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# Cloning and Characterization of *cspL* and *cspP*, Two Cold-Inducible Genes from *Lactobacillus plantarum*

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**Two cold shock genes, *cspL* and *cspP*, have been cloned from two *Lactobacillus plantarum* strains. These genes, which are nonallelic, were present in all strains tested. The genes encode 66-amino-acid polypeptides related to each other and to the cold shock Csp family. Transcription of *cspP* rendered a single mRNA, while two *cspL* mRNAs were found with common 5′ ends. The amounts of these transcripts increased moderately upon exposure of the cultures to cold.**

Living cells exposed to a temperature downshift induce adaptive responses characterized by the arrest or decrease in the synthesis of most proteins and the incrementation of some others (the cold shock proteins) (10). In *Escherichia coli*, a small acidic protein, CspA, which is almost undetectable at 37°C becomes transiently overexpressed, to about 200-fold, upon transfer of the cultures to 10°C (4, 8). Six structural homologs (*cspB* to *cspG*) have been identified (12, 14, 23), and their products constitute the Csp family of proteins in *E. coli*. Similar families have been detected in *Pseudomonas fragi* (7), *Bacillus subtilis* (6, 21), and *Bacillus cereus* (13). Only some components of these families increase in concentration after cold shock. However, all contain two well-conserved RNA-binding sequences (motifs RNP1 and RNP2) characteristic of eukaryotic Y-box proteins (22), and both CspA of *E. coli* and CspB of *B. subtilis* have been shown to recognize the so-called Y-box sequence ATTGG (5, 9, 11). CspA-like proteins are thought to be involved in regulation of gene expression, possibly acting as RNA chaperones, thereby allowing macromolecular synthesis and growth to resume or be sustained at low temperature (9, 11, 23).

Lactic acid bacteria are widely used in dairy and other food fermentations (3); they commonly suffer diverse stress challenges during manufacturing and ripening, including low temperatures. *Lactobacillus plantarum* is one of the most widespread lactic acid bacteria in the environment and is largely used for the production of fermented products of animal and vegetal origin (20). In this report, we describe two genes, present in a series of *L. plantarum* strains, that map in different loci and whose products are members of the Csp family of cold shock proteins.

**Cloning and sequencing of *cspL* and *cspP*.** Molecular biology techniques were performed essentially as described by Sambrook et al. (16). In the course of independent experiments, two open reading frames (ORFs), whose deduced amino acid sequences showed extensive homology to the cold shock protein CspA of *E. coli*, were obtained. The *cspL* gene was cloned from the total DNA of *L. plantarum* Lp80, a silage inoculant strain, while *cspP* was obtained from the total DNA of *L. plantarum* C3.8, a strain isolated from a dairy environment. The nucleotide sequences of both genomic fragments were obtained by the dideoxy method of Sanger et al. (17) and are presented in Fig. 1. Both ORFs are 198 nucleotides long and are preceded by ribosome binding sites complementary to the 3′ end of *L. plantarum* 16S rRNA (1). The deduced protein sequences span 66 amino acids (Fig. 1), have predicted molecular weights of 7,299 (CspL) and 7,257 (CspP), and have calculated pIs of 4.31 (CspL) and 4.34 (CspP). The coding regions of *cspL* and *cspP* differ by only 37 nucleotides (nt), which corresponds to an overall homology of 81.4%. Most of the nt changes (27 of 37) occur at the third position of the coding triplets, which reduces the amino acid substitutions to 8 of 66 (3 are nonconservative). However, outside the ORFs, the nt sequences differ markedly (Fig. 1). CspL and CspP are closely related to the members of the Csp family (67 and 65% identity with CspA, respectively) and display the expected RNP1 and RPN2 signatures of RNA-binding proteins.

The transcription starting points were established by primer extension (18). They were located at adenosine residues 89 and 71 nt upstream of the translation start codons of *cspL* and *cspP*, respectively. They are preceded by −35 and −10 consensus sequences (Fig. 1). In the case of *cspL*, additional contact with the RNA polymerase may be provided by two TG dinucleotides present immediately upstream of the −10 sequence (15). Y-box motifs were observed in the vicinity of *cspL* and *cspP* promoters, suggesting that transcriptional regulation may play a role in their expression.

Downstream of *cspL*, two sets of inverted repeats were observed at about 300 nt ($\Delta G = -13$ kcal/mol) and 750 nt ($\Delta G = -30.2$ kcal/mol) from the +1 site. An inverted repeat ($\Delta G = -18$ kcal/mol) was found immediately after *cspP*. The *cspP* and the proximal *cspL* inverted repeats are followed by several T's, indicating that they probably act as rho-independent transcriptional terminators (see below).

***cspL* and *cspP* are nonallelic.** To determine whether *cspL* and *cspP* are polymorphic alleles or nonallelic loci encoding related proteins, the total chromosomal DNA from *L. plantarum* Lp80 and C3.8, but also that from ATCC strains 14917, 8014, and 10241 and from LL 441, was subjected to pulsed-field gel electrophoresis and hybridized with *cspL* and *cspP* probes under high-stringency conditions. All six strains contained *cspP* in a 25-kbp *Apa*I fragment, distinct from the polymorphic *Apa*I fragment hybridizing to the *cspL* probe (30 kbp

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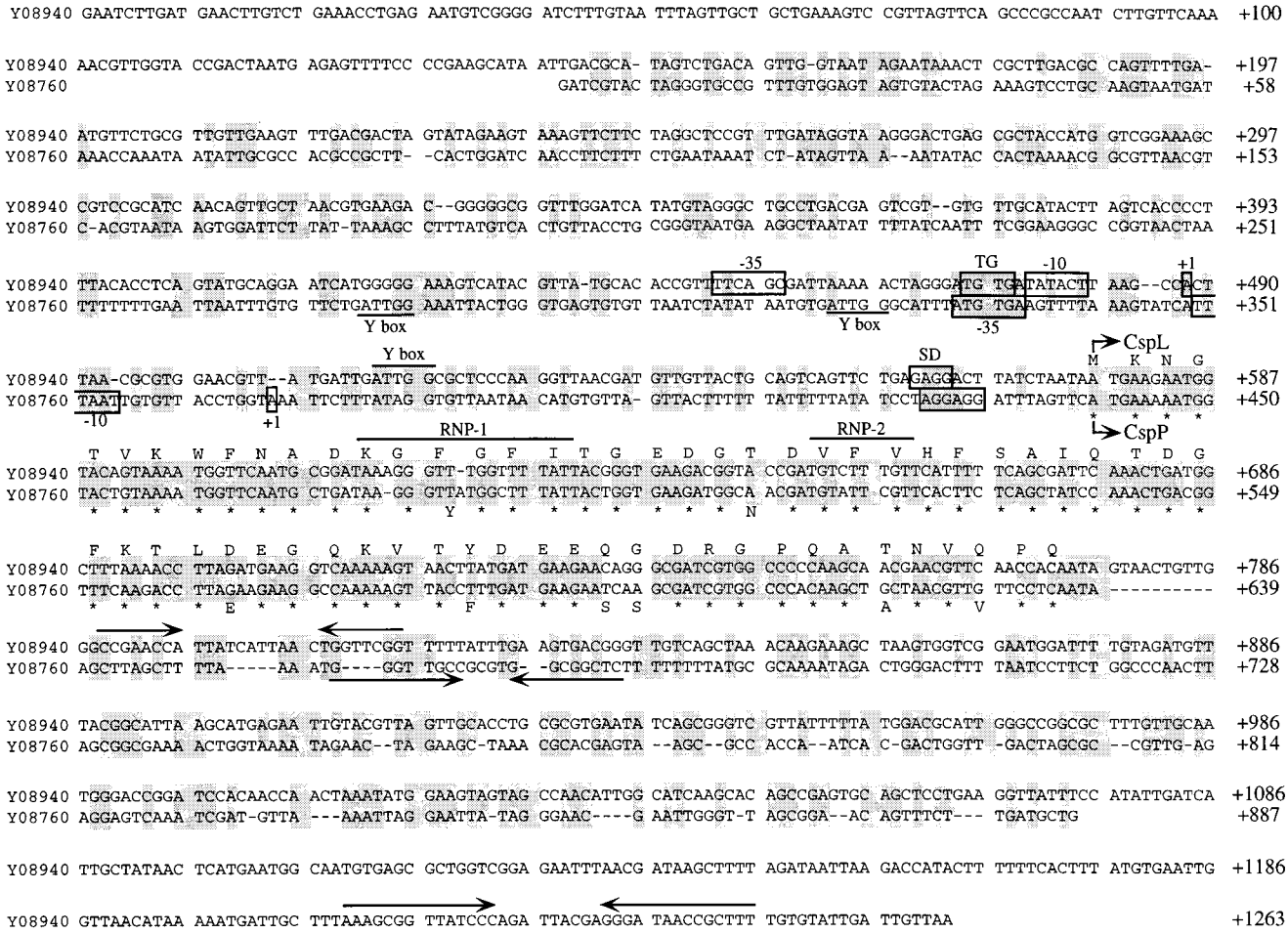


FIG. 1. Nucleotide and deduced CspL and CspP amino acid sequences. Identical nucleotides are shaded, and gaps are indicates by dashes; identical amino acids are indicated by asterisks. The putative −35 and −10 boxes of the promoters are indicated. The twin TG nucleotides preceding the −10 box in the *cspL* promoter are also boxed and labeled. The transcription starts are boxed and are indicated by +1. The RNP1 and RNP2 signatures are overlined, and the Y boxes are indicated above (*cspL*) or under (*cspP*) the sequence. The putative transcriptional terminators are indicated by facing arrows. The numbers on the right are the coordinates of the nucleotides as used in the GenBank database.

in Lp80, LL 441, ATCC 14917, and ATCC 8014; 40 kbp in C3.8 and ATCC 10241) (Fig. 2). This demonstrates that *cspL* and *cspP* belong to two distinct loci and, very likely, that they are common elements of the genetic program of *L. plantarum.*

**Effect of cold shock on *cspL* and *cspP* mRNA abundance.** Northern blot analyses were undertaken to determine the amounts of *cspL* and *cspP* mRNAs both at 37°C and after a cold shock to 10°C. The probes were derived from different parts of the divergent 5′ regions of *cspL* and *cspP*, with runoff extension products from specific oligonucleotide primers being used to ensure strict specificity of hybridization. Surprisingly, two distinct transcripts (330 and 760 nt) were detected in the case of *cspL*, whereas *cspP* gave a single mRNA of 330 nt (Fig. 3). Since we detected a single transcription start site in both cases, the most likely explanation for the occurrence of two *cspL* mRNAs is the involvement of two alternative transcription terminators, as suggested by the presence of two inverted repeats following the *cspL* ORF (Fig. 1). This was confirmed by Northern hybridization to a set of probes spanning various regions of the *cspL* locus. These experiments demonstrated that the 760-nt mRNA shared its 5′ moiety with the 330-nt transcript but extended about 400 nt further down to the region where the second putative transcriptional terminator was found (data not shown).

Shifting *L. plantarum* from 37 to 10°C resulted in a moderate increase (three- to fivefold) in *cspL* and *cspP* mRNAs which was already observed after 1 h at 10°C (Fig. 3). Interestingly, the two *cspL* transcripts displayed the same time course of incrementation. No drastic change in mRNA abundance was seen afterwards. When cells were shifted back to 37°C for 1 h, the intensities of all Northern signals declined to levels similar to those seen before the cold stress.

It has been postulated that the enormous incrementation of CspA produced upon transfer of *E. coli* cells to low temperatures was the result of gene induction (4, 19). However, Fang et al. (2) have shown that expression of *cspA* is constitutive but that the mRNA produced at 37°C is extremely unstable. It then becomes possible that *cspL* and *cspP* mRNAs are produced constitutively as well but that they are quite stable even at 37°C. Transfer of *L. plantarum* Lp80 cultures from this temperature to 10°C resulted in a gradual increase of the doubling time (from 50 to 1,400 min [data not shown]) rather than in a lag period after which the growth resumed, as is observed in *E. coli* (8). It might be possible, then, that growth does not be-

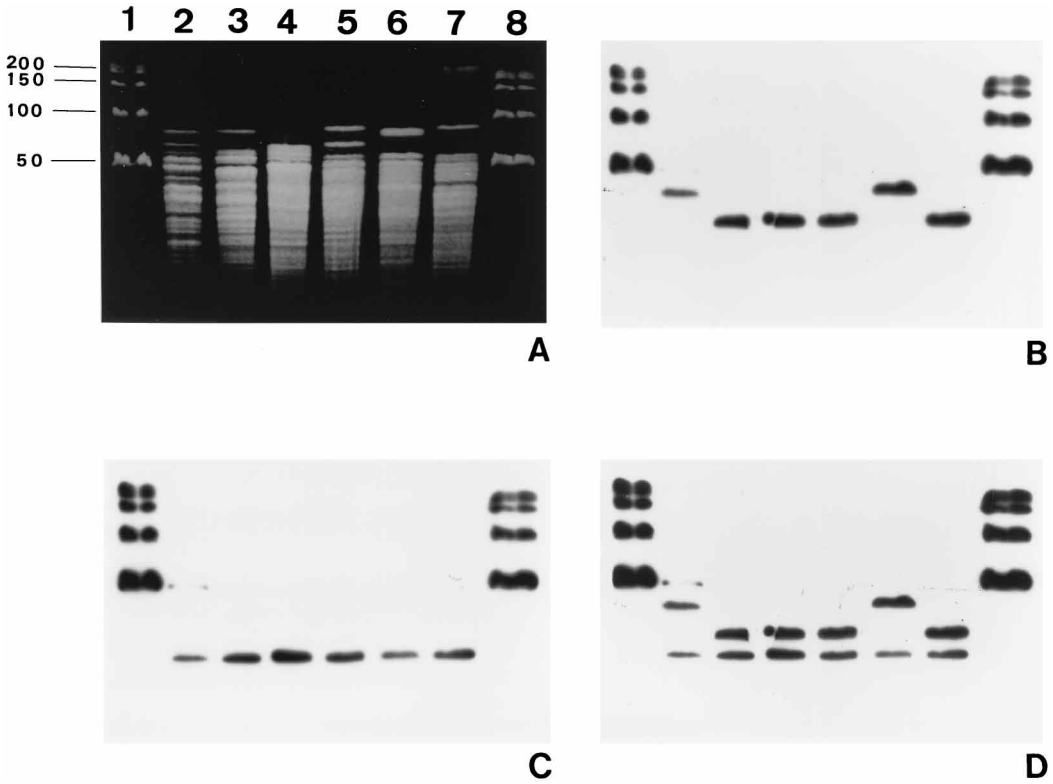


FIG. 2. Southern blot analysis of *csp* sequences present in the DNAs of different *L. plantarum* strains. The DNA was cut with *Apa*I and subjected to pulsed-field electrophoresis (A). DNA probes specific for *cspL* (B) and *cspP* (C) were used. The photograph labeled (D) is the composite picture resulting from the superposition of the two autoradiograms shown in panels B and C. Lanes: 1 and 8, λ DNA ladder; 2, *L. plantarum* C3.8; 3, Lp80; 4, LL 441; 5, ATCC 8014; 6, ATCC 10241; 7, ATCC 14917.

come arrested in the lactobacilli because CspL and/or CspP is already present at the time of the downshift, while in *E. coli*, stabilization of *cspA* mRNA would be necessary before growth could be reinitiated.

The understanding of the cold shock response in lactic acid bacteria is of outmost importance to the food industry, since cold is the most popular conservation system for fermented foods. Consequently, knowledge of the processes that are trig-

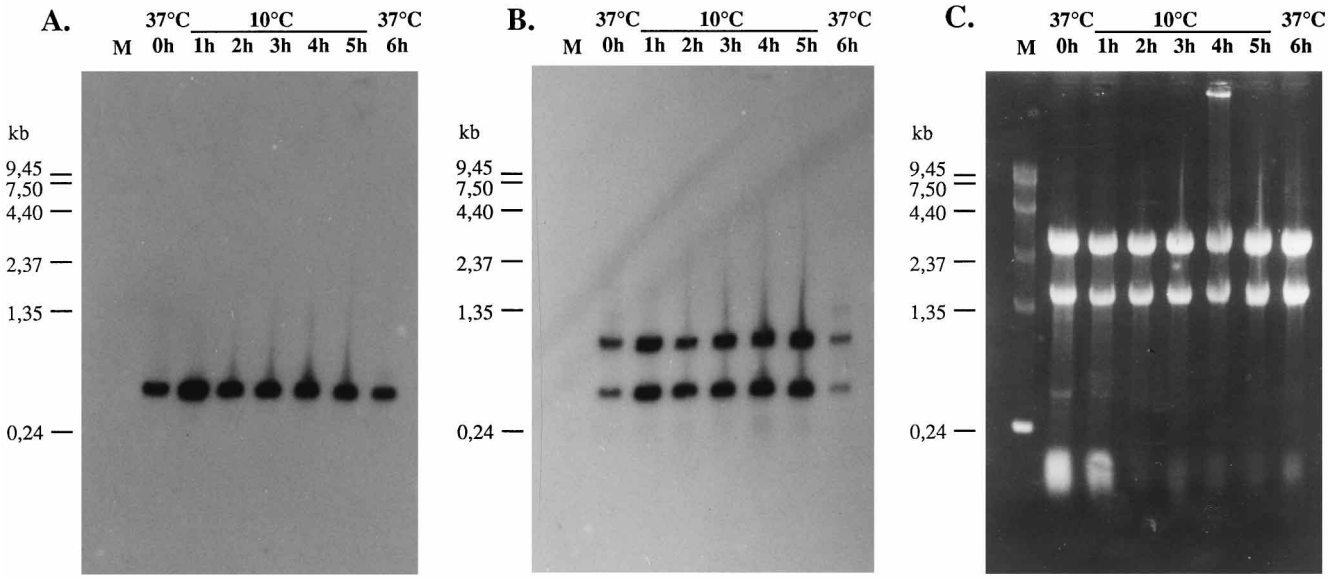


FIG. 3. Northern blot analysis of *cspP* (A) and *cspL* (B) mRNAs. The same blot has been hybridized successively with the *cspP* and *cspL* probes. (C) A picture of the ethidium bromide-stained agarose gel before blotting. RNA ladder (sizes [in kilobase pairs] are indicated on the left) is shown in lane M. Samples have been taken from cultures grown at 37°C until mid-exponential phase (lane 37°C, 0 h) and then shifted to 10°C for 1 to 5 h (as indicated on the top of the corresponding lanes); a culture shifted back to 37°C for 1 h following the 5-h cold shock (lane 37°C, 6 h) has also been examined.

gered under these circumstances might contribute to maintenance of a live microbiota which is essential to generate the organoleptic properties of the products and their extended shelf lives. Additionally, the development of low-temperature-inducible systems would facilitate the regulation of homologous and heterologous gene expression, which could be of help in the construction of genetically improved strains for industrial applications.

**Nucleotide sequence accession numbers.** The sequences of *cspL* and *cspP* have been submitted to the GenBank database and given the accession numbers Y08940 and Y08760, respectively.

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