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The nine peptidoglycan hydrolases genes in *Lactobacillus helveticus* are ubiquitous and early transcribed

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ABSTRACT

Peptidoglycan hydrolases (PGHs) are bacterial enzymes that can hydrolyze the peptidoglycan in bacterial cell wall leading to autolysis. By releasing intracellular enzymes, autolysis of *Lactobacillus helveticus* has important applications in cheese ripening as its extent varied from strain to strain. Nine genes coding PGHs were previously annotated in the genome of the high autolytic strain *L. helveticus* DPC 4571. This study was conducted to evaluate the clone diversity of the nine PGHs genes within a collection of 24 *L. helveticus* strains, highly diverse in terms of origin, biotope and autolytic activity. Pulsed field gel electrophoresis was applied to assess the genomic diversity of the 24 strains. The presence or absence of nine PGHs genes was verified for all *L. helveticus* strains. Nucleotide and deduced amino acid sequence were compared for six relevant strains. Finally, gene expression was monitored by reverse transcription during growth and by zymogram for 12 strains. The nine PGHs genes are ubiquitous and transcripted early during growth. Zymograms were similar in terms of molecular size of the bands, but exhibited strain to strain variations in the number of bands revealing from 2 to 5 lytic bands per strain.

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1. Introduction

Lactobacillus helveticus is a thermophilic lactic acid bacterium (LAB) used in the dairy industry as a starter or an adjunct culture for extra-hard, semi-hard and Swiss type cheese manufacture as well as for some types of fermented milk processing (Beresford et al., 2001; Gobetti et al., 2010; Slattery et al., 2010). By releasing intracellular enzymes, autolysis of *L. helveticus* has many impacts on cheese ripening and its extent is known to be strain dependent (Crow et al., 1995; Hannon et al., 2003, 2006, 2007; Lortal and Chapot-Chartier, 2005; Valence et al., 2000).

Autolysis is provoked by an enzymatic hydrolysis of the cell wall peptidoglycan, the three dimensional network protecting and maintaining shape of the bacterial cell, by endogenous enzymes called peptidoglycan hydrolases (PGHs) or autolysins. These are involved in numerous modifications of the peptidoglycan during growth and division as for example; regulation of peptidoglycan synthesis, cell wall turnover during growth, cell separation, sporulation and formation of flagella (Vollmer et al., 2008). Several PGHs, with similar or distinct specificity can coexist in a cell. Based on the cleavage specificities on peptidoglycan, the PGHs are divided into: (i) β-N-acetylmuramidases, (ii) β-N-acetyl glucosamidases, (iii) N-acetylmuramyl-L-amidases, and (iv) peptidase (endopeptidases, carboxypeptidases) (Layec et al., 2008; Scheurwater et al., 2008).

For LAB, the characterization of PGHs has been performed at the genome level for *Lactococcus lactis* (Buist et al., 1995; Lepeuple et al., 1998a, 1998b; Pillidge et al., 2002). For that species 5 PGHs were found: AcmA to AcmD with a catalytic domain homologous to that of enterococcal muramidase and YjgB a putative endopeptidase. The differences of the level of transcription suggested that these enzymes could be involved in different functions during growth (Huard et al., 2003, 2004). For the sequenced strain, *L. helveticus* DPC 4571, nine PGHs genes were annotated on the genome (Callanan et al., 2008).

In vitro screening as well as cheese manufacture shows that autolysis varies from strain to strain, for *L. lactis* as well as for *L. helveticus.* However the mechanisms involved are still not fully elucidated. Prophage expression seems crucial for *L. lactis* lysis whereas a prophage cured derivative lysed with the same extent as its mother strain for *L. helveticus,* suggesting different mechanisms (Deutsch et al., 2002; Kozakova et al., 2010; Kenny et al., 2005; Lortal et al., 1997a; Valence et al., 2000).

The variation in autolysis extent of *L. helveticus* strains could be due to PGHs complement and/or to cell wall structural differences. The aim of this study was to explore the diversity of genes coding for PGHs in a collection of 24 strains of *L. helveticus*, the most diverse possible in terms of several origins and biotopes.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

The origin of the strains used in this study is given in Table 1. Strains were stored at −20 °C in cryotubes (Biovalley, Marne-La-Vallée, France). Cultures were performed at 43 °C in MRS broth (Difco Laboratories, USA), growth was monitored at 650 nm using a spectrophotometer (Beckman, DU 7400). Viability of *L. helveticus* strains was determined by the pour plate technique on MRS agar (pH 5.4) under anaerobic conditions (Anaerocult, Merck, Darmstadt, Germany) at 43 °C for 48 h. Duplicate samples were taken and the average results were expressed in CFU/ml.

2.2. Strains identification

Strains were identified by multiplex PCR (Ventura et al., 2000) with modification of annealing temperature (55 °C instead 60 °C) and by whole 16S rRNA gene sequencing using W001 and W002 primers (Godon et al., 1997). Assembly of sequences was performed by Vector NTI (Invitrogen, Carlsbad, CA, USA). Analysis of whole 16S rRNA sequence was performed by Blast on NCBI and on RDP classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) (Wang et al., 2007).

2.3. Pulsed field gel electrophoresis (PFGE)

The culture and the agarose blocks were prepared as described previously (Lortal et al., 1997b). The blocks were equilibrated for one hour in a restriction buffer at 4 °C and transferred to 300 µl fresh digestion buffer containing 15 U of *Sma*I, 25 U of *Sgr*AI or 30 U of *Sbf*I endonucleases (New England BioLabs, Beverly, MA, USA). The blocks were incubated overnight at 25 °C for *Sma*I and at 37 °C for *Sgr*AI and *Sbf*I. PFGE was performed in a Bio-Rad CHEF DRII electrophoretic cell on 1% (w/v) agarose gel (Ultrapur, Gibco-BRL, Scotland) in running buffer (45 mmol/L Tris, 45 mmol/L boric acid, 1 mmol/L EDTA, pH 8.0) at 200 V and at 14 °C with following pulsed times and total running time: *Sma*I (initial time — 10 s, final time — 10 s, total running time — 24 h), *Sgr*AI (10 s, 15 s, 22 h) and *Sbf*I (10 s, 20 s, 22 h). The TL marker was used in this study (Lortal et al., 1997b). After electrophoresis, gels were stained by ethidium bromide, visualized using UV light, photographed and analyzed by the BioNumerics (Applied Math, Austin, TX, USA). Clustering of strain profiles obtained from genome restriction

Table 1
Original of the strains used in this study.

Species	Strain	N°CJRM-BIA	Biotope	Origin	Reference
L. helveticus	CCDM BAII	nd	nd	Czech Republic	
	CCDM 112	nd	Highland isolate	Czech Republic	
	CCDM 121	nd	*L. helveticus* var. *pragenesis,* starter	Czech Republic	
	CCDM 125	nd	Starter	Czech Republic	
	CCDM 136	nd	Highland isolate	Czech Republic	
	CCDM 140	nd	Highland isolate	Czech Republic	
	CCDM 380	nd	nd	Czech Republic	
	CCDM 466	nd	Highland isolate	Czech Republic	
	CCDM 467	nd	Highland isolate	Czech Republic	
	CCDM 468	nd	Highland isolate	Czech Republic	
	CCDM 499	nd	Highland isolate	Czech Republic	
	CP 615	CIRM-BL4 105	Isolate Japan	Japan	Lortal et al., 1997a, 1997c.
	CIP 103 146^T	CIRM-BIA 101	Starter, Emmental production	France	Lortal et al., 1997a, 1997c.
	CNRZ 32	CIRM-BLA 103	Starter, Comté production	France	Lortal et al., 1997a, 1997c; Deutsch et al., 2002; Hannon et al., 2006.
	CNRZ 241	CIRM-BLA 102	Starter, Comté production	France	Lortal et al., 1997a, 1997c; Deutsch et al., 2002.
	CNRZ 303	CIRM-BIA 100	Starter, Comté production	France	Lortal et al., 1997a, 1997c; Deutsch et al., 2002; Hannon et al., 2006
	CNRZ 414	CIRM-BLA 106	Isolate from Koumis (cow's milk)	Russia	Lortal et al., 1997a, 1997c; Deutsch et al., 2002
	DPC 4571*	nd	Swiss chesse whey	Ireland	Hannon et al., 2003, 2007; Hickey et al., 2007.
	ICT BROI*	nd	Isolate from raw cow's milk	Czech Republic	
	ICT CH1	nd	Commercial starter	Czech Republic	
	ICT KUM*	nd	Isolate from Koumis (mare's milk)	Kazakhstan	
	ISLC 5	CIRM-BIA 104	Starter, Parmasan production	Italy	Valence and Lortal, 1995; Lortal et al., 1997a, 1997c.
	ITG LH1*	CIRM-BL4 107	Serum	France	Valence and Lortal, 1995; Lortal et al., 1997c; Hannon et al., 2006.
	ITG LH77*	CIRM-BLA 99	Starter, Comté production	France	Valence and Lortal, 1995; Lortal et al., 1997c.
L. acidophilus	CNRZ 204	CIRM-BIA 440	Human	France	
L. delbrueckii subsp. *bulgaricus*	CNRZ 207^T	CIR.M-BIA 220	Emmenthal Swiss cheese	France	
L. delbrueckii subsp. *lactis*	CNRZ 208^T	CIRM-BIA 658	Bulgarian yogurt	France	
L. crispatus	CIP 102990	CIRM-BIA 664	Conjunctivitis	France	

[A]CCDM, Czech Collection of Dairy Microorganisms, Prague, the Czech Republic.
[B]CIRM-BIA, Centre International de Ressources Microbiennes-Bacteries d'intérét alimentaire, INRA Rennes, France.
[C]CNRZ, Centre National de Recherche Zootechnique collection, INRA Jouy-en-Josas, France.
[D]CIP, Collection of Institut Pasteur, Paris, France.
[E]ICT, Institute of Chemical Technology, Prague, Czech Republic.
[F]ISLC, Istituto Sperimentale Lattiero Caseario di Lodi, Italy.
[G]ITG, Institut Technique de Gruyère, La Roche-sur-Foron, France.
[H]nd, not determined.
[I]The strains in bold type were studied by RT-PCR.
[J]The PGH genes of the strains marked with star (*) were sequenced.

by three endonucleases was accomplished by using unweighted pair group method with arithmetic averages (UPGMA) and standard deviation 3.3%.

2.4. DNA and RNA extraction

Genomic DNA (gDNA) was extracted from strains grown for 12 h in MRS broth by DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) using the manufacturer's protocol for Gram-positive bacteria with two modifications: (i) initial enzymatic lysis step with lysozyme (20 mg/mL, Tris–HCl 20 mmol/L pH 8.0, EDTA 2 mmol/L, Triton X-100 1.2%) was prolonged for 1 h at 37 °C and (ii) the temperature for cleaving proteins with proteinase K was increased to 70 °C for 30 min.

Total RNAs were extracted from strains grown for 4, 8, 12, 16, 24 and 48 h in MRS broth by RNeasy Mini Kit (QIAGEN) also following the manufacturer's instructions. Enzymatic lysis was performed by addition of 100 µL lysis buffer (lysozyme 20 mg/mL, Tris–HCl 10 mmol/L pH 8.0, EDTA 1 mmol/L) for 15 min at room temperature. Thereafter, the cells were disintegrated by addition of zinc beads (100 mg) and processed twice for 45 s at 30 s^{-1} in bead mill Retsch MM301 (Retsch, Haan, Germany). For elimination of gDNA residues from RNA extracts, the samples were digested by DNAse ready-to-use in Dnase Ambion kit (Ambion, Austin, TX, USA). DNA and RNA concentrations were estimated at a wavelength of 260 nm with a Nanodrop ND-1000 spectrophotometer (Labtech, Palaiseau, France).

2.5. Primer design

The identical regions between orthologue genes of *L. helveticus* DPC 4571 and *L. acidophilus* NCFM were determined by multiple sequence alignment using CLUSTLW (http://clustalw.genome.jp/). In these regions the primers (Table 2) for each of nine *L. helveticus* genes were designed using the Primer 3 (http://frodo.wi.mit.edu) with the following parameters: (i) G + C content from 36% to 60% and (ii) primer size between 20 and 25 bp. All studied genes were available in the GenBank: *L. helveticus* (NC_010080) and *L. acidophilus* (NC_006814).

2.6. Reverse transcription (RT) and PCR experiments

Total RNAs were converted into DNA (cDNA) by qScript™ cDNA Synthesis kit (Quanta Bioscience, Gaithersburg, MD, USA). Negative PCR control was achieved with extracted RNA to verify the total absence of DNA before conversion into cDNA for each experiment. The PCR was done with gDNA and cDNA. The composition of each PCR reaction was as follows: 20 ng of genomic DNA, 2.5 µl of 10× buffer containing $MgCl_2$ (QIAGEN), 1 µl of 5 mmol/L dNTP Mix (QIAGEN), 1 µl of each primer (50 µmol/L) and 2.5 U of *Taq* DNA Polymerase (QIAGEN) in a final volume of 25 µl. Each PCR was carried out with Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) under following conditions: the initial denaturation step at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at $T_{annealing}$ (Table 2) for 30 s and extention at 72 °C for $t_{extention}$ (Table 2), final extension step was followed by an additional 10 min elongation period and then kept at 4 °C. Negative and positive (DPC 4571) controls were included in all PCR sets. All PCR products (5 µl) were separated by electrophoresis in 1.0% (w/v) agarose gel (Interchim, Montlucon, France) in 0.5× TBE buffer (Sigma-Aldrich, Lyon, France) at 100 V for 30 min in a OneRun system (Biorad, San Diego, CA, USA). The ready-to-use O'RangeRuler 100-bp DNA ladder (Fermentas, St. Remy les Chevreuse, France) was used as a molecular marker. After electrophoresis, gels were stained by ethidium bromide, visualized by transillumination with UV light and photographed.

2.7. DNA sequencing and sequence analysis

The PCR products were sequenced by the Sanger method by AGOWA (Berlin, Germany). Sequence assembly was performed by Vector NTI (Invitrogen, Carlsbad, CA, USA). For six strains of *L. helveticus* (CNRZ 32, ITG LH1, ITG LH77, ITC BROI, ITC KUM and DPC 4571) sequence alignment and comparisons were performed with the ClustalW algorithm in MEGA 4 software (The Biodesign Institut, Tempe, AZ, USA). The number of differences in nucleotide sequence, amino acid sequence and amino acid sequence in conserved region was determined between strains and reference strain DPC 4571.

2.8. Zymogram analysis

Zymogram was carried out as described by (Valence and Lortal, 1995; Lortal et al., 1997c). Samples were prepared from pellets (corresponding to 4 mg of dry weight) of washed whole cells and 100 µl Laemmli buffer (62.5 mmol/L Tris–HCl, pH 6.8 containing 10% glycerol, 2% SDS and 5% 2-mercaphoethanol), mixed and heated for 2 min at 100 °C. SDS-polyacrylamide separating gels (12%) containing 0.2% (w/v) g autoclaved whole cells of *L. helveticus* (CCDM 466, CNRZ 414, ICT BROI, ICT KUM, ISLC5, DPC4571) were used as substrate to

Table 2
Specific primers designed for each of the PGH genes identified in *L. helveticus* DPC4571 genome using Primer3 software.

Locus	Primer ID	Sequence (5′-3′)	PCR parameters		
			$T_{annealing}$(°C)	$t_{extension}$(s)	PCR product size (bp)
Lhv_190	Lhv_190 Fwd	ATGAAGAAGAGACTTTTGACCA	50	120	1197
	Lhr_190 Re	TTGACGGATACCAATTCTGT			
Lhv_191	Lhv_191 Fwd	GTTTTATCCAGATTGAGCTTTGTGA	57	15	442
	Lhv 191 Re	TAGCCCATCACAACAACAGG			
Lhv_549	Lhv_549 Fwd	AGCGTAAAGACTGTTGTTCGTGT	58	15	346
	Lhv_549 Re	TCGTCATCACCGCAGAAGTA			
Lhv_649	Lhv_649 Fwd	GGGATCGAGCTGAATCAAGA	53	30	510
	Lbv_549 Re	GTACATCGTAATGCCCTTTTC			
Lhv_1059	Lhv_1059 Fwd	TGAGAAGCGAAGTTCACAGG	57	30	400
	Lhv_1059 Re	GCCAACATTGACGTTTGAAG			
Lhv_1295	Lhv_1295 Fwd	GAATACTGCTCGGCCACAAA	57	30	510
	Lhv_1295 Re	AGTGGCAAGGAACACCATGT			
Lhv_1307	Lhv_1307 Fwd	GTGCTATCCATGTTAAAGCTTGTG	58	30	216
	Lhv_1307 Re	AACATCCTAAACCGCTGTCG			
Lhv_1433	Lhv_1433 Fwd	GGGACTACTTACCGCAATCCTAA	57	60	885
	Lhv_1433 Re	ACGCCAGTTAGCACGAGAAT			
Lhv_2053	Lhv_2053 Fwd	CGGCAACAATATCAACATGG	57	15	281
	Lhv_2053 Re	CGTTAAGACCACGCCAGTTA			

detect lytic activities. After electrophoresis (constant voltage 120 V for first 10 min and 180 V for 65 min), the gels were perfused for 30 min in distilled water at 37 °C, then transferred to renaturing buffer (50 mmol/L Tris–HCl, 1% Trition-X100, pH 8.6) and shaken gently for 3 h at 37 °C. In order to determine the apparent molecular weight, low molecular weight marker — LMW (Amersham, GE healthcare, Little Chalfont, UK) and pre-stained molecular weight marker — PMW (Sigma, St. Luis, MO, USA) were used. The renatured autolysins appeared as clear translucent bands on the opaque background. The contrast was enhanced by staining the gels in 0.1% (w/v) methylene blue in potassium hydroxide 0.01% (w/v). Photos of the gels were taken by using a luminous table and digital single lens reflex camera Pentax (K20, HOYA Corp., Tokyo, Japan).

3. Results

3.1. Strains identification and typing

L. helveticus strains were chosen according to their geographic origin and biotopes and to their difference in autolytic properties when data were available in literature (Table 1). Thus the collection of 24 strains comprises very high autolytic strains like DPC 4517 as well as low autolytic strains such as ITG LH1. Identity of all 24 tested strains was confirmed as *L. helveticus* using two methods, species-specific PCR and sequencing of 16S rRNA gene (data not shown).

Diversity of 24 strains was confirmed by PFGE using three rare-cutting restriction endonucleases *Sma*I, *Sgr*AI and *Sbf*I that produced a discrete pattern of bands for the fingerprinting. The final dendrogram (Fig. 1), assembling restriction patterns of *Sma*I, *Sgr*AI and *Sbf*I for all strains, showed a genomic diversity from 75% to 100%. Except for duplicate samples of CNRZ 32, there are two identical strains CNRZ 303 and CNRZ 414. More than 95% of similarity was found for the eight strains (CCDM 121 and CCDM 125, CCDM 112 and ICT CH1, CNRZ 32 and CNRZ 241, CCDM 136 and CCDM 140) and the other strains could be divided into four groups (profile similarity below 80%).

3.2. PCR detection of PGHs

The nine genes identified as peptidoglycan hydrolases were analyzed *in silico* (Table 3). Autolytic system of *L. helveticus* would consist of proteins from 18.1 to 42.9 kDa and the main autolytic specificities, muramidases, peptidases and amidases, are encountered. Their isoelectric point is from to 8.38 to 10.09. To detect in the 24 strains (Table 1) the presence or absence of those nine potential PGHs genes annotated in the genome of *L. helveticus* DPC 4571, nine pairs of primers in conserved regions were designed (Table 2). Their specificities were tested towards corresponding PGHs genes of *L. helveticus* DPC 4571 and some of the phylogenetically close lactobacilli (*L. acidophilus* CNRZ 204, *L. delbrueckii* subsp. *bulgaricus* CNRZ 207^T, *L. delbrueckii* subsp. *lactis* CNRZ 208^T and *L. crispatus* CIP 102990). The nine pairs of primers were specific of *L. helveticus* with amplicons of the expected size (Table 3). Primers were then applied to the 24 strains of *L. helveticus*. For all strains, PCR product of the expected size was found suggesting that the nine genes are present in the 24 strains of *L. helveticus*, and that they are ubiquitous. For the strain ITG LH77 and gene Lhv_1295, the specific PCR product of 482 bp plus one fragment about 1500 bp was obtained. Sequencing of the extra product showed that it was a non-specific PCR product (data not shown).

3.3. Sequencing of PGHs amplicons

The sequencing of the nine PCR products was performed to ensure they correspond to the nine PGHs genes and to study further clone variability for six relevant strains DPC 4571, CNRZ 32, ITG LH1, ITG LH77, ICT BROI, and ICT KUM. The size of the PCR products is close to the gene length for genes Lhv_190 and Lhv_1295. For genes Lhv_191, Lhv_549, Lhv_649, Lhv_1059, Lhv_1307, and Lhv_1433 the size of the PCR products represents 50% of the gene length and only 30% for the gene Lhv_2053. The analysis of the sequences revealed high conservation of PGH genes with an average genes identity from 98.2% to 99.4% comparing to DPC4571 genes. This leads to average

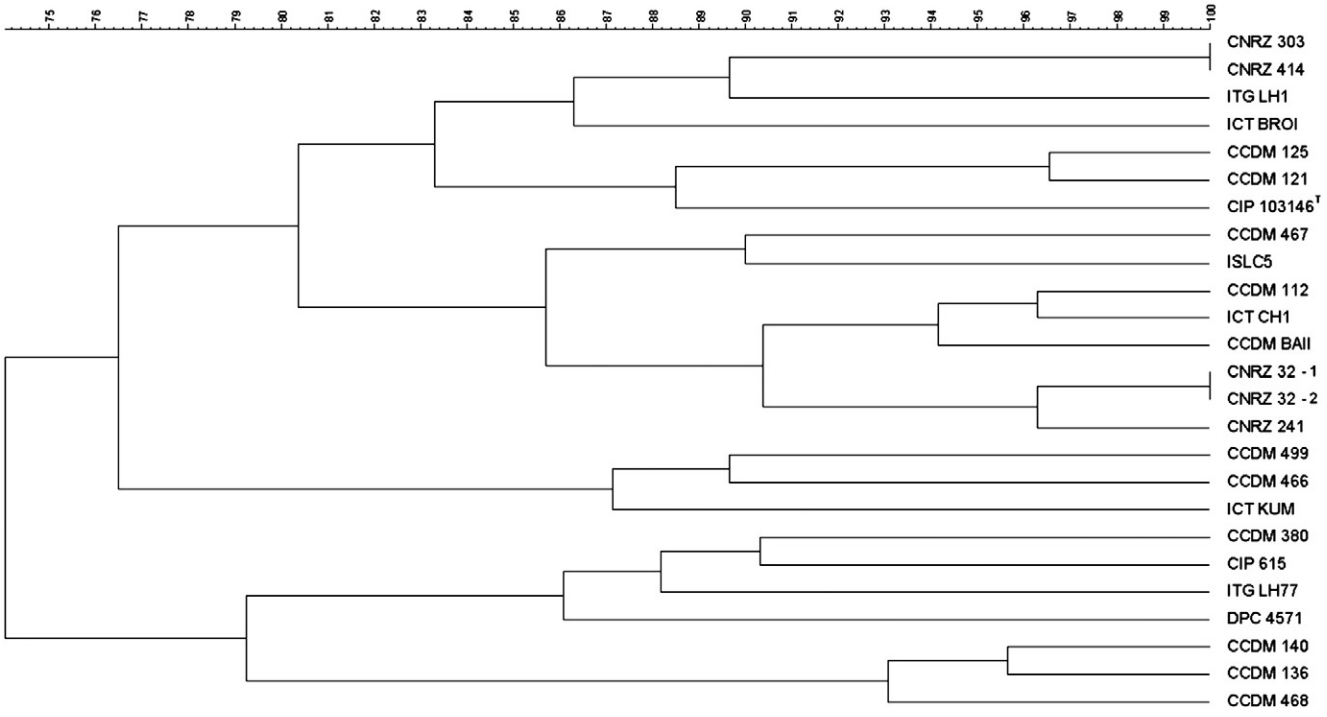

Fig. 1. Dendrogram based on UPGMA clustering of Dice association coefficients for normalized patterns obtained after assembling of restriction patterns of *Sma*I, *Sgr*AI and *Sbf*I PFGE with total genomic DNA from 24 *L. helveticus* strains. Strain CNRZ 32 was analyzed in duplicate (CNRZ32-1 & CNRZ32-2).

Table 3
In silico analysis of protein specificity and characteristics of the nine peptidoglycan hydrolases predicted from the genome of *L. helveticus* DPC 4571.

Gene	Protein	Specificity	Protein length (aa)	SS cleaving position (aa-aa)	SS cleaving probability (%)	Molecular weight (Da)	Isoelectric point
Lhv_190	N-acetyl-muramidase	Two regions: 1-Muramidase 2-SLAP-Bacterial surface layer protein	407	22-23/38-39	45/55	42,944/41,252	9.65/9.62
Lhv_191	Amidase	Two regions: 1-N-acetylmuramoyl-L-alanine amidase, 2-SLAP-Bacterial surface layer protein	363	29-30	100	38,152	8.38
Lhv_549	N-acetyl-muramidase	Muramidase	215	29-30/34-35	30/70	21,554/20,950	9.17/9.18
Lhv_649	Hypotetical protein	Glyco_25 hydrolase family	234	31-32/43-44	70/30	23,450/22,125	9.70/9.65
Lhv_1059	Putative N-acetyl-muramidase	LysM domain	199	31-32	100	18,495	9.96
Lhv_1295	Putative enterolysin	Peptidase_23 family (Gly-Gly endopeptidase)	218	21-23/33-34	80/20	22,789/21,442	9.67/9.67
Lhv_1307	Putative enterolysin	Peptidase_23 family (Gly-Gly endopeptidase)	181	22-23	100	18,171	9.53
Lhv_1433	Lysin	Four regions: 1, 2, 3-Glyco_25 hydrolases family, 4-SLAP-Bacterial surface layer protein	406	36-37	100	40,007	9.41
Lhv_2053	Lysin	Three regions: 1, 2, 3-G1yco_25 hydrolases family	276	35-36	100	25,754	10.09

[A]SS, signal sequence.
[B]aa, amino acid.

sequence differences from 1 to 9 amino acids for the sequences deduced from the nine PGH genes analyzed (Table 4).

Interestingly, the gene Lhv_190 forming an operon with Lhv_191, is the only one that showed a high variability for its amino acids sequence (from 8 to 14 differences) depending of the strain considered. Four genes, Lhv_649, Lhv_1059, Lhv_1295, and Lhv_1433, showed variation only for one strain (ICT BROI, CNRZ 32, ITG LH77 or ICT KUM respectively).

3.4. Detection of PGHs transcripts

The presence of nine PGH genes in 24 *L. helveticus* strains raises to the question of their functionality. In order to select an optimal time for detection of PGHs transcription, two extreme strains in terms of growth rate (ITG LH1, ICT BROI) and reference strain DPC 4571 were chosen to observe the transcription of all nine PGHs genes in MRS broth during the growth at 4, 8, 12, 16, 24 and 48 h. All annotated PGHs genes were transcribed after 4 h of growth (beginning of the exponential growth phase) until 24 h (stationary phase). Transcription of PGHs seems to be growth rate dependent (Fig. 2). In case of quickly growing strains ITG LH1 transcription is stopped after 24 h and by contrast for slowly growing strain ICT BROI, which is able to transcribe all PGHs until 48 h.

In order to determine if all genes are transcribed for 12 *L. helveticus* strains (Table 1) RNA was extracted at the end of the exponential growth phase (12 h). A transcript for each of the nine genes was detected in all of the tested strains (data not shown).

3.5. Detection of PGHs expression using zymogram

The activity of PGHs was detected by renaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with substrate-containing gels (zymogram). By using six autoclaved bacterial cells of *L. helveticus* as substrate (CCDM 466, CNRZ 414, ICT BROI, ICT KUM, ISLC5, DPC4571), lytic activity in the exponential growth phase of 12 *L. helveticus* strains was tested.

Irrespective of the substrate, similar profile on zymogram was obtained. Only the intensity of bands differed, the best results were obtained using CCDM 466 and CNRZ 414 as substrate (Fig. 3).

The zymogram revealed a slightly different profile for each strain. Generally, two categories of translucent zones were observed as described by Valence and Lortal, 1995: Cluster A — higher molecular weight product around 40 kDa (43–38 kDa) and Cluster B — lower molecular weight product from 30 kDa (25–32 kDa). Bands are distributed as follows: two or three bands in cluster A and from one to six bands in cluster B (Fig. 3). For strain BROI two more bands at 35 kDa and 20 kDa respectively, were clearly observed. It was not possible to determine accurately the apparent molecular weight for cluster A because of the predominant S-layer protein located just above and disrupting the area of molecular weight 43–45 kDa. The total number of bands in each profile never exceeded nine. *In silico* predictions (Table 3) revealed molecular weight of 18.1 to 42.9 kDa for PGHs and an isoelectric point systematically above 8.

Table 4
Homology of peptidoglycan hydrolytic genes sequence and hydrolases protein sequence between five strains of *L. helveticus* (CNRZ 32, ITG LH1, ITG LH77, ICT BROI, ICT KUM) and reference strain *L. helveticus* DPC 4571.

Gene	Gene length (bp)	PCR product length (bp)	Difference between strain and DPC 4571										Average	
			32J		LH1		LH77		BROI		KUM			
			N_{NS}	N_{AAS}	N_{NS}	N_{AAS}	N_{NS}	N_{AAS}	N_{NS}	N_{AAS}	N_{NS}	N_{AAS}	N_{NS}	N_{AAS}
190	1224	1197	16	8	16	8	45	14	19	8	12	8	22	9
191	1092	442	4	2	4	2	5	3	3	2	4	2	4	2
549	648	345	2	1	2	1	0	0	2	1	4	2	2	1
649	705	510	2	2	2	2	2	2	12	10	5	4	5	4
1059	600	400	15	10	6	3	4	2	4	2	4	2	7	4
1295	657	500	4	4	4	4	16	8	4	4	4	4	6	5
1307	546	216	4	3	4	3	0	0	2	1	2	1	2	2
1433	1221	885	1	1	1	1	2	1	0	0	22	6	5	2
2053	831	281	6	4	6	4	0	0	5	4	4	3	4	3

[A]$N_{NS,}$ number of differences in nucleotidic sequence.
[B]N_{AAS}, number of differences in amino acid sequence.

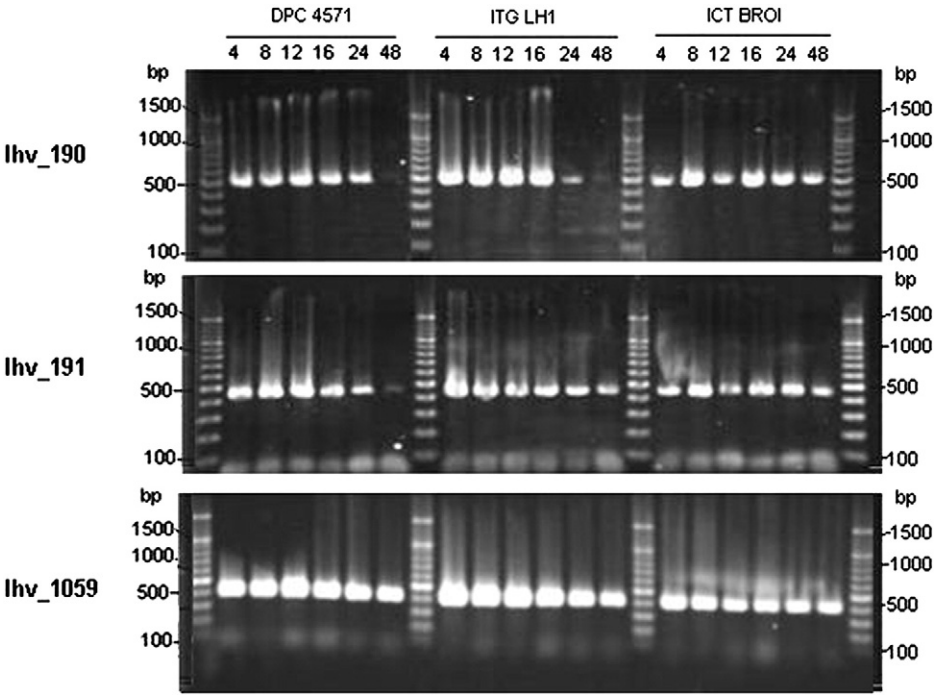

Fig. 2. Example of transcription for three of nine PGH genes (Lhv_190, Lhv_191 and Lhv_1059) for three strains of *L. helveticus* (ITG LH1, ICT BROI, DPC4571) during growth in MRS broth at 4, 8, 12, 16, 24 and 48 h, detected using RT-PCR.

The bands, which do not correspond with predicted size, could be the lytic enzymes modified during translation. Moreover, not all the enzymes can renature and be active on zymogram using this methodology, making uncertain any correlation between the deduced (Table 3) and the observed apparent molecular weight of the lytic bands (Fig. 3).

4. Discussion

As autolysis of lactic acid bacteria, and in particular *L. helveticus*, is important for cheese ripening, an improved starter selection relative to this technological property would be useful. Indeed, *in vitro* screenings do not really predict the behavior in cheese (Boutrou et al., 1998; O'Donovan et al., 1996; Treimo et al., 2006). Different hypotheses exist regarding strain dependence of autolysis, variation in lytic genes complement or their expression/regulation, or differences in cell wall composition between strains, or both. In order to test the first hypothesis, the peptidoglycan hydrolases gene content of 24 strains of *L. helveticus* was compared to the one of the sequenced strain DPC 4571 (Callanan et al., 2008) which is also known as a very autolytic strain (Hannon et al., 2007). The 24 strains were coming from various biotopes and their genomic profile similarity was first estimated by pulsed field gel electrophoresis using three restriction enzymes. The nine putative PGH genes of DPC 4571 were present in all strains; in addition, for three strains they were shown to be transcribed very early during growth (4 h) much before the start of lysis, suggesting they contribute first to the elongation/change in the peptidoglycan network. This early expression is in accordance with results obtained for AcmC and YjgB genes of *L. lactis* where the quantitative analysis of the transcript showed their expression at the beginning of the log phase (Huard et al., 2004). The presence of nine ubiquitous PGH genes by comparison to DPC 5471 does not exclude the presence of supplementary non related genes in the other strains; complete genome sequencing of more strains of *L. helveticus* will be necessary to ensure the total number of PGH genes in that species. By comparison, 5 PGH genes were found in *L. lactis,* 35 in *Bacillus subtilis*, and 38 in *Escherichia coli*. But it is important to notice that not all PGHs are potential autolytic enzymes causing lysis of the bacterial cells by

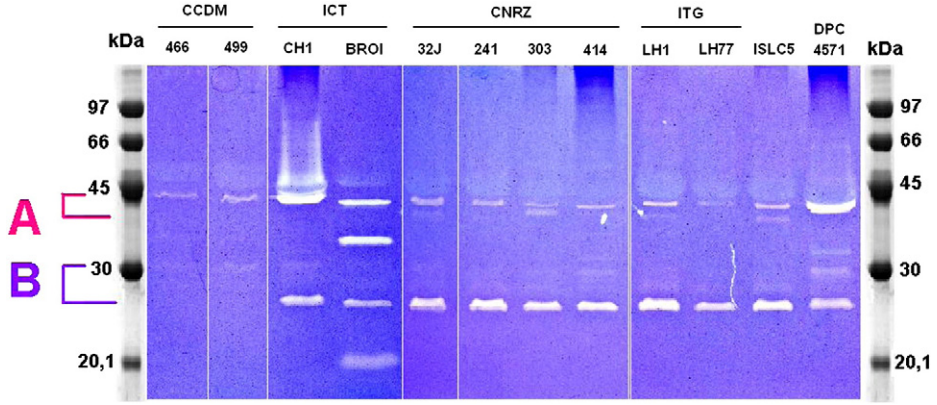

Fig. 3. Lytic gene activity on zymogram at 12 h (autoclaved cells of CCDM 466 were included in polyacrylamide separating gel as a substrate) for 12 strains of *Lactobacillus helveticus.*

splitting bonds in the peptidoglycan (Höltge, 1995; Huard et al., 2004; Smith et al., 2000).

Comparative analysis of sequenced PCR fragments revealed extensive conservation of all PGH genes among the strains (more than 98.2% to more than 99.4% depending on the gene considered; Lhv_190 being the most variable). This corresponds to very few significant amino acid changes. This high conservation of PGH genes in *L. helveticus*, whatever the strain and its origin, confirmed what was suggested before from zymogram analysis (Lortal et al., 1997c). Comparative analysis of the PGH AcmA amino acid sequence revealed similarity of 98% between two *L. lactis* subsp. *cremoris* strains, MG1363 and 2250, whereas between *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1363 the similarity for AcmA was only 42% (Pillidge et al., 2002). This author mentioned the ubiquitous presence of one gene — AcmA for *L. lactis* while the relative influence of AcmA on starter autolysis varied in a strain-dependent manner. Regarding the clonal diversity of PGH genes this study is the first on such a large number of strains combining expression and transcription. The steadily increasing number of genome sequences available for some species should allow us to study at least *in silico* the clonal diversity of PGH genes in the years to come.

Several lytic bands were detected using zymograms, in the molecular weight range expected from *in silico* deduction. It is interesting to notice that the total number of bands obtained by sum of bands of all strains never exceeds nine, which is the number of PGHs. However the correlation between lytic bands and genes cannot be made without targeted mutants. The profiles between strains were rather similar as observed before (Lortal et al., 1997c) however few strains exhibited unique bands like CCDM 466.

The large number of enzymes in *L. helveticus* and the absence of mutants do not facilitate the attribution of any role during lysis and the attribution of zymogram lytic band to one particular gene.

In conclusion, there was no diversity in PGH gene content, even if the strains had different autolytic activities in cheese. Highly autolytic strain DPC 4571 (Hannon et al., 2007) and low autolytic strain ITG LH1 (Valence et al., 2000) possess similar genes as well as the others strains. The explanation of difference in autolysis among *L. helveticus* strains can be due to post-transcriptional steps.

It can be also due to differences in cell wall composition. This hypothesis is supported by the fact that changing the *L. helveticus* strain used as substrate within the gel can led to more intense bands. Further research will be focused on cell wall polymers content like teichoic acid or neutral polysaccharides.

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