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Changes in *cspL*, *cspP*, and *cspC* mRNA Abundance as a Function of Cold Shock and Growth Phase in *Lactobacillus plantarum*

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An inverse PCR strategy based on degenerate primers has been used to identify new genes of the cold shock protein family in *Lactobacillus plantarum*. In addition to the two previously reported *cspL* and *cspP* genes, a third gene, *cspC*, has been cloned and characterized. All three genes encode small 66-amino-acid proteins with between 73 and 88% identity. Comparative Northern blot analyses showed that the level of *cspL* mRNA increases up to 17-fold after a temperature downshift, whereas the mRNA levels of *cspC* and *cspP* remain unchanged or increase only slightly (about two- to threefold). Cold induction of *cspL* mRNA is transient and delayed in time as a function of the severity of the temperature downshift. The cold shock behavior of the three *csp* mRNAs contrasts with that observed for four unrelated non-*csp* genes, which all showed a sharp decrease in mRNA level, followed in one case (*bglH*) by a progressive recovery of the transcript during prolonged cold exposure. Abundance of the three *csp* mRNAs was also found to vary during growth at optimal temperature (28°C). *cspC* and *cspP* mRNA levels are maximal during the lag period, whereas the abundance of the *cspL* transcript is highest during late-exponential-phase growth. The differential expression of the three *L. plantarum csp* genes can be related to sequence and structural differences in their untranslated regions. It also supports the view that the gene products fulfill separate and specific functions, under both cold shock and non-cold shock conditions.

When exposed to abrupt temperature downshifts, microorganisms undergo severe physiological disturbances such as a reduction in membrane fluidity, changes in the level of DNA supercoiling, and the formation of stable secondary structures in DNA and RNA that impair replication, transcription, and protein synthesis. To overcome the deleterious effects of cold shock and ensure that cellular activity will resume or be maintained at low temperature, bacteria have developed a transient adaptive response, the cold shock response, during which the expression of a subset of specific proteins is induced (reviewed in references 19, 36, and 42). The most strongly induced proteins include a family of closely related small (~7.5-kDa) single-stranded DNA- and RNA-binding proteins termed cold shock proteins (Csp). These proteins have been proposed to function as general RNA or DNA chaperones that stabilize single-stranded regions in RNA and DNA and thereby contribute to efficient translation, transcription, and DNA replication at low temperature (19, 24, 50). Their structure, a β barrel made up of five antiparallel β strands, is the archetype of a very common protein domain referred to as the oligonucleotide/oligosaccharide-binding fold (19, 38, 39).

Csp proteins are widespread among bacteria, and many species have been found to contain multiple variants of this large family of proteins (19, 50). Although the specific functions of these iso-Csp proteins remain unclear, most recent findings indicate that they not only are required for the cold shock adaptative response but also play a more general role in adapting cellular functions to various growth conditions. Of the nine Csp proteins from *Escherichia coli* (CspA to CspI), only four (CspA, CspB, CspG, and CspI) are cold inducible, with CspA being induced at the highest level and over the broadest temperature range (45, 50). Recent work has shown that CspA is also highly expressed during early exponential growth at optimal temperature (6), whereas expression of CspD is triggered at the onset of stationary phase and upon nutrient starvation (49). CspC and CspE are constitutively produced at 37°C and have been implicated in cellular processes taking place during normal growth, such as transcriptional regulation and chromosome condensation (3, 21, 48). The expression patterns and roles of CspH and CspF, the two most distant members of the *E. coli* Csp family, remain unexplored (50).

In contrast to *E. coli*, *Bacillus subtilis* contains three Csp proteins (CspB, CspC, and CspD) which are all induced after a temperature downshift (17). Like synthesis of *E. coli* CspD, synthesis of *B. subtilis* CspB and CspC is found to increase upon entry into stationary phase (20). Single and combined deletion of the three *csp* genes in *B. subtilis* has shown that the presence of at least one Csp protein is essential for cell viability and that the three Csp variants individually and complementarily contribute to efficient cell growth and survival at both low and optimal temperatures (17, 18).

Regulation of the expression of the *E. coli* and *B. subtilis* Csp proteins is complex, taking place at the transcriptional and posttranscriptional levels (18, 27, 36, 42). Furthermore, several observations indicate that iso-Csp proteins belonging to the same species self- and/or cross-regulate their own synthesis (2, 3, 10, 18).

Csp proteins are also found in different species of lactic acid bacteria (28). For example, a family of five *csp* genes (*cspA* to *cspE*) is present in *Lactococcus lactis*. Their expression is strongly induced upon cold shock, except for CspE, which is already abundant at optimal temperature (46, 47).

Lactobacillus plantarum is one of the most widespread lactic acid bacteria in the environment. This bacterium is also largely used as a starter for the production of fermented products of animal and vegetal origin (43). Strains that are used in fermen-

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tation and ripening processes undergo multiple stresses including drastic changes in temperature, pH, and carbon source. However, little is known about the mechanisms used by *L. plantarum* to adapt to environmental fluctuations. A better understanding of these mechanisms should provide important insight into how to improve current industrial starter strains.

We have previously reported the presence of two *csp* genes, *cspL* and *cspP*, in *L. plantarum* (32). In the present study, an inverse PCR strategy was used to isolate new members of the *csp* gene family from strain NC8. A new gene, *cspC*, has been cloned and characterized. Comparative transcriptional analyses of the three genes show that expression of *cspL* mRNA differs from that of *cspC* and *cspP*, both after a temperature downshift and during growth under optimal conditions. The cold shock behavior of the three *csp* transcripts also contrasts with that of non-*csp* genes, indicating that regulation of the *csp* genes involves cold-specific mechanisms acting at the level of transcription and/or stability of the mRNA. The data are consistent with the view that the three *csp* gene products of *L. plantarum* fulfill separate and specific functions in cold adaptation, as well as during normal growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *L. plantarum* silage strain NC8 (1) was grown in MRS broth (Difco Laboratories, Detroit, Mich.) without shaking. Cold shocks were performed by transferring NC8 cultures grown at 28°C into water baths precooled at the appropriate temperature. The *E. coli* strain TG1 was grown in Luria broth at 37°C (37). When necessary, ampicillin was added to a final concentration of 250 μg/ml.

DNA amplification, cloning, and sequencing. PCR amplifications were carried out using *Taq* (Advanced Biotechnologies, Leatherhead, United Kingdom) or Dynazyme (Finnzymes Oy, Espoo, Finland) DNA polymerase. Chromosomal DNA from NC8 was prepared as previously described (4). NC8 DNA was partially digested with *Mbo*I and then circularized by ligation to be used as a template in inverse PCR amplification. The degenerate primers used to amplify borders of the NC8 *csp* genes are CSPIF2 (5′-GGITWCAAAWCIYTRCAIGA AGGYCA-3′) and PIR1 (5′-GCYYTGRATMGCTGAGAARTGWAC-3′). Inverse PCR was carried out with 40 amplification cycles of 0.25 min at 95°C, 6 min at 50°C, and 4 min at 72°C.

The central region of the NC8 *cspC* gene was amplified using the degenerate primer CSPU3 (5′-GGTTACGTTASCWGCTKSHGGDCC-3′) (14) and the specific primer LPCF (nucleotides [nt] 290 to 310 of the *cspC* sequence). The resulting PCR fragment was radiolabeled with [α-^{32}P]ATP (Amersham, Buckinghamshire, United Kingdom) by random-primer DNA labeling (GIBCO-BRL, Gaithersburg, Md.) and used as a probe in Southern blot analysis of NC8 DNA cut by different restriction enzymes. The same probe was used to screen a genomic library of *L. plantarum* Lp80 (partial *Sau3*AI restriction fragments cloned into the *Bam*HI site of pGI4010) (26) transformed into TG1. Colony hybridization identified a plasmid (pGIS003) carrying a copy of the Lp80 *cspC* gene that was sequenced using CSPIF2, PIR1, and a set of specific primers. Two primers, CSPCG (5′-CACCGCTCAAGATTGGAC-3′) and PECA (5′GCCTT CAAGCAAGTCGCAAT-3′), were then derived from this sequence in order to amplify and sequence the NC8 *cspC* gene. Southern blot analysis and colony hybridization were performed using standard procedures (37).

Pairs of primers used to amplify and sequence the NC8 *cspL* (PEL91, nt 1 to 22; PEL17, nt 1263 to 1284) and *cspP* (PEP22, nt 1 to 19; PEPA, nt 687 to 703) genes were chosen based on the Lp80 *cspL* and C3.8 *cspC* sequences, respectively. Standard conditions used for direct PCR amplification are 30 cycles of 1 min at 92°C, 1 min at 50°C, and 1 min at 72°C, followed by an elongation step of 10 min at 72°C. Amplified products were extracted from agarose gels using a Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). DNA sequencing of purified PCR fragments and plasmid dsDNA was performed by Eurogentec S.A., Seraing, Belgium.

RNA hybridization and primer extension analysis. NC8 total RNA was prepared from 25-ml cultures using a Tri reagent kit (Sigma, St. Louis, Mo.). Cells were harvested by centrifugation and mechanically broken with 0.18-mm-diameter glass beads in a Braun homogenizer (four 1-min periods of homogenization with 1-min intervals on ice). Primer extensions were performed as previously described (15). Oligonucleotides used to map the 5′ termini of *L. plantarum csp* mRNAs are PEC5 (nt 357 to 381) and PECC (nt 443 to 463) for *cspC*, PEL60 (nt 554 to 575) for *cspL*, and PEP40 (nt 447 to 465), PEP1 (nt 404 to 430), and PEP2 (nt 420 to 444) for *cspP*. Radiolabeled elongation products were analyzed on 7% (wt/vol) polyacrylamide–urea sequencing gels, next to DNA sequencing reactions performed with the same primers. Single-stranded DNA templates were generated by λ exonuclease-mediated digestion of the 5′-phosphorylated strand of PCR products (Pharmacia Biotech, Uppsala, Sweden). Sequencing reactions were performed using T7 DNA polymerase (Pharmacia Biotech).

For quantitative Northern blot analysis, RNA samples were separated by electrophoresis in 1.2% (wt/vol) agarose–0.6% (wt/vol) formaldehyde gels in MOPS (morpholinepropanesulfonic acid) buffer. Gels were stained with ethidium bromide to ensure that equivalent amounts of total RNA were loaded in each sample. RNA was transferred to Hybond-N nylon membranes (Amersham) and fixed by heat treatment (80°C, 2 h). Hybridization was performed as previously reported (15), using α-^{32}P-radiolabeled PCR fragments as specific probes for *cspC* (nt 290 to 549), *cspL* (nt 317 to 1284), *cspP* (nt 1 to 703), *alr* (nt 653 to 1295), *bglH* (nt 661 to 1262), *cbh* (nt 116 to 945), and *lldh* (nt 654 to 1115). Radioactive bands were quantified with an Instant Imager (Packard Instruments, Meriden, Conn.) and visualized by autoradiography. When necessary, the relative amounts of transcript were standardized by hybridization with an *L. plantarum* 16S rRNA-specific probe (nt 87 to 1102).

Nucleotide sequence accession numbers. The EMBL accession numbers for the *L. plantarum* NC8 strain sequences reported here are Y19217 (*cspC*), Y19218 (*cspL*), and Y19219 (*cspP*). The nucleotide sequences for Lp80 *cspL* and C3.8 *cspP* can be found in the GenBank database with accession no. Y08940 and Y08760, respectively. The GenBank accession numbers for *L. plantarum alr*, *bglH*, *cbh*, *lldh*, and 16S rRNA sequences are Y08941, Y15954, M96175, X70926, and D79210, respectively.

RESULTS

Cloning of *cspC*, a new member of the *csp* gene family in *L. plantarum*. We have recently reported the cloning of *cspL* and *cspP*, two cold shock protein genes from *L. plantarum* Lp80 and C3.8, respectively (32). In the present study, an inverse PCR approach based on degenerate primers was used with the aim of amplifying new members of the *csp* family from strain NC8. This strain was chosen as a model for genetic studies because it displays interesting features such as being plasmid free and highly transformable (1). The nucleotide sequences of the degenerate primers PIR1 and CSPIF2 (see Materials and Methods) were determined based on highly conserved regions in *csp* genes from more than 30 different bacterial species (14; K. P. Francis, unpublished data). Total DNA from NC8 was partially digested with *Mbo*I, and the resulting fragments were circularized by ligation to serve as a template in PCR amplification. Six different amplification products were obtained and sequenced. One of them contained a sequence identical to the previously identified *cspL* gene of Lp80, while two other amplicons carried DNA fragments corresponding to the *cspP* gene from strain C3.8. The remaining three PCR products were found to contain a sequence belonging to a new member of the *csp* family that we named *cspC*. Although the primers and conditions used in the amplification were chosen so as to amplify the largest number of specific fragments, the possibility that *L. plantarum* contains additional and more distant Csp variants cannot be totally ruled out.

The central region of the NC8 *cspC* gene was amplified using a specific primer complementary to the 5′ end of the gene and a degenerate primer corresponding to a conserved region at the 3′ end (U3CSP) (14). This fragment was used as a probe to screen an Lp80 genomic library (26) by colony hybridization. A clone carrying a complete copy of the *cspC* gene was identified. PCR primers were then derived from the sequence of this clone to amplify and determine the complete sequence of the NC8 *cspC* gene. Southern blot analysis demonstrated that this gene is present as a single copy in the NC8 genome (data not shown). PCR amplification and DNA sequencing of the two other *csp* genes from NC8 showed that the *cspL* gene is 100% identical to that of Lp80, whereas 19 nucleotide differences were found between the NC8 and C3.8 sequences in the noncoding regions of *cspP* (data not shown).

The three *csp* genes encode small and closely related 66-amino-acid proteins, with a calculated pI value close to 4 (Fig. 1). They contain two RNA-binding motifs called RNP1 and

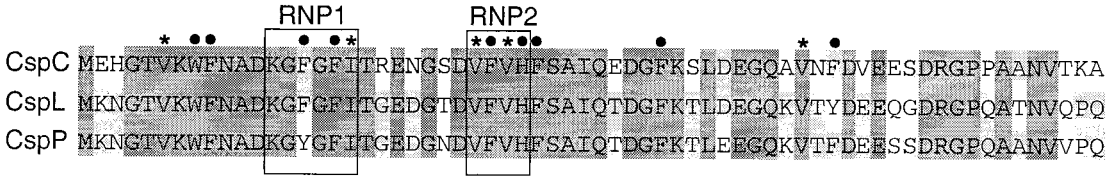

FIG. 1. Comparison of *L. plantarum* CspC, CspL, and CspP sequences. Amino acids residues that are identical in all three or just two Csp proteins are shaded in dark and light grey, respectively. The RNA-binding motifs RNP1 and RNP2 are boxed. Conserved residues that are critical for forming the hydrophobic core of the protein (asterisks) or for nucleic acid binding (dots) are indicated.

RNP2 (30, 34, 40), together with additional conserved residues that are important for the formation of the β-barrel core of the protein and for nucleic acid binding activity (Fig. 1) (12, 34, 40). The newly identified CspC protein is the most distant member of the family since it displays 73 and 74% identity with CspL and CspP, respectively, while CspL and CspP are 88% identical.

The three *csp* genes exhibit long 5′ untranslated leader regions (5′ UTRs). Primer extension analysis was performed on NC8 total RNA in order to map the transcription start point of *cspC*. Independent elongation reactions were performed using two different primers to selectively identify products arising from specific hybridization. Multiple signals were obtained with either primer individually, but only three of them were common to both reactions (Fig. 2A). A similar analysis of the NC8 *cspL* transcript identified a single 5′ end mapping at the same position as previously reported for the Lp80 *cspL* gene (Fig. 2B) (32), whereas two specific elongation products were detected for *cspP* (Fig. 2A). The smallest product corresponds to the *cspP* transcription start initially mapped in C3.8 (32). However, in light of the present results, it appears more likely that the minor signals observed for both *cspC* and *cspP* arise from specific degradation of the mRNA and that the actual transcription initiation sites of these two genes correspond to the 5′ ends of the longest and more abundant extension products (Fig. 2A).

The proposed transcription start of the three *csp* genes is preceded by a sequence that is 67% (*cspP*) or 89% (*cspC* and *cspL*) identical to the extended −10 box consensus sequence (TNTGNTATAAT) of the σ^A promoters from gram-positive bacteria (22). The presence of TG dinucleotides in this box has been found to increase contacts between the RNA polymerase and DNA (44). In *cspP*, which has the less well conserved −10 box, a putative −35 box sequence is found at the expected position of the promoter region, while no obvious −35 boxes are found in *cspC* and *cspL* (Fig. 2B).

This analysis reveals that *cspC* and *cspP* have similarly long predicted 5′ UTRs that are 49% identical, whereas *cspL* displays a slightly shorter 5′ UTR that is only 22% identical to that of *cspC* and 27% identical to that of *cspP* (Fig. 2B and data not shown). The sequence similarities in the 5′ UTRs contrast with the degree of homology between the proteins since, as mentioned above, CspL and CspP are closer to each other than CspC and CspP. In addition, *cspC* and *cspP* genes exhibit a typical monocistronic structure containing a single transcription terminator at the 3′ end, while two consecutive putative terminators are found downstream of the *cspL* coding region, as previously reported for the Lp80 *cspL* gene (Fig. 2B) (32).

Changes in *csp* and non-*csp* mRNA abundance upon cold shock. Quantitative Northern blot analysis was used to monitor the levels of *cspC*, *cspL*, and *cspP* mRNA after transferring exponentially growing NC8 cultures (optical density at 600 nm [OD_{600}] = 0.8) from 28°C to 8°C (Fig. 3). Those values were set as optimal and medium cold shock temperatures, respectively, on the basis of the *L. plantarum* NC8 Arrhenius plot of growth (S. Derzelle, B. Hallet, T. Ferain, J. Delcour, and P. Hols, submitted for publication). The expression patterns of the *csp* genes were compared to those obtained for four non-*csp* genes which fulfill separate and unrelated functions in *L. plantarum*. These include the alanine racemase gene (*alr*), which is responsible for the production of D-alanine, an essential component of the bacterial cell wall (23); the phospho-β-glucosidase gene (*bglH*), involved in utilization of specific aromatic β-glucosides as a carbon source (31); the L-lactate dehydrogenase gene (*ldhL*), which catalyzes the last step of L-lactic acid production by fermentation (13); and the *cbh* gene, which encodes a conjugated bile acid hydrolase (7).

We identified a single transcript of the expected size (330 nt) for both *cspC* and *cspP*, whereas two signals were observed for *cspL*, as previously reported for the Lp80 *cspL* gene (Fig. 3). The shorter transcript (330 nt) has been shown to contain the *cspL* open reading frame whereas the longer transcript (760 nt) extends further downstream to encompass a putative 77-amino-acid open reading frame bearing no similarity to known proteins (32). It remains unclear whether these two transcripts arise from alternative transcriptional termination (Fig. 1B) or whether they arise through posttranscriptional processing of the mRNA.

The amount of *cspC* and *cspP* transcripts remained unchanged after the temperature downshift (Fig. 3A and B). In contrast, the level of *cspL* mRNAs was found to increase up to eightfold after 3 h at 8°C. The amount of transcripts subsequently decreased to a steady-state level that remained five times higher than observed before the cold shock (Fig. 3C). When taken separately, the long and short transcripts displayed similar kinetics (Fig. 3C and data not shown). These results clearly differentiate the *csp* genes from the four non-*csp* genes examined, since all of them showed an abrupt diminution in their mRNA level immediately after cold shock (Fig. 3D). However, in the case of *bglH*, the amount of transcript increased again after 3 h of cold exposure to about the same level as before the temperature shift (Fig. 3D).

Cold induction of *cspL* mRNA is delayed according to the severity of the temperature downshift. To further characterize the influence of cold shock on the expression of *cspL*, Northern blot analysis was performed on exponentially growing NC8 cultures shifted to different temperatures (Fig. 4). According to the Arrhenius plot of *L. plantarum* NC8 growth (Derzelle et al., submitted), these temperatures correspond to nonstressed suboptimal growth conditions (15°C) or to mild (10°C), medium (8°C), and severe (6°C) cold shocks. The level of the large *cspL* transcript was found to increase at all temperatures tested. The small transcript in this set of experiments was barely detectable for unknown reasons. However, a delay in induction was observed with decreasing temperatures, the highest level of the *cspL* transcript being detected after 1 h at 10°C, after 3 h at 8°C, and after 5 h at 6°C (Fig. 4). After 1 h at 15°C, the amount of the *cspL* transcript was already six to

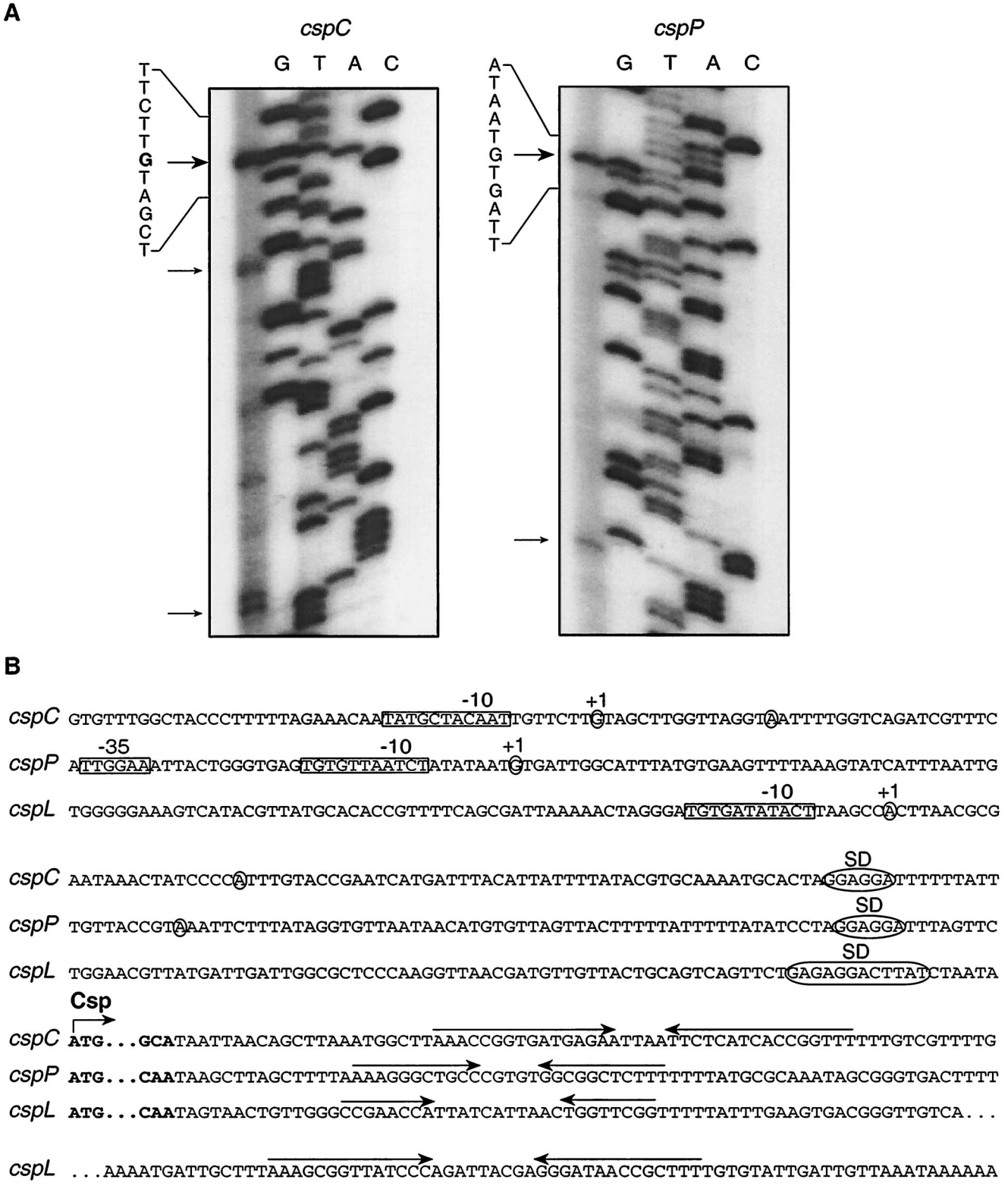

FIG. 2. (A) Primer extension analysis of the *cspC* (left) and *cspP* (right) transcripts. RNA samples were extracted from cold-shocked cultures (8°C). Bold and thin arrows indicate the positions of major and minor extension products, respectively, obtained with independent primers. Additional signals are nonspecific products that are detected only with individual primers. (B) Nucleotide sequences of the 3′ and 5′ regions of *L. plantarum* NC8 *cspC*, *cspL*, and *cspP* genes. Only the first and last codons of the Csp-coding sequence are shown. Transcription starts (+1) are circled, as are the 5′ ends of shorter and less abundant primer extension products detected for *cspC* and *cspP*. The promoter −35 and −10 boxes and the Shine-Dalgarno sequences (SD) are boxed and circled, respectively. Horizontal arrows indicate the positions of putative transcription terminators.

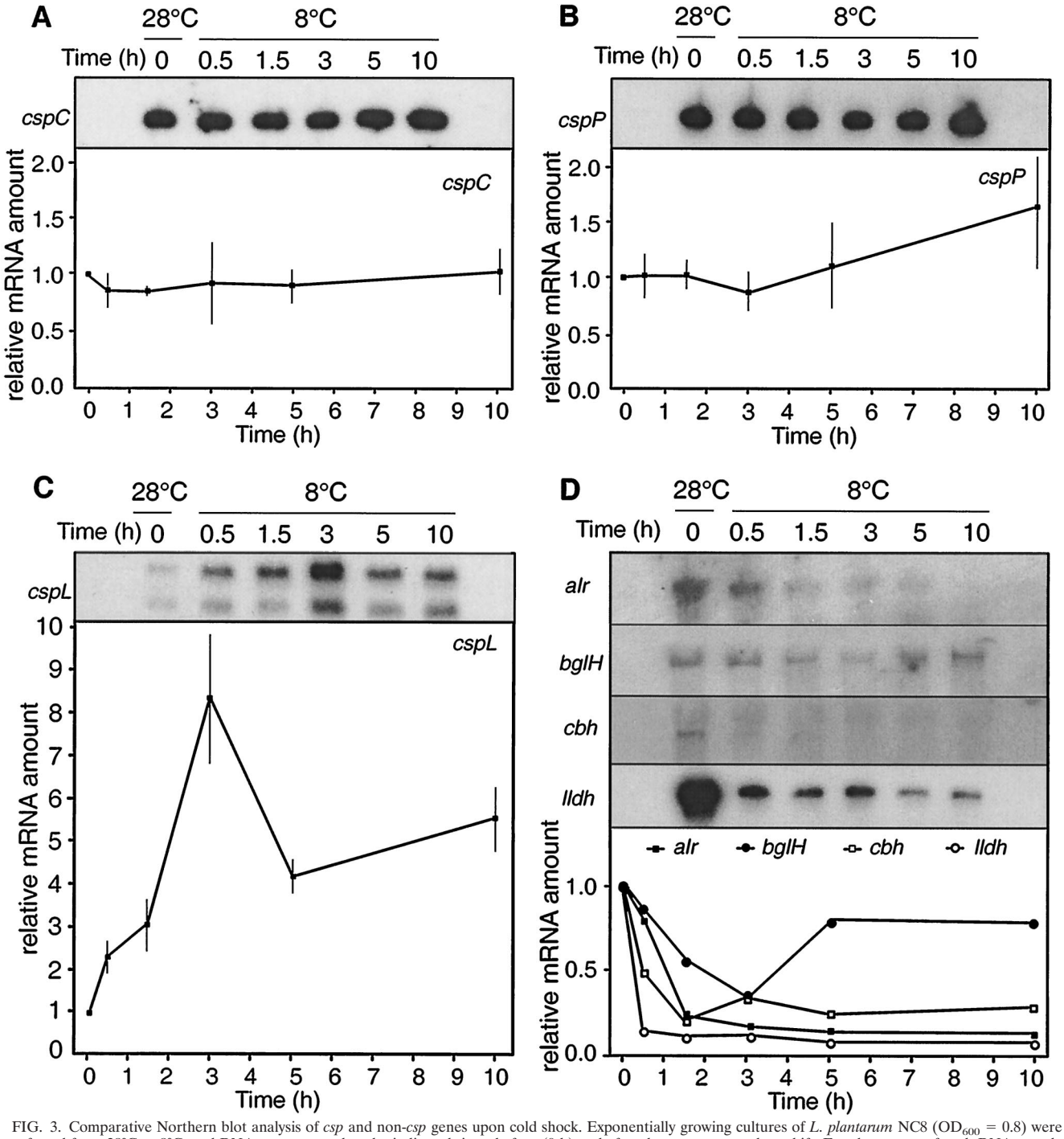

FIG. 3. Comparative Northern blot analysis of *csp* and non-*csp* genes upon cold shock. Exponentially growing cultures of *L. plantarum* NC8 (OD_{600} = 0.8) were transferred from 28°C to 8°C, and RNA was prepared at the indicated times before (0 h) and after the temperature downshift. Equal amounts of each RNA sample were run on 1.2% (wt/vol) agarose–0.6% (wt/vol) formaldehyde gels and hybridized with specific radioactive probes for *cspC* (A), *cspP* (B), *cspL* (C), and the four non-*csp* genes *alr*, *bglH*, *cbh*, and *lldh* (D). The same membranes were rehybridized with the different probes. One representative result is shown for each gene. Relative mRNA amounts were calculated from the radioactivity measured in the transcript bands at each time point with respect to that found at 0 h. *cspL* quantification was performed by summing the radioactivities of the short and long transcripts. Data presented for *cspC*, *cspL*, and *cspP* are from three independent experiments.

seven times higher than in precooled cells, and it stayed at about the same level after 3 h and 5 h of cold exposure. These findings indicate that there is a dose effect of cold on expression of the *cspL* transcript. Hybridization of the same RNA samples with *cspC* and *cspP* probes showed that their mRNA levels are only slightly (two- to threefold) higher at 10 and 15°C than at 6 and 8°C (data not shown).

Changes in *cspC*, *cspP*, and *cspL* mRNA abundance during growth at optimal temperature. The level of *cspC*, *cspL*, and *cspP* transcripts was examined over a complete culture cycle performed at 28°C (Fig. 5A). This analysis revealed that early growing cells contain substantial amounts of *cspC* and *cspP* mRNAs, which rapidly decline to become 10 times less abundant at the entry into stationary phase (Fig. 5A). A very similar

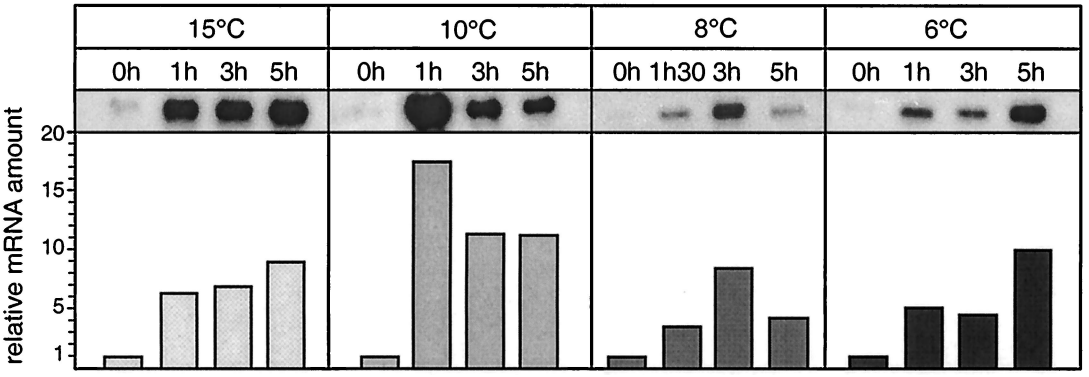

FIG. 4. Delayed cold induction of *cspL* mRNA level with decreasing temperatures. RNA was extracted from an exponentially growing NC8 culture (OD_{600} = 0.8) before and after different times following a temperature downshift from 28°C to 15, 10, 8, or 6°C. The resulting RNA samples were analyzed by hybridization with a *cspL* probe as described for Fig. 3. As the smaller transcript was barely detectable, quantification was performed only on the large transcript.

behavior has recently been reported for CspA, the major Csp protein in *E. coli*, and to some extent for CspE, which is not cold induced (3, 6). By contrast, a different pattern is observed for the *L. plantarum cspL* gene (Fig. 5A). The amount of *cspL* transcripts increases to reach a plateau during exponential growth and then decreases during stationary phase to return to its initial level (Fig. 5A). This pattern also distinguishes *cspL* from the *cspD* gene of *E. coli*, or the *cspB* and *cspC* genes of *B. subtilis*, the expression of which is induced at the beginning of stationary phase (20, 49).

In *E. coli*, the extent of *cspA* cold induction is inversely proportional to the preexisting concentration of CspA protein at the time of the cold shock (6). To see whether a similar effect can be observed in *L. plantarum*, the amount of *cspC*, *cspL*, and *cspP* transcripts was quantified before and after transferring a stationary-phase culture (OD_{600} = 7) from 28°C to 10°C. After 2 h of cold exposure, all three genes showed a very limited (between 1.1- and 1.6-fold) increase, if any, of their transcript levels (Fig. 5B). For *cspL*, this contrasts with the strong induction observed after a cold shock of exponentially growing cells.

DISCUSSION

Using an inverse PCR approach based on degenerate primers, we demonstrate that strain NC8 of *L. plantarum* contains at least three Csp-encoding genes. Two of them, *cspL* and *cspP*, were previously identified from separate strains (32). The

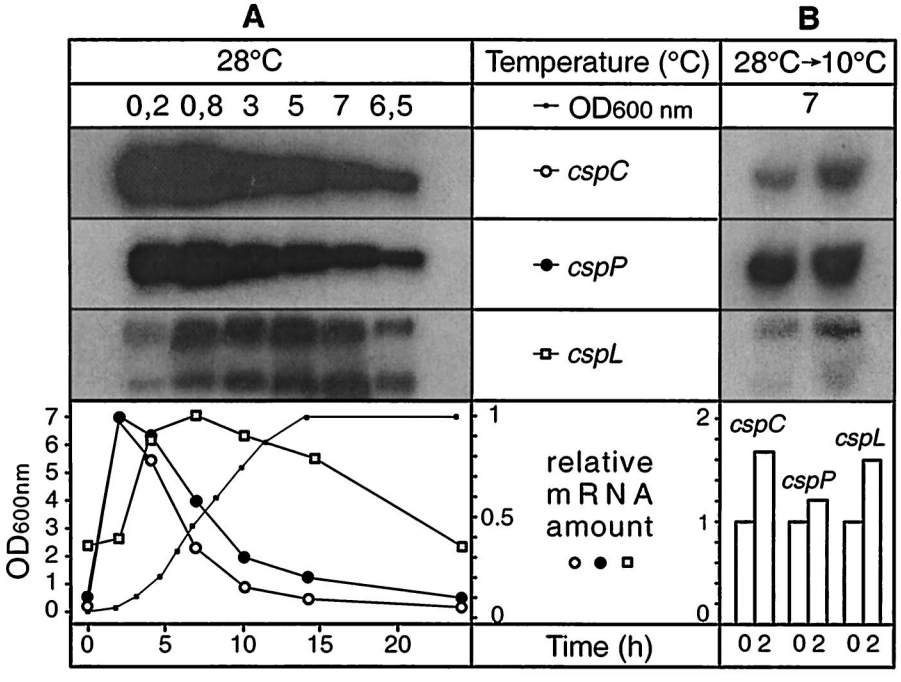

FIG. 5. (A) Growth phase-dependent expression of *cspC*, *cspL*, and *cspP* mRNAs. Stationary-phase NC8 cells were diluted with fresh MRS medium and allowed to grow for 25 h at 28°C. RNA samples were taken at different times of culture and analyzed by Northern blot hybridization with *cspC*, *cspP*, and *cspL* probes, as indicated. Results obtained after hybridization of the same membrane with the three different probes are shown. Relative mRNA amount is expressed with respect to the highest level of each transcript during the culture. Radioactivities in the short and large *cspL* transcripts were summed. (B) Increase of *cspC*, *cspP*, and *cspL* mRNA levels in cold-shocked stationary-phase cells. Relative amounts of *cspC*, *cspP*, and *cspL* transcripts were examined 2 h after transferring stationary-phase NC8 cultures (OD_{600} = 7) from 28°C to 10°C.

newly isolated *cspC* gene represents the most distant member of the *csp* family identified thus far in *L. plantarum*.

Comparative Northern blot analysis reveals that the relative abundance of *cspC*, *cspL*, and *cspP* transcripts differentially varies after a temperature downshift as well as during growth in optimal conditions. Upon cold shock of exponentially growing cells, *cspL* undergoes a significant and transient induction, whereas the amount of *cspC* and *cspP* mRNAs remains unchanged or increases slightly. These two different patterns distinguish the *csp* genes from non-*csp* genes, the mRNA level of which is found to rapidly decrease after cold shock. This is a key result of the present study, as it demonstrates that *csp* transcripts may be more stable and/or more efficiently expressed than other cellular messengers, even if their absolute level does not significantly increase during cold exposure.

Rapid disappearance of non-*csp* transcripts is likely to arise from the general inhibition of both RNA and protein synthesis that follows an abrupt temperature downshift (29, 41). In particular, impairment of translation initiation would leave RNA molecules unprotected, and consequently these become more sensitive to degradation although cellular RNAse activity itself is reduced at low temperature (35). However, the *bglH* gene can be distinguished from the three other non-*csp* genes examined since its mRNA level rises again after 3 h of cold exposure. Interestingly, this corresponds to the time at which *cspL* mRNA is most abundant. One may therefore suggest that resumption of *bglH* mRNA expression could be a consequence of cold adaptation mechanisms involving CspL and presumably other cold-induced proteins. As this enzyme provides a means of using a broader range of carbon sources, its early expression during cold acclimation may represent a valuable contribution to cell survival and growth at low temperature. This raises the possibility that separate classes of genes respond differently to continuous growth at cold temperatures as a function of their biological roles.

It becomes clear that Csp proteins are not only involved in cold adaptation but also play an important role during growth at optimal temperature. Recent reports have shown that cold-inducible Csp proteins may also be expressed at specific stages of the normal growth cycle. CspA, the major cold shock protein of *E. coli*, is transiently and massively expressed during the early phase of the growth curve at 37°C, whereas expression of CspB and CspC from *B. subtilis* is triggered at the onset of stationary phase (6, 20). Here, we demonstrated that the relative amounts of *L. plantarum cspC*, *cspL*, and *cspP* transcripts strongly fluctuate during growth at 28°C, indicating that specific regulation mechanisms operate on the three genes to direct their expression at different times of the culture. The growth phase is also found to determine the extent of *cspL* cold induction, since a cold shock of exponentially growing cells results in a much stronger increase in *cspL* transcript level than a cold shock performed in stationary phase. This suggests that stationary-phase cells could adapt to cold without requiring further expression of *cspL*.

Based on (i) the results presented here, (ii) our analysis of the *L. plantarum cspL*, *cspP*, and *cspC* sequences, and (iii) what is known about cold shock regulation in other bacteria, we can make a series of predictions on the regulation mechanisms that are experimentally testable. In *E. coli* and *B. subtilis*, although a moderate increase in the transcription of *csp* genes is observed, Csp protein expression is regulated primarily at a post-transcriptional level. In particular, cold-induced *csp* genes usually contain a long 5′ UTR which, in the case of *E. coli cspA*, has been found to destabilize the mRNA at 37°C and to enhance its stability and translation efficiency after cold shock (11, 16, 33, 51). Comparison of the *L. plantarum csp* genes shows that the 5′ UTRs of *cspC* and *cspP* are longer and more similar to each other than to the 5′ UTR of *cspL*. Furthermore, *cspC* and *cspP* 5′ UTRs are predicted to form similar hairpin structures, whereas the 5′ UTR of *cspL* exhibits a distinctive Y-like secondary structure (data not shown). Additionally, both *cspC* and *cspP* display minor extension signals ending close to bulge loops in the predicted hairpin structure of their 5′ UTRs (data not shown), suggesting an increased susceptibility to cleavage by a specific RNase and a higher instability of respective mRNAs compared to *cspL*. These differences in the *csp* gene 5′ UTRs relate to the finding that the mRNA expression patterns of *cspC* and *cspP* diverge from that of *cspL*, both at low and at optimal temperatures. The fact that the degree of similarity between the 5′ UTRs differs from that of the protein sequences suggests that the coding and untranslated regulatory regions of the *csp* genes have evolved independently in order to allow functionally distinct Csp proteins to be expressed in different conditions.

Cold induction of CspA and other *E. coli* Csp proteins requires the presence of a 14-nt sequence, referred to as the downstream box (DB), located 12 nt downstream of the initiation codon (8, 33). More recently, a second *cis*-acting element, the upstream box (UB), has been identified within the 5′ UTR, 14 nt upstream of the Shine-Dalgarno sequence (51). Although the actual roles of the DB and UB sequences remain unclear (for recent debates in the literature, see references 5 and 9), both boxes are complementary to specific regions of the 16S rRNA 3′ end, and it is proposed that they thereby contribute to translation initiation by increasing the affinity of the ribosome for mRNA. Enhanced translation may in turn contribute to the stabilization of the transcripts by protecting them from degradation (35).

Close examination of the *L. plantarum csp* transcripts reveals that each contains a DB-like box at the N terminus of the coding region (consensus, 5′-uGGuACAGUaAAAUGGUU-3′) that is complementary to the *L. plantarum* 16S rRNA 3′-end sequence (nt 1472 to 1487). Interestingly, a highly related DB-like sequence (5′-GUUCCCAGAAGGAU-3′) is also found in the coding region of the *bglH* gene, although in this case, the N-terminal amino acid sequence of the protein is totally divergent from that of the Csp proteins. As for the *csp* genes, the presence of this box may enhance the translation efficiency, and therefore the stability, of *bglH* mRNA compared to other cellular transcripts.

An upstream sequence that is complementary to a different region of the 16S rRNA 3′ end (nt 1049 to 1061) is also found in the 5′ UTR of *cspL* (5′-CCCAAGGUUAACG-3′) but not in *cspC* or in *cspP*. This UB-like sequence is entrapped within the arms of the proposed Y-like structure of the *cspL* 5′ UTR (data not shown). Therefore, we would predict that formation of this structure must be prevented to give access to the UB region and to permit translation-mediated stabilization of the transcript. This postulated mechanism would explain the temperature-dependent delay observed for *cspL* cold induction, since decreasing cold shock temperatures are expected to enhance the stability of the RNA duplex. Destabilization of the 5′-UTR structure could be promoted by specific cold-inducible RNA helicases analogous to the *E. coli* CsdA protein (27). It may also require the RNA chaperone activity of the Csp proteins themselves.

Whichever mechanisms are responsible for regulation of the *csp* genes in *L. plantarum*, their differential expression at low and optimal temperatures is consistent with the view that they fulfill specific and perhaps complementary functions in adapting cell physiology to different conditions. It remains to be determined whether fluctuations in the mRNA level observed

for the three genes correlate with variations in the relative amounts of their products. The deletion or the constitutive overexpression of *L. plantarum csp* genes should also provide important insight on their respective contribution to cell survival and growth.

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