Clustered organization and transcriptional analysis of a family of five csp genes of Lactococcus lactis MG1363

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A family of genes encoding cold-shock proteins, named cspA, cspB, cspC, cspD and cspE, was cloned and sequenced from Lactococcus lactis MG1363. The genes cspA and cspB and the genes cspC and cspD are located in tandem repeats, an organization of csp genes that has never been encountered before. The five genes encode small (7 < 1–7 < 6 kDa) proteins with high mutual sequence identities (up to 85%) and high identities (about 45–65%) with the major cold-shock proteins from Escherichia coli (CspA) and Bacillus subtilis (CspB). Northern-blot analysis revealed single transcripts of about 300 nucleotides for each csp gene and showed that cspA, cspB, cspC and cspD mRNA levels were strongly increased upon cold shock to 10 °C (about 10-, 40-, 10- and 30-fold compared to 30 °C, respectively), whereas the cspE mRNA level was not increased. The expression of the cold-induced csp genes was highest in the 6–8 h lag phase after cold shock. A differential expression in time, in which cspA and cspC were maximally expressed at 2 h and cspB and cspD at 4 h after cold shock, was observed. The 35 and 10 regions of the five promoters were identified and transcriptional start sites were mapped in each case by primer extension at different temperatures which confirmed that regulation takes place at the transcriptional level. Significant differences were observed between the 5′-untranslated leader regions of the four cold-induced csp genes and the corresponding region of the non-cold-induced cspE gene.

Keywords: csp genes, low-temperature adaptation, transcription, Lactococcus lactis

INTRODUCTION

Lactococcus lactis plays an important role in many dairy fermentations. During processing and ripening of fermented dairy products these bacteria have to deal with different environmental stresses, such as low pH, high salt concentrations and temperature extremes (Rallu et al., 1996). Several stress responses of L. lactis have been studied and stress-induced genes could be identified (Rallu et al., 1996; Sanders et al., 1995; Van Asseldonk et al., 1993). However, low temperature stress has received less attention. Cold stress might be important for the survival of starter cultures after frozen storage and for fermentations taking place at low temperatures.

The response to cold shock has been extensively studied in Escherichia coli and has been shown to result in the induction of a specific set of 14 proteins. These proteins play a role in various cellular processes and include, among others, NusA, RecA, H-NS, GyrA, polynucleotide phosphorylase and CspA (further referred to as CspA E) (Jones et al., 1987, 1996; Jones & Inouye, 1994, 1996). Maximal induction after cold shock was detected for CspA E, which is transiently overexpressed (200-fold induction) and then represents 13% of the newly synthesized proteins (Goldstein et al., 1990; Jones et al., 1987). A highly similar protein, CspB (further referred to as CspB B), has been described in Bacillus subtilis (Willimsky et al., 1992).

CspA E (Goldstein et al., 1990) and CspB B (Willimsky et
al., 1992) are small proteins with a molecular mass of 7-4 kDa and a low isoelectric point (pI 5.9 and 4.3, respectively). CspA\textsuperscript{E} acts as a transcriptional activator of at least two other genes encoding the cold-induced proteins GyrA (Jones et al., 1992) and H-NS (LaTeana et al., 1991), both involved in DNA supercoiling. The crystal structures of CspA\textsuperscript{E} and CspB\textsuperscript{E} have been resolved and both proteins are able to bind specifically to single-stranded DNA containing a Y-box motif (ATTTG) or its complementary sequence (CCAT). The synthesis of the major CSPs is still unclear but it seems to be regulated by at least three of the nine genes of respectively nine, three and six members have been identified (Lee et al., 1996; Nakashima et al., 1996; Yamanaka & Dunn, 1997) and two in Lactobacillus lactis (Chapot-Boerrigter et al., 1997). CspB\textsuperscript{E} are also considered to be RNA-binding proteins because they both possess highly conserved RNA-binding motifs, i.e. RNP-1 (ribonucleoprotein) and a rudimentary RNP-2 motif (Jones & Inouye, 1994; Schindelin et al., 1993), and it appears that CspA\textsuperscript{E} can act as an RNA chaperone (Jiang et al., 1997). For CspB\textsuperscript{E} a function as an anti-freeze protein has been suggested because a lower survival has been observed after freezing of cells in which the cspB gene was disrupted (Willimsky et al., 1992). The regulation of the synthesis of the major CSPs is still unclear but it seems to take place at the level of both transcription (Lee et al., 1994) and translation (Brandi et al., 1996). Recently, it was shown that the absent presence of CspA\textsuperscript{E} after cold shock is due to increased stability of its mRNA at low temperature (Fang et al., 1997).

In E. coli, B. subtilis and Bacillus cereus, families of csp genes of respectively nine, three and six members have been found (Graumann et al., 1996; Lee et al., 1994; Mayr et al., 1996; Nakashima et al., 1996; Yamanaka & Inouye, 1997). In E. coli at least three of the nine identified csp genes are cold induced (Lee et al., 1994; Nakashima et al., 1996). The csp genes of E. coli appeared to be scattered on the chromosome (Lee et al., 1994) and also for other bacteria only non-clustered csp genes have been reported (Graumann et al., 1996; Mayr et al., 1996). A recent study by Graumann et al. (1997) using a triple csp deletion mutant of B. subtilis revealed that CSPs are essential for cellular growth and for efficient protein synthesis at both optimal and low temperatures.

The cold-shock response of L. lactis IL1403 was studied by Panoff et al. (1994), revealing that 12 proteins were overexpressed after cold shock. Recently, one cold-induced csp gene was identified in L. lactis (Chapot-Chartier et al., 1997; Kim & Dunn, 1997) and two in another lactic acid bacterium, Lactobacillus plantarum (Mayo et al., 1997). In this study, a family of five genes encoding CSPs of L. lactis MG1636 was characterized. A clustered organization of csp genes has been observed for the first time: two tandems of two csp genes. Transcriptional analysis of the L. lactis csp genes revealed cold induction for four of these genes and a differential expression of the respective genes during the adaptation phase after cold shock.

**METHODS**

**Bacterial strains and growth conditions.** E. coli MC1061 (Casadaban & Cohen, 1980) was used as a host strain in cloning experiments and was grown in Tryptone Yeast (TY) medium with aeration at 37°C (Sambrook et al., 1989). Antibiotics were used in the following concentrations: ampicillin 50 μg ml\textsuperscript{-1}; chloramphenicol 10 μg ml\textsuperscript{-1}. L. lactis MG1636, a plasmid-free and prophage-cured derivative of L. lactis NCDO 712 (Gasson, 1983), was grown in M17 broth (Difco) supplemented with 0.5% (w/v) glucose at 30°C without aeration. Growth curves of L. lactis were obtained by measuring the OD\textsubscript{600} at various time points by diluting the sample fourfold in M17 broth.

**DNA techniques and sequencing.** Chromosomal DNA of L. lactis was isolated as described previously (Vos et al., 1989). L. lactis cells were transformed by electroporation (Wells et al., 1993). E. coli cells were transformed by the CaCl\textsubscript{2} procedure and plasmid isolations were carried out according to established procedures (Sambrook et al., 1989). E. coli plasmid DNA was isolated on a large scale using Qiagen columns. Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were purchased from Gibco-BRL Life Technologies, New England Biolabs or Promega and used as recommended by the manufacturers. Cloning procedures, radiolabelling of DNA fragments, agarose gel electrophoresis and Southern-blot hybridizations were performed according to established procedures (Sambrook et al., 1989). DNA fragments were isolated from agarose gels by using the GlassMAX DNA Isolation Matrix System (BRL Life Technologies). PCR was carried out according to conditions described previously (Kuipers et al., 1991). Nucleotide sequences of plasmid DNA were analysed with an ALF automatic sequencer (Pharmacia Biotech) in combination with an AutoRead sequencing kit (Pharmacia Biotech) with fluorescein-labelled primers. Oligonucleotides used as primers in sequencing reactions, primer extension experiments and PCR, were purchased from Pharmacia Biotech.

**Cloning of csp genes.** PCR with primers based on homologous regions of CspA\textsuperscript{E} (Goldstein et al., 1990) and CspB\textsuperscript{E} (Willimsky et al., 1992; Table 2) with chromosomal DNA of L. lactis MG1636 as a template resulted in the amplification of a fragment of about 200 bp (PCR1) with primers 1 and 2 (both containing an EcoRI site). When primers 3 and 4 were used, a fragment of about 500 bp (PCR2) was amplified. The fragments were cloned in pUC18 (pUC18PCR1) and pGEM-T (purchased from Promega; pGEM-TPCR2), respectively. The fragments were sequenced and appeared to contain parts of putative csp genes. By use of PCR1 as a probe in Southern hybridization, four hybridizing fragments (HindIII chromosomal DNA digest) were detected (Fig. 1a). The first hybridizing band was cloned as a 3.3 kb EcoRI–HpaII fragment into the EcoRI and AccI sites (after calf intestine alkaline phosphatase treatment) of pUC19, resulting in pUC19Cspa/A (Table 1). The second hybridizing band was cloned as a HindIII–BglII fragment (2.1 kb) in the HindIII- and the BamHI-sites of pUC19 (pUC19Cspa/D; Table 1) and sequenced by primer walking. Attempts to clone the third hybridizing fragment either as a 3.5 kb HindIII fragment or as a 4.5 kb EcoRI–SacI fragment in both a high-copy (pUC19) and a low-copy vector (pNZ284, a pACYC derivative; Van Alen-Boerrieter et al., 1991) failed. The fourth hybridizing
Table 1. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>pUC18PCR1</td>
<td>pUC18 containing a PCR fragment (PCR1) of about 200 bp obtained with primers 1 and 2</td>
</tr>
<tr>
<td>pGEM-TPCR2</td>
<td>pGEM-T containing a PCR fragment (PCR2) of about 550 bp obtained with primers 3 and 4</td>
</tr>
<tr>
<td>pUC19CspA/B</td>
<td>pUC19 containing a 3.3 kb EcoRI–HpaII fragment including cspA/cspB</td>
</tr>
<tr>
<td>pUC19CspC/D</td>
<td>pUC19 containing a 2.1 kb HindIII–BglII fragment including cspC/cspD</td>
</tr>
<tr>
<td>pUC19CspE</td>
<td>pUC19 containing a 1.1 kb HindIII–PstI fragment including cspE and its upstream region</td>
</tr>
<tr>
<td>pLEX</td>
<td>pUC18 containing a 0.95 kb PCR fragment which is cloned in the Sau3AI and HincII sites including cspE and its downstream region</td>
</tr>
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</table>

Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>CGGAATTCCGGIA(A/T)TGTTTTA</td>
</tr>
<tr>
<td>Primer 2</td>
<td>CGGAATTCTGTIAC(A/G)TGTTTTA</td>
</tr>
<tr>
<td>Primer 3</td>
<td>GGNANNGTNAA(A/G)TGTTTTA</td>
</tr>
<tr>
<td>Primer 4</td>
<td>(G/A/T)AT(A/G)AANCC(A/G)AANCC(C/T)TT</td>
</tr>
<tr>
<td>pAMILEX</td>
<td>GAAACCATTAGTGTCTGTGAGC</td>
</tr>
<tr>
<td>pAMI4</td>
<td>TGAACGGGGCCCTAACC</td>
</tr>
<tr>
<td>PEcspA</td>
<td>GCCATACCGCTTGTCCATATTG</td>
</tr>
<tr>
<td>PEcspB</td>
<td>GCCAAAATCCCCCTTATCTGGAG</td>
</tr>
<tr>
<td>PEcspC</td>
<td>CTTGGCATATCATCTTGCCAG</td>
</tr>
<tr>
<td>PEcspD</td>
<td>ACCAATCCTTTAGTACGGC</td>
</tr>
<tr>
<td>PEcspE</td>
<td>TGTGCGAGAACGTCGTTT</td>
</tr>
</tbody>
</table>

band was cloned as a HindIII–PstI fragment (1.1 kb) in the HindIII and PstI sites of pUC19 (pUC19CspE; Table 1). On this fragment a putative csp gene was located; to obtain its downstream region the following inverse PCR strategy was used. Chromosomal DNA was digested with HpaII and self-ligated. PCR was performed with this template and with pAMILEX and pAMI4 (Table 2) as primers. A 950 bp fragment was obtained which was cloned in the blunt HindIII and the BamHI site (compatible with Sau3AI) of pUC18 after digestion with Sau3AI (resulting in pLEX; Table 1).

DNA and deduced protein sequence analysis. Computer analysis of DNA sequences and the deduced amino acid sequences was performed with the program PC/Gene (version 6.70; IntelliGenetics) and Clone (Version 4.0; Clone Manager). The EMBL/GenBank and SWISS-PROT/PIR databases were used to search for amino acid sequence similarities.

RNA techniques and primer extension experiments. RNA isolation, Northern blotting and subsequent hybridization with radiolabelled probes was performed as described previously (Kuipers et al., 1993). For cold-shock experiments, cultures were grown at 30 °C to mid-exponential phase, after which they were spun down by centrifugation and resuspended in medium precooled to 10 °C. After exposure to 10 °C for various time periods (0, 0.5, 1, 2, 4 and 24 h) total RNA was isolated. The same oligonucleotides were used as probes in Northern blotting and as primers in primer extension experiments (PEcspA to PEcspE; Table 2). Quantification of the csp transcripts in Northern blotting was performed using the Dynamics Phosphor Imaging System. Cross-hybridization of the probes to the other csp genes was checked using Southern blotting, quantified with the same system. As a control for the RNA quantity the usp45 gene, which is constitutively expressed (Van Asseldonk et al., 1990), was used and correction factors were calculated by using the Phosphor Imaging System. Primer extension experiments on the csp genes were carried out as described previously (Kuipers et al., 1993), with the same RNA samples as used for Northern blotting. The resulting cDNA was subjected to electrophoresis alongside nucleotide sequencing ladders generated with the same primers using the deoxy chain-termination method (Sanger et al., 1977) and [α-32P]dATP as radiolabel.

RESULTS

Cloning of genes encoding putative CSPs

Using different primers based on the homologous sequences of CspA and CspB (Table 2) two PCR products of about 200 bp (PCR1) and 550 (PCR2) bp
Fig. 1. (a) Southern hybridization, with PCR1 used as a probe, of chromosomal DNA of *L. lactis* MG1363 digested with *Hind*III (lane 1), *Hind*III and *Eco*RI (lane 2), *Hind*III and *Pst*I (lane 3), *Hind*III and *Bgl*II (lane 4), and *Hind*III and *Bam*HI (lane 5). Marker sizes are indicated on the right, and arrows indicate the hybridizing *Hind*III fragments. (b) Organization and nomenclature of the *csp* genes found in *L. lactis* MG1363. The large arrows indicate the ORFs, the smaller arrows indicate the transcription starts and the major terminators are indicated by a hairpin structure.

were amplified with *L. lactis* MG1363 chromosomal DNA as a template. After cloning and sequencing it appeared that these PCR products contained parts of genes homologous to the major *csp* genes. In a Southern-blotting experiment, using PCR1 as a probe, four hybridizing fragments were detected in different digests of *L. lactis* chromosomal DNA (Fig. 1a). Two *csp* genes, named *cspA* and *cspB*, are located on an *Eco*RI–*Hpa*II fragment (cloned in pUC19 resulting in pUC19CspA/B). Another fragment (cloned in pUC19 resulting in pUC19CspC/D) also contained two *csp* genes (named *cspC* and *cspD*), organized in a tandem repeat. A single *csp* gene, named *cspE* (cloned in pUC19 resulting in pUC19CspE), is located on a *Hind*III–*Pst*I fragment and its downstream region was cloned by an inverse PCR strategy (pLEX). The organization of the different *csp* genes is shown in Fig. 1(b). In Southern hybridization with PCR2 as a probe only two fragments, identical to fragments that hybridized with PCR1 as a probe, could be detected (data not shown). When the different *csp* genes were used as probes in Southern hybridization (different chromosomal-DNA digests) no extra hybridizing bands could be detected compared to the four bands obtained when using PCR1 as a probe. In an *Eco*RI digest all *csp* homologues were located on only two fragments, indicating a clustered organization on the *L. lactis* MG1363 chromosome. No hybridization was observed using plasmid DNA (isolated from several *L. lactis* strains) and PCR1 as a probe, indicating that these *csp* genes are chromosomally encoded and that no homologues are located on plasmids (data not shown).

A remarkably high nucleotide sequence identity was found for the two tandem repeats: 79% over 800 nt containing both ORFs. In the tandem repeats the first ORFs (*cspA* and *cspC*) and the second ORFs (*cspB* and *cspD*) are highly similar (81% and 82% identity, respectively). Also the spacing between the two adjacent ORFs is similar (268 nt for *cspA* and *cspB*, 277 nt for *cspC* and *cspD*).

The five CSPs of *L. lactis* have a mutual identity of 52–85% at the amino acid level. The identity to the major CSPs, CspA<sup>E</sup> and CspB<sup>B</sup>, is about 45–65% and is lowest for CspA and CspC (Fig. 2, Table 3). The calculated molecular masses of the *L. lactis* CSPs range from 7.1 kDa for CspE to 7.6 kDa for CspA and CspC (Table 3). CspA and CspC have an unusually high pI (approximately 9) compared to other CSPs (approximately 5).

**Cold induction of *csp* genes**

Cells of *L. lactis* were cultured to the mid-exponential phase at 30 °C, after which they were subjected to a cold shock by resuspending in precooled GM17 medium (10 °C). The growth characteristics of the cold-shocked
Table 3. Identity (%), size of ORF in amino acids, molecular mass and pl of the *L. lactis* CSPs, CspA and CspB

<table>
<thead>
<tr>
<th></th>
<th>CspA</th>
<th>CspB</th>
<th>CspC</th>
<th>CspD</th>
<th>CspE</th>
<th>CspA&lt;sup&gt;e&lt;/sup&gt;</th>
<th>CspB&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tr>
<td>Identity (%)</td>
<td>62</td>
<td>76</td>
<td>59</td>
<td>60</td>
<td>45</td>
<td>50</td>
<td>66</td>
</tr>
<tr>
<td>Size (aa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
<td>69±2</td>
<td>34±9</td>
<td>69±6</td>
<td>24±4</td>
<td>14±6</td>
<td>45±9</td>
<td></td>
</tr>
<tr>
<td>pI</td>
<td></td>
<td></td>
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**Culture**

The results of the Northern blotting of the *csp* genes, using *usp45* as an internal control, established that *cspB* and *cspD* were induced about 40- and 30-fold, respectively, at 10 °C, whereas *cspA* and *cspC* were induced about 10-fold compared to the level at 30 °C (Fig. 4b). At 30 °C (t = 0) a relatively high *cspE* mRNA level was detected compared to the other four *csp* genes, but *cspE* seemed not to be induced significantly at low temperature (Fig. 4b). Strikingly, the time at which maximal mRNA levels were found was different for the cold-induced *csp* genes. *cspA* and *cspC* reached maximal accumulation at 1–2 h after cold shock whereas for *cspB* and *cspD* maximal accumulation occurred at about 2–4 h after cold shock (Fig. 4b). The mRNA levels of *cspA*, *cspB*, *cspC* and *cspD* were decreased at 8 h after cold shock (data not shown), when exponential growth was resumed (Fig. 3). Other stress conditions, such as...
heat stress (10 min 42 °C), salt stress (10 min 0.5 M NaCl), low-pH stress (10 min pH 4.0, adjusted with lactic acid) or stationary-phase conditions (2 h after reaching stationary phase) did not result in increased mRNA levels of any of the csp genes (data not shown).

Identification of promoter regions

Using the primer extension technique, transcription start points of the csp genes were identified (Fig. 5; only shown for cspC and cspD); they are indicated in Fig. 6. For cspD a double transcription start was found: a major start at the indicated A-residue and a minor start at the T-residue three bases downstream. For each csp gene, transcripts were detectable at 30 °C, and for cspA, cspB, cspC and cspD increased amounts of transcript were found at 10 °C. The same transcription start points were identified at high and low temperature. Northern blotting showed that the mRNA size for the different csp genes is about 300 nt, which corresponds well with the detected transcription starts and the putative terminators [ΔG = −6, −10, −8, −8 and −8 kcal mol⁻¹ (−25, −42, −33, −33 and −33 kJ mol⁻¹)] for cspA, cspB, cspC, cspD and cspE, respectively. For cspA and cspC hairpin structures [ΔG = −10 and −14 kcal mol⁻¹ (−42 and −59 kJ mol⁻¹), respectively] were found further downstream the ORFs, for which the size of the mRNA corresponds with larger transcripts that were detected in small amounts (only detected after prolonged exposure of the blots to X-ray films). When DNA fragments containing parts of the csp genes and the region between the clustered csp genes were used as probes, only transcripts of about 300 nt were detected, indicating that the csp genes located in tandem repeats are monocistronic.

The detected transcription start sites allowed identification of −35 and −10 promoter regions of the csp genes (Fig. 6). The promoter regions are 67–92%
identical to the established consensus sequences of *L. lactis* (De Vos & Simons, 1994). The consensus 17 bp spacing between the −35 and −10 regions is found for all lactococcal csp promoters (Fig. 6). The non-cold-induced cspE gene has the lowest similarity (4 nt mismatches) with the consensus promoters, whereas the promoter regions of the cold-induced csp genes are less different from the consensus promoter regions (3, 3, 2 and 1 nt mismatches for cspA, cspC, cspD and cspB, respectively). In the promoter regions of cspC and cspB, complementary sequences (CCAAT) of the Y-box motifs (ATTGG) are present (Fig. 6). Several of these motifs were also found further up- and downstream of the promoter regions of the other lactococcal csp genes.

The 5′-untranslated leader regions (5′ UTRs) of the cold-induced cspA, cspB, cspC and cspD genes are highly identical (approximately 60%) whereas the identity with this region of the non-cold-inducible cspE is much lower (about 30%; Fig. 6). Furthermore, the 5′ UTR of cspE (94 nt) is slightly longer than those of the other lactococcal csp genes (86, 84, 83 and 87 nt for cspA, cspB, cspC and cspD, respectively). The 5′ UTRs of all lactococcal csp genes appear to be rich in secondary structure, encompassing the entire region as calculated by the method of Zuker & Stiegler (1981).

**DISCUSSION**

A family of five genes, named cspA, cspB, cspC, cspD and cspE, encoding putative CSPs was cloned from *L. lactis* MG1363 and it appeared that these csp genes were organized in clusters. cspA and cspB as well as cspC and cspD are located in a tandem repeat whereas cspE was found as a single gene. To our knowledge, a clustered organization of csp genes has never been observed before (Graumann et al., 1996; Lee et al., 1994; Mayo et al., 1997; Mayr et al., 1996; Willinsky et al., 1992). cspB is identical to the cspE gene that was recently obtained from *L. lactis* AM2 using an inverse PCR strategy (Chapot-Chartier et al., 1997).

The five csp genes can be grouped based on sequence analysis: a group consisting of cspA and cspC (the first genes in the tandem repeats); and a group consisting of cspB, cspD and cspE. Members within these groups code for highly similar proteins (about 80% identity) whereas the identity between these two groups is only about 55%. High similarity (45-65% identity) was also observed with the sequences of the major CSPs CspA<sup>E</sup> and CspB<sup>E</sup>, and was lowest for CspA and CspC. The residues important for single-stranded DNA binding of CspA<sup>E</sup> and CspB<sup>E</sup> (Newkirk et al., 1994; Schröder et al., 1995) are highly conserved in CspB, CspD and CspE, whereas in CspA and CspC some additional residues are different from the CspA<sup>E</sup> and CspB<sup>E</sup> DNA-binding residues. The RNA-binding RNP-1 (consensus KGFGF) and RNP-2 (consensus VFVH) motifs (Jones & Inouye, 1994; Schindelin et al., 1993; Schröder et al., 1995) are also found in the *L. lactis* CSPs although some differences are observed. Interestingly, the pl values of CspA and CspC (9.2 and 9.6, respectively) are much higher than those of the other CSPs (approximately 4.5) due to the presence of more basic residues (8 and 11 for CspA and CspC, respectively, compared to 7 for CspB, CspD and CspE) and the presence of 4 tyrosine residues for CspA and CspC and no tyrosine residues in CspB, CspD and CspE. This high pl of CspA and CspC might result in an improved nucleic acid binding capacity since these proteins do not need to overcome charge repulsion when approaching nucleic acids (Schröder et al., 1995).

Furthermore, protein 3-D modelling based on the crystal structure of CspA<sup>E</sup> and CspB<sup>E</sup> (Schindelin et al., 1993, 1994) revealed a similar β-barrel structure formed by five β-strands for all five lactococcal CSPs (J. A. Wouters, unpublished results).

For all csp genes transcripts of about 300 bp were found and no combined transcripts were found for the csp genes located in tandem repeats. Furthermore, Northern blotting revealed increased mRNA levels for the csp genes at different times after cold shock, indicating that regulation of these genes takes place at the transcriptional level. Maximal induction of mRNA was approximately 40- and 30-fold for cspB and cspD, respectively, whereas the mRNA level of cspA and cspC increased approximately 10-fold. cspE was not induced at 10°C. A differential expression in time after cold shock was observed for the different csp genes. mRNA levels of cspA and cspC increase shortly after cold shock (in the first 2 h) whereas cspB and cspD mRNA levels are highest at 4 h after cold shock. Possibly the more basic CSPs, CspA and CspC, are involved in the regulation of the expression of their counterparts CspB and CspD located further downstream. Since no mRNA induction was observed upon exposure to other stress conditions, such as heat, salt, low pH and stationary phase, it is concluded that these csp genes, with the exception of cspE, might play a specific role in low-temperature adaptation. Recently, it was shown that the non-cold-induced cspD gene of the *E. coli* CspA family is in fact induced under stationary-phase conditions (Yamanaka & Inouye, 1997).

Recent studies indicate that the 5′ UTR plays an important role in the stability of the *E. coli* cspA transcript (Fang et al., 1997) and the regulation of CspA<sup>E</sup> expression after cold shock (Jiang et al., 1996). Although the 5′ UTRs of the lactococcal csp genes are not as exceptionally long (83–94 nt) as this region of the *E. coli* cspA (159 nt; Goldstein et al., 1990), they might play a similar role. Most intriguing in this respect is the finding that the 5′ UTRs of the four cold-induced lactococcal csp genes are highly similar but that clear differences are observed in this sequence of the non-cold-induced cspE gene, indeed suggesting a regulatory function of this leader.

Future research will focus on the differential expression, the clustered organization and the regulation of the newly described csp genes in *L. lactis*. The physiological role of the *L. lactis* CSPs will be studied using single and multiple overexpression constructs and using strains with disrupted csp genes.
ACKNOWLEDGEMENTS

We thank Tomasz Oczkowski and Girbe Buist for their technical assistance with cloning of cspE.

REFERENCES


Received 16 January 1998; revised 15 June 1998; accepted 7 July 1998.