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Cold Shock Induction of the *cspL* Gene in *Lactobacillus plantarum* Involves Transcriptional Regulation

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Fragments of the *cspL* promoter region were fused to the *gusA* reporter and reintroduced into *Lactobacillus plantarum* cells, either on multicopy plasmids or through single-copy chromosomal integration. β-Glucuronidase activity and primer extension data demonstrate that the *cspL* promoter is induced in response to cold shock and that multicopy constructs quench the induction of the resident *cspL* gene.

Small acidic cold shock proteins (CSPs) are known to be highly induced in response to cold shock. These proteins are found in a wide range of bacteria, and multiple paralogs are often present. In *Escherichia coli*, for example, nine CSPs (CspA to CspI) are known (32); of these, CspA, CspB, CspG, and CspI are cold inducible, with CspA (an RNA chaperone) being the most strongly induced (21). CspA binds to RNA in a cooperative manner and prevents the formation of stable secondary structures. Since RNA duplexes are more stable at low temperatures, an increased level of CSPs may be essential for protein synthesis to proceed under these conditions (18, 21, 32). CspA is also a transcriptional antiterminator, playing an important role in cold shock adaptation by reprogramming gene expression to induce other CSPs at low temperatures (4). CspA can thus affect the translation rate of a range of proteins in addition to the transcription rate of some genes (e.g., *gyrA* and *hns*) (32).

The mechanism of cold shock induction of *E. coli cspA* has been extensively studied. It occurs mainly posttranscriptionally, although a moderate increase in the level of transcription is also observed (16, 25). An important feature shared by the four cold-induced *csp* genes of *E. coli* is the presence of an unusually long 5′ untranslated region (5′UTR). This region is crucial for *csp* mRNA destabilization at 37°C and for transient stabilization upon cold shock in *E. coli* (13, 14, 16, 25, 34). CspA induction also depends on very efficient translation at low temperatures (5, 11, 31). Indeed, the inhibitory effect of cold on the translational apparatus is bypassed in the case of *cspA* mRNA (16, 31). *cspA* mRNA contains two *cis*-acting translation enhancer elements that are suggested to be complementary to sequences of the 16S rRNA: a downstream box (DB) located downstream of the translation initiation codon (11, 25) and an upstream box (UB) located upstream of the Shine-Dalgarno sequence (34). Both elements are conserved in all *E. coli* cold-inducible *csp* genes. The relative amount of CSPs is also regulated at the level of protein stability (5, 28). CspA abundance also seems to be controlled via transcriptional attenuation that takes place through the binding of CspA to the 5′ end (cold box) of *cspA* mRNA (3, 6, 12, 14). It has recently been reported that polynucleotide phosphorylase, by selectively degrading *cspA* mRNA, represses CspA production at the end of the cold shock acclimation phase (33).

Few reports have dealt with the mechanisms involved in CSP induction in gram-positive bacteria (17, 18, 22). In *Bacillus subtilis*, *cspB* mRNA is stabilized (30-fold) after a temperature downshift from 37 to 15°C (22), implying that cold shock induction takes place at the posttranscriptional level. Transcriptional fusion of the *cspB* coding region to a non-cold shock promoter still allows induction of the *cspB* transcript, whereas expression of the β-galactosidase reporter transcriptionally fused to the *cspB* promoter is only slightly increased upon cold shock (17, 22). Long 5′UTRs are present in *cspB* and *cspC* of *B. subtilis* and in the four cold-induced *csp* genes of *E. coli.* CSPs of *B. subtilis* have a high binding affinity for the first 25 bases of their mRNAs' 5′UTRs, which suggests that autoregulatory control of *csp* expression in *B. subtilis* is similar to that seen in *E. coli* (17).

The lactic acid bacterium *Lactobacillus plantarum* is widely used as a starter for the production of fermented products of animal and vegetal origins (24). During the manufacture of lactic acid-fermented products, low-temperature challenges are common; therefore, understanding the cold shock response in this microorganism is of great industrial importance. Three *csp* genes (*cspL*, *cspC*, and *cspP*) were recently identified in *L. plantarum* (9). The *cspL* transcript is induced 10-fold by a temperature downshift from 27 to 8°C. *cspL* displays unique features, such as a σ^A promoter devoid of a −35 box and a 5′UTR with a potential Y-like secondary structure in addition to putative DB and UB elements (Fig. 1A) (9). The *cspL* promoter drives the transcription of two cold-inducible transcripts likely to result from terminator readthrough and/or RNase processing: the shorter one (330 nucleotides [nt]) covers the *cspL* open reading frame (ORF), whereas the longer one (760 nt) extends further downstream into a region encoding a 77-amino-acid (aa) ORF (*orfX*) of unknown function (9, 23). Overproduction of CspL prior to cold shock preadapts *L. plantarum* to growth at cold temperatures (S. Derzelle, B. Hallet, T. Ferain, J. Delcour, and P. Hols, unpublished data).

In this study, the regulation of *cspL* expression upon cold

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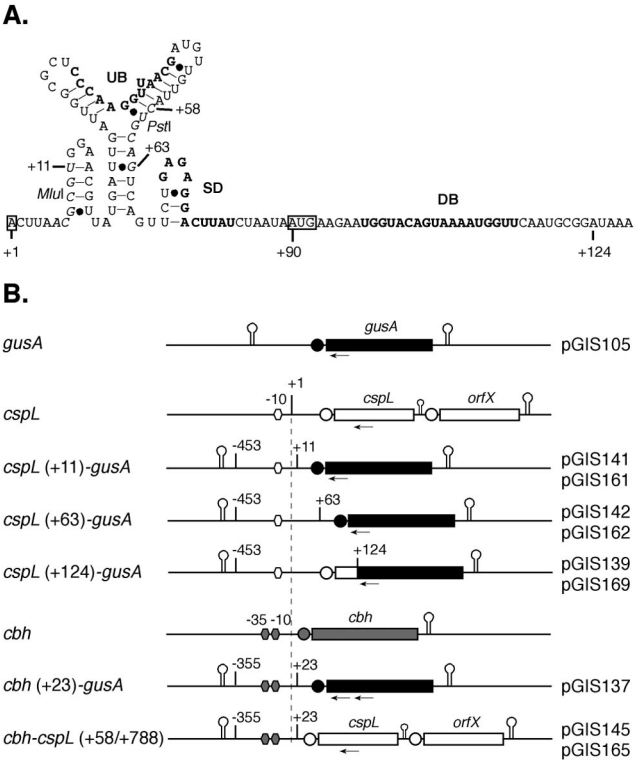

FIG. 1. Molecular structure of the genetic constructs. (A) Schematic map of the predicted mRNA secondary structure of the 5′ untranslated *cspL* region, showing the *Mlu*I and *Pst*I restriction sites used in constructing the fusions, the Shine-Dalgarno sequence (SD, in bold), and the putative DB and UB (in bold). The *cspL* ORF (AUG codon, boxed) starts 89 nt downstream of the transcription start site (boxed +1). The predicted RNA secondary structure was obtained by free-energy minimization with the MFOLD program, version 2.0 (19, 20, 35). (B) Schematic drawing of the various genetic fusions used to study *cspL* regulation. The top line shows the *gusA* reporter carried by pGIS105. The next lines show schematic maps of the intact *cspL* and *cbh* genes (lanes 2 and 6, respectively), as well as those of the *cspL-gusA*, *cbh-gusA*, and *cbh-cspL* fusions (lanes 3 to 5, 7, and 8, respectively). Hairpins, hexagons, circles, and boxes represent, respectively, the terminators, promoters, Shine-Dalgarno sequences, and coding regions of *gusA*, *cspL*, or *cbh.* The *cspL*, *gusA*, and *cbh* fragments are indicated in white, black, and grey, respectively. The putative 77-aa ORF (*orfX*) downstream of *cspL* is also indicated by a white box (lanes 2 and 8). The fusion points are indicated by black straight lines and are numbered. Nucleotide numbers start from the transcription initiation site (+1), as determined previously (9). Arrows locate the specific *cspL* and *gusA* primers used to perform primer extension analyses.

shock was investigated by fusing fragments of the *cspL* promoter of various lengths with the *gusA* reporter gene (β-glucuronidase).

Construction of *cspL* and *cbh* fusions. The *gusA* reporter was used to study *cspL* regulation, as the *lacZ* reporter could not be used in *L. plantarum* due to high endogenous β-galactosidase activity (15). It was previously demonstrated that the *gusA* reporter is suitable for the comparison of the efficiencies of various promoters in this species (26). Plasmid constructs were made using pGIS105 (Fig. 1B) as the starting vector. pGIS105 is a derivative of pNZ272, a multicopy vector (26) that contains a strong transcriptional terminator upstream of the *gusA* reporter gene to prevent transcriptional activation of *gusA* by upstream promoters. pGIS105 was constructed by insertion of the Ω module (containing the terminator) from plasmid pHP45Ω (27) as a 2.0-kb *Bam*HI fragment into the unique *Bgl*II site of the multiple cloning site (MCS) of plasmid pNZ272.

A first set of multicopy fusions was created between the expression signals of *cspL* and the *gusA* reporter gene (Fig. 1B). The first *cspL-gusA* fusion (pGIS141) is transcriptional and contains the fragment of the *cspL* promoter region extending from positions −453 to +11 (numbering from the transcription start site), which was constructed as follows. The *cspL* promoter region was PCR amplified with the oligonucleotides 5′-TCGGGATCCGG GGATCTTTGTAATTTAGTT-3′ (*Bam*HI site is underlined) and 5′-TTCCCAAGCTTTCCGCATTGAACCATTTTACTG T-3′. The PCR fragment was digested with *Bam*HI-*Mlu*I and inserted into pMTL23P (7) so as to allow cloning of a *Bam*HI-*Pst*I fragment into the MCS of pGIS105. A second *cspL-gusA* transcriptional fusion (pGIS142) contains the *cspL* promoter fragment from positions −453 to +63. It was constructed by digesting the above-described PCR fragment with *Bam*HI and *Pst*I and then cloning the fragment into pGIS105, yielding pGIS142. A translational fusion (pGIS139) was also constructed between a *cspL* gene fragment extending from −453 to +124 bp (aa 12) and the *gusA* reporter (starting at aa 3). The above-described PCR fragment was digested with *Bam*HI and *Hin*dIII (positions −453 to +124) and subcloned into pMTL23P. The *gusA* ORF was PCR amplified with oligonucleotides gus1 (5′-CAGTTAAGCTT GGTCCGTCCTGTAGAAACC-3′) (*Hin*dIII site is underlined) and gus3 (5′-AGGAATTCGATTCATTGTTTGCCTC CTG-3′) (*Eco*RI site is underlined) and was then digested with *Hin*dIII-*Eco*RI before being cloned into pMTL23P downstream of the *cspL* fragment. A *Bam*HI-*Xho*I fragment containing the translational fusion was then inserted between the same restriction sites in pGIS105. The absence of mutations in these fusions was confirmed by DNA sequencing.

A second set of multicopy fusions was made with the promoter of the *cbh* gene (conjugated bile salts hydrolase). The *cbh* promoter was chosen as a control, as previous transcriptional analyses had revealed that *cbh* mRNA abundance was reduced approximately threefold upon cold shock (9). The *cbh* transcription start site was mapped by primer extension analysis downstream of a putative σ^A promoter sequence (TAGA TT-15 bp-TGNTTTTAT), 50 nt upstream of the translation start codon (data not shown). pGIS137 was used as a control for analyses involving the *gusA* reporter gene and was obtained as follows. The *cbh* promoter region (−355 to +23) was PCR amplified with the oligonucleotides cbh1 (5′-GGAATTCGGC CAACGCTAATGGTGTTA-3′) (*Eco*RI site is underlined) and cbh3 (5′-TCAGCTGCAGATTTGATAAGTTAATAAG CTT-3′) (*Pst*I site is underlined). The *cbh* PCR fragment was digested with *Eco*RI-*Pst*I and cloned into pMTL23P. A *Bgl*II-*Pst*I fragment from the last-named plasmid was subsequently cloned between the *Bam*HI and *Pst*I sites of pGIS105, resulting in pGIS137. pGIS145 is a pGIS142 derivative where the *cspL* promoter was exchanged with the *cbh* promoter and was constructed as follows. The *cspL* gene was isolated as a *Pst*I-*Ssp*I fragment from plasmid pGIS001 (23), cloned between *Pst*I and *Sma*I into pMTL23P, and recovered as a *Pst*I-*Xho*I fragment for cloning into pGIS142 to substitute for the *gusA* gene. The *cbh* promoter was isolated as an *Sma*I-*Pst*I fragment from a

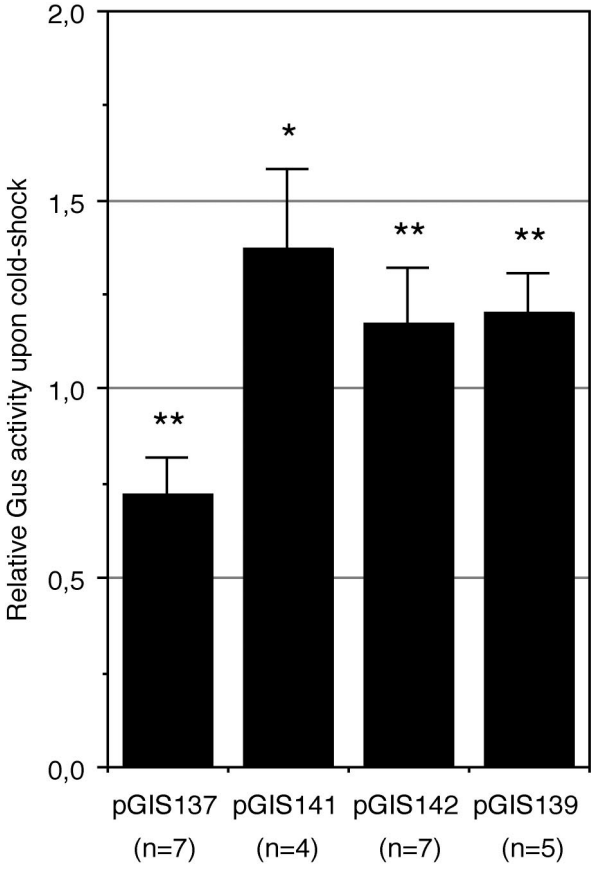

FIG. 2. Relative Gus activities of the various recombinant strains after a cold shock of 3 h at 8°C (values are averages of results of the numbers of experiments indicated below the bars). The reference level of 1 corresponds to the Gus activity measured before the cold shock in cells grown at 27°C and collected at an optical density (OD) (600 nm) of 0.8. Error bars represent 1 standard error of the mean. Statistical analysis was performed with the Student *t* test. *, significant difference ($P < 0.05$); **, highly significant difference ($P < 0.01$).

pMTL23P derivative and cloned between the *Pvu*II and *Pst*I sites in pGIS142 to replace the *cspL* promoter, resulting in pGIS145. The above-named plasmids were introduced into *L. plantarum* NC8 by electroporation (2).

These various fusions were also single-copy integrated into the 3′ end of the $tRNA^{Ser}$ gene by site-specific recombination (10). The fusions were recovered as *Sal*I-*Xho*I fragments from the pGIS105 derivatives and cloned into the unique *Sal*I restriction site of the pMC1 integration vector (10) to generate plasmids pGIS161, pGIS162, pGIS169, and pGIS165 (Fig. 1B). Integration at the $tRNA^{Ser}$ locus was confirmed by Southern blot analysis (data not shown).

Changes in Gus activities upon cold shock. β-Glucuronidase (Gus) activity in cold-shocked cells was measured (26) relative to that in control cultures kept at 27°C. No Gus activity could be detected in the recombinant strain harboring the basal replicon pGIS105, demonstrating the absence of transcriptional activation of *gusA* by vector promoters. The control *cbh-gusA* fusion (pGIS137) showed a 1.4-fold reduction of Gus activity upon cold shock (Fig. 2), as per previous observations that *cbh* mRNA abundance decreases in wild-type cells under similar conditions (9). Cold shock repression of this fusion may therefore result from down-regulation of the *cbh* promoter, instability and/or impaired translation of the *gusA* mRNA, or incorrect folding of the Gus protein at cold temperatures.

All three multicopy *cspL-gusA* fusions (pGIS139, pGIS141, and pGIS142) showed an increase in Gus activity upon cold shock (Fig. 2), demonstrating that the *cspL* promoter is induced by cold. The observed 1.2- to 1.4-fold induction of reporter activity is 1 order of magnitude lower than the 10-fold increase in *cspL* mRNA abundance observed in wild-type cells (9; see below). This may result from posttranscriptional repression of reporter expression at low temperatures, as discussed above for pGIS137, but it may also indicate that cold shock induction of *cspL* mRNA relies in part on posttranscriptional regulatory mechanisms, as will be discussed later.

No Gus activity was detected in any of the chromosomal integrants, as expected from the scarcity of the corresponding mRNAs (see below).

Changes in mRNA abundance upon cold shock. Total RNA was extracted from mid-exponential cells grown at 27°C or from cells cold shocked for 3 h at 8°C, and primer extension was performed as previously described (9). The reliability of using primer extension followed by InstantImager analysis (Packard Instruments, Meriden, Conn.) for quantitating changes in mRNA abundance upon cold shock was first established in the wild-type NC8 strain with a *cspL* primer (5′-CC CGTAATAAAACCAAACCCTT-3′). The 10-fold induction of the *cspL* extension signal in cold-shocked cells observed in the present study (Fig. 3B, graph, lane NC8) is in close agreement with that indicated by previous Northern blot analyses (9). RNAs isolated from the recombinant strains harboring either the multicopy (Fig. 3) or the monocopy (Fig. 4) fusions were subjected to primer extension analyses with a *gusA* primer (gus1, 5′-GGTTGGGGTTTCTACAGGAC-3′), together with the *cspL* primer shown above, so as to allow simultaneous detection of the *gusA* reporter mRNA and the *cspL* mRNA arising from the resident *cspL* gene (see the following section). Experiments were performed at least twice with each fusion, yielding consistent results. Extension products of the expected sizes were obtained with all multicopy fusions, except with pGIS137, where the *gusA* signal was partially masked by nonspecific extension products. The assay was therefore repeated for this construct with the downstream gus0 primer (5′-CCC ACAGGCCGTCGAGTTTT-3′), which gave the extension product shown in Fig. 3.

mRNA abundance increased two- to threefold upon cold shock for the three *cspL-gusA* fusions (Fig. 3A, lanes pGIS141, pGIS142, and pGIS139), and this is in line with the above-reported Gus activities. The levels of induction were similar in the three fusion constructs tested, indicating that neither the secondary structure of the 5′ untranslated *cspL* mRNA region nor the UB and DB play a significant role in *cspL* cold shock induction. The effects of cold shock on mRNA abundances were stronger than those seen on enzymatic activities, suggesting that low temperatures impair β-glucuronidase expression, as discussed previously. These data demonstrate that cold shock induction of the *cspL* gene results, at least in part, from transcriptional activation taking place on a *cis* element(s) located between coordinates −453 and +11 of the promoter region.

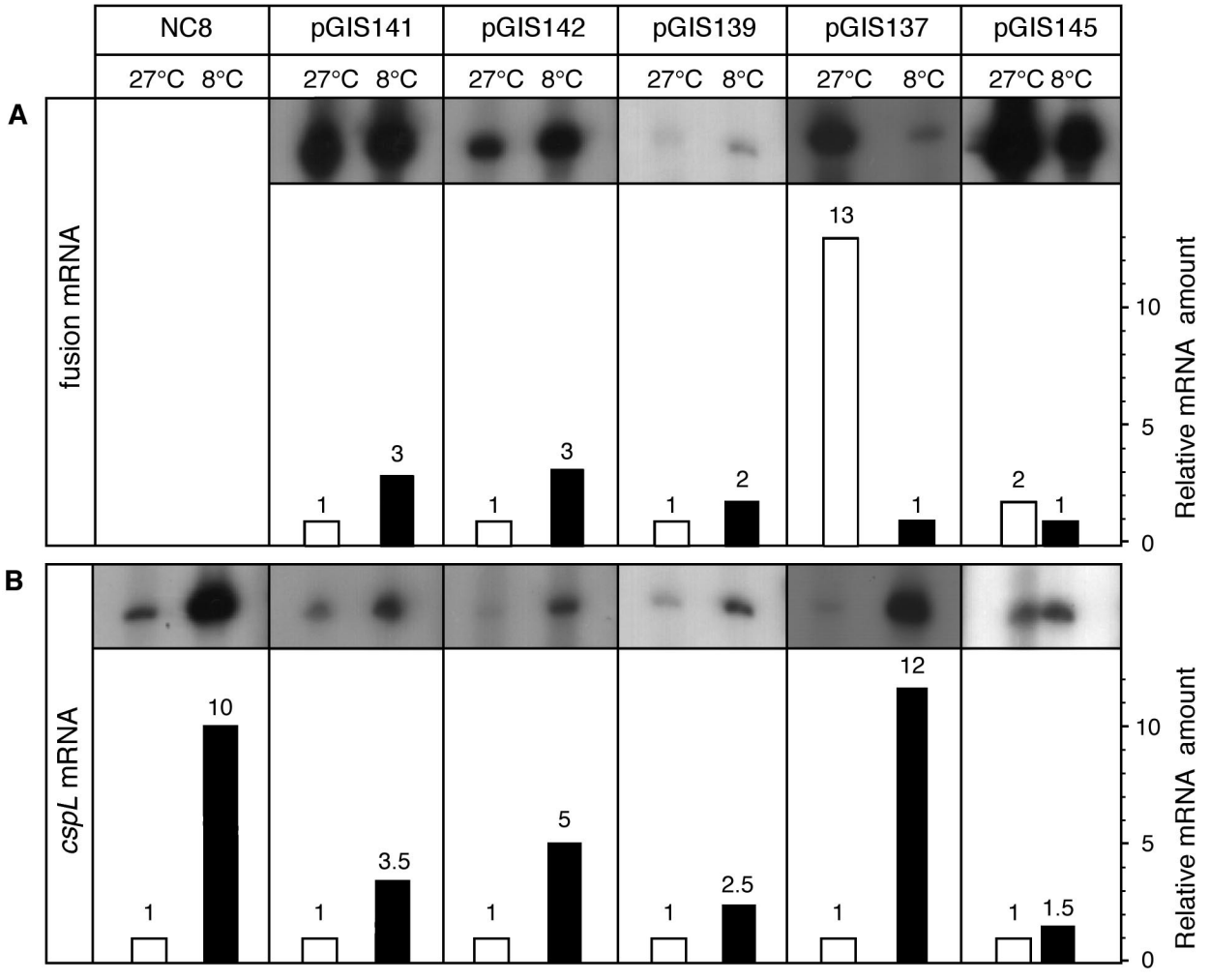

FIG. 3. Primer extension analysis of cold shock induction (multicopy constructs). (A) Primer extension analysis of cold shock induction of the various autoreplicative constructs. Cells were grown at 27°C to an OD (600 nm) of 0.8 and shifted to 8°C for 3 h. Samples for RNA extraction were taken before (lane 1) and after (lane 2) the shift. Graphical presentation of the relative mRNA abundances of the various fusions are shown, with the lowest mRNA level taken as 1. (B) Similar analysis performed on mRNAs transcribed from the resident *cspL* gene in wild-type cells (NC8) and in cells carrying the various multicopy plasmids.

In contrast, a reduction in mRNA abundance was observed upon cold shock, with the *cbh-gusA* and *cbh-cspL* constructs used as controls (Fig. 3A, lanes pGIS137 and pGIS145, respectively), suggesting again that the *cbh* promoter is down-regulated. However, this reduction was 1 order of magnitude lower with the *cbh-cspL* construct than with the reciprocal *cbh-gusA* fusion (compare signals in lanes pGIS145 and pGIS137 in Fig. 3A), which may indicate that *cspL* mRNA is more stable at low temperatures (9). The dramatic (13-fold) reduction in the amount of *gusA* mRNA transcripts arising from the *cbh-gusA* fusion (pGIS137) under cold shock conditions, as opposed to the moderate (3-fold) reduction of the *cbh* mRNA abundance reported in cold-shocked wild-type cells (9), suggests that the 2- to 3-fold induction levels seen with the three *cspL-gusA* fusions (pGIS141, pGIS142, and pGIS139) were largely underestimated.

Monocopy fusions yielded extension products of lower intensities than those seen with their multicopy counterparts, as expected because of the lower gene dosage (Fig. 4, upper panels). Nevertheless, *cspL-gusA* transcriptional fusions maintained enhanced primer extension signals under cold shock conditions (Fig. 4, upper panels, lanes pGIS162 and pGIS169). As before, *cspL* mRNA arising from the the reciprocal *cbh-cspL* transcriptional fusion was less abundant in cold-shocked cells (Fig. 4, upper panel, lane pGIS165). Data obtained with the monocopy fusions support the observations made with the autoreplicative constructs.

Multicopy fusions quench cold shock induction of the resident *cspL* gene. Cold shock induction of the *cspL* mRNA transcribed from the resident *cspL* gene was strongly reduced in cells harboring multiple copies of the *cspL-gusA* fusions (Fig. 3B, graphs, lanes pGIS141, pGIS142, and pGIS139). This quenching effect was not seen with the monocopy constructs (Fig. 4, lower panels, lanes pGIS161, pGIS162, and pGIS169), as cold shock induction of the resident *cspL* gene was still in the wild-type 10-fold range (data not shown). This gene dosage effect could not be ascribed to the *gusA* moiety of the fusions, as cold shock induction of the resident *cspL* gene remained in

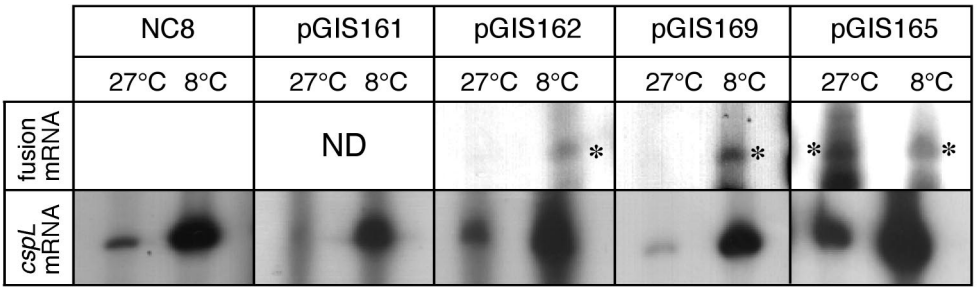

FIG. 4. Primer extension analysis of cold shock induction (integrated constructs). (Upper panel) Primer extension analysis of cold shock induction of the various single-copy constructs integrated in the $tRNA^{Ser}$ gene. Cells were grown at 27°C to an OD (600 nm) of 0.8 and shifted to 8°C for 3 h. Samples for RNA extraction were taken before (lane 1) and after (lane 2) the shift. ND, not detected. The asterisks indicate the position of the extension products showing the expected size. (Lower panel) Similar analysis performed on mRNAs transcribed from the resident *cspL* gene in wild-type cells (NC8) and in cells carrying the various single-copy constructs.

the wild-type range in cells harboring the multicopy *cbh-gusA* construct (Fig. 3, lanes pGIS137). A likely explanation for the quenching effect is that cold-specific transcriptional activators may be titrated out by multiple copies of their targets when provided in *trans.* DNA footprinting and gel mobility shift analyses that were performed with the *E. coli cspA* promoter region demonstrated that a segment upstream of the core promoter is specifically protected upon cold shock, due to the binding of a yet-unknown factor synthetized de novo at cold temperatures (30).

Quenching of resident *cspL* cold shock induction was also observed in cells harboring the reciprocal *cbh-cspL* fusion (Fig. 3B, lane pGIS145). The possibility of titration of *trans*-acting transcriptional activators can be excluded in this situation, as multiple copies of the *cbh* promoter segment present in the pGIS137 recombinant strain did not bring about such an effect. It is conceivable that the CspL protein itself, which is likely to be more abundant at both 27 and 8°C due to the presence of multiple copies of the pGIS145 plasmid, may act as a transcriptional repressor. In *E. coli*, cold shock induction of *cspA* was shown to be inversely proportional to CspA concentration (6), and negative autoregulation is thought to be important for ensuring the transience of *cspA* induction (12). Alternatively, we cannot rule out the possibility that multiple copies of the putative OrfX protein could be responsible for the pGIS145-related quenching effect.

Concluding remarks. By fusing *cspL* promoter fragments to the *gusA* reporter, it was possible to demonstrate that a regulatory *cis* element(s) responsible for cold shock induction is present in the segment of the *cspL* promoter region extending from −453 to +11. Replacement of this region with the promoter from the non-cold shock *cbh* gene led to cold shock repression. Multiple copies of the *cspL* promoter fragments were shown to quench cold shock induction of the resident *cspL* gene, indicating that a titratable *trans*-acting inducer(s) is likely to be involved. These data indicate that *cspL* induction in cold-shocked *L. plantarum* cells is mediated, at least in part, by transcriptional regulatory mechanisms. The fact that the *cspL* gene is transcribed from a noncanonical promoter, devoid of a −35 box but containing an extended −10 box, supports this claim (8). Recent reports on cold shock induction in *Synechocystis* sp. strain PCC 6803 and *B. subtilis* clearly demonstrate the involvement of a two-component signal transduction system (1, 29). Whether this would hold true for the *cspL* gene in *L. plantarum* remains to be investigated.

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