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MINIREVIEW

The Role of Cold-Shock Proteins in Low-Temperature Adaptation of Food-Related Bacteria

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Summary

There is a considerable interest in the cold adaptation of food-related bacteria, including starter cultures for industrial food fermentations, food spoilage bacteria and food-borne pathogens. Mechanisms that permit low-temperature growth involve cellular modifications for maintaining membrane fluidity, the uptake or synthesis of compatible solutes, the maintenance of the structural integrity of macromolecules and macromolecule assemblies, such as ribosomes and other components that affect gene expression. A specific cold response that is shared by nearly all food-related bacteria is the induction of the synthesis so-called cold-shock proteins (CSPs), which are small (7 kDa) proteins that are involved in mRNA folding, protein synthesis and/or freeze protection. In addition, CSPs are able to bind RNA and it is believed that these proteins act as RNA chaperones, thereby reducing the increased secondary folding of RNA at low temperatures. In this review established and novel aspects concerning the structure, function and control of these CSPs are discussed. A model for bacterial cold adaptation, with a central role for ribosomal functioning, and possible mechanisms for low-temperature sensing are discussed.

Key words: Cold adaptation – cold-shock proteins – ribosome adaptation – food-related bacteria – pathogens – spoilage bacteria – lactic acid bacteria

Low-temperature adaptation

Processing and storage of food products at low temperature is rather common in food technology. For that reason, the low-temperature behaviour of food-related bacteria including lactic acid bacterium starter cultures, food spoilage bacteria and food-borne pathogens, is an important issue. Starter organisms, such as the lactic acid bacteria *Lactococcus lactis*, *Lactobacillus plantarum* and *Streptococcus thermophilus*, are widely used for the production of a large variety of foods. These organisms are exposed to low temperature during frozen storage, as well as during low-temperature fermentation and/or storage of fermented products. In addition, the use of freezing as preservation method, the extended use of chilled (convenience) foods, and the increased popularity of fresh or minimally processed food all created the need for understanding the cold-adaptation response of spoilage micro-organisms and food pathogens, in partic-

ular. Due to the increased time intervals between production and consumption of food products and the extended use of refrigerators, notably the risks of foodborne psychrotrophic pathogens, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus* and non-proteolytic *Clostridium botulinum* increased (ABEE and WOUTERS, 1999). Research on cold adaptation of bacteria focuses on the genetic and physiological processes involved in low-temperature adaptation (BERRY and FOEGEDING, 1997; YAMANAKA et al., 1998; GRAUMANN and MARAHIEL, 1998). A thorough understanding of the cold-adaptation process can be instrumented in optimiz-

Abbreviations:

- CSP – (7 kDa) cold-shock protein
- CIP – (non-7 kDa) cold-induced protein
- 5'-UTR – 5'-untranslated leader
- CS-box – cold-shock box
- DB – downstream box
- RNP-1 (and 2) – RNA-binding region 1 (and 2)

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ing fermentations at low temperature and may offer insight into methods to control the growth of spoilage and pathogenic bacteria, which will positively affect the shelf-life and safety of refrigerated foods.

Mechanisms that permit low-temperature growth of microorganisms include modifications in DNA supercoiling, maintaining membrane fluidity, regulating uptake and synthesis of compatible solutes, production of cold-shock proteins, modulating mRNA secondary structure and, more generally, maintaining the structural integrity of macromolecules and macromolecule assemblies, such as ribosomes (see reviews by RUSSELL, 1990; JAENICKE, 1991; BERRY and FOEGEDING, 1997; GRAUMANN and MARAHIEL, 1998). Negative DNA supercoiling of the bacterial nucleoid increases at low temperature and this has important consequences for the regulation of transcription (DRLICA, 1992). It has been shown for *E. coli* that DNA topoisomerase activity, DNA gyrase activity and the histon-like HU protein have an important role in the process of controlling transcription at low temperature (MIZUSHIMA et al., 1997). In general, as the growth temperature decreases, an increase is observed in the proportion of shorter and/or unsaturated fatty acids in membrane lipids allowing an optimal degree in fluidity of the cytoplasmic membrane. One of the most important consequences of these membrane lipid changes is the modulation of the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (RUSSELL, 1990; RUSSELL and FUKANAGA, 1990). Compatible solutes, such as betaine, proline and carnitine, play a crucial role in osmoprotection and could also be involved in cold adaptation. For different compatible solutes, such as betaine, carnitine, ectoine and mannitol, a protective effect during freeze-drying has been reported (LOUIS et al., 1994). It was found that growth of *L. monocytogenes* was stimulated at 7 °C in the presence of betaine and that these cells transport betaine 15-times faster at 7 °C than at 30 °C (KO et al., 1994). These data suggest that there might be a functional link between osmoprotection and cold adaptation, however, the exact mode of action of compatible solutes in cold adaptation remains to be elucidated.

Using proteomics approaches the rapid induction of specific sets of proteins upon cold shock is observed for a variety of bacteria. The number of these so-called cold-induced proteins (CIPs) may vary from approximately 18 for *Escherichia coli*, 12 for *L. monocytogenes*, 22 for *L. lactis* to 37 for *Bacillus subtilis* (JONES et al., 1987; GRAUMANN et al., 1996; BAYLES et al., 1996; PANOFF et al., 1994; WOUTERS et al., 1999A), whereas the synthesis of the majority of proteins is blocked. The *E. coli* CIPs play a role in various cellular processes and include 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), CsdA and RbfA (a ribosome associated helicase and a ribosome binding factor A which are both important for ribosomal structure) (JONES et al., 1987; JONES and INOUE, 1994; JONES and INOUE, 1996). For *B. subtilis* CIPs are described that are involved in a variety of cellular processes, such as

chemotaxis (CheY), sugar uptake (HPr), translation (ribosomal proteins S6 and L7/L12), protein folding (PpiB) and general metabolism (CysK, IlvC, Gap and triosephosphate isomerase) (GRAUMANN et al., 1996). Similarly, for *L. lactis* CIPs have been identified that are also involved in the translation process (ribosomal protein L9), sugar metabolism (β -PGM, HPr, CcpA), chromosome structuring (histon-like HU-protein) and signal transduction (WOUTERS et al., submitted A; WOUTERS et al., submitted B).

In recent years, emphasis in research on cold adaptation was on the specific and high induction of a set of low-molecular weight (7 kDa) proteins at low temperature: the so-called cold-shock proteins (CSPs). Because of the implications of CSPs for the process of translation at low temperature and their observed role as regulatory proteins of other cold-induced proteins, this review will describe these proteins in more detail. Novel aspects about the functioning of CSPs will be discussed, specifically in relation to food-borne bacteria, including *E. coli* of which certain species are highly pathogenic, like *E. coli* O157:H7 as the cause of outbreaks of hemorrhagic colitis (RILEY et al., 1983), and *B. subtilis* that is used as a starter in food fermentations (HARWOOD and ARCHIBALD, 1990). Research focussed on the so-called cold-shock response, in which a cold-shock is defined as a rapid decrease in growth temperature, a condition that is taken as a model to study low-temperature adaptation.

Cold-shock proteins and their mode of action

CSPs (molecular weight of approximately 7 kDa) are observed in a variety of Gram-positive and Gram-negative bacteria (Table 1) in which they share a high degree of sequence similarity (>45%). For most bacterial species, families of CSPs consisting of two to nine members have been found. The concomitant presence of *csp* gene families probably resulted from gene duplications within the organism. FRANCIS et al. (1997) characterized *csp* genes in a variety of bacteria, including among others also *Aeromonas hydrophila* and *Staphylococcus aureus*, by use of a PCR method and used this method for the discrimination of bacterial species. However, CSPs are not observed in the complete genomes of all bacteria, e.g. in *Helicobacter pylori* (TOMB et al., 1997), *Campylobacter jejuni* (HAZELEGER et al., 1998) and *Mycoplasma genitalium* (GRAUMANN and MARAHIEL, 1996). For *L. lactis*, *Y. enterocolitica* as well as for *E. coli* a clustered organization of *csp* genes on the chromosome was observed (WOUTERS et al., 1998; YAMANAKA et al., 1998; NEUHAUS et al., 1999) and probably originated from gene duplications (YAMANAKA et al., 1998). For *L. lactis* and *Y. enterocolitica* tandem repeats of two adjacent *csp* genes, separated by an interval of only approximately 300 bp, were observed. The physiological or genetic significance of the *csp* gene duplications and the clustered organization of these genes remain to be elucidated.

The most extensively studied CSPs are CspA of *E. coli* (CspA^E) and CspB of *B. subtilis* (CspB^B). The determi-

Table 1. Alignment of CSPs of food-related microorganisms.

CSP ¹	Amino acid sequence ²	aa ³	pI ⁴
	<div> RNP-1 RNP-2⁷ </div>		
Gram-positive	<div> + ** * ** *** * * + *⁸ </div>		
CspA Llac	MINGTVKWFNMDKGYGFITTEDMQ-DVFAILLSIQNGFKKYNEGQKVTDFVTMTARGRYASNIDKV ⁹	66	9.2
CspB Llac	MTKGTVKWFNPDKGFGITSEDGQ-DVFAHFSQIQTSQFKTLDEGQKVTDFVEAGQGRGPQAVNIEKA ⁹	66	4.9
CspC Llac	MNKGKINWFNADKGYGFIMADDMQ-DVFAYILLSIQNDFKKYDEGQKVTDFDIKMTSRGRYASNVHKK ⁹	66	9.6
CspD Llac	MANGTVKWFNATKGFGITSEDGQ-DLFAHFSAIQSDGFKSLDEGQKVEFDVEEGQGRGPQAVNITKA ⁹	66	4.4
CspE Llac	MAQGTVKWFNATKGFGITTEEGN-DVFAHFSAIQTDGFKTLDEGQKVTDFVEEGPRGPQAVNIQK ⁹	65	4.6
CspC Lpla	MEHGTVKWFNADKGFGITRENGS-DVVFHFSAIQEDGFKSLDEGQAVNFDVEESDRGPPAANVTKA ¹⁰	66	4.4
CspL Lpla	MKNGTVKWFNADKGFGITGEDGT-DVVFHFSAIQTDGFKTLDEGQKVTYDEEQDGRGPQATNVQPO ¹¹	66	4.3
CspP Lpla	MKNGTVKWFNADKGYGFITGEDGN-DVVFHFSAIQTDGFKTLDEGQKVTDFDEESSDRGPPAANVVPQ ¹¹	66	4.4
CspB Bsub	MLEGKVKWFNSEKGFGEIEVEGQD-DVVFHFSAIQEGGFKTLEEGQAVSFEIVEGNRGPQAAANVTKEA ¹²	67	4.5
CspC Bsub	MEQGTVKWFNADKGFGFIERENG-DVVFHFSAIQSDGFKSLDEGQKVSFDVEEQARGAQAANVQKA ¹²	66	4.6
CspD Bsub	MQNGKVKWFNNEKGFGEIEVEGGD-DVVFHFTAIEGDGKYKSLDEEGQEVSEIVEGNRGPQASNVVKL ¹²	66	4.4
CspA Bcer	MTVTGQVKWFNNEKGFGEIEVPGEN-DVVFHFSAIETDGFKSLDEEGQKVSFEIEDGNRGPQAKNVIK ¹³	67	4.7
CspB Bcer	MNGKVKWFNNEKGFGEIEMEGSE-DVVFHFSAIQSDGYKALEEGQEVSDITEGNRGPQAAANVAKL ¹³	65	4.5
CspC Bcer	MQGRVKWFNADKGFGFIEREDGD-DVVFHFSAIQDGYKSLDEEGQVEFDIVDGARGPQAAANVVKL ¹³	65	4.4
CspD Bcer	MQTGKVKWFNNEKGFGEIEVEGGE-DVVFHFSAIQDGYKSLDEEGQEVSEIVDGNRGPQAAANVTKN ¹³	66	4.5
CspE Bcer	MTLTGKVKWFNSEKGFGEIEVADGS-DVVFHFSAITDGFKSLDEEGQEVSEFEV ¹³	54	4.1
CspB Lmon	MQGTGTVKWFNNEKGFGEIEVEGGD-DVVFHFSAIQEGGFKTLEEGQSVFEIVEEGQGRGPQAEKVTKL ¹⁴	66	4.4
CspL Lmon	MNMEQGTGTVKWFNADKGFGFIERENG-DVVFHFSAIQDGYKSLDEEGQAVTFDVEEGQGRGPQAAANVQKA ¹⁵	68	4.3
CspA Sthe	MANGTVKWFNATKGFGITSEDGQ-DLFAHFSIQSDGFKSLDEQKVEFDVEVGQGRGPQAVNITKA ^{16, 17}	66	4.5
CspB Saur	MNNGTVKWFNADKGFGEIECENG-DVVFHFSGIASDGYKTLDEEGQKVTFEITEGQRGDQAVNVQTV ¹⁸	66	4.4
CspC Saur	MNNGTVKWFNADKGFGEIEREDGS-DVVFHFSIAIEDGYKSLDEEGQKVEFDIVEGDRGEQAANVVKM ¹⁸	66	4.3
Gram-negative			
CspA Ecol	MSGKMTGIVKWFNADKGFGITPDGSKDVFVHFSAIQNDGYKSLDEGQKVSFTIESGAKGPAAGNVTS ¹⁹	70	5.5
CspB Ecol	MSNKMTGLVKWFNADKGFGITPDGSKDVFVHFSAIQNDNYRTLFEGQKVTFSIESGAKGPAANVTITD ¹⁹	71	7.6
CspC Ecol	MAKIKGQVKWFNNEKGFGITPDGSKDVFVHFSAIQNGFKTLAEGQNVFEIQDQKGPAAANVTAT ¹⁹	69	7.6
CspD Ecol	MEKGTGTVKWFNADKGFGEIEGGEDIFAHYSTIQMDGYKTLKAGQSVQFDVHQGPKNHASVIVPVEVEA ^{19, 24}	74	5.8
CspE Ecol	MSKIGKTVKWFNNEKGFGITPDGSKDVFVHFSAIQNTNGFKTLAEGQVFEITNGAKGPAANVTIAL ¹⁹	69	9.4
CspF Ecol	MSRKMTGIVKTFDCKSGKGLITPSDGRIDVQLHVSALNLRDAEITTLGRVEFCRINGLRGPSAANVYLS ¹⁹	70	9.9
CspG Ecol	MSNKMTGLVKWFNADKGFGITPDGSKDVFVHFTAISNEFRTLNENQKVEFSIEQGRGPAAANVTIL ¹⁹	70	5.6
CspH Ecol	MSRKMTGIVKTFDRKSGKGLITPSDGRKEVQVHISAFTPRDAEVLIPGLRVEFCRVNGLRGPTAANVYLS ¹⁹	70	10.7
CspI Ecol	MSNKMTGLVKWFNNEKGFGITPKDGSKDVFVHFSAIQSDNFKTLTENQEVEFGIENGPKGPAAVHVAL ¹⁹	70	9.1
CspA Styp	MSGKMTGIVKWFNADKGFGITPDGSKDVFVHFSAIQNDGYKSLDEGQKVSFTIESGAKGPAANVTSL ²⁰	70	5.7
CspB Styp	MTTKITGLVKWFNNEKGFGITPKDGSKDVFVHFSAIQSDNFKTLTENQEVEFSVEQGPQKPSAVNVVAL ²¹	70	5.8
CspH Styp	MSRKMTGIVKTFDCKSGKGLITPSDGRKDVQVHISACRQHEATEALIPGIRVEFCRINGLRGPTAANVYLS ²²	70	9.5
CapA Pfra	MSQRQSGTVKWFNDEKGFGITPDGSDLLFVHFKAIESDGFKSLKEGQVTSFVAEKQKGMQA ²³	64	7.1
CapB Pfra	MSNRQGTGTVKWFNDEKGFGITPDGSDLLFVHFKAIQSDGFKSLKEGQVTSFIATRGQKGMQAEVQVI ²³	69	7.2
TapA Pfra	MSNRQGTGTVKWFNDEKGFGITPDGSDLLFVHFRAIQNGGFKSLKEGQKVTFFIAVQGGQKGMQADKVQAE ²³	70	9.3
TapB Pfra	MSQRQSGTVKWFNDEKGFGITPDGSDLLFVHFRAIQNGGFKSLKEGQKVTFFIAVQGGQKGMQA ²³	63	9.7
CspA1 Yent	MSNKMTGLVKWFNADKGFGITPDGSKDVFVHFSAIQSDNFKTLDEGQKVEFSIENGAKGPSAVNVVAL ²⁴	70	5.7
CspA2 Yent	MSNKMTGLVKWFNADKGFGITPDGSKDVFVHFSAIQSDNFKTLDEGQKVEFSIENGAKGPSAVNVIAL ²⁴	70	5.7
	<div> β1²⁵ β2 β3 β4 β5 </div>		

1. Llac – *Lactococcus lactis*; Lpla – *Lactobacillus plantarum*; Bsub – *Bacillus subtilis*; Bcer – *Bacillus cerues*; Lmon – *Listeria monocytogenes*; Sthe – *Streptococcus thermophilus*; Saur – *Staphylococcus aureus*; Ecol – *Escherichia coli*; Styp – *Salmonella typhimurium*; Pfra – *Pseudomonas fragi*; Yent – *Yersinia enterocolitica*.

2. Missing residues are indicated with hyphens.

3. Total length of CSP in amino acids.

4. Calculated iso-electric point.

5. + indicates induction of CSP upon cold shock at mRNA or protein level; – indicates no induction of CSP upon cold shock at mRNA or protein level.

6. Induction of CSP upon exposure to other stresses than cold. Stat – stationary phase induction; UHP – induction by Ultra High Pressure treatment; GPD – growth phase dependent expression.

7. RNP-1 and RNP-2 indicate the conserved RNA binding motifs (boxed).

8. Typical amino acid residues conserved in CSPs. + indicated the hydrophobic residues that form the hydrophobic core in the β-barrel structure of CspA of *E. coli*. * indicates the aromatic residues located on the surface of CspA of *E. coli* (conserved residues are indicated in bold).

9. WOUTERS et al., 1998. 10. DERZELLE, S., HOLS, P., DELCOUR, J., personal communication. 11. MAYO et al., 1997. 12. GRAUMANN et al., 1996. 13. MAYR et al., 1997. 14. BERNARD, F. M., FRANCIS, K. P., REES, C. E. D., STEWART, G. S. A. B., unpublished data. 15. FRANCIS, K. P., REES, C. D., STEWART, G. S. A. B., unpublished data. 16. WOUTERS et al., 1999B. 17. VARCAMONTI, M., personal communication. 18. FRANCIS, K. P., unpublished data. 19. YAMANAKA et al., 1998. 20. JEFFRIES, A. G., LAW, R. M., BEJ, A. K., unpublished data. 21. CRAIG et al., 1998. 22. FRANCIS, K. P., and STEWART, G. S. A. B., unpublished data. 23. MICHEL et al., 1995. 24. NEUHAUS et al., 1999. 25. β1–β5 indicate the five anti-parallel β-strands that form a β-barrel structure with two β-sheets as observed for CspA of *E. coli* and CspB of *B. subtilis*.

nation of their crystal structures revealed that both proteins consist of five antiparallel β -strands which together form a β -barrel structure (SCHINDELIN et al., 1993; SCHINDELIN et al., 1994; NEWKIRK et al., 1994). It was observed that CspA^E contains a set of surface exposed aromatic amino acids (W11, F12, F18, F20, F31, F34, Y42 and F53), which appeared to be essential for RNA or DNA binding (see below) and a set of hydrophobic residues (V9, I21, V30, V32 and V51) forming a hydrophobic core of the protein (NEWKIRK et al., 1994) (Table 1). CSPs contain regions highly homologous to the cold-shock domain of eukaryotic DNA-binding proteins, like YB1 and FRGY2, that are known to act as transcription factors. Both CspA^E and CspB^B are able to bind specifically to single-stranded DNA containing the Y-box motif (ATTGG) or its complementary sequence (NEWKIRK et al., 1994; GRAUMANN and MARAHIEL, 1994) thereby regulating gene expression (JONES and INOUE, 1994). For the majority of thus far characterized CSPs the iso-electric point is acidic (Table 1). However, several CSPs have a more basic iso-electric point (8-10), a characteristic that might have major consequences on the RNA and DNA-binding characteristics of CSPs.

Strikingly, it has been shown that CspA^E acts as a transcriptional activator of at least two genes encoding CIPs, possibly by stabilization of the open complex formation by RNA polymerase. These proteins, GyrA (JONES et al., 1992B; BRANDI et al., 1994) and H-NS (LATEANA et al., 1991), are both involved in DNA supercoiling, which is significantly modified at low temperature. Next, heterologous expression of CspB^B in *E. coli* induced a protein

pattern that strongly resembled that upon cold shock, indicating that also CspB^B functions of as a regulatory protein (GRAUMANN and MARAHIEL, 1997). For *B. subtilis* as well as *L. lactis* changes in protein patterns were observed upon deletion of CSPs (GRAUMANN et al., 1996; WOUTERS et al., submitted A). Two-dimensional gel electrophoresis of cell-free extracts of *L. lactis* strains carrying deletions in their *csp* genes or specifically overexpressing one of the *csp* genes, revealed repression or induction of CIPs, respectively, indicating that CSPs regulate other proteins involved in cold adaptation. Strikingly, it was observed that the different lactococcal CSPs each regulate different CIPs which might provide a rational explanation for the existence of a CSP family (WOUTERS et al., submitted A; WOUTERS et al., submitted C).

Since CspA^E and CspB^B both possess highly conserved RNA-binding motifs, i.e. RNP-1 and RNP-2, they are also considered to be RNA-binding proteins (JONES and INOUE, 1994; SCHINDELIN et al., 1993) (Table 1). It has been shown that CspA^E and CspB^B bind to mRNA with a broad sequence specificity (GRAUMANN et al., 1997; JIANG et al., 1997). The RNP motifs are located on one sheet of CspA^E and contain several highly solvent exposed Phe residues (F18, F20, F31, F34; Table 1). The nucleic acid binding capacity of CspB^B was largely reduced upon mutation of Phe residues in the RNP motifs to Ala, indicating an essential role for these regions in DNA/RNA binding (SCHRÖDER et al., 1995). Mutation of Phe residues in the RNP motifs of both CspB^B and CspA^E not only resulted in a reduction of the nucleic acid binding capacity but also in a decreased protein stability (HILLIER et al., 1998; SCHINDLER et al., 1998). Since CSPs are able to bind RNA it is proposed that these proteins act as RNA chaperones, thereby minimising the in-

Fig. 1. Model of the mode of action of CSPs during the initiation process of translation. At normal 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 (B1). Easy-translatable mRNAs of cold-shock genes are translated because of the presence of downstream boxes [DB] by partially intact or by a minority of intact ribosomal structures (B2). 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). After GRAUMANN et al. (1997) and JONES et al. (1996). See text for details.

creased secondary folding of nascent mRNA at low temperature. By this action, CSPs facilitate the initiation of translation for which RNA should be in a linear form. The binding of CSPs to RNA is only moderately strong and it is assumed that the ribosome can detach the CSPs from the linear, nascent mRNA molecules (GRAUMANN et al., 1997; JIANG et al., 1997; Fig. 1).

Single deletions in the genes encoding CspA^E or CspB^B did not reveal a distinct phenotype in relation to growth at low temperature (BAE et al., 1997; WILLIMSKY et al., 1992). The disruption of *cspE*^E, a gene that is transiently induced during the growth lag after dilution of stationary phase cells, resulted in a longer lag period after dilution. However, the exact role of CspE^E in this phenomenon is not yet elucidated (BAE et al., 1999). However, 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. In contrary, for *L. lactis* a triple deletion of *cspABE* did not affect growth characteristics (WOUTERS et al., submitted A), which was explained by the increased expression of remaining *csp* genes. It appeared that loss of one or two of the CSPs led to an increase in the synthesis of the remaining CSP(s) at high as well as at low temperatures for *E. coli*, *B. subtilis* as well as *L. lactis* (BAE et al., 1997; GRAUMANN et al., 1997; WOUTERS et al., submitted A).

For CspB^B a function as an anti-freeze protein has been suggested because a lower survival was observed after freezing of *B. subtilis* cells in which the *cspB* gene was disrupted (WILLIMSKY et al., 1992). Similarly, for *L. lactis* a freeze-sensitive phenotype was observed upon deletion of *cspA*, *cspB* and *cspE*. Maximal freeze-protection upon exposure to low temperature could still be obtained for the triple mutant strain but the freeze-protective response was significantly delayed (WOUTERS et al., submitted A). Strikingly, also upon overproduction of CspB, CspD or CspE increased survival to freezing of *L. lactis* is obtained (WOUTERS et al., 1999A; WOUTERS et al., submitted C). It is speculated that the CSPs can stabilise RNA and DNA during the freezing process but the exact role of CSPs in these processes remains to be proven.

Interestingly, 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 *cspA* primers, PCR products were only obtained for strains belonging to the psychrotrophic *B. cereus* group (FRANCIS et al., 1998). Next, differences are also observed between the ribosomal RNA subunits (detrimental for protein synthesis at low temperature) of the psychrotrophic and non-psychrotrophic strains, in combination possibly explaining the differences in the minimum growth temperature. These specific sequence differences in the *cspA* gene, 16S rDNA, 23S rDNA and the 16S-23S rDNA spacer region between mesophilic and psychrotolerant *B. cereus* species, resulted in the definition of a new species, *Bacillus weihenstephanensis* sp. nov., compromising psychrotolerant *B. "cereus"* strains (LECHNER et al., 1998).

The psychrotolerant species can be of great importance in both food spoilage and food poisoning.

Regulatory elements involved in CSP synthesis

The regulation of the production of CSPs is controlled at several levels. The regulation of production of CspA^E has been characterized in most detail and it appeared that regulation of its expression after cold shock takes place at the level of transcription, at the level of translation as well as at the level of mRNA and protein stability, involving several characteristic genetic elements (TANABE et al., 1992; JIANG et al., 1993; BRANDI et al., 1996; GOLDENBERG et al., 1996; MITTA et al., 1997; Fig. 2). An AT-rich sequence (UP-element) upstream the -35 region of the *cspA*^E promoter enhances *cspA*^E transcription at low temperature (MITTA et al., 1997; GOLDENBERG et al., 1997). *cspA*^E mRNA is highly unstable at 37 °C but is stabilized upon cold shock. This increase in stability at low temperature is dependent on the unusually long, untranslated 5'-mRNA leader region (5'-UTR) of *cspA*^E, which is rich in secondary structure (JIANG et al., 1997). The mRNA stabilization of *cspA*^E upon cold shock appeared to be transient and is lost once cells have adapted to low temperature (GOLDENBERG et al., 1996). For *B. subtilis* it was shown that the CSPs have high affinity to bind to the first 25 bases of their 5'-UTRs, named cold-shock box (CS-box). In addition, it was found that CspA^E negatively regulates its gene expression through a similar CS-box on its 5'-UTR (JIANG et al., 1996; BAE et al., 1997). In this way, CSPs could down-regulate translation of their messengers by which they limit their cellular concentrations. This might be an important regulatory mechanism since a too high level of CspB^B has a growth inhibitory effect on *B. subtilis* as was shown using artificial overproduction (GRAUMANN and MARAHIEL, 1997; GRAUMANN and MARAHIEL, 1998). For *L. lactis* a highly different 5'-UTR and CS-box are observed for the non-cold induced *cspE* gene in comparison to these regions of the cold-induced *csp* genes. It was speculated that CspE destabilizes the mRNA of the cold-induced genes at high temperature by which no translation can occur. Indeed, upon disruption of CspE in *L. lactis* an increased synthesis of CspC and CspD was observed, indicating a central role for CspE in repression of the synthesis of these CSPs at normal growth temperature (WOUTERS et al., submitted A).

It has been suggested that upon cold shock, a period during which protein synthesis is blocked as a result of ribosomal malfunctioning, the mRNAs of CSPs are still translatable because of the presence of a downstream box (DB). This DB, located in the coding region of the protein, is complementary to a sequence proximal to the ribosome binding site-decoding region in 16S rRNA and was shown to be required for efficient translation under cold-shock conditions (MITTA et al., 1997). For *cspD* of *L. lactis* a highly complementary DB was found that may

Fig. 2. Schematic representation of the regulatory elements involved in the expression of CspA of *E. coli*. UP indicates the AT-rich UP-element, -35 and -10 indicate the respective promoter regions, *csp* ORF (blocked region) indicates the *csp* open reading frame, the hairpin indicates the terminator region, CS-box indicates the cold-shock box, RBS indicates the ribosome binding site, DB indicates the downstream box. See text for details.

be functional since *cspD* is the highest expressed *csp* gene upon cold shock (WOUTERS, J. A., unpublished data).

Strikingly, it has been observed that not all members of CSP families are cold induced (LEE et al., 1994; WOUTERS et al., 1998; YAMANAKA et al., 1998; WANG et al., 1999; Table 1) and it is assumed that the different CSPs might play a role in a variety of cellular processes. For example, CspD^E of *E. coli* and CspB^B and CspC^B of *B. subtilis* were shown to be induced during stationary phase conditions (YAMANAKA and INOUE, 1997; GRAUMANN and MARAHIEL, 1999). Moreover, CspC^E and CspE^E are two non-cold-induced members of the CspA family of *E. coli*, and these proteins have been implicated in chromosomal condensation and/or cell division (YAMANAKA et al., 1994; YAMANAKA et al., 1998). Recently, HANNA and LIU (1998) showed that CspE^E interacts with nascent RNA in transcription complexes, indicating a role for this protein in the transcription process. Next to its cold induction CspA^E is also induced upon exposure to ultra high-pressure treatments (WELCH et al., 1993; Table 1). BRANDI et al. (1999) reported that expression of *cspA^E* is high upon dilution of a stationary phase culture and that the *cspA^E* mRNA level decreases with increasing cell density. The extent of the cold-shock induction of *cspA^E* is inversely proportional to the pre-existing level of CspA^E. Furthermore, it is reported that the expression of *cspA^E* under non-stress conditions is regulated by the antagonistic effects of the DNA-binding proteins Fis and H-NS on transcription, variation of *cspA^E* mRNA stability and, possibly, autoregulation (BRANDI et al., 1999). Consequently, it is not necessarily to be expected that CSPs play a role solely in cold adaptation (BRANDI et al., 1999).

The role of ribosomes in cold adaptation

The structure and function of the ribosomes seem to play a central role in the 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. VANBOGELEN and NEIDHARDT (1990) stated that the ribosome might be the temperature sensor in bacteria. During a cold-shock treatment the translational capacity is strongly reduced by which the concentration of charged tRNA would be too high, blocking the A-site of the ribosome. This in turn would lower 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 (VANBOGELEN and NEIDHARDT, 1990; JONES et al., 1992A; GRAUMANN and MARAHIEL, 1996). In addition, it has been observed that upon incubation of cells of *E. coli* and *B. subtilis* with chloramphenicol a response similar to the cold-shock response develops, with the specific induction of certain CSPs and CIPs. This response was related to the inactivation of the ribosomes that are specifically blocked by chloramphenicol. It is believed that the mRNAs of cold-induced genes are still translatable during cold shock because of the presence of DB elements that ensure additional binding to the ribosome. The induction of cold-shock specific ribosomal factors, such as CsdA and RbfA, leads to restoration of the ribosomal structure and the ability to form intact translation initiation complexes for translation of non cold-shock mRNAs (JONES and INOUE, 1996; MITTA et al., 1997;

Fig. 1). An important role in the regulation of the translation at low temperature has been shown for CsdA, that is essential for the unwinding of stable secondary structures formed at low temperature (JONES et al., 1996).

Furthermore, it was observed that not only the ribosomal proteins but also the structure and the number of the *rrn* operons determine the ability of a bacterium to adapt to nutrient and temperature changes. For *E. coli* it is observed that the time to adapt to a temperature shift increased with decreasing numbers of intact operons (CONDON et al., 1995). For *B. cereus* differences in the structure of the *rrn* operons were observed between mesophilic and psychrotolerant species of this bacterium, and more specifically in the small (30S) ribosomal subunit involved in the early steps of translation initiation. Mutations are found from G and C in the mesophilic strains to A and T in the psychrotolerant strains which might result in a state of the ribosome capable of translation at low temperature, caused by the melting point reduction of the A-T bonds (PRÜB et al., 1999).

Perspectives

The increasing number of studies on the cold-shock response of a variety of organisms allows an overview and an opportunity for comparison of their responses. The synthesis of the 7-kDa CSPs upon cold shock has been elucidated in most detail but the exact functioning of this group of proteins remains to be elucidated. The regulation of synthesis, the role of elements involved in the regulation and other aspects regarding the functioning of CSPs have been elucidated, although, also a large number of questions have remained unanswered. The reasons for the existence of CSP families of which the members show highly similar primary and three-dimensional structures, is still unclear. It was shown that the different *csp* genes are induced during different growth conditions. Upon deletion of the genes encoding CSPs compensatory effects of the remaining counterparts are noted, which points to the presence of a tightly controlled internal network to control expression. For their action as RNA chaperones the need for a combined action and dimerization of CSPs has been reported (SCHINDLIN et al., 1993; MAYR et al., 1996; GRAUMANN et al., 1997). It is shown that different CSPs function as transcriptional regulators and in this way they might regulate different proteins, thereby indicating specific functions for each of the counterparts of the CSP family. Many aspects of the presence of CSP families and their role in bacterial evolution are currently being investigated mainly by use of single and multiple *csp* deleted strains. The increasing number of complete genome sequences, the development of the micro-array technology and the further development of proteomics analysis will undoubtedly significantly contribute to the unraveling of CSP functioning, and in particular to the exploration of their role in global regulatory phenomena.

Another point of major interest is the way of sensing low temperature signals. In recent years a limited amount

of data regarding this aspect has become available and indicated a central role for the ribosome, of which the macromolecular structure is affected at low temperature. A central role has also been assigned for the cytoplasmic membrane in which many changes occur upon low-temperature exposure. For the cold-induced *cspA* and *cspB* genes of *E. coli* the expression is differentially regulated at low temperature. It has been proposed that different biothermostats or thermoregulators play a role in the induction of these genes and in low temperature adaptation (ETCHEGARAY et al., 1996). Recently, WOUTERS et al. (submitted A) identified a cold-induced signal transduction protein in *L. lactis*, which might be involved in temperature sensing. Moreover, the observed cold-induction of alternative (stress) sigma factors also includes these proteins to the cold-responsive regulon. Transcription of σ^S of *E. coli* and σ^B of *L. monocytogenes* have been shown to be induced upon low-temperature exposure but σ^B of *B. subtilis* is not induced at low temperature as observed using *lacZ* promoter fusions and Western blotting (LOEWEN et al., 1998; BECKER et al., 1998).

The research on cold adaptation might yield direct applications with respect to food preservation methods and fermentation technology. Upon different cold-shock treatments prior to freezing clear differences are observed in survival capacity of several bacteria after freezing and a role for CSPs in this response has been shown (WILLIMSKY et al., 1992; WOUTERS et al., submitted A; WOUTERS et al., submitted C). This may result in high survival rates of bacteria in frozen food products or of starter strains during frozen storage. Furthermore, genetic elements involved in the increased synthesis of CSPs at low temperature might provide valuable tools for the expression of enzymes at low temperature. For example, the *cspA^E* promoter was used to express recombinant proteins at low temperature in *E. coli* allowing 3 to 5-fold induction using different promoter fragments. However, it should be noted that the *cspA^E* promoter became repressed after two hours of exposure to low temperature, which makes it not very suitable for use as a high-yield expression system yet (VASINA and BANEYX, 1996; VASINA and BANEYX, 1997). From the increased knowledge regarding CSPs useful information has been gained to understand low temperature adaptation and the deleterious effects of cold shock for certain bacteria. This may also result in the development of methods to control the growth of microorganisms that continue to challenge the shelf-life and safety of refrigerated foods.

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