoding a putative two-component response regulator system, Knudsen et al. (47) found this gene to be important for regulating cell motility through temperature-dependent induction of flaA transcription at low temperature (25°C). Although the roles of flaA induction in cold stress adaptation are not clear, it is plausible that temperature-dependent increased transcription regulation by degU also regulates other as yet unidentified response factors that mediate some cold stress adaptation responses.

Research in other bacterial organisms also supports a role for some proteins that are part of bacterial two-component regulatory systems in cold-adaptation responses. Notable examples include the DesK-DesR two-component system of B. subtilis, which regulates the expression of a cold-inducible des gene. The histidine kinase Hik33 of cyanobacterium Synechocystis species is involved in sensing low temperatures and regulation of cold-inducible gene expression (1, 5, 87). The BglG-like transcription antiterminator of L. monocytogenes is yet another regulatory protein whose transcription is also enhanced under cold stress (30, 52). However, its potential transcription regulatory functions in cold stress adaptation remains unknown. Cold stress–associated induction of L. monocytogenes psr mRNA production was also detected by Liu and coworkers (52). This repressor protein has been implicated in regulation of its own expression as well as that of penicillin binding protein 5, and its deletion leads to various cell-wall alterations, including decreased rhamnose content in Enterococcus hirae (50, 55). Its potential functions in L. monocytogenes are presently unknown, but its induction might also lead to alterations in cell-surface protein expressions, and future studies should thus elucidate its potential roles in cold stress adaptation mechanisms.

OTHER PROTEINS WITH POTENTIAL INVOLVEMENT IN COLD STRESS ADAPTATION MECHANISMS OF L. MONOCYTOGENES

Several molecular studies have detected other genetic elements putatively involved in L. monocytogenes cold stress adaptation. A transposon mutagenesis–based study reported by Zheng and Kathariou (109) described three L. monocytogenes cold-sensitive mutants able to grow at 8°C or above but, interestingly, unable to grow at 4°C. This phenotype was linked to the inactivation of three low-temperature requirement genes, ltrA, ltrB, and ltrC (109). These genetic elements seem to be essential for growth at low temperatures (4°C) but are dispensable at temperatures of 8°C or above (109). However, evaluation of the actual molecular contributions by products encoded by these three genes in cold adaptation awaits further elucidation.

Induction of general stress resistance proteins appears to be another nonspecific strategy widely used by microbes to counteract different adverse environmental conditions, including cold stress. Liu et al. (52) also detected increased transcription of the groEL, clpP, and clpB genes during cold acclimation in L. monocytogenes cells. This molecular response may reflect cellular adaptation to cold stress–associated alterations in protein synthesis. Compromised transcription and ribosomal activity probably results in increased production of abnormal proteins under cold stress. Therefore, cells try to counteract this increasing production of chaperones and proteases to remove the large amounts of abnormal proteins that would otherwise interfere with normal functions if left to accumulate. GroEL chaperone induction is also observed in response to several other forms of stress, including heat shock, low pH, salt, and bile salts (39, 75). Under cold stress, its production in L. monocytogenes cells might be designed to maintain protein solubility and functions in the cytoplasm. Alternatively, it might simply be induced as part of more generalized stress responses that are triggered by various stimuli (52).

Caseinolytic proteases, which are involved in degradation of damaged polypeptides and salvage of amino acids, also seem to be induced during cold acclimation of L. monocytogenes (52), although current literature reports that deletion of either ClpP or ClpB has no marked influence on L. monocytogenes growth at low temperatures (16, 27). Furthermore, cold stress adaptation mechanisms have also been linked to changes in other cell-surface metabolic processes. In particular, oxidative stress seems to be one of the main metabolic changes that accompany the cold-adaptation process in L. monocytogenes cells. Elevated transcription of both flp and trxB genes was associated with growth of L. monocytogenes at low temperatures (52). These transcripts encode Flp and thioredoxin reductase, respectively. L. monocytogenes Flp has been implicated in molecular responses designed to overcome cellular oxidative stress in the organism (52). Thioredoxin reductase enzyme is part of the thiol-dependent oxidation-reduction system that contributes to maintenance of reducing environments in bacterial cytoplasm (reviewed in reference (41)). The cold acclimation process in L. monocytogenes cells is also associated with increased transcription of genes involved in amino acid synthesis. Transcription of the hisJ, trpG, cysS, and aroA genes that encode amino acid biosynthetic enzymes are upregulated under cold stress, indicating cellular starvation in certain amino acids (52). Consequently, the stressed microbes respond by increasing the production of enzymes needed to increase the production of deficient amino acids.
Increased transcription from genetic factors that indicate altered degradative metabolism responses also accompanies cold stress acclimation of *L. monocytogenes* (52). Enhanced transcription of *eutB* and *celD* was detected in response to cold stress. These two genes are predicted to encode proteins involved in ethanolamine and cellulose degradation, respectively (52). The roles of such an adaptation strategy in view of cold stress are not yet clear. It has been suggested as a strategy designed to prepare *L. monocytogenes* for survival as free-living bacteria (52). It thus seems that *L. monocytogenes* takes advantage of cold stress as an environment-derived signal to trigger metabolic changes that enable it to persist in various natural reservoirs, including food-related environments. The *L. monocytogenes mela* gene is yet another metabolic gene whose transcription is also up-regulated in response to cold stress (52). This gene encodes the malolactic enzyme, which is involved in malolactate fermentation in some lactic acid bacteria. Thus several metabolic changes seem to be induced in response to cold stress in *L. monocytogenes*, and future studies focusing on these different metabolic pathways may enhance our understanding of how these organisms adapt their physiology to counteract molecular constraints of cold stress, as well as other food-related environments.

**IMPLICATIONS OF *L. MONOCYTOGENES* MOLECULAR COLD-ADAPTATION CAPABILITIES IN VIEW OF FOOD SAFETY**

The ability of *L. monocytogenes* organisms to adapt to various stresses in food and food-processing environments is a serious threat to human food safety. These microorganisms possess robust molecular adaptation capacities that enable them to respond when faced with food-associated environment challenges. This ultimately allows resistance to common food-preservation techniques, including refrigeration, routinely used to prevent the growth of most foodborne microbes. It is therefore envisaged that advances in our understanding of the molecular basis behind cold-adaptation mechanisms of *L. monocytogenes* in food environments might also open up avenues for their exploitation in view of developing practical food-safety applications. Challenges remain: molecular knowledge must be gathered, and molecular concepts need to be channeled into practical control methods during food processing and storage. Notable examples include the revelation that the survival and growth of *L. monocytogenes* on foods depends in part on its ability to exploit the food environment for cryptoprotec- tants. *L. monocytogenes* growth at low temperatures is enhanced by its molecular ability to accumulate cryoprotective osmolytes and short peptides from the growth medium. Clearly, the abundance of these substances in foods provides a ready source for the microbes' cryoprotection. This type of knowledge might also open up avenues for control strategies that can inhibit the uptake of such substances from food environments by microbes, and thereby slow or prevent their growth on food products.

Molecular studies may also provide important information, such as overlapping molecular response mecha-

**CONCLUSIONS AND PERSPECTIVES**

Understanding the molecular mechanisms behind cold tolerance of *L. monocytogenes* provides an insight into how these microbes achieve their psychrotolerance and other forms of stress resistance. In view of cold stress, many studies to date have shown that one of the main response mechanisms involves the induction of several cold stress proteins in *L. monocytogenes* cells. However, the current knowledge is limited in terms of identity of such protein factors or how they functionally contribute to alleviation of cold stress constraints at the molecular level. We hope that more knowledge of protein identity will come with more approaches based on application of gene expression, such as subtractive hybridization or microarrays. The study by Liu et al. (52) has revealed a number of genes and provides a starting point for further elucidation of molecular factors involved in the cold acclimation process of *L. monocytogenes*.

Similar approaches in the future will aid in highlighting additional potential target genes of cold stress resistance in this organism. This also paves way for further molecular analysis of potential targets at the protein and functional levels. Therefore, many possible molecular cold response targets presently await further exploration in view of cold stress adaptation in *L. monocytogenes*. There are also several key areas where very little is known, especially in terms of how the cold stress threats are actually sensed and communicated within and between the microbial cell, and the eventual induction of cold tolerance measures and their coordination in *L. monocytogenes* and other microbes.

**REFERENCES**


COLD STRESS TOLERANCE OF LISTERIA MONOCYTOGENES


