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Adaptation to Cold and Proteomic Responses of the Psychrotrophic Biopreservative *Lactococcus piscium* Strain CNCM I-4031

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Abstract:

There is considerable interest in the use of psychrotrophic bacteria for food biopreservation and in the understanding of cold adaptation mechanisms. The psychrotrophic biopreservative *Lactococcus piscium* strain CNCM I-4031 was studied for its growth behavior and proteomic responses after cold shock and during cold acclimation. Growth kinetics highlighted the absence of growth latency after cold shock, suggesting a very high promptness in cold adaptation, a behavior that has never been described before for lactic acid bacteria (LAB). A comparative proteomic analysis was applied with two-dimensional gel electrophoresis (2-DE), and upregulated proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Both cold shock and cold acclimation triggered the upregulation of proteins involved in general and oxidative stress responses and fatty acid and energetic metabolism. However, 2-DE profiles and upregulated proteins were different under both conditions, suggesting a sequence of steps in cold adaptation. In addition, the major 7-kDa Csp protein was identified in the *L. piscium* CNCM I-4031 genome but was not cold regulated. The implication of the identified cold shock proteins and cold acclimation proteins in efficient cold adaptation, the possible regulation of a histidyl phosphocarrier protein, and the roles of a constitutive major 7-kDa Csp are discussed.

1. Introduction

Lactic acid bacteria (LAB) usually become the dominant flora during the storage of refrigerated preserved meat or fish products. Some of them have been widely described for their beneficial role in food preservation (42) and psychrotrophic strains present a real advantage for a biopreservation strategy (29). Matamoros et al. (35) have isolated psychrotrophic lactic acid bacteria from seafood products and examined them for their ability to inhibit pathogenic and spoiling bacteria in model medium. One of these strains, *Lactococcus piscium* CNCM I-4031, previously named EU2241, has been shown to extend the sensory shelf-life of vacuum-packed shrimp and cold-smoked salmon, and to limit the development of *Listeria monocytogenes*, *Staphylococcus aureus* and *Brochothrix thermosphacta* (12, 13, 34). This bacterium shows an uncommon temperature-related growth profile. Unlike most of the LAB, the growth of *L. piscium* EU2241occurs from 0 to 29°C with an optimum at 26°C and is inhibited at temperatures higher than 29°C suggesting a specific cold adaptation behavior.

Low temperatures usually result in a wide range of molecular and cellular disruptions: decrease in membrane fluidity and in enzymatic activity (Arrhenius), inefficient folding of some proteins, increase in RNA and DNA secondary structure leading to a decreased efficiency of transcription and translation (51). Cold environments are widespread on Earth and, under these conditions, the microbial communities are dominated by various psychrophilic and psychrotrophic bacteria that have developed diverse cold adaptation strategies (38). Proteomic analysis is a powerful tool to investigate adaptation strategies to various environmental changes for numerous species including LAB (7). According to the current knowledge, after a downshift in temperature, psychrotrophic and mesophilic bacteria synthesize a set of cold shock proteins (Csps) in amounts related to the severity of the shock (22). In *Escherichia coli*, *Bacillus subtilis* and *Lactococcus lactis*, the major up-regulated Csp belongs to a 7-kDa protein family (19, 47, 51). These proteins present a highly conserved nucleic acid binding domain and are implicated in numerous cellular functions including gene regulation, freeze protection and RNA chaperones. 7-kDa CSP-like proteins have been reported in the genome of over 50 bacterial species(51).

Despite their impact on foodstuffs, the cold shock and cold acclimation responses of psychrotrophic LAB are poorly documented. Bacteria in technological starters face a great variety of stresses throughout food processing. A better understanding of the *L. piscium* response to refrigerated temperatures could be helpful to optimize its application as a biopreservation agent in chilled products. The aim of this study was to describe the growth behavior of the bioprotective strain *L. piscium* CNCM I-4031 at chilled temperature and after cold shock, and to analyze protein responses to both cold shock and cold acclimation using 2-dimensional gel electrophoresis (2-DE). The principal cold-induced proteins, detected by comparing gel electrophoresis profiles between the optimal growth of *L. piscium* CNCM I-4031 at 26°C and after cold acclimation or cold shock at 5°C, were identified and are discussed according to their molecular and cellular functions.

2. Materials and methods

Bacterial strain, growth conditions and cold shock treatments. *Lactococcus piscium* EU2241, deposited in the national collection of microorganisms of the Pasteur Institute (Paris, France) under the name CNCM I-4031, was stored at -80°C in Elliker (ELK) broth with 10% glycerol. Subcultures were performed for 20 h at optimal temperature (26°C) in 10 ml common LAB complex nutrient-rich medium ELK broth (Biokar Diagnostics, Beauvais, France). One liter of ELK contains 20 g tryptone, 5 g yeast extract, 2.5 g gelatin, 5 g glucose, 5 g sucrose, 5 g lactose, 1.5 g Na acetate, 4 g NaCl and 0.5 g ascorbic acid, pH was adjusted to 7 (11). Cultures were obtained by inoculating 1% (v/v) of the subculture in 200 ml ELK and incubating without shaking at 26°C.

Growth kinetics were investigated using the automated microbial growth analyzer system Bioscreen C (Labsystem, Helsinki, Finland) in 96-well microtiter plates with 400 µl per well of the bacterial culture and without shaking. Cells were harvested from cultures at 26°C at mid-exponential phase (OD_{600nm} = 0.5) then diluted to an OD_{600nm} = 0.2 with Elliker broth at the appropriate temperature. Growth kinetics were performed in Elliker broth at 5°C for acclimation experiments or at 26°C for standard conditions. For cold shock experiments, diluted cultures were submitted to 1 or 2 h incubation at 0°C or 5°C then growth kinetics were performed at 26°C. All experiments were carried out in triplicate. Growth curves were fitted with the Gompertz model for estimation of µ_max (54).

For the proteomic study, 200 ml of mid-exponential phase (OD_{600nm} = 0.5) cultures were obtained as described above at either 26°C for standard conditions or 5°C for acclimation conditions. For cold shock experiments, cells were cultivated at 26°C and submitted to 1 h cold shock treatment at 5°C in fresh Elliker broth. Each culture sample was centrifuged at 10,000 x *g* for 20 min at 26°C or 5°C, according to the temperature of the culture. Each pellet was washed in 10 mM Tris-HCl, pH 7.2, 5 mM EDTA, resuspended in 10 ml of the same buffer containing 200 mM glycine and quickly frozen in liquid nitrogen before storage at -80°C. Three independent cultures were performed for each condition.

Protein extraction. For each condition (standard, acclimation and cold shock), cytosoluble proteins were extracted from frozen cell pellets. Cells were pulse sonicated using a Vibra-Cell® 72434 sonicator (Bioblock, Illkirch, France) in an ice bath during 5 cycles of 6 min in the presence of a protease inhibitor (Tablets COMPLETE Roche Diagnostics, Mannheim, Germany). After ultracentrifugation (188,000 x *g* for 60 min), extracts were treated with RNase and DNase solutions, and dialyzed against water using a 2 kDa cut-off membrane (Sigma-Aldrich, Saint-Quentin Fallavier, France).

2-Dimensional Electrophoresis (2-DE). Each extract was analyzed on analytic 2-DE gels using the adapted O`Farrell method (39). A pH gradient of 4-7 was chosen for isoelectric focusing (IEF) as a large proportion of the putative hydrosoluble proteins from the draft genome sequence of *Lactococcus lactis* are observed in this range of pH (7) and confirmed for *L. piscium* in pH 3-10 IEF experiments in our lab (data not shown). The second-dimension electrophoresis was performed on 13% SDS polyacrylamide gels to optimize the separation of proteins with a molecular weight ranging from 5 to 150 kDa. Aliquots containing 50 µg of protein for analytic gels and 600 µg for preparative gels were concentrated in 15 µl final volume and diluted in 275 µl rehydration buffer containing 6 M urea, 2 M thio-urea, 4% CHAPS, 2% Biolyte 3/10, 0.01% bromophenol blue and 5% DTT. After 18 h of active rehydration of IPG dry strips 4-7 (Bio-Rad, Marnes-la-Coquette, France) at 50 V, IEF was performed using the Bio-Rad Protean IEF Cell at 66,000 V.h. The strips were next treated with buffer containing 6 M urea, 2% SDS, 0.05 M Tris-HCl pH 8.8, 30% glycerol and supplemented with 2% DTT, and then again with the same buffer containing 4% iodoacetamide. Finally, proteins were visualized by the silver staining method for analytic gels and Bio-Safe colloidal Coomassie Blue (Bio-Rad, Marnes la Coquette, France) for preparative gels. For each condition, at least two replicates from each of the three independent cultures were carried out.

Image and statistical analysis. Images of analytic gels were recorded on a Bio-Rad GS800 densitometer. Gels were analyzed with the Progenesis SameSpots version 3.0 software (Nonlinear Dynamics Ltd, Newcastle, UK). The quality of the gels was ensured using the Quality Control (QC) of the software. This includes an analysis of image compression, image saturation (spot exposition), image bit depth, available dynamic range and intensity levels in use, alteration due to edited image and intensity level resolution. Only those images which passed the available quality control were included in the analysis. Available dynamic range and intensity levels were at least 95% and 96% for all gels, respectively. Automatic pixel level geometric alignments of the gels, followed by manual corrections, were performed with the vector alignment tool of the SameSpots workflow. In addition, an automatic management of spots and spot volume measurement was carried out. The background-corrected abundance of each spot was calculated and the abundance ratio determined by dividing the sample abundance by the reference abundance. Spot volumes were normalized to calibrate data between different sample runs then normalized spots were analyzed statistically using the statistical module implemented in SameSpots. Principal component analysis (PCA) was used to separate the gels according to variations in the normalized volume of the spots. After that, each cold-induced condition (cold adaptation and cold shock) was compared to the control at 26°C using an analysis of variance (ANOVA) which was reduced to a t-test in the software for comparison of one independent factor. Significant over-expressed spots were detected at a 5% significance level (p-value < 0.05). Finally, these significant over-expressed spots were refined using a q-value < 0.05 to discard false positives, a power > 0.8 to ensure reproducibility among gels of the same condition and a fold > 1.8 for the biological significance.

Protein identification. Detectable up-regulated spots were excised from preparative gels. In-gel digestion was performed with the Progest system (Genomic Solution) according to a standard trypsinolysis protocol. Gel plugs were first washed twice with 10% (v/v) acetic acid, 40% (v/v) ethanol in water, and then with acetonitrile. They were further washed with 25 mM NH_4CO_3 and dehydrated in acetonitrile (two alternating cycles). Digestion was performed for 6 h at 37°C with 125 ng of modified trypsin (Promega) dissolved in 20% (v/v) methanol in 20 mM NH_4CO_3. Tryptic peptides were first extracted with 50% (v/v) acetonitrile, 0.5% trifluoroacetic acid in water, and then with pure acetonitrile. Both peptide extracts were pooled, dried in a vacuum speed concentrator and suspended in 25 µL of 2% (v/v) acetonitrile, 0.08% (v/v) trifluoroacetic acid in water.

LC-MS/MS analysis was performed on an Ultimate 3000 LC system (Dionex, Voisins-le-Bretonneux, France) connected to an LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher, USA) via a nanoelectrospray ion source. After 4 min, the pre-column was connected to the separating nanocolumn Pepmap C18 (0.075 x 15 cm, 100 Å, 3 µm) and the linear gradient was started from 2 to 36% of buffer B (80% acetonitrile, 0.1% formic acid) in buffer A (2% acetonitrile, 0.1% formic acid) at 300 nl/min over 50 min. The double- and triple-charged precursor ions were subjected to MS/MS fragmentation with a 3-min exclusion window, and with classical peptide fragmentation parameters (Qz = 0.22, activation time = 50 ms, collision energy = 35%).

The raw mass data were first converted to mzXML format with the ReAdW software (http://tools.proteomecenter.org/software.php). In the absence of genomic data for *L. piscium*, protein identification was performed by querying MS/MS data against a Firmicute protein database (UniProtKB, 2009.06.08) together with an in-house contaminant database, using the X!Tandem software (X!Tandem Tornado 2008.02.01.3, http://www.thegpm.org) with the following parameters: one trypsin missed cleavage allowed, alkylation of cysteine and conditional oxidation of methionine, precursor and fragment ion set at 10 ppm and 0.5 Da, respectively.

A refined search was added with similar parameters except that semitryptic peptides and possible N-ter acetylation of proteins were searched for.

All peptides that matched with an E-value lower than 0.05 were parsed with an in-house program (http:/PAPPSO.inra.fr/bioinformatique.html). Proteins identified with at least three unique peptides and a log (E-value) lower than 10^{-8} were validated.
For the samples matching less than three unique peptides per protein, mass spectra with a quality score higher than 0.2 were interpreted by *de novo* sequencing, which was performed using PepNovo software (version 3). Enzymatic cleavage and modifications were as previously indicated.
Generated sequence tags were submitted to homology searches in *Lactococcus*, Firmicute and bacterial protein databases (UniProtKB, 2009.06.08) with Fasts software (v.34.26) (31) using the MDM20 matrix in an iterative process. Only homologies with a protein E-value lower than 10^{-5}, identified with at least two different peptides, were selected.
In all cases, the automatic *de novo* interpretation of MS/MS spectra was manually validated.
Identification of a 7-kDa CSP gene. The nucleic sequence of a putative 7-kDa CSP identified by LC-MS/MS was investigated. A degenerated nucleotide sequence obtained by reverse transcription of the amino acid sequence was compared to the gene sequences of other bacterial 7-kDa CSPs retrieved from online databases. PCR primers were designed from the conserved regions of these sequences: CspD1-f: 5'-GGCAAATGGAACAGTAAAATGG-3'; CspD3-r: 5'-TCCATCAGATTGGATTGCTGAG-3'. PCR reactions were performed in a total volume of 50 µl containing 1X PCR buffer, 1.5 mM $MgCl_2$, 50 ng DNA, 0.8 µM each primer, 0.2 mM each dNTP and 1U Taq DNA polymerase. Amplification consisted of a first cycle of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and 5 min of final extension at 72°C. The nucleotide sequence of the amplified gene fragment was determined with an ABI 3730 automated sequencer using the Taq Dye-Deoxy TM terminator cycle sequencing method (Genome Express company, Meylan, France) and the cspD1-f and cspD3-r primers. Anticipated errors of PCR and sequencing reactions were avoided by sequencing both DNA strands. The sequence was then compared to databases using the nucleotide-nucleotide BlastN program (http://www.ncbi.nlm.nih.gov/BLAST/).
Contiguous parts of the gene were obtained using an inverse-PCR strategy. Briefly, separate samples containing 10 µg of total DNA were digested using twelve restriction enzymes *EcoRI, SalI, EcoRV, BsmAI, NcoI, DpnI, BspHI, XhoI, MseI, HindIII, HaeIII and HhaI* (Ozyme, Saint-Quentin-en-Yvelines, France) as recommended by the supplier. None of these enzymes cut inside the previously identified gene fragment. Re-circularization of the digested products (40 µl) was performed with 800 U of T4 DNA ligase in a final volume of 400 µl. PCR amplification was then applied as described above using reverse primers Icq1f (5'-CGAAGCCTTTTTCAGCGTTA-3') and Icq2r (5'- TCATCACTGGTCAGACGGAA-3') and re-circularized DNA as a template. PCR products were cloned with the Invitrogen pcrII-TOPO-TA cloning kit (Invitrogen, Carlsbad, California, USA) and sequenced as described above using primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3').

3. Results

Effect of temperature on bacterial growth. Growth kinetics were performed at 26 and 5°C and μ_{max} was 0.6 h^{-1} and 0.06 h^{-1} respectively (Figure 1). These results are in accordance with those of Matamoros et al. (30). It is noteworthy that, at 5°C, μ_{max} was reached instantly without a lag time. No difference was observed in the growth rate at 26°C (μ_{max} = 0.6 h^{-1}) between the control cultures and the cultures submitted to cold shock during 1 or 2 hours at either 0°C or 5°C. Moreover, no lag time was observed whatever the severity of the cold shock.
Differential proteome analysis. In order to characterize protein expression variation in response to *L. piscium* cold acclimation and cold shock, 2-DE gel profiles of cytosoluble protein contents of cultures at 26°C (standard culture), at 5°C (cold acclimation) and cultures

transferred from 26°C to 5°C for 1 h (cold shock) were compared. The PCA analyses of the complete data set (22 gels) clearly showed three distinct clusters of gels corresponding to biological reproductions from the three experimental conditions (Figure 2), indicating specific responses of *L. piscium* CNCM I-4031 to cold shock and cold acclimation conditions.

Figure 3 displays the 2-DE gels of cytosoluble silver-stained proteins extracted from *L. piscium* in the three temperature conditions. In cold shock conditions, 28 spots were significantly over-expressed and three were down-expressed as compared to the standard conditions (26°C). In cold acclimation conditions, 31 spots were significantly over-expressed and 32 were down-expressed. Among these spots, four were over-expressed and two were down-expressed in both cold shock and cold acclimation conditions. Over-expressed spots after cold shock and during cold acclimation were named hypothetical Csps (h-Csps) and hypothetical Caps (h-Caps), respectively, and over-expressed spots in both cold conditions were named early Caps according to the definition of Hébraud and Potier (22).

Over-expressed protein identification. 33 spots, including 20 h-Csps, one early Cap and 12 h-Caps, were detected from preparative gels and submitted to identification using LC-MS/MS. Overall, 23 proteins were identified from MS-MS data (Table 1). Among them, six proteins were identified with several spot occurrences. Differences in migration for the same identified protein may be due to post-translational modifications, isoforms, truncated or degraded forms. Moreover, only truncated forms were identified for three proteins (GAPDH, EF-Tu, DnaK). Identified proteins were classified into four metabolic groups.

In the energetic metabolic processes, the histidyl phosphocarrier protein (HPr) (h-Cap03 and h-Csp16) was up-regulated after cold shock and during cold acclimation. Fba, GAPDH and Pgk, implicated successively in glycolysis, were up-regulated after cold shock. The enzyme of heterolactic acid fermentation and pyruvate metabolism, AdhE, was up-regulated 2-fold after cold shock and up-regulated 5.1-fold during cold acclimation. Three ubiquitous bacterial enzymes involved in fatty acid metabolism were identified: FabH involved in the initiation phase of type II fatty acid synthesis (27) and FabF and FabG involved in fatty acid elongation (52). FabF was up-regulated after cold shock, FabG during cold acclimation and FabH in both cold shock and acclimation conditions.

Proteins implicated in several steps of gene expression, including nucleoside and amino acid biosynthetic pathways (UPP and DAHP synthetase), tRNA modifications (Tgt and TrmU), RNA metabolic pathway (RhlB and putative Sigma 54 modulation protein), formation and methylation of ribosomes (RplE, RsmG) and peptide elongation (EF-G, EF-Tu), were identified. UPP, DAHP synthetase, Tgt, RplE, RsmG, RhlB and EF-Tu were up-regulated after cold shock and TrmU and EF-G during cold acclimation.

Among the five identified proteins involved in prokaryotic general stress responses, three are associated with cell redox homeostasis. OsmC-like protein and AhpF were induced after cold shock and thioredoxin was over-expressed during cold acclimation. The ubiquitous Heat Shock Protein chaperone DnaK was up-regulated after cold shock and the universal stress protein A (UspA) was up-regulated during cold acclimation.

Furthermore, exploring the major low molecular weight proteins, a non-cold-regulated protein migrating at 7 kDa was identified as a putative 7-kDa CSP.

Identification of a putative 7-kDa CSP gene (HM114314). PCR amplification with primers CspD1-f and CspD3-r using DNA from strain *L. piscium* CNCM I-4031 as a template resulted in the amplification of a fragment of approximately 100 bp as expected. Sequencing of this fragment and comparison of the nucleotide sequence with an online database confirmed the presence of a putative *csp* gene. PCR amplification with reverse PCR primers Icq1f and Icq2r, designed upon this sequence using the DNA from strain CNCM I-4031 digested by enzyme *HhaI* and ligated as a template, resulted in the amplification of a 350 bp fragment. Analysis of this fragment with the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) revealed the presence of a 162 bp open reading frame comprising the original PCR fragment. This ORF encodes a partial protein of 54 amino acids (Figure 4) that shares 85, 84 and 81% identity with cold shock proteins from *Enterococcus faecium*, *Ec. faecalis* and *L. lactis subsp. lactis*, respectively. The protein also contains the two RNA-binding domains RNP1 and RNP2. Because of its similarity to the *L.*

lactis 7-kDa CSPE protein (closest protein of a bacterium from the same genus), the protein from *L. piscium* CNCM I-4031 was named CspE.

4. Discussion

Psychrotrophic bacteria, like *Pseudomonas fragi*, *L. monocytogenes* and *Arthrobacter globiformis*, respond to temperature downshifts by a lag period before growth according to the amplitude of the cold shock (5, 6, 37). In psychrophilic *Vibrio* sp. and mesophilic bacteria *L. lactis*, *Vibrio vulnificus* (36) or *B. subtilis*, a lag period was not observed; however, the growth rate measured during the first hours was lower than the maximal growth rate (2, 30, 40). *L. piscium* CNCM I-4031 is able to endure cold shock within the permissive growth range without a lag period or intermediate growth rate. These original results suggest that the determinant factors of cold shock protection are constitutively expressed. Because comparative analysis of 2-DE gel profiles highlighted specific responses to cold shock and cold acclimation *versus* standard conditions, a molecular regulation should be required when *L. piscium* CNCM I-4031 is submitted to a cold shock although no effect was observed on growth. Mesophilic bacteria react to a sudden downshift in temperature by a halt in general protein expression whereas psychrotrophic bacteria do not. In *L. piscium* CNCM I-4031, most of the spots on 2-DE gels were apparently not cold-regulated, which seems to confirm the psychrotrophic behavior of the strain. In mesophilic and psychrotrophic bacteria, cold shock proteins (Csps) are over-expressed for a limited time after cold shock. They assist in overcoming the immediate effects of temperature downshifts. In psychrotrophic bacteria during prolonged growth at low temperature, cold acclimation proteins (Caps) are over-expressed and allow growth at chilled temperatures (17, 22). Cold-induced proteins (Csps and Caps) play roles in a variety of cellular processes such as fatty acid metabolism, chromosome structuring, transcription, translation, general metabolism, energetic metabolism and stress response (19, 49, 51). In contrast to what has been observed in several species including *E. coli*, *B. subtilis* and *L. lactis* after cold shock, this report reveals the absence of a major cold-induced protein of the 7-kDa Csp family. However, one non-cold-inducible 7-kDa Csp-E was identified which is probably involved in cryoprotection in *L. lactis* (50). The role of Csp-E at optimal and chilled temperatures remains to be evaluated but constitutive expression of this 7-kDa Csp could be deeply implicated in the efficient cold adaptation of this strain.

To maintain membrane fluidity, synthesis of the normal unsaturated fatty acids is required, involving enzymes of the Fab (16) family, some of which are cold-regulated. FabF is cold-activated in *E. coli* (32) and controls the temperature-dependent regulation of the branched-chain fatty acid pathway in *L. monocytogenes* (53). In *L. piscium* CNCM I-4031, cold regulation of FabF, FabG and FabH indicates a global increase in fatty acid metabolism correlated with low temperature. In order to overcome the efficient decrease in transcription and translation, proteins involved in these cellular processes have been identified in several species after cold shock (18), and particularly in our study.

The decreased enzyme-catalyzed reaction rates due to cold temperatures can be offset by an induction of some glycolytic enzymes to compensate for an overall lower energetic capacity. For example, the maximal glycolytic activity measured at 30°C in *L. lactis* increased approximately 2.5-fold following a cold shock (48). Cold induction has been observed for glycolytic enzymes including GAPDH in *B. subtilis*, and GAPDH Fba and PGK in *L. piscium* CNCM I-4031. A role in the control of glycolysis in *L. lactis* has been assigned to GAPDH, which was shown to be a rate-limiting enzyme in the glycolytic pathway during starvation (41). Transcriptional regulation of glycolysis has been strongly linked to HPr phosphorylation (10). Moreover, Hpr is involved in the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) implicated in the concomitant uptake and phosphorylation of carbohydrates. Cold induction of this protein has been observed in *B. subtilis, L. sakei* (18, 20) and *L. piscium* CNCM I-4031. In our study, two very close spot occurrences were up-

regulated, first after cold shock and second during acclimation. We suggest that these two spots are related to different phosphorylated forms and that HPr phosphorylation could be a key to glycolysis regulation in cold adaptation. In addition, alternative fermentative pathways have been described in lactic acid bacteria under nutritional stress conditions (9) and, in our study, AdhE was strongly cold up-regulated suggesting the induction of possible mixed acid fermentation.

Cold stress provokes protein mis-folding and oxidative compounds as well as inducing the expression of general stress proteins including antioxidants and protein chaperones. DnaK is described as the major heat shock protein (HSP) molecular chaperone in *E. coli*. HSPs are commonly induced by multiple stresses and play an important role in withstanding and surviving harmful conditions (44). In *L. lactis*, the expression of DnaK is induced by heat, saline and acid stresses (4, 14, 21, 25, 46) and it was reported that *in vitro* over-expression of DnaK conferred freeze tolerance on *E. coli* (8). Until now, cold induction of DnaK has only been described in *Leuconostoc mesenteroides* (43). We speculate that this chaperone is also involved in the cold shock response of *L. piscium* CNCM I-4031.

Previous studies on *Shewanella oneidensis* and *L. sakei* reported the cold induction of universal stress proteins Usp (15, 33) as shown in this report during cold acclimation. Expression of this small cytoplasmic protein is enhanced in *E. coli* when cellular viability is challenged with any unfavorable agents, such as heat shock, nutrient starvation, DNA-damaging agents and stress agents which inhibit cell growth (26). The oxidative stress protein Ahp is required by *B. subtilis* upon oxidative heat and salt stresses (1, 23), by *Staphylococcus aureus* upon osmotic stress (3), by LAB in aero tolerance (24) and by *Shewanella putrefaciens* upon osmotic stress and during cold acclimation (28). In *L. piscium* CNCM I-4031, three oxidative stress proteins were cold-induced including Ahp, OsmC and TrX. In *L. lactis*, Trx is implicated in aerobic environment life and it was assumed to trigger induction of several mechanisms acting at the membrane and of metabolic levels, including GAPDH activation (45). It is noteworthy that GAPDH is also cold-induced in *L. piscium* CNCM I-4031. On the other hand, OsmC is known to belong to the general stress regulon of *E. coli* and *B. subtilis,* enhanced by stress sigma factor. In *L. lactis*, despite the absence of sigma factor, the OsmC homologue was up-regulated upon cold shock and in *L. piscium* CNCM I-4031, a putative sigma 54 modulation protein was 4-fold up-regulated during cold acclimation.

In *L. piscium* CNCM I-4031, comparative analysis of 2-DE gel profiles highlighted specific responses to cold shock and cold acclimation *versus* standard conditions. The proteome was not analyzed kinetically but it was assumed that cold shock is a transient state before cold acclimation. Among other things, specific stress proteins were observed for cold shock and cold acclimation. This suggests dynamic protein regulation during cold adaptation, with Csps (DnaK, OsmC, Ahp) involved in stress responses being time relayed by Caps (UspA, Thioredoxin) involved in the same pathways.

In conclusion, we suggest that *L. piscium* CNCM I-4031 adapts quickly to low temperatures by a constitutive expression of the potentially cryoprotective Csp-E, and by an increase in glycolysis with potential Hpr phosphorylation regulation. In a biopreservation strategy, the protective strains are generally cultivated at their optimum temperature, eventually freeze-dried and then directly inoculated into products that are stored at chilled temperature. Cold acclimation thus constitutes a real advantage in bacterial competition against spoiling and pathogenic psychrotrophic bacteria in terms of food preservation. It would enable cells to multiply at optimal temperatures and be inoculated into chilled foodstuffs without delaying the food preservative effect.

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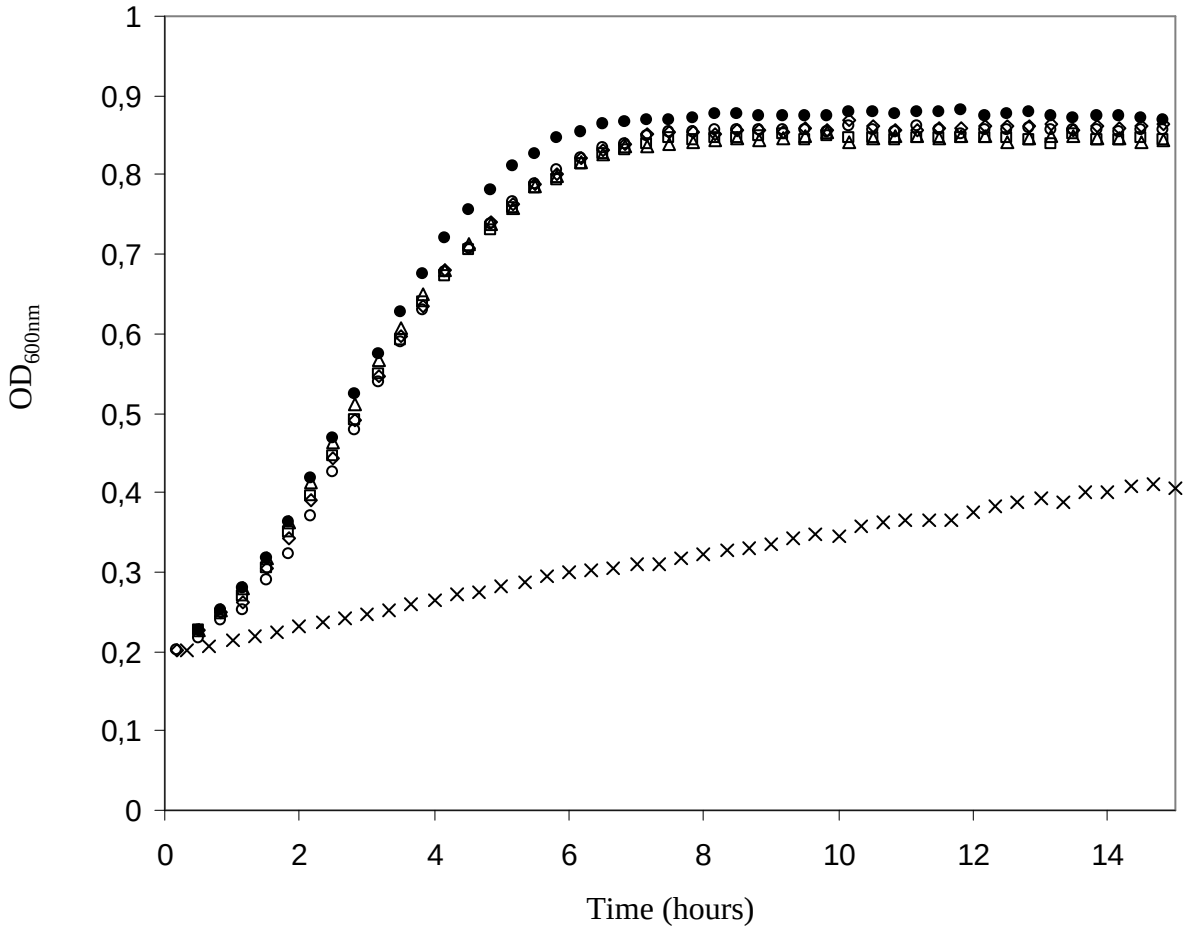
Figures

Figure 1: Effects of cold shock and cold acclimation at 5°C on the growth of *Lactococcus piscium* CNCM I-4031 in Elliker broth. Cells were harvested from standard cultures at 26°C at mid exponential phase and grown at 5°C (×), at 26°C without cold shock (•),at 26°C after 1h at 0°C (○), 2 h at 0°C (□),1h at 5°C (◇) and 2 h at 5°C(△). Standard deviation values were lower than 0.03 and were not indicated on the graph (n = 9 ; 3 replicates of 3 independent cultures).

Figure 2: Principal component analysis performed on the complete data set of the 22 2-DE gels according to variations in the normalized volume of the spots. Standard temperature conditions (●); cold shock (○); cold acclimation (□).

Figure 3: Two dimensional electrophoresis gel of cytosoluble silver stained proteins from *Lactococcus piscium* EU2241. Proteins were extracted from exponential cultures at 5°C. Identified cold shock proteins (h-Csp) cold acclimation proteins (h-Cap) and the non cold induced 7 Kda Csp are localized.

Figure 4: Alignment of *Lactococcus piscium* EU2241 CSPE protein with close 7-KDa CSPs from various bacteria. Consensus symbols : ! is anyone of IV; $ is anyone of LM; % is anyone of FY; # is anyone of NDQEBZ. Boxed: RNP1 and RNP2 motives.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0,5
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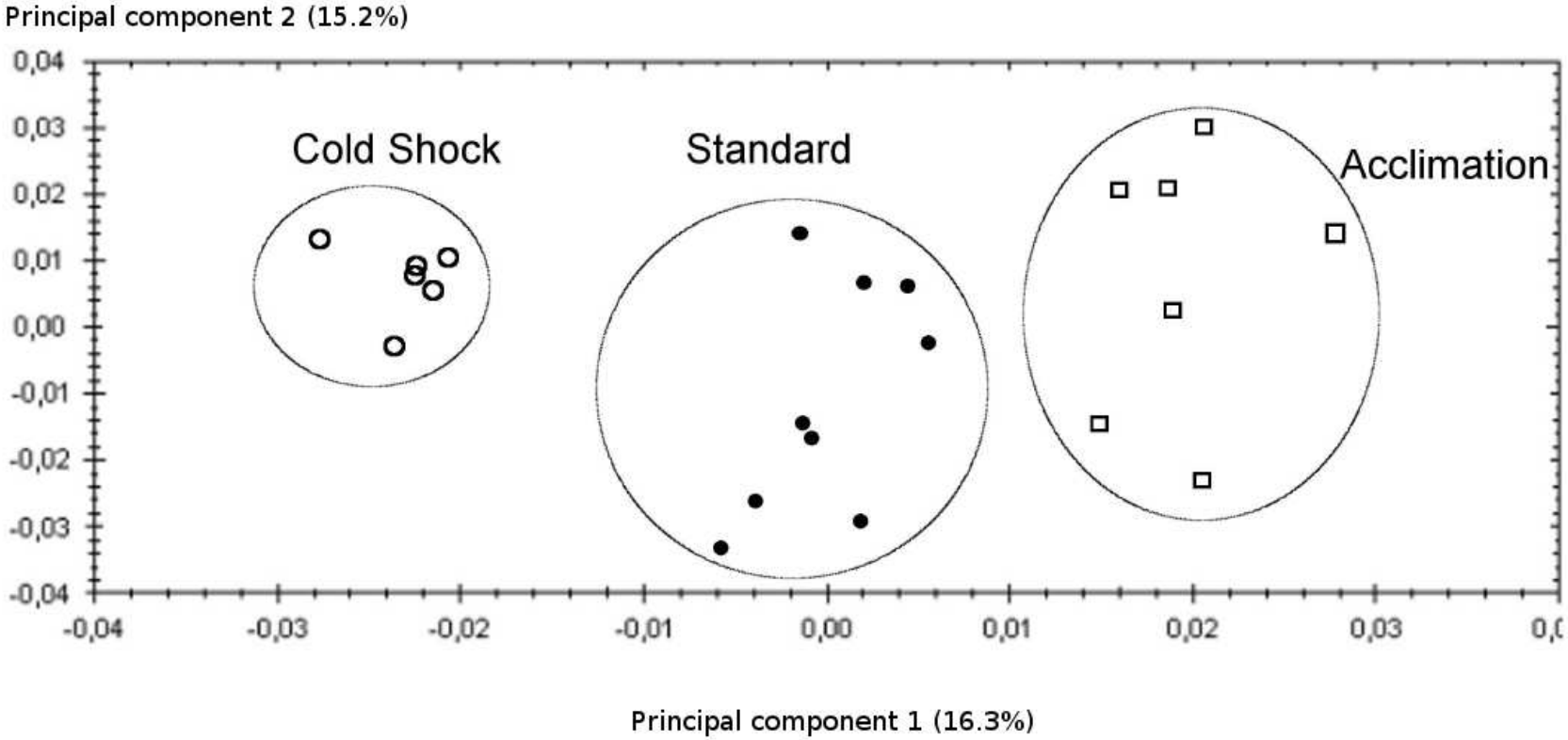
0,2
0,1
0
OD600nm
0
2
4
6
8
10
12
14
Time (hours)


Principal component 2 (15.2%)
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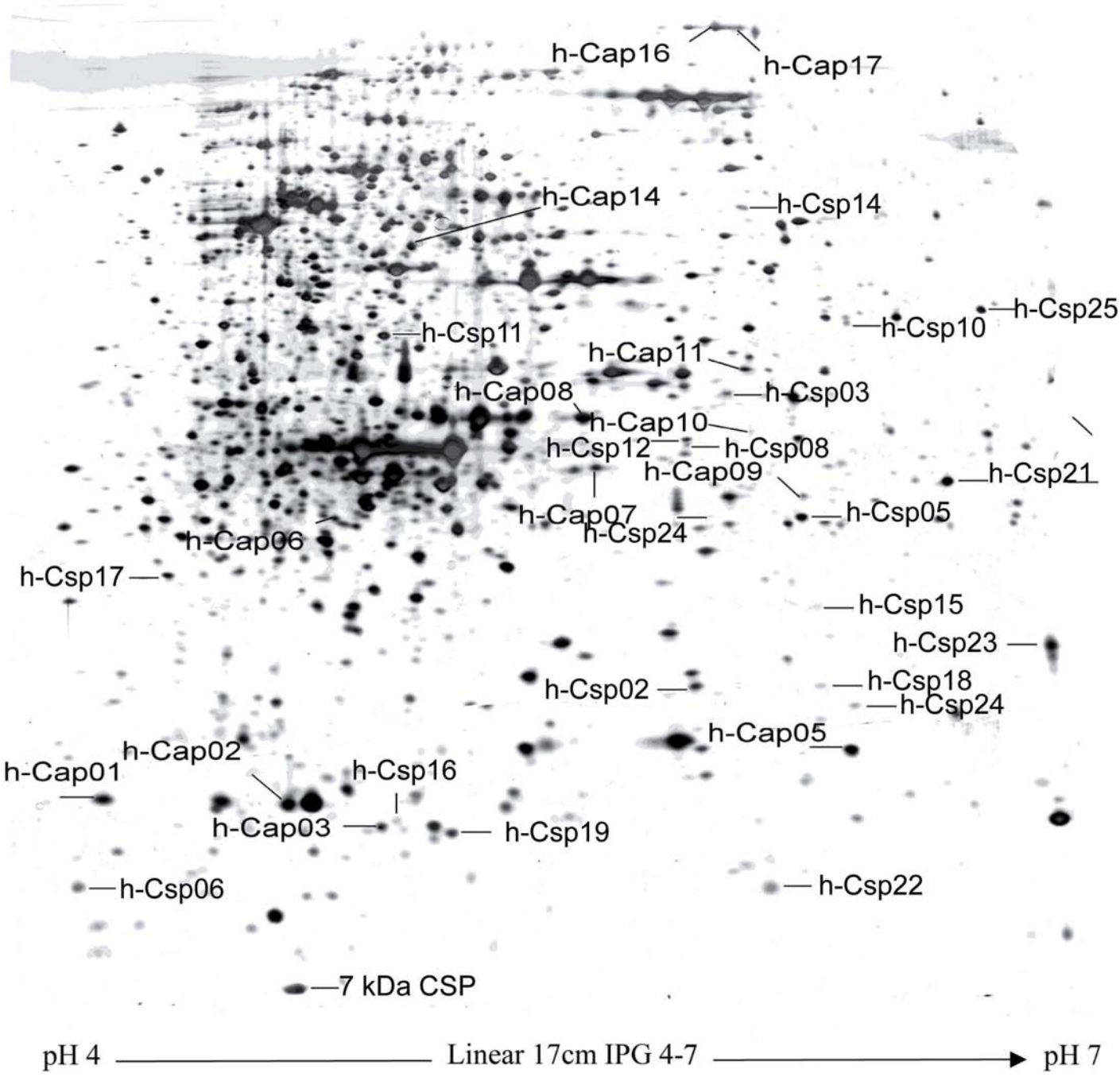
0,03
0,02
0,01
0,00
-0,01
-0,02
-0,03
-0,04
Cold Shock
Standard
Acclimation
-0,04
-0,03
-0,02
-0,01
0,00
0,01
0,02
0,03
Principal component 1 (16.3%)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h-Cap10
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h-Csp21
h-Cap07
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h-Csp24
h-Cap06
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h-Csp15
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h-Csp02
h-Csp18
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h-Cap02
h-Cap01
h-Csp16
h-Cap03
h-Csp19
h-Csp06
h-Csp22
7 kDa CSP
pH 4
Linear 17cm IPG 4-7
pH 7


1
50
69
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cspC_B_subtilis ..MEQGTVKWFNAEKGFGFIERENGDDVFVHFSAIQSDGFKSLDEGQKVSFDVEQGARGAQAANVQKA.
Consensus ..Ma#GTVKWFNAeKGFGFItge#G.DVFvHFSAIQt#GFKtL#EGQkV.%DvE#G.rgpqa.n..k..
RNP1
RNP2