Molecular characterization of a family of cold-shock proteins of *Lactococcus lactis*

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Contents

Stellingen

1. Het feit dat Phadtare *et al.* (1999) gegevens van *Lactococcus lactis* en *Lactobacillus plantarum* combineren tot de koude schok respons van *Lactobacillus lactis* geeft aan dat niet alle wetenschappers de verschillende melkzuurbacteriën juist onderscheiden.

   Phadtare, S., Alsina, J., and Inouye, M. 1999. Cold-shock response and cold-shock proteins. Curr. Opinion Microbiol. **2:**175-180.

2. De specifieke identificatie van bacteriën op basis van de karakterisatie van *csp*-genen (Francis en Stewart, 1997) gaat voorbij aan het feit dat niet alle bacteriën *csp* genen bezitten (o.a. Tomb *et al.*, 1997; Hazeleger *et al.*, 1998).

   Francis, K. P., and G. S. A. B. Stewart. 1997. Detection and speciation of bacteria through PCR using universal major cold-shock protein primer oligomers. J. Ind. Microbiol. Biotechnol. **19:**286-293.
   Hazeleger, W. C., J. A. Wouters, F. M. Rombouts, and T. Abee. 1998. Physiological activities of *Campylobacter jejuni* far below the minimal growth temperature. Appl. Environ. Microbiol. **64:**3917-3922.
   Tomb, J.-F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann *et al.* 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

3. In relatie tot de eigenschappen van koude-schok eiwitten kan beter worden gesproken over vorstbeschermende eiwitten (freeze-protective proteins) dan over anti-vries eiwitten (anti-freeze proteins).

4. Het is opmerkelijk dat voor de bepaling van de genoomsequentie van *Bacillus subtilis* gekozen is voor een stam die is blootgesteld aan röntgenstraling (Kunst *et al.*, 1997).

   Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo *et al.* 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390:**249-256.

5. De vooruitgang binnen de moleculaire biologie is in hoge mate afhankelijk van de ontwikkeling van de informatietechnologie.

6. Het focusseren van onderzoek binnen een onderzoeksgroep op een gering aantal onderwerpen of organismen verhoogt de efficiëntie en de impact van dit onderzoek meer dan evenredig.

7. Naast het onderzoek naar de voedingsstatus van ouderen, zou ook het onderzoek naar de reactie op hitte en koude van deze bevolkingsgroep meer aandacht verdienen, gezien het grote aantal sterfgevallen tijdens extreem hete en koude perioden van het jaar.

8. De "Schwalbe" is een essentieel, waardevol en mooi onderdeel van de voetbalsport.

9. Indien deeltijdwerk leidt tot een verhoogde efficiëntie, kan beter worden gesproken van vermenigvuldigtijdwerk.

10. De beste methode van studentenwerving voor de Wageningen Universiteit is de aanleg van een directe treinverbinding naar de kennisstad Wageningen.

Stellingen behorende bij het proefschrift
"Molecular characterization of a family of cold-shock proteins of *Lactococcus lactis*"

Jeroen A. Wouters
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Abstract

Lactic acid bacteria (LAB) are widely used as starter cultures in fermentation processes. The stress response of LAB during different industrial processes, and during low-temperature conditions in particular, requires a better understanding. For that reason a research project on the cold adaptation of *Lactococcus lactis* MG1363, a model LAB strain, was initiated. Research focused on the identification and characterization of a family of five *csp* genes, named *cspA, cspB, cspC, cspD* and *cspE*, encoding highly similar cold-shock proteins (CSPs; 65-85% identity). On the *L. lactis* MG1363 chromosome two tandem groups of *csp* genes (*cspA/cspB* and *cspC/cspD*) were identified, whereas *cspE* was found as a single gene. Transcription analysis showed that *cspE* is the only non-cold-induced *csp* gene, whereas the other *csp* genes are induced 10- to 40-fold at different time points after cold shock. The 7-kDa CSPs, corresponding to the *csp* genes of *L. lactis* MG1363, were the highest induced proteins upon cold shock to 10°C as was shown by two-dimensional gel electrophoresis. Using the nisin-inducible expression system CspB, CspD and CspE could be overproduced to high levels. For CspA and CspC a limited overproduction was obtained, that could be explained by low stability of *cspC* mRNA and by low stability of CspA. For *L. lactis* NZ9000ΔAB (deleted in *cspAB*) and NZ9000ΔABE (deleted in *cspABE*) no differences in growth at normal and at low temperature were observed, compared to that of the wild-type strain *L. lactis* NZ9000. The deletion of *csp* genes was compensated by increased expression of the remaining *csp* genes. These data indicate that the expression of *csp* genes in *L. lactis* is regulated by a tightly controlled transcription network.

When *L. lactis* cells were shocked to 10°C for 4 h the survival to freezing increased approximately 100-fold compared to mid-exponential phase cells grown at 30°C. *L. lactis* cells overproducing CspB, CspD or CspE at 30°C show a 2-10 fold increased survival after freezing compared to control cells. The adaptive response to freezing conditions by prior exposure to 10°C was significantly delayed in strain NZ9000ΔABE compared to strains NZ9000 and NZ9000ΔAB. In combination, these data indicate that 7-kDa CSPs of *L. lactis* enhance the survival capacity after freezing. CSPs either might have a direct protective effect during freezing, e.g. by RNA stabilization, and/or induce other factors involved in the freeze-adaptive response. A group of strongly cold-induced 7-kDa proteins was also identified for *Streptococcus thermophilus* and, indeed, enhanced production of these proteins coincided with increased survival to freezing of this bacterium.

Using two-dimensional gel electrophoresis, induction of several (non-7 kDa) cold-induced proteins (CIPs) of *L. lactis* was observed upon overproduction of CSPs. Furthermore, several CIPs were no longer cold induced in the *csp*-deleted strains, which indicates that CSPs might activate the expression of certain CIPs. A selection of CIPs of *L. lactis* was identified and appears to be implicated in a variety of cellular processes, e.g. transcriptional and translational control, sugar metabolism and signal sensing. Furthermore, it was shown that the maximal glycolytic activity measured at 30°C increases (approximately 2.5-fold) upon incubation at 10°C for 2 to 4 h, a process for which protein synthesis is required. Based on their cold induction and involvement in cold adaptation of glycolysis, it is proposed that the CcpA/HPr control circuit regulates a (unidentified) factor involved in the increased glycolytic activity.

The research described in this thesis contributes to the understanding of the response of lactic acid bacteria to low temperatures and might yield applications for dairy industry, especially with respect to fermentation performance and the survival of starter bacteria during freezing.

Outline

Since low temperatures are involved in various steps of production and storage of fermented dairy products, there is considerable interest in the activities of starter lactic acid bacteria in response to temperatures below their optimum temperature of growth. The aim of the work described in this thesis is the characterization of the response to low-temperature conditions of lactic acid bacteria, and of the model starter lactic acid bacterium *Lactococcus lactis* MG1363 in particular. Research mainly focussed on the expression of so-called cold-shock proteins (CSPs) and their functioning at low temperature in *L. lactis* MG1363.

In **Chapter 1** an introduction of the role of CSPs in the low-temperature adaptation of food-related bacteria is given.

In **Chapter 2** a family of five genes encoding CSPs of *L. lactis* MG1363 is identified and characterized. The transcriptional analysis of these genes at normal and at low growth temperatures is described. The structural characteristics of the genes and their encoded proteins are discussed.

Chapter 3 reports the increased survival to freezing of *L. lactis* MG1363 upon prior exposure to 10°C compared to control cells grown at 30°C. Using two-dimensional gel-electrophoresis the proteins encoded by the previously described *csp* genes could be identified. It was speculated that the CSPs, the highest induced proteins upon exposure to 10°C for 2 to 4 h, play a role in cryoprotection. Indeed, *L. lactis* cells overproducing CspD show a maximally 10-fold increased survival to freezing.

In **Chapter 4** a group of strongly cold-induced 7-kDa proteins is identified in the thermophilic lactic acid bacterium *Streptococcus thermophilus* using two-dimensional gel electrophoresis. Also for *S. thermophilus* the survival to freezing increases by prior exposure to low temperature, a response for which protein synthesis is required. By using a PCR method a *csp* gene was identified for *S. thermophilus*.

In **Chapter 5** the physiological and regulatory aspects of the overproduction of the CSPs of *L. lactis* are reported. It was shown that also the overproduction of CspB and CspE increased the survival after freezing of *L. lactis* cells. Furthermore, the overproduction of specific CSPs resulted in increased production of certain non-7 kDa cold-induced proteins (CIPs) of *L. lactis*, suggesting that CSPs function as activators of the production of these proteins.

In **Chapter 6** the regulatory and physiological effects of the disruption of *csp* genes of *L. lactis* MG1363 are described. Deletion of three counterparts of the *csp* gene family did not result in growth defects but did result in a delayed cryoprotective response of this strain. The deletion of *csp* genes resulted in compensatory expression of the remaining *csp* genes and a tightly controlled network of the production of CSPs is proposed. Moreover, in the *csp* deleted strains the expression of CIPs was reduced further indicating the role of CSPs in the production of CIPs.

Chapter 7 focuses on the effects of low temperature on the glycolytic activity of *L. lactis*. A 2.5-fold increase in acidification rate is observed upon pre-exposure to low temperature for several hours. It is shown that HPr and CcpA are involved in this reaction and a regulatory role for (the complex of) these proteins is discussed.

In **Chapter 8** the findings of this thesis in relation to the role and functioning of the CSPs of *L. lactis* in low-temperature adaptation are integrated and discussed.

Chapter 1

The role of cold-shock proteins in low-temperature adaptation of food-related bacteria

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ABSTRACT

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 of 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.

Submitted for publication

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 particular. 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; Graumann and Marahiel, 1998; Yamanaka *et al.*, 1998). A thorough understanding of the cold-adaptation process can be instrumented in optimizing 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 micro-organisms 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). Evidence is accumulating that there is a functional link between osmoprotection and cold adaptation, however, the exact mode of action of compatible solutes in cold adaptation remains to be elucidated.

By 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; Panoff *et al.*, 1994; Bayles *et al.*, 1996; Graumann *et al.*, 1996; Chapter 3), 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 Inouye, 1994; Jones and Inouye, 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 an 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 (Chapters 6 and 7).

COLD-SHOCK PROTEINS AND THEIR MODE OF ACTION

In recent years, research on cold adaptation mainly focussed the specific and high induction of a set of low-molecular weight proteins at low temperature: the so-called cold-shock proteins (CSPs). These proteins have a size of approximately 7 kDa and share a high degree of sequence similarity (>45%) in a variety of Gram-positive and Gram-negative bacteria (Table 1). 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 by use of a PCR method and used this method for the discrimination of bacterial species. However, *csp* genes are not present in all bacteria, e.g. in the complete genomes sequences of *Helicobacter pylori* (Tomb *et al.*, 1997), *Campylobacter jejuni* (Hazeleger *et al.*, 1998) and *Mycoplasma genitalium* (Graumann and Marahiel, 1996) no *csp* genes were found. For *L. lactis*, *Y. enterocolitica* as well as for *E. coli* a

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

CSP[1]	Amino acid sequence[2]	aa[3]	pI[4]	Cold[5]	Stress[6]	
	RNP-1 RNP-2[7]					
Gram-positive	+ ** * *+ +*+ * * + *[8]					
CspA Llac	MINGTVKWFNMDKGYGFIITEDMQ-DVFAYLLSIQGNGFKKYNEGQKVTFDVTMTARGRYASNIDKV[9]	66	9.2	+		
CspB Llac	MTKGTVKWFNPDKGFGFITSEDGQ-DVFAHFSQIQTSGFKTLDEGQKVTFDVEAGQRGPQAVNIEKA[9]	66	4.9	+		
CspC Llac	MNKGKINWFNADKGYGFIMADDMQ-DVFAYLLSIQGNDFKKYDEGQKVTFDIKMTSRGRYASNVHKR[9]	66	9.6	+		
CspD Llac	MANGTVKWFNATKGFGFITSEDGQ-DLFAHFSAIQSDGFKSLDEGQKVEFDVEEGQRGPQAVNITKA[9]	66	4.4	+		
CspE Llac	MAQGTVKWFNATKGFGFITTEEGN-DVFAHFSAIQTDGFKTLDEGQKVTFDVEEGPRGPQAVNIQK[9]	65	4.6	-		
CspC Lpla	MEHGTVKWFNADKGFGFITRENGS-DVFVHFSAIQEDGFKSLDEGQAVNFDVEESDRGPPAANVTKA[10]	66	4.4	+		
CspL Lpla	MKNGTVKWFNADKGFGFITQEDGT-DVFVHFSAIQTDGFKTLDEGQKVTYDEEQGDRGPQATNVQPQ[11]	66	4.3	+		
CspP Lpla	MKNGTVKWFNADKGYGFITGEDGN-DVFVHFSAIQTDGFKTLEEGQKVTFDEESSDRGPQAANVVPQ[11]	66	4.4	+		
CspB Bsub	MLEGKVKWFNSEKGFGFIEVEGQD-DVFVHFSAIQGEGFKTLEEGQAVSFEIVEGNRGPQAANVTKEA[12]	67	4.5	+	Stat	
CspC Bsub	MEQGTVKWFNAEKGFGFIERENGD-DVFVHFSAIQSDGFKSLDEGQKVSFDVEQGARGAQAANVQKA[12]	66	4.6	+	Stat	
CspD Bsub	MQNGKVKWFNNEKGFGFIEVEGGD-DVFVHFTAIEGDGYKSLEEGQEVSFEIVEGNRGPQASNVVKL[12]	66	4.4	+		
CspA Bcer	MTVTGQVKWFNNEKGFGFIEVPGEN-DVFVHFSAIETDGFKSLEEGQKVSFEIEDGNRGPQAKNVIKL[13]	67	4.7	+		
CspB Bcer	MNGKVKWFNNEKGFGFIEMEGSE-DVFVHFSAIQSDGYKALEEGQEVSFDITEGNRGPQAANVAKL[13]	65	4.5	+		
CspC Bcer	MQGRVKWFNAEKGFGFIERENGD-DVFVHFSAIQQDGYKSLEEGQQVEFDIVDGARGPQAANVVKL[13]	65	4.4	-		
CspD Bcer	MQTGKVKWFNGEKGFGFIEVEGGE-DVFVHFSAIQGDGFKTLEEGQEVSFEIVDGNRGPQAANVTKN[13]	66	4.5	-		
CspE Bcer	MTLTGKVKWFNSEKGFGFIEVADGS-DVFVHFSAITGDGFKSLDEGQEVSFEVED[13]	54	4.1	-		
CspB Lmon	MQTGTVKWFNSEKGFGFIEVEGGD-DIFVHFSAIEGEGFKTLDEGQSVEFEIVEGQRGPQAEKVTKL[14]	66	4.4	-		
CspL Lmon	MNMEQGTVKWFNAEKGFGFIERENGD-DVFVHFSAIQGDGFKSLDEGQAVTFDVEEGQRGPQAANVQKA[15]	68	4.3	+		
CspA Sthe	MANGTVKWFNATKGFGFITSEDGQ-DLFAHFSSIQSDGFKSLDEDQKVEFDVEVGQRGPQAVNITKA[16, 17]	66	4.5	+		
CspB Saur	MNNGTVKWFNAEKGFGFIEQENGG-DVFVHFSGIASDGYKTLEEGQKVTFEITEGQRGDQAVNVQTV[18]	66	4.4	?		
CspC Saur	MNNGTVKWFNAEKGFGFIERENGS-DVFVHFSAIAEDGYKSLEEGQKVEFDIVEGDRGEQAANVVKM[18]	66	4.3	?		
Gram-negative						
CspA Ecol	MSGKMTGIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDGYKSLDEGQKVSFTIESGAKGPAAGNVTSL[19]	70	5.5	+	UHP/GPD	
CspB Ecol	MSNKMTGLVKWFNADKGFGFISPVDGSKDVFVHFSAIQNDNYRTLEEGQKVTFSIESGAKGPAAANVIITD[19]	71	7.6	+		
CspC Ecol	MAKIKGQVKWFNESKGFGFITPADGSKDVFVHFSAIQGNGFKTLAEGQNVEFEIQDGQKGPAAVNVTAI[19]	69	7.6	+		
CspD Ecol	MEKGTVKWFNNAKGFGFICPEGGGEDIFAHYSTIQMDGYRTLKAGQSVQFDVHQGPKGNHASVIVPVEVEAAVA[19]	74	5.8	-	Stat	
CspE Ecol	MSKIKGNVKWFNESKGFGFITPEDGSKDVFVHFSAIQTNGFKTLAEGQRVEFEITNGAKGPSAANVIAL[19]	69	9.4	-		
CspF Ecol	MSRKMTGIVKTFDGKSGKGLITPSDGRIDVQLHVSALNLRDAEEITTGLRVEFCRINGLRGPSAANVYLS[19]	70	9.9	-		
CspG Ecol	MSNKMTGLVKWFNADKGFGFITPDDGSKDVFVHFTAIQSNEFRTLNENQKVEFSIEQGQRGPAAANVVTL[19]	70	5.6	+		
CspH Ecol	MSRKMTGIVKTFDRKSGKGFIIPSDGRKEVQVHISAFTPRDAEVLIPGLRVEFCRVNGLRGPTAANVYLS[19]	70	10.7	-		
CspI Ecol	MSNKMTGLVKWFNPEKGFGFITPKDGSKDVFVHFSAIQSNDFKTLTENQEVEFGIENGPRGPAAVHVVAL[19]	70	9.1	+		
CspA Styp	MSGKMTGIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDGYKSLDEGQKVSFTIESGAKGPAAANVTSL[20]	70	5.7	?		
CspB Styp	MTTKITGLVKWFNPEKGFGFITPKDGSKDVFVHFSAIQSNEFRTLNENQEVEFSVEQGPKGPSAVNVVAL[21]	70	5.8	+		
CspH Styp	MSRKMTGIVKTFDCKSGKGLITPSDGRKDVQVHISACRQHETEALIPGIRVEFCRINGLRGPTAANVYLS[22]	70	9.5	?		
CapA Pfra	MSQRQSGTVKWFNDEKGFGFITPQGGGDDLFVHFKAIESDGFKSLKEGQTVSFVAEKGQKGMQA[23]	64	7.1	+		
CapB Pfra	MSNRQTGTVKWFNDEKGFGFITPQSG-DDLFVHFKAIQSDGFKSLKEGQQVSFIATRGQKGMQAEEVQVI[23]	69	7.2	+		
TapA Pfra	MSNRQTGTVKWFNDEKGFGFITPESGP-DLFVHFRAIQGNGFKSLKEGQKVTFIAVQGQKGMQADKVQAEA[23]	70	9.3	+		
TapB Pfra	MSQRQSGTVKWFNDEKGFGFITPESGP-DLFVHFRAIQGNGFKSLKEGQKVTFIAVQGQKGMQA[23]	63	9.7	+		
CspA1 Yent	MSNKMTGLVKWFNADKGFGFITPADGSKDVFVHFSAIQSNDFKTLDEGQKVEFSIENGAKGPSAVNVVAL[24]	70	5.7	+		
CspA2 Yent	MSNKMTGLVKWFNADKGFGFITPADGSKDVFVHFSAIQSNDFKTLDEGQKVEFSIENGAKGPSAVNVIAL[24]	70	5.7	+		
	β1[25] β2 β3 β4 β5					

1. Llac = *Lactococcus lactis*, Lpla = *Lactobacillus plantarum*, Bsub = *Bacillus subtilis*, Bcer = *Bacillus cereus*, 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. + indicates 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).
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15. Francis, K. P., Rees, C. D., Stewart, G. S. A. B., unpublished data.
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17. Varcamonti, M., personal communication.
18. Francis, K. P., unpublished data.
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21. Craig *et al.*, 1998.
22. Francis and Stewart, 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. subilis*.

clustered organization of *csp* genes on the chromosome was observed (Yamanaka *et al.*, 1998; Neuhaus *et al.*, 1999; Chapter 2). For *L. lactis* and *Y. enterocolita* 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 determination 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 (Graumann and Marahiel, 1994; Newkirk *et al.*, 1994) thereby regulating

gene expression (Jones and Inouye, 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. Remarkably, 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, supporting the function of $CspB^B$ as a regulatory protein (Graumann and Marahiel, 1997). Upon deletion of *csp* genes of *B. subtilis* as well as *L. lactis* changes in protein patterns were observed (Graumann *et al.*, 1996; Chapter 6). 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 (Chapters 5 and 6).

Since $CspA^E$ and $CspB^B$ both posses highly conserved RNA-binding motifs, i.e. RNP-1 and RNP-2, they are also considered to be RNA-binding proteins (Jones and Inouye, 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 β-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 the 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 $CspA^E$ resulted not only in a reduction of the nucleid 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 increased 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 response is not yet elucidated (Bae *et al.*, 1999). However, multiple deletion analysis of the *csp*

gene 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 (Graumann *et al.*, 1997). In contrary, for *L. lactis* a triple deletion of *cspABE* did not affect growth characteristics (Chapter 6), which was explained by the increased expression of remaining *csp* genes. It appeared that loss of one or two of the CSPs leads 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; Chapter 6).

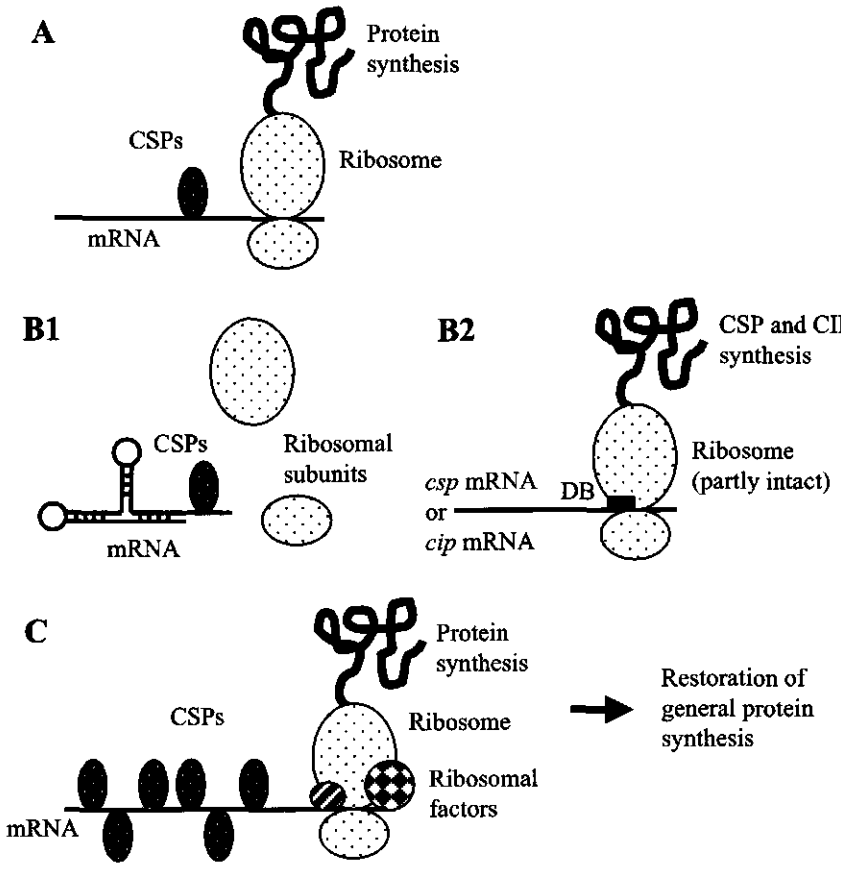

Fig. 1. Model of the mode of action of CSPs during the initiation process of translation. (A) At normal temperature mRNA molecules are hardly folded and translation takes place at a maximum rate. (B) Upon cold shock the ribosomal structure is disrupted and the secondary structure of mRNA molecules is drastically increased (1). 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 (2). (C) 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. After Graumann *et al.* (1997) and Jones *et al.* (1996). See text for details.

For $CspB^B$ a function as anti-freeze protein has been suggested because a lower survival after freezing was observed for *B. subtilis* cells in which the *cspB* gene was disrupted (Willimsky *et al.*, 1992). Similarily, 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 (Chapter 6). Strikingly, also upon overproduction of CspB, CspD or CspE increased survival to freezing of *L. lactis* is obtained (Chapters 5 and 6). It is speculated that the CSPs can stabilize RNA and DNA during the freezing process but the exact role of CSPs in these processes remains to be determined.

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). However, 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.

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 5'-untranslated 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 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).

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 (Chapter 6).

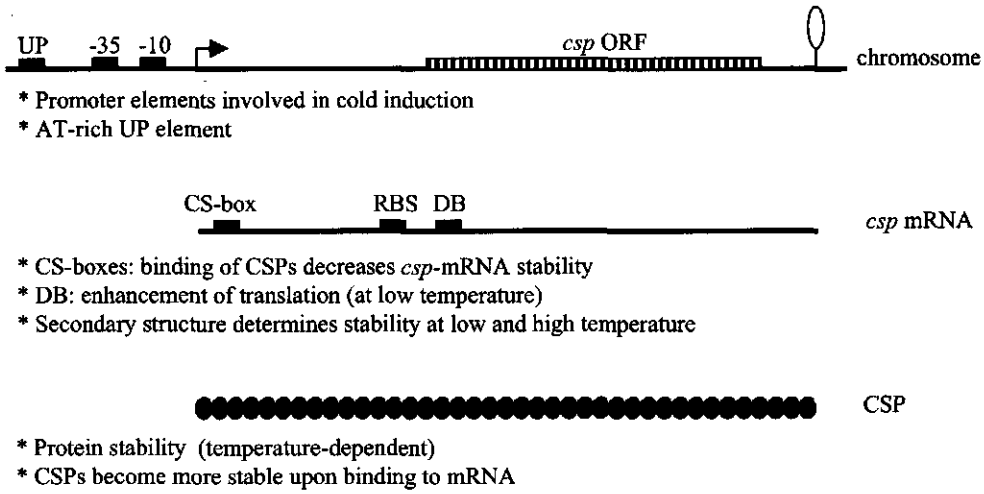

Fig. 2. Schematic representation of the regulatory elements involved in the expression of CspA of *E. coli*. UP indicates the AT-rich UP-element, -35 and -10 indicate the respective promoter regions, *csp* ORF (blocked region) indicates the *csp* open reading frame, the hairpin indicates the terminator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

Strikingly, it has been observed that not all members of CSP families are cold induced (Lee *et al.*, 1994; Yamanaka *et al.*, 1998; Wang *et al.*, 1999; Chapter 2; Table 1). It is assumed that the different CSPs might play a role in a variety of cellular processes. Furthermore, the expression of several *csp* genes increased upon exposure to other stress conditions than cold, e.g. $CspD^E$, $CspB^B$ and $CspC^B$ were shown to be induced during stationary phase conditions (Yamanaka and Inouye, 1997; Graumann and Marahiel, 1999). Moreover, $CspA^E$ is also induced upon exposure to ultra high-pressure treatments (Welch *et al.*, 1993; Table 1). Next, $CspC^E$ and $CspE^E$ are two non-cold-induced members of the $CspA^E$ 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 $CspE^E$ of *E. coli* interacts with nascent RNA in transcription complexes, indicating a role for this protein in the transcription process. Finally, 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. Consequently, it is not necessarily to be expected that CSPs play a role solely in cold adaptation (Brandi *et al.*, 1999).

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 be functional since *cspD* is the highest expressed *csp* gene upon cold shock (Chapter 8).

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 also 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 Inouye, 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).

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 (Schindelin *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 regarding the presence of CSP families and their role in bacterial evolution are currently being investigated 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. Recently, a cold-induced signal transduction protein was identified in *L. lactis*, which might be involved in temperature sensing (Chapter 6). 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; Chapters 5 and 6). 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 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 2 h 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.

Chapter 2

Clustered organization and transcriptional analysis of a family of five *csp* genes in *Lactococcus lactis* MG1363

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ABSTRACT

A family of genes encoding cold-shock proteins, named *cspA, cspB, cspC, cspD* and *cspE,* was cloned and sequenced from *Lactococcus lactis* MG1363. The genes *cspA* and *cspB* and the genes *cspC* and *cspD* are located in tandem repeats, an organization of *csp* genes that has never been encountered before. The five genes encode small (7.1 to 7.6 kDa) proteins with high mutual sequence identities (up to 85%) and high identities (about 45-65%) with the major cold-shock proteins from *Escherichia coli* (CspA) and *Bacillus subtilis* (CspB). Northern-blot analysis revealed single transcripts of about 300 nucleotides for each *csp* gene and showed that *cspA-, cspB-, cspC-,* and *cspD*-mRNA levels were strongly increased upon cold shock to 10°C (about 10, 40, 10 and 30 fold compared to 30°C, respectively), whereas the *cspE*-mRNA level was not increased. The expression of the cold-induced *csp* genes was highest in the 6-8 h lag phase after cold shock. A differential expression in time, in which *cspA* and *cspC* were maximally expressed at two hours and *cspB* and *cspD* at 4 h after cold shock, was observed. The -35 and -10 regions of the five promoters were identified and transcriptional start sites were mapped in each case by primer extension at different temperatures which confirmed that regulation takes place at the transcriptional level. Significant differences between the 5'-untranslated leader regions of the four cold-induced *csp* genes and the corresponding region of the non-cold-induced *cspE* gene were observed.

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INTRODUCTION

Lactococcus lactis plays an important role in many dairy fermentations. During processing and ripening of fermented dairy products these bacteria have to deal with different environmental stresses, such as low pH, high salt concentrations and temperature extremes (Rallu *et al.*, 1996). Several stress responses of *L. lactis* have been studied and stress-induced genes could be identified (Van Asseldonk *et al.*, 1993; Sanders *et al.*, 1995; Rallu *et al.*, 1996). However, low temperature stress gained less attention. Cold stress might be of importance for the survival of starter cultures after frozen storage and for fermentations taking place at low temperatures.

The response to cold shock has been extensively studied in *Escherichia coli* and was shown to result in the induction of a specific set of 14 proteins. These proteins play a role in various cellular processes and include, among others, NusA, RecA, H-NS, GyrA, polynucleotide phosphorylase and CspA (further referred to as $CspA^E$)(Jones *et al.*, 1987; Jones and Inouye, 1994; Jones and Inouye, 1996; Jones *et al.*, 1996). Maximal induction after cold shock was detected for $CspA^E$ which is transiently overexpressed (200-fold induction) and then represents 13% of the newly synthesized proteins (Jones *et al.*, 1987; Goldstein *et al.*, 1990). A highly similar protein, CspB (further referred to as $CspB^B$), has been described in *Bacillus subtilis* (Willimsky *et al.*, 1992).

$CspA^E$ (Goldstein *et al.*, 1990) and $CspB^B$ (Willimsky *et al.*, 1992) are small proteins characterized by a molecular mass of 7.4 kDa and a low isoelectric point (pI is 5.9 and 4.3, respectively). $CspA^E$ 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. The crystal structures of $CspA^E$ and $CspB^B$ have been resolved and both proteins are able to bind specifically to single-stranded DNA containing a Y-box motif (ATTGG) or its complementary sequence (CCAAT)(Graumann and Marahiel, 1994; Newkirk *et al.*, 1994; Schindelin *et al.*, 1994). Cold shock proteins (CSPs) contain sequence regions highly homologous to the cold-shock domain of eucaryotic DNA-binding proteins, designated Y-box factors (Landsman, 1992). $CspA^E$ and $CspB^B$ are also considered RNA-binding proteins because they both possess highly conserved RNA-binding motifs, i.e. RNP-1 (ribonucleoprotein) and a rudimentary RNP-2 motif (Schindelin *et al.*, 1993; Jones and Inouye, 1994) and it appears that $CspA^E$ can act as an RNA chaperone (Jiang *et al.*, 1997). For $CspB^B$ a function as an anti-freeze protein has been suggested because a lower survival has been observed after freezing of cells in which the *cspB* gene was disrupted (Willimsky *et al.*, 1992). The regulation of the synthesis of the major cold shock proteins is still unclear but it seems to take place both at the level of transcription (Lee *et al.*, 1994) and translation (Brandi *et al.*, 1996). Recently, it was shown that the abundant presence of $CspA^E$ after cold shock is due to increased stability of its mRNA at low temperature (Fang *et al.*, 1997).

In *E. coli, B. subtilis,* and *Bacillus cereus,* families of *csp* genes of respectively 9, 3 and 6

members have been found (Lee *et al.*, 1994; Graumann *et al.*, 1996; Mayr *et al.*, 1996; Nakashima *et al.*, 1996; Yamanaka and Inouye, 1997). In *E. coli* at least three of the nine identified *csp* genes are cold induced (Lee *et al.*, 1994; Nakashima *et al.*, 1996). The *csp* genes of *E. coli* appeared to be scattered on the chromosome (Lee *et al.*, 1994) and also for other bacteria only non-clustered *csp* genes have been reported (Graumann *et al.*, 1996; Mayr *et al.*, 1996). A recent study of Graumann *et al.* (1997) using a triple *csp* deletion mutant of *B. subtilis* revealed that CSPs are essential for cellular growth and for efficient protein synthesis at both optimal and low temperatures.

The cold-shock response of *L. lactis* IL1403 was studied by Panoff *et al.* (1994) and revealed that 12 proteins were overexpressed after cold shock. Recently, one cold-induced *csp* gene was identified in *L. lactis* (Chapot-Chartier *et al.*, 1997; Kim and Dunn, 1997) and two in another lactic acid bacterium, *Lactobacillus plantarum* (Mayo *et al.*, 1997).

In this study, a family of five genes encoding cold-shock proteins of *L. lactis* MG1363 is characterized. For the first time a clustered organization of *csp* genes is observed: two tandems of two *csp* genes. Transcriptional analysis of the *L. lactis csp* genes revealed cold induction for four of these genes and a differential expression of the respective genes during the adaptation phase after cold shock.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* MC1061 (Casadaban and Cohen, 1980) was used as a host strain in cloning experiments and was grown in Tryptone Yeast (TY) medium with aeration at 37°C (Sambrook *et al.*, 1989). Antibiotics were used in the following concentrations: ampicillin 50 μg ml^{-1}; chloramphenicol 10 μg ml^{-1}. *L. lactis* MG1363, a plasmid-free and prophage cured derivative of *L. lactis* NCDO712 (Gasson, 1983), was grown in M17 broth (Difco) supplemented with 0.5% (w/v) glucose at 30°C without aeration. Growth curves of *L. lactis* were obtained by measuring the optical density at 600 nm (OD_{600}) at various time points by diluting the sample four times in M17 broth.

DNA techniques and sequencing. Chromosomal DNA of *L. lactis* was isolated as described previously (Vos *et al.*, 1989). *L. lactis* cells were transformed by electroporation (Wells *et al.*, 1993). *E. coli* cells were transformed by the $CaCl_2$ procedure and plasmid isolations were carried out according to established procedures (Sambrook *et al.*, 1989). *E. coli* plasmid DNA was isolated at a large scale using Qiagen columns (Qiagen). Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were purchased from GIBCO/BRL Life Technologies, New England Biolabs, or Promega and used as recommended by the manufacturers. Cloning procedures, radiolabeling of DNA fragments, agarose gel electrophoresis and Southern-blot hybridizations were performed according to established procedures (Sambrook *et al.*, 1989). DNA fragments were isolated from agarose gels by

using the GlassMAX DNA Isolation Matrix System (BRL Life Technologies). PCR was carried out according to conditions described previously (Kuipers *et al.*, 1991). Nucleotide sequences of plasmid DNA were analyzed with an ALF automatic sequencer (Pharmacia Biotech) in combination with an AutoRead sequencing kit (Pharmacia Biotech) with fluorescein-labeled primers. Oligonucleotides, used as primers in sequencing reactions, primer extension experiments and PCR, were purchased from Pharmacia Biotech.

Cloning of *csp* genes. PCR with primers based on homologous regions of $CspA^{E}$ (Goldstein *et al.*, 1990) and $CspB^{B}$ (Willimsky *et al.*, 1992; Table 1) with chromosomal DNA of *L. lactis* MG1363 as a template resulted in the amplification of a fragment of about 200 bp (PCR1) with primers 1 and 2 (both containing an *Eco*RI-site). When primers 3 and 4 were used a fragment of about 550 bp (PCR2) was amplified. The fragments were cloned in pUC18 (pUC18PCR1) and pGEM-T (purchased from Promega; pGEM-TPCR2), respectively. The fragments were sequenced and appeared to contain parts of putative *csp* genes. By use of PCR1 as a probe in Southern hybridization, four hybridizing fragments (*Hin*dIII-chromosomal-DNA digest) were detected (Fig. 1A). The first hybridizing band was cloned as a 3.3 kb *Eco*RI/*Hpa*II fragment into the *Eco*RI- and *Acc*I-sites (after calf intestine alkaline phosphatase treatment) of pUC19, resulting in pUC19CspA/B (Table 1). The second hybridizing band was cloned as a *Hin*dIII/*Bgl*II fragment (2.1 kb) in the *Hin*dIII- and the *Bam*HI-sites of pUC19 (pUC19CspC/D; Table 1) and sequenced by primer walking. Attempts to clone the third hybridizing fragment either as a 3.5 kb *Hin*dIII fragment or as an 4.5 kb *Eco*RI/*Sac*I fragment in both a high copy (pUC19) and a low copy vector (pNZ84, a pACYC derivative; Van Alen-Boerrigter *et al.*, 1991) failed. The fourth hybridizing band was cloned as a *Hin*dIII/*Pst*I fragment (1.1 kb) in the *Hin*dIII- and *Pst*I-sites of pUC19 (pUC19CspE; Table 1). On this fragment a putative *csp* gene was located and to obtain its downstream region the following inverse PCR strategy was used. Chromosomal DNA was digested with *Hpa*II and self-ligated. PCR was performed with this template and with pAMILEX and pAMI4 (Table 1) as primers. A 950 bp fragment was obtained which was cloned in the blunt *Hinc*II and the *BamH*I-site (compatible with *Sau*3AI) of pUC18 after digestion with *Sau*3AI (resulting in pLEX; Table 1).

DNA- and deduced protein sequence analysis. Computer analysis of DNA sequences and the deduced amino acid sequences was performed with the programs PC/GENE (version 6.70; IntelliGenetics) and Clone (Version 4.0; Clone Manager). EMBL/Genbank and Swissprot/PIR databases were used to search for amino acid sequence similarities.

RNA techniques and primer extension experiments. RNA isolation, Northern blotting and subsequent hybridization with radiolabeled probes was performed as described previously (Kuipers *et al.*, 1993). For cold-shock experiments cultures were grown at 30°C to mid-exponential phase after which they were spun down by centrifugation and resuspended in 10°C-precooled medium. After

Table 1. Plasmids and oligonucleotides used in this study

Plasmids	
pUC19PCR1	pUC19 containing a PCR fragment (PCR1) of about 200 bp obtained with primers 1 and 2
pGEM-TPCR2	pGEM-T containing a PCR fragment (PCR2) of about 550 bp obtained with primers 3 and 4
pUC19CspA/B	pUC19 containing a 3.3 kb *Eco*RI/*Hpa*II fragment containing *cspA/cspB*
pUC19CspC/D	pUC19 containing a 2.1 kb *Hin*dIII/*Bgl*II fragment containing *cspC/cspD*
pUC19CspE	pUC19 containing a 1.1 kb *Hin*dIII/*Pst*I fragment containing *cspE* and its upstream region
pLEX	pUC18 containing a 0.95 kb PCR fragment which is cloned in the *Sau*3AI and *Hin*cII-sites containing *cspE* and its downstream region
Oligonucleotides (5'-3')	
Primer 1	CGGAATTCGGIA(A/T)IGTIAA(A/G)TGGTT(T/C)AA
Primer 2	CGGAATTCGTIAC(A/G)TTIGCIGC(C/T)TGIGGICC
Primer 3	GGNANNGTNAA(A/G)TGGTT(C/T)AA
Primer 4	(G/A/T)AT(A/G)AANCC(A/G)AANCC(C/T)TT
pAMILEX	GAACGCAATGAGTCCTG
pAMI4	TGACAGCGGGCCTAACC
PE*cspA*	GCCATAGCCTTGTCCATATTG
PE*cspB*	GCCAAATCCTTTATCTGGA
PE*cspC*	CTTGCATATCATCTGCCA
PE*cspD*	ACCAAATCCTTTAGTAGC
PE*cspE*	TGTGCGAAAACGTCGTTT

exposure to 10°C for various time periods (0, 0.5, 1, 2, 4 and 24 h) total RNA was isolated. The same oligonucleotides were used as probes in Northern blotting and as primers in primer extension experiments (PE*cspA* to PE*cspE*; Table 1). Quantification of the *csp* transcripts in Northern blotting was performed using the Dynamics Phosphor Imaging System. Cross-hybridization of the probes to the other *csp* genes was checked using Southern blotting, quantified with the same system. As a control for the RNA quantity the *usp45* gene, which is constitutively expressed (Van Asseldonk *et al.*, 1990), was used and correction factors were calculated by using the Phosphor Imaging System. Primer extension experiments of the *csp* genes were carried out as described previously (Kuipers *et al.*, 1993) with the same RNA samples as used for Northern blotting. The resulting cDNA was subjected to electrophoresis alongside nucleotide sequencing ladders generated with the same primers using the dideoxy chain-termination method (Sanger *et al.*, 1977) and [α-^{32}P]dATP as radiolabel.

Accession numbers: The EMBL accession numbers for the reported sequences in this paper are Y17215 (for *cspA* and *cspB*), Y17216 (for *cspC* and *cspD*) and Y17217 (for *cspE*).

RESULTS

Cloning of genes encoding putative cold-shock proteins. Using different primers based on the homologous sequences of CspA^{E} and CspB^{B} (Table 1) two PCR products of about 200 (PCR1) and 550 (PCR2) bp were amplified with *L. lactis* MG1363 chromosomal DNA as a template. After

cloning and sequencing it appeared that these PCR products contained parts of genes homologous to the major *csp* genes. In a Southern-blotting experiment, using PCR1 as a probe, four hybridizing fragments were detected in different digests of *L. lactis* chromosomal DNA (Fig. 1A). Two *csp* genes, named *cspA* and *cspB*, are located on an *Eco*RI/*Hpa*II fragment (cloned in pUC19 resulting in pUC19CspA/B). Another fragment (cloned in pUC19 resulting in pUC19CspC/D) also contained two *csp* genes (named *cspC* and *cspD*), organized in a tandem repeat. A single *csp* gene, named *cspE* (cloned in pUC19 resulting in pUC19CspE), is located on a *Hin*dIII/*Pst*I fragment and its downstream region was cloned by an inverse PCR strategy (pLEX). The organization of the different *csp* genes is shown in Fig. 1B. In Southern hybridization with PCR2 as a probe only two fragments, identical to fragments that hybridized with PCR1 as a probe, could be detected (data not shown). When the different *csp* genes were used as probes in Southern hybridization (different chromosomal-DNA digests) no extra hybridizing bands could be detected compared to the four bands obtained when using PCR1 as a probe. In an *Eco*RI digest all *csp* homologs were located on only two fragments indicating a clustered organization on the *L. lactis* MG1363 chromosome. No hybridization was observed using plasmid DNA (isolated from several *L. lactis* strains) and PCR1 as a probe, indicating that these *csp* genes are chromosomally encoded and that no homologs are located on plasmids (data not shown).

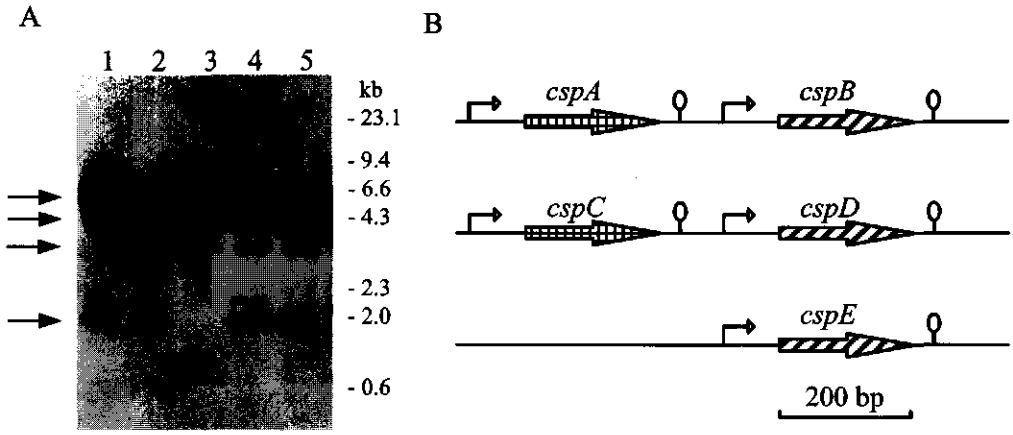

Fig. 1. Identification and organization of *csp* genes in *L. lactis* MG1363. (A) Southern hybridization of chromosomal DNA of *L. lactis* MG1363 digested with *Hin*dIII (lane 1), *Hin*dIII and *Eco*RI (lane 2), *Hin*dIII and *Pst*I (lane 3), *Hin*dIII and *Bgl*II (lane 4) and *Hin*dIII and *Bam*HI (lane 5) with PCR1 used as a probe. Marker sizes are indicated on the right and arrows indicate the hybridizing *Hin*dIII fragments. (B) Organization and nomenclature of the *csp* genes found in *L. lactis* MG1363. The large arrows indicate the ORFs, the smaller arrows indicate the transcription starts and the terminators are indicated by a hairpin structure (only the major terminators are indicated).

A remarkably high nucleotide sequence identity was found for the two tandem repeats: 79% over 800 nt containing both ORFs. In the tandem repeats the first ORFs (*cspA* and *cspC*) and the second ORFs (*cspB* and *cspD*) are highly similar (81% and 82% identity, respectively). Also the spacing between the two adjacent ORFs is similar for both tandem repeats (268 nt for *cspA* and *cspB*, 277 nt for *cspC* and *cspD*).

The five CSPs of *L. lactis* have a mutual identity of 52 to 85% at amino acid level. The identity to the major cold-shock proteins, $CspA^E$ and $CspB^B$, is about 45-65% and is lowest for CspA and CspC (Fig. 2; Table 2). The calculated molecular weights of the *L. lactis* CSPs range from 7.1 for CspE to 7.6 kDa for CspA and CspC (Table 2). CspA and CspC have an unusually high pI (approximately 9) compared to other CSPs (approximately 5).

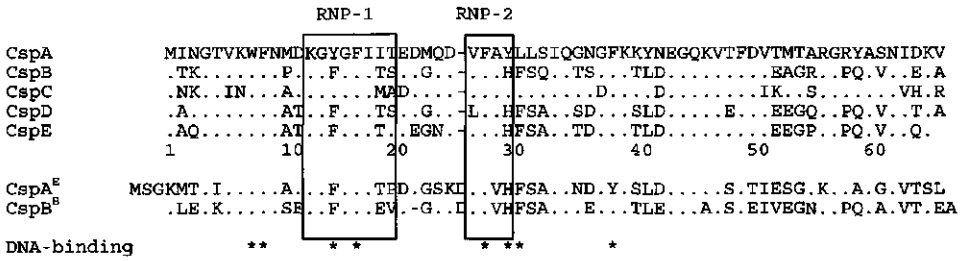

Fig. 2. Alignment of the deduced amino acid sequences of lactococcal CSPs and the amino acid sequences of $CspA^E$ and $CspB^B$. Identical amino acids are indicated with dots, gaps are indicated with dashes. Important regions for DNA binding are indicated with asterisks and RNA-binding motifs (RNP-1 and RNP-2) are boxed.

Table 2. Identity (%), size of ORF in amino acids (AA), molecular weight in kDa (MW) and isoelectric point (pI) of the *L. lactis* CSPs, $CspA^E$ and $CspB^B$.

	Identity (%) CspA	CspB	CspC	CspD	CspE	$CspA^E$	$CspB^B$	Size (AA)	MW (kDa)	pI
CspA	*	62	76	59	60	45	50	66	7.6	9.2
CspB		*	56	80	82	59	62	66	7.3	4.9
CspC			*	52	52	48	47	66	7.6	9.6
CspD				*	85	64	65	66	7.2	4.4
CspE					*	61	63	65	7.1	4.6
$CspA^E$						*	61	70	7.4	5.9
$CspB^B$							*	67	7.4	4.3

Cold induction of *csp* genes. Cells of *L. lactis* were cultured until mid-exponential phase at 30°C after which they were subjected to a cold shock by resuspending in precooled GM17 medium (10°C). Growth characteristics of the cold-shocked culture are shown in Fig. 3. A lag time of about six to eight h after cold shock was observed, after which exponential growth is resumed with a lower

growth rate (6-fold reduction) as compared to 30°C. The amount of mRNA of the *csp* genes was monitored by Northern blotting at various times after cold shock (Fig. 4B; only shown for *cspB*). Probes specific for each cold-shock gene were used (Table 1) and the cross-hybridization for all probes was calculated and appeared to be maximally 6% with primer PE*cspE* and *cspC* (data not shown). Transcripts of about 300 nt were detected for all *csp* genes whereas for *cspA* and *cspC* also larger transcripts (about 450 and 350 nt, respectively) were detected in small amounts (<5%; see below).

cspB and *cspD* are induced about 40- and 30-fold at 10°C, respectively, whereas c*spA* and *cspC* are induced by a factor of about 10 compared to the level at 30°C. At 30°C (t = 0) a high *cspE* mRNA level was detected compared to other *csp* genes, but *cspE* seems not to be induced at low temperature. Strikingly, the time at which maximal mRNA levels were found was different for the cold-induced *csp* genes. *cspA* and *cspC* reach maximal accumulation at 1-2 h after cold shock whereas for *cspB* and *cspD* maximal accumulation occurs at about 2-4 h after cold shock (Fig. 4B). The mRNA levels of *cspA, cspB, cspC* and *cspD* are decreased at 8 h after cold shock (data not shown), when exponential growth is resumed (Fig. 3). Other stress conditions like heat stress (10 min 42°C), salt stress (10 min 0.5 M NaCl), low pH stress (10 min pH 4.0, adjusted with lactic acid) or stationary phase conditions (2 h after reaching stationary phase) did not result in increased mRNA levels of any of the *csp* genes (data not shown).

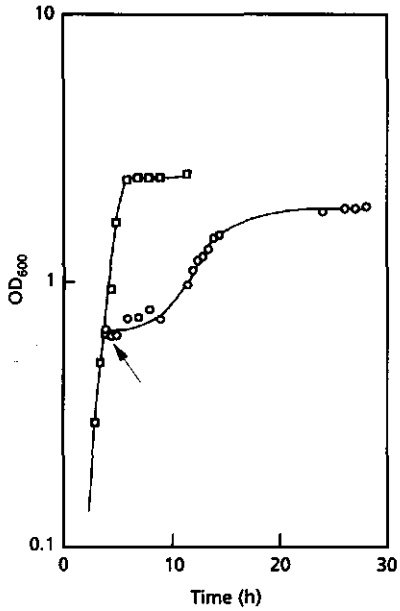

Fig. 3. Growth of *L. lactis* MG1363 at 30°C (squares) and after cold shock to 10°C (circles). The arrow indicates the time point of cold shock.

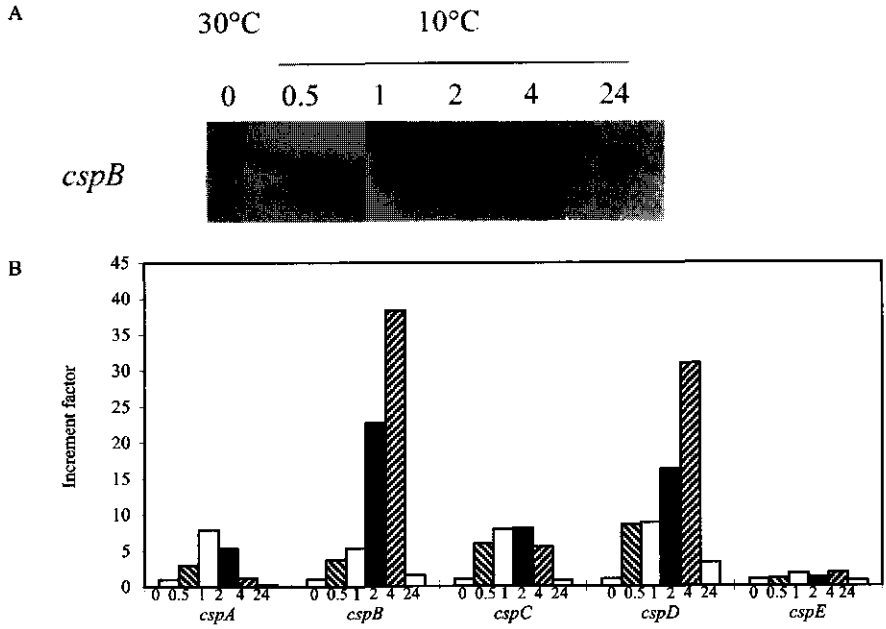

Fig. 4. Transcriptional analysis of *csp* genes of *L. lactis* MG1363. (A) Northern blot of RNA extracted at 0, 0.5, 1, 2, 4 and 24 h after cold shock hybridized with a probe specific for *cspB* (Table 1). The transcript size is about 300 nt. (B) Increment of mRNA-levels at different times after cold shock relative to t=0 (30°C). Correction for mRNA amounts was performed using *usp45* (Van Asseldonk *et al.*, 1990) as a standard.

Identification of promoter regions. Using the primer extension technique transcription start points of the *csp* genes were identified (Fig. 5; only shown for *cspC* and *cspD*) and are indicated in Fig. 6. For *cspD* a double transcription start was found, a major start at the indicated A-residue and a minor start at the T-residue three bases downstream. For each *csp* gene transcripts were detectable at 30°C and for *cspA*, *cspB*, *cspC* and *cspD* increased amounts of transcript were found at 10°C. The same transcription start points were identified at high and low temperature. Northern blotting showed that the mRNA size for the different *csp* genes is about 300 nt which corresponds well with the detected transcription starts and the putative terminators (ΔG= -6 kcal/mol, -10 kcal/mol, -8 kcal/mol, -8 kcal/mol and -8 kcal/mol for *cspA*, *cspB*, *cspC*, *cspD* and *cspE*, respectively). For *cspA* and *cspC* hairpin structures (ΔG= -10 kcal/mol and -14 kcal/mol, respectively) were found further downstream the ORFs, for which the size of the mRNA corresponds with larger transcripts which were detected in small amounts (only detected after prolonged exposure of the blots to X-ray films). When DNA fragments, containing parts of the *csp* genes and the region between the clustered *csp* genes, were used as probes only transcripts of about 300 nt were detected, indicating that also the *csp* genes located in tandem repeats are monocistronic.

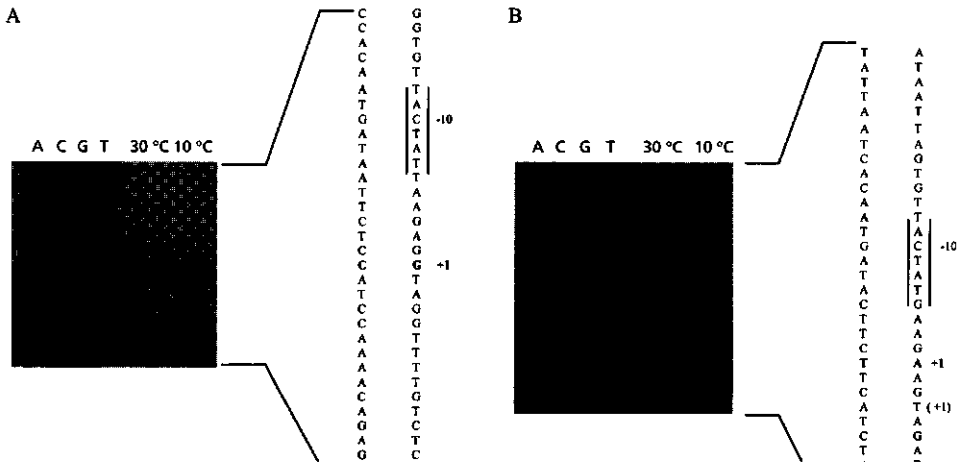

Fig. 5. Primer extension experiments for (A) *cspC* and (B) *cspD*. Sequence ladders are indicated on the left. RNA samples were taken at mid-exponential growth phase at 30°C and at 2 h after cold shock to 10°C. The nucleotide sequences, the -10 promoter regions and the transcription starts are indicated on the right.

The detected transcription start sites allowed to identify -35 and -10-promoter regions of the *csp* genes (Fig. 6). The promoter regions are 67 to 92% identical to the established consensus sequences of *L. lactis* (De Vos and Simons, 1994). The consensus 17-bp spacing between the -35 and -10 regions is found for all lactococcal *csp* promoters (Fig. 6). The non-cold-induced *cspE* gene has the lowest similarity (4 nt mismatches) with the consensus promoters, whereas the promoter regions of the cold-induced *csp* genes are less different from the consensus promoter regions (3, 3, 2 and 1 nt mismatches for *cspA*, *cspC*, *cspD* and *cspB*, respectively). In the promoter regions of *cspC* and *cspB* complementary sequences (CCAAT) of the Y-box motifs (ATTGG) are present (Fig. 6). Several of these motifs were also found further up- and downstream of the promoter regions of the other lactococcal *csp* genes.

The 5'-untranslated leader regions (5'-UTR) of the cold-induced *cspA*, *cspB*, *cspC* and *cspD* genes are highly identical (approximately 60%) whereas the identity with this region of the non-cold inducible *cspE* is much lower (about 30%; Fig. 6). Furthermore, the 5'-UTR of *cspE* (94 nt) is slightly longer than the ones of the other lactococcal *csp* genes (86, 84, 83 and 87 nt for *cspA*, *cspB*, *cspC* and *cspD*, respectively). The 5'-UTR of all lactococcal *csp* genes appear to be rich in secondary structure, encompassing the entire region as calculated by the method of Zuker and Stiegler (1981).

DISCUSSION

A family of five genes, named *cspA*, *cspB*, *cspC*, *cspD* and *cspE*, encoding putative cold-shock proteins was cloned from *L. lactis* MG1363 and it appeared that these *csp* genes were organized in clusters. *cspA* and *cspB* as well as *cspC* and *cspD* are located in a tandem repeat whereas *cspE* was

found as a single gene. A clustered organization of *csp* genes has never been observed before (Willimsky *et al.*, 1992; Lee *et al.*, 1994; Graumann *et al.*, 1996; Mayr *et al.*, 1996; Mayo *et al.*, 1997). *cspB* is identical to the *cspB* gene that was recently obtained from *L. lactis* AM2 using an inverse PCR strategy (Chapot-Chartier *et al.*, 1997).

The five *csp* genes can be grouped based on sequence analysis: a group consisting of *cspA* and *cspC* (the first genes in the tandem repeats) and a group consisting of *cspB, cspD* and *cspE*. Members within these groups code for highly similar proteins (about 80% identity) whereas the identity between these two groups is only about 55%. High similarity (45-65% identity) was also observed with the sequences of the major cold-shock proteins $CspA^E$ and $CspB^B$ and was lowest for $CspA^L$ and $CspC^L$. The residues important for single-stranded DNA binding of $CspA^E$ and $CspB^B$ (Newkirk *et al.*, 1994; Schröder *et al.*, 1995) are highly conserved in CspB, CspD and CspE, whereas in CspA and CspC some additional residues are different from the $CspA^E$ and $CspB^B$ DNA-binding residues. The RNA-binding RNP-1 (consensus KGFGF) and RNP-2 motifs (consensus VFVH)(Schindelin *et al.*, 1993; Jones and Inouye, 1994; Schröder *et al.*, 1995) are also found in the *L. lactis* CSPs although some differences are observed. Interestingly, the pI of CspA and CspC (9.2 and 9.6, respectively) are much higher than the pI of the other CSPs (approximately 4.5) due to the presence of more basic residues (8 and 11 for CspA and CspC, respectively, compared to 7 for CspB, CspD and CspE) and the presence of 4 tyrosine residues for CspA and CspC and no tyrosine residues in CspB, CspD and CspE. This

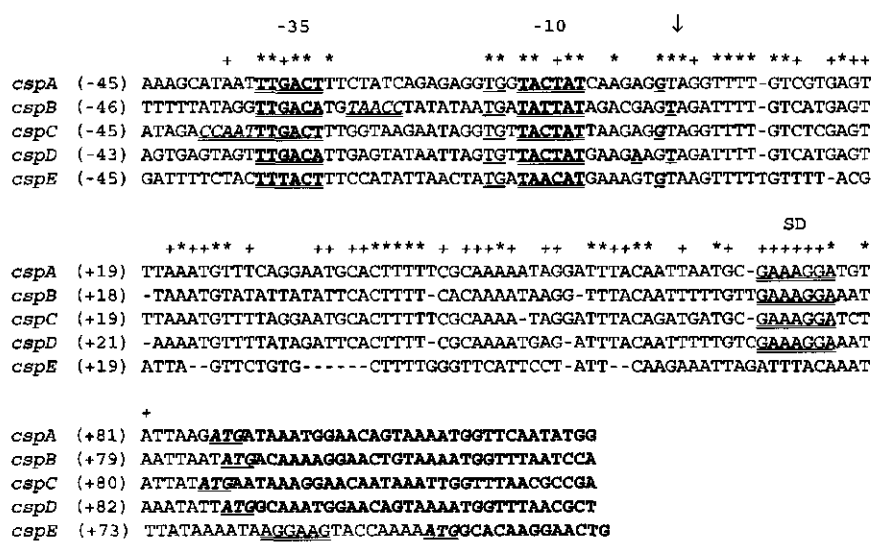

Fig. 6. Alignment of the nucleotide sequence of the *csp* promoters and the 5'-untranslated leader sequences of the lactococcal *csp* genes. The translated regions are indicated in bold. Shine-Dalgarno-sequences are double-underlined, transcription starts are underlined and in bold (and indicated with an arrow), -35 and -10-regions are double underlined and in bold, TG-dinucleotides (-16 region) are underlined, startcodons are underlined, in bold and in italic. Y-box motifs are double underlined and in italics. Identical nucleotides in all five *csp* genes are indicated with *; identical nucleotides in the four cold-induced *csp* genes only are indicated with +. Nucleotides are numbered from their +1 transcription starts.

high pI of CspA and CspC might result in an improved nucleic acid binding capacity since these proteins do not need to overcome charge repulsion when approaching nucleic acids (Schröder *et al.*, 1995). Furthermore, protein 3-D modelling based on the crystal structure of $CspA^E$ and $CspB^B$ (Schindelin *et al.*, 1993; Schindelin *et al.*, 1994) revealed a similar β-barrel structure formed by five β-strands for all five lactococcal CSPs (Chapter 8).

For all *csp* genes transcripts of about 300 bp were found and no combined transcripts were found for the *csp* genes located in tandem repeats. Furthermore, Northern blotting revealed increased mRNA levels for the *csp* genes at different times after cold shock indicating that regulation for these genes takes place at the transcriptional level. Maximal induction of mRNA levels was approximately 40 and 30 fold for *cspB* and *cspD,* respectively, whereas the mRNA level of *cspA* and *cspC* increased approximately 10 fold. *cspE* was not induced at 10°C. A differential expression in time after cold shock was observed for the respective *csp* genes. mRNA levels of *cspA* and *cspC* increase shortly after cold shock (in first two h) whereas *cspB* and *cspD* mRNA levels are highest at 4 h after cold shock. Possibly the more basic CSPs, CspA and CspC, are involved in the regulation of the expression of their further downstream located counterparts CspB and CspD. Since no mRNA induction was observed upon exposure to other stress conditions, such as heat, salt, low pH and stationary phase, it is concluded that these *csp* genes, with the exception of *cspE,* might play a specific role in low temperature adaptation. Recently, it was shown that the non-cold induced *cspD* gene of the *Escherichia coli* CspA family is in fact induced under stationary phase conditions (Yamanaka and Inouye, 1997).

Recent studies indicate that the 5'-UTR plays an important role in the stability of the *E. coli cspA* transcript (Fang *et al.,* 1997) and the regulation of $CspA^E$ expression after cold shock (Jiang *et al.*, 1997). Although the 5'-UTRs of the lactococcal *csp* genes are not as exceptionally long (83 to 94 nt) as this region of the *E. coli cspA* (159 nt; Goldstein *et al.*, 1990), they might play a similar role. Most intriguing in this respect, is the finding that the 5'-UTRs of the four cold-induced lactococcal *csp* genes are highly similar but that clear differences are observed in this sequence of the non-cold-induced *cspE* gene, indeed suggesting a regulatory function of this leader.

Future research will focuss on the impact of the differential expression, the clustered organization and on the regulation of the newly described *csp* genes in *L. lactis*. The physiological role of the *L. lactis* CSPs will be studied using single and multiple overexpression constructs and using strains with disrupted *csp* genes.

ACKNOWLEDGEMENTS

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Chapter 3

Analysis of the role of 7 kDa cold-shock proteins of *Lactococcus lactis* MG1363 in cryoprotection.

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ABSTRACT

Low-temperature adaptation and cryoprotection were studied in the lactic acid bacterium *Lactococcus lactis* MG1363. An approximately 100-fold increased survival after freezing was observed when cells were shocked to 10°C for 4 h compared to mid-exponential phase cells grown at 30°C, indicating an active protection against freezing. Using two-dimensional gel electrophoresis we were able to identify a group of 7 kDa cold-induced proteins (CSPs) that corresponds to a previously described family of *csp* genes of *L. lactis* MG1363 (Chapter 2). The 7-kDa CSPs appeared to be the highest induced proteins upon cold shock to 10°C. Northern blotting and two-dimensional gel electrophoresis showed that the *csp* genes were maximally expressed at 10°C, while induction was lower at 20 and 4°C. However, pre-incubation at 20 and 4°C, as well as stationary phase conditions, also induced cryoprotection (approximately 30-, 130- and 20-fold compared to 30°C mid-exponential phase, respectively). For all treatments leading to an increased freeze survival (low temperature exposure to 4, 10 and 20°C and stationary phase conditions) increased levels of three proteins (26, 43 and 45 kDa) were observed for which a role in cryoprotection might be suggested. Increased freeze survival coincides with increased CSP expression, except for stationary phase conditions. However, the level of observed freeze protection does not directly correlate to the *csp* gene expression levels. In addition, for the first time specific overproduction of a CSP in relation to freeze survival was studied. This revealed that *L. lactis* cells overproducing CspD at 30°C show a 2-10 fold increased survival after freezing compared to control cells. This indicates that the 7 kDa cold-shock protein CspD may enhance the survival capacity after freezing but that other factors supply additional cryoprotection.

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INTRODUCTION

Lactic acid bacteria (LAB) play an important role in the food industry, because of their wide application as starter cultures in many fermentation processes. The stress responses of LAB during different industrial processes require a better understanding (Rallu *et al.*, 1996). Many LAB fermentations are started with the addition of frozen cultures and therefore there is a great interest to increase the freeze-survival capacity of starter strains. Freezing is a complex stress condition since cells may not only be damaged by ice crystal formation, but also by high osmolarity due to high concentrations of internal solutes during the freezing process. Membrane integrity and macromolecule denaturation have been mentioned as factors that determine the survival after freezing (El-kest and Marth, 1992; Franks, 1995; Thammavongs *et al.*, 1996).

A remarkable phenomenon is the ability of bacteria to adapt to temperatures that are far below their optimum growth temperature. It has been well established that after a rapid decrease in the temperature of the culture medium (cold shock) a set of proteins is preferentially expressed (reviews by Jones and Inouye, 1994; Graumann and Marahiel, 1996; Yamanaka *et al.*, 1998). It has been found in *Escherichia coli* that about 15 proteins are overproduced after a cold shock from 37 to 10°C. Among these proteins, CspA (cold-shock protein A, 7 kDa) shows the highest induction (Goldstein *et al.*, 1990). For *Bacillus subtilis* a homologous cold-shock protein, termed CspB, has been identified (Willimsky *et al.*, 1992). A family of nine CspA homologues is present in *E. coli* of which only CspA, CspB and CspG are cold induced (Lee *et al.*, 1994; Nakashima *et al.*, 1996; Yamanaka *et al.*, 1998). In addition, a family of three cold-induced cold-shock proteins (CSPs) has been observed in *B. subtilis* (Graumann *et al.*, 1996). Recently, a family of five *csp* genes, named *cspA, cspB, cspC, cspD* and *cspE*, encoding highly similar CSPs (65-85% identity), has been described in the lactic acid bacterium *Lactococcus lactis* MG1363 (Chapter 2). On the *L. lactis* MG1363 chromosome two tandem groups of *csp* genes (*cspA/cspB* and *cspC/cspD*) were identified, whereas *cspE* was found as a single gene. Transcription analysis showed that *cspE* is the only non-cold-induced *csp* gene, whereas the other *csp* genes are induced 10- to 40-fold at different times after cold shock (Chapter 2).

For CSPs several functions have been shown at low as well as at elevated growth temperatures. CSPs may function as RNA chaperones as they are able to bind to mRNA molecules and minimize secondary structure by which they facilitate the translation process (Graumann *et al.*, 1997; Jiang *et al.*, 1997). CspA of *E. coli* also appeared to function as a transcriptional activator as is described for two genes of which the products, H-NS and GyrA, are both involved in DNA-supercoiling (LaTeana *et al.*, 1991; Jones *et al.*, 1992B). Interestingly, it was noted that many organisms develop an increased ability to survive freezing after cold-shock treatment (Goldstein *et al.*, 1990; Willimsky *et al.*, 1992; Panoff *et al.*, 1995; Thammavongs *et al.*, 1996; Kim and Dunn, 1997). CspB of *B. subtilis* appeared to be implicated in increased tolerance to freezing, as was shown using a strain in which the gene encoding this protein was disrupted (Willimsky *et al.*, 1992). However, in previous studies a direct

relation of the actual CSP levels with functionality of these proteins was never established.

In this study we provide evidence for an active adaptation response of *L. lactis* MG1363 to a repetitive freezing challenge after exposure to low temperature. Protein synthesis is required for this adaptation and major differences in the pattern of synthesised proteins are found in the class of 7-kDa CSPs. The specific effect of overproduction of CspD on freeze survival is studied and the correlation between the expression of CSPs and the survival after freezing of *L. lactis* MG1363 will be discussed.

MATERIALS AND METHODS

Growth, cold-shock treatment, and freeze-thaw challenge. *L. lactis* MG1363, a plasmid-free and prophage cured derivative of *L. lactis* NCDO712 (Gasson, 1983) was cultured at 30°C in M17 medium containing 0.5% glucose (GM17). For studying growth kinetics, 1% inoculated cultures were grown at different temperatures. Growth was monitored by measuring OD_{600}. For cold-shock treatments, 50 ml cultures were grown until mid-exponential phase (OD_{600} = 0.5) at 30°C, after which 25 ml of each culture was pelleted and resuspended in the same volume of pre-cooled medium (20, 10 and 4°C). The cultures were incubated at these temperatures for 50 h, during which the OD_{600} was measured. To study the freeze-thaw survival capacity, cells of *L. lactis* were frozen at mid-exponential phase and at 2 and 4 h after cold shock to 4, 10 and 20°C. 1 ml aliquots were spun down and resuspended in 1 ml fresh GM17 medium. Subsequently, these samples were frozen at –20°C for exactly 24 h and thawed during 4 min at 30°C. The number of c.f.u. was determined just before freezing and after each of four consecutive freeze-thaw cycles (24 h freeze periods, thawing for 4 min at 30°C) by spread plating decimal dilutions. After 2 days of incubation on GM17 plates at 30°C the numbers of c.f.u. were counted. The freezing experiments were performed in triplicate with samples from individual cultures.

Protein analysis using two-dimensional gel electrophoresis (2D-EF) and one-dimensional SDS-PAGE. Total cellular proteins were extracted from 10 ml cultures by homogenizing with a MSK cell homogenizer (B. Braun Biotech International, Germany) and zirconium beads (0.1 mm, Biospec Products, USA) 8 times for 1 min (cooled on ice between treatments). After homogenizing the zirconium beads were allowed to sediment by gravity, after which the supernatant, containing the cellular proteins, was analysed by 2D-EF or SDS-PAGE. The protein content of the extract was determined using the bicinchoninic acid method as provided by the supplier (Sigma Chemicals, USA) and equal amounts of protein were applied on the protein gels.

2D-EF was essentially performed as described by O'Farrell (1975) using a Pharmacia 2D-EF system (Pharmacia Biotech, Sweden). Prior to loading of the samples on the IEF gel, 20 µl of protein solution (40 µg protein) was treated with 20 µl lysis solution (9 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 4-7L [Pharmacia Biotech, Sweden], 2% Triton X-100, 8 mM phenylmethyl-

sulfonylfluoride) at 37°C for 5 min, after which 60 µl of sample solution (8 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 4-7L, 0.5% Triton X-100, few grains of bromophenolblue) was added. The total volume (100 µl) was loaded on the acidic end of the first dimensional IEF gel with linear iso-electric point (pI) ranges from 4 to 7 or from 3 to 10 (Immobiline Dry strips, Pharmacia Biotech, Sweden). For the second dimension, 15% homogeneous SDS-PAGE gels were used to obtain optimal separation at the low molecular weight region. Two molecular weight markers (Pharmacia Biotech, Sweden) were used with band sizes of 67, 43, 30, 22.1 and 14.4 kDa and of 16.9, 14.4, 10.7, 8.2, 6.2 and 2.5 kDa, respectively. The gels were silver stained according to Blum *et al.* (1987) and were analysed using GEMINI software (Applied Imaging, England). The intensity of each spot was calculated as a percentage of the total intensity of the spots visualised on a gel and subsequently, induction factors were calculated. One-dimensional tricine-SDS-PAGE for the separation of low molecular weight proteins was performed as described by Schägger and Von Jagow (1987).

Freeze survival of *L. lactis* exposed to different stress conditions. *L. lactis* was grown until mid-exponential phase at 30°C after which the cells were centrifuged and resuspended in GM17 medium in which they were exposed to the relevant stress conditions. Cells were exposed to either heat stress (10 min 42°C), salt stress (10 min 0.5 M NaCl), pH stress (10 min at pH of 4, adjusted with lactic acid) or stationary phase stress [4 h after reaching mid-exponential phase (OD_{600} 0.5) which means that cells were at maximum OD_{600} (approximately 2.4) for 2 h] and subsequently analysed for freeze stability. After stress exposure a 1 ml-sample was taken, spun down and resuspended in fresh GM17 medium. The number of c.f.u. was determined before freezing and after each of four repetitive freeze-thaw cycles.

Northern-blot analysis. For analysis of the *csp*-mRNA levels in *L. lactis* after exposure to several stress conditions, total RNA was extracted as described by Kuipers *et al.* (1993). For Northern blot analysis, 20 µg RNA was glyoxylated and fractionated using a 1% agarose gel as described by Van Rooijen and De Vos (1990). Equal amounts of RNA were loaded on the gel and RNA was stained using ethidium bromide. A 0.24-9.5 kb RNA ladder (Gibco/BRL Life Technologies, The Netherlands) was used to determine the transcript size. RNA was blotted on a GeneScreen Plus Membrane (Dupont, Nen Research Products, USA). The blot was hybridised using a mix of probes each specific for one of the five *csp* genes of *L. lactis* (Chapter 2) that were labelled simultaneously. The blots were exposed to X-ray films (X-Omat MS, Kodak, USA).

Overproduction of CspD using a nisin controlled expression system. CspD was overexpressed using the nisin controlled expression system as described by Kuipers *et al.* (1995) and De Ruyter *et al.* (1996B). Using the oligonucleotides OECspDFor (5'- GCTGCCATGGCAAATGG-AACAGTAAAATGG-3') and OECspDRev (5'-CACGAAGCTTTTTCCTCTTGCTGGCTAAGT-3'), containing a *Nco*I- and a *Hin*dIII-site (underlined), respectively, the *cspD* gene could be amplified using PCR. The obtained fragment was digested with *Nco*I and *Hin*dIII and subsequently cloned in

vector pNZ8032 (De Ruyter *et al.*, 1996A), thereby replacing the *gusA* gene that was originally present in pNZ8032. In this way, a translational fusion was obtained at the ATG start codon of the *nisA* promoter and the *cspD* gene. The generated plasmid (pNZOECspD) was transformed to *L. lactis* NZ3900, yielding *L. lactis* NZ3900 harbouring pNZOECspD. *L. lactis* NZ3900 is a derivative of *L. lactis* MG1363 in which the two-component regulatory *nisR/nisK* pathway is integrated on the chromosome. Upon addition of different concentrations of the inducer M17W-nisin (0, 0.1, 0.2 and 0.5 ng/ml; Kuipers *et al.*, 1995) CspD could be overproduced in high quantities as was analysed using SDS-PAGE. To study the freeze survival of *L. lactis* NZ3900 harbouring OECspD, this strain was cultured at 30°C to OD_{600} of 0.3 after which different concentrations of nisin were added. After 90 min of incubation (final OD_{600} approximately 1.5) protein samples and samples to analyse the freeze survival (performed in triplicate) were taken. As a control, a freeze-challenge was performed with *L. lactis* NZ3900 containing pNZ8020, a plasmid carrying the *nisA* promoter without any fused gene (De Ruyter *et al.*, 1996A).

RESULTS

Growth characteristics of *L. lactis* at low temperature. The minimal temperature of growth for *L. lactis* MG1363 in liquid media is just below 4°C. The maximal growth rate significantly decreases at low temperatures: μ = 0.20, 0.18, 0.11, 0.03 and 0.02 h^{-1} at 20, 15, 10, 7 and 4°C, respectively. In liquid GM17 medium a lag-time of 9 days is observed at 4°C. When growth of *L. lactis* is analysed at GM17 plates no growth is observed after 14 days of incubation at 4°C, whereas slow growth is observed at 7°C. When at mid-exponential phase the temperature is rapidly downshifted, growth of *L. lactis* is blocked. An adaptation time of about 6 h is observed after a shock from 30 to 10°C (Fig. 1). When the temperature is shifted to 20°C hardly any growth delay is observed and when the growth temperature is 4°C no adaptation is observed within the first 45 h after cold shock.

Increased survival after freezing following a low-temperature treatment. The survival after freezing was determined for cells grown to mid-exponential phase at 30°C and for cells that were exposed to 10°C for 2 and 4 h. The survival after freezing (four repeated freeze-thaw cycles) increased by a factor of about 10 and 100 when the cells were shocked to 10°C for 2 and 4 h prior to freezing, respectively (Fig. 2). When chloramphenicol (100 $\mu g\ ml^{-1}$) was added during the cold-shock treatment no increased survival after freezing was observed, indicating that protein synthesis is required for the adaptation process (Fig. 2).

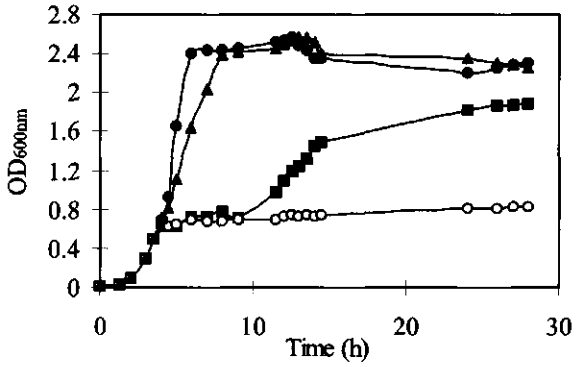

Fig. 1. Growth of *L. lactis* at 30°C (closed circles) and following cold shock to 20°C (triangles), 10°C (squares) or 4°C (open circles).

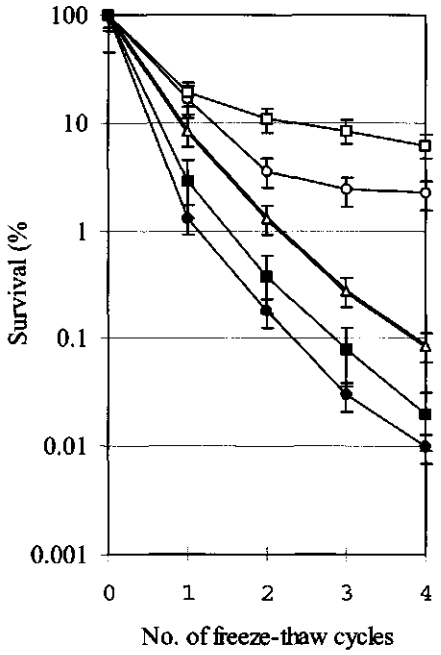

Fig. 2. The survival after freezing of *L. lactis* after exposure to different cold-shock conditions. The survival (% of surviving cells relative to the number of cells prior to freezing) of *L. lactis* cells cultured without (triangles) or with a cold-shock treatment from 30°C to 10°C for 2 (open circles) or 4 h (open squares) and in the presence of chloramphenicol for 2 (open circles) or 4 (open squares) h is depicted. The freezing experiments were performed in triplicate and the averages of the three experiments are shown. The error bars represent the standard deviation. The number of cells before freezing is set at 100% (OD_{600} = 0.5, about 4 x 10^8 cells).

Identification and expression analysis of CSPs. In order to investigate the presence of 7-kDa CSPs in *L. lactis* MG1363 total cellular proteins were extracted from a mid-exponential culture of *L. lactis* at 30°C and from a culture at 4 h after cold shock to 10°C. Following separation by 2D-EF approximately 150 spots could be identified. Careful analysis of the 2D-EF gels revealed that *L. lactis* contains a group of six cold-induced proteins with molecular masses of approximately 7 kDa. Major induction was observed for three proteins with molecular masses of approximately 7 kDa and pIs of approximately 4.5-5.0 (spot B = CspB, spot D = CspD and spot F; Fig. 3). In addition, the spots

representing CspB, CspD and CspE could be assigned based on the calculated pI and in comparison with specific overexpressed CSPs of *L. lactis* (unpublished results). The new protein F, which has a pI of approximately 4.7 could represent a previously not identified member of the lactococcal CSP family (Chapter 2). Finally, CspA and CspC of *L. lactis,* which have an pI of 9.2 and 9.6, respectively (Chapter 2), could be visualised using a different range for IEF (pI 3 to 10). CspA and CspC also appeared to be cold induced (Fig. 3). CspE appears to be the major CSP present at 30°C (E; Fig. 3A) and it is slightly induced after cold shock (approximately 2-fold; Fig. 3; Table 1). In agreement with the mRNA levels of the *csp* genes (Chapter 2) major induction was observed for CspB and CspD at the protein level (B and D; Fig. 3B; Table 1). After cold shock CspB, CspD, CspE and spot F make up nearly 10% of all proteins present (calculated on basis of the silver stained gels; Table 1). For CspD a 50-fold induction was observed, whereas CspB and spot F should be subject to an even higher induction level because these spots were undetectable at 30°C (Table 1). 2D-EF analysis of protein extracts of cultures exposed to a cold shock in the presence of chloramphenicol revealed no induction of 7-kDa CSPs (data not shown).

Further analysis of the 2D-EF gels revealed both increased (approximately 22 spots, Fig. 3, Table 2) and decreased protein levels (approximately 30 spots; minimally 2-fold induction or reduction). For three proteins with molecular weights of approximately 33, 43 and 45 kDa at least a 10-fold induction was detected at 4 h after cold shock to 10°C (spot 10, 12 and 15; Fig. 3; Table 2).

Table 1. The levels of the CSPs of *L. lactis* at 30°C and at 10°C. The relative amounts (% of total amount of protein measured in a silver stained 2D-EF gel) of the 7-kDa CSPs of *L. lactis* at 30°C at mid-exponential phase and at 4 h after cold shock to 10°C are given (lettering according to CSP family of *L. lactis* and Fig. 3A and B)

Spot	30 °C	10 °C
A	n.d.*	n.d.
B	0 %	1.49 %
C	n.d.	n.d.
D	0.07 %	3.50 %
E	1.55 %	3.09 %
F	0 %	1.65 %

* n.d. = not determined

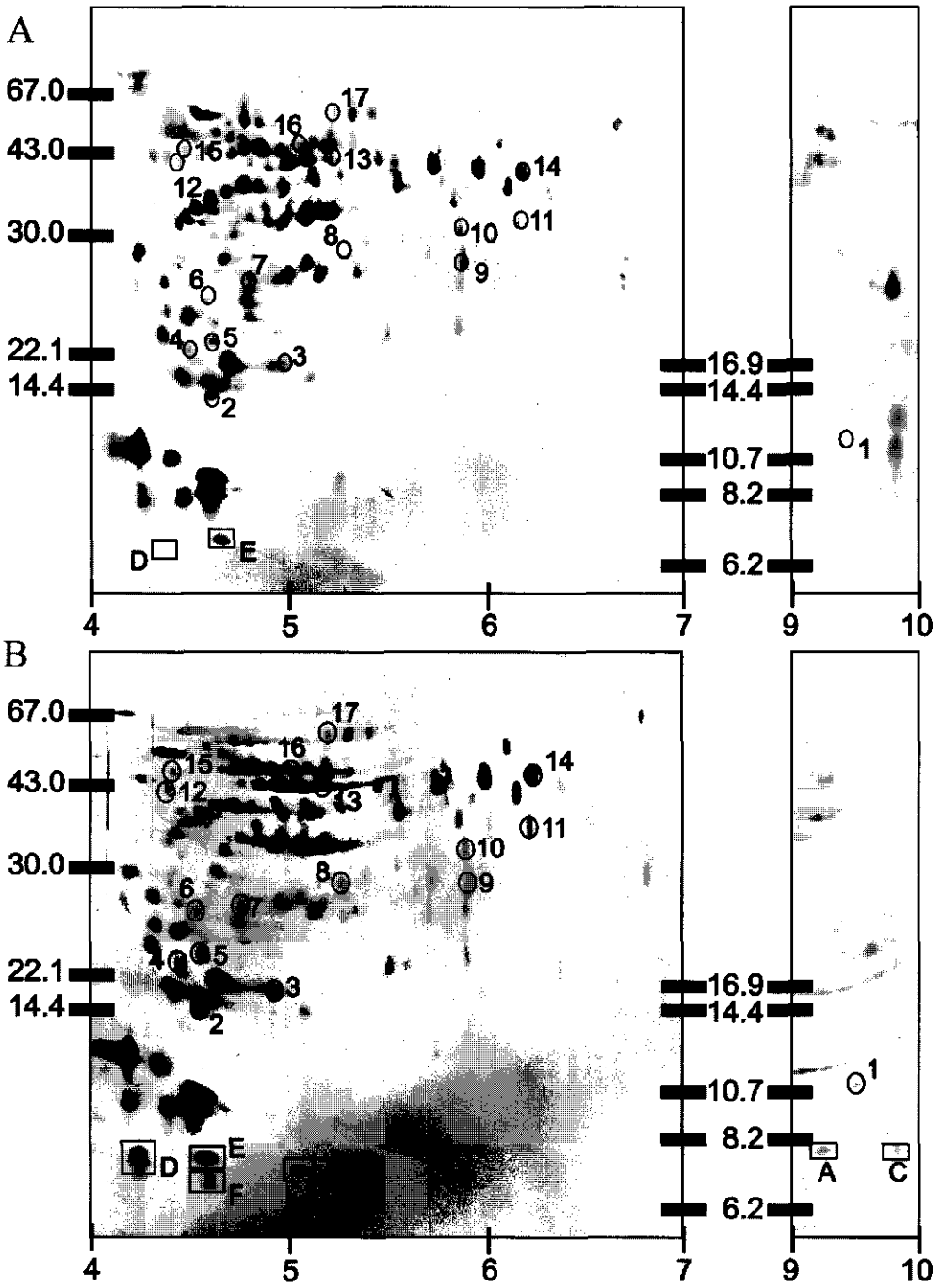
A
67.0
43.0
30.0
22.1
14.4
16.9
14.4
10.7
8.2
6.2
4
5
6
7
9
10
B
67.0
43.0
30.0
22.1
14.4
16.9
14.4
10.7
8.2
6.2
4
5
6
7
9
10
A
C
D
E
F

Table 2. Proteins (numbering according to Fig. 3) with increased expression levels at 4 h after cold shock to 10°C. Of each spot the approximate molecular weight (MW in kDa), the induction factor (x) at 4 h after cold shock from 30 to 10°C and the induction (+ = at least a 2-fold induction, - = no induction or reduction) after cold shock to 20°C, after cold shock to 4°C and at stationary phase conditions is given. For spots with identical molecular weights also the approximate pI is given. Three spots (6, 12 and 15) with increased expression under all conditions are shown in bold.

	MW (in kDa)	10°C (x)	20°C (+/-)	4 C (+/-)	Stat (+/-)
1	11	n.d.	-	-	-
2	14	5	-	-	-
3	20	2	-	-	-
4	22	2.5	-	-	-
5	23	2	-	+	-
6	**26**	**3**	**+**	**+**	**+**
7	27	3	-	-	-
8	29 (pI ± 5.3)	6	+	-	-
9	29 (pI ± 6.0)	2.5	-	-	-
10	33	10	-	-	-
11	35	4	+	-	+
12	**43 (pI ± 4.5)**	**14**	**+**	**+**	**+**
13	43 (pI ± 5.2)	2	+	-	+
14	44	2	-	-	-
15	**45 (pI ± 4.5)**	**10**	**+**	**+**	**+**
16	45 (pI ± 5.0)	7	+	-	+
17	66	3	+	-	+

n.d. = not determined

Survival after freezing of *L. lactis* following different temperature shocks. The mRNA levels of the *csp* genes of *L. lactis* were analysed at different times after cold shock from 30 to 20°C and from 30 to 4°C (Fig. 4A). It appeared that the total mRNA level for the *csp* genes is only slightly increased after 30 min after cold shock to 20°C. After longer incubation at this temperature, no *csp* mRNA could be detected. After a temperature downshock from 30 to 4°C clear induction of *csp* genes is observed at 1 h after cold shock. This induction is approximately 10-fold lower than observed at 4 h after cold shock to 10°C (Fig. 4A, lane 9). At 2 h after cold shock to 4°C *csp* mRNA levels were decreased (Fig. 4A). 2D-EF analysis reveals that only CspB and CspD were slightly induced at 4 h after cold shock to 4°C and slight induction of CspD was observed at 4 h after cold shock to 20°C (Fig. 4B). Furthermore, no induction of CspA and CspC was observed after cold shock from 30°C to 20 or 4°C (data not shown). These results indicate that the lactococcal CSPs are tightly temperature-

Fig. 3. Two-dimensional gel electrophoresis of cell-free extracts of *L. lactis* grown at 30°C (A) and after cold shock to 10°C for 4 h (B). Molecular weight marker bands are indicated on the left (high molecular weight marker) or on the right (low molecular weight marker) and a pI scale is given at the bottom. CSPs of *L. lactis* are boxed and the lettering is according the CSP family (B = CspB, D = CspD, E = CspE, F = spot F). At the right of each gel a part of the 2D-EF gels separated with a pI range from 3 to 10 is shown on which CspA (A) and CspC (C) of *L. lactis* are indicated (for the second dimension the same separation procedure is used). Proteins with increased levels (minimally 2-fold) after cold shock to 10°C (other than the 7-kDa CSPs) are circled and numbered (see also Table 2).

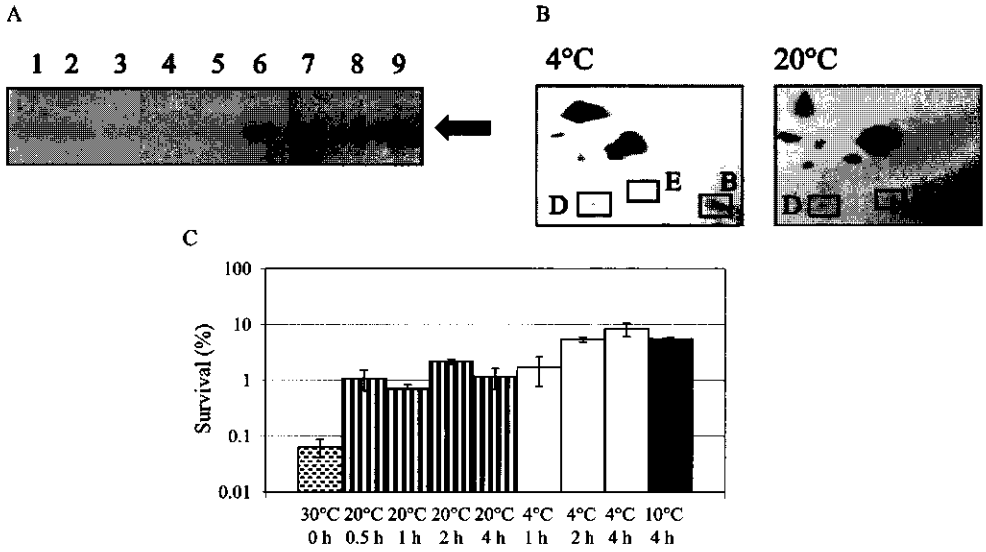

Fig. 4. *csp*-mRNA levels, protein levels and freeze survival of *L. lactis* cells exposed to different cold-shock conditions. (A) Northern blot analysis with total RNA isolated from *L. lactis* at mid-exponential phase at 30°C (lane 1), at 0.5, 1, 2 and 4 h after cold shock to 20°C (lane 2, 3, 4 and 5), at 1, 2 and 4 h after cold shock to 4°C (lane 6, 7 and 8) and at 4 h after cold shock to 10°C (lane 9). *csp* mRNA levels were analysed using a mix of probes specific for *cspA, cspB, cspC, cspD* and *cspE* of *L. lactis* (Chapter 2). The arrow indicates the *csp* transcripts of approximately 300 nt. (B) 2D-EF of cell-free extracts of *L. lactis* after cold shock from 30 to 4°C (left) and after cold shock from 30 to 20°C (right). Only fragments of the gel containing the relevant CSPs are shown. The CSPs are boxed and indicated with B (CspB), D (CspD) or E (CspE). (C) The survival after freezing of *L. lactis* after the cold-shock treatments as described for panel A. The percentage of surviving cells after four freeze-thaw cycles is shown. The freezing experiments were performed in triplicate and the averages of the three experiments are shown. The error bars represent the standard deviation. The number of cells before freezing is set at 100% (OD_{600} = 0.5, about 4 x 10^8 cells).

regulated and that the expression is highest after cold shock to 10°C. After cold shock to 20°C and 4°C for 4 h, 20 and 10 other induced proteins are observed, respectively, apart from the five 7-kDa CSPs (data not shown).

Remarkably, after exposure of the *L. lactis* cells to 4°C for 2 and 4 h significant cryoprotection was also observed. The survival after 4 repetitive freeze-thaw cycles was approximately 130-fold higher than the survival of mid-exponential grown cells. When *L. lactis* was shifted to 20°C also an adaptive response to freezing was found which leads to an approximately 30-fold increased survival after 4 freeze-thaw cycles compared to mid-exponential cells grown at 30°C. This adaptive response was already observed at 30 min after cold shock (Fig. 4C). These data show that an increased freeze survival coincides with increased CSP expression and in more detail coincides with CspD expression for which induction is observed at all tested cold-shock conditions. However, the percentage of surviving cells after freezing is quantitatively not directly correlated to the expression level of the CSPs or one specific CSP.

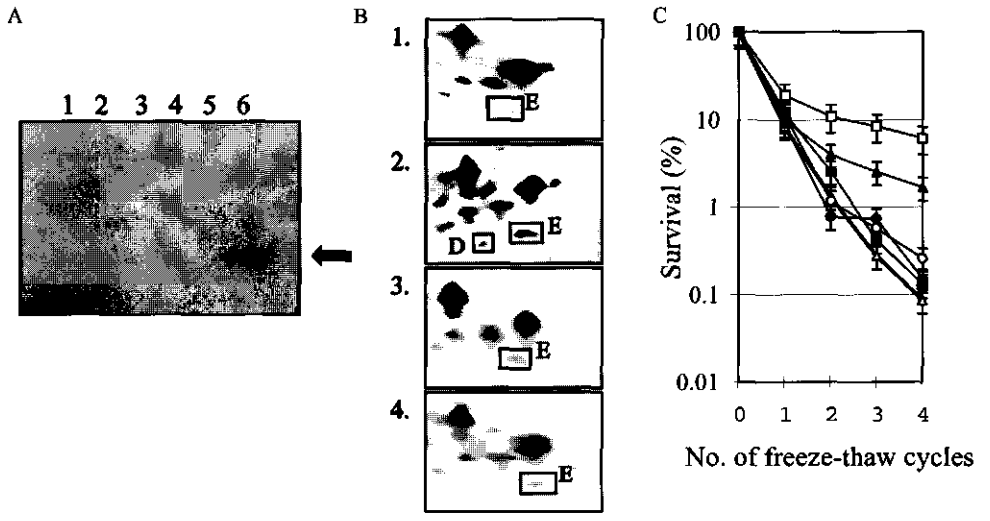

Fig. 5. *csp*-mRNA levels, protein levels and freeze survival of *L. lactis* cells exposed to different stress conditions. (A) Northern blot analysis with total RNA isolated from *L. lactis* at different growth or stress conditions: mid exponential phase (lane 1, 30°C), heat shock (lane 2; 10 min. 42°C), salt shock (lane 3; 10 min. 0.5 M NaCl), acid shock (lane 4; 10 min. pH=4 adjusted with lactic acid), stationary phase (lane 5; 2 h in stationary phase at 30°C), cold shock (lane 6; 4 h at 10°C). *csp* mRNA levels were analysed using a mix of probes specific for *cspA, cspB, cspC, cspD* and *cspE* of *L. lactis* (Chapter 2). The arrow indicates the *csp* transcripts of approximately 300 nt. (B) 2D-EF of cell-free extracts of *L. lactis* after exposure to heat (1), salt (2), acid (3) and stationary phase (4) stress. Only fragments of the gel containing the relevant CSPs are shown. The CSPs are boxed and indicated with D (CspD) or E (CspE). (C) The survival after freezing of *L. lactis* after exposure to several stress conditions: mid-exponential phase (open triangles), heat shock (open circles), salt shock (closed circles), acid shock (closed squares), stationary phase (closed triangles) and cold shock (open squares). The freezing experiments were performed in triplicate and the averages of the three experiments are shown. The error bars represent the standard deviation. The number of cells before freezing is set at 100% (OD_{600} = 0.5, about 4 x 10^8 cells).

Survival after freezing of *L. lactis* following different stress exposures. Following cold shock from 30 to 10°C a high *csp* mRNA induction was observed (Fig. 5A, lane 6). However, after exposures to either heat, pH, acid or stationary phase stress no significant increase of the *csp* mRNA levels was observed (Fig. 5A). Also at the protein level no up or down regulation of the CSPs was found after the different stress exposures. Only a slight induction of CspD is observed under salt stress conditions. CspE could be detected under all conditions tested and appears to be constitutively expressed (Fig. 5B). The survival after freezing upon exposure to heat, salt and acid stress was similar to the survival of mid-exponential phase cells cultured at 30°C. Surprisingly, stationary phase cells showed a 20-fold higher survival after 4 repetitive freeze-thaw cycles than the control cells (30°C mid-exponential phase; Fig. 5C). For stationary phase cells no increased CSP levels were observed and apparently other factors are involved in the increased freeze survival. 2D-EF gels revealed induction of 19 proteins (at least 2-fold induction) at stationary phase (data not shown). Three of these proteins were also induced at 4 h after cold shock to 20, 10 and 4°C (spot 6, 12 and 15; Table 2).

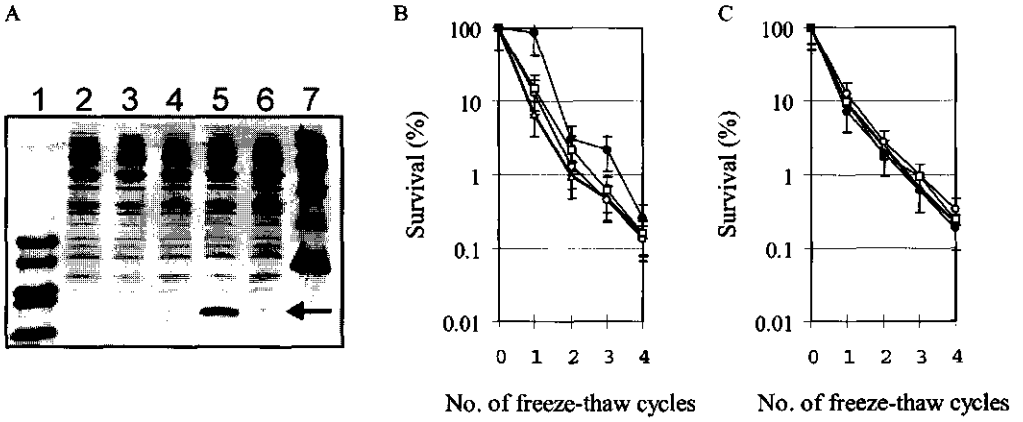

Fig. 6. Overproduction of CspD in *L. lactis* at 30°C. (A) Coomassie-stained SDS-PAGE of protein extracts from *L. lactis* NZ3900 containing pNZOECspD induced with different concentrations of nisin. In lane 1 and lane 7 a low and a high molecular weight marker are applied, respectively (band sizes of 16.9, 14.4, 10.7, 8.2, 6.2 kDa and of 67, 43, 30, 22.1 and 14.4 kDa (top to bottom), respectively). In lane 2, 3, 4 and 5 cell-free extracts of *L. lactis* NZ3900 containing pNZOECspD induced with 0, 0.1, 0.2 and 0.5 ng M17Wnisin/ml, respectively, are applied. In lane 6 a cell-free extract of *L. lactis* NZ3900 containing pNZ8020 (contains the *nisA* promoter without any fused gene) induced with 0.5 ng M17Wnisin/ml is run. (B) The survival after freezing of *L. lactis* NZ3900 containing pNZOECspD upon induction with 0 (triangles), 0.1 (open circles), 0.2 (squares) and 0.5 (closed circles) ng M17Wnisin/ml, respectively. The freezing experiments were performed in triplicate and the averages of the three experiments are shown. The error bars represent the standard deviation. The number of cells before freezing is set at 100 % (OD_{600} = 1.5, about 1 x 10^9 cells). (C) As a control also the survival after freezing of *L. lactis* NZ3900 containing pNZ8020 upon induction with 0 (triangles), 0.1 (open circles), 0.2 (squares) and 0.5 (closed circles) ng M17Wnisin/ml, respectively, is shown.

Overproduction of CspD and its effects on freeze survival. Under all cold-shock conditions CspD is induced and this coincides with increased survival to freezing. For this reason the effect of specific overexpression of CspD on survival was studied. Using *L. lactis* NZ3900 containing pNZOECspD considerable amounts of CspD were produced upon addition of nisin (Fig. 6A). Upon addition of 0.1 or 0.2 ng nisin/ml CspD levels comparable to cold-shock conditions were obtained. However, upon addition of 0.5 ng nisin/ml it appeared that extremely high CspD quantities could be obtained (approximately 12.6% of total protein). When cells overproducing CspD, were exposed to a repeated freeze-thaw challenge a slight increase in freeze survival can be observed, depending on the added concentration of nisin (Fig. 6B). Upon addition of 0.1 and 0.2 ng nisin/ml the survival to freezing hardly increases. However, when 0.5 ng nisin/ml is added the survival is approximately 2-10 times higher than control cells. Indeed, no effect on growth (data not shown) and survival to freezing (Fig. 6C) are noted upon the addition of nisin for *L. lactis* NZ3900 containing pNZ8020. This indicates that the increase in survival of *L. lactis* NZ3900 harbouring pNZOECspD is due to the increased CspD level.

DISCUSSION

Analysis of growth characteristics of many LAB has resulted in the grouping of LAB in psychrophilic, mesophilic and thermophilic strains. The strain used in this study is the mesophilic *L. lactis* MG1363 of which the minimal growth temperature is slightly lower than 4°C. The maximal growth rate was significantly reduced when the growth temperature was lowered. *L. lactis* MG1363 is able to recover from a cold-shock treatment with a temperature drop of 20°C within 6-8 h, indicating that this strain has the capacity to efficiently adapt to low temperatures (Fig. 1).

Since many starter LAB are stored frozen prior to use in fermentations there is much interest in the survival of these strains after freezing. It was observed that the survival after freezing of *L. lactis* increased approximately 100-fold when this bacterium was exposed to 10°C for 4 h (Fig. 2). The addition of chloramphenicol to the growth medium during a cold-shock treatment blocks the cryprotection process. These results indicate that within a few hours of incubation at low temperature significant cryoprotection is obtained for which protein synthesis is required. Since 2D-EF analysis revealed that the members of the 7 kDa CSP family of *L. lactis* are the highest induced proteins after cold shock, it is tempting to speculate that these proteins are directly involved in the protection against freezing. Willimsky *et al.* (1992) showed that deletion of the gene encoding CspB of *B. subitilis* resulted in a decreased freeze survival and the authors suggested a role as anti-freeze proteins for CSPs, because off their low molecular weight and their abundant presence. Our study shows that the *csp* mRNA and CSP expression levels in *L. lactis* increased upon cold shock to 20, 10 as well as to 4°C. However, the induction of CSPs was highest after cold shock to 10°C. Upon cold shock to 20°C induction of CspD was observed and upon cold shock to 4°C the level of both CspB and CspD increased. However, these cold-shock conditions did not result in induction levels comparable to exposure to 10°C. In contrast, the survival after freezing was similarly increased after exposure to 20 and 4°C compared to exposure to 10°C, indicating that neither the *csp*-mRNA level nor the CSP level quantitatively correlates to improved cryoprotection. Since CspD was induced at all cold-shock conditions, the specific impact of CspD on the freeze survival was monitored using controlled overproduction. This revealed a slight increase in freeze survival at high overproduction levels and no protective effect at CspD levels comparable to cold-shock conditions. In conclusion, the 7 kDa cold-shock protein CspD may enhance the survival capacity after freezing but this protein is evidently not the only factor determining cryoprotection. Probably other proteins are needed for this protective effect and a concerted action of several proteins can not be excluded. Overproduction of CspD results in increased levels of several proteins, however, it does not induce the expression of the proteins induced by both low temperature as well as stationary phase conditions (Table 2, data not shown).

In contrast to observations for CspA, CspB and CspG of *E. coli* (Etchegaray and Inouye, 1999), no induction of the lactococcal CSPs was observed in the presence of chloramphenicol (100 μg ml^{-1}) at 10°C. Our investigations show that growth is blocked, no new proteins are synthesised and no

increase in survival to freezing is observed upon addition of chloramphenicol. We speculate that the translational machinery in *L. lactis* is not intact and that during this condition also *csp* genes cannot be translated.

Since the actual CSP levels do not directly correlate with increased freeze survival, it might be speculated that one (or more) member(s) of the CSP family regulates the adaptive response to freezing by regulating the expression of other proteins. It has been reported that CspA of *E. coli* regulates the expression of genes belonging to the cold-shock stimulon (LaTeana *et al.*, 1991; Jones *et al.*, 1992). In *L. lactis* MG1363 various other proteins than the 7-kDa CSPs were found to be induced following cold shock, ranging from 16, 20 and 10 induced proteins after shock to 10, 20 and 4°C, respectively. Strikingly, the levels of three proteins (spot 6, 12 and 15, Table 2) were increased at all cold-shock treatments and at stationary phase conditions, but not at any of the other stress conditions. For all these conditions increased survival after freezing was observed and it is tempting to speculate that these unidentified proteins may play a role in cryoprotection.

Upon exposure of *L. lactis* to other stresses than cold stress (e.g. heat, salt, acid and stationary phase stress) the *csp* mRNA levels and the expression of CSPs were not affected. For CspD of *E. coli* (Yamanaka and Inouye, 1997) and CspB and CspC of *B. subtilis* (Graumann *et al.*, 1997) stationary phase induction has been observed. *L. lactis* cells that were in stationary phase for more than 2 h exhibited increased survival after freezing as compared to mid-exponential cells, whereas other stress exposures did not result in protection to freezing. These observations indicate that during stationary phase other factors than CSPs are important as cryoprotectant. Generally, many organisms show increased resistance to stress conditions during stationary phase (Kolter *et al.*, 1993). Starved *L. lactis* IL1403 showed enhanced resistance to heat, ethanol, acid, osmotic, and oxidative stress (Hartke *et al.*, 1994) and our data show also enhanced resistance to freezing stress. For *L. lactis* MG1363 we observed increased levels of 19 proteins at stationary phase conditions. A central position for mediating the stress responses might be assigned to alternative sigma factors, but thus far only a vegetative factor is described for *L. lactis* (Gansel *et al.*, 1993). In related Gram-positive organisms, like *B. subtilis* and *Listeria monocytogenes*, alternative sigma factors were shown to coordinate responses to a variety of signals such as temperature, pH, osmolarity and stationary phase (Hecker *et al.*, 1996; Becker *et al.*, 1998). To study cross-protection at different stress conditions it is of great interest to elucidate the presence of such general stress proteins and to verify whether the stress response in *L. lactis* involves similar sigma factors or is co-ordinated via alternative pathways.

For the first time the actual expression levels of CSPs are correlated to a physiological function in low temperature adaptation, in this case freeze survival of the industrially important lactic acid bacterium *L. lactis*. This study shows that exposure to 4, 10 and 20°C for several hours leads to increased freeze survival and this coincides with increased CSP expression. However, the level of observed freeze protection does not quantitatively correlate to the *csp* gene expression levels. In

addition, *L. lactis* cells specifically overproducing CspD at 30°C show a 2-10 times increased survival after freezing compared to control cells. This indicates that the 7-kDa cold-shock protein CspD may enhance the survival capacity after freezing but that this protein is probably not the only factor determining cryoprotection. The exact functioning of the members of the CSP family in *L. lactis* in relation to freeze adaptation is not known yet. In order to gain more insight in this respect, *csp* disruption mutants of *L. lactis* will be constructed.

Chapter 4

Cold-shock proteins and low-temperature response of *Streptococcus thermophilus* CNRZ302

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ABSTRACT

Low-temperature adaptation and cryoprotection were studied in the thermophilic lactic acid bacterium *Streptococcus thermophilus* CNRZ302. *S. thermophilus* actively adapts to freezing during a pretreatment at 20°C, resulting in an approximately 1000-fold increased survival after four freeze-thaw cycles compared to mid-exponential phase cells grown at an optimal temperature of 42°C. No adaptation is observed when cells are exposed to a temperature (10°C) below its minimal growth temperature (just below 15°C). By using two-dimensional gel electrophoresis several 7-kDa cold-induced proteins were identified, which are the major induced proteins after a shift to 20°C. These cold-shock proteins were maximally expressed at 20°C, while induction was low upon cold shock to 10°C. To confirm the presence of *csp* genes in *S. thermophilus* a PCR strategy was used which yielded products of different sizes. Sequence analysis revealed *csp*-like sequences that were up to 95% identical to those of *csp* genes of *S. thermophilus* ST1-1, *S. dysgalactiae*, *S. pyogenes* and *Lactococcus lactis*. Northern-blot analysis revealed 7 to 9-fold induction of *csp* mRNA upon a temperature shift to 20°C showing that this thermophilic bacterium indeed contains at least one cold-inducible *csp* gene and that its regulation takes place at the transcriptional level.

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INTRODUCTION

Lactic acid bacteria (LAB) play an important role in food industry, because of their wide application as starter cultures in many fermentation processes. The genetic and physiological stress response of the thermophilic yoghurt starter strain *Streptococcus thermophilus* thus far has been hardly studied and has been mainly directed to the acid and heat stress response (Auffray *et al.*, 1995; Gonzaléz-Márquez *et al.*, 1997). Low temperature adaptation is highly relevant from a practical point of view since many LAB fermentations are initiated by the addition of frozen starter cultures that should benefit from a high freeze-survival capacity. The postfermentation acidification taking place at low temperature in the cooperative yoghurt fermentation of *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, is a well-known undesired property. This results in a product that contains too much lactic acid and is therefore unfit for consumption (De Vos, 1996). Understanding the cold-adaptation of *S. thermophilus* could provide the basis for targeted strain improvement to overcome post-processing acidification and to increase the number of viable cells after freezing.

Bacteria are able to adapt to temperatures far below their optimum growth temperature and a set of 7-kDa proteins (named cold-shock proteins [CSPs]) is strongly induced in response to a rapid decrease in growth temperature (reviews by Graumann and Marahiel, 1998; Jones and Inouye, 1994; Yamanaka *et al.*, 1998). CSPs are found in a wide variety of Gram-positive and Gram-negative bacteria, such as in *Escherichia coli* (Yamanaka *et al.*, 1998), *Bacillus subtilis* (Graumann and Marahiel, 1998) and *Lactococcus lactis* (Chapter 2). Moreover, Francis and Stewart (1997) monitored a wide variety of bacteria and observed that *csp* genes were present in all species tested. However, CSPs were not observed in all bacteria, e.g., in *Helicobacter pylori* (Tomb *et al.*, 1997) and *Campylobacter jejuni* (Hazeleger *et al.*, 1998) they were absent.

CSPs may function as RNA chaperones, as they possess binding sites for single-stranded nucleic acids. In this way they could minimize secondary folding of mRNA thereby facilitating the translation process (Graumann *et al.*, 1997; Jiang *et al.*, 1997). CspA of *E. coli* also appears to function as a transcriptional activator as has been described for two genes of which the products, GyrA and H-NS, are both involved in DNA-supercoiling (Jones *et al.*, 1992B; La Teana *et al.*, 1992). Furthermore, CspB of *B. subtilis* appeared to be implicated in freezing tolerance, as was shown using a strain in which the *cspB* gene was disrupted (Willimsky *et al.*, 1992). It was noted that many organisms develop an increased ability to survive freezing after a cold-shock treatment. Maintaining membrane integrity and prevention of macromolecule denaturation have been mentioned as key factors increasing the freeze-survival (El-kest and Marth, 1992; Franks, 1995; Thammavongs *et al.*, 1996). However, the exact function of CSPs in cryoprotection remains to be elucidated.

In this study we provide evidence for an active adaptation response of the thermophilic starter lactic acid bacterium *S. thermophilus* to a freezing challenge after exposure to low temperature. Protein synthesis is required for this adaptation and major differences in the pattern of synthesized

proteins are found in the class of 7-kDa CSPs. Furthermore, a *csp* gene is characterized and its expression is studied upon exposure to low temperature.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *S. thermophilus* CNRZ302 was cultured at 42°C in M17 broth (Difco) containing 0.5% (w/v) lactose (LM17). For studying growth kinetics 1% inoculated cultures were grown at different temperatures. Growth was monitored by measuring the optical density at 600 nm (OD_{600}). *E. coli* MC1061 (Casabadan and Cohen, 1980) was used as a host strain in cloning experiments and was grown in Tryptone Yeast (TY) medium with aeration at 37°C (Sambrook *et al.*, 1989). Ampicillin was used at a concentration of 50 μg ml^{-1}.

Cold-shock treatment and freeze-thaw challenge. For cold-shock treatments 50-ml cultures were grown in LM17 medium until mid-exponential phase (OD_{600} 0.5) after which 25 ml of the culture was pelleted (10 min. 3660 rpm) and resuspended in the same volume of precooled medium (25, 20, 15 or 10°C). The cultures were incubated at the different temperatures for 50 h, during which the OD_{600} was measured. To study the freeze-thaw survival capacity, cells of *S. thermophilus* were frozen at mid-exponential phase (OD_{600} 0.5) and at 2 and 4 h after cold shock to 10 and 20°C. 1-ml aliquots were spun down (5 min. 13000 rpm) and resuspended in 1 ml fresh LM17 medium and subsequently frozen at −20°C for exactly 24 h and thawed during exactly 4 min at 30°C in a waterbath. The number of CFU was determined just before freezing and after four consecutive freeze-thaw challenges (24-h freeze periods, thawing for 4 min at 30°C) by spread plating decimal dilutions. After 2 day incubations on LM17 plates at 42°C the numbers of CFU were counted. The experiments were performed in duplicate and the data are presented as a mean (coefficient of variation < 10%).

Two-dimensional gelelectrophoresis (2D-EF). Total cellular proteins were extracted from 10 ml cultures by homogenizing them with a MSK cell homogenizer (B. Braun Biotech International, Germany) and zirconium beads (0.1 mm, Biospec Products, USA) 6 times for 1 min (cooled on ice between treatments). After homogenizing, the zirconium beads were allowed to sediment by gravity. The supernatant, containing the cellular proteins, was analysed by 2D-EF. The protein content of the extracts was determined using the bicinchoninic acid method (Sigma Chemicals, USA) and equal amounts of protein were applied on 2D-EF gels. 2D-EF was essentially performed as described by O'Farrell (1975) using a Pharmacia 2D-EF system (Pharmacia Biotech, Sweden). Prior to loading of the samples on the IEF gel, 20 μl protein solution (40 μg protein) was treated with 20 μl lysis solution (9 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 4-7L (Pharmacia Biotech, Sweden), 2% Triton X-100 and 8 mM phenylmethylsulfonylfluoride) at 37°C for 5 min, after which 60 μl of sample solution (8 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 4-7L, 0.5% Triton X-100 and a few grains of bromophenolblue) was added. The total volume (100 μl) was loaded on the acidic end of the first

dimensional IEF gel with a linear iso-electric point (pI) range from 4 to 7 (Immobiline Dry strips, Pharmacia Biotech, Sweden). For the second dimension, 15% homogeneous SDS-PAGE gels were used to obtain an optimal separation at the low molecular weight region. Two molecular weight markers were used with band sizes of 67, 43, 30, 22.1 and 14.4 kDa and of 16.9, 14.4, 10.7, 8.2, 6.2 and 2.5 kDa, respectively. The gels were silver stained according to Blum *et al.* (1987) and the gels were analyzed using GEMINI software (Applied Imaging, United Kingdom).

PCR and cloning of *csp* genes of *S. thermophilus*. For the identification of *csp* genes in *S. thermophilus* a PCR approach was chosen. PCR was carried out according to conditions described by Kuipers *et al.* (1991) in a total volume of 50 µl containing 10 mM TRIS/HCl (pH 8.8), 50 mM NaCl, 2 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate, 1 U *Pwo* polymerase (Gibco/BRL Life Technologies, The Netherlands), 10 pmol of each primer and 10 to 100 ng of template chromosomal DNA. PCR was performed in 25 cycles, consisting of a denaturation step at 95°C for 1 min, a primer annealing step at 42°C for 90 seconds and a primer extension step at 72°C for 2 min.

Primers were either based on the homologous regions of several *csp* genes (CSPU5 containing an *Eco*RI-site and CSPU3 containing a *Bam*HI-site; Francis and Stewart, 1997) or on the sequence of the cold-induced *cspB* gene of *L. lactis* MG1363 (Chapter 2; CspBFOR: 5'-ATTGGTTTAATCCAG-ATAA-3'; CspBREV: 5'-TTTTATGCTTTTTCGATA-3'; primers were purchased from Gibco/BRL Life Technologies, The Netherlands). Total DNA of *S. thermophilus* was extracted according to Vos *et al.* (1989). The PCR products were analysed on a 2% agarose gel. PCR products obtained with CSPU3 and CSPU5 were cloned in the *Bam*HI and *Eco*RI-sites of pUC18. Plasmid DNA was sequenced using an ALF DNA sequencer (Pharmacia Biotech). All manipulations with recombinant DNA were carried out following standard procedures (Sambrook *et al.*, 1989) and according to the specifications of the enzyme manufacturers (Gibco/BRL Life Technologies, The Netherlands). Computer analysis of DNA sequences and the deduced amino acid sequences were performed with Clone (Version 4.0; Clone Manager) and sequence comparisons were performed using Blast and the EMBL/GenBank and SWISS-PROT/PIR databases.

mRNA analysis. Total RNA was isolated at optimal growth temperature (42°C) at mid-exponential phase and at 2 and 4 h after cold shock to 20 and 10°C, respectively, as described previously (Kuipers *et al.*, 1993). RNA was denatured and equal amounts of RNA were applied on the gel. RNA was fractionated on a 1%-agarose gel containing formaldehyde according to Sambrook *et al.* (1989) and the RNA was stained by ethidium bromide. A 0.24-9.5 kb RNA ladder (Gibco/BRL Life Technologies, The Netherlands) was used to determine the transcript size. The gel was blotted to a Nylon membrane (GeneScreen, New England Nuclear, USA) following the recommendations of the manufacturer. The streptococcal *csp* fragment, [^{32}P]-labelled by random priming, was used as a probe and hybridisation was performed at 65°C. Blots were exposed to X-ray film (Kodak Scientific Imaging Film Biomax MR, USA) and quantification of the *csp* transcript was performed using the Dynamics

Phosphor Imaging System (Dynamics, USA).

Nucleotide sequence accession number. The EMBL accession number for the sequence reported in this paper is Y18814.

RESULTS

Effect of temperature down shock on growth of *S. thermophilus* CNRZ302. *S. thermophilus* CNRZ302 grows optimally at 42°C and has a minimal growth temperature just below 15°C. At 15°C a lag-time of 12 days is observed (data not shown). The growth rate (μ_m, defined as 1/D) significantly decreases at low temperatures (from 0.4 h^{-1} at 42°C to 0.008 h^{-1} at 15°C). Using these values the theoretical minimum temperature for growth of *S. thermophilus* was calculated and appeared to be 10.6°C (Ratkowsky *et al.*, 1982; Wijtzes *et al.*, 1995). When a *S. thermophilus* culture is shifted from 42 to 20°C this strain is able to adapt relatively quickly (within 1 to 2 h [Fig. 1]). However, this culture is not able to recover from a cold shock from 42 to 10°C within 50 hours (Fig. 1; data not shown).

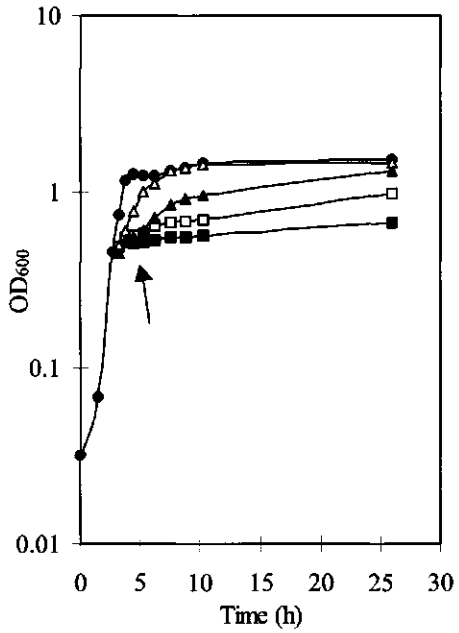

Fig. 1. Growth of *S. thermophilus* CNRZ302 following a cold shock to different temperatures. Growth is measured as OD_{600} for cells grown at 42°C (closed circles) and following cold shock from 42°C to 25°C (open triangles), 20°C (closed triangles), 15°C (open squares) or 10°C (closed squares), respectively. The arrow indicates the time point of cold shock.

Increased survival after freezing following a low temperature treatment. The survival after freezing was determined for cells grown to mid-exponential phase at 42°C and for cultures which were exposed to low temperature (10 or 20°C) for 2 and 4 h. After four consecutive freeze-thaw cycles

approximately 0.01% of cells cultured at 42°C survived. Exposure of cells to 20°C for 2 or 4 h results in an increased survival of approximately a factor 100 or 1000, respectively, compared to the survival of mid-exponential cells cultured at 42°C (Fig. 2A). However, exposure to 10°C for 2 or 4 h results in only a small increase (5- and 10-fold, respectively) in freeze-survival (Fig. 2B). Upon addition of chloramphenicol (100 μg ml^{-1}) during the cold-shock treatment the adaptive response to freezing is completely blocked, indicating that protein synthesis is required in the adaptation process (Fig. 2). Only exposure for 4 h to 20°C in the presence of chloramphenicol did not result in a complete block of the adaptive response (10-fold increment compared to control cells [Fig. 2A]).

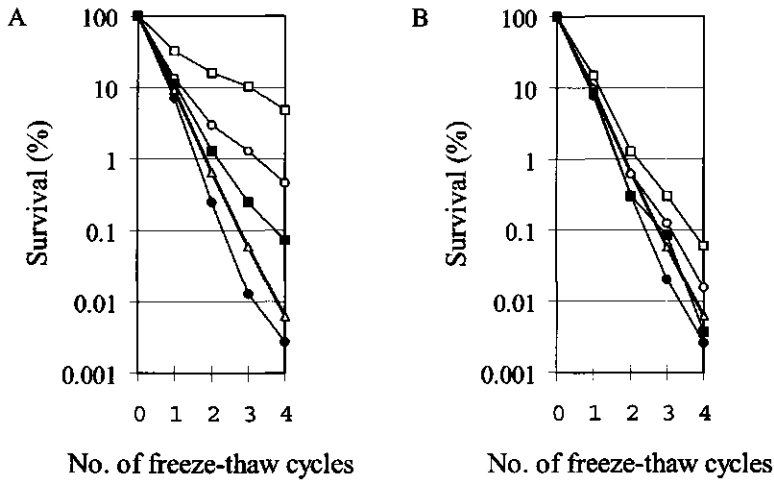

Fig. 2. Survival after freezing of *S. thermophilus* CNRZ302. Survival is given as the percentage of surviving cells relative to the amount prior to freezing (100%). (A) Freeze survival of *S. thermophilus* cells shocked from 42 to 20°C. (B) Freeze survival of *S. thermophilus* cells shocked from 42 to 10°C. For each panel the freeze-survival curves are depicted for cells without exposure (squares) or with exposure to a cold shock for 2 (open circles) or 4 h (open squares) and in the presence of chloramphenicol for 2 (closed circles) or 4 (closed squares) h during the cold-shock treatment.

Identification and expression analysis of CSPs. Cell-free extracts from mid-exponential cultures (42°C) and from cultures cold-shocked for 2 and 4 h at 10 or 20°C, respectively, were isolated and separated using 2D-EF. By using the described separation and staining conditions approximately 150 spots could be identified. Major cold induction was observed at 20°C for a group of proteins with a molecular size of approximately 7 kDa (Fig. 3). Analysis of the 2D-EF gels outside the 7-kDa region revealed an additional 14 and 18 induced proteins (more than 2-fold induction) after cold shock to 20°C for 2 and 4 h, respectively (Fig. 3B and 3C; circled spots). Eight of these proteins appeared to be induced at 2 h as well as at 4 h after cold shock to 20°C. In comparison, only four proteins were induced at 4 h after cold shock to 10°C (Fig. 3D; circled spots). Three proteins were induced at all cold-shock conditions tested and highest induction (> 5-fold) was observed for a protein of

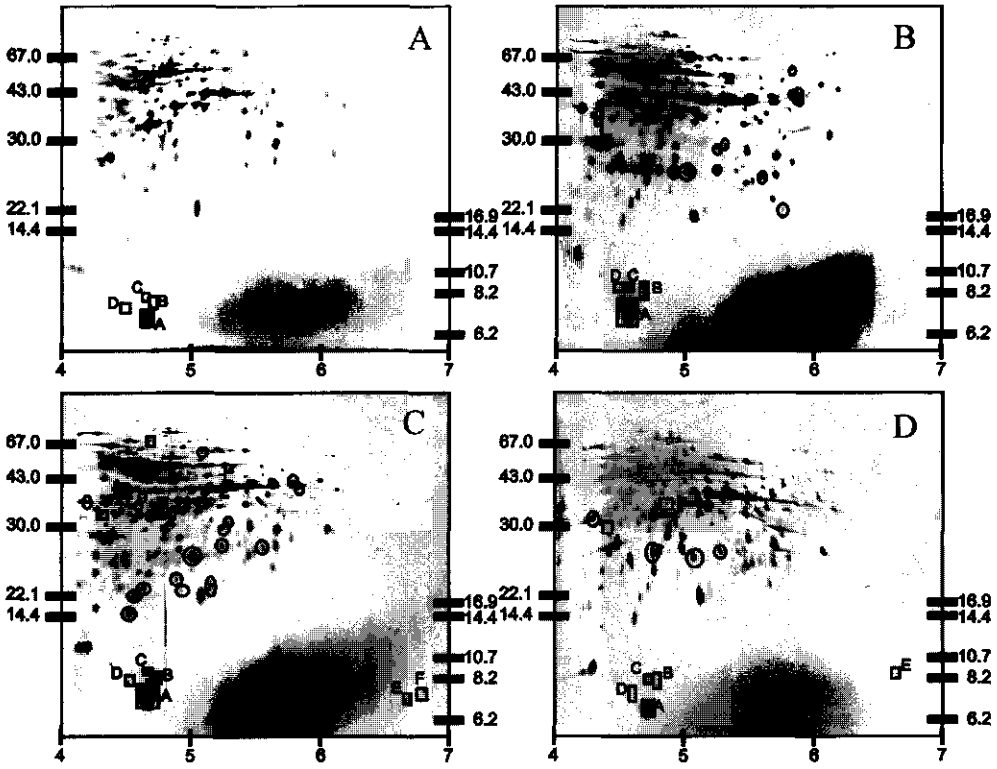

Fig. 3. 2D-EF of cell-free extracts of *S. thermophilus* CNRZ302. (A) Total protein extracted from cells grown at 42°C. (B) Total protein extracted from cells exposed to cold shock from 42 to 20°C for 2 h. (C) Total protein extracted from cells exposed to cold shock from 42 to 20°C for 4 h. (D) Total protein extracted from cells exposed to cold shock from 42 to 10°C for 4 h. Spots in the 7-kDa CSP region are boxed and lettered as described in the text and Table 1. Cold-induced proteins outside the 7-kDa region are circled. Proteins outside the 7-kDa region that are repressed after cold shock are boxed (without lettering). Molecular size marker bands are indicated on the left (high molecular mass marker) or on the right (low molecular mass marker) and a pI scale is given at the bottom.

approximately 25 kDa and a pI of approximately 5 (Fig. 3B, 3C and 3D). Furthermore, also repressed proteins (more than two-fold reduction) were observed: three, four and two spots upon cold shock at 20°C for 2 h, at 20°C for 4 h and at 10°C for 4 h, respectively. Of this group two spots were repressed for all cold-shock conditions tested (Fig. 3B, 3C, 3D; boxed spots without lettering).

The position of the CSPs on the 2D-EF gels of *L. lactis* MG1363 (Chapter 3) enabled the identification of spots in the same region for *S. thermophilus*. For *S. thermophilus* four spots were observed in the CSP region at 42°C. One of these proteins (spot A) was highly expressed at 42°C and further induced upon cold shock to 20°C (about three times; Table 1). Furthermore, two induced spots (B and C) and two new spots (E and F) could be identified in the CSP region after cold shock to 20°C. Spot D appeared not to be induced at low temperature (Fig. 3; Table 1). While spots A-D have a pI of approximately 5, spot E and F have a much higher pI of approximately 7. The proteins in the CSP

region make up about 11% of all protein present after cold shock to 20°C for 4 h (calculated on basis of the silver stained gels; Table 1). After exposure to a cold shock from 42°C to 10°C for 4 h low induction of was observed for spot C and spot E whereas no induction for the other proteins in the 7 kDa CSP region was observed (Fig. 3D). 2D-EF analysis of protein extracts of cultures exposed to a cold shock to either 10 or 20°C in the presence of chloramphenicol revealed no increased expression of proteins in the CSP region (data not shown).

Table 1. Relative amounts of the proteins in the CSP region for *S. thermophilus* at 42°C, at 2 and 4 h after cold shock to 20°C and at 4 h after cold shock to 10°C (lettering according to Fig. 3). The relative amounts are depicted as a percentage of the total amount of protein measured in a silver-stained 2D-EF gel.

Spot	42°C t = 0	20°C t = 2	20°C t = 4	10°C t = 4
A	3.00	7.45	8.92	3.98
B	0.58	1.23	1.56	0.40
C	0.10	0.13	0.40	0.25
D	0.32	0.36	0.35	0
E	0	0	0.52	0.15
F	0	0	0.37	0

Identification of a putative *csp* gene. Using primers based on the homologous regions of the *cspB* gene of *L. lactis* (Chapter 2) fragments of the expected size (approximately 180 nt) were amplified when chromosomal DNA of *S. thermophilus* was used as a template. Also using the universal primers CSPU5 and CSPU3 (Francis and Stewart, 1997) products of the expected size (180 nt) were obtained for *S. thermophilus* (data not shown). Next to these fragments also a fragment of about 450 nt was amplified for *S. thermophilus*. The amplification of this larger fragment could have been an indication for the presence of a clustered organisation of *csp* genes in *S. thermophilus* similarly as has been observed for *L. lactis* (Chapter 2). However, after sequencing this DNA fragment showed high homology to genes encoding the 50S ribosomal protein L27 in *Bacillus* species.

The PCR product obtained with CSPU3 and CSPU5 was cloned in pUC18. Three plasmids were sequenced and yielded the same sequence: a *csp* homologue, named *cspA*, in *S. thermophilus*. This partial sequence was highly identical (up to 95% identity) to partial *csp* sequences of *S. thermophilus* ST1-1 (Kim *et al.*, 1998), *Streptococcus dysgalactiae*, *Streptococcus pyogenes* (Francis and Stewart, 1997) and to *cspB*, *cspD* and *cspE* of *L. lactis* (Chapter 2). The amino acid sequence of the encoded protein is given in Fig. 4 and revealed the presence of the conserved RNA-binding motifs, RNP-1 and RNP-2, in CspA of *S. thermophilus*.

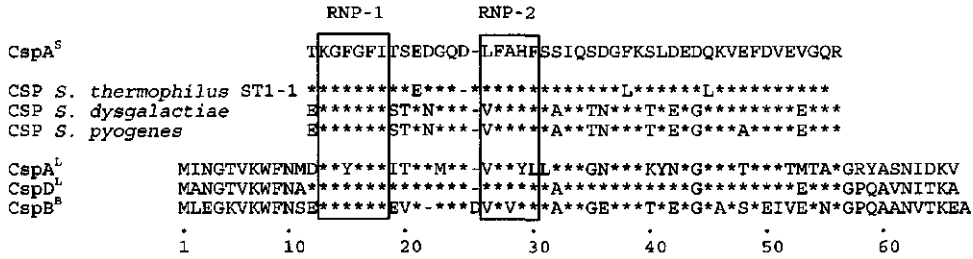

Fig. 4. Alignment of the deduced amino acid sequence of CspA of *S. thermophilus* CNRZ302 (CspA^S) and the amino acid sequences of *S. thermophilus* ST1-1 (Kim *et al.*, 1998), *S. dysgalactiae*, *S. pyogenes* (Francis and Stewart, 1997), CspA and CspD of *L. lactis* (Chapter 2) and CspB of *B. subtilis* (Willimsky *et al.*, 1992). Identical amino acids to CspA of *S. thermophilus* CNRZ302 are indicated with an asterisk and gaps are indicated with a dash. The RNA-binding motifs (RNP-1 and RNP-2) are boxed.

Analysis of *cspA* mRNA levels after cold shock. By using the *S. thermophilus cspA* sequence as a probe, hybridisation was observed with a single transcript of approximately 280 nt. The mRNA level of this *csp* gene is low at 42°C and is induced 7 to 9-fold upon cold shock to 20°C after 2 hours as well as after 4 hours. It can, however, not be excluded that mRNA of *csp* genes other than the *cspA* gene are hybridising to the probe used. Upon cold shock to 10°C increased mRNA levels are also observed, however, the induction reaches only a factor of approximately 3 to 4 (Fig. 5).

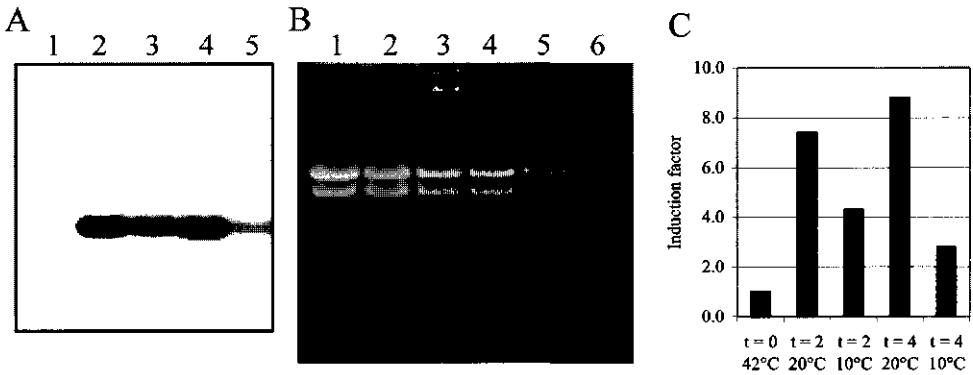

Fig. 5. mRNA analysis of *cspA* of *S. thermophilus* CNRZ302 upon exposure to cold shock. (A) Northern blot of RNA extracted at 42°C (lane 1), at 2 h after cold shock to 20°C (lane 2) and 10°C (lane 3) and at 4 h after cold shock to 20°C (lane 4) and 10°C (lane 5), hybridized with the streptococcal *cspA* probe. The transcript is approximately 280 nt. (B) Formaldehyde-agarose gels with ethidium bromide stained RNA. A RNA-ladder (lane 6) is used containing RNA fragments of 0.24, 1.35, 2.37, 4.40, 7.46 and 9.49 kb, respectively. (C) Increase in mRNA levels (relative to t=0 at 42°C) for the respective cold-shock conditions.

DISCUSSION

Analysis of growth characteristics of LAB has resulted in a grouping into psychrophilic, mesophilic and thermophilic strains. The strain used in this study is the thermophilic *S. thermophilus* CNRZ302 which has an optimal growth temperature of approximately 42°C. The minimal growth temperature of *S. thermophilus* CNRZ302 was shown to be slightly lower than 15°C, whereas the theoretical minimal growth temperature appeared to be 10.6°C. *S. thermophilus* was able to recover from a cold-shock treatment with a temperature drop of about 20°C within 1-2 h, indicating that this strain has the capacity to efficiently adapt to low temperatures. However, a cold shock from 42 to 10°C resulted in a growth block.

Dairy fermentations are often started by addition of frozen starters and therefore the freeze-survival capacity is a very important parameter. This study shows that 0.01% of the *S. thermophilus* cells cultured at 42°C survive four repetitive freeze-thaw cycles. However, a 100- and 1000-fold increase in survival was observed after preincubation at 20°C for 2 and 4 h, respectively. Kim and Dunn (1997) tested several LAB, including *S. thermophilus*, for survival after freezing. For *S. thermophilus* TS2 the survival after freezing was only slightly increased, which can be explained by the low adaptation temperature (10°C) used in their study. However, our study shows that effective protection of *S. thermophilus* CNRZ302 can be achieved by pretreatment at 20°C.

The adaptation to freezing by low temperature exposure is blocked by the addition of chloramphenicol. This indicates that protein synthesis is required in the adaptation process. Apparently, newly synthesized proteins have a protective effect during the freezing challenge or cause changes that lead to cryoprotection. However, the freeze adaptation upon pretreatment for 4 h at 20°C could not be completely blocked by chloramphenicol. This might be explained by either an incomplete block of protein synthesis or by the induction of specifically 7-kDa CSPs by chloramphenicol as is reported for CspA of *E. coli* (Van Bogelen *et al.*, 1990). However, no induction of 7-kDa CSPs is observed upon exposure to chloramphenicol for *S. thermophilus*.

For *S. thermophilus* a set of proteins of approximately 7 kDa was induced upon cold shock. These proteins were 3 to 4-fold induced at 2 and 4 h after cold shock at 20°C but were hardly induced upon cold shock to 10°C. One of these proteins (spot A) was highly present at 42°C and was induced approximately three times upon cold-shock from 42 to 20°C. Also four other spots (spots B, C, E and F; Fig. 3; Table 1) in the low molecular mass region were induced after cold shock, indicating the presence of a 7-kDa CSP family in *S. thermophilus*. Furthermore, spot D appeared not to be induced upon cold shock to 20°C and was not detectable upon cold shock to 10°C (Fig. 3, Table 1). Non-cold induced 7 kDa CSPs, better referred to as CSP-like proteins, are also observed for *E. coli* (Lee *et al.*, 1994) and the more related *L. lactis* (Chapter 2). Since the members of the 7-kDa CSP family of *S. thermophilus* are highest induced proteins after cold shock, it is tempting to speculate that these proteins are involved in the protection against freezing. A *B. subtilis* strain deleted in the *cspB* gene

showed a decreased level of freeze survival and it is speculated that CSPs have an antifreeze function minimizing cell damage (Willimsky *et al.*, 1992). However, next to the proteins in the 7-kDa region, a set of approximately 18 proteins was also shown to be induced in *S. thermophilus* upon cold shock to 20°C and these might also play a role in cryoprotection.

By using a PCR strategy the presence of a *csp* homologue in *S. thermophilus* could be verified. At the nucleotide level the homology with the *csp* sequences of *S. dysgalactiae* and *S. pyogenes* is 78% (30 differences in 136 residues) and 74% (35 differences in 136 residues), respectively. The difference with *cspD* and *cspB* of *L. lactis* MG1363 is much smaller: only 5 and 27 residues, respectively (96 and 80% identity). This is an indication for the close relation of *S. thermophilus* to *L. lactis* (formerly described as *Streptococcus lactis*) and might be an indication for a similar evolutionary history of these bacteria. At the amino acid level the CspA sequence of *S. thermophilus* CNRZ302 was up to 95% identical to CSP sequences of *S. thermophilus* ST1-1 (Kim *et al.*, 1998), *S. dysgalactiae*, *S. pyogenes* (Francis and Stewart, 1997) and CspB, CspD and CspE of *L. lactis* (Chapter 2). A recently identified CSP of *S. thermophilus* ST1-1 (Kim *et al.*, 1998) appeared to be three amino acids different (an Asp-Glu substitution at position 22 and Lys-Leu-substitutions at positions 39 and 46 [Fig. 4]) from CspA of *S. thermophilus* CNRZ302. The RNP motifs are highly conserved in CSPs which suggests a structural importance. It was shown that these regions are involved in RNA binding (Graumann and Marahiel, 1998; Jiang *et al.*, 1997). The RNP-1 motif of CspA of *S. thermophilus* (KGFGFI) is highly conserved, although in other LAB also the KGYGFI sequence is observed (Francis and Stewart, 1997; Chapter 2). The RNP-2 motif of CspA of *S. thermophilus* (LFAHF) is distinctly different from the consensus sequence (VFVHF). However, similar differences have been observed for the RNP-2 motifs of other streptococcal CSP sequences and CspD of *L. lactis* (Francis and Stewart, 1997; Kim *et al.*, 1998; Chapter 2).

By Northern blotting a transcript of 280 nt was observed for *cspA*. Furthermore, increased *cspA*-mRNA levels were observed upon cold shock, indicating that its up-regulation after low-temperature exposure takes places at the transcriptional level. The mRNA-induction was approximately 7 to 9-fold after cold shock to 20°C. Also upon cold shock to 10°C (below the minimal growth temperature) an increased *cspA* mRNA-level was observed (4-fold) although this level declined upon longer incubation at this temperature. Furthermore, at 10°C the increased *csp* mRNA level does not lead to increased CSP expression. This might be explained by the low translational efficiencies as are also reported for *E. coli* tested below its minimal growth temperature (Yamanaka *et al.*, 1998).

This study provides evidence for an active low-temperature adaptation response for the thermophilic starter LAB *S. thermophilus*, resulting in a 1000-fold increased freeze-survival. For the first time the presence of cold-shock proteins in a thermophilic lactic acid bacterium is shown at the DNA as well as at the protein level and by using Northern blotting and 2D-EF cold induction could be

shown. The observed increased survival after freezing of this industrially important LAB can be of great importance for the conservation methods of this strain prior to use in dairy processing. However, the exact functioning of the members of the CSP family in *S. thermophilus* in relation to freeze survival and low-temperature adaptation remains to be elucidated.

Chapter 5

Physiological and regulatory effects of the controlled overproduction of five cold-shock proteins of *Lactococcus lactis* MG1363.

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ABSTRACT

The physiological and regulatory effects of overproduction of five cold-shock proteins (CSPs) of *Lactococcus lactis* were studied. CspB, CspD and CspE could be overproduced to high levels (up to 19% of total protein), whereas for CspA and CspC limited overproduction (0.3-0.5% of total protein) was obtained. Northern-blot analysis revealed low abundance of the *cspC* transcript, indicating that the stability of *cspC* mRNA is low. The limited overproduction of CspA is likely to be caused by a low stability of CspA since upon an Arg-Pro mutation at position 58 its stability significantly increased. Using two-dimensional gel electrophoresis it was found that upon overproduction of the respective CSPs several proteins were induced, including a number of cold-induced proteins of *L. lactis*. Strikingly, upon overproduction of CspC also induction of CspB, putative CspF and putative CspG was observed. Overproduction of CspB or CspE results in an increased survival to freezing of *L. lactis* (maximally 10 and 5-fold after 4 freeze-thaw cycles, respectively). It is concluded that CSPs in *L. lactis* play a regulatory role in the cascade of events that are initiated by a cold-shock treatment and they either have a direct protective effect during freezing, e.g. by RNA stabilization, and/or induce other factors involved in the freeze-adaptive response.

Submitted for publication

INTRODUCTION

In a variety of bacteria, cold-shock proteins (CSPs) are the major induced proteins upon exposure to cold shock. Different functions have been appointed to CSPs, e.g. functions as transcriptional activators, RNA-chaperones and anti-freeze proteins, (reviews by Graumann and Marahiel, 1998 and Yamanaka *et al.*, 1998). CspA of *Escherichia coli* ($CspA^E$) and CspB of *Bacillus subtilis* ($CspB^B$) have been shown to bind ssDNA and $CspA^E$ has been shown to act as a transcriptional activator for the genes *hns* and *gyrA*, encoding proteins involved in DNA-supercoiling (LaTeana *et al.*, 1991; Jones *et al.*, 1992B; Brandi *et al.*, 1994). $CspA^E$ and $CspB^B$ show a highly similar five stranded β-barrel structure with several outward-facing residues important for ssDNA binding. Furthermore, CSPs contain two highly conserved RNA-binding motifs, named RNP-1 and RNP-2, and, indeed, for $CspA^E$ and $CspB^B$ mRNA-binding capacity has been demonstrated (Graumann *et al.*, 1997; Jiang *et al.*, 1997). It has been proposed that members of the CSP family bind to RNA in a co-operative manner and function as RNA-chaperones, thereby facilitating the translation process (Graumann *et al.*, 1997). Disruption of $cspB^B$ causes a freeze-sensitive phenotype (Willimsky *et al.*, 1992) and also affects the level of induction of other cold-induced proteins upon temperature down shock in *B. subtilis* (Graumann *et al.*, 1996). Deletion of three CSPs in *B. subtilis* was shown to be lethal (Graumann *et al.*, 1997). Since not all members of the CSP family are cold induced it has been suggested that CSPs play a role in multiple cellular processes, such as chromosomal condensation and/or cell division (Yamanaka *et al.*, 1998).

The mesophilic lactic acid bacterium *Lactococcus lactis* is widely used to start industrial food fermentations. A variety of genes involved in the stress-response that probably are important for cell survival under stress conditions have been studied for this organism (reviews by Sanders *et al.*, 1999 and Duwat *et al.*, 1999). The *L. lactis* MG1363 chromosome was found to contain two tandemly located and cold-inducible *csp* genes (*cspA/cspB* and *cspC/cspD*) and a single, constitutively expressed *cspE* gene. The encoded CSPs can be divided in two groups based on iso-electric point (pI) and homology. One group consists of CspA and CspC that share 80% identical residues and these CSPs have a pI of 9 whereas the other group includes CspB, CspD and CspE that share 85% identical residues and these CSPs have a pI of 5 (Chapter 2). Upon cold shock of *L. lactis* IL1403 from 30°C to 15°C a 10-fold induction of *cspB* directed ß-galactosidase activity is observed (Chapot-Chartier *et al.*, 1997). Similar cold-induced expression has been reported for $cspA^E$ and revealed that the transient induction of $CspA^E$ is achieved at the level of transcription (Jiang *et al.*, 1993) and at the level of mRNA stabilization (Brandi *et al.*, 1996; Goldenberg *et al.*, 1996; Fang *et al.*, 1997). Furthermore, it has been reported that mRNAs of cold-induced proteins are still translated at cold-shock conditions because of the presence of a so-called downstream box (DB), which enhances the capacity to form the translation initiation complex with non-adapted ribosomes at low temperature (Mitta *et al.*, 1997). The presence of CSPs in the cell is also determined by the stability of the proteins. The CSPs of *B. subtilis*

undergo very rapid folding and unfolding transitions and they exhibit low conformational stability in solution. These CSPs are rapidly degraded by proteases *in vitro* but are protected against proteolysis by the binding to RNA (Graumann *et al.*, 1997).

Overproduction of $CspA^E$ lead to an increase in the levels of three cold-induced proteins (CIPs; Jones *et al.*, 1992A). Moreover, the heterologous expression of $CspB^B$ in *E. coli* resulted in a reduction of cellular growth and in differences in the production of several proteins that resembles the cold-shock response (Graumann and Marahiel, 1997). For *csp* deleted *B. subtilis* strains compensatory effects of the remaining CSPs have been reported (Graumann *et al.*, 1997) and a similar response might be expected for *L. lactis*. Moreover, the physiological effects of overproduction of different members of a CSP family have never been studied. For these reasons, we used the nisin controlled expression system (Kuipers *et al.*, 1998) to overproduce the CSPs of *L. lactis* and, subsequently, to monitor their physiological and regulatory effects. CspB, CspD and CspE could be overproduced to high levels whereas for CspA and CspC only low overproduction levels were detected, due to low protein and mRNA stability at 30°C, respectively. Overproduction of specific CSPs revealed major induction of other CSPs and CIPs, indicating a regulatory function for these proteins. *L. lactis* strains overproducing CspB or CspE did not show a shorter lag time upon cold shock, but did show enhanced survival to freezing.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. The *L. lactis* strains used in this study were cultured at 30°C without aeration in M17-medium containing 0.5% glucose. *L. lactis* was transformed by electroporation as described by Wells *et al.* (1993). *E. coli* M1601 was used as a host for cloning experiments and was grown in tryptone-yeast medium with aeration at 37°C (Casabadan and Cohen, 1980). Chloramphenicol was used as a selection marker at a concentration of 10 $\mu g\ ml^{-1}$. Growth of *L. lactis* was monitored by measurement of the optical density at 600 nm (OD_{600}).

DNA techniques and DNA sequence analysis. PCR amplifications were performed as previously described by Kuipers *et al.* (1991) in 25 cycles (denaturation step at 95°C for 30 s, primer annealing step at an appropiate temperature for 1 min and primer extension step at 72°C for 2 min). All manipulations with recombinant DNA were carried out following standard procedures (Sambrook *et al.*, 1989). Plasmid DNA and chromosomal DNA of *L. lactis* were isolated as described previously (Vos *et al.*, 1989). DNA sequences were determined on both strands using an ALF DNA sequencer (Pharmacia Biotech, Uppsula, Sweden) and were analyzed using Clone (Version 4.0; Clone Manager) and Lasergene (DNASTAR Inc., USA) software.

Construction of plasmids for overexpression. *cspA*, *cspB*, *cspC* and *cspE* were amplified by PCR employing the primers listed in Table 1, containing either a *Nco*I site (forward primers) or a

*Hin*dIII site (reverse primers, except for OECspERev, which contains a *Xho*I site because of the presence of a *Hin*dIII site 6 bp downstream of the structural *cspE* gene). The obtained PCR products were digested with *Nco*I and *Hin*dIII (or *Xho*I) and cloned in pNZ8032 (De Ruyter *et al.*, 1996A) digested with the same restriction enzymes. In this way, a translational fusion is obtained of the *nisA* promoter to the start codon of the respective *csp* gene that hereby replaces the *gusA* gene that was originally present in pNZ8032. The constructs were made in such a way that each *csp* gene contained its own putative terminator. Each of the resulting plasmids (Table 1) was transformed to *L. lactis* NZ3900, that contains the *nisR* and *nisK* genes of the two-component nisin transduction pathway integrated on the chromosome (De Ruyter *et al.*, 1996B). Overproduction of CspD was obtained using *L. lactis* NZ3900 containing pNZOECspD as was described previously (Chapter 3). To obtain overproduction the appropriate strain was cultured to an OD_{600} of 0.3 followed by the addition of M17W-nisin which has a higher induction capacity and a lower growth inhibitory effect compared to wild-type nisin (Kuipers *et al.*, 1995). For optimal overexpression the strains were incubated at 30°C for 90 min. By the insertion of a *Nco*I site one bp was mutated in the second codon of *cspA*, *cspB* and *cspC*. Consequently, the second amino acid was changed from isoleucine to valine, from threonine to alanine and from asparagine to aspartic acid for CspA, CspB and CspC, respectively.

Table 1. Plasmids and oligonucleotides used in this study.

Plasmid or oligonucleotide	Relevant property	Reference
Plasmids		
pNZ8020	Cm^R	De Ruyter *et al.*, 1996A
pNZ8032	Cm^R	De Ruyter *et al.*, 1996A
pNZ8048	Cm^R	Kuipers *et al.*, 1998
pNZOECspA	Cm^R; overexpression *cspA*	This study
pNZOECspB	Cm^R; overexpression *cspB*	This study
pNZOECspC	Cm^R; overexpression *cspC*	This study
pNZOECspD	Cm^R; overexpression *cspD*	Chapter 2
pNZOECspE	Cm^R; overexpression *cspE*	This study
pNZOECspA*	Cm^R; overexpression *cspA**	This study
Oligonucleotides	Sequence	
OECspAFor	5'-GCTGC CATGG TAAAT GGAAC AGTAA AATGG-3'	
OECspARev	5'-GGTCA AGCTT ATAAA CTGTT AGGAA AGCAA-3'	
OECspBFor	5'-GCTGC CATGG CAAAA GGAAC TGTAA AATGG-3'	
OECspBRev	5'-CGACA AGCTT GGAAA GCAAC TAATC TTTCC-3'	
OECspCFor	5'-GCTGC CATGG ATAAA GGAAC AATAA AATTGG-3'	
OECspCRev	5'-GCTGA AGCTT AGGGA AGTGT GAGTT TCCTC-3'	
OECspEFor	5'-GCTGC CATGG CACAA GGAAC TGTTA AATGG-3'	
OECspERev	5'-GCAGC TCGAG TGTTA AGGCT TTCAT TATAA G-3'	
OECspA*Rev	5'-GCTGG GTACC CAAAA TTTCT ACTTA ATCTA TATTT GAAGC ATANG GCCCT CGACG-3'	

A CspA mutant was constructed in which the Arg residue at position 58 was replaced by Pro. Using the primers OECspAFor and CspA*Rev (containing a *KpnI* site; Table 1) the mutated *cspA* gene was amplified, digested with *NcoI* and *KpnI* and was subsequently cloned in the vector pNZ8048 (Table 1; Kuipers *et al.*, 1998). The generated plasmid was sequenced on both strands, revealing the presence of the desired mutation leading to the Arg-58-Pro substitution. Moreover, a frame-shift occurred in the penultimate codon 65 that resulted in a C-terminal deletion of Lys and Val, resulting in a 64 residue R58P-CspAΔK65V66 mutant, designated CspA*.

Freeze challenge. To study the effects of CSPs on the freeze survival of *L. lactis* a freeze-thaw challenge with strains overproducing the CSPs to different levels was performed as described previously (Chapter 3). In short, 1 ml samples of cultures were withdrawn, spun down, resuspended in fresh medium, the number of c.f.u. was determined and the samples were directly frozen at –20°C for 24 h. After this freezing period the samples were thawn at 30°C for 4 min and at this time point the number of c.f.u. was determined. Next, the sample was frozen again at –20°C and this cycle was repeated for an additional 3 times. Freeze-thaw challenges were performed in duplicate and the results are indicated as the percentage of cells remaining alive relative to the number of cells before the first freeze period (set at 100%).

Protein extraction and protein analysis using one-dimensional gel electrophoresis (1D-EF) and two-dimensional gelelectrophoresis (2D-EF). Proteins were extracted by homogenizing using a MSK cell homogenizer (B. Braun Biotech International, Melsungen, Germany) and zirconium beads (0.1 mm Biospec Products, Bartlesville, OK). Protein analysis was performed using tricine-SDS-PAGE (one-dimensional) as described by Schägger and Von Jagow (1987) or using 2D-EF as described previously (Chapter 3). Equal amounts of protein were loaded on the 1D-EF (20 μg) and 2D-EF gels (40 μg). A low molecular size marker (protein bands of 16.9, 14.4, 10.7, 8.2, and 6.2 kDa) and a high molecular size marker (protein bands of 94, 68, 43, 29, 18.4 and 14.4 kDa) were used as size markers (both purchased from Pharmacia Biotech, Uppsula, Sweden). 1D-EF gels were stained using Coomassie Brilliant Blue and 2D-EF gels were colored using silver staining. The 2D-EF gels were analyzed using GEMINI software (Applied Imaging, Sunderland, England).

Determination of N-termini. 500 μg of protein was loaded on the 2D-EF gels for determination of the N-terminus of specific spots, using identical conditions as for the running of analytical gels. The proteins were blotted on an Immobilon-P-Transfer membrane (Millipore, Bedford, USA) using a Trans Blot unit following the instruction of the manufacturer (Biorad, Richmond, USA). The proteins were stained with Coomassie Brilliant Blue and fragments of the blot were subjected to the Edman procedure and subsequent analysis using the Model 476A Protein Sequencing System (Applied Biosystems, USA) at the Sequence Center (University Utrecht, The Netherlands). By using the BlastP database the derived N-termini were screened for sequence similarities.

mRNA analysis. RNA was isolated and Northern-blot analysis was performed as described

previously (Kuipers et al., 1993). Equal amounts of RNA were separated on 1%-agarose gels and blotted on GeneScreen Plus Membrane (Dupont, Nen Research Products, USA). The blots were hybridized with γ-^{32}P-ATP labelled probes specific for the respective *csp* genes (Chapter 2).

RESULTS

Overproduction of CSPs in *L. lactis*. To elucidate the role of lactococcal CSPs in cold adaptation, *L. lactis* strains overproducing these specific CSPs were constructed (Table 1). Considerable overproduction was achieved for CspB, CspD and CspE, which yielded overproduction to 11%, 13% and 19% of total protein, respectively. Strikingly, for CspA and CspC a much lower overproduction level was obtained (approximately 0.5% and 0.3% of total protein, respectively) even using a 4-fold higher concentration of nisin (Fig. 1A). By using the nisin-controlled expression system stepwise overproduction of the CSPs could be achieved by the

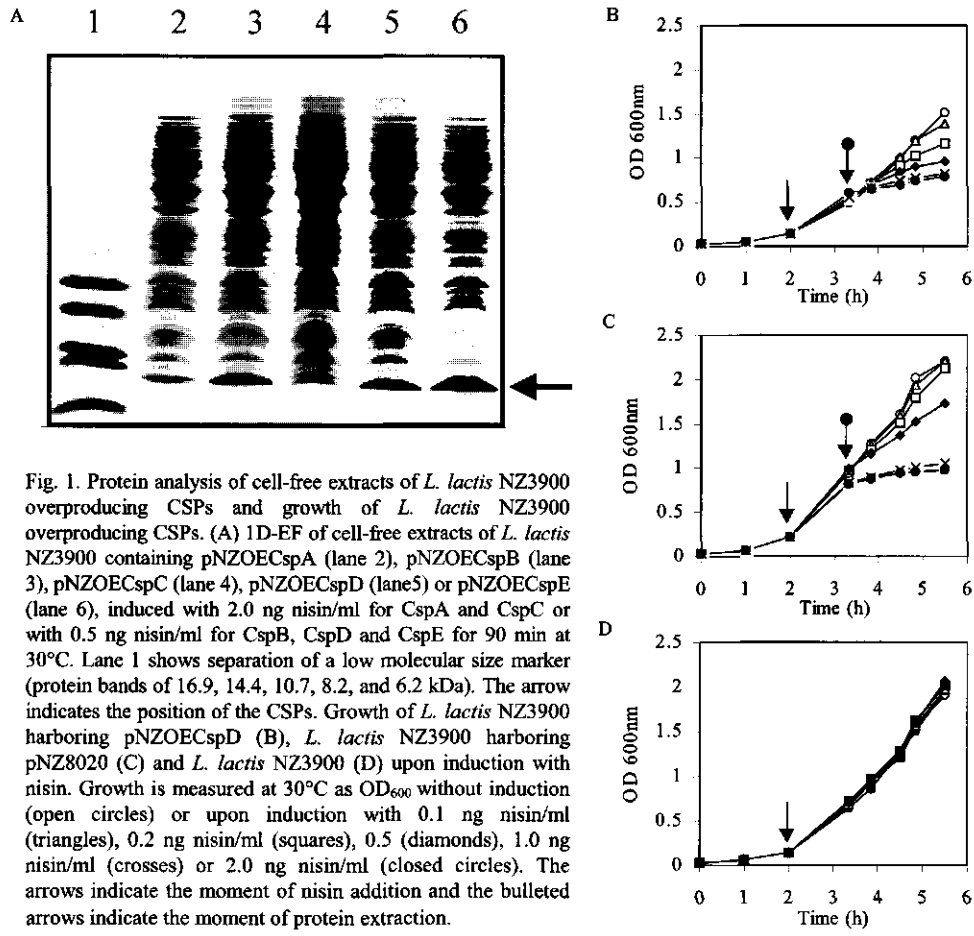


Fig. 1. Protein analysis of cell-free extracts of *L. lactis* NZ3900 overproducing CSPs and growth of *L. lactis* NZ3900 overproducing CSPs. (A) 1D-EF of cell-free extracts of *L. lactis* NZ3900 containing pNZOECspA (lane 2), pNZOECspB (lane 3), pNZOECspC (lane 4), pNZOECspD (lane5) or pNZOECspE (lane 6), induced with 2.0 ng nisin/ml for CspA and CspC or with 0.5 ng nisin/ml for CspB, CspD and CspE for 90 min at 30°C. Lane 1 shows separation of a low molecular size marker (protein bands of 16.9, 14.4, 10.7, 8.2, and 6.2 kDa). The arrow indicates the position of the CSPs. Growth of *L. lactis* NZ3900 harboring pNZOECspD (B), *L. lactis* NZ3900 harboring pNZ8020 (C) and *L. lactis* NZ3900 (D) upon induction with nisin. Growth is measured at 30°C as OD_{600} without induction (open circles) or upon induction with 0.1 ng nisin/ml (triangles), 0.2 ng nisin/ml (squares), 0.5 (diamonds), 1.0 ng nisin/ml (crosses) or 2.0 ng nisin/ml (closed circles). The arrows indicate the moment of nisin addition and the bulleted arrows indicate the moment of protein extraction.

addition of increasing concentrations of nisin (see below). Upon addition of increasing concentrations of nisin the growth rate of *L. lactis* NZ3900 harboring the overexpression constructs decreased significantly (Fig. 1B, shown for CspD) while that of the control strain was slightly reduced (Fig. 1C). The growth rate of NZ3900 (plasmid-free) was not reduced upon incubation with the same concentrations of nisin (Fig. 1D), excluding an antimicrobial effect of nisin at the concentrations used.

Limited overproduction of CspA and CspC is explained by low protein and mRNA stabilty. To further investigate the differences in overproduction levels obtained for the two groups of lactococcal CSPs, the mRNA levels of lactococcal *cspA*, *cspC* and *cspD* (positive control) were monitored upon overexpression (Fig. 2A). For *cspA* and *cspD* a major increase of the mRNA level was observed upon induction with 0.5 and 2.0 ng nisin/ml. Thus, despite the similar high mRNA levels for *cspA* and *cspD*, only a concomitantly high protein level is obtained for CspD. For *cspC* a low level of mRNA induction is observed compared to the mRNA levels of *cspA* and *cspD* which offers an explanation for the low amount of CspC obtained. Since the transcription signals are identical for all *csp* overexpression constructs, this low mRNA level is most likely caused by the low stability of the *cspC* transcript at 30°C.

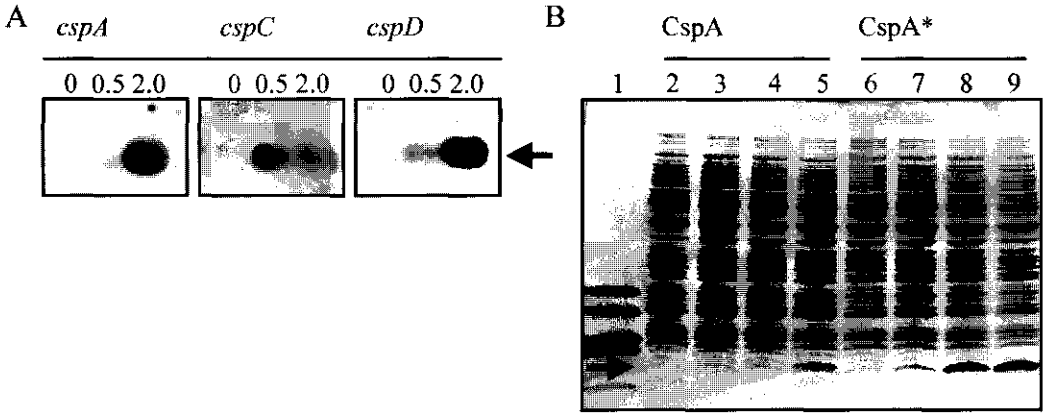


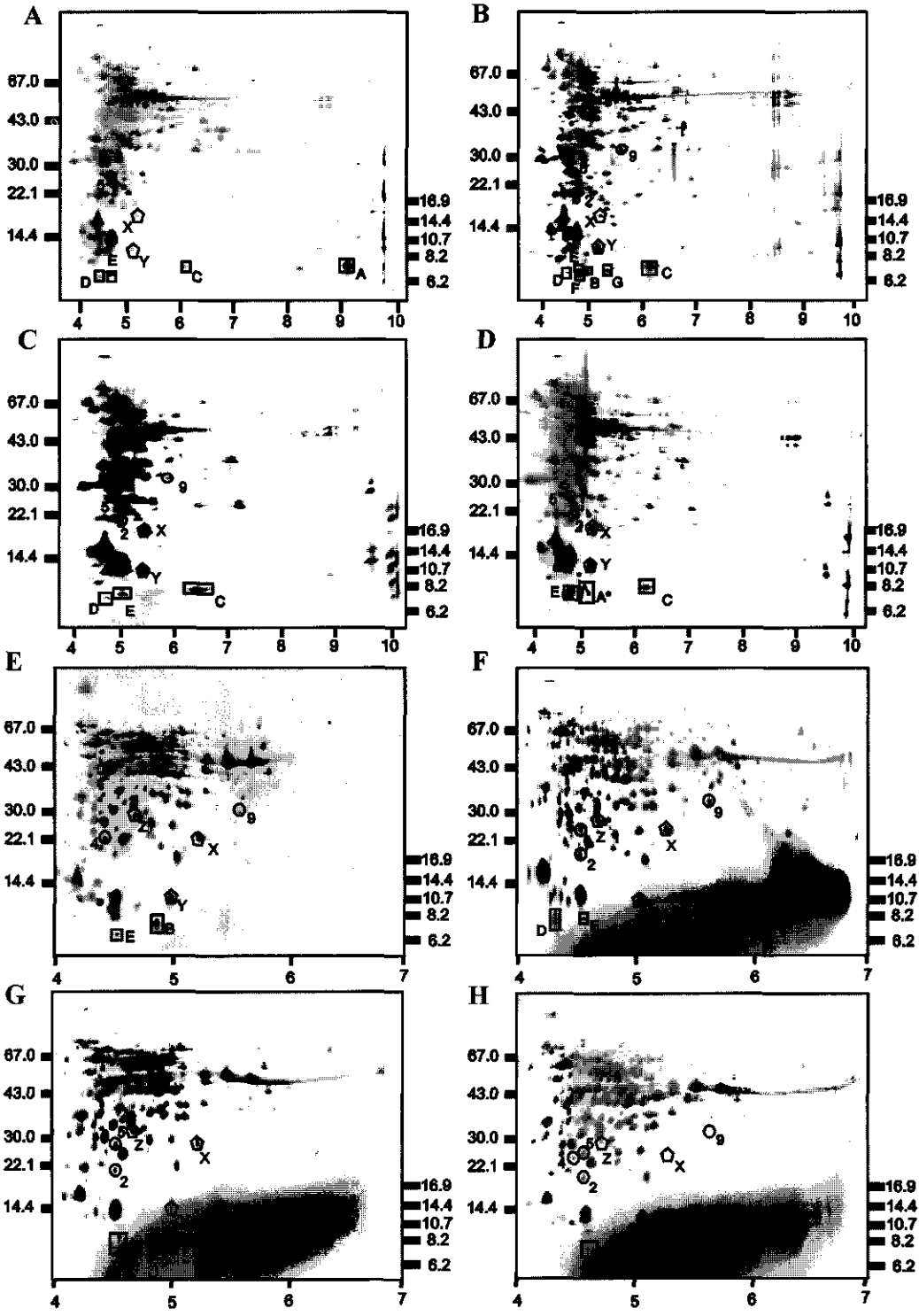
Fig. 2. Analysis of *L. lactis* NZ3900 overproducing CspA or CspC at mRNA and protein level. (A) Northern analysis of *cspA* (left), *cspC* (center) and *cspD* (right) in *L. lactis* NZ3900 containing pNZOECspA, pNZOECspC or pNZOECspD, respectively, upon induction with 0, 0.5 and 2.0 ng nisin/ml for 90 min at 30°C. Equal amounts of total RNA were loaded on the gel and the arrow indicates the *csp* transcripts. The film for the detection of the *cspC* transcript was exposed four times longer to the blot compared to films for *cspA* and *cspD*. (B) 1D-EF of cell-free extracts of *L. lactis* NZ3900 containing pNZOECspA or pNZOECspA* without induction (lane 2 and 6) and upon induction with 0.2 (lane 3 and 7), 0.5 (lane 4 and 8) and 2.0 ng nisin/ml (lane 5 and 9), respectively. In lane 1 a low molecular weight marker (16.9, 14.4, 10.7, 8.2 and 6.2 kDa) is separated. Equal amounts of protein were loaded on the gel and the arrow indicates the position of CSPs.

CspA and CspC contain an Arg residue at position 58 whereas CspB, CspD and CspE contain a Pro residue at this position. The absence of the Pro residue might result in a lower conformational stability of CspA and CspC because Pro residues are known to reduce the entropy of unfolded proteins (Schindler *et al.*, 1999). The stability of *L. lactis* CspA was investigated by constructing a CspA-mutant in which the Arg residue at position 58 was replaced by Pro. A much higher production level of CspA* (approximately 20-fold upon induction with 2.0 ng nisin/ml) was detected compared to the overproduction of native CspA, resulting in CspA* amounting to 9% of total protein (Fig. 2B). It was calculated that the Arg-Pro substitution results in a change of the calculated pI of CspA from 8.5 to 6.5. The mutation of Ile to Val in the second codon did not affect the CspA pI but the C-terminal deletion of Lys and Val, further reduced the calculated pI of CspA* to 4.8 (see below and Materials and Methods).

Regulatory role of CSPs in cold-shock adaptation. To study the effect of the overproduction of CSPs on protein synthesis patterns in *L. lactis*, 2D-EF was performed. The overproduced CSPs migrated approximately at their calculated pIs, except for CspC, as was confirmed by N-terminal sequencing (Fig. 3). Overproduction of CspC in *L. lactis* results in induction of CspB, protein F (putative CspF) and a previously not identified protein G (putative CspG) in the 7 kDa region. Upon induction of control cells (*L. lactis* harboring pNZ8020) with 2.0 ng nisin/ml increased production levels of CspC, CspD and CspE were observed compared to non-induced cells (data not shown). Overproduction of CspA did not result in induction of CSPs whereas overproduction of CspA* resulted in a dramatic increase in the CspE level compared to control cells. Furthermore, overproduction of CspB, CspD or CspE did not affect the level of any of the other CSPs (Fig. 3E-H; data not shown for CspA that is only visualized on gels of the pI 3-10 range).

Upon analysis of the 2D-EF gels outside the 7 kDa region several induced and repressed proteins were observed (Fig. 3). Several CIPs of *L. lactis* (Chapter 3) could be identified among the induced proteins. Overproduction of CspA results in the induction of CIP2 (hypothetical 50S-ribosomal protein L9) and CIP5 (N-terminal block) while upon overproduction of CspC also CIP9 (N-terminus not determined) and several other proteins were induced. For the overproduction of CspA*

Fig. 3. 2D-EF analysis of cell-free extracts of *L. lactis* NZ3900 overproducing the lactococcal CSPs. Cell-free extracts were isolated from *L. lactis* NZ3900 containing pNZOECspA (A), pNZOECspC (B), pNZ8020 (C) or pNZOECspA* (D) upon induction with 2.0 ng nisin/ml and were separated on a pI range of 3-10. Cell-free extracts were also isolated from *L. lactis* NZ3900 containing pNZOECspB (E), pNZOECspD (F), pNZOECspE (G) or pNZ8020 (H) induced with 0.1 ng nisin/ml and were separated on a pI range from 4 to 7. Equal amounts of protein were loaded on the gel and visualized using silver staining. Molecular size markers are indicated on the left (high molecular weight marker) or on the right (low molecular weight marker). CSPs of *L. lactis* are boxed and the lettering is according to the CSP family (F = putative CspF, G = putative CspG) and the calculated pIs are 8.5, 5.0, 9.1, 4.5 and 4.6 for CspA, CspB, CspC, CspD and CspE, respectively. Spots induced by the overproduction of the CSPs are circled and numbered if they belong to the previously identified CIPs (Chapter 3) or are pentagonized and lettered (X, Y, Z), if they are induced during the specific overproduction of CSPs.


A
B
C
D
E
F
G
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67.0
43.0
30.0
22.1
14.4
16.9
14.4
10.7
8.2
6.2
4
5
6
7
8
9
10

induction of both CIP2 and CIP5 is observed. Furthermore, upon overproduction of CspB the levels of CIP4 (N-terminus not determined) and CIP9 increased with a factor of 2.5 and 3 (compared to values observed in the control gel), respectively. The overproduction of CspD resulted in increased levels (2.5-, 2- and 4-fold) for CIP2, CIP5 and CIP9, respectively, while overproduction of CspE resulted in increased levels of CIP2 (2-fold) and CIP5 (2-fold). The changes in the expression levels of these CIPs indicate that CSPs might play a regulatory role in the induction of specific proteins involved in the cold-adaptation process. On the other hand, increased production is also observed for three non-cold induced proteins (proteins X, Y [homologous to CelA of *B. subtilis*] and Z) upon overproduction of CspB, CspD or CspE. The production of these proteins did not increase upon overproduction of CspA or CspC (Fig. 3).

Effect of overproduction of CSPs on cold adaptation and freeze-survival of *L. lactis*. The effect of overproduction of CspB or CspD, the major cold-induced CSPs of *L. lactis*, on the adaptation to cold shock was tested. These CSPs were overproduced using relatively low nisin concentrations (0.05 and 0.1 ng nisin/ml), yielding expression levels comparable to cold-shock conditions, after which the cultures were exposed to 10°C (without the addition of nisin). For all conditions an identical adaptation time of 6-7 h is observed (data not shown) indicating no beneficial effect on cold adaptation of elevated CspB or CspD expression prior to cold shock.

The effect of CSPs on survival to freezing was tested by exposure of cultures overproducing one of the CSPs to a freeze-thaw challenge (Fig. 4). The overproduction of CspB (induced with 0.5 ng nisin/ml) resulted in approximately 10-fold increased survival compared to that of non-induced cells after four repetitive freeze-thaw cycles. Following overproduction of CspE a smaller positive effect (5-fold increment maximally) on freeze survival is observed, whereas for control cells (*L. lactis* harboring pNZ8020) no effect of higher concentrations of nisin is noted (data not shown). For *L. lactis* cells overproducing CspA, CspC or CspA* no additional freeze-protective effect was observed compared to control cells (data not shown).

DISCUSSION

In this report, we describe the effects of the overproduction of five different CSPs of the LAB *L. lactis*. The family of CSPs of *L. lactis* can be divided into two groups, consisting of CspA and CspC on one hand and CspB, CspD and CspE on the other, based on amino acid composition and pI (Chapter 2). Overproduction of CSPs of the latter group resulted in much higher overproduction levels (11-19% versus 0.3-0.5% of total protein) compared to the levels obtained for CspA and CspC. The CSPs migrated on the 2D-EF gels at their calculated pIs, except for CspC that has a calculated pI of 8.4 (instead of 9.1) in the overproduction construct because of the Asp-Asn substitution at the second codon. However, the CspC protein was found to migrate at a pI of approximately 6 as was confirmed by N-terminal sequencing. No formylation of the N-terminus, as

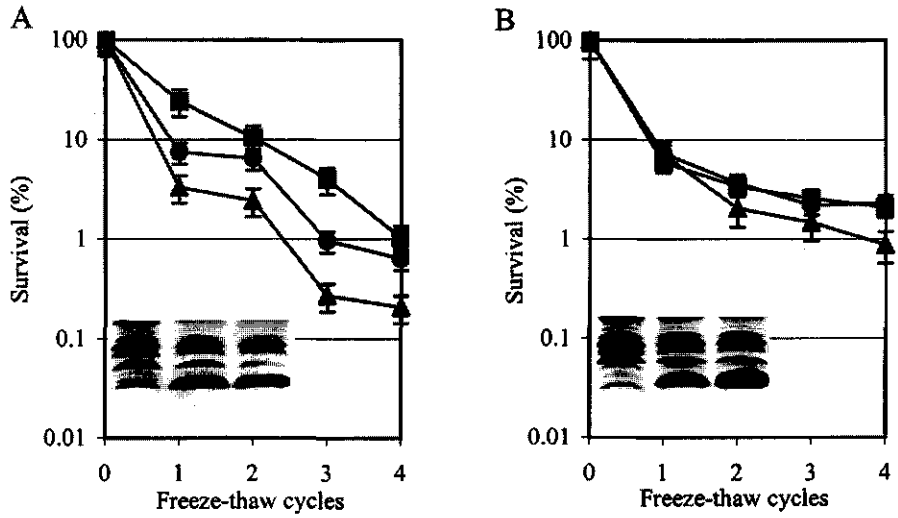

Fig. 4. Survival to freezing of *L. lactis* NZ3900 upon overproduction of CspB (A) and CspE (B). The freeze survival is depicted as the percentage of surviving cells compared to the number of cells prior to freezing for *L. lactis* NZ3900 harboring pNZOECspB or pNZOECspE without induction (triangles) or with induction of 0.2 ng nisin/ml (circles) or 0.5 ng nisin/ml (squares). Inserts show fragments of 1D-EF gels on which the cell-free extracts of the cultures are separated (the region of 7-kDa CSPs is shown; left, center and right: 0, 0.2 or 0.5 ng nisin/ml, respectively).

found for $CspB^B$ (Graumann *et al.*, 1996), was observed and it is speculated that the pI-shift is caused by post-translational modifications. Furthermore, two new proteins in the 7-kDa region were identified for *L. lactis*: one designated CspF that was also induced upon cold shock as described previously (Chapter 3) and another designated CspG with a pI of 5.5. These putative CSPs might be encoded by genes located on an uncharacterized 3.5 kb *Hin*dIII-hybridizing fragment on the *L. lactis* chromosome (Chapter 2). The growth rate of *L. lactis* is reduced upon overproduction of CspB, CspD and CspE at 30°C. Also for control cells (*L. lactis* NZ3900 harboring pNZ8020) a slightly reduced growth rate was observed upon incubation with nisin, but the overproduction of CSPs further reduced growth and this might be explained by energy consumption and more intensive occupation of the transcription and translation machinery. Similar growth inhibitory effects were observed for the heterologous expression of $CspB^B$ in *E. coli* at 37°C (Graumann and Marahiel, 1997).

The observation that CspB, CspD and CspE of *L. lactis* can be overproduced to high quantities at 30°C is remarkable. The artificial overexpression of the $cspA^E$ gene was very low at 37°C due to its low mRNA stability (Goldenberg *et al.*, 1996). The low quantity of overexpression obtained for the lactococcal *cspC* gene is indeed explained by low stability of the transcript at 30°C as was shown using Northern blotting. Since for *cspA* a high mRNA induction is observed, the low quantity of CspA overproduction should be explained by other factors. CspC of *B. subtilis* contains an Ala residue at position 58 whereas CspB and CspD of *B. subtilis* contain a Pro residue at this

position and, indeed, $CspB^B$ and $CspD^B$ were far more stable than $CspC^B$ (Schindler *et al.*, 1999). CspA* can be overproduced to much higher quantities at 30°C compared to CspA which can be explained by the high stability of the protein as a consequence of the reduced entropy. Next to the specific Arg-Pro mutation, also the decrease in pI may contribute to CspA* stability. Recently, it was shown that the stability of the CSPs of *B. subtilis* significantly increased in the presence of a nucleic acid ligand. It is suggested, that the stability of these CSPs *in vivo* is mediated by the binding to mRNA (Schindler *et al.*, 1999), which might be largely dependent on the overall charge of the protein. Schröder *et al.* (1995) suggested that CSPs may act as RNA-chaperones since they possess a positively charged RNA binding epitope that is backed by a negatively charged surface that would prevent approach of RNA by charge repulsion. Mutation of the surface-exposed Phe residues, important for nucleic acid binding, at positions 15, 17 and 27 has been found to result in decreased stability of the protein, probably due to a decreased nucleic acid binding capacity that makes the protein more susceptible to protease action (Schindler *et al.*, 1998; Schindler *et al.*, 1999). This would offer an alternative explanation for the increased stability of CspA* and could also explain why the CSPs of *L. lactis* that have a low pI can be overproduced up to high quantities. Overproduction was also examined at 10°C and still no overproduction of CspA and CspC was observed, indicating that mRNA and/or proteins are not sufficiently stabilized to yield detectable protein levels (data not shown). For *L. lactis* NZ3900 harboring pNZOECspD moderate CspD overproduction was observed at 10°C compared to control cells (data not shown), which shows that the nisin controlled expression system is functional at low temperature, however, with a reduced efficiency.

Upon overproduction of CspC the level of CspB, putative CspF and putative CspG increased, whereas the overproduction of CspA, CspB, CspD or CspE did not affect the expression of other CSPs. CspC might directly stimulate the expression of the other CSPs by transcriptional activation as has been reported for genes regulated by $CspA^E$ for which Y-box motifs (ATTGG or CCAAT) have been shown to be important (Jones *et al.*, 1992B; LaTeana *et al.*, 1991; Brandi *et al.*, 1994). Several of these elements are observed in the upstream regions of the lactococcal *csp* genes (Chapter 2). The observation that all lactococcal CSPs induce the expression of certain proteins, including several CIPs, indicates a regulatory function for this group of proteins. The production of CIP2, CIP4, CIP5 and CIP9 seems to be regulated by several CSPs indicating overlap in regulatory pathways. The N-terminus of CIP2 was identified and shows homology to the 50S ribosomal L9 of *B. subtilis*. Cold-induced ribosomal proteins are reported for both *E. coli* (S1, S6, L7/L12; Jones *et al.*, 1992A) as well as *B. subtilis* (S6, L7/L12; Graumann *et al.*, 1996) and are suggested to be essential for the correct assembly of rRNA at low temperatures. For the overproduction of CspB, CspD and CspE also induction is observed for non-cold induced proteins X, Y (CelA) and Z. The observed induction of protein Y, homologous to a cellobiose-specific enzyme II subunit of the

phosphotransferase-system of *B. subtilis* (Sadaie *et al.*, 1997), might be an indication for a role of CSPs in other processes than cold adaptation, e.g. sugar metabolism.

Overproduction of CSPs did not stimulate adaptation of *L. lactis* to cold-shock conditions. However, similar to the overproduction of CspD (Chapter 3), also overproduction of CspB and CspE yielded 10-fold and 5-fold increased freeze survival compared to control cells, respectively. For *B. subtilis* disruption of $cspB^{B}$ resulted in a decreased freeze survival (14-fold compared to wild-type cells) indicating an essential role for this gene in freeze protection (Willimsky *et al.*, 1992). CspB, CspD and CspE may enhance the freeze survival of *L. lactis* either directly by protecting RNA or DNA by the moderate, aspecific binding activities mentioned for CSPs (e.g. RNA stabilization) or indirectly by the induction of other factors that supply cryoprotection. The proteins that are induced upon overproduction of the lactococcal CSPs might be involved in cryoprotection of *L. lactis*.

In this report the overproduction of specific CSPs of *L. lactis* and several physiological effects are studied. Overproduction to different quantities could be obtained for all CSPs depending on mRNA and protein stability. 2D-EF revealed that overproduction of CSPs at 30°C resulted in the induction of a specific group of proteins, including CIPs. It is concluded that CSPs of *L. lactis* play a regulatory role in the cold-shock response and that they control the production of both CSPs and CIPs.

Chapter 6

Multiple disruptions of cold-shock genes in *Lactococcus lactis* MG1363 show the direct involvement of cold-shock proteins in gene regulation

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ABSTRACT

We report several regulatory and physiological effects of the deletion of multiple *csp* genes of the lactic acid bacterium *Lactococcus lactis* MG1363. *L. lactis* strains NZ9000ΔAB, deleted in *cspA* and *cspB*, and NZ9000ΔABE, deleted in *cspA*, *cspB* and *cspE*, were constructed by double cross-over recombination. Both strains showed no differences in growth at normal and at low temperature compared to that of the wild-type strain *L. lactis* NZ9000. However, the adaptive response to freezing by prior exposure to 10°C was significantly delayed in strain NZ9000ΔABE compared to strains NZ9000 and NZ9000ΔAB, indicating a possible role of CspE in freeze adaptation. Northern analysis showed that deletion of *csp* genes was compensated by an increased expression of the remaining *csp* genes, indicating a tightly controlled transcriptional network. CspA and/or CspB may act as repressor(s) of *cspE* expression at low temperature and it is suggested that CspE is involved in repressing the expression of *cspC* and *cspD* either at the transcriptional level or by decreasing their mRNA stabilities. Furthermore, it was established by a proteomics approach that in the wild-type situation CSPs regulate the expression of cold-induced proteins that are no longer cold-induced in the CSP-deleted strains. For these regulatory circuits no compensatory mechanisms seem to have evolved, in part explaining the presence of a family of CSPs that consists of very similar members.

Submitted for publication

INTRODUCTION

Several bacteria react to a sudden down-shift in temperature by the production of a set of proteins, together forming the cold-shock stimulon, that includes small (7 kDa) cold-shock proteins (CSPs) such as CspA in *E.coli* ($CspA^E$) and CspB in *B. subtilis* ($CspB^B$). In a variety of bacteria, families of CSPs, consisting of 3 to 9 members, have been described. $CspA^E$ and $CspB^B$ show a similar β-barrel structure and residues important for DNA and RNA-binding have been determined. Both proteins contain two motifs, named RNP-1 and RNP-2, that are involved in RNA binding (reviews by Graumann and Marahiel, 1998 and Yamanaka *et al.*, 1998). For $CspA^E$ a broad range mRNA-binding capacity was determined (Jiang *et al.*, 1997) while CspB, CspC and CspD of *B. subtilis* bind to RNA in a co-operative and interactive manner suggesting that CSPs function as RNA chaperones that facilitate the initiation of translation (Graumann *et al.*, 1997). Furthermore, $CspA^E$ acts as a transcriptional activator for the *hns* and the *gyrA* genes of which the gene products, H-NS and GyrA, are both involved in DNA-supercoiling (LaTeana *et al.*, 1991; Jones *et al.*, 1992B; Brandi *et al.*, 1994). Disruption of $cspB^B$ results in a decreased freeze survival of *B. subtilis* cells (Willimsky *et al.*, 1992) and causes reduced expression of several proteins upon cold shock (Graumann *et al.*, 1996). Remarkably, deletions of $cspA^E$ and $cspB^B$ show no effect on the growth characteristics of *E. coli* and *B. subtilis*, respectively (Graumann *et al.*, 1996; Bae *et al.*, 1997). Multiple deletion of *csp* genes in *B. subtilis* revealed that the presence of at least one CSP is essential for cellular growth and efficient protein synthesis at optimal and low temperatures. Deletion of one or two counterparts of the CSP family resulted in a higher level of induction of the remaining CSP(s), suggesting that CSPs directly or indirectly downregulate the production of certain members of this protein family (Graumann *et al.*, 1997).

Regulation of *csp* genes takes place at several levels and for $CspA^E$ it was shown that cold-shock induction is achieved at the transcriptional level (Tanabe *et al.*, 1992; Goldenberg *et al.*, 1997) as well as at the level of mRNA stabilization (Brandi *et al.*, 1996; Goldenberg *et al.*, 1996; Fang *et al.*, 1997). The AT-rich sequence immediately upstream of the $cspA^E$ promoter functions as the UP-element to enhance transcription (Goldenberg *et al.*, 1997; Mitta *et al.*, 1997) while the mRNA stability is largely dependent on the presence of the unusually long 5'-untranslated leader (5'-UTR) of the $cspA^E$ genes. The cold-shock box (CS-box), located at the 5'-end of the UTR, is a binding site for a possible repressor and plays a negative role in $cspA^E$ transcription in cold-shock adapted cells (Fang *et al.*, 1998). A downstream box (DB) located 12 bases downstream the initiation codon of the $cspA^E$ mRNA is complementary to a region near the decoding region of the 16S rRNA. This element is essential for the mRNA translation during the adaptation time upon cold shock by the formation of a stable initiation complex (Mitta *et al.*, 1997). The increased presence of $CspA^E$ upon cold shock is also dependent on an increment of its stability at low temperature (Goldenberg *et al.*, 1996).

Lactic acid bacteria (LAB) are frequently used to start industrial food fermentations. Preceding and during production of these starter cultures and during manufacture and storage of the fermented products, LAB experience a variety of temperature changes. For these reasons, the cold adaptation response of LAB is gaining a lot of interest. Recently, a CSP family has been identified in the mesophilic *Lactococcus lactis* MG1363, consisting of at least five *csp* genes. The *L. lactis* chromosome was found to contain two tandemly located and cold-inducible *csp* genes (*cspA/cspB* and *cspC/cspD*) and a single, constitutively expressed *cspE* gene (Chapter 2). In recent years, the knowledge regarding *csp* gene expression and regulation significantly increased for a variety of bacteria. However, the understanding and the physiological role of CSPs and the explanation for the presence of CSP families remain to be elucidated. In this work, we characterize the regulatory and cold-response effects of multiple disruptions of *csp* genes of *L. lactis*. Major compensatory effects in expression are observed for the remaining CSPs upon the deletion of their counterparts of the CSP family, indicating a tightly controlled network for the expression of the lactococcal *csp* genes. Several cold-induced proteins (CIPs) were no longer cold-induced in the strains deleted in CspA and CspB or in CspA, CspB and CspE, indicating a regulatory role for these CSPs in the cold-shock response.

MATERIALS AND METHODS

Strains and culturing conditions. *L. lactis* NZ9000 (Kuipers *et al.*, 1998; Table 1) was used as the wild-type strain for the generation of *csp* deleted strains and has been generated from strain MG1614 which is a rifampicine and streptomycin-resistant derivative of strain MG1363 (Gasson, 1983). *L. lactis* was cultured at 30°C or at lower temperatures (as indicated) on M17-medium containing 0.5% glucose (GM17). Growth was monitored by measuring the optical density at 600 nm (OD_{600}). *E. coli* MC1061 (Casabadan and Cohen, 1980) was used as a host strain in cloning experiments and was grown in tryptone yeast extract medium with aeration at 37°C (Sambrook *et al.*, 1989). Antibiotics were used in the following concentrations: ampicillin 50 $\mu g\ ml^{-1}$; erythromycine 2.5 $\mu g\ ml^{-1}$.

Generation of *csp* deletions. *L. lactis* strains carrying deletions in their *csp* genes were constructed using a double cross-over replacement strategy (Leenhouts and Venema, 1993). For the generation of *L. lactis* strains deleted in their *csp* genes the regions flanking (approximately 800 bp) the respective genes were amplified using PCR as described by Kuipers *et al.* (1991). All manipulations with recombinant DNA were carried out following standard procedures (Sambrook *et al.*, 1989) and according to the specifications of the manufacturer (GIBCO/BRL Life technologies, Breda, The Netherlands). The oligonucleotides used for the PCR (Table 1) contain various restriction sites: the forward fragment 1 primers contain an *Eco*RI site, the reverse fragment 1

primers a *KpnI* site, the forward fragment 2 primers a *XbaI* site and the reverse fragment 2 primers contain a *SalI* site. The cloning of the amplified fragments in pUC18ERY (Kranenburg *et al.*, 1997) resulted in the plasmids pUCEryΔAB12 and pUCEryΔE12 (Amp^R, Ery^R; Table 1). The followed replacement strategy results in deletion of the *cspA-cspB* tandem repeat starting from the codon for the 12th amino acid (Asp) of CspA to 8 bp downstream of the coding region of *cspB*. For the deletion of *cspE* the region from the 8th amino acid residue (Trp) to 18 bp downstream of the coding region was removed. Using this strategy the promoter regions of the upstream *csp* genes remain intact. The obtained plasmids were transformed to *L. lactis* NZ9000 by electroporation (Wells *et al.*, 1993) and selected for erythromycine resistance in which the first integration took place. The erythromycine-resistant strains were cultured without selective pressure of erythromycine for 200 generations and were analysed for the occurrence of the second cross-over event. Candidates were verified using PCR and ultimately in a Southern blotting experiment using PCR-fragment 1 as a probe as described previously (Chapter 2). Using this strategy *L. lactis* strains deleted for *cspA* and *cspB* (NZ9000ΔAB) and deleted for *cspA*, *cspB* and *cspE* (NZ9000ΔABE) were obtained.

Table 1. Strains, plasmids and oligonucleotides used in this study.

Strain/plasmid/oligonucleotide	Relevant property	Reference
Strains		
L. lactis NZ9000		Kuipers *et al.* (1998)
L. lactis NZ9000ΔAB	NZ9000 derivative; Δ*cspA*, Δ*cspB*	This study
L. lactis NZ9000ΔABE	NZ9000 derivative; Δ*cspA*, Δ*cspB*, Δ*cspE*	This study
Plasmids		
pUC18ERY	Amp^R, Ery^R	Van Kranenburg *et al.* (1997)
pUC18ERYΔAB12	Amp^R, Ery^R	This study
pUC18ERYΔE12	Amp^R, Ery^R	This study
Oligonucleotides	Sequence	
CspAB1For	5'-GCAGG AATTC ATGCT CGCTC AGGCT CTTTT-3'	
CspAB1Rev	5'-AGCCG GTACC CATAT TGAAC CATTT TACTG-3'	
CspAB2For	5'-AGCGT CTAGA TAATT AATGA AAAAC GGAGC-3'	
CspAB2Rev	5'-CACGG TCGAC AAATC ACCAA ATTGA GGCAA-3'	
CspE1For	5'-CGCCG AATTC ATATG GATAA AGTTC GCCGT-3'	
CspE1Rev	5'-CGTCG GTACC ATTAA CAGTT CCTTG TGCCA-3'	
CspE2For	5'-GCTGT CTAGA CACTG ACAAA ATTGT CAGTG-3'	
CspE2Rev	5'-TCCCG TCGAC ATTCC GAGTC TAGCA TGGTG-3'	
PromCspE	5'-TCCTA TTCAA GAAAT TAGAT-3'	

Freeze-thaw challenge. The generated mutant strains and the wild-type strain were tested for their susceptibility towards freezing in a previously described freeze-thaw challenge (Chapter 3). In short, cells were cultured in GM17 medium until mid-exponential phase (OD_{600} 0.5) at 30°C and subsequently, rapidly downshifted in growth temperature from 30 to 10°C. The cultures were exposed to 10°C for different time periods (0, 2, 4, 6, 8 h) and, subsequently, 1 ml of these cultures was frozen at –20°C. After a 24 h freezing period the number of remaining viable cells (c.f.u.) was determined using plate counting following incubating for 48 h at 30°C. This freeze-thaw cycle was performed for four times in total.

mRNA analysis. RNA isolation, Northern blotting and subsequent hybridization with radiolabelled probes was performed as described previously (Vos *et al.*, 1989; Kuipers *et al.*, 1993). For the specific detection of the mRNAs of the *csp* genes previously described primers were used (Chapter 2). Quantification of the *csp* transcripts in Northern blotting was performed using the Dynamics Phosphor Imaging System (Dynamics, Rochester, NY). RNA was extracted from mid-exponential cultures and from cultures exposed to a cold shock to 10°C for several time periods. Equal amounts of total RNA were applied on the gel and a probe specific for lactococcal 16S rRNA (5'-ATCTACGCATTTCACCGCTAC-3'; Klijn *et al.*, 1991) was used for the quantification of the amount of RNA supplied on the gel.

Protein analysis. The protein composition of cell-free extracts was determined using two-dimensional gel electrophoresis (2D-EF) as described previously (Chapter 3). Total protein was extracted from cultures growing at mid-exponential phase at the optimal temperature (30°C) and from cultures exposed to a cold shock to 10°C for several hours using a cell MSK cell homogenizer (Braun Biotech International, Melsungen, Germany) and zirconium beads (Biospec Products, Bartlesville, OK). Equal amounts of protein were analysed using the Multiphor 2D-electrophoresis system (Pharmacia Biotech, Uppsula, Sweden) and protein spots were visualized using silver staining (Blum *et al.*, 1987). The 2D-EF gels were compared using the GEMINI-programme (Applied Imaging, Sunderland, United Kingdom). 500 μg of protein was loaded on the 2D-EF gels for determination of the N-terminus of specific spots, using identical conditions as for the running of analytical gels. The proteins were blotted on an Immobilon-P-Transfer membrane (Millipore, Bedford, USA) using a Trans Blot unit following the instruction of the manufacturer (Biorad, Richmond, USA). The proteins were stained with Coomassie Brilliant Blue and fragments of the blot were subjected to the consecutive Edman degradation and subsequent analysis using the Model 476A Protein Sequencing System (Applied Biosystems, USA) at the Sequence Center (University Utrecht, The Netherlands). Using the BlastP database the derived N-terminal sequences were screened for sequence similarities.

RESULTS

Generation and growth characteristics of *csp*-deleted strains. Using a double cross-over replacement strategy *L. lactis* strains deleted in the tandem *cspA* and *cspB* genes (NZ9000ΔAB) as well as carrying an additional deletion in *cspE* (NZ9000ΔABE) were obtained. The chromosomal configuration was confirmed by PCR (data not shown) and Southern hybridization with a radiolabelled internal *cspB* fragment as a probe, sharing homology to the known lactococcal *csp* genes (Fig. 1). Deletion of *cspAB* or *cspABE* did not affect the growth rate at optimal growth temperature (30°C) nor at other temperatures (4, 7, 10, 15, 20 or 42°C). Upon cold-shock treatment at 10°C, the adaptation level was found to be identical for the wild-type strain and the mutants (data not shown). The stationary phase and lysis behavior was monitored by following the OD_{600} but no differences were found in the development of absorbance for the *cspAB* and *cspABE* disrupted strains compared to the wild-type strain, in contrast to the situation for *B. subtilis* (Graumann *et al.*, 1997). Moreover, no differences in the number of colonies, the estimated lag time and the size and exterior of the colonies were observed upon incubation of the *csp* mutants on GM17-plates at the different temperatures. Finally, the *csp* mutants and the wild-type cells showed similar size, chain length and appearance (data not shown).

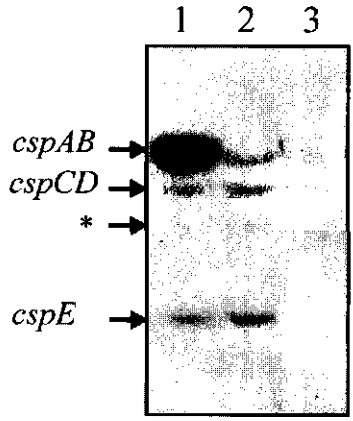


Fig. 1. Southern hybridization with PCR1 as a probe (Chapter 2) of chromosomal DNA of strains *L. lactis* NZ9000 (lane 1), NZ9000ΔAB (lane 2) and NZ9000ΔABE (lane 3) digested with *Hin*dIII. The arrows point to the hybridizing *Hin*dIII-fragments carrying the indicated *csp* genes. The asterisk indicates a possible sixth member of the lactococcal CSP family.

The cryoprotective response is delayed in the *cspABE* disrupted strain. *L. lactis* is able to adapt to freezing conditions by pre-exposure to 10°C, yielding increased survival to freezing (Chapter 3). Exponentially growing cells of strains NZ9000, NZ9000ΔAB and NZ9000ΔABE were exposed to 10°C for several hours and, subsequently, tested for survival to freezing (Fig. 2). For strain NZ9000, pre-exposure to 10°C for 2 and 4 h increased the survival to freezing approximately 50- and nearly 1000-fold, respectively, after 4 repetitive freeze-thaw cycles. Longer pre-exposure to 10°C (6 and 8 h) did not increase the survival any further (Fig. 2A). Strain NZ9000ΔAB showed an identical response to freezing as the wild-type strain, resulting in nearly 1000-fold increased

survival upon exposure to 10°C for 4 h compared to cells that were not exposed to low temperature (Fig. 2B). The freeze survival capacity of strain NZ9000ΔABE increased only approximately 10- and 100-fold after exposure to 10°C for 2 h and 4 h, respectively. Upon exposure to 10°C for 6 h the survival was found to be further increased and after exposure for 8 h the maximally 1000-fold cryoprotective effect was observed (Fig. 2C). This indicates that strain NZ9000ΔABE is able to pre-adapt to freezing conditions, but that this adaptation process is significantly delayed in comparison to wild-type cells.

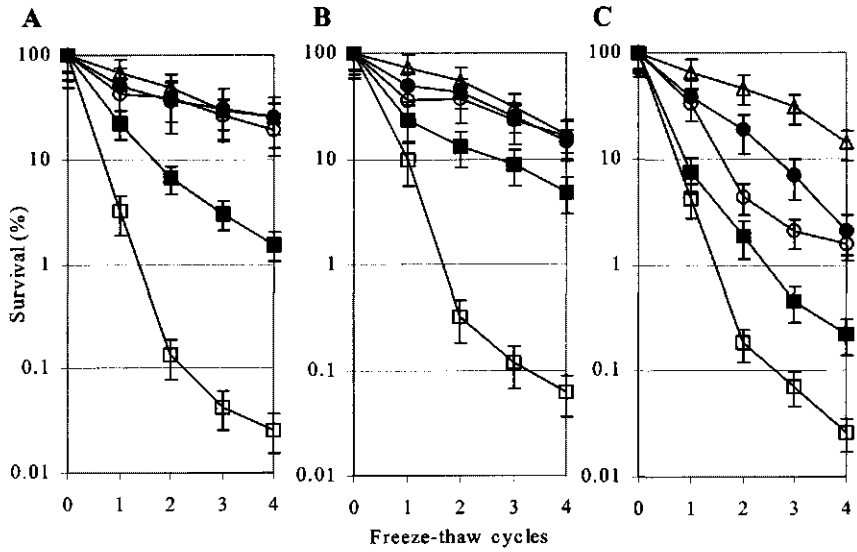


Fig. 2. Freeze survival of strains *L. lactis* NZ9000 (A), NZ9000ΔAB (B) and NZ9000ΔABE (C) after pre-treatment at 10°C for several hours. Survival to freezing (number of viable cells prior to freezing is set at 100%) of *L. lactis* after pre-exposure to 10°C for 0 h (open squares), for 2 h (closed squares), for 4 h (open circles), for 6 h (closed circles) and for 8 h (triangles). The data are shown as an average of two independent experiments and the error bars indicate the variation for each sample point.

Analysis of *csp*-mRNA levels in *csp*-disrupted strains. The mRNA levels of the five previously characterized *csp* genes were monitored using specific probes after different exposure times to cold shock to 10°C in wild-type and *csp*-deleted strains (Fig. 3). In strain NZ9000 cold-induced expression (up to 20-fold) was observed for *cspA, cspB* and *cspD* (Fig. 3), whereas *cspE* was only slightly induced upon cold shock (2-3 fold), similar as was reported previously for the parental strain *L. lactis* MG1363 (Chapter 2). For *cspC* hardly any mRNA could be detected at high as well as low temperature, probably due to its low expression. At 24 h after cold shock very low mRNA levels were observed for all *csp* genes. In strain NZ9000ΔAB the *cspE*-mRNA level increased upon exposure to low temperature compared to wild-type cells: at 2 h after cold shock a 6-

fold induction was observed, compared to a 3-fold induction for strain NZ9000. The expression of *cspC* and *cspD* in strain NZ9000ΔAB was similar to the expression in wild-type cells and no *cspA* and *cspB* transcripts were detected in this strain. For strain NZ9000ΔABE a major induction was noted in the quantity of *cspD* mRNA. At 30°C an increased *cspD*-mRNA level (5-fold) was detected compared to strains NZ9000 and NZ9000ΔAB and this level increased by 3-fold at 1 h after cold shock after which the *cspD*-mRNA level remained high until 4 h after cold shock. In contrast, a more gradual increase in *cspD* expression was observed for wild-type cells (Fig. 3). To analyze the regulatory effects of the disruption of *cspE* on the expression of *cspE* itself, the *cspE* mRNA level was also monitored using a probe specific to the region from base 44 to 63, a part of the 5'-UTR of the *cspE* mRNA that is not deleted from the chromosome of strain NZ9000ΔABE (Fig. 4D). In both strain NZ9000 and strain NZ9000ΔAB the *cspE*-mRNA level was at an approximately constant level with a slight increase (up to 3-fold) at 3, 4 and 24 h after cold shock (Fig. 4A and B). Strikingly, no *cspE*-leader mRNA of the expected size (115 nt) could be detected in strain NZ9000ΔABE (Fig. 4C), suggesting that expression of CspE is required for the expression of the *cspE* gene.

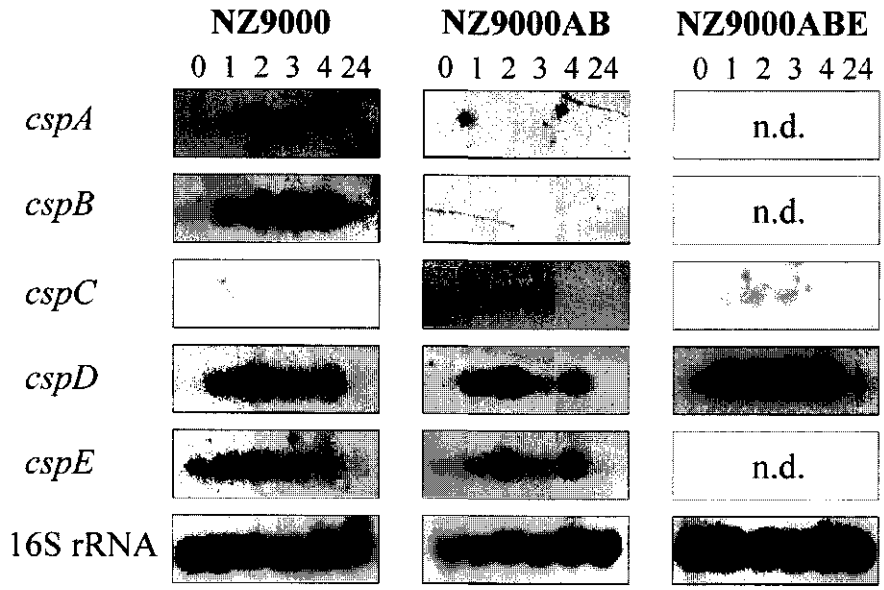
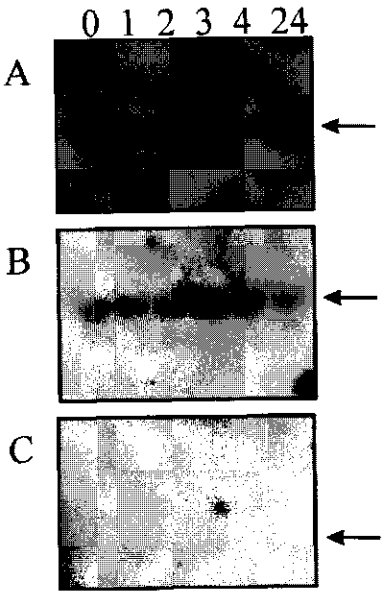


Fig. 3. mRNA levels of the *csp* genes in strains *L. lactis* NZ9000, NZ9000ΔAB and NZ9000ΔABE. Northern blot of RNA extracted at 0, 1, 2, 3, 4 and 24 h after cold shock from 30 to 10°C of strains NZ9000 (left), NZ9000ΔAB (center) and NZ9000ΔABE (right). Transcript sizes are about 300 nt for all *csp* genes and approximately 1500 nt for 16S rRNA. n.d. = not determined.



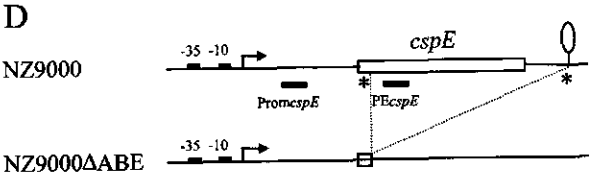
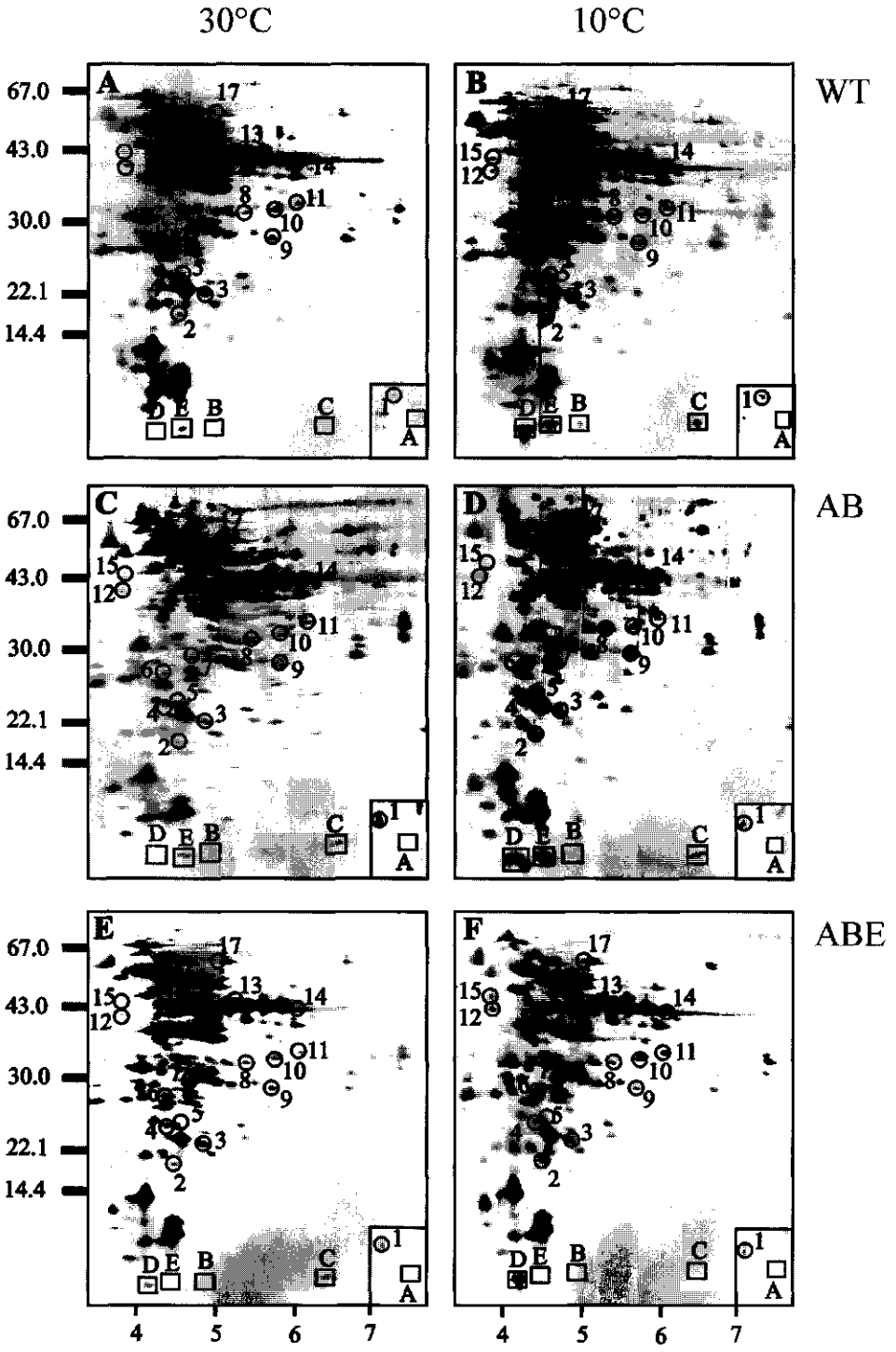


Fig. 4. mRNA analysis of *cspE* at the 5'-UTR. Northern blot of RNA extracted at 0, 1, 2, 3, 4 and 24 h after cold shock from 30 to 10°C of *L. lactis* strains NZ9000 (A), NZ9000ΔAB (B) and NZ9000ΔABE (C). The transcript size is about 300 nt or presumably 115 nt in strain NZ9000ΔABE (indicated with arrows). (D) Schematic diagram of the *cspE* chromosomal region in *L. lactis*. The black bars indicate the sequence complementary to the probes Prom*cspE* and PE*cspE*. The transcription initiation site is indicated with an arrow and the -10 and -35 sequences of the promoter are depicted as black boxes. The terminator site is indicated with a hairpin structure. The region between the asterisks is deleted in strain NZ9000ΔABE.

Effect of deletion of *cspAB* or *cspABE* on the protein levels of the remaining CSPs. To study whether differences occur in the level of the remaining CSPs upon deletion of the CSPs AB or ABE, the proteins were analyzed by 2D-EF (Fig. 5). Cell-free extracts were prepared from strains NZ9000, NZ9000ΔAB and NZ9000ΔABE growing at mid-exponential phase at 30°C and at 2 and 4 h upon exposure to cold shock to 10°C. For strain NZ9000 the only observed CSP at mid-exponential phase at 30°C was CspE. Upon exposure to 10°C, induction of CspD and, to a lower extent, of CspA, CspB, CspC and CspE was observed (Fig. 5A and B). In absence of CspA and CspB, the production of CspE was slightly increased at 30°C, while also CspC was detected at this temperature. Upon cold shock, the amount of CspC and CspD was similar to that found in strain NZ9000, whereas the CspE level slightly increased (Fig. 5C and D). Both CspC and CspD were present at mid-exponential phase at 30°C in strain NZ9000ΔABE. Upon exposure to low temperature, the level of CspD increased and the CspC level slightly decreased (Fig. 5E and F).

Identification of CIPs in *L. lactis* and the effects of the disruption of *csp* genes on their expression. Separation of cell-free extracts by 2D-EF revealed different protein patterns and the production level of several CIPs was found to be clearly affected in the strains NZ9000ΔAB and NZ9000ΔABE compared to the wild-type strain. In a previous study we identified a group of 17 CIPs in *L. lactis* (Chapter 3) and for a subset of proteins belonging to this group the N-termimal amino acid sequence was determined (Table 2). CIP1 (a histon-like protein) and CIP5 were cold-induced in wild-type cells but were no longer induced at low temperature in strains NZ9000ΔAB


30°C
10°C
WT
AB
ABE
67.0
43.0
30.0
22.1
14.4
A
B
C
D
E
F
4
5
6
7

and NZ9000ΔABE. CIP8 (a hypothetical signal transduction protein) and CIP9 were found to be still cold induced in strain NZ9000AB but not anymore in strain NZ9000ΔABE. For the remaining CIPs no differences were observed in their presence between the wild-type strain and the *csp*-deleted strains (Table 2).

Table 2. List of CIPs of strain NZ9000 (WT) and their expression in strains NZ9000ΔAB (ΔAB) and NZ9000ΔABE (ΔABE) 4 h after cold shock to 10°C. N-terminal sequence, the molecular weight (MW) and highest similarities for several CIPs are indicated. For CIP10 to CIP17 no differences in expression were observed in the strains NZ9000ΔAB or NZ9000ΔABE and no N-termini were determined.

CIP	WT	ΔAB	ΔABE	Protein	MW (kDa)	Highest similarity
1	+	-	-	(1) ANKQDLIAEV (10) (2) ANKQDLIAKV (11)	11 10	HlpA of *Streptococcus mutans*
2	+	+	+	(1) ANISKASAHEDTLENFTIE (19) (60)AELEQAKSLKETLEKLTVE (78)	14 15	Ribosomal protein L9 of *B. subtilis*
3	+	+	+	n. d.		
4	+	+	+	n. d.		
5	+	-	-	N-terminally blocked		
6	+	+	+	(1) MFKAVLFDLDGVI (14) (1) MFKAVLGCLCGVI (14)	26 26	β-PGM of *L. lactis*
7	+	+	+	n.d.		
8	+	+	-	(1) XXXILVVNDNTPI (13) (2) DKKILVVDDEKPI (14)	29 27	hypothetical signal transduction protein of *B. subtilis*
9	+	+	-	N-terminally blocked		

\+ = induction of spot after cold shock from 30 to 10°C for 4 h.
\- = no induction of spot after cold shock from 30 to 10°C for 4 h.
n.d. = N-terminus not determined

Fig. 5. Protein analysis of cell-free extracts of strains NZ9000 (A and B), NZ9000ΔAB (C and D) and NZ9000ΔABE (E and F) by 2D-EF. Cell-free extracts were isolated from these strains prior to cold shock (30°C mid-exponential phase; A, C and E) and at 4 h after cold shock to 10°C (B, D and F) and were separated on a pI range of 3-10. Equal amounts of protein were loaded on the gel and the proteins were visualized using silver staining. A molecular size marker is indicated on the left and a pI scale is given at the bottom. The CSPs of *L. lactis* are boxed and have been identified previously (Chapters 3 and 5). CIPs are circled and numbered as described previously (Chapter 3). The box in the lower right corner of each gel indicates the region between pI values of approximately 9 and 10 of the same molecular mass region. The N-termini of a number of spots is indicated in Table 2.

DISCUSSION

In this report we describe physiological and regulatory effects of the disruption of a set of genes encoding CSPs of *L. lactis*. A *L. lactis* strain deleted for the tandem *cspA-cspB* genes, and a strain additionally deleted in *cspE* were generated. In absence of these CSPs the growth characteristics (lag time and growth rate) of *L. lactis* were not affected and the lack of these CSPs is probably compensated by the increased production of the remaining CSPs. A proteomics approach based on 2D-EF showed that deletion of CspAB is compensated by increased production of CspE while deletion of CspABE is compensated by increased CspC and CspD production, mainly at 30°C. In *E. coli* and *B. subtilis* no differences in growth characteristics were observed upon deletion of $cspA^E$ and $cspB^B$ (Bae *et al.*, 1997; Graumann *et al.*, 1996). However, for *B. subtilis* deletion of the genes for two members of the three-member CSP family revealed decreased growth rates at 30 and 10°C. Deletion of the genes for all three members revealed a lethal phenotype (Graumann *et al.*, 1997).

L. lactis adapts to freezing conditions during prior exposure to 10°C for several hours, resulting in increased survival and it has been speculated that 7-kDa CSPs play a role in this response (Chapter 3). Indeed, cells overproducing CspB, CspD or CspE show improved survival to freezing (Chapter 5). In this work we show that strain NZ9000ΔAB adapts equally well as strain NZ9000 to freezing conditions and we suggest that deletion of *cspA* and *cspB* is compensated by the observed increased production of CspE. For strain NZ9000ΔABE the maximum freeze-protective effect is reached after 8 h compared to 4 h for strain NZ9000, indicating that the freeze protective response is significantly delayed. This suggests that CspE is important for the survival to freezing for *L. lactis*. CspE may either have a direct effect during freezing, e.g. by stabilizing RNA, or regulate the expression of other factors involved in the cryoprotective response.

The effect of deletion of *csp* genes on the expression of the remaining *csp* genes was examined and showed that some CSPs regulate the production of other CSP-family members. Upon deletion of *cspA* and *cspB*, the *cspE* mRNA level increased at low temperature and resulted in increased production of CspE whereas the transcription of *cspC* and *cspD* was not altered in comparison to the wild-type strain. This indicates that in *L. lactis* NZ9000ΔAB either the transcription initiation of *cspE* is derepressed or the *cspE*-transcript stability is increased. Additional deletion of CspE resulted in significantly increased *cspC* and *cspD* mRNA levels at 30°C and 10°C, accompanied by an increased CspC and CspD production. This indicates that CspE is involved in the regulation of these genes and probably acts as a negative regulator either acting on the *cspC* and *cspD* promoters or on their mRNA stability. For $CspA^E$ and for the CSPs of *B. subtilis* binding to the CS-box at the 5'-UTR has been observed (Bae *et al.*, 1997; Graumann *et al.*, 1997). $CspA^E$ has been shown to bind to the 5'-UTR of *csp* mRNA by which these transcripts are tagged, and consequently cannot be translated and are rapidly degraded by RNases (Bae *et al.*, 1997). Similar

CS-boxes have been observed in the 5'-UTRs of the lactococcal *csp* genes and careful inspection revealed that the four cold-induced *csp* genes (*cspA*, *cspB*, *cspC* and *cspD*) contain highly similar CS-boxes, which differ significantly from the one of the non-cold-induced *cspE* gene (Fig. 6). It is tempting to speculate that CspE can bind with high affinity to the 5'-UTRs of these four *csp* genes, thereby preventing their transcription at 30°C and accelerating their degradation. CspE probably binds with lower affinity to its own 5'-UTR which makes it the only significantly expressed *csp* gene at 30°C in *L. lactis*. Northern blot analysis of *cspE* expression in strain NZ9000ΔABE showed no detectable *cspE* mRNA using a probe for the undeleted 5'-UTR, indicating that reduced or no directed transcription by the *cspE* promoter took place. This might indicate that CspE promotes transcription of its own gene and, thus, seems to be subject to positive autoregulation, a mechanism that has not been noted previously for *csp* genes. However, it should be noted that the *cspE* transcript might also be destabilized due to the absence of an internal part of the transcript. In conclusion, it is shown that the CSP production in *L. lactis* is tightly regulated. The compensatory effects of CSPs upon deletion of the *csp* genes indicate that it is essential to keep the cumulative expression of specific *csp* genes at a minimal level. On the other hand, the CSP level should stay well below a maximum level, since growth inhibitory effects of these proteins have been observed upon overproduction in high quantities (Chapter 5). It is proposed that a tight regulatory circuit controls the production of CSPs in *L. lactis*.

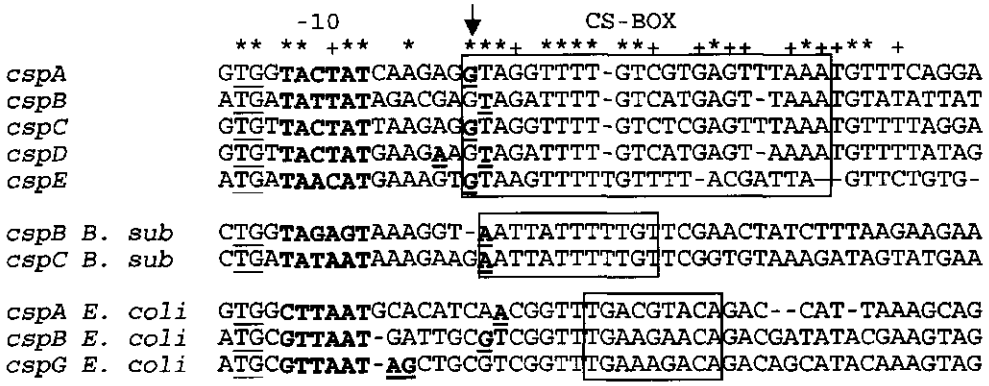


Fig. 6. Alignment of the CS-box at the 5'-UTR of the mRNAs of the *csp* genes of *L. lactis*, those of *cspB* and *cspC* of *B. subtilis* and those of *cspA*, *cspB* and *cspG* of *E. coli*. The transcription starts sites are underlined and in bold (and indicated with an arrow), while the -10 regions are in bold, and the TG dinucleotides (-16-region) are underlined. Identical nucleotides in all five *L. lactis csp* genes are indicated with *; identical nucleotides in the four cold-induced *csp* genes are indicated with +. Gaps are indicated with dashes.

Amino-terminal analysis of the CIPs of *L. lactis* revealed that proteins involved in a variety of cellular processes are induced upon cold shock. CIP1 was identified as a histon-like protein and showed the highest sequence similarity to HlpA of *Streptococcus mutans* (Stinson *et al.*, 1998). Remarkably, H-NS of *E. coli* was also found to be cold-induced and a role for this protein in optimizing DNA-supercoiling, which increases at low temperature, has been suggested (LaTeana *et al.*, 1991; Brandi *et al.*, 1994). CIP2 showed homology to the 50S ribosomal protein L9 of *B. subtilis* (Ogasawara *et al.*, 1994). For both *E. coli* and *B. subtilis* cold-induced ribosomal proteins have been identified (S1, S6, L7/L12 and S6 and L7/L12, respectively; Jones *et al.*, 1992A; Graumann *et al.*, 1996) and it is suggested that these proteins are required for the correct assembly of rRNA at low temperatures (Graumann *et al.*, 1996). Furthermore, CIP6 was identified as β-phosphoglucomutase of *L. lactis* (β-PGM; Qian *et al.*, 1997) and CIP8 was found to be homologous to a hypothetical signal transduction protein of *B. subtilis* (Ogasawara *et al.*, 1994). This indicates that the low-temperature response includes adaptation at several levels. In this work we show that the cold-induced expression of CIP1 and CIP5, and CIP8 and CIP9 was affected upon disruption of *cspAB* or *cspABE*, respectively, indicating that these CSPs regulate other proteins involved in cold adaptation. Previously, we observed that the overproduction of the lactococcal CSPs in *L. lactis* induces a variety of proteins, among which also a number of CIPs (CIP2, CIP4, CIP5 and CIP9; Chapter 5). Collectively, these data indicate that the production of CIP5 and CIP9 is reduced upon deletion of CspABE and production of these CIPs is increased upon overproduction of CspA and CspE (Chapter 5). This strongly suggests a regulatory role of CspA, CspB or CspE in the production of CIP5 and CIP9. It has been reported that $CspA^E$ functions as a transcriptional activator of several cold induced genes by interacting with Y-boxes located in their promoter regions (LaTeana *et al.*, 1991; Brandi *et al.*, 1994). A similar regulation may be operating for the CSPs of *L. lactis*. The reduced expression of CIP1, 5, 8 and 9 upon cold shock in *L. lactis* NZ9000ΔABE does not result in growth defects but might cause the delayed response for freeze survival as was noted. Apparently, these regulatory functions can not be compensated by the increased expression of the remaining CSPs and it is likely that they are highly specific for each CSP. This may also provide a rational explanation for the existence of a CSP family.

In this report we describe the effects of the deletion of multiple *csp* genes of the lactic acid bacterium *L. lactis*. Deletion of 3 of the 5 known members of the lactococcal CSP family does not result in growth defects, but for strains NZ9000ΔABE a delayed response in its freeze-protective response is noted. Transcription and protein analysis revealed that CSPs compensate for the absence of the deleted CSPs and it is shown that the CSP production in *L. lactis* is tightly regulated. Furthermore, CSPs are found to regulate the expression of CIPs that are no longer cold induced in CSP-deleted strains, indicating a regulatory role for CSPs in the cold-adaptive response of *L. lactis*.

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Chapter 7

Changes in glycolytic activity of *Lactococcus lactis* induced by low temperature

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ABSTRACT

The effects of low-temperature stress on the glycolytic activity of the lactic acid bacterium *Lactococcus lactis* were studied. The maximal glycolytic activity measured at 30°C increased approximately 2.5-fold following a shift from 30 to 10°C for 4 h in a process that required protein synthesis. Analysis of cold adaptation of strains disrupted in genes involved in sugar metabolism showed that both HPr and CcpA are involved in the increased acidification at low temperature. In contrary, a strain disrupted in Enzyme I showed a similar increased acidification as the wild-type strain. This indicates that the phosphoenolpyruvate-dependent sugar phosphotransferase system is not directly involved in this response whereas the regulatory function of 46-seryl phosphorylated HPr (HPr(Ser-P)) probably is involved. Protein analysis showed that the production of both HPr and CcpA was induced several times (up to 2-3 fold) upon exposure to low temperature. The *las* operon, which is subject to catabolite activation by the CcpA/HPr(Ser-P) complex, was not induced upon cold shock and no increased LDH activity was observed. Similarly, the rate-limiting enzyme of the glycolytic pathway during starvation conditions GAPDH, was not induced upon cold shock. This indicates that another factor than LDH or GAPDH will be rate determining for the increased glycolytic activity upon exposure to low temperature. Based on their cold induction and involvement in cold adaptation of glycolysis, it is proposed that the CcpA/HPr(Ser-P) control circuit regulates this factor(s) and hence couples catabolite repression and cold-shock response in a functional and mechanistic way.

Submitted for publication

INTRODUCTION

Lactic acid bacteria (LAB) are widely used to start industrial fermentations of foods during which they face a variety of stress conditions. The adaptation responses of *Lactococcus lactis* to these stress conditions have been investigated (reviews by Rallu *et al.*, 1996 and Sanders *et al.*, 1999). Starter LAB are exposed to low temperature during frozen storage, as well as during low-temperature fermentation. The survival and fermentation capacity of LAB during these conditions will determine the end result of the fermentations. Many of the fermentations are stopped by storage at low temperature and during this storage the fermentation may continue slowly, resulting in an overacidified product. For these reasons, it is of interest to study the cold-adaptive response of LAB in relation to acidification characteristics.

Recent research on low-temperature response of various bacteria has resulted in the identification of a group of 7-kDa proteins that appears to represent the highest induced proteins upon a rapid down-shift in temperature and are for that reason called cold-shock proteins (CSPs). It has been shown that CSPs can function as RNA chaperones, transcriptional activators and freeze-protective compounds in *Escherichia coli* and *Bacillus subtilis* (reviews by Graumann and Marahiel, 1998 and Yamanaka *et al.*, 1998). Also in *L. lactis* MG1363 a CSP family, consisting of five members, has been identified (Chapter 2). Moreover, a variety of other cold-induced proteins (CIPs) has been characterized in several bacteria. In *E. coli* and *B. subtilis* approximately 20 and 35 CIPs, respectively, were observed and these proteins are involved in a variety of cellular processes, like chromosomal condensation, chemotaxis, general metabolism, transcription and translation (Jones and Inouye, 1994; Jones and Inouye, 1996; Graumann *et al.*, 1996; Jones *et al.*, 1996). Strikingly, for *B. subtilis* also cold induction was observed for glyceraldehyde-phosphate dehydrogenase (GAPDH) and HPr, both involved in glycolysis (Graumann *et al.*, 1996). *L. lactis* MG1363 showed induction of 17 CIPs, including β-phosphoglucomutase, a hypothetical signal transduction protein, ribosomal protein L9 and a histon-like protein (Chapters 3 and 6).

For *L. lactis* the main pathway for energy generation is the glycolysis in which two substrate level phosphorylation reactions, involving phosphoglycerate kinase and pyruvate kinase, operate to yield energy. During growth of *L. lactis* on glucose or lactose, more than 90% of the fermented sugar is converted into L-lactate (Thomas *et al.*, 1980). Pyruvate is the end-product of glycolysis and is either converted into L-lactate (homolactic fermentation) or into a mix of fermentation products, such as L-lactate, acetate, ethanol or formate (mixed acid fermentation), depending on growth rate (Garrigues *et al.*, 1997; Qian *et al.*, 1997). Glucose and lactose are transported in *L. lactis* by the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) that mediates concomitant the uptake and phosphorylation of these carbohydrates. This group translocation process is catalyzed by the non-sugar-specific proteins Enzyme I and HPr in combination with the sugar specific Enzyme II, that can consist of one or more proteins (Luesink *et al.*, 1999). The genes

encoding phosphofructokinase (*pfk*), pyruvate kinase (*pyk*) and lactate dehydrogenase (*ldh*), have been cloned and were shown to be located in the *las* (lactic acid synthesis) operon, that is under control of a single promoter (Llanos *et al.*, 1992; Llanos *et al.*, 1993). HPr is not only involved in sugar uptake but also plays a regulatory role in the sugar metabolism and catabolite repression, depending on its phosphorylation. For *B. subtilis* it has been reported that 46-seryl phosporylated HPr can form a complex with catabolite control protein A (CcpA) in the presence of glycolytic intermediates such as fructose-diphosphate or glucose-6-phosphate (Fujita *et al.*, 1995). Recently, it has been shown that the lactococcal 46-seryl phosphorylated HPr (HPr(Ser-P)) functions as a co-activator in the catabolite activation of *pyk* and *ldh* genes in cooperation with CcpA (Luesink *et al.*, 1999). Furthermore, a role for the control of the glycolysis in *L. lactis* has been appointed to GAPDH which was shown to be rate limiting in the glycolytic activity of starved cells (Poolman *et al.*, 1987A). The gene encoding GAPDH, *gap*, has been cloned and is expressed on a monocistronic transcript while no other glycolytic pathway genes were observed adjacent to *gap* (Cancilla *et al.*, 1995).

Despite the increased knowledge of the cold-shock response in recent years, still limited knowledge of the physiological role of cold-induced proteins exists. In this work, we present data on the glycolytic activity at low temperature and we report on new cold-induced proteins involved in the glycolytic pathway. The glycolytic activity measured at 30°C shows a marked increase upon prior exposure of the cells to 10°C for several hours. This response seems to involve the regulatory CcpA/HPr(Ser-P) complex and the role of this control circuit in the glycolytic pathway is discussed.

MATERIALS AND METHODS

Strains and growth conditions. *L. lactis* NZ9800 (De Ruyter *et al.*, 1996A) was used as wild-type strain in this study and was cultured in M17 medium containing either 0.5% glucose or 0.5% maltose at 30°C or as indicated otherwise. Strains that were disrupted in the *ptsI* (*L. lactis* NZ9881 [Luesink *et al.*, 1999]) or *ccpA* gene (*L. lactis* NZ9870 [Luesink *et al.*, 1998]) were cultured on M17 broth containing 0.5% glucose and a strain disrupted in the *ptsH* gene (*L. lactis* NZ9880 [Luesink *et al.*, 1999]) was grown on M17 broth containing 0.5% maltose. Growth of *L. lactis* was monitored by measuring the optical density at 600 nm (OD_{600}). *L. lactis* cells were exposed to a cold-shock treatment as described previously (Chapter 2).

Determination of maximal glycolytic activity. The glycolytic activity was assessed by measuring the initial rate of acidification essentially as described by Poolman *et al.* (1987A). In short, cells were cultured, centrifuged and resuspended in 0.5 mM Kmes/50 mM KCl buffer (pH 6.5) to OD_{600} of 20. Subsequently, 0.4 ml of this suspension was added to 9 ml of the same buffer equilibrated at 30°C. The acidification of the medium was measured upon addition of glucose or maltose (0.5% final concentration) using a Schott pH electrode and a pH meter connected to a

recorder. Changes in pH were converted into nmol H^+/min/mg protein by calibration of the cell suspension with 10-µl portions of 100 mM NaOH. The protein content of the extract was determined using the bicinchoninic acid method as provided by the supplier (Sigma Chemicals, St. Louis, MO). Using an Aminex Ion Exclusion Column (Biorad, Hercules, CA) the end-products of the acidification analysis (lactate, acetate and formate) were determined by HPLC as described previously (Starrenburg and Hugenholtz, 1991).

mRNA analysis. RNA was isolated and Northern-blot analysis was performed as described previously (Kuipers *et al.*, 1993). RNA was denatured and equal amounts of RNA were separated on 1%-agarose gels containing formaldehyde according to the method of Sambrook *et al.* (1989) and blotted on GeneScreen Plus Membrane (Dupont, Wilmington, DE). A 0.24-9.5 kb RNA ladder (GIBCO/BRL Life Technologies, Breda, The Netherlands) was used to determine the transcript size and the RNA was stained with ethidium bromide. The blots were hybridized with a probe specific for *ptsH* (5'-CTGCAACGATGTGGAATCTTTAG-3'), *ptsI* (5'-GATGGATTGTAAGGTTGATA-3'), *ccpA* (5'-GTGCCACATCATAAATTGTTGTTGTTG-3') or *ldh* (5'-GCATCAGAGTAGTCTGCA-GAG-3')(Luesink *et al.*, 1998; Luesink *et al.*, 1999) that were end-labelled with γ-^{32}P-dATP. For the detection of *gap* mRNA a PCR fragment, obtained using the primers GAPFOR (5'-GTTGGTATTAACGGTTTTGGTCG-3') and GAPREV (5'-GAGTGGACAGTAGTCATTGTCCC-3')(Cancilla *et al.*, 1995), was labelled with α-^{32}P-ATP. The total amount of RNA loaded on the gels was analyzed using a 16S-rRNA probe (5'-ATCTACGCATTTCACCGCTAC-3') specific for *L. lactis* (Klijn *et al.*, 1991).

Protein extraction and protein analysis using two-dimensional gel electrophoresis (2D-EF). Proteins were extracted using a MSK cell homogenizer (B. Braun Biotech International, Germany) and zirconium beads (0.1 mm, Biospec Products, OK). Protein analysis was performed by 2D-EF as described in Chapter 3 and equal amounts of protein (40 µg) of the cell-free extracts were separated on an iso-electric point (pI) region from 4 to 7 and, subsequently, on 15%-polyacrylamide homogenous SDS gels together with a molecular size marker. Proteins were visualized by silver staining and the spots were analyzed by GEMINI software (Applied Imaging, Sunderland, England).

LDH activity and GAPDH activity. LDH activities of cell-free extracts of *L. lactis* were analyzed by NADH consumption as described by Hillier and Jago (1982). GAPDH activity was analyzed by measuring the increase at 340 nm in a double-beam spectrophotometer as a result of NADH production as described previously by Poolman *et al.* (1987A).

RESULTS

Acidification rates of *L. lactis* cells incubated at low temperature. To relate low-temperature incubation to physiological response, the glycolytic activity was determined for *L. lactis*

cultures grown under different conditions. Mid-exponential cells (OD_{600} 0.5) cultured at 30°C showed a maximal glycolytic activity of approximately 600 nmol/min/mg protein that was increased to approximately 1600 nmol/min/mg protein upon exposure to 10°C for several hours. This increase in glycolytic activity was maximal (2.3-fold) after 4 to 5 h incubation at 10°C. Upon longer exposure, the maximum acidification rate decreased to approximately 900 nmol/min/mg protein (Fig. 1). In the presence of chloramphenicol, which inhibits protein synthesis and consequently inhibits cell growth (data not shown), during cold incubation cells did not show an increase in the maximum glycolytic activity (Fig. 1). After prolonged incubation with chloramphenicol the glycolytic activity was very low (60 nmol/min/mg protein at 20 h after cold shock), indicating the necessity of constant protein synthesis to maintain glycolytic activity.

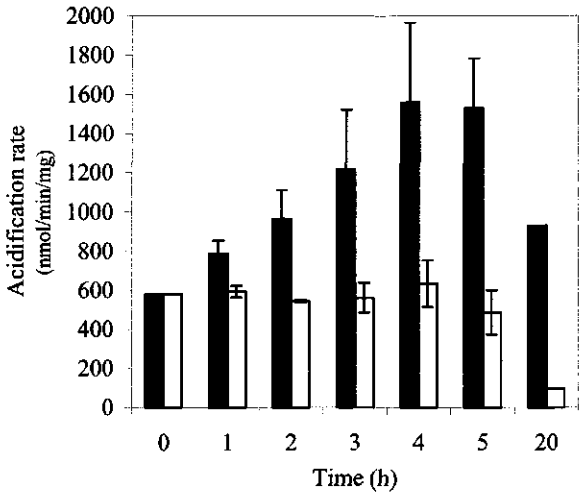


Fig. 1. Effect of cold shock on the maximal glycolytic activity of *L. lactis* NZ9800. Cells were grown until OD_{600} 0.5 at 30°C and subsequently exposed to 10°C in absence (dark bars) or presence of 100 µg chloramphenicol/ml (open bars). At various time points after cold shock (0, 1, 2, 3, 4, 5 and 20 h) the maximal glycolytic activity (nmol H^+/min/mg protein) was determined at 30°C in duplicate. The error bars indicate the standard deviation.

No increased acidification for *L. lactis* NZ9880(Δ*ptsH*) and *L. lactis* NZ9870(Δ*ccpA*). To further elucidate the mechanism of the increased maximum glycolytic activity of *L. lactis* cells exposed to low temperature, acidification rates were also determined for *L. lactis* NZ9880(Δ*ptsH*), *L. lactis* NZ9881(Δ*ptsI*) and *L. lactis* NZ9870(Δ*ccpA*). For *L. lactis* NZ9880(Δ*ptsH*) the acidification rate is significantly reduced (nearly 3-fold) in mid-exponential cells compared to wild-type cells in this growth phase (Fig. 2A). Analysis of the end-products revealed that the production of acetic acid increased in comparison to wild-type cells, indicating characteristics of a mixed-acid fermentation. Upon low-temperature exposure of *L. lactis* NZ9880(Δ*ptsH*) cells no increase in maximum glycolytic activity was observed (Fig. 2A). For *L. lactis* NZ9881(Δ*ptsI*) a five-fold reduction of the acidification rate was observed for cells grown at 30°C compared to wild-type cells, that is most likely explained by the reduced sugar uptake by *L. lactis* NZ9881(Δ*ptsI*) (Fig. 2B).

Similar to wild-type *L. lactis* also an approximately 2-fold increase in acidification is observed for *L. lactis* NZ9881(Δ*ptsI*) upon exposure to 10°C after 2-3 h (Fig. 2B). The maximum glycolytic activity of *L. lactis* NZ9870(Δ*ccpA*) cells was also strongly reduced at 30°C compared to wild-type cells (Fig. 2C), which might be explained by the reduced activity of the *las* operon. Upon exposure to 10°C no increased acidification is observed for *L. lactis* NZ9870(Δ*ccpA*) cells compared to wild-type cells (Fig. 2C). Also for *L. lactis* NZ9870(Δ*ccpA*) an increased formation of acetic acid was observed, similarly as observed by Luesink *et al.* (1998). HPLC analysis also revealed that the ratio of the products (lactate, acetate, formate) formed by cells cultured at high or at low temperature was identical. In conclusion, these data indicate that that both HPr and CcpA are involved in the increased acidification at low temperature, in contrast to Enzyme I. This indicates that the PTS is not directly involved in this response whereas the regulatory function of HPr(Ser-P) probably is involved.

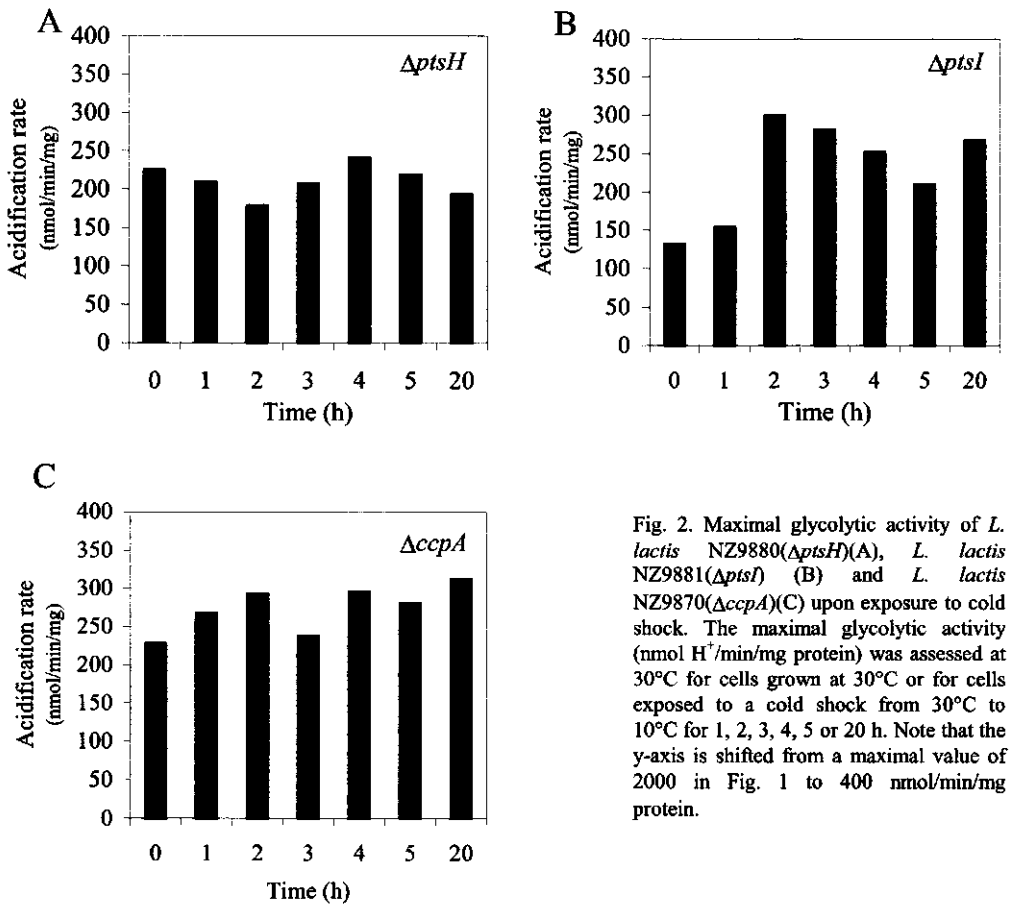


Fig. 2. Maximal glycolytic activity of *L. lactis* NZ9880(Δ*ptsH*)(A), *L. lactis* NZ9881(Δ*ptsI*) (B) and *L. lactis* NZ9870(Δ*ccpA*)(C) upon exposure to cold shock. The maximal glycolytic activity (nmol H^+/min/mg protein) was assessed at 30°C for cells grown at 30°C or for cells exposed to a cold shock from 30°C to 10°C for 1, 2, 3, 4, 5 or 20 h. Note that the y-axis is shifted from a maximal value of 2000 in Fig. 1 to 400 nmol/min/mg protein.

Analysis of *ptsH*, *ptsI* and *ccpA* upon cold shock. Using specific probes the mRNA levels of *ptsH*, *ptsI* and *ccpA* were analyzed in *L. lactis* NZ9800 after cold shock. The 2.0-kb *ptsHI* transcript (Luesink *et al.*, 1999) appeared to be induced upon cold shock to 10°C (2-fold maximally after 4 h). Using a probe specific for *ptsH* two transcripts of 2.0 and 0.3 kb identical as described by Luesink *et al.* (1999) were detected that were also induced upon exposure to 10°C (maximally 2 and 1.5-fold after 4 h, respectively). Next, the expression of the 1.2-kb *ccpA* transcript (Luesink *et al.*, 1998) was slightly induced upon cold shock (maximally 1.5-fold at 4 h)(Fig. 3A).

The effect of low-temperature exposure on the levels of the proteins encoded by *ptsH*, *ccpA* and *ptsI* was analyzed using cell-free extracts of *L. lactis* NZ9800 cells before and after cold shock (2 and 4 h). Based on 2D-EF gels for cell-free extracts of *L. lactis* NZ9870, *L. lactis* NZ9880 and *L. lactis* NZ9881 and based on the calculated molecular masses and pIs of HPr (MW 9.1; pI 4.9), Enzyme I (MW 62.6; pI 4.6) and CcpA (MW 36.6; pI 5.0) the spots representing the respective proteins, could be determined for *L. lactis* NZ9800. HPr is one of the highest produced proteins (5% of the total visualized proteins on the 2D-EF gels) in mid-exponential cells. The quantity of HPr slightly increased upon cold shock for 2 or 4 h (1.5 to 2-fold; Fig. 3B) which is in agreement with the increased *ptsH*-mRNA level. For Enzyme I no induction upon exposure to low temperature was observed (Fig. 3B). For CcpA also cold induction was observed (Fig. 3B) which was confirmed by use of a *Bacillus megaterium* CcpA-antibody that revealed 2 to 3-fold induction upon cold shock (data not shown).

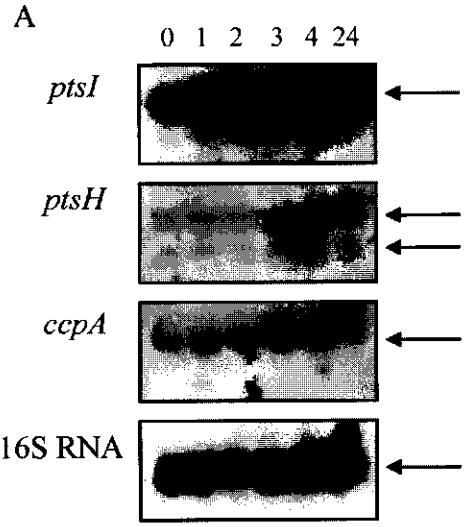
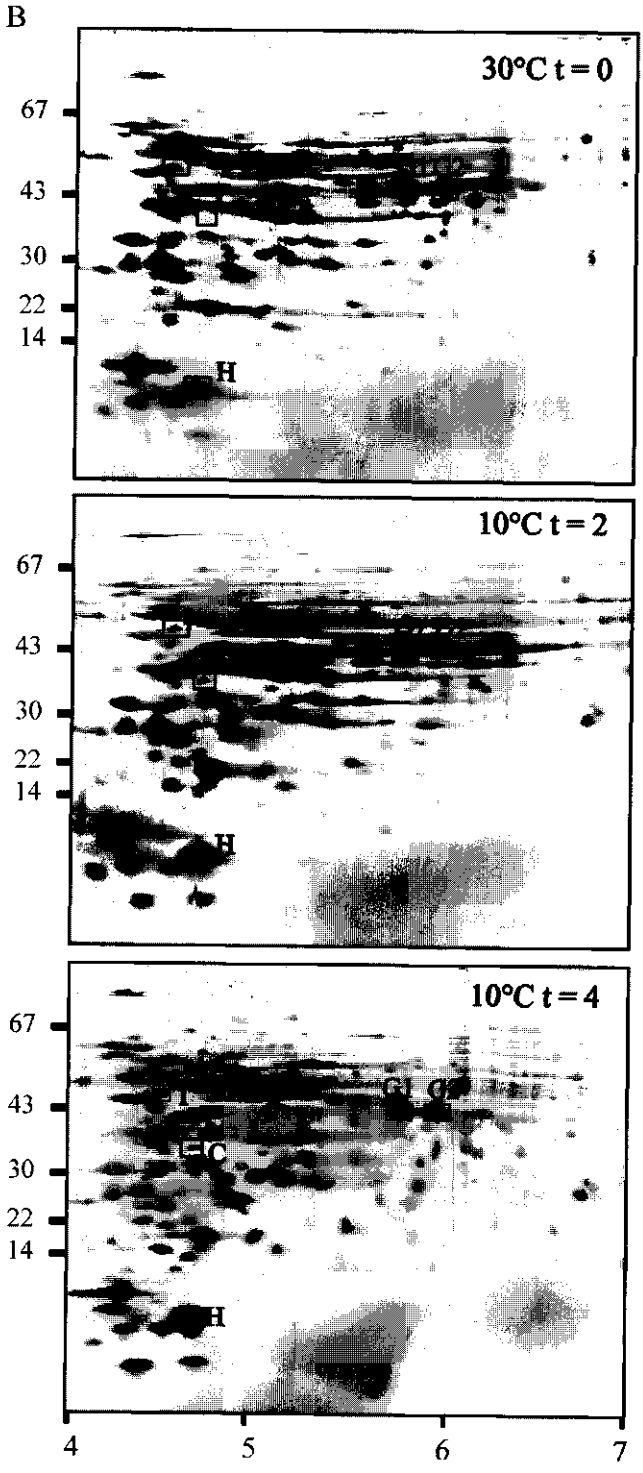


Fig. 3. Analysis of the cold induction of HPr, Enzyme I and CcpA. (A) The mRNA levels of the *ptsH, ptsI* and *ccpA* genes in *L. lactis* NZ9800 were analyzed prior cold shock and upon exposure to cold shock for several time periods using Northern blotting. Total RNA was extracted at 0, 1, 2, 3, 4 and 20 h after cold shock from 30 to 10°C of *L. lactis* NZ9800. The blots were hybridized with the specific *ptsHI, ccpA* and 16S rRNA probes. Transcript sizes are about 2.0, 0.3, 1.2 and 1.5 kb for the *ptsHI, ptsH* and *ccpA* genes and 16S rRNA, respectively, and are indicated with arrows. (B) 2D-EF gels of cell-free extracts of *L. lactis* NZ9800 isolated prior to cold shock (30°C mid-exponential phase; upper panel) and at 2 h (central panel) and at 4 h (lower panel) after cold shock to 10°C. Equal amounts of protein were loaded on the gels and the proteins were visualized using silver staining. Molecular size marker bands are indicated on the left and a pI scale is given at the bottom. The putative spots representing HPr (H), Enzyme I (I), CcpA (C) and GAPDH (G1 and G2) are indicated.


B
30°C t = 0
67
43
30
22
14
H
10°C t = 2
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67
43
30
22
14
H
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5
6
7

mRNA analysis of the *las* operon and *gap* and analysis of LDH and of GAPDH activity. No cold-temperature induced acidification is observed for strains deleted in the genes encoding HPr and CcpA. Hence, the complex that is assumed to be formed between HPr and CcpA might play a role in the increased acidification upon incubation at 10°C by inducing specific genes. To investigate this assumption the mRNA level of the *las* operon, that is known to be positively regulated by the putative CcpA/HPr(Ser-P) complex (Luesink *et al.*, 1999), was monitored upon exposure to cold shock. For none of these transcripts (sizes of 4, 3 and 1 kb) cold induction was observed (Fig. 4A), whereas also the LDH activity did not increase upon exposure to cold shock. In the presence of chloramphenicol a significant reduction in LDH activity was measured at 0.5, 2 and 4 h after cold shock indicating that *de novo* protein synthesis is required to maintain the LDH activity (Fig. 4B). This also indicates that LDH can not be the rate-limiting step in glycolysis since the maximum glycolytic activity in these cells stays at a constant level during this time period (Fig. 1).

The conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, catalyzed by GAPDH, was previously identified as the rate-limiting step in the glycolysis of starved *L. lactis* cells (Poolman *et al.*, 1987A). The monocistronic transcript of *gap* has a size of 1.3 kb and was constant during the first hours upon cold shock (Fig. 4A). Strikingly, the transcript is induced at 20 h after cold shock (approximately 3-fold), whereas for the other genes analyzed here (*ptsI, ptsH, ccpA* and the *las* operon) the transcripts can hardly be determined at this time point, a condition during which *L. lactis* are probably starved. Upon cold shock for 0.5, 2 and 4 h the GAPDH activity was identical to the activity prior to cold shock (approximately 4 μmol/min/mg; Fig. 4B). Similar to the LDH activity, the GAPDH activity was reduced upon cold shock in the presence of chloramphenicol, indicating that also the GAPDH activity is not a rate-limiting step during these conditions. Comparison of the 2D-EF gels of Fig. 3B with a gel of *L. lactis* MG1363 revealed the position of GAPDH, which appears to be a double spot, as previously reported (Kilstrup *et al.*, 1997). Upon exposure to 10°C for 4 h none of these two spots was cold induced.

DISCUSSION

Since *L. lactis* is extensively used in dairy fermentations, it is of great importance to be able to control metabolic pathways. In recent years, metabolic engineering proved a valuable tool for the optimization of fermentation processes and the design of novel fermentation pathways (De Vos *et al.*, 1998). Expanding the knowledge of the stress response in this respect will contribute to the benefits of these new approaches. In this report the relation between the glycolytic pathway and the cold-stress response of *L. lactis* was investigated and it was revealed that upon exposure to low temperature the acidification rate of *L. lactis* cells increases. At low temperature enzyme-catalyzed

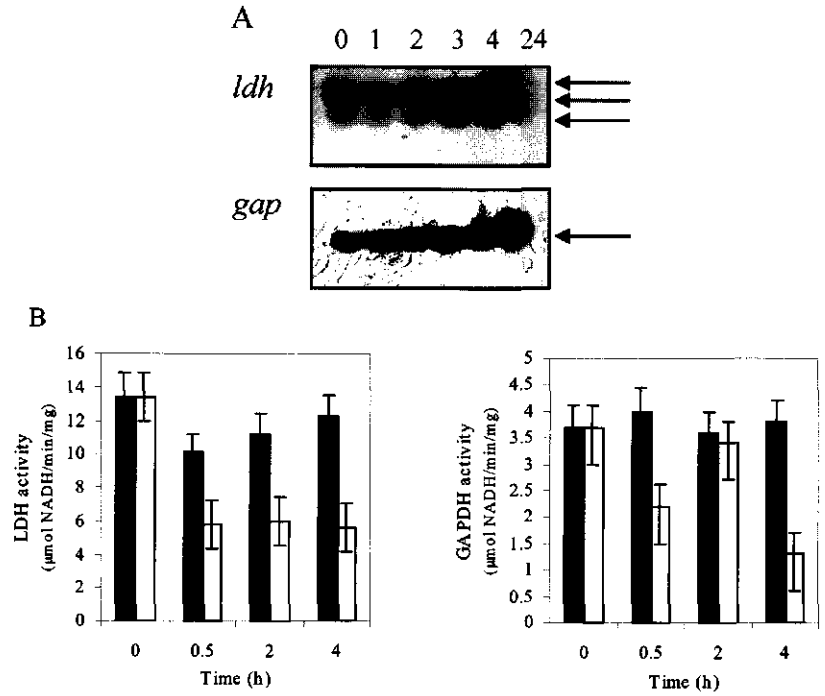

Fig. 4. Analysis of mRNA levels of the *las* operon and *gap* and LDH and GAPDH activity of *L. lactis* NZ9800 upon exposure to cold shock to 10°C. (A) Total RNA was extracted at 0, 1, 2, 3, 4 and 20 h after cold shock. Equal amounts of RNA were run on the gel. Blots were hybridized with a specific *ldh* probe that detects three transcripts of 4, 3 and 1 kb encoding *ldh*, *pfk* and *pyk*, or with a specific *gap* probe that detects a 1.3 kb *gap* transcript (indicated with arrows). (B) LDH (µmol NADH/min/mg protein; left panel) and GAPDH (µmol NADH/min/mg protein; right panel) activity of *L. lactis* NZ9800 upon exposure to cold shock for 0, 0.5, 2 and 4 h in the absence (closed bars) and in the presence (open bars) of 100 µg chloramphenicol/ml. Average values of two determinations and the standard deviations are depicted.

reaction rates are known to decrease and it is assumed that during these conditions induction of certain factors is required to compensate for this loss in activity. It is conceivable, that exposure to 10°C results in induction of glycolytic enzymes to compensate for an overall lower glycolytic capacity. In the presence of chloramphenicol during low-temperature exposure no increased acidification is observed indicating that protein synthesis is required. This observation also excludes the possibility of deregulation of glycolysis at low temperature by uncoupling of regulatory mechanisms as described by Poolman *et al.* (1987B).

For *L. lactis* strains deleted in the genes encoding HPr or CcpA, two important regulators of the glycolytic activity, no increase in maximal glycolytic activity is observed upon exposure to low temperature. In the absence of *ptsI* that encodes the Enzyme I subunit of the PTS still an increased acidification is observed, excluding a rate-limiting role of the PTS and also indicating that the regulatory function of HPr(Ser-P) probably is involved. Strikingly, mRNA analysis revealed induction of *ccpA, ptsH* as well as *ptsI* (encoding both HPr and Enzyme I) upon cold shock. *L. lactis*

CcpA and HPr were both cold induced at the protein level. Strikingly, the importance of HPr in the cold-adaptive response is further stressed by the observation that *L. lactis* NZ9880(*ΔptsH*) is not able to grow at low temperature (Wouters *et al.*, unpublished data). It has been described that the putative CcpA/HPr(Ser-P) complex can either positively (e.g. the *las* operon) or negatively (e.g. the *gal* operon) control certain key steps in metabolic pathways (Luesink *et al.*, 1998; Luesink *et al.*, 1999). However, no cold-induction was observed for the transcripts of the *las* operon and it is concluded, that despite the increased level of CcpA and HPr, the CcpA/HPr(Ser-P) complex does not induce the *las* operon expression at these conditions. Next, it was shown that the LDH activity is not the rate-limiting step of glycolysis at these conditions. Poolman *et al.* (1987A) showed that GAPDH is the rate-limiting step in the glycolytic activity of starved *L. lactis* cells. Analysis of GAPDH at low temperature revealed that neither the *gap* mRNA level nor the GAPDH activity increased upon exposure to low temperature. Furthermore, incubation of *L. lactis* cells at low temperature in the presence of chloramphenicol revealed that also GAPDH activity was not rate limiting in glycolysis at these conditions. It is speculated that CcpA and HPr control several other steps of the glycolysis by their specific interaction with the catabolite responsive element (CRE). These CREs are found throughout the *L. lactis* chromosome and are divergent in their homology to the consensus sequence (Luesink *et al.*, 1999). It can be postulated that more of these elements are found in the genes of the glycolytic pathway, by which an expanded regulatory role of HPr and CcpA can be expected. Apparently, an unidentified factor(s) is required for the increased glycolytic activity upon exposure to low temperature and it is proposed that the CcpA/HPr(Ser-P) complex will regulate the factor(s) required for this increase.

In conclusion, the maximal glycolytic activity measured at 30°C showed a marked increase upon incubation of *L. lactis* cells at 10°C for several hours. However, for the rate-limiting steps of glycolyis, i. e. the enzymes encoded by the *las* operon and GAPDH, no induction was observed upon cold shock. This indicates that another factor than LDH or GAPDH will be rate determining for the increased glycolytic activity upon exposure to low temperature. Based on their cold induction and involvement in cold adaptation of glycolysis, it is proposed that the CcpA/HPr(Ser-P) control circuit regulates this factor(s) and hence couples catabolite repression and cold-shock response in a functional and mechanistic way.

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Chapter 8

General discussion

Lactic acid bacteria (LAB) have a long history of safe and beneficial use in the production of fermented foods. The fermentation capacity of these bacteria is of utmost importance for the desired rapid initiation and, hence, the final result of fermentations. As a consequence, the responses of starter cultures to stressful processing conditions has received a considerable amount of attention (Rallu *et al.*, 1996; Sanders *et al.*, 1999). However, only a limited number of data is available on the cold-adaptive response and the survival of LAB during low-temperature conditions. For several reasons the low-temperature response of LAB is of great importance. First, for many dairy fermentations the inoculum is produced with (frozen) starter cultures and the rate of survival of these bacteria during storage is a determining factor of the economic efficiencies of fermentations. Next, many fermentations are stopped by storage of the product at low temperature (e.g. storage of yoghurt at 4-7°C). At this low temperature the lactic acid production might slowly continue resulting in an overacidified product, as has been well described for yoghurt fermentation using *Streptococcus* and *Lactobacillus* strains (De Vos, 1996). The ripening of Gouda cheese, for which *Lactococcus* strains are used to start the fermentation, is taking place at approximately 13°C. At this temperature the ripening process slowly proceeds, yielding the required organoleptic characteristics. The biochemical processes involved in cheese ripening and the rate-determining steps of substrate conversions and protective mechanisms at low temperature are not fully understood. Next, the extended consumption of probiotic food products, containing LAB strains such as *Lactobacillus* and *Bifidobacterium* species, also increased the interest in studying the survival of these strains during low-temperature storage, since consumption of a high number of viable cells is assumed to be essential for the beneficial action of these products. The use of freezing as preservation method, the extended use of chilled (convenience) foods, and the increased popularity of fresh or minimally processed foods, often preservative-free, stimulated studies of cold-adaptation behaviour of beneficial but also of spoilage or pathogenic microorganisms, including LAB such as *Lactobacillus* species (Abee and Wouters, 1999).

The objective of the work described in this thesis is to elucidate the mechanisms by which LAB adapt to low-temperature conditions. For this purpose *Lactococcus lactis* MG1363 was chosen as a model organism, because of the well-known available genetic tools and its relatively easy accessibility. The research focused on the characterization of a family of genes encoding 7-kDa cold-shock proteins (CSPs). By using strains deleted in two or three *csp* genes and by using strains specifically overproducing a specific CSP, the regulation of expression of these genes and the functionality of the

encoded proteins is partly elucidated. Aspects regarding the structure/function relation of the characterized proteins of *L. lactis* are discussed below. A tentative model for the regulation of *csp* expression at normal as well as at low growth temperatures is discussed in relation to recent literature, including information about other bacteria, notably *Escherichia coli* and *Bacillus subtilis*.

CHARACTERIZATION OF A FAMILY OF GENES ENCODING CSPS IN *L. LACTIS*.

A family of genes, named *cspA, cspB, cspC, cspD* and *cspE,* encoding CSPs in *L. lactis* MG1363 was identified and characterized. The genes *cspA* and *cspB* as well as the genes *cspC* and *cspD* are located in tandem repeats, separated by approximately 270 bp within each repeat (Fig. 1; Chapter 2). Recently, such a clustered organization of *csp* genes has also been reported for *E. coli* and *Yersinia enterocolitica* (Yamanaka *et al.*, 1998; Neuhaus *et al.*, 1999). In *E. coli* the single *cspA* gene is located at 80 min. on the *E. coli* chromosome whereas the other *csp* genes are clustered around 34 min. The genes in the tandem of *cspB* and *cspF* and the tandem of *cspG* and *cspH* of *E. coli* are separated by intervals of 300 and 285 bp, respectively. The *E. coli csp* genes within each tandem are transcribed divergently, in contrast to the configuration of the lactococcal *csp* gene tandems. For *Y. enterocolitica* a *csp* gene tandem was described in which the *csp* genes are separated by an interval of approximately 300 bp. For this gene tandem both monocistronic and bicistronic transcripts could be detected (Neuhaus *et al.*, 1999), in contrast to the situation in *L. lactis* for which only monocistronic *csp* transcripts are observed (Chapter 2, see below). The gene organization in *L. lactis*, *E. coli* and *Y. enterocolitica* probably resulted from gene duplications. On the other hand the locations of the single *csp* genes found for *L. lactis* and *E. coli* are unique which indicates that these genes might have a more independent history (Yamanaka *et al.*, 1998; Chapter 2). The presence of multiple *csp* genes within one organism might ensure functionality in case one (or more) *csp* gene(s) happen(s) to get damaged.

The five lactococcal *csp* genes encode small proteins (7.1 to 7.6 kDa) with high mutual sequence identities (up to 85%) and high identities (about 45-65%) with the major CSPs from other bacteria. Northern-blot analysis revealed single transcripts of about 300 nucleotides for each *csp* gene. Furthermore, it was shown that *cspA*-, *cspB*-, *cspC*-, and *cspD*-mRNA levels were strongly increased upon cold shock of *L. lactis* from 30 to 10°C (about 10, 40, 10 and 30-fold induction, respectively), whereas the *cspE*-mRNA level was not increased (Fig. 1). The expression of the cold-induced *csp* genes was highest in the 6-8 h lag phase after cold shock. A differential expression in time was observed, in which *cspA* and *cspC* were maximally expressed after 2 h and *cspB* and *cspD* after 4 h following cold shock. For each gene the transcriptional start site was mapped by primer extension experiments with RNA isolated at different temperatures and this confirmed that regulation takes place at the transcriptional level (Chapter 2). By using two-dimensional gel electrophoresis (2D-EF) and overproduction studies the 7-kDa CSPs corresponding to all five *csp* genes could be identified. In addition to these CSPs two spots in the 7 kDa region were observed, designated CspF and CspG (N-

terminal sequence not determined; Chapters 3 and 5). The genes corresponding to these proteins may be located on a 3.5-kb *Hin*dIII fragment that hybridizes to an internal *cspB*-gene fragment as was observed using Southern blotting. Several attempts to clone this fragment failed (Chapter 2). All together, these data indicate that *L. lactis* MG1363 possibly contains a family of maximally 7 genes.

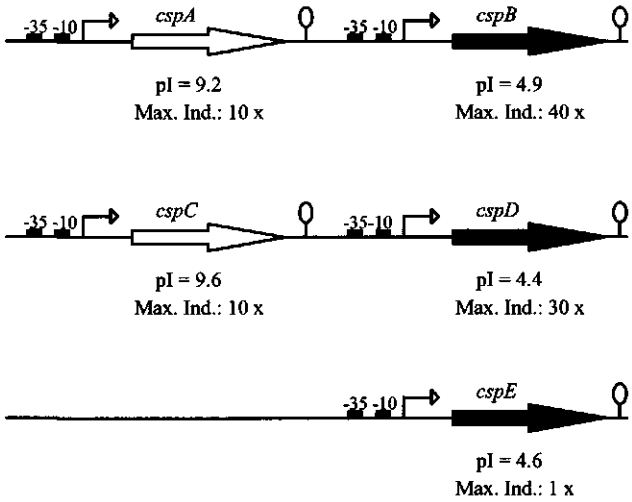

Fig. 1. Organization and nomenclature of the *csp* genes found in *L. lactis* MG1363. The large arrows indicate the open reading frames and the smaller arrows the transcription starts. The -35 and -10 regions are indicated by boxes and the putative terminators are indicated by hairpin structures. For each *csp* gene the isoelectric point (pI) and the maximal factor of mRNA induction (max. ind.) are given (after Chapter 2).

The CSPs were found to be the highest induced proteins of *L. lactis* upon exposure to a cold shock to 10°C. Northern blotting and 2D-EF showed that the *csp* genes were maximally expressed at 10°C, while induction was lower at 20 and 4°C (Chapter 3). *L. lactis* strains NZ9000ΔAB, deleted in *cspA* and *cspB*, and NZ9000ΔABE, deleted in *cspA*, *cspB* and *cspE*, were constructed by double cross-over recombination. Both strains showed no differences in growth at normal and at low temperature compared to the wild-type strain *L. lactis* NZ9000. This can be explained by the increased production of the remaining CSPs in the *csp*-deleted strains as was observed using 2D-EF (Chapter 6). Preliminary attempts failed to generate a *L. lactis* strain from which all *csp* genes were deleted (Frenkiel, H., and J. A. Wouters, unpublished data). This might indicate that the absence of *csp* genes is lethal for *L. lactis* similar as was observed for *B. subtilis* for which a (total) triple *cspBCD*-deleted mutant could only be generated in the presence of $cspB^{B}$ *in trans* on a plasmid (Graumann *et al.*, 1997).

REGULATORY ASPECTS OF *CSP* EXPRESSION IN *L. LACTIS*.

The low-temperature induction of the *csp* genes seems, among others, to take place at the

transcriptional level, and may be explained by stimulated promoter activity and/or increased mRNA stability at low temperature. By using *gusA*-promoter fusions it was attempted to assess the cold inducibility of the *csp* gene promoters but no cold-induction could be revealed (Wouters, J. A., unpublished data). This is probably explained by titration effects since the high-copy number plasmid pNZ273 was used (Platteeuw *et al.*, 1994). Similarly, Chapot-Chartier *et al.* (1997) only observed cold-induced *cspB*-directed galactosidase activity using a low copy plasmid (pIL derivative) in *L. lactis* IL1403 and not using a high copy number plasmid. The *cspB* gene of *L. lactis* MG1363 is identical to that of *L. lactis* IL1403, a strain for which 3 *csp* genes have been described (Chapot-Chartier *et al.*, 1997; Bolotin *et al.*, 1999).

The increased expression of the remaining *csp* genes upon deletion of *csp* genes of *L. lactis* shows that CSPs regulate the production of other CSP family members (Chapter 6). Upon deletion of *cspA* and *cspB*, the *cspE* mRNA level increased at low temperature and this resulted in increased production of CspE, whereas the transcription of *cspC* and *cspD* was not altered in comparison to the wild-type strain. In *L. lactis* NZ9000ΔAB either the transcription initiation of *cspE* is derepressed or the *cspE* transcript stability is increased. Additional deletion of CspE resulted in significantly increased *cspC* and *cspD* mRNA levels at 30°C and 10°C in *L. lactis* NZ9000ΔABE, resulting in increased CspC and CspD production. This indicates that CspE is directly or indirectly involved in the regulation of these genes and probably acts as a negative regulator either acting on the *cspC* and *cspD* promoters or on their mRNA stability (Chapter 6). For CspA from *E. coli* ($CspA^E$) and CspB from *B. subtilis* ($CspB^B$) binding to the so-called cold-shock box (CS-box) at the 5'-untranslated leader (5'-UTR) of *csp* mRNA has been found (Bae *et al.*, 1997; Graumann *et al.*, 1997) by which these transcripts are tagged. Consequently, these mRNAs cannot be translated and are rapidly degraded by RNases (Bae *et al.*, 1997). Significant differences between the 5'-UTR of the four cold-induced *csp* genes and the corresponding region of the non-cold-induced *cspE* of *L. lactis* gene were observed (Chapter 2). Also, the CS-boxes of the four cold-induced *csp* genes differ significantly from the one of the non-cold-induced *cspE* gene. It is speculated that CspE can bind with high affinity to the CS-box of these four cold-induced *csp* genes, thereby preventing their translation at 30°C and probably accelerating their degradation. CspE probably binds with lower affinity to its own 5'-UTR which makes it the only significantly expressed *csp* gene at 30°C in *L. lactis* (Fig. 2). Upon more detailed analysis of the truncated *cspE* gene expression in strain NZ9000ΔABE no mRNA at the undeleted 5'-UTR was detected, indicating absence of transcription at the promoter. This might indicate that CspE promotes transcription of its own gene and, thus, seems to be subject to positive autoregulation, a mechanism that has not been noted previously for *csp* genes (Fig. 2). However, it should be noted that the *cspE* transcript might also be destabilized due to the absence of an internal part of the transcript (Chapter 6).

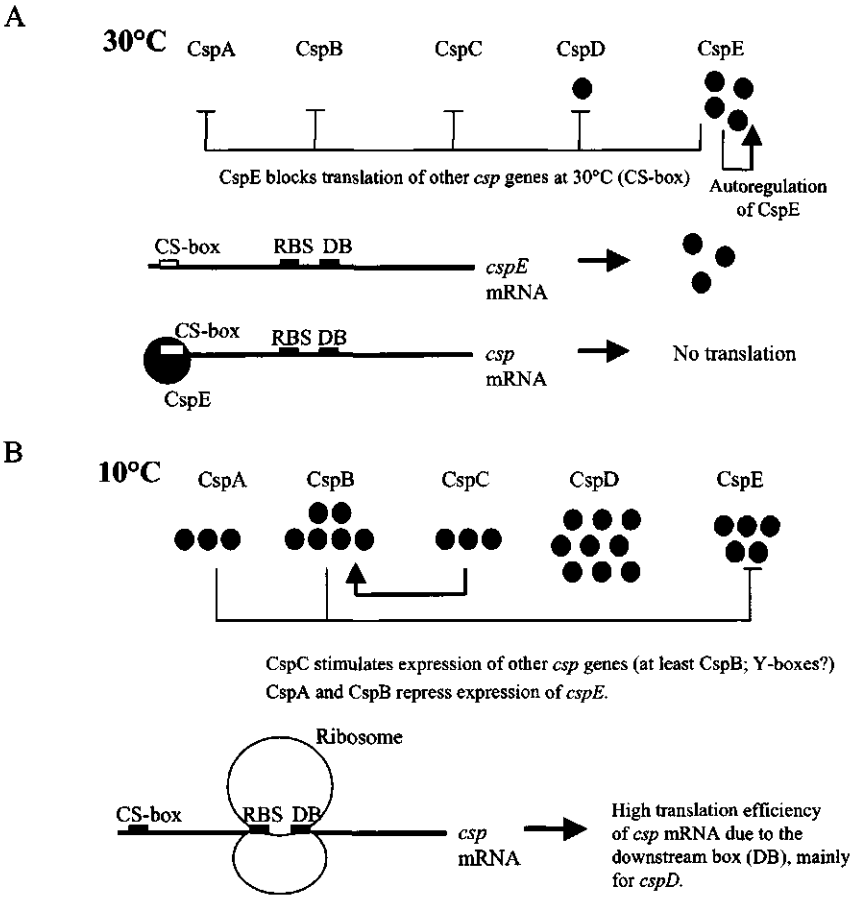

Fig. 2. Schematic representation of the regulation of *csp* gene expression in the CSP family of *L. lactis* and the genetic elements involved at normal (A; 30°C) and at low growth temperature (B; 10°C). See text for details.

Strikingly, upon overproduction of CspC the level of CspB increased and so was the amount of putative CspF and putative CspG. In contrast, overproduction of CspA, CspB, CspD or CspE did not affect the production of other CSPs (Chapter 5). CspC might directly stimulate the expression of the other *csp* genes by transcriptional activation as has been reported for gene regulation by $CspA^E$ for which Y-box motifs (ATTGG or CCAAT) have been shown to be important (LaTeana *et al.*, 1991; Jones *et al.*, 1992B; Brandi *et al.*, 1994). The presence of several of these elements is observed in the upstream regions of the lactococcal *csp* genes (Chapter 2), but a direct relation to the presence of these Y-boxes and transcriptional activation of the respective *csp* genes remains to be elucidated.

For *E. coli*, the mRNAs encoding CSPs and several cold-induced proteins (CIPs) are better translated than other mRNAs during cold-shock conditions (Mitta *et al.*, 1997). For non-cold shock

mRNAs additional cold-inducible ribosomal factors, such as RfbA and CsdA, are required for translation at low temperature (Jones and Inouye, 1996; Jones *et al.*, 1996). The translatability of mRNAs at low temperature has been related to a region designated as the down-stream box (DB), as has been shown for *cspA* of *E. coli* (Mitta *et al.*, 1997). This DB accounts for additional binding to the anti-downstream box (anti-DB) of 16S rRNA, thereby enhancing the formation of initiation complexes. In the 16S rRNA of *L. lactis* a possible anti-DB-sequence could be identified showing 53% identity to the anti-DB 16S rRNA of *E. coli* (Brosius *et al.*, 1978; Chiaruttini and Milet, 1993 [Table 1]). Alignment of the *csp* genes of *L. lactis* to the lactococcal anti-DB box showed that the DB of *cspD* contains the highest number of matching nucleotides (9), whereas the lowest number of matching nucleotides (5) is found for *cspB*. For *cspA*, *cspC* and *cspE* six matching nucleotides are observed in the DB (Table 1) that may explain the intermediate production of these proteins upon cold shock. Indeed, in *L. lactis* major cold induction is observed for CspD and to a lower extent for CspA, CspB, CspC and CspE (Chapter 3). Despite the similar induction at the mRNA level, the production of CspD is higher than that of CspB upon cold shock, suggesting marked differences in translational efficiency, possibly explained by differences in the DB. However, it should be noted that for *E. coli* biochemical support for a DB-anti DB interaction is lacking and that the conclusions are primarily based on mRNA-rRNA sequence complementarities. It has been argued that other explanations must be sought to explain the observed effect of the DB sequence on gene expression (Etchegaray and Inouye, 1999; Bläsi *et al.*, 1999).

Table 1. Alignment of the anti-DB of 16S rRNA of *L. lactis* (Chiaruttini and Milet, 1993) and *E. coli* (Brosius *et al.*, 1978) and the DBs of the *csp* genes of *L. lactis* and *cspA* of *E. coli* (Mitta *et al.*, 1997). The nucleotides of the DB complementary to the anti-DB of the 16S rRNA are shown in bold and are underlined and the start codons of the *csp* genes are depicted in italics. The number of potential base pairs, and the type of base pairing between the DB and the anti-DB are given.

Gene	(Anti)-downstream box	Complementary nucleotides			
16S RNA anti-DB *L. lactis*	1489-AGUAGCCAGAAUGGAAUC-1472	AU	GC	GU	Total
cspA	85-AG**AU**GAUAAA**UG**GAAC**AG**-102	4	1	1	6
cspB	84-A**UAU**GACAAAA**G**GAACU**G**-101	2	1	2	5
cspC	83-A**UAU**GAA**U**AAA**G**GAAC**A**A-100	4	0	2	6
cspD	87-**UUAU**G**G**CAAA**UG**GAAC**AG**-104	5	2	2	9
cspE	95-AAA**UG**G**C**A**C**AA**G**GAACU**G**-112	2	2	2	6
16S RNA anti-DB *E.coli*	1483-AGUACUUAGUGUUUCACC-1466				
cspA E. coli	146-AA**AUGA**C**U**GG**UA**UC**GU**AA-163	6	2	2	10

In conclusion, it is shown that the CSP production in *L. lactis* is tightly regulated at the transcriptional and the translational level as well as at the level of mRNA and protein stability. This type of multi-level regulation is similar to the regulation of expression as reported for $cspA^E$ (Yamanaka *et al.*, 1998). The increased production of the remaining CSPs upon deletion of the *csp* genes emphasizes that it is of outmost importance to keep the *csp* expression at a minimal level. On the other hand, the CSP level should stay below a maximum level since growth inhibitory effects of these proteins have been observed upon overproduction to high quantities (Chapter 5). It has also been shown that certain CSPs (e.g. CspC and CspE) are involved in the regulation of their counterparts of the CSP family (Chapters 5 and 6), which might explain the essence of the presence of more than one CSP in *L. lactis* for instance to be able to fine-tune the response to variable changing environmental conditions. It is observed that a tightly controlled regulatory circuit controls the production of CSPs in *L. lactis*. The role of the *csp* gene duplications on the regulation of expression remains to be elucidated, e.g. by studying the effects of deletion of the counterparts of a *csp* gene tandem. It was observed that the genes located in the tandems were subject to a differential expression in time, in which *cspA* and *cspC* were maximally expressed at 2 h and *cspB* and *cspD* at 4 h after cold shock (Chapter 2). This might also indicate a different role for the respective *csp* genes in the cold-adaptive response.

STRUCTURAL CHARACTERISTICS OF CSPS

The most extensively studied CSPs are $CspA^E$ and $CspB^B$ and the determination 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; Newkirk *et al.*, 1994; Schindelin *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 appear to be essential for nucleic acid binding, and a set of hydrophobic residues (V9, I21, V30, V32 and V51) forming a hydrophobic core of the protein (Newkirk *et al.*, 1994). 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 (Graumann and Marahiel, 1994; Newkirk *et al.*, 1994) thereby regulating gene expression (Jones and Inouye, 1994). For $CspA^E$ and $CspB^B$ it was shown that they act as broad-range specificity RNA-binding proteins for which the highly conserved RNA-binding motifs, i.e. RNP-1 and RNP-2, play an important role (Schindelin *et al.*, 1993, Graumann *et al.*, 1997; Jiang *et al.*, 1997). The residues important for single-stranded DNA binding of $CspA^E$ and $CspB^B$ (Newkirk *et al.*, 1994; Schröder *et al.*, 1995) are highly conserved in CspB, CspD and CspE of *L. lactis*, whereas in CspA and CspC some additional residues are different from the $CspA^E$ and $CspB^B$ DNA-binding residues. The RNP-1 and RNP-2 motifs are also found in the lactococcal CSPs although some differences are observed (Chapter 2). Protein three-dimensional modelling based on the crystal structure of $CspA^E$ and $CspB^B$ (Schindelin

et al., 1993; Schindelin *et al.*, 1994) revealed a similar β-barrel structure formed by five β-strands for all five lactococcal CSPs. The amino acid backbone has a similar folding for all CSPs of *L. lactis*. Furthermore, it was observed that most residues important for DNA and RNA binding (basic: K7, K13, H29 and R56 or aromatic: W8, F15, F17, F27, F30) are located on one side of the surface of the respective CSPs (Wouters, J.A., B. Renckens, and R. J. Siezen, unpublished data [Fig. 3]). Interestingly, the iso-electric points (pI) of CspA and CspC (9.2 and 9.6, respectively [Fig. 1]) are much higher than the pI of the other lactococcal CSPs (approximately 4.5 [Fig. 1]) due to the presence of more basic (8 and 11 for CspA and CspC, respectively, compared to 7 for CspB, CspD and CspE) and tyrosine residues (4 for CspA and CspC and none for CspB, CspD and CspE). The high pI of CspA and CspC might result in altered nucleic acid binding capacities since these proteins do not need to overcome charge repulsion when approaching nucleic acids, similar as discussed for mutants of $CspB^{B}$ (Schröder *et al.*, 1995). However, recently, it has also been stated that acidic CSPs have a ideal structure to act as RNA chaperones since they possess a positively charged RNA-binding epitope that is backed by a negatively-charged surface that would prevent approach of other parts of RNA by charge repulsion (Graumann and Marahiel, 1998).

The observation that CspB, CspD and CspE of *L. lactis* can be overproduced to high quantities at 30°C is remarkable (Chapter 5). The artificial overexpression of the $cspA^{E}$ gene was very low at 37°C due to its low mRNA stability (Goldenberg *et al.*, 1996). The low quantity of

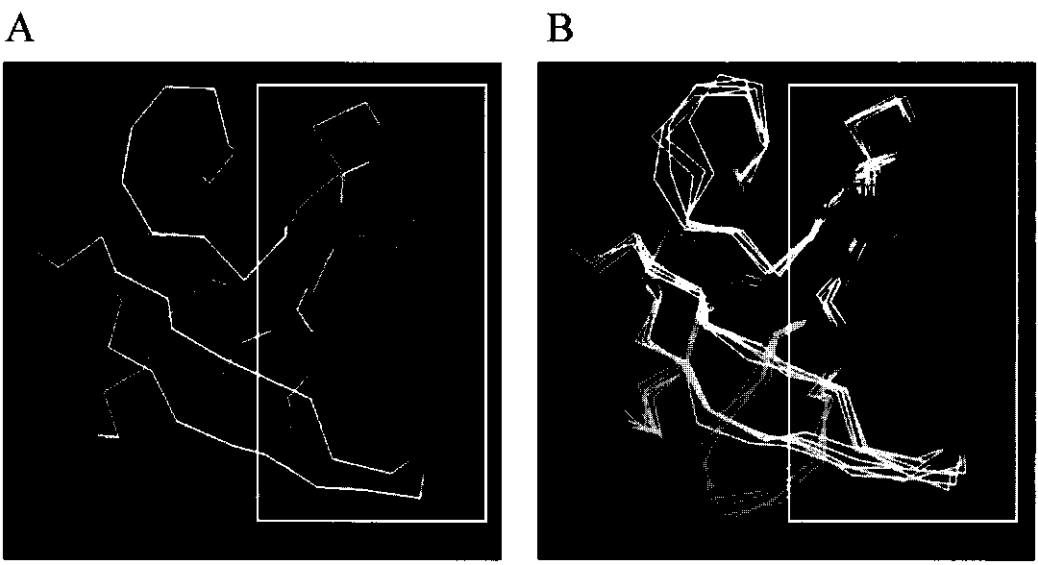

Fig. 3. Modeling of the three-dimensional folding of CspA of *L. lactis* (A) and an overlay of the modeling of the 3D-folding structures of CspB, CspC, CspD and CspE of *L. lactis* and CspA of *E. coli* (B). The white line indicates the backbone structure of the respective CSPs. Important residues for RNA and DNA binding are boxed and indicated grayish (basic: K7, K13, H29 and R56 or aromatic: W8, F15, F17, F27, F30). The modeling was based on the 3D-crystal structures of $CspA^{E}$ and $CspB^{B}$ (Schindelin *et al.*, 1993; Schindelin *et al.*, 1994) using Quanta/Charmm (Molecular Simulations Inc., San Diego, Ca., USA).

overexpression obtained for the lactococcal *cspC* gene is indeed explained by low stability of the transcript at 30°C as was shown using Northern blotting. Since for *cspA* a high mRNA induction is observed, the low quantity of CspA overproduction should be explained by other factors. CspA* (a CspA mutant that contains an Arg-Pro substitution at position 58 and a C-terminal deletion of Lys65 and Val66) can be overproduced in much higher quantities than CspA. This can be explained by the high stability of the protein as a consequence of the reduced entropy. Next to the specific Arg-Pro mutation, also the decrease in pI may contribute to CspA* stability (Chapter 5). Recently, it was shown that the stability of the CSPs of *B. subtilis* was significantly increased by binding to a nucleic acid ligand, which might be dependent on the charge of the protein (Schindler *et al.*, 1999). This would offer an alternative explanation for the increased stability of CspA* and could also explain why the CSPs of *L. lactis* that have a low pI can be overproduced up to high quantities.

CSPs are relatively small proteins and thus they are ideal substrates to study their single stranded RNA and DNA-binding characteristics. Other striking characteristics are the large non- polar patch found on the exterior of the proteins and the fact that the proteins are all β-sheet proteins that perform extremely rapid folding transitions (Schindler *et al.*, 1995; Hillier *et al.*, 1998; Reid *et al.*, 1998). For these reasons, the structural characteristics of CSPs received a considerable amount of attention. In future research the structural characteristics of the CSPs of *L. lactis* could be studied in more detail by using site-directed mutagenesis and may supply information about: *i.* DNA and RNA-binding capacities of the different counterparts of the CSP family in relation to their overall charge, and *ii.* binding characteristics of CSPs in relation to protection of the cell and cellular molecules during freezing (see below).

CSPs AND THEIR ROLE IN CRYOPROTECTION

Maintaining membrane integrity and prevention of macromolecule denaturation have been mentioned as key factors increasing the freeze-survival of microorganisms (El-kest and Marth, 1992; Franks, 1995; Thammavongs *et al.*, 1996). It was noted that many microorganisms develop an increased ability to survive freezing after a cold-shock treatment. Also, for *L. lactis* MG1363 as well as *Streptococcus thermophilus* CNRZ302, the survival to freezing increased when cells were exposed to low temperature (approximately 100-fold and 1000-fold increase upon exposure to 10 or 20°C for 4 h, respectively), compared to mid-exponential phase cells grown at 30°C. For both strains it was shown that for this freeze-adaptive process protein synthesis is required, indicating an active protection mechanism against freezing (Chapters 3 and 4). Pre-incubation of *L. lactis* cells at 20 and 4°C, as well as at stationary phase conditions at 30°C, also induced cryoprotection (approximately 30-, 130- and 20-fold compared to 30°C mid-exponential phase, respectively). Strikingly, the increased survival to freezing of *L. lactis* coincides with increased CSP production, except for stationary phase conditions (Chapter 3). In addition, it was observed that for *L. lactis* cells overproducing CspB, CspD or CspE at

30°C the survival to freezing increased 2-10 fold compared to control cells (Chapters 3 and 5). Next, the adaptive response to freezing conditions by prior exposure to 10°C was significantly delayed in strain NZ9000ΔABE compared to strains NZ9000 and NZ9000ΔAB, indicating a possible role of at least CspE in the kinetics of freeze adaptation (Chapter 6). In combination these data show that CSPs in *L. lactis* play a role in the adaptive response to freezing. Also for $CspB^{B}$ a freeze-protective effect is described: a *B. subtilis* strain deleted in the *cspB* gene showed a decreased freeze survival (Willimsky *et al.*, 1992). Recently, also for *Lactobacillus plantarum* a freeze-protective effect was reported upon overproduction of its CSPs (Derzelle, S., P. Hols, and J. Delcour, personal communication). It has been speculated that CSPs have an anti-freeze function, protecting against cell damage similar as has been observed for anti-freeze proteins, e.g. in insects, plants or polar fish. However, except for their high production levels and their low molecular weights, no further similarities in sequence or structure were found between anti-freeze proteins and CSPs. Anti-freeze proteins function in minimizing ice-crystal formation by the formation of additional H-bridges, resulting in lowering of the freezing point of internal fluids (Yang *et al.*, 1988; Sicheri and Yang, 1995). On the other hand, the exact function of CSPs in cryoprotection remains to be elucidated but a freeze-protective rather than an anti-freeze function should be expected. Since CSPs are known to have RNA and DNA binding characteristics they might have a direct protective effect during freezing, e.g. by RNA or DNA stabilization. On the other hand, CSPs might have an indirect role in the freeze protective response since they act as activators of other proteins (see below) involved in cryoprotection. It has been observed that three proteins (26, 43 and 45 kDa) are induced during all conditions leading to increased survival to freezing (low temperature exposure to 20, 10 and 4°C and stationary phase conditions) and for these proteins a role in cryoprotection is suggested (Chapter 3).

CSPs ACT AS ACTIVATORS OF COLD-INDUCED PROTEINS

By using two-dimensional gel electrophoresis (2D-EF), 17 CIPs could be identified for *L. lactis*, besides the 7 kDa CSPs (Chapter 3). N-terminal analysis of the CIPs of *L. lactis* revealed that proteins involved in a variety of cellular processes are induced upon cold shock. CIP1 (11 kDa) was identified as a histon-like protein and showed the highest homology to HlpA of *Streptococcus mutans* (Stinson *et al.*, 1998). H-NS of *E. coli* was also found to be cold induced and a role for this protein in optimizing DNA-supercoiling, which increases at low temperature, has been suggested (LaTeana *et al.*, 1991; Brandi *et al.*, 1994). CIP2 (14 kDa) showed homology to the 50S ribosomal protein L9 of *B. subtilis* (Ogasawara *et al.*, 1994). For both *E. coli* and *B. subtilis* cold-induced ribosomal proteins have been identified (S1, S6, L7/L12 and S6 and L7/L12, respectively; Jones *et al.*, 1992A; Graumann *et al.*, 1996) and it is suggested that these proteins are required for the correct assembly of rRNA at low temperatures (Graumann *et al.*, 1996). Furthermore, CIP6 (26 kDa) was identified as β-phosphoglucomutase of *L. lactis* (β-PGM; Qian *et al.*, 1997) and CIP8 (29

kDa) was found to be homologous to a hypothetical signal transduction protein of *B. subtilis* (Ogasawara *et al.*, 1994), indicating that the low-temperature response includes adaptation of several cellular processes. In Chapter 6 it is described that CIP1 and CIP5 were cold induced in wild-type cells but were no longer induced at low temperature in *L. lactis* NZ9000ΔAB and *L. lactis* NZ9000ΔABE. CIP8 and CIP9 were found to be still cold-induced in strain NZ9000ΔAB but not anymore in NZ9000ΔABE. The overproduction of the lactococcal CSPs in *L. lactis* induces a variety of proteins, among which also a number of CIPs (CIP2, CIP4, CIP5 and CIP9; Chapter 5). These data indicate that these CSPs regulate other proteins involved in cold adaptation. In combination, the production of CIP5 and CIP9 is reduced upon deletion of CspABE and production of these CIPs increases upon overproduction of CspA and CspE (Chapter 5), strongly suggesting a regulatory role of these CSPs on the production of CIP5 and CIP9. For *E. coli* it has been reported that $CspA^E$ functions as a transcriptional activator of several cold-induced genes by interacting with Y-boxes located in their promoter regions (LaTeana *et al.*, 1991; Brandi *et al.*, 1994) and a similar regulation might be expected for the CSPs of *L. lactis*. The reduced expression of CIP1, 5, 8 and 9 upon cold shock does not result in growth defects but might cause the delayed response for freeze survival as was noted in *L. lactis* NZ9000ΔABE. It is striking to note that these regulatory functions can not be compensated by the increased expression of the remaining CSPs. Probably, these regulatory aspects are highly specific for each CSP and this might be an indication for the necessity of the presence of a CSP family, thereby indicating specific functions for the counterparts. A more detailed identification of promoter elements involved in the regulation by the *csp* genes might provide a better insight in the total set of genes regulated by the lactococcal *csp* genes.

In Chapter 7 the effects of low temperature on the glycolytic activity of *L. lactis* were studied. The maximal glycolytic activity measured at 30°C increased (approximately 2.5-fold) upon incubation at 10°C for several hours. For this adaptative response protein synthesis is required. Using strains disrupted in several genes in sugar metabolism it was revealed that both HPr and CcpA were involved in the increased glycolysis rate, whereas Enzyme I was not involved in this response. These data indicate that the major sugar-uptake system, the phosphoenolpyruvate-dependent sugar phosphotransferase system (PEP-PTS) is not involved in this response whereas the regulatory function of 46-seryl phosphorylated HPR (HPr(Ser-P)) probably is involved. Protein analysis showed that the production of both HPr and CcpA was slightly (up to 2-3 fold) induced upon exposure to low temperature for several hours. The *las* operon, that is subject to catabolite activation by the CcpA/HPr(Ser-P) complex was not induced upon cold shock and no increased LDH activity was observed for these conditions. Also another rate-limiting enzyme of the glycolytic pathway, GAPDH, was not induced upon cold shock. In combination, these data indicate that another factor(s) is responsible for the increased glycolytic activity upon exposure to low temperature. It is proposed that the CcpA/HPr(Ser-P) complex regulates this factor(s).

The use of 2D-EF proved to be a powerful tool to study the effects of stress exposure on overall cell adaptation or to study the specific effects of disruption or overexpression of certain genes on total protein production (Chapters 3, 5, 6 and 7). It was attempted to monitor the actual production level of the cellular proteins, by use of silver staining as a detection method. However, it should be noted that the analysis of the total "proteome" has several restrictions, such as the identification of membrane proteins, proteins with extreme pI values and highly unstable proteins. In future, the use of 2D-EF will be expanded with the identification of proteins using mass spectrometry or N-terminal sequencing, the increased number of 2D-EF protein databases, and the further development of bio-informatics. The increasing number of genome sequences and the development of micro-array technologies will significantly contribute to the exploration of global gene expression. The application of these techniques will undoubtedly contribute to a better understanding of global responses during cellular stress response. In more detail, the availability of these techniques for *L. lactis* (Bolotin *et al.*, 1999; Kuipers, 1999) will contribute to the unraveling of CSP functioning, and in particular to the exploration of the global regulatory functioning of the lactococcal CSPs.

PERSPECTIVES OF RESEARCH ON LOW-TEMPERATURE ADAPTATION

The research on cold adaptation might yield direct applications for food preservation 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; Chapters 5 and 6). This might result in a high survival 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 recombinant proteins at low temperature. For example, the $cspA^E$ promoter was used to express recombinant proteins at low temperature in *E. coli* and a 3 to 5-fold induction using different promoter fragments was found. However, it should be noted that the $cspA^E$ promoter became repressed after 2 h at low temperature, which makes it not very suitable for use in high-yield expression systems (Vasina and Baneyx, 1996; Vasina and Baneyx, 1997). From the increased knowledge on CSPs useful information can be gained to understand low-temperature adaptation and the deleterious effects of cold shock for certain bacteria. This may also offer perspectives for methods to control the growth of microorganisms that limit the shelf life and safety of refrigerated foods.

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Summary

Lactic acid bacteria (LAB) are widely used as starter cultures in fermentation processes world wide. The stress response of LAB during different industrial processes, and especially low-temperature conditions, requires a thorough understanding to be able to optimize these processes. Many LAB fermentations are started with the addition of frozen cultures and therefore there is a great interest to better control the freeze-survival capacity of starter strains. A number of these fermentation processes, like yoghurt or cheese production, take place at low temperature and/or the fermented products are stored at low temperatures after fermentation by which the product might be overacidified resulting in spoilage of the product. Additionally, the increased consumption of probiotic food products significantly enhanced the interest in methods for better survival of the probiotic LAB strains during (low temperature) storage, since consumption of a high dose of viable cells is required for the beneficial action of these products. This research project focuses on the low-temperature adaptation of LAB and on that of *Lactococcus lactis* MG1363, a model LAB strain, in particular.

It has been well established that in a wide variety of bacteria a set of proteins is preferentially expressed after a rapid decrease in the temperature of the culture medium (cold shock). Chapter 2 describes the cloning and sequencing of a family of five *csp* genes of *L. lactis* MG1363, named *cspA, cspB, cspC, cspD* and *cspE*, encoding highly similar CSPs (65-85% identity). On the *L. lactis* MG1363 chromosome two tandem groups of *csp* genes (*cspA/cspB* and *cspC/cspD*) were identified, whereas *cspE* was found as a single gene. Transcription analysis showed that *cspE* is the only non-cold-induced *csp* gene, whereas the other *csp* genes are induced 10- to 40-fold at different times after cold shock. In Chapter 3 the identification of a group of 7-kDa CSPs, corresponding to the *csp* genes of *L. lactis* MG1363, is described. These proteins were the highest induced proteins upon cold shock to 10°C. Northern blotting and two-dimensional gel electrophoresis showed that the *csp* genes were maximally expressed at 10°C, while induction was lower at 20 and 4°C.

Physiological and regulatory aspects of the lactococcal CSPs were studied in more detail by the controlled overexpression and the specific deletion of *csp* genes. In Chapter 5 it is reported that CspB, CspD and CspE can be overproduced to high levels (up to 19% of total protein) using the nisin-inducible expression system, whereas for CspA and CspC limited overproduction (0.3-0.5% of total protein) was obtained. Northern-blot analysis revealed low abundance of the *cspC* transcript, indicating that the stability of *cspC* mRNA at 30°C is low. The limited overproduction of CspA is likely to be caused by a low stability of CspA since upon an Arg-Pro mutation at position 58 its stability significantly increased. Strikingly, upon overproduction of CspC induction of CspB, putative CspF and putative CspG was also observed. Chapter 6 describes two *L. lactis* strains: NZ9000ΔAB, deleted in *cspA* and *cspB*, and NZ9000ΔABE, deleted in *cspA*, *cspB* and *cspE*, that were constructed by

double cross-over recombination. For both strains no differences in growth at normal and at low temperature were measured, compared to that of the wild-type strain *L. lactis* NZ9000. Deletion of *csp* genes was compensated by increased expression of the remaining *csp* genes, indicating a tightly controlled transcription network. In strain NZ9000ΔAB *cspE* was found to be expressed to a higher extent than in wild-type cells in response to low temperature, indicating that CspA and/or CspB may act as repressor(s) of *cspE* expression at low temperature. In strain NZ9000ΔABE the mRNA levels of *cspC* and *cspD* significantly increased at all temperatures investigated, suggesting that CspE decreases their expression either by transcriptional repression or by decreasing their mRNA stabilities.

During freezing bacterial cells may be damaged by ice crystal formation and/or by high osmolarity caused by high concentrations of internal solutes during the freezing process. Resistance to disruption of the membrane integrity and macromolecule denaturation have been mentioned as factors that determine the survival of bacterial cells after freezing. An approximately 100-fold enhanced survival after freezing was found when *L. lactis* cells were shocked to 10°C for 4 h compared to mid-exponential phase cells grown at 30°C. For this adaptive response protein synthesis is required, indicating that an active mechanism is able to protect cells against freezing. Strikingly, it was observed that the freeze-survival of *L. lactis* was highest during maximal CSP production (Chapter 3). To study the effects of CSPs on the survival after freezing in more detail, *L. lactis* strains specifically overproducing one of the CSPs or strains deleted in *csp* genes were tested on their susceptibility to freezing (Chapters 5 and 6). *L. lactis* cells overproducing CspB, CspD or CspE at 30°C show a 2-10 fold increased survival after freezing compared to control cells. The adaptive response to freezing conditions by prior exposure to 10°C was significantly delayed in strain NZ9000ΔABE compared to strains NZ9000 and NZ9000ΔAB. In combination, these data indicate that 7-kDa CSPs enhance the survival capacity after freezing. It is concluded that CSPs in *L. lactis* either have a direct protective effect during freezing, e.g. by RNA stabilization, and/or induce other factors involved in the freeze-adaptive response.

In Chapter 4 several 7-kDa proteins that are strongly induced upon cold shock from 42 to 20°C are reported for the thermophilic LAB *Streptococcus thermophilus* using two-dimensional gel electrophoresis. Also for *S. thermophilus* the survival to freezing increases by prior exposure to low temperature, albeit that the optimum adaptation temperature was significantly higher than that for *L. lactis*. It was shown that protein synthesis is required for the adaptive response to freezing. Using a PCR method a *csp* gene was identified for *S. thermophilus*.

In Chapter 5 it was found that upon overproduction of specific CSPs at 30°C the production of several non-7 kDa cold-induced proteins (CIPs) of *L. lactis* increased. Interestingly, in Chapter 6 it was observed that several CIPs were no longer cold induced in the *csp*-deleted strains, indicating that CSPs might activate the production of certain CIPs. For these regulatory circuits no compensatory

mechanisms seem to have evolved, in part explaining the necessity of a CSP family, consisting of very similar members. The N-terminal sequences of several CIPs of *L. lactis* were identified and these CIPs appeared to be implicated in a variety of cellular processes, including transcriptional control (a protein homologous to a histon-like protein of *Streptococcus mutans*), translational control (a protein homologous to the ribosomal L9 protein of *B. subtilis*), sugar metabolism (a protein identical to β-phosphoglucomutase of *L. lactis*) and signal sensing (a protein homologous to a signal transduction protein of *B. subtilis*). It remains to be established which function these proteins fulfill in the context of cold adaptation or other stress conditions.

In Chapter 7 we show that the maximal glycolytic activity measured at 30°C increases (approximately 2.5-fold) upon pre-incubation at 10°C for several hours, a process for which protein synthesis is required. Using strains disrupted in several genes in sugar metabolism it was shown that both HPr and CcpA were involved in the increased acidification, whereas Enzyme I was not involved in this response. This indicates that the major sugar uptake system, the phosphoenolpyruvate-dependent sugar phosphotransferase system (PEP-PTS) is not directly involved in this response whereas the regulatory function of 46-seryl phosphorylated HPR (HPr(Ser-P)), working in combination with CcpA, probably is involved. Protein analysis showed that the production of both HPr and CcpA was slightly (up to 2-3 fold) induced upon exposure to low temperature for several hours. The *las* operon, that is subject to catabolite activation by the CcpA/HPr(Ser-P) complex was not induced upon cold shock and no increased LDH activity was observed for these conditions. Also another rate-limiting enzyme of the glycolytic pathway, GAPDH, was not induced upon cold shock. In combination, these data indicate that other factors will be rate-determining for the increased glycolytic activity upon exposure to low temperature. It is proposed that the CcpA/HPr(Ser-P) complex regulates these factor(s).

In this work a family of cold-induced *csp* genes is identified and characterized. It is shown that CSPs are implicated in freeze-protection of *L. lactis* cells and that CSPs regulate the production of other proteins involved in cold adaptation (CIPs). The regulation of expression of the lactococcal *csp* genes is controlled at several levels via a tightly controlled network in which the counterparts of the *csp* family play a critical role. The research on cold adaptation might yield direct applications for food preservation and fermentation technology. Upon different cold-shock treatments a significant increase in the freeze-survival capacity of several bacteria is observed and a role for CSPs in this response has been shown. This might result in an enhanced survival rate 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 production of enzymes at low temperature. From the increased knowledge regarding CSPs, useful information can be gained to understand low temperature adaptation and the deleterious effects of

cold shock on certain bacteria. This may also offer insight into methods to control the growth of microorganisms that continue to challenge the shelf life and safety of refrigerated foods.

Samenvatting

Melkzuurbacteriën spelen een belangrijke rol in de levensmiddelenindustrie door hun wijdverspreide gebruik in vele fermentatie-processen. De stress-respons, de reactie op ongunstige groeiomstandigheden, van melkzuurbacteriën tijdens industriële processen behoeft een beter begrip. Veel melkzuurbacterie-fermentaties worden gestart door de toevoeging van bevroren startercultures en daarom is er veel belangstelling om de overleving van startercultures na invriezen beter te controleren. Een aantal van deze melkzuurbacterie-fermentaties, zoals de yoghurt- of kaasproductie, vindt plaats bij lage temperatuur terwijl de fermentatie producten bij lage temperatuur bewaard worden. De toegenomen consumptie van probiotische levensmiddelen heeft de interesse in de overleving van probiotische melkzuurbacteriën tijdens productie en bewaring doen toenemen. De consumptie van een hoge dosis levende cellen kan benodigd zijn voor de mogelijke gezondheidsbevorderende effecten van dergelijke producten. Dit onderzoeksproject spits zich toe op de aanpassing van melkzuurbacteriën aan lage temperatuur, met *Lactococcus lactis* MG1363 als modelorganisme.

Het is bekend dat bacteriën tijdens blootstelling aan lage temperatuur zogenaamde koude-schok-eiwitten (CSPs) synthetiseren. Hoofdstuk 2 beschrijft de identificatie en karakterisatie van een familie van vijf *csp*-genen van *L. lactis* MG1363, genaamd *cspA*, *cspB*, *cspC*, *cspD* en *cspE*, die coderen voor sterk op elkaar gelijkende CSPs (65 tot 85% identiek). Op het chromosoom van *L. lactis* MG1363 werden twee tandems van *csp*-genen, (*cspA/cspB* en *cspC/cspD*) geïdentificeerd. Daarentegen werd *cspE* als alleenliggend gen gevonden. Analyse van de transcriptie toonde aan dat *cspE* het enige niet koude-geïnduceerde gen is, terwijl voor de andere genen 10- tot 40-voudige toename van expressie gevonden werd op verschillende tijdstippen na blootstelling aan een abrupte temperatuurdaling (koude schok). In Hoofdstuk 3 is de groep van 7-kDa CSPs, behorend bij de eerder gekarakteriseerde *csp*-genen, geïdentificeerd. De CSPs zijn de sterkst geïnduceerde eiwitten van *L. lactis* MG1363 na een koude schok van 30 naar 10°C. Northern-blotting en twee-dimensionale gelelectroforese toonden aan dat de *csp* genen tot maximale expressie komen bij 10°C in vergelijking tot 20 en 4°C.

Fysiologische en regulatoire aspecten van de productie van CSPs van *L. lactis* MG1363 werden in meer detail bestudeerd door gebruik te maken van gecontroleerde overexpressie en specifieke uitschakeling van de *csp*-genen. In Hoofdstuk 5 is beschreven dat CspB, CspD en CspE tot grote hoeveelheden kunnen worden overgeproduceerd (tot 19% van de totale hoeveelheid intracellulair, oplosbaar eiwit) met behulp van een nisine-gecontroleerd overexpressie-systeem. Daarentegen kon slechts een zeer geringe overproductie worden verkregen voor CspA en CspC (0.3-0.5% van de totale hoeveelheid eiwit). Northern-blot-analyse toonde een geringe hoeveelheid van het *cspC* transcript aan, hetgeen erop duidt dat dit transcript erg instabiel is. De geringe

overproductie van CspA wordt waarschijnlijk veroorzaakt door een lage stabiliteit van het CspA-eiwit omdat na plaatsspecifieke mutatie van een arginine-residue in een proline-residue op positie 58 de stabiliteit van CspA sterk verhoogd bleek. Opmerkelijk is dat na overproductie van CspC een toegenomen productie werd waargenomen van CspB en van twee andere mogelijke CSPs, genaamd CspF en CspG. Hoofdstuk 6 beschrijft twee *L. lactis* stammen: *L. lactis* NZ9000ΔAB, gedeleteerd in de genen *cspA* en *cspB*, en *L. lactis* NZ9000ΔABE, gedeleteerd in de genen *cspA*, *cspB* en *cspE*, die werden geconstrueerd door middel van dubbel-cross-over-recombinatie. Voor beide stammen werden geen verschillen waargenomen in hun groeigedrag bij normale en lage temperatuur in vergelijking met de wild-type stam *L. lactis* NZ9000. De uitschakeling van de *csp*-genen werd gecompenseerd door een verhoogde expressie van de resterende *csp*-genen. Dit is een indicatie voor een strikt-gecontroleerd transcriptie-netwerk. In stam *L. lactis* NZ9000ΔAB kwam *cspE* sterker tot expressie bij lage temperatuur dan in de wild-type stam, hetgeen aangeeft dat CspA en/of CspB mogelijk werkzaam zijn als repressor van CspE bij lage temperatuur. In stam *L. lactis* NZ9000ΔABE namen de mRNA-niveau's van *cspC* en *cspD* significant toe bij alle onderzochte temperaturen. Dit suggereert dat CspE de expressie van deze genen vermindert, hetzij door transcriptionele remming danwel door het verlagen van de stabiliteit van de transcripten.

Tijdens invriezen kunnen bacteriële cellen beschadigd worden door de vorming van ijskristallen en/of door de hoge osmolariteit van het cytoplasma. De integriteit van de membraan en denaturatie van macromoleculen zijn genoemd als de bepalende factoren voor de overleving van bacteriële cellen na invriezen. Voor *L. lactis* werd waargenomen dat de overleving na invriezen ongeveer 100 maal hoger is van cellen blootgesteld aan 10°C gedurende 4 uur vergeleken met mid-exponentiële (niet-blootgestelde) cellen. Voor deze aanpassingsreactie bleek eiwitsynthese benodigd te zijn, hetgeen aangeeft dat het hier om een actief aanpassingsmechanisme gaat. Opvallend was dat de bescherming tegen invriezen door *L. lactis* cellen maximaal was bij een maximale CSP-productie. Om de effecten van CSPs tijdens invriezen in meer detail te bestuderen, werden stammen die specifiek één van de CSPs overproduceren en stammen waarvan een aantal *csp*-genen is uitgeschakeld, getest op hun gevoeligheid voor invriezen (Hoofdstuk 5 en 6). *L. lactis*-cellen die CspB, CspD of CspE overproduceren blijken het invriezen 2- tot 10-keer beter te overleven in vergelijking met controle cellen. De aanpassingsrespons op invriezen van *L. lactis* NZ9000ΔABE was significant vertraagd in vergelijking met *L. lactis* NZ9000 en *L. lactis* NZ9000ΔAB. In combinatie geven deze waarnemingen aan dat CSPs betrokken zijn bij de bescherming tijdens invriezen. De CSPs in *L. lactis* hebben ofwel een direct beschermend effect, bijvoorbeeld door de bescherming van RNA, ofwel een indirect effect door de inductie van andere factoren betrokken bij de bescherming tegen invriezen.

In Hoofdstuk 4 wordt beschreven dat voor de thermofiele melkzuurbacterie *Streptococcus thermophilus* een groep van sterk koude-geïnduceerde 7-kDa CSPs is waargenomen met behulp van

twee-dimensionale gelelectrophorese. Ook voor *S. thermophilus* wordt een verhoogde overleving na invriezen waargenomen na blootstelling van de cellen aan lage temperatuur. Voor dit aanpassingsproces gedurende de blootstelling aan lage temperatuur is *de novo* eiwitsynthese noodzakelijk. Met behulp van een PCR-methode kon een *csp*-gen van *S. thermophilus* worden geïdentificeerd.

In Hoofdstuk 5 wordt beschreven dat de overproductie van CSPs bij 30°C leidt tot de inductie van een aantal andere eiwitten, waaronder ook koude-geinduceerde eiwitten (CIPs). Daarnaast, wordt in Hoofdstuk 6 beschreven dat een aantal CIPs niet meer door koude wordt geïnduceerd in stammen gedeleteerd in hun *csp*-genen. Dit geeft aan dat CSPs mogelijk de productie van sommige CIPs stimuleren. Voor deze regulatoire mechanismen is geen compensatie waargenomen, hetgeen een gedeeltelijke verklaring zou kunnen vormen voor de noodzakelijke aanwezigheid van een CSP-familie, die uit zeer sterk op elkaar gelijkende leden bestaat. Van een aantal CIPs werd de N-terminale aminozuurvolgorde bepaald en deze CIPs blijken betrokken te zijn bij een verscheidenheid van celprocessen, zoals controle van transcriptie (een eiwit homoloog aan een histon-achtig eiwit van *Streptococcus mutans*), controle van translatie (een eiwit homoloog aan het ribosomale L9-eiwit van *B. subtilis*), suikermetabolisme (een eiwit identiek aan β-fosfoglucomutase van *L. lactis*) en signaalperceptie (een eiwit homoloog aan een signaal transductie eiwit van *B. subtilis*).

Vervolgens wordt in Hoofdstuk 7 beschreven dat de maximale glycolytische activiteit bij 30°C van *L. lactis* cellen, die enkele uren zijn blootgesteld aan 10°C, verhoogd is vergeleken met niet-blootgestelde cellen (2.5-voudige toename). Voor dit proces blijkt *de novo* eiwitsynthese noodzakelijk te zijn. Door gebruik te maken van *L. lactis*-stammen waarvan een aantal genen betrokken bij het suikermetabolisme zijn uitgeschakeld, kon worden vastgesteld dat zowel HPr als CcpA betrokken zijn bij de verhoogde verzuringssnelheid na koude schok, maar dat Enzym I niet betrokken is. Dit wijst erop dat het belangrijkste suikeropname-systeem, het fosfoenolpyruvaat-afhankelijke suiker-fosfotransferase-systeem niet betrokken is bij de verhoogde verzuringssnelheid na blootstelling aan lage temperatuur maar dat de regulatoire functie van 46-seryl gefosforyleerd HPr (HPr(Ser-P)) hierbij waarschijnlijk wel betrokken is. Eiwitanalyse toonde aan dat de productie van zowel HPr als CcpA licht verhoogd is na incubatie van *L. lactis*-cellen bij lage temperatuur (2-3 voudige toename). Het *las* operon, dat gereguleerd wordt door het CcpA/HPr(Ser-P)-complex werd niet geïnduceerd na koude schok en er werd ook geen toename in de lactaat-dehydogenase activiteit waargenomen onder deze condities. Ook een ander snelheidsbepalend enzym van de glycolyse, glyceraldehyde-fosfaat dehydrogenase, werd niet geïnduceerd na koude schok. Gecombineerd tonen deze gegevens aan dat een andere factor(en) snelheidsbepalend is voor de toegenomen glycolytische activiteit na blootstelling aan lage temperatuur. Verondersteld wordt dat het CcpA/HPr(Ser-P) complex betrokken is bij de regulatie van deze factor(en).

In dit onderzoek is een familie van koude-geïnduceerde genen geïdentificeerd en gekarakteriseerd. Het is aangetoond dat CSPs betrokken zijn bij de bescherming van *L. lactis* cellen tegen invriezen en dat CSPs de productie van andere eiwitten betrokken bij koude-adaptatie (CIPs) reguleren. De expressie van de *L. lactis csp* genen wordt op verschillende niveau's gereguleerd door een strikt gecontroleerd netwerk waarin de leden van de *csp*-familie een kritieke rol spelen. Het onderzoek aan koude-adaptatie van bacteriën kan directe toepassingen opleveren voor de conservering van levensmiddelen en de fermentatie-technologie. Na verschillende koude-schokbehandelingen voorafgaand aan blootstelling aan invriezen wordt een significante verhoging van de overleving van bacteriën waargenomen en het is aangetoond dat CSPs hierbij betrokken zijn. Dit kan resulteren in een verhoogde overleving van bacteriën in bevroren levensmiddelen of van startercultures tijdens ingevroren bewaring. Verder kunnen de genetische elementen betrokken bij koude-geïnduceerde productie van CSPs waardevolle gereedschappen opleveren voor de productie van enzymen bij lage temperatuur. Uit de toegenomen kennis betreffende CSPs kan bruikbare informatie worden geëxtraheerd om het effect van blootstelling aan lage temperatuur en de koude-adaptatie van bacteriën beter te begrijpen. Dit kan ook inzicht opleveren in nieuwe methoden voor het controleren van de groei van micro-organismen die de bewaartijd en veiligheid van gekoelde voedingsmiddelen bedreigen.

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Curriculum vitae

Jeroen Wouters werd geboren op 4 oktober 1970 in Ankeveen. De middelbare schoolopleiding begon hij aan het St.-Vituscollege in Bussum, waarna hij in 1989 het VWO-diploma behaalde aan het Marnix College te Ede. In hetzelfde jaar startte hij met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen met als oriëntaties Levensmiddelenchemie en Levensmiddelenmicrobiologie. Tijdens de doctoraalfase verrichtte hij onderzoek bij de Leerstoelgroep Levensmiddelenchemie LUW (Prof. Dr. Ir. A. G. J. Voragen; Dr. Ir. J.-P. Vincken), de Vakgroep Vleeskunde UU (Prof. Dr. Ir. B. Krol; Dr. Ir. J. Houben) en de Leerstoelgroep Levensmiddelenmicrobiologie LUW (Prof. Dr. Ir. F. M. Rombouts, Dr. T. Abee, Dr. Ir. A. Verheul). Van juli tot december 1994 liep hij stage op de Research Department van H.J. Heinz Company (Pittsburgh, USA). In juni 1995 werd het doctoraaldiploma behaald.

Van juli 1995 tot juni 1999 deed hij een promotie-onderzoek aan de Wageningen Universiteit in samenwerking met NIZO food research te Ede. Het in dit proefschrift beschreven onderzoek werd zowel bij de Leerstoelgroep Levensmiddelenmicrobiologie (WU) als bij de Microbial Ingredients Section (NIZO food research) uitgevoerd onder begeleiding van Dr. T. Abee, Prof. Dr. O. P. Kuipers, Prof. Dr. W. M. De Vos en Prof. Dr. Ir. F. M. Rombouts. Vanaf juli 1999 is hij werkzaam als postdoctoraal onderzoeker bij het Wageningen Centre for Food Sciences binnen het project "Microbial stress response in minimal processing".

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