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

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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 a group of 7 kDa cold-induced proteins (CSPs) was identified that corresponds to a previously described family of *csp* genes of *L. lactis* MG1363 (Wouters *et al.*, 1998, *Microbiology* 144, 2885–2893). The 7 kDa CSPs appeared to be the most strongly 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 (approx. 30-, 130- and 20-fold, respectively, compared to 30 °C mid-exponential phase). For all treatments leading to an increased freeze survival (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 with 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.

Keywords: *Lactococcus lactis*, low-temperature adaptation, cryoprotection, cold-shock proteins

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 great interest in increasing the freeze-survival capacity of starter strains. Freezing is a complex stress condition since cells may be damaged not only 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 & Marth, 1992; Franks, 1995; Thammavongs *et al.*, 1996).

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Abbreviations: 2D-EF, two-dimensional gel electrophoresis; CSP, cold-shock protein; LAB, lactic acid bacteria.

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 (see reviews by Jones & Inouye, 1994; Graumann & 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 (Willmsky *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 (Wouters *et al.*, 1998). 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 not cold induced, whereas the other *csp* genes are induced 10- to 40-fold at different times after cold shock (Wouters *et al.*, 1998).

Several functions have been shown for CSPs 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.*, 1992). Interestingly, it was noted that many organisms develop an increased ability to survive freezing after cold-shock treatment (Goldstein *et al.*, 1990; Kim & Dunn, 1997; Panoff *et al.*, 1995; Thammavongs *et al.*, 1996; Willmsky *et al.*, 1992). CspB appeared to be implicated in increased tolerance to freezing, as was shown using a strain in which the gene encoding this protein was disrupted (Willmsky *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 synthesized proteins are found in the class of 7 kDa CSPs. The specific effect of overproduction of CspD on freeze survival was studied and the correlation between the expression of CSPs and the survival after freezing of *L. lactis* MG1363 will be discussed.

METHODS

Growth, cold-shock treatment and freeze-thaw challenge.

Lactococcus lactis MG1363, a plasmid-free and prophage-cured derivative of *L. lactis* NCDO 712 (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 OD₆₀₀. For cold-shock treatments, 50 ml cultures were grown to mid-exponential phase (OD₆₀₀ 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 OD₆₀₀ 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. Aliquots of 1 ml 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 d 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 an MSK cell homogenizer (B. Braun Biotech International) and zirconium beads (0.1 mm, Biospec Products) eight 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) 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. 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), 2% Triton X-100, 8 mM PMSF] at 37 °C for 5 min, after which 60 µl sample solution (8 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 4-7L, 0.5% Triton X-100, a few grains of bromophenol blue) was added. The total volume (100 µl) was loaded on the acidic end of the first-dimension IEF gel with linear pI ranges from 4 to 7 or from 3 to 10 (Immobiline Dry strips, Pharmacia Biotech). For the second dimension, 15% homogeneous SDS-PAGE gels were used to obtain optimal separation in the low-molecular-mass region. Two molecular mass markers (Pharmacia Biotech) 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). The intensity of each spot was calculated as a percentage of the total intensity of the spots visualized on a gel, and subsequently, induction factors were calculated. One-dimensional Tricine-SDS-PAGE for the separation of low-molecular-mass proteins was performed as described by Schägger & von Jagow (1987).

Freeze survival of *L. lactis* exposed to different stress conditions.

L. lactis was grown to 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 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} (approx. 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 & 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) was used to determine the transcript size. RNA was blotted on a GeneScreen Plus Membrane (Dupont, NEN Research Products). The blot was hybridized using a mix of probes each specific for one of the five *csp* genes of *L. lactis* (Wouters *et al.*, 1998) that were labelled simultaneously. The blots were exposed to X-ray films (X-Omat MS, Kodak).

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.* (1996). Using the oligonucleotides OECspDFor (5'-GCTGCCATGGCAAATGGAACAGTAAAATGG-3') and OECspDRev (5'-CACGAAGCTTTTCCTCTTGCTG-GCTAAGT-3'), containing a *Nco*I site and a *Hind*III site (underlined), respectively, the *cspD* gene could be amplified using PCR. The obtained fragment was digested with *Nco*I and *Hind*III and subsequently cloned in vector pNZ8032 (De Ruyter *et al.*, 1996), thereby replacing the *gusA* gene that was originally present in pNZ8032. In this way, a translational fusion was obtained of the *nisA* promoter to the ATG start codon of the *cspD* gene. The plasmid generated (pNZOECspD) was transformed into *L. lactis* NZ3900, a derivative of *L. lactis* MG1363 in which the two-component regulatory *nisR/nisK* pathway is integrated into the chromosome. Upon addition of different concentrations of the inducer M17W nisin (0, 0.1, 0.2 and 0.5 ng ml⁻¹; 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(pNZOECspD), this strain was cultured at 30 °C to OD_{600} 0.3, after which different concentrations of nisin were added. After 90 min of incubation (final OD_{600} approx. 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(pNZ8020); pNZ8020 carries the *nisA* promoter without any fused gene (de Ruyter *et al.*, 1996).

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: $\mu = 0.20, 0.18, 0.11, 0.03$ and 0.02 h⁻¹ at 20, 15, 10, 7 and 4 °C, respectively. In liquid GM17 medium a lag-time of 9 d is observed at 4 °C. When growth of *L. lactis* is analysed on GM17 plates no growth is observed after

Fig. 1. Growth of *L. lactis* at 30 °C (●) and following cold shock to 20 °C (▲), 10 °C (■) or 4 °C (○).

Fig. 2. Survival after freezing of *L. lactis* after exposure to different cold-shock conditions. The survival [% of surviving cells relative to $x = 0$ (100%)] of *L. lactis* cells cultured without (△) or with a cold-shock treatment from 30 °C to 10 °C for 2 h (○) or 4 h (□) and in the presence of chloramphenicol for 2 h (●) or 4 h (■) is depicted. The freezing experiments were performed in triplicate and the means of the three experiments are shown. The error bars represent standard deviation. The number of cells before freezing is set at 100% (OD_{600} 0.5, about 4×10^8 cells).

14 d incubation at 4 °C, whereas slow growth is observed at 7 °C. When the temperature is rapidly downshifted at mid-exponential phase, growth of *L.*

Fig. 3. For legend see facing page.

Table 1. 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 (numbering according to CSP family of *L. lactis* and Figs 4a and 4b). ND, Not determined.

Spot	Relative amount (%)	
	30 °C	10 °C
A	ND	ND
B	0	1.49
C	ND	ND
D	0.07	3.50
E	1.55	3.09
F	0	1.65

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 µg 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).

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 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 unidentified member of the lactococcal CSP family (Wouters *et al.*, 1998). Finally, CspA and CspC of *L. lactis*, which have pI values of 9.2 and 9.6, respectively (Wouters *et al.*, 1998), could be visualized using a different range for IEF (pI 3–10). 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 (approx. twofold; Fig. 3; Table 1). In agreement with the mRNA levels of the *csp* genes (Wouters *et al.*, 1998), major induction was observed for CspB and CspD at the protein level (B and D; Fig. 3b; Table 1). After cold shock, CspB, CspD 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 (approx. 22 spots, minimally twofold induction; Fig. 3, Table 2) and decreased protein levels (approx. 30 spots; minimally twofold reduction). For three proteins with molecular masses of approximately 33, 43 and 45 kDa at least a 10-fold induction was detected at 4 h after cold shock to 10 °C (spots 10, 12 and 15; Fig. 3; Table 2).

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 was only slightly increased at 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 was observed at 1 h after cold shock. This induction was approximately 10-fold lower than observed at 4 h after cold shock to

Fig. 3. 2D-EF of cell-free extracts of *L. lactis* grown at 30 °C (a) and after cold shock to 10 °C for 4 h (b). Molecular mass 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. 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 twofold) after cold shock to 10 °C (other than the 7 kDa CSPs) are circled and numbered (see also Table 2). Spots 6, 12 and 15 are numbered in bold; these proteins are induced at 4 h after cold shock to 20, 10 and 4 °C as well as after exposure to stationary phase (see Discussion).

Table 2. Proteins (numbering according to Fig. 3) with increased expression levels at 4 h after cold shock to 10 °C

For each spot the approximate molecular mass, the induction factor (-fold) at 4 h after cold shock from 30 to 10 °C, and the induction (+, at least a twofold induction; –, no induction or reduction) after cold shock to 20 °C, after cold shock to 4 °C and under stationary-phase conditions is given. For spots with identical molecular masses the approximate pI is also given. Data for the three spots (6, 12 and 15) with increased expression under all conditions are shown in bold. ND, Not determined.

Spot	Mol. mass (kDa)	Induction			
		10 °C (-fold)	20 °C (+/-)	4 °C (+/-)	Stat. (+/-)
1	11	ND	–	–	–
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	+	–	+

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 revealed 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-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, respectively, are observed 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 four repetitive freeze–thaw cycles was approximately 130-fold higher than the survival of mid-exponential-phase cells. When *L. lactis* was shifted to 20 °C an adaptive response to freezing was also found, which led to an approximately 30-fold increased survival after four freeze–thaw cycles com-

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 (lanes 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* (Wouters *et al.*, 1998). 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 from 30 to 20 °C (right). Only the parts of the gel containing the relevant CSPs are shown. The CSPs are boxed and labelled B (CspB), D (CspD) and E (CspE). (c) Survival after freezing of *L. lactis* following the cold-shock treatments as described for (a): percentage of surviving cells after four freeze–thaw cycles. The freezing experiments were performed in triplicate and the means of the three experiments are shown. The error bars represent standard deviation. The number of cells before freezing is set at 100 % (OD₆₀₀ 0.5, about 4×10^8 cells).

pared to mid-exponential-phase 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 under all cold-shock conditions tested. However, the percentage of surviving cells after freezing is quantitatively not directly correlated with 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* under 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* (Wouters *et al.*, 1998). 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 the parts of the gel containing the relevant CSPs are shown. The CSPs are boxed and labelled D (CspD) and E (CspE). (c) Survival after freezing of *L. lactis* following exposure to several stress conditions: mid-exponential phase (control, Δ), heat shock ($\circ$), salt shock ($\bullet$), acid shock ($\blacksquare$), stationary phase ($\blacktriangle$) and cold shock ($\square$). The freezing experiments were performed

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 was 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 following 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 four 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 twofold 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 (spots 6, 12 and 15; Table 2).

Overproduction of CspD and its effects on freeze survival

Under all cold-shock conditions CspD is induced and this coincides with increased survival after freezing. For this reason the effect of specific overexpression of CspD on survival was studied. Using *L. lactis* NZ3900(pNZOECspD) considerable amounts of CspD were produced upon addition of nisin (Fig. 6a). Upon addition of 0.1 or 0.2 ng nisin ml⁻¹, CspD levels comparable to cold-shock conditions were obtained. However, upon addition of 0.5 ng nisin ml⁻¹ extremely high CspD quantities could be obtained (approx. 12.6 % of total protein). When cells overproducing CspD were exposed to a repeated freeze-thaw challenge a slight increase in freeze survival could be observed, depending on the added concentration of nisin (Fig. 6b). Upon addition of 0.1 and 0.2 ng nisin ml⁻¹ the survival after freezing hardly increased. However, when 0.5 ng nisin ml⁻¹ was added the survival was approximately 2–10 times higher than that of control cells. No effect on growth (data not shown) or survival after freezing (Fig. 6c) was noted upon the addition of nisin for the control strain *L. lactis* NZ3900(pNZ8020). This indicates that the increase in survival of *L. lactis* NZ3900(pNZOECspD) is due to the increased CspD level.

in triplicate and the means of the three experiments are shown. The error bars represent standard deviation. The number of cells before freezing is set at 100% (OD₆₀₀ 0.5, about 4×10^8 cells).

Fig. 6. (a) Overproduction of CspD in *L. lactis* at 30 °C: Coomassie-stained SDS-PAGE of protein extracts from *L. lactis* NZ3900(pNZOECspD) induced with different concentrations of nisin. Lanes 1 and 7 contain a low- and a high-molecular-mass marker, respectively [band sizes of 16.9, 14.4, 10.7, 8.2 and 6.2 kDa and of 67, 43, 30, 22.1 and 14.4 kDa (top to bottom), respectively]. Lanes 2, 3, 4 and 5 contain cell-free extracts of *L. lactis* NZ3900(pNZOECspD) induced with 0, 0.1, 0.2 and 0.5 ng M17W nisin ml⁻¹, respectively. Lane 6 contains a cell-free extract of *L. lactis* NZ3900(pNZ8020) (carrying the *nisA* promoter without any fused gene) induced with 0.5 ng M17W nisin ml⁻¹. (b) Survival after freezing of *L. lactis* NZ3900(pNZOECspD) upon induction with 0 (△), 0.1 (○), 0.2 (□) and 0.5 (●) ng M17W nisin ml⁻¹, respectively. The freezing experiments were performed in triplicate and the means of the three experiments are shown. The error bars represent standard deviation. The number of cells before freezing is set at 100% (OD₆₀₀ 1.5, about 1 × 10⁹ cells). (c) As a control the survival after freezing of *L. lactis* NZ3900(pNZ8020) upon induction with 0 (△), 0.1 (○), 0.2 (□) and 0.5 (●) ng M17W nisin ml⁻¹, respectively, is shown.

DISCUSSION

Analysis of growth characteristics of many LAB has resulted in the grouping of these bacteria into 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 adapt efficiently 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. The survival after freezing of *L. lactis* MG1363 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 blocked the cryoprotection 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 most strongly induced proteins after cold shock, it is tempting to speculate that these proteins are directly involved in the protection against freezing. Willmsky *et al.* (1992) showed that deletion of the gene encoding CspB of *B. subtilis* resulted in a decreased freeze survival and the authors suggested a role as anti-freeze proteins for CSPs, because of their low molecular mass and their abundant presence. Our study shows that the *csp* mRNA and CSP expression levels in *L. lactis* increased upon cold shock to 20 and 10 °C 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 those seen after 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 with improved cryoprotection. Since CspD was induced under 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 & 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 synthesized and no increase in survival after 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 regulate(s) 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 (spots 6, 12 and 15; Table 2) were increased after all cold-shock treatments and under stationary-phase conditions, but not under 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 stresses other than cold stress (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 & 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 against freezing. These observations indicate that during stationary phase, factors other than CSPs are important as cryoprotectants. 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 under 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 has been described for *L. lactis* (Gansel *et al.*, 1993). In related Gram-positive organisms, like *B. subtilis* and *Listeria monocytogenes*, alternative sigma factors have been 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 under 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.

In this study, the actual expression levels of CSPs have for the first time been correlated with a physiological function in low-temperature adaptation, in this case

freeze survival of the industrially important bacterium *L. lactis*. Exposure to 4, 10 and 20 °C for several hours leads to increased freeze survival and this coincides with increased CSP expression. However, the observed level of freeze protection does not quantitatively correlate with the *csp* gene expression levels. In addition, *L. lactis* cells specifically 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 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 yet known. In order to gain more insight into this aspect, *csp* disruption mutants of *L. lactis* will be constructed.

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