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Members of the group of 7-kDa cold-shock proteins (CSPs) are the proteins with the highest level of induction upon cold shock in the lactic acid bacterium *Lactococcus lactis* MG1363. By using double-crossover recombination, two *L. lactis* strains were generated in which genes encoding CSPs are disrupted: *L. lactis* NZ9000ΔAB lacks the tandemly orientated *cspA* and *cspB* genes, and NZ9000ΔABE lacks *cspA, cspB,* and *cspE.* Both strains showed no differences in growth at normal and at low temperatures compared to that of the wild-type strain, *L. lactis* NZ9000. Two-dimensional gel electrophoresis showed that upon disruption of the *cspAB* genes, the production of remaining CspE at low temperature increased, and upon disruption of *cspA, cspB,* and *cspE,* the production of CspD at normal growth temperatures increased. Northern blot analysis showed that control is most likely at the transcriptional level. Furthermore, it was established by a proteomics approach that some (non-7-kDa) cold-induced proteins (CIPs) are not cold induced in the *csp*-lacking strains, among others the histon-like protein HslA and the signal transduction protein LlrC. This supports earlier observations (J. A. Wouters, M. Mailhes, F. M. Rombouts, W. M. De Vos, O. P. Kuipers, and T. Abee, Appl. Environ. Microbiol. 66:3756–3763, 2000). that the CSPs of *L. lactis* might be directly involved in the production of some CIPs upon low-temperature exposure. Remarkably, the adaptive response to freezing by prior exposure to 10°C was significantly reduced in strain NZ9000ΔABE but not in strain NZ9000ΔAB compared to results with wild-type strain NZ9000, indicating a notable involvement of CspE in cryoprotection.

Lactic acid bacteria (LAB) are frequently used to start industrial food fermentations. 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-adaptive response of LAB attracts increasing interest. *Lactococcus lactis* is a mesophilic LAB that is widely used in the manufacturing of cheese. In recent years, this bacterium has become the model organism for LAB because of its relevance in the food industry and the development of a wide variety of genetic systems.

Several bacteria react to a sudden downshift 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). In a variety of bacteria, families of CSPs, consisting of three to nine members, have been described of which CspA in *Escherichia coli* (CspA[E]) and CspB in *Bacillus subtilis* (CspB[B]) are the best characterized (see reviews in references 15, 37, and 39). CspA[E] and CspB[B] are capable of binding to single-stranded DNA and RNA, and based on these characteristics, several functions for CSPs have been suggested, such as transcriptional activators (7, 19, 25), RNA chaperones that facilitate the initiation of translation (16, 18),

and freeze-protective proteins (34). Recently it has been shown that CSPs might regulate the expression of cold-induced genes as antiterminators (3). 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 (13, 29) as well as at the level of mRNA and protein stability (6, 10, 12). For *L. lactis* MG1363, a family of five *csp* genes has been identified. The *L. lactis* chromosome was found to contain two sets of two tandemly located and cold-inducible *csp* genes (*cspA/cspB* and *cspC/cspD*) and a single, constitutively expressed *cspE* gene (38). By using *L. lactis* strains specifically overproducing the respective CSPs, it was found that these proteins protect against freezing and might be involved in the regulation of (non-7-kDa) cold-induced proteins (CIPs) (36).

Because of the implications of CSPs in freeze protection and their presumed central role in cold adaptation, it is of great interest to further investigate the role of these proteins for *L. lactis*, especially in relation to food or dairy production processes. In this work, we characterized the effects of multiple *csp* gene disruptions on adaptation to cold and gene regulation of *L. lactis*. Deletion of *csp* genes affects freeze survival of *L. lactis*, the production of the remaining counterparts of the lactococcal CSP family as well as the production of several CIPs.

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MATERIALS AND METHODS

Strains and culturing conditions. *L. lactis* NZ9000 (24) (Table 1) was used as the wild-type strain for the generation of *csp*-lacking strains and has been generated from strain MG1614, which is a rifampin- and streptomycin-resistant derivative of strain MG1363 (11). *L. lactis* was cultured at 30°C or at lower

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

Strain, plasmid, or oligonucleotide	Relevant properties or sequence	Reference
Strains		
L. lactis NZ9000		24
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	30
pUC18ERYΔAB12	Amp^r Ery^r	This study
pUC18ERYΔE12	Amp^r Ery^r	This study
Oligonucleotides		
CspAB1For	5′-GCAG<u>G AATTC</u> ATGCT CGCTC AGGCT CTTTT–3′	
CspAB1Rev	5′-AGCC<u>G GTACC</u> CATAT TGAAC CATTT TACTG–3′	
CspAB2For	5′-AGCG<u>T CTAGA</u> TAATT AATGA AAAAC GGAGC–3′	
CspAB2Rev	5′-CACG<u>G TCGAC</u> AAATC ACCAA ATTGA GGCAA–3′	
CspE1For	5′-CGCC<u>G AATTC</u> ATATG GATAA AGTTC GCCGT–3′	
CspE1Rev	5′-CGTC<u>G GTACC</u> ATTAA CAGTT CCTTG TGCCA–3′	
CspE2For	5′-GCTGT CTAGA CACTG ACAAA ATTGT CAGTG–3′	
CspE2Rev	5′-TCCC<u>G TCGAC</u> ATTCC GAGTC TAGCA TGGTG–3′	

temperatures (as indicated) on M17 medium containing 0.5% glucose (GM17). Growth was monitored by measuring the optical density at 600 nm. *E. coli* MC1061 (8) was used as a host strain in cloning experiments and was grown in tryptone yeast extract medium with aeration at 37°C (28). Antibiotics were used in the following concentrations: ampicillin, 50 μg ml^{-1}; erythromycin, 2.5 μg ml^{-1}.

Generation of *csp* deletions. *L. lactis* strains carrying deletions in their *csp* genes were constructed using a double-crossover replacement strategy (26). For the generation of *L. lactis* strains with deletions in their *csp* genes, the regions flanking (approximately 800 bp) the respective genes were amplified using PCR as described by Kuipers et al. (23). 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 contain a *Kpn*I site, the forward fragment 2 primers contain an *Xba*I site, and the reverse fragment 2 primers contain a *Sal*I site. The cloning of the amplified fragments in pUC18ERY (30) resulted in the plasmids pUCEryΔAB12 and pUCEryΔE12 (Amp^r Ery^r) (Table 1). The subsequent 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. The plasmids obtained were transformed into *L. lactis* NZ9000 by electroporation (33) and selected for erythromycin resistance in which the first integration took place. The erythromycin-resistant strains were cultured without selective pressure of erythromycin for 200 generations and were analyzed for the occurrence of the second crossover event. Candidates were verified using PCR and ultimately in a Southern blotting experiment using PCR fragment 1 as a probe as described previously (38). Using this strategy, an *L. lactis* strain lacking *cspA* and *cspB* (NZ9000ΔAB) was created and, sequentially, also a *L. lactis* strain additionally lacking *cspE* (NZ9000ΔABE) was obtained. Attempts to generate *L. lactis* strains lacking *cspE* only or lacking the *cspA*, *cspB*, *cspC*, *cspD*, and *cspE* genes using a similar strategy failed at the second crossover event. All manipulations with recombinant DNA were carried out following standard procedures (28) and according to the specifications of the manufacturer (GIBCO/BRL Life technologies, Breda, The Netherlands).

Protein analysis. The protein composition of cell extracts was determined using two-dimensional gel electrophoresis (2D-E) as described previously (35). 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 h using a cell MSK cell homogenizer (Braun Biotech International, Melsungen, Germany) and zirconium beads (Biospec Products, Bartlesville, Okla.). Equal amounts of protein were analyzed using the Multiphor 2D electrophoresis system (Pharmacia Biotech, Uppsala, Sweden), and protein spots were visualized using silver staining (4). For the 2-DE, a representative gel for two independent samples is shown. The spots on the 2D-E gels were compared, calibrated, and calculated using the GEMINI program (Applied Imaging, Sunderland, England). N-terminal sequences of specific spots in 2-DE were determined as described previously (35), and by using the BlastP database and the *L.*

lactis IL1403 genome database (5) (http://spock.jouy.inra.fr/cgi-bin/blast.cgi) the derived N-terminal sequences were screened for sequence similarities.

mRNA analysis. RNA isolation, Northern blotting, and subsequent hybridization with radiolabeled probes was performed as described previously (22, 32). For the specific detection of the mRNAs of the *csp* genes, previously described primers were used (38). Quantification of the *csp* transcripts in Northern blotting was performed using the Dynamics Phosphor Imaging System (Dynamics, Rochester, N.Y.). Equal amounts of total RNA were applied on the gel as was shown using a probe specific for lactococcal 16S rRNA (5′-ATCTACGCATTTCACC GCTAC-3′) (21).

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 (35). In short, cells were cultured in GM17 medium until mid-exponential phase (optical density at 600 nm, 0.5) at 30°C and subsequently were rapidly downshifted in growth temperature from 30 to 10°C. The cultures were exposed to 10°C for different time periods (0, 2, and 4 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 (CFU) was determined using plate counting following incubating for 48 h at 30°C. This freeze-thaw cycle was performed four times in total.

RESULTS

Generation and growth characteristics of strains lacking *csp*. Using a double-crossover replacement strategy, *L. lactis* strains with deletions in the tandem *cspA* and *cspB* genes (NZ9000ΔAB) and carrying an additional deletion in *cspE* (NZ9000ΔABE) were obtained. The chromosomal configuration was confirmed by PCR and Southern hybridization (data not shown). Deletion of *cspAB* or *cspABE* did not affect the growth rate at optimal growth temperature (30°C) or at other temperatures (4, 7, 10, 15, 20, or 42°C). Upon cold-shock treatment at 10°C, identical adaptation times and growth rates were observed for the wild-type strain and the mutants (Fig. 1). Moreover, no differences in the number of CFU, the estimated lag time, and the appearance of the colonies were observed upon incubation of the *csp* mutants on GM17 plates at different temperatures for a 14-day period. Furthermore, the *csp* mutants and the wild-type cells showed similar sizes and chain lengths (data not shown).

Deletion of *cspAB* or *cspABE* affects the production of the remaining CSPs. Since no changes in the growth of *L. lactis*
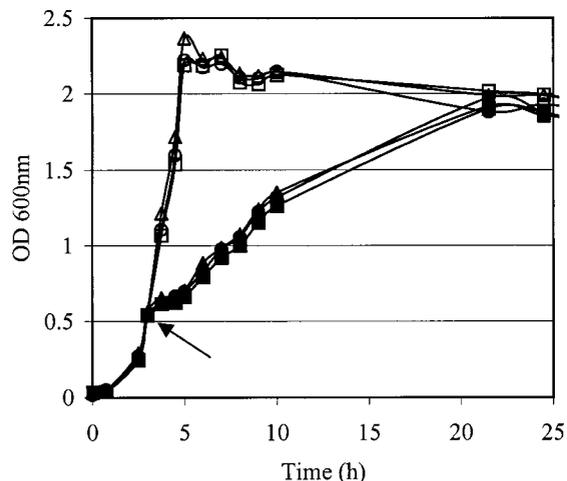
FIG. 1. Growth of NZ9000 (triangles), NZ9000ΔAB (circles), and NZ9000ΔABE (squares) at 30°C (open symbols) and after cold shock to 10°C (closed symbols). The arrow indicates the time point of cold shock.

were observed upon deletion of *csp* genes, the production levels of the remaining CSPs were analyzed by 2D-E with *L. lactis* NZ9000ΔAB and *L. lactis* NZ9000ΔABE (Fig. 2A). The positions of the CSPs on the 2D-E gels have been determined previously using overproduction of the individual CSPs and analysis of their N-terminal sequences (35, 36). For wild-type 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 lesser extent, of CspA (Fig. 3), CspB, CspC, and CspE was observed (Fig. 2A). For strain *L. lactis* NZ9000ΔAB, identical production levels of CspD were found at high and low temperatures as observed for the wild-type strain, while CspE production increased slightly (twofold) at cold-shock conditions. At 30°C the level of CspC was higher for NZ9000ΔAB than for NZ9000, but it reached similar production levels at cold-shock conditions for NZ9000ΔAB and NZ9000. Strikingly, in strain NZ9000ΔABE, CspD was present at mid-exponential phase at 30°C, whereas it is not detectable at these conditions in wild-type cells. At low temperature the level of CspD increased but did not exceed the level found in wild-type cells. Remarkably, the CspC level slightly decreased upon low-temperature incubation with strain NZ9000ΔABE compared to results with wild-type cells (Fig. 2A). Thus, the loss of CspA and CspB is compensated for by an increased production of CspE at low temperature, and the additional deletion of *cspE* is compensated for by increased production of CspD at 30°C. At 10°C the production of the remaining CSPs, CspC, and CspD, in NZ9000ΔABE was not greater than in wild-type cells. To further assess the changes in the production levels of CspD and CspE, the mRNA levels of these genes also were analyzed in wild-type cells and in the strains with *csp* deleted (Fig. 2B). Indeed, an increased amount of *cspE* transcript (twofold induction) was observed for NZ9000ΔAB compared to its level in wild-type cells. By analogy, also for the *cspD* transcript an increased amount was found at 30°C compared to results for both wild-type cells and NZ9000ΔAB cells (fivefold induction). The *cspD* mRNA level also increased

(similar fivefold induction) upon low-temperature incubation. However, at these conditions no significant increase in CspD production was noted (Fig. 2A). As a control, the 16S rRNA mRNA level was determined, and the levels showed maximally 9% variance (Fig. 2B).

Disruption of *csp* genes affects the production of CIPs. In a previous study we identified a group of 17 CIPs in *L. lactis* (35). We also showed that the levels of some CIPs increased upon specific overproduction of CSPs, which might point to the involvement of CSPs in the regulation of CIPs (36). To further study this phenomenon, we analyzed the levels of the lactococcal CIPs in the mutants with *csp* deleted. Separation of cell extracts by 2D-E revealed that the production level of several CIPs was affected in the strains NZ9000ΔAB and NZ9000ΔABE compared to results with the wild-type strain (Fig. 3). For a subset of proteins belonging to this group, the N-terminal amino acid sequence was determined (Table 2). CIP1 (histon-like protein HslA) and CIP5 (unidentified) were cold induced in wild-type cells but were no longer induced at low temperature for strains NZ9000ΔAB and NZ9000ΔABE. For HslA (CIP1), a higher production level was observed for strain NZ9000ΔAB than for wild-type cells at 30°C, which might point to derepression of this protein upon deletion of CspA and/or CspB. CIP8 (signal transduction protein LlrC) and CIP9 (unidentified) were not cold induced in strain NZ9000ΔABE. For the other CIPs, including OsmC (CIP2) and β-PGM (CIP6), no differences were observed in their production levels between the wild-type strain and the strains in which *csp* was deleted (Table 2).

Deletion of *cspABE* decreases the freeze survival of *L. lactis*. Since LAB starter cultures are frequently stored frozen, it is of practical relevance to monitor the abilities of these bacteria to survive freezing temperatures and to determine the critical factors for survival. *L. lactis* is able to adapt to freezing conditions by preexposure to 10°C, yielding increased survival in freezing conditions (20, 35). For strain NZ9000, preexposure to 10°C for 2 and 4 h increased the survival of freezing approximately 50- and nearly 1,000-fold, respectively, after four repetitive freeze-thaw cycles (Fig. 4A). Strain NZ9000ΔAB showed a response to freezing identical to the wild-type strain's (Fig. 4B). However, the capacity of strain NZ9000ΔABE to survive a freeze increased only approximately 10- and 100-fold after exposure to 10°C for 2 and 4 h, respectively (Fig. 4C). This indicates that the strain NZ9000ΔABE is less well able to adapt to freezing conditions at 10°C, possibly explained by the combined effect of the absence of CspE, the lower total amount of CSPs present, and/or the decreased production of certain CIPs.

DISCUSSION

In this report we describe the effect of the disruption of two or three genes encoding CSPs in *L. lactis* on the cold-adaptive response of this bacterium. An *L. lactis* strain lacking the tandem *cspA*-*cspB* genes and a strain additionally lacking *cspE* were generated. In the absence of the encoded CSPs, the growth characteristics (lag time and growth rate) of *L. lactis* were not affected. By analogy, deletions of *cspA*[E] and *cspB*[B] show no effect on the growth characteristics of *E. coli* and *B. subtilis*, respectively, which can possibly be explained by in-
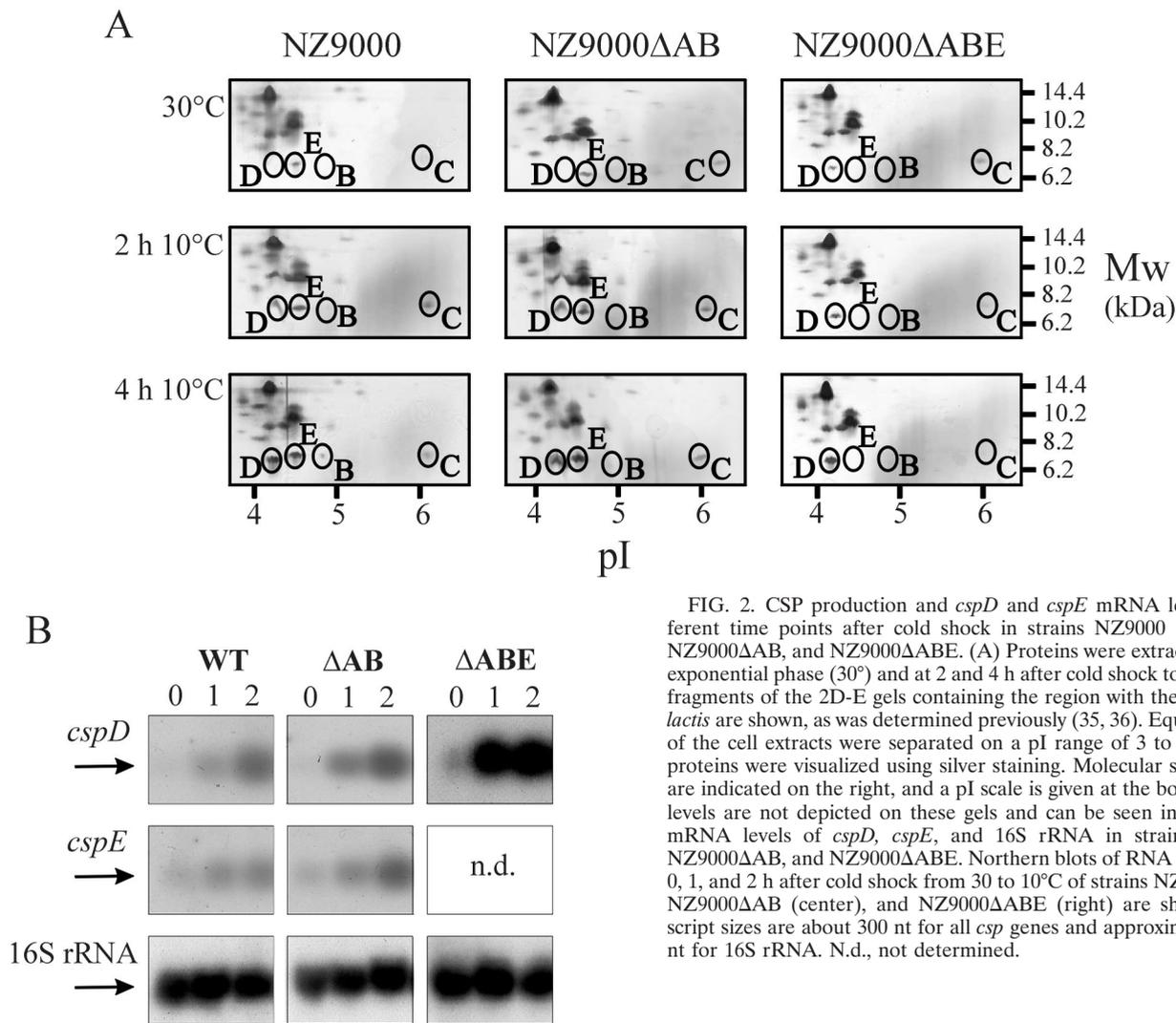
FIG. 2. CSP production and *cspD* and *cspE* mRNA levels at different time points after cold shock in strains NZ9000 (wild type), NZ9000ΔAB, and NZ9000ΔABE. (A) Proteins were extracted at mid-exponential phase (30°) and at 2 and 4 h after cold shock to 10°C. Only fragments of the 2D-E gels containing the region with the CSPs of *L. lactis* are shown, as was determined previously (35, 36). Equal amounts of the cell extracts were separated on a pI range of 3 to 10, and the proteins were visualized using silver staining. Molecular size markers are indicated on the right, and a pI scale is given at the bottom. CspA levels are not depicted on these gels and can be seen in Fig. 3. (B) mRNA levels of *cspD*, *cspE*, and 16S rRNA in strains NZ9000, NZ9000ΔAB, and NZ9000ΔABE. Northern blots of RNA extracted at 0, 1, and 2 h after cold shock from 30 to 10°C of strains NZ9000 (left), NZ9000ΔAB (center), and NZ9000ΔABE (right) are shown. Transcript sizes are about 300 nt for all *csp* genes and approximately 1,500 nt for 16S rRNA. N.d., not determined.

creased production of the remaining CSPs (1, 16). We suggest that the presence of the remaining CSPs is sufficient to allow growth of *L. lactis* NZ9000ΔABE at both low and high temperatures. Indeed, deletion of *cspA-cspB* is compensated for by increased production of CspE and CspC, while deletion of *cspA-cspB-cspE* is compensated for by increased CspC and CspD production, mainly at 30°C. For *cspD* and for *cspE*, an increase was seen in the mRNA level at these conditions, indicating that the increased production is regulated at the transcriptional level. The increased production of CSPs upon deletion of their counterparts would point to an overlap in their functioning and to the necessity for a minimal CSP level in a cell. The latter aspect might also explain the inability to further inactivate the remaining *cspC* and *cspD* genes in strain *L. lactis* NZ9000ΔABE, similar to observations for *B. subtilis* (16). Multiple deletion of *csp* genes in *B. subtilis* revealed that the presence of at least one CSP is essential for growth at optimal and low temperatures (16). Next, the increased production of the remaining CSPs upon deletion of *csp* genes also suggests that CSPs directly or indirectly down-regulate the

production of their family members. Based on the 2D-E observations for *L. lactis* NZ9000ΔABE, we speculate that CspE negatively regulates the production of at least CspC and CspD at 30°C. Also, for *E. coli* it has been found that CSPs are involved in the regulation of their family members: CspE functions as a negative regulator of *cspA* expression (2).

In a variety of bacteria, non-7-kDa cold-induced proteins have been identified which play a role in a variety of cellular processes, such as chromosome structuring, transcription, translation, general metabolism, sugar metabolism, and stress response (see reviews in references 15, 37, and 39). In this study, the amino-terminal sequences of a selection of these CIPs were determined, and in *L. lactis* also these proteins seem to be involved in a variety of cellular processes. CIP1 was identified as the histon-like protein HslA of *L. lactis*. Remarkably, H-NS of *E. coli* was also found to be cold induced, and a role for this protein in optimizing DNA supercoiling at low temperature has been suggested (7, 25). CIP2 was identical to OsmC in the *L. lactis* IL1403 genome (5), which is 49% identical to the osmotically inducible protein OsmC of *E. coli* (17). OsmC of *E. coli* and its ortholog, YkzA of *B. subtilis*, belong to the RpoS and SigB stress regulons, respectively (14, 31), which indicates that this type of protein is involved in stress adapta-
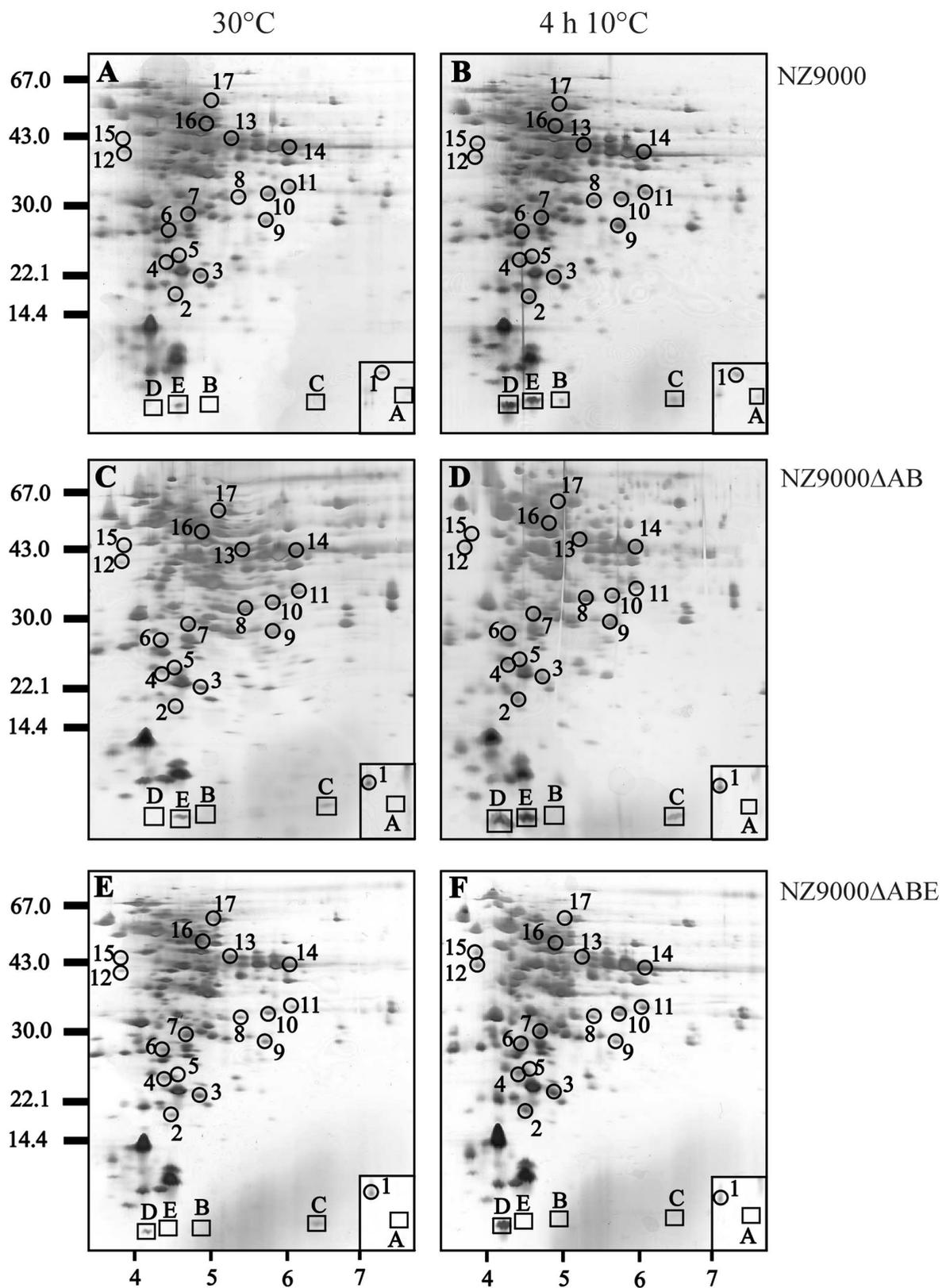
FIG. 3. Protein analysis of cell extracts of strains NZ9000 (A and B), NZ9000ΔAB (C and D), and NZ9000ΔABE (E and F) by 2D-E. Cell extracts were isolated from these strains prior to cold shock (30°C mid-exponential phase) (A, C, and E) and at 4 h after cold shock to 10°C (B, D, and F) and were separated on a pI range of 3 to 10. Equal amounts of protein were loaded on the gel, and the proteins were visualized using silver staining. Molecular size markers are 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 (35). CIPs are circled and numbered as described previously (35). 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 weight. The N-terminal sequences of a number of spots are given in Table 2.

TABLE 2. List of CIPs of strain NZ9000 (WT) and their production in strains NZ9000ΔAB (ΔAB) and NZ9000ΔABE (ΔABE) 4 h after cold shock to 10°C

CIP[a]	WT[b]	ΔAB[b]	ΔABE[b]	N-terminal sequence[c]	MW[d] (kDa)	Protein
1	+	−	−	(1) ANKQDLIAEV (10)	11	
				| | | | | | | |+|		
				(2) ANKQDLIAKV (11)	10	HslA
2	+	+	+	(1) KNISTASAHXEDTLENF (17)	14	OsmC
				||||||||| |||||||		
				(1) MKNISTASAHWEDTLENF (18)	14	OsmC
3	+	+	+	n.d.	20	
4	+	+	+	n.d.	22	
5	+	−	−	N-terminally blocked	23	
6	+	+	+	(1) MFKAVLFDLDGVI (14)	26	
				|||||||||||||		
				(1) MFKAVLFDLDGVI (14)	26	β-PGM
7	+	+	+	n.d.	27	
8	+	+	−	(1) XXXILVVDDEKPI (13)	29	
				|||||||||		
				(1) MKKILVVDDEKPI (14)	27	LlrC
9	+	+	−	N-terminally blocked	29	

[a] For CIP10 to CIP17, no differences in production were observed for the strains NZ9000ΔAB or NZ9000ΔABE, and no N-terminal sequences were determined.

[b] + indicates induction (at least twofold) of spot after cold shock from 30 to 10°C for 4 h. − indicates no induction of spot after cold shock from 30 to 10°C for 4 h.

[c] The upper sequence is the N-terminal derived sequence, and the lower sequence represents the N-terminal sequence for the respective proteins in the *L. lactis* IL1403 genome sequence (X indicates unidentified residues in N-terminal sequences).

[d] MW, molecular mass. The upper molecular mass indicates the molecular mass based on the migration on a 2D-E gel, and the lower molecular mass indicates the calculated molecular mass based on the encoded gene sequence. n.d., N-terminal sequence not determined.

tion. Remarkably, in the *L. lactis* genome no stress sigma factor is found (5), which necessarily points to an alternative regulatory mechanism for lactococcal OsmC. The precise function of OsmC and its orthologs remains to be established. Furthermore, CIP6 was identified as β-phosphoglucomutase of *L. lactis* (β-PGM) (27), and CIP8 was identified as LlrC in the *L. lactis* IL1403 genome (5). LlrC was found to be homologous to YycF, a member of a two-component signal transduction system of *B. subtilis*, which is probably involved in temperature sensing and is essential for growth of *B. subtilis* (9). It is tempting to speculate that the LlrC two-component signal transduction is involved in a temperature-sensing pathway of *L. lactis*.

These data indicate that the low-temperature response of *L. lactis* includes adaptation at several levels and support the observation of multilevel cold adaptation for other organisms (15, 39).

In this work we show that the cold-induced production of HslA (CIP1) and CIP5 and of LlrC (CIP8) and CIP9 was affected upon disruption of *cspAB* or *cspABE*, respectively, indicating that CSPs regulate proteins most likely involved in cold adaptation. Previously we observed that overproduction of CSPs in *L. lactis* induces a variety of proteins, among which also are a number of CIPs: CIP2 (OsmC), CIP4, CIP5, and CIP9 (36). Collectively, these data indicate that the production
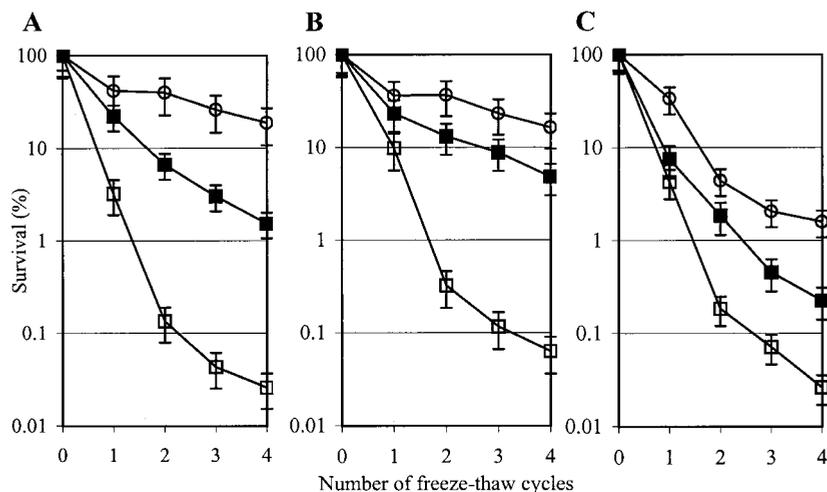

FIG. 4. Abilities of NZ9000 (A), NZ9000ΔAB (B), and NZ9000ΔABE (C) to survive a freeze after pretreatment at 10°C for several hours. Survival of freezing (number of viable cells prior to freezing is set at 100%) of *L. lactis* after preexposure to 10°C for 0 (open squares), 2 (closed squares), and 4 h (open circles) is shown. The data are shown as an average from two independent experiments, and the error bars indicate the variation for each sample point.

of CIP5 and CIP9 is reduced upon deletion of CspABE, and production of these CIPs is increased upon overproduction of CspA and CspE (36). This would strongly suggest a regulatory role of CspA or CspE in 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, possibly by interacting with Y-boxes located in their promoter regions (7, 25). A similar regulation may be operating for the CSPs of *L. lactis*; however, no Y-boxes (ATTGG or the complementary CCAAT) were observed in the upstream regions of the genes encoding the identified proteins HslA (CIP1), OsmC (CIP2), β-PGM (CIP6), or LlrC (CIP8) of *L. lactis*.

In view of practical applications, it is an important finding that *L. lactis* adapts to freezing conditions during prior exposure to 10°C for several hours, resulting in increased survival rates (20, 35). More detailed analysis revealed that cells specifically overproducing CspB, CspD, or CspE show improved survival under freezing conditions (35, 36). In this work, we show that the level of freeze protection of strain NZ9000ΔABE is lower than that of strains NZ9000 and NZ9000ΔAB upon exposure to 10°C for 2 and 4 h. We suggest that deletion of *cspA* and *cspB* in NZ9000ΔAB is compensated for by the observed increased production of CspE. For strain NZ9000ΔABE, the decreased freeze survival can be explained by the absence of CspE, the lower total amount of CSPs present, and/or the decreased production of certain CIPs. These data suggest that CSPs are important for the survival of freezing for *L. lactis*. CSPs may either have a direct effect during freezing, e.g., by stabilizing RNA and/or DNA, or may regulate the expression of other factors involved in the cryoprotective response (i.e., CIP8 and CIP9). Further elucidation of gene regulation by CSPs and identification of the CIPs of *L. lactis* will undoubtedly result in an improved understanding of low-temperature adaptation of this organism.

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