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Conservation of the Major Cold Shock Protein in Lactic Acid Bacteria

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Abstract. Primers designed from consensus regions of the major cold shock gene of different bacterial species were used in PCR amplification of Lactic Acid Bacteria (LAB). An appropriately-sized PCR product was obtained from *Lactococcus lactis* subsp. *lactis* LL43-1 and MG1363; *Lactococcus lactis* subsp. *cremoris* LC10-1, LC11-1, and LC12-1; *Streptococcus thermophilus* ST1-1; *Enterococcus faecalis* EF1-1; *Lactobacillus acidophilus* LA1-1; *Lactobacillus helveticus* LH1-1; *Pediococcus pentosaceus* PP1-1; and *Bifidobacterium animalis* BA1-1. The PCR products were cloned and sequenced. The deduced amino acid sequences displayed high sequence similarity with the major cold shock proteins of *Escherichia coli* and *Bacillus subtilis* and the human Y-box factor. The amino acid residues of the cold shock domain implicated in nucleic acid binding in several unrelated species were also highly conserved in the LAB strains. It is possible, therefore, that this protein in LAB may also act as a transcriptional enhancer to other cold shock genes and/or act as an RNA chaperone unwinding tightly folded RNA molecules.

Cold shock-induced proteins have been identified in a number of different organisms, including *Escherichia coli* [4] and *Bacillus subtilis* [19], and they have been implicated in the adaptive processes required for cell viability at low temperatures [7]. The major cold shock proteins of *E. coli* (CspA) and *B. subtilis* (CspB) have been characterized. These small hydrophilic proteins consist of 70 and 67 amino acids respectively, and share about 61% sequence homology [19]. These proteins also show high sequence homology (43%) with the Y-box factors [19], which are a family of eukaryotic nucleic acid-binding proteins. The domain of these proteins involved in the nucleic acid binding is referred to as the cold shock domain. The domain preferentially binds to the Y-box, which is an element in the promoter region of mammalian major histocompatibility complex class II genes. The Y-box is characterized by the highly conserved sequence ATTGG [20]. This sequence has also been shown to exist in the promoter regions of at least two cold shock genes, *hns* [11] and *gyrA* [8]. *hns* encodes the nucleoid protein H-NS, and *gyrA* encodes the α subunit of DNA gyrase. It has been demonstrated that CspA binds to the ATTGG element in the promoter region of *gyrA* [8]. It has also been demonstrated that CspB binds to single-stranded DNA that contains the ATTGG element as well as the complementary CCAAT sequence [5]. Therefore, it has been suggested that CspA and CspB could act as a transcriptional enhancer to cold shock genes by recognizing the putative ATTGG sequence.

Further evidence of the protein–nucleic acid interaction comes from the three-dimensional structures of CspA and CspB, which have been determined by 2D-NMR and X-ray studies [15–17]. First, the two structures were found to be very similar. Second, it was revealed that the surface of the proteins is rich in aromatic and basic amino acid residues characteristic of proteins that interact with nucleic acids. Third, it was revealed that the proteins contain sequence motifs typical of RNA-binding proteins. It has been suggested that the highly conserved amino acids located at the binding site (Lys^{7}, Trp^{8}, Lys^{13}, Phe^{15}, Phe^{17}, Phe^{27}, His^{29}, Phe^{30}, Phe^{38}, and Arg^{56}) of CspB, as well as the corresponding amino acids of CspA and Y-box factors, are involved in DNA binding and possibly also in RNA binding [13, 16].

Although the major cold shock protein has been studied in *E. coli* and *B. subtilis*, little is known in the industrially important lactic acid bacteria, despite the

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significance of viability of these starter bacteria at cold temperatures. In this study, PCR was used to identify the major cold shock gene in several strains of LAB, and the deduced amino acid sequences were analyzed.

Materials and Methods

Bacterial strains. The strains used were *Lactococcus lactis* subsp. *lactis* (*L. lactis*) LL43-1, MG1363; *Lactococcus lactis* subsp. *cremoris* (*L. cremoris*) LC10-1, LC11-1, LC12-1; *Streptococcus thermophilus* ST1-1; *Enterococcus faecalis* EF1-1; *Lactobacillus acidophilus* LA1-1; *Lactobacillus helveticus* LH1-1; *Pediococcus pentosaceus* PP1-1; and *Bifidobacterium animalis* BA1-1. The strains were obtained from Gist-brocades Australia, except MG1363 [3].

Medium and bacterial growth. *Lactococcus*, *Streptococcus*, and *Enterococcus* were grown on M17 medium [18] containing 0.5% glucose (M17G), and other species were grown on MRS medium [1] at pH 5.4 for *Lactobacillus* and *Pediococcus*, and at pH 7.2 for *Bifidobacterium*. All strains were grown anaerobically at 30°C for *Lactococcus* and at 37°C for all other strains.

PCR amplification. The total genomic DNA was isolated as previously described [12]. The degenerate PCR primers used were cspla: 5′GGTTTAATGTAGACAARGGNTTYGGNTTYAT3′ and csp1b: 5′TAGTAGGTACCRTTNGCNGCYTGNGGNCC3′ [9]; and csp3a: 5′GGTTYAAYGCNGARAARGGNTT3′ and csp3b: 5′GTNACRTTNGCNGCYTGNGG3′. The PCR was carried out in 50-µl volumes. The optimal PCR conditions for each strain were as follows: *L. lactis*, *L. cremoris*, and *Pediococcus pentosaceus*, 1.5 mM $MgCl_2$, 200 ng template DNA, and 58°C annealing temperature for 2 min; *Streptococcus thermophilus*, 4.5 mM, 500 ng, and 52°C respectively; *Enterococcus faecalis* and *Lactobacilus* spp., 3.0 mM, 200 ng, and 55°C respectively; and *Bifidobacterium animalis*, 1.5 mM, 200 ng and 56°C respectively. The other PCR parameters were same for all strains and were as follows: 100 pmol of each primer, 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim), denaturation (94°C, 1 min), extension (72°C, 1 min), and 30 cycles. The PCR product was visualized following electrophoresis on 6% polyacrylamide gels.

Cloning and sequence analysis. The PCR product was purified, digested with *Acc*I and *Kpn*I (the primers csp1a and csp1b, respectively, were designed to contain these sites), and ligated to pBluescript II vector (Stratagene), or alternatively the PCR product was blunt-end ligated to the vector. The recombinant DNA was transformed into *E. coli* XL1-Blue cells (Stratagene). DNA sequencing was done with an automated sequencer (Applied Biosystems) following the manufacturer's instructions. The deduced amino acid sequences were analyzed with a evolutionary analysis program called Eprotpars from the WebANGIS GCG package.

Results and Discussion

The amino acid sequences of previously characterized major cold shock proteins were aligned, and highly conserved stretches of the proteins were chosen as templates in order to design degenerate primers. A set of 22-mer oligonucleotides with 256-fold degeneracy (coding-strand primer: csp3a) and a set of 20-mer oligonucleotides with 1024-fold degeneracy (complementary-strand primer: csp3b) were generated. The relatively low G+C contents of LAB were considered in the designing of the first ten nucleotides (5′ end), but not in the rest because perfect homology on the 3′ end is desirable for good elongation. These primers, along with the primers previously designed [9], were used in the PCR amplification of the genomic DNA isolated from 11 LAB strains. The primers csp3a and csp3b were appropriate for the *Bifidobacterium animalis* strain, and the primers csp1a and csp1b for all others. A PCR product of approximately 178 bp was obtained from all strains tested. Therefore, PCR amplification of the gene homologous to the major cold shock proteins is possible in the LAB strains tested.

The PCR product that was generated from the strains was cloned into the pBluescript vector, then transformed into *E. coli*. Sequencing was performed on both strands of the DNA obtained. All sequences were deposited into the GenBank database, and the accession numbers are as follows: *L. lactis* LL43-1, AF023485; *L. lactis* MG1363, AF023486; *L. cremoris* LC10-1, AF023487; *L. cremoris* LC11-1, AF023488; *L. cremoris* LC12-1, AF023489; *Streptococcus thermophilus* ST1-1, AF023492; *Enterococcus faecalis* EF1-1, AF047608; *Lactobacillus acidophilus* LA1-1, AF023490; *Lactobacillus helveticus* LH1-1, AF023491; *Pediococcus pentosaceus* PP1-1, AF047609; and *Bifidobacterium animalis* BA1-1, AF049710. The nucleotide sequences obtained represent approximately 75% of the open reading frame and were highly homologous with one another.

The deduced amino acid sequence from all of the LAB strains displayed high sequence similarity with the major cold shock proteins of *B. subtilis*, *E. coli*, and the human Y-box factor (Fig. 1). The amino acid residues of the cold shock domain implicated in the nucleic acid binding in *B. subtilis*, *E. coli*, and the human Y-box factor were also conserved in the LAB strains. Of the ten amino acid residues indicated to be important in the protein function, eight were contained in the fragments sequenced. Phe^{17}, Phe^{27}, and His^{29} were conserved in all species examined. Lys^{13}, Phe^{15}, and Phe^{30} were conserved in all cold shock proteins but not in the Y-box factor. Phe^{38} was replaced by Tyr in *E. coli*, *L. acidophilus*, and *B. animalis*, and by Leu in human. Arg^{56} was replaced by Lys in *E. coli* and human. The four contiguous amino acid residues (PRKY) present in the human Y-box factor were absent in all bacterial species examined. Within the same subspecies of *lactis* and *cremoris* there were some differences among the strains, but the important amino acid residues were completely conserved. The homologous gene identified in the LAB strains was designated as *cspL*.

When the amino acid sequences were analyzed

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                    20          30              40          50          60
                     .           .               .           .           .
B. subtilis         KGFGFIEVE:GQDDVFVHFSAIQGEG::::FKTLEEGQAVSFEIVEGNRGPQAAN
E. coli             ------TPDD-SK----------ND-::::Y-S-D---K---T-ES-AK--A-G-
H. sapien           N-Y---NRNDTKE-----QT--KKNNPRKYLRSVGD-ET-E-DV---EK-EE---
L. lactis 43        ------TT-E-N:---A------TD-::::----D---K-T-DVE--P-------
L. lactis M         ------TS-D--:---A---Q--TS-::::----D---K-T-DVEA-Q-------
L. crem 10          ------TS-E-K:-L-A------SD-::::----D---K-E-DVE--Q-------
L. crem 11          ------TS-D--:---A---Q--TS-::::----D---K-T-DVEA-Q-------
L. crem 12          ------TS-E-K:-L-A------SD-::::----D---K-E-DVE--Q-------
S. thermo           ------TS-E--:-L-A---S--SD-::::-LS-D-D-L-E-DVEV-Q-------
E. faecal           ------SP-D-N:----------D-::::----------T-DVED-H-------
L. acidop           ------TG-D--:---------N---::::Y-S-D------YDVEQSD-------
L. helvet           ------TGSDNK:---------KTD-::::--S-----K--YDVEQ-G-------
P. pentos           ------TR-D-S:----------SD-::::--------S---DVE-SD-------
B. animal           ------TG-D--:---------N---::::Y-S-DK-----YNVKOSD-------
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Fig. 1. Sequence alignment of the cold shock proteins from the LAB strains (*L. lactis* 43: *L. lactis* LL43-1; *L. lactis* M: *L. lactis* MG1363; *L. crem* 10: *L. cremoris* LC10-1; *L. crem* 11: *L. cremoris* LC11-1; *L. crem* 12: *L. cremoris* LC12-1; *S. thermo*: *Streptococcus thermophilus* ST1-1; *E. faecal*: *Enterococcus faecalis* EF1-1; *L. acidop*: *Lactobacillus acidophilus* LA1-1; *L. helvet*: *Lactobacillus helveticus* LH1-1; *P. pentos*: *Pediococcus pentosaceus* PP1-1; *B. animal*: *Bifidobacterium animalis* BA1-1), *E. coli* (CspA), *B. subtilis* (CspB), and a Y-box factor from human; the residues that are identical to the residues of CspB are indicated by dashes; and insertions are indicated by colons.

phylogenetically, a distinct group was formed (Fig. 2). It consisted of all the *L. cremoris* strains, *L. lactis* MG1363, and the *S. thermophilus* strain. *L. lactis* LL43-1, although not part of the group, was still closely related to the group. This grouping is reflective of the relatedness of the *Lactococcus* and *Streptococcus* genera based on the 16S rRNA sequences. No other groups were formed. All of the Gram-positive strains were highly homologous to the Gram-negative *E. coli*, indicating that the cold shock protein may have existed before the divergence of the two divisions of bacteria.

A few proposals have been made about the function of the protein at low temperature. One is that during initiation of transcription it is involved in converting the closed complex to an open complex. Another is that it acts as an RNA chaperone, facilitating the unwinding of tightly folded RNA molecules [7]. It has also been demonstrated in *B. subtilis* that the protein plays a role in preserving cell viability at cold temperatures [19]. The preservation quality of LAB at cold temperatures is important because of the significant loss of cell viability during storage in cold conditions of commercially prepared starter cultures. It is believed that the cause of cell injury and death during freezing is due to the growth of ice crystals. Another hypothesis proposed is that these cold shock proteins act like ‘‘antifreeze proteins’’ [4, 8, 10]. Antifreeze proteins are low-molecular-weight proteins [21], and they are commonly found at high concentrations in organisms that live in extremely cold environments [2, 6, 14]. However, the cold shock proteins do not share any significant amino acid sequence similarity with the antifreeze proteins, and whether they are similar at the tertiary structure level is yet to be determined.

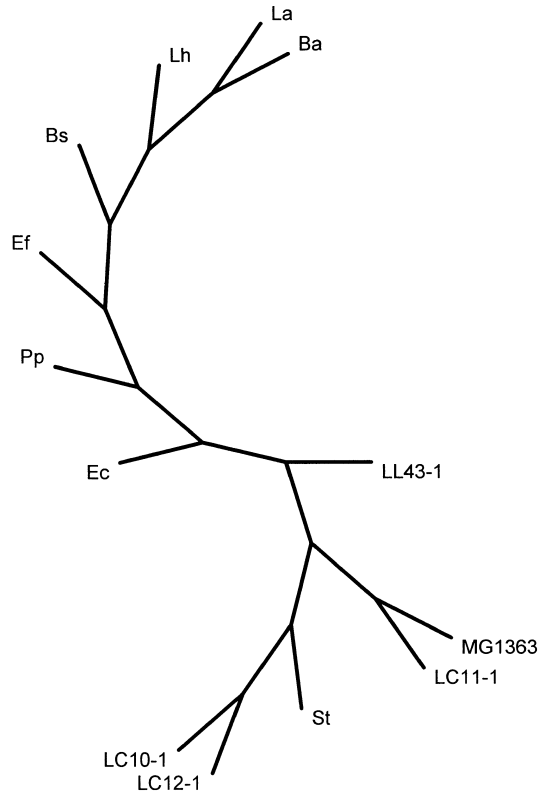

Fig. 2. A phylogenetic analysis of the cold shock protein amino acid sequences of *B. subtilis* (Bs), *E. coli* (Ec), *L. lactis* LL43-1 (LL43-1), *L. lactis* MG1363 (MG1363), *L. cremoris* LC10-1 (LC10-1), *L. cremoris* LC11-1 (LC11-1), *L. cremoris* LC12-1 (LC12-1), *Streptococcus thermophilus* ST1-1 (St), *Enterococcus faecalis* EF1-1 (Ef), *Lactobacillus acidophilus* LA1-1 (La), *Lactobacillus helveticus* LH1-1 (Lh), *Pediococcus pentosaceus* PP1-1 (Pp), and *Bifidobacterium animalis* BA1-1 (Ba) (the evolutionary tree was drawn, with the program Eprotpars, as a radial cladogram).

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