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prtH2, Not *prtH*, Is the Ubiquitous Cell Wall Proteinase Gene in *Lactobacillus helveticus*▽

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Lactobacillus helveticus **strains possess an efficient proteolytic system that releases peptides which are essential for lactobacillus growth in various fermented dairy products and also affect textural properties or biological activities. Cell envelope proteinases (CEPs) are bacterial enzymes that hydrolyze milk proteins. In the case of *L. helveticus*, two CEPs with low percentages of amino acid identity have been described, i.e., PrtH and PrtH2. However, the distribution of the genes that encode CEPs still remains unclear, rendering it difficult to further control the formation of particular peptides. This study evaluated the diversity of genes that encode CEPs in a collection of strains of *L. helveticus* isolated from various biotopes, both in terms of the presence or absence of these genes and in terms of nucleotide sequence, and studied their transcription in dairy matrices. After defining three sets of primers for both the *prtH* and *prtH2* genes, we studied the distribution of the genes by using PCR and Southern blotting experiments. The *prtH2* gene was ubiquitous in the 29 strains of *L. helveticus* studied, whereas only 18 of them also exhibited the *prtH* gene. Sequencing of a 350-bp internal fragment of these genes revealed the existence of intraspecific diversity. Finally, expression of these two CEP-encoding genes was followed during the growth in dairy matrices of two strains, ITG LH77 and CNRZ32, which possess one and two CEP-encoding genes, respectively. Both genes were shown to be expressed by *L. helveticus* at each stage of growth in milk and at different stages of mini-Swiss-type cheese making and ripening.**

Lactobacillus helveticus belongs to the group of lactic acid bacteria (LAB) and is widely used as a starter in the manufacture of dairy products such as hard cheeses like Emmental, Grana Padano, or Provolone (9, 11) and in many fermented milks (14, 15, 17, 28, 37, 38) because of its ability to produce lactic acid by catabolizing lactose.

L. helveticus strains are among the most nutritionally fastidious LAB as they show multiple amino acid auxotrophies. In order to overcome nutritional requirements when grown in milk, *L. helveticus* possesses a strong proteolytic system capable of producing short peptides and liberating amino acids from the casein matrix. This proteolytic system is composed of (i) cell envelope proteinases (CEPs) that hydrolyze caseins into oligopeptides, (ii) transport systems that allow uptake of oligopeptides, and (iii) various intracellular peptidases with differing and partly overlapping specificities, leading to a pool of free amino acids (13, 26).

Many LAB typically possess only one CEP-encoding genes (7, 12, 23, 32), but the presence of two or more different genes that encode CEPs has been reported in *L. bulgaricus* and *L. helveticus* (22, 31, 34). Among LAB, the proteolytic system of *L. helveticus* is considered to be one of the most efficient in cheese proteolysis (25), as well as in generating various bioactive peptides from caseins that are antihypertensive, are antimicrobial, reduce the risk of colon cancer, or are immunomodulators in fermented milk (15, 17, 19, 28, 37, 38) and in cheese (2). Several studies have biochemically characterized the CEPs in different strains of *L. helveticus* such as CP53, Zuc2, or L89 (16, 20, 27), but little information is available at the genetic level despite the widespread use of *L. helveticus* in dairy products. The *prtH* gene of *L. helveticus* CNRZ32 was identified and showed significant similarity to known lactococcal *prtP* genes (22). Moreover, the *L. helveticus* CNRZ32 strain possesses at least two distinct CEPs, as a *prtH* deletion mutant was indistinguishable from the wild type in growth rate and acid production in milk (22). The existence of a second CEP-encoding gene, named *prtH2*, in strain CNRZ32 has been reported, as well as the presence of two other putative genes that encode CEPs, named *prtH3* and *prtH5*, which are significantly induced during growth in milk versus MRS medium (Difco, Detroit, MI) (31). Only one complete genome of *L. helveticus* is currently available, that of strain DPC4571 (3). At least one gene that encodes a serine proteinase, lhv_1641, was found but was annotated as a pseudogene (3). Finally, analysis of the substrate specificities of proteinases for caseins in eight *L. helveticus* strains has confirmed the existence of CEP biodiversity in this species (18).

All of these data suggest that CEP-encoding genes could be variable among *L. helveticus* strains, and this biodiversity could influence some parameters of technological interest such as the growth rate in milk, acid production, or peptide liberation.

The aims of this study were to evaluate the diversity of genes that encode CEPs in a collection of strains of *L. helveticus* isolated from various biotopes and to study their transcription in dairy matrices.

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TABLE 1. *L. helveticus* strains used in this study

Strain	Biotope	Origin	Reference or source
CNRZ32[a]	Artisanal starter used in Comté	France	INRA of Jouy-en-Josas
CNRZ32JS	Artisanal starter used in Comté	France	J. Steele, University of Wisconsin, Madison
CNRZ32A	Mutant derived from CNRZ32JS	United States	J. Steele
CNRZ32B	Mutant derived from CNRZ32JS	United States	J. Steele
CNRZ32C	Mutant derived from CNRZ32JS	United States	J. Steele
CNRZ67	Artisanal lactic starter of Gruyère de Comté	France	
CNRZ240	Artisanal starter of Gruyère de Comté	France	
CNRZ241	Artisanal starter used in Comté	France	
CNRZ303	Artisanal starter used in Comté	France	6
CNRZ328	Artisanal starter of Emmental	Finland	
CNRZ414	Cow milk Koumis	Russia	
CNRZ450	Commercial starter of Emmental	France	
CNRZ498	Yogurt	France	
CNRZ762	Artisanal starter of Gruyère de Comté	France	
CNRZ891	Commercial starter	France	
CNRZ1080	ND[c]	China	
CNRZ1095	ND	Finland	
CNRZ1111	Commercial starter	France	
CNRZ1317	Commercial starter used in Tallegio	Italy	
ISLC5[b]	Artisanal lactic starter of Grana Padano	Italy	36
CP615	ND	Japan	
CIP 103146T[d]	Artisanal starter used in Emmental	France	
ROSELL 5088	ND	Canada	
ROSELL 5089	ND	Canada	
DPC4571	Swiss cheese whey	Ireland	T. Beresford, Teagasc
ITG LH1	Whey	France	5
ITG LH2	Aminopeptidase-deficient mutant strain derived from ITG LH1	France	1
ITG LH3	Aminopeptidase-deficient mutant strain derived from ITG LH1	France	1
ITG LH77	Artisanal starter	France	5

[a] CNRZ, Centre National de Recherche Zootechnique collection, INRA Jouy-en-Josas, France. The strains in bold were studied by RT-PCR and Southern hybridization.
[b] ISLC, Istituto Sperimentale Latiero-Caesario, Italy.
[c] ND, not determined.
[d] CIP, Collection of Institut Pasteur, France.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Twenty-nine strains of *L. helveticus* were chosen according to their technological significance in dairy products, as well as their geographic origins (Table 1). *L. helveticus* strains were propagated three times without shaking at 43°C in MRS medium (Difco, Detroit, MI) from a cryoball stored at −80°C. For growth in milk, 10 ml of ultra-high-temperature (UHT) milk was inoculated with an overnight MRS culture at different concentrations (1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001%). After 12 h of culture at 43°C without shaking, the first nonclotted dilution was used to inoculate 10 ml of UHT milk at 5%, which was then incubated at 43°C without shaking until the appropriate pH was reached.

Small-scale Swiss-type cheese making. Two small-scale Swiss-type cheeses (800 g) were made according to a standardized Emmental cheese-making process (24) from thermized skim milk and from an overnight culture in UHT milk of *L. helveticus* strain ITG LH77 or CNRZ32. A subculture was made at 44°C until 1.1% acidity was achieved in Phagex medium (Phagex LB; Laboratoires Standa, Caen, France) reconstituted at 13.5% (wt/vol) and heat treated at 85°C for 30 min. Three milliliters of this culture was added to the cheese milk (~10^5 CFU/ml). A freeze-dried starter culture of *Streptococcus thermophilus* (PAL-ITG ST20-87; Laboratoires Standa) for vat inoculation was also prepared in 13.5% (wt/vol) reconstituted Marstar 412A (Danisco, Dangé Saint Romain, France) until 0.95% acidity was achieved. Twelve milliliters of this culture (~10^6 CFU/ml) was added to 1 liter of the cheese milk.

Briefly, the milk was ripened with starters (*S. thermophilus* and either strain ITG LH77 or CNRZ32 of *L. helveticus*) at 32°C for 60 min and coagulated by the addition of calf rennet. The gel was cut into curd grains with a mean diameter of 4 mm. After 15 min of fore working, the whey-curd mixture was cooked at 53.5°C for 33 min. The stir-out duration was 45 min. The curd was then molded in 12-cm-diameter molds. The pressing step (6 h) and the acidification step (14 h) were conducted in thermostat-controlled ovens at 48 and 36°C, respectively, in order to reproduce the cooling rate of the center of a full-size Emmental wheel. The cheeses were then cooled and salted for 5 h in NaCl-saturated brine at 7°C. The cheeses were wrapped under vacuum in BK1L ripening bags (F-28; Cryovac, Epernon, France) and ripened at 12°C for 12 days.

Primer design and PCR experiments. Previous results have shown that *L. helveticus* CNRZ32 has at least two genes that encode CEPs (22, 31). We decided to align the two genes to highlight specific regions. At the nucleotide level, no significant identity was observed between the two genes (accession no. AF133727 and DQ826130). When the amino acid sequences (accession no. AAD50643 and ABI13574) were aligned, only 22% identity was observed. A homology search for both proteins was carried out with the BLAST program (http://www.ncbi.nlm.nih.gov/blast/; February 2008). PrtH of *L. helveticus* CNRZ32 was homologous to the cell wall-associated serine proteinase of *L. johnsonii* NCC533 (60% identity, 74% similarity), to the PII-type proteinase precursor of *Lactococcus lactis* subsp. *cremoris* (51% identity, 65% similarity), and to the cell wall-associated serine proteinase of *L. casei* ATCC 334 and BL23 (50% identity, 64% similarity), whereas PrtH2 of *L. helveticus* CNRZ32 was homologous to PrtP of *L. acidophilus* NCFM (60% identity, 74% similarity), to PrtR of *L. rhamnosus* BGT10 (40% identity, 53% similarity), and to PrtR of *L. casei* BL23 (35% identity, 51% similarity).

Specific primers for the *prtH* gene were chosen by aligning its sequence with the gene that encodes the cell wall-associated serine proteinase of *L. johnsonii* NCC533 and with *prtH2* of CNRZ32. Alignments were achieved with the BioEdit software. Primers were chosen both in regions conserved between *L. helveticus* CNRZ32 and *L. johnsonii* NCC533 genes to increase the hybridization probability in all of the strains to be tested and in regions not conserved between *prtH* and *prtH2* to avoid cross-hybridization. The same methodology was used to choose *prtH2*-specific primers by alignment with the gene that encodes the *L. acidophilus* NCFM PrtP proteinase. One of the primer pairs chosen corresponded to primers used previously to amplify the *prtH* gene (22).

The sequences and localizations of the different primers used are shown in Table 2 and Fig. 1. Each PCR was carried out with a Veriti 96-well thermal cycler

TABLE 2. Sequences and localizations of primers used in this work

Primer	Sequence (5′–3′)	Target sequence
PrtH-for-1	GGTACTTCAATGGCTTCTCC	1816–1835[a]
PrtH-rev-1	GATGCGCCATCAATCTTCTT	2282–2301[a]
PrtH-for-3	CTCAGCACCAGGTGGACATA	1755–1774[a]
PrtH-for-2	CGATGATAATCCTAGCGAGC	786–805[a,c]
PrtH-rev-2	TGGCAGAACCTGTGCCTA	1405–1422[a,c]
PrtH2-for-1	CCAGCTAATAATCAAGACCA	547–566[b]
PrtH2-rev-1	AACAGCATATTGAACTGCTC	1083–1102[b]
PrtH2-for-2	GAAGACAAGGTGCTGGTCAA	1592–1611[b]
PrtH2-rev-2	TAGCATTTTGGTCAAAGACA	2044–2063[b]
PrtH2-for-3	GTTGGTGCCGCAACTAAATC	1618–1637[b]

[a] *L. helveticus* CNRZ32 *prtH* gene; accession no. AF133727.
[b] *L. helveticus* CNRZ32 *prtH2* gene; accession no. DQ826130.
[c] These primers were used by Pederson et al. in 1999 (22).

(Applied Biosystems, Foster City, CA) in a final volume of 20 μl with 1.25 U of *Taq* DNA polymerase (Invitrogen, Cergy-Pontoise, France), 20 ng of genomic DNA, each deoxynucleoside triphosphate (Invitrogen, Cergy-Pontoise, France) at 200 μmol liter^{-1}, and each primer (Sigma-Aldrich, Lyon, France) at 25 μmol liter^{-1}. After a first denaturation step of 4 min at 95°C, the mixture was subjected to 30 cycles of three steps of 30 s (95°C, 58°C, and 72°C). A final extension at 72°C for 10 min was performed. PCR products were checked on a 2% (wt/vol) agarose gel (Interchim, Montluçon, France) in 1× Tris-borate-EDTA (TBE 1x; Sigma-Aldrich, Lyon, France) at 100 V for 45 min in an i-Mupid system (Eurogentec, Angers, France). The ready-to-use O'RangeRuler 100-bp DNA ladder (Fermentas, St. Rémy Lès Chevreuse, France) was used as a molecular weight marker.

RNA and DNA extraction. Total RNA extraction was carried out either from an overnight UHT milk culture (10 ml at 43°C) or from 10 g of mini-Emmental cheeses in order to have more than 10^8 CFU ml^{-1}. RNAs were obtained as described by Ulvé et al. (35), with the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) after adding 2 U of RNase-free DNase I (Ambion, Courtaboeuf, France) and incubation for 30 min at 37°C. DNA was extracted from 10 ml of *L. helveticus* strains cultivated for 16 h in MRS medium as previously described (8). DNA and RNA concentrations were estimated at a wavelength of 260 nm with a Nanodrop ND-1000 spectrophotometer (Labtech, Palaiseau, France).

Reverse transcription (RT)-PCR experiments. Total RNAs obtained from strains grown on MRS broth, milk, or cheeses were converted into cDNA with the QScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). PCR was achieved on cDNA with primers PrtH-for-1/PrtH-rev-1 and PrtH2-for-3/PrH2-rev-2 as described above for genomic DNA. For each experiment, a PCR negative control was achieved with extracted RNA to verify the total absence of DNA before conversion into cDNA.

Southern blotting. Internal fragments of the *prtH* and *prtH2* genes were used as probes. These fragments were amplified from the genomic DNA of strain CNRZ32 with primers PrtH-for-1/PrtH-rev-1, PrtH-for-2/PrtH-rev-2, PrtH2-for-1/PrtH2-rev-1, and PrtH2-for-3/PrtH2-rev-2 (Table 2 and Fig. 1) and labeled with the DIG DNA labeling and detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The probes were hybridized against EcoRV (New England BioLabs, Ozyme, Saint-Quentin, France) restriction digestion of the genomic DNAs of 16 *L. helveticus* strains separated on a 0.8% (wt/vol) agarose gel (Interchim, Montluçon, France) in TBE 1x (Sigma-Aldrich, Lyon, France) at 40 V for 16 h after transfer onto a Hybond-N membrane (GE Healthcare, Orsay, France). The ready-to-use O'GeneRuler 1-kb DNA ladder (Fermentas, Saint-Rémy Lès Chevreuse, France) was used as a molecular weight marker. The Southern blotting, prehybridization, and hybridization conditions used were described previously (33). Hybridization was carried out at 65°C for high-stringency conditions and at 42°C in the presence of 10% formamide for low-stringency conditions. Detection of specific hybrids was performed with the DIG DNA labeling and detection kit under the conditions recommended by the supplier (Roche Molecular Biochemicals, Mannheim, Germany).

DNA sequencing. DNA sequencing was performed on a double-stranded template from a PCR product by the Sanger method by AGOWA (Berlin, Germany). Homology searching was done with the BLAST program (http://www.ncbi.nlm.nih.gov/blast/), and sequence comparisons and alignments were performed with the BioEdit software and the ClustalW algorithm.

RESULTS

Organization of PrtH and PrtH2. PrtH of *L. helveticus* CNRZ32 is a 1,849-amino-acid (aa) protein with a deduced molecular mass of 204 kDa (Fig. 1) (22). It is synthesized as a preproenzyme and becomes mature after removal of the N-terminal 176 aa containing the 25-aa signal sequence and the 151-aa prodomain. It belongs to the subtilase family characterized by the catalytic triad Asp30-His94-Ser432 (Fig. 1) (30). It contains A, B, H, and W domains homologous to those of other subtilisin-like serine proteinases and an insertion domain in the catalytic domain. It lacks the LPxTG anchor motif but

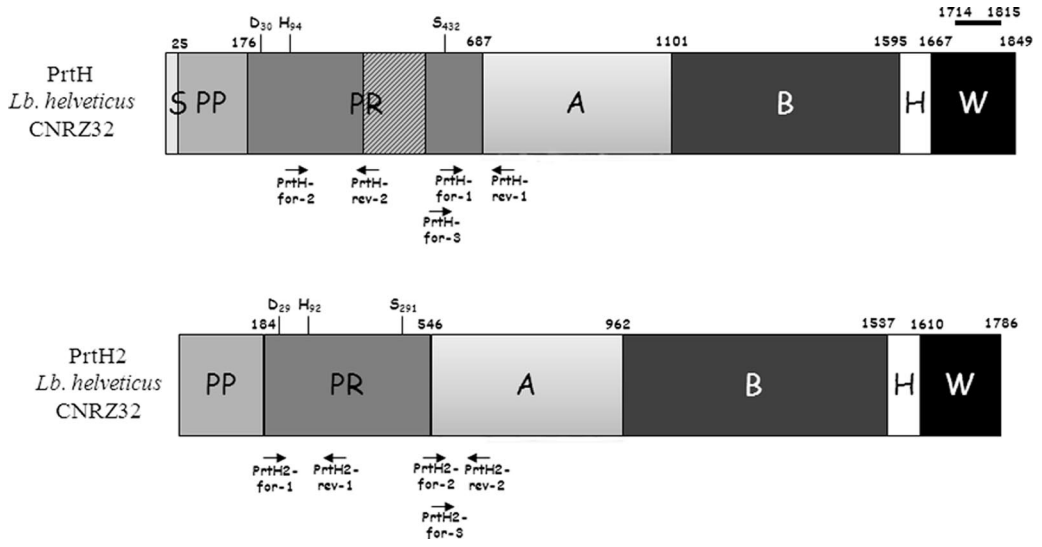

FIG. 1. Schematic representation of the predicted domains of PrtH (22) and PrtH2 of *L. helveticus* CNRZ32. Each number refers to the last amino acid of a domain. The catalytic triad and the amino acid number corresponding in the catalytic domain are indicated. The localization of the primers used in this work is shown by arrows. S, signal sequence; PP, preprodomain; PR, catalytic proteinase domain; A, A domain; B, B domain; H, helical domain; W, cell wall domain. The hatched part of the PrtH catalytic domain corresponds to insertion domain I. The C-terminal 101-aa region of PrtH showing homology with S-layer proteins is overlined.

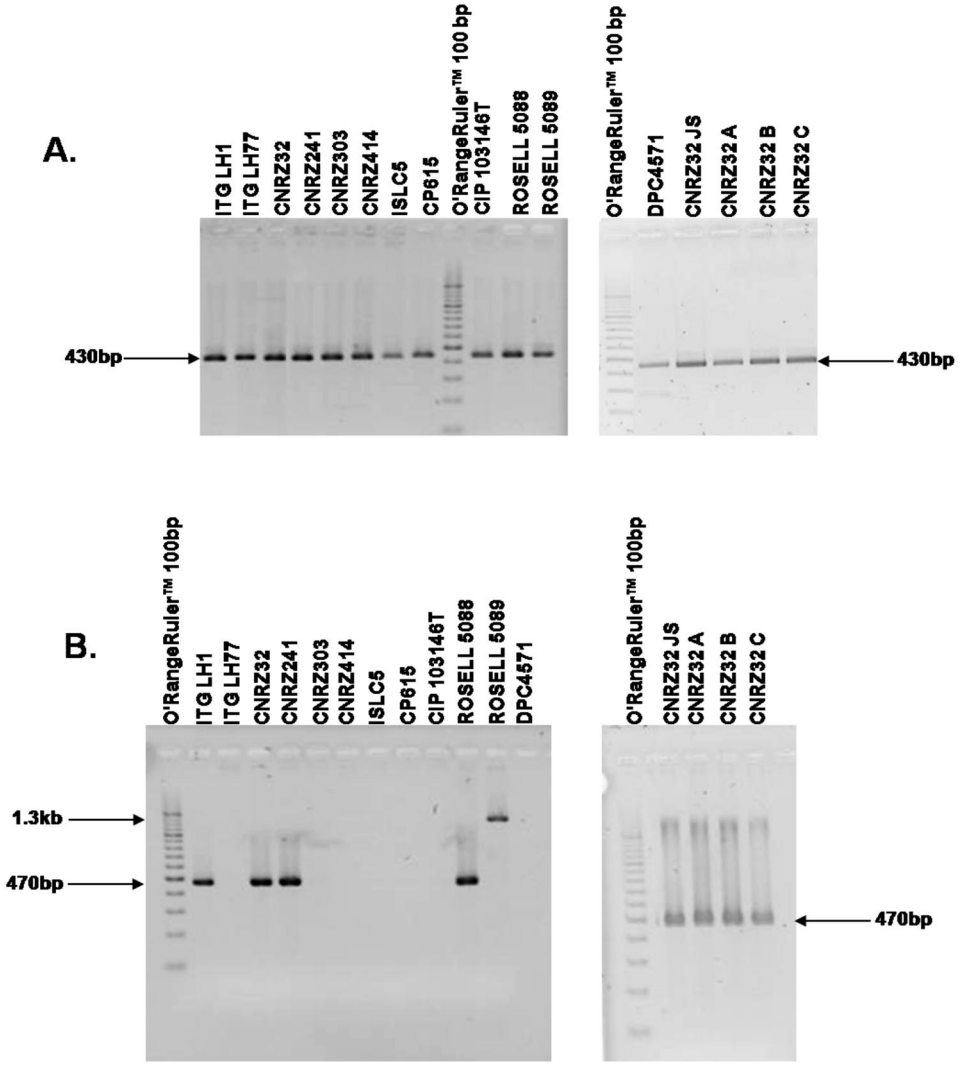

FIG. 2. Results of amplification of the *prtH2* (A) and *prtH* (B) genes of 16 strains of *L. helveticus*. PCR products were obtained with the PrtH2-for-3/PrtH2-rev-2 (A) and PrtH-for-1/PrtH-rev-1 (B) primers and separated on a 2% (wt/vol) agarose gel in TBE 1x at 100 V for 45 min. The O'RangeRuler 100-bp DNA ladder was used as a molecular weight marker.

has a C-terminal 101-aa region (from aa 1714 to aa 1815) homologous to that of S-layer proteins. When compared with PrtH and other members of the subtilisin-like serine proteinase family, PrtH2 exhibited a putative prosequence of 184 aa (Fig. 1) with a putative cleavage site most probably located between Ala184 and Asn185, as found in PrtR of *L. rhamnosus* BGT10 (21). PrtH2 could be synthesized as a proenzyme leading to a mature proteinase of 1,602 residues. As in PrtH, the N-terminal region of the mature PrtH2 proteinase showed the characteristics of subtilisin-like serine proteinases with the catalytic triad Asp29, His92, and Ser291 where residue 1 defines the first residue of the mature protein (Fig. 1). The oxyanion hole residue well conserved among CEPs was present in the catalytic domain of PrtH2, at position Asn189. Unlike PrtH of *L. helveticus* CNRZ32, PrtB of *L. bulgaricus* NCDO1489, or PrtP of *L. paracasei* 151, PrtH2 did not contain the I domain inserted in the catalytic domain (Fig. 1). The putative A domain, located just after the catalytic domain, could contain 416 aa. The nine well-conserved segments identified in the A domain of other characterized CEPs and of PrtR of *L. rhamnosus* BGT10 were also present in PrtH2, i.e., 643-LVEGFLRF-652, 661-VPYLSYYGDM-672, 733-AFSPNGD-741, 770-VRVLADS-778, 781-KSYHS-787, 806-WDGKLYDA-815, 823-DGNYTYRF-832, 848-PVIIDTTAPVL-860, and 941-FSDVADN-949 (21, 29). In the B domain, sequences were poorly conserved and the domain limits for PrtH2 became unclear. Compared with PrtH, the B domain could spread from aa 962 to aa 1536 and the H domain could spread from aa 1537 to aa 1609 (Fig. 1). As for PrtH, no LPxTG anchor motif can be identified in the C terminus of PrtH2. However, as found for PrtH, homology with S-layer proteins was found, suggesting a similar attachment mechanism.

Detection of the *prtH* and *prtH2* genes by PCR. To determine the presence or absence of the *prtH* and *prtH2* genes among various strains of *L. helveticus*, six pairs of primers (PrtH-for-1/PrtH-rev-1, PrtH-for-2/PrtH-rev-2, PrtH-for-3/PrtH-rev-1, PrtH2-for-1/PrtH2-rev-1, PrtH2-for-2/PrtH2-rev-2, and PrtH2-

for-3/PrtH2-rev-2; Table 2 and Fig. 1) were used (see Materials and Methods). In all of the cases, either one product of the expected size or no product was obtained, confirming the specificity of the primers used. As illustrated in Fig. 2A for the PrtH2-for-3/PrtH2-rev-2 primers, the 16 *L. helveticus* strains tested (in bold in Table 1) exhibited a band of about 430 bp likely corresponding to the *prtH2* gene whereas only 8 of the 16 strains also exhibited a band with the expected size (470 bp) corresponding to the *prtH* gene (Fig. 2B). For the ROSELL 5089 strain, a band of about 1.3 kb was obtained that could correspond to the *prtH* gene but was different from the other strains or could be a nonspecific product (see below).

These results suggest that *prtH2*, not *prtH*, is the ubiquitous CEP-encoding gene in *L. helveticus*. These results were further confirmed by using primers PrtH-for-1/PrtH-rev-1 and PrtH2-for-3/PrH2-rev-2 for 13 supplementary strains of *L. helveticus* (reported with normal typing in Table 1). All of the strains exhibited a band corresponding to the *prtH2* gene, and nine of them also exhibited a band corresponding to the *prtH* gene, i.e., ITGLH2, ITGLH3, CNRZ67, CNRZ450, CNRZ498, CNRZ891, CNRZ1080, CNRZ1111, and CNRZ1317.

Detection of the *prtH* and *prtH2* genes by Southern blotting. To confirm our PCR results, we performed Southern blotting analyses. Two probes corresponding to internal fragments of the *prtH* and *prtH2* genes were obtained from the genomic DNA of strain CNRZ32 with primers PrtH-for-2/PrtH-rev-2 and PrtH2-for-3/PrtH2-rev-2 (Table 2 and Fig. 1) and hybridized against EcoRV restriction digests of genomic DNAs of 16 of the 29 *L. helveticus* strains (in bold in Table 1) under high-stringency conditions (65°C). With the PrtH2-for-3/PrtH2-rev-2 PCR product as a probe, one band with a molecular size of greater than 10 kb was detected in all of the strains (Fig. 3A) and there was a slight variation in the molecular mass of the band in ITG LH77, CP615, CIP 103146T, and DPC4571. This result showed that all of the *L. helveticus* strains tested showed sequences homologous to that of the *prtH2* gene. With the PrtH-for-2/PrtH-rev-2 PCR product as a probe, a band of about 9 kb was detected in ITG LH1, CNRZ32JS, and the mutants CNRZ32A, CNRZ32B, and CNRZ32C while a band of 2.7 kb was present in CNRZ32 and CNRZ241 (Fig. 3B). These bands probably correspond to the *prtH* gene and highlight a restriction polymorphism in the latter two strains compared to the other five. Moreover, they were present only in strains for which an amplicon had been obtained for the *prtH* gene. No band corresponding to the *prtH* gene was detected in ROSELL 5088 and ROSELL 5089, despite the fact that they have been detected by PCR.

To increase the probability of detection of *prtH* in strains ROSELL 5088 and ROSELL 5089 and to highlight the potential presence of additional genes that encode CEPs of the subtilisin family, we used low-stringency conditions (42°C) and mixed four probes corresponding to internal fragments of the *prtH* and *prtH2* genes and obtained from the genomic DNA of strain CNRZ32 with primers PrtH-for-1/PrtH-rev-1, PrtH-for-2/PrtH-rev-2, PrtH2-for-1/PrtH2-rev-1, and PrtH2-for-3/PrtH2-rev-2 (Table 2 and Fig. 1). These probes were hybridized against the EcoRV restriction enzyme-digested genomic DNAs of the 16 *L. helveticus* strains. As shown in Fig. 3C, a band with a molecular size of greater than 10 kb was present in all of the restriction patterns and probably corresponded to the *prtH2* gene, as detected in Fig. 3A. Moreover, the virtual EcoRV digestion of the DPC4571 genome with RestrictionMapper version 3 (www.restrictionmapper.org) predicted a 16-kb fragment containing lhv_1641, the expected *prtH2* gene in strain DPC4571. The band of about 9 kb detected in Fig. 3B and corresponding to the *prtH* gene was present in the ITG LH1, CNRZ32JS, CNRZ32A, CNRZ32B, and CNRZ32C digestion patterns, whereas the band of about 2.7 kb, due to a polymorphism of EcoRV restriction around this gene, was detected for CNRZ32 and CNRZ241. Another band of 1.2 kb was always associated with and probably corresponded to the EcoRV fragments recognized by the PrtH-for-1/PrtH-rev-1 probe. The 9-kb band was also present in ROSELL 5088, while ROSELL 5089 showed a band of about 9.8 kb. This coincides with the PCR results in which this strain showed an amplicon 800 bp longer than that of other strains that possess the *prtH* gene. However, in both strains, no band at 1.2 kb was detected, suggesting that the PrtH-for-1/PrtH-rev-1 probe did not hybridize, unlike the PrtH-for-2/PrtH-rev-2 probe.

Finally, two other bands of about 3.8 and 0.75 kb (except for ISCL5 [0.9 kb]) were detected in the ITG LH1, CNRZ32, CNRZ32JS, CNRZ32A, CNRZ32B, CNRZ32C, CNRZ241, ISLC5, ROSELL 5088, and ROSELL 5089 digestion patterns. This suggests that another sequence in the genomes of these strains possessed homology with the *prtH* or *prtH2* genes which can be detected only under low-stringency conditions.

Southern blotting experiments confirmed that all of the strains possess the *prtH2* gene, while strains ITG LH1, CNRZ32, CNRZ32JS, CNRZ32A, CNRZ32B, CNRZ32C, CNRZ241, ROSELL 5088, and ROSELL 5089 also had the *prtH* gene. However, the latter two strains seemed to have a more divergent *prtH* allele, as it was detected only with one of two probes and only under low-stringency conditions. Finally, another band with homology to *prtH* or *prtH2* was present in 10/16 strains.

PCR product sequencing. In order to ensure that the PCR products obtained for all of the strains actually corresponded to the *prtH* and *prtH2* genes and to observe a possible biodiversity in the sequences of these genes, the nine PCR products obtained with the PrtH-for-1/PrtH-rev-1 primers and the 16 products obtained with the PrtH2-for-3/PrtH2-rev-2 primers were sequenced.

Analyses of the *prtH2* sequences of the 16 strains revealed that strains CIP 103146T, CP615, DPC4571, ITG LH77, ROSELL 5088, and ROSELL 5089 led to PCR products with 100% identity (99% for strains ROSELL 5088 and ROSELL 5089) with a region of the DPC4571 genome (accession no. NC_010080; from nucleotide [nt] 1617908 to nt 1617607) that corresponds to lhv_1641. This gene is annotated as a pseudogene with a predicted function of proteinase PrtP (3). The 1% divergence between the sequences of ROSELL 5088 and ROSELL 5089 corresponds to a G-to-A substitution at position 1617790 of the DPC4571 genome (NC_010080). This substitution could provoke a Glu-to-Lys modification at the amino acid level.

Strains ITG LH1, CNRZ32, CNRZ32JS, CNRZ32A, CNRZ32B, CNRZ32C, CNRZ241, CNRZ303, CNRZ414, and ISLC5 led to PCR products showing 100% identity with the published sequence of the *prtH2* gene of CNRZ32 (accession no. DQ826130). A comparison of the *prtH2* sequences of these two groups of strains reveals 60% identity at both the nucleotide and amino

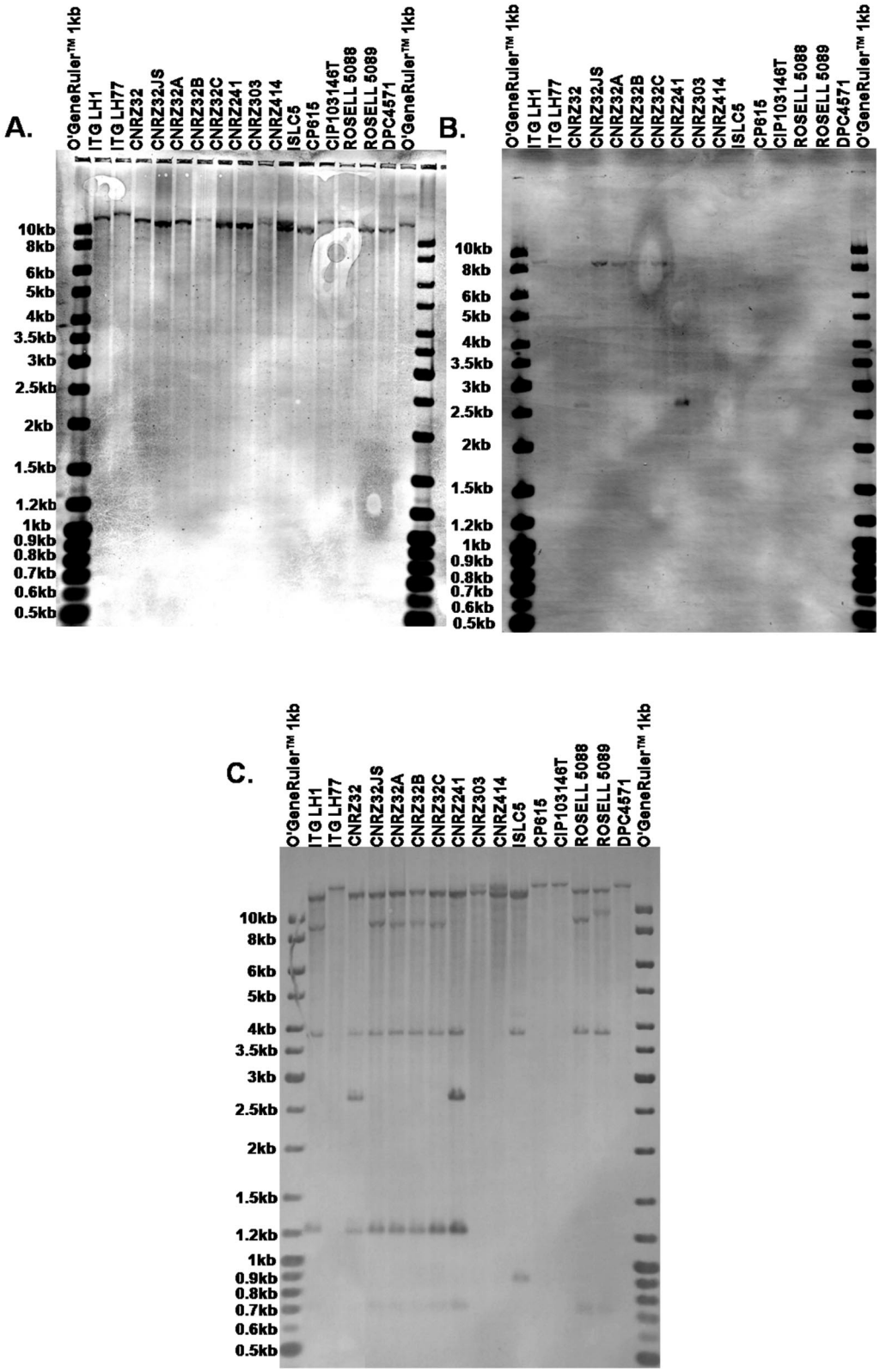

FIG. 3. Southern hybridization of the EcoRV-digested genomic DNAs of 16 *L. helveticus* strains with probes corresponding to internal fragments of *prtH2* and *prtH* obtained from the genomic DNA of the CNRZ32 strain with the PrtH2-for-3/PrtH2-rev-2 (A) and PrtH-for-2/PrtH-rev-2 (B) primers under high-stringency conditions (65°C). (C) Southern hybridization of the EcoRV-digested genomic DNAs of the 16 *L. helveticus* strains with probes corresponding to internal fragments of *prtH2* and *prtH* obtained from the genomic DNA of the CNRZ32 strain with the PrtH2-for-1/PrtH2-rev-1, PrtH2-for-3/PrtH2-rev-2, PrtH-for-1/PrtH-rev-1, and PrtH-for-2/PrtH-rev-2 primers under low-stringency conditions (42°C). A labeled O'GeneRuler 1-kb DNA ladder was used to estimate molecular weights.

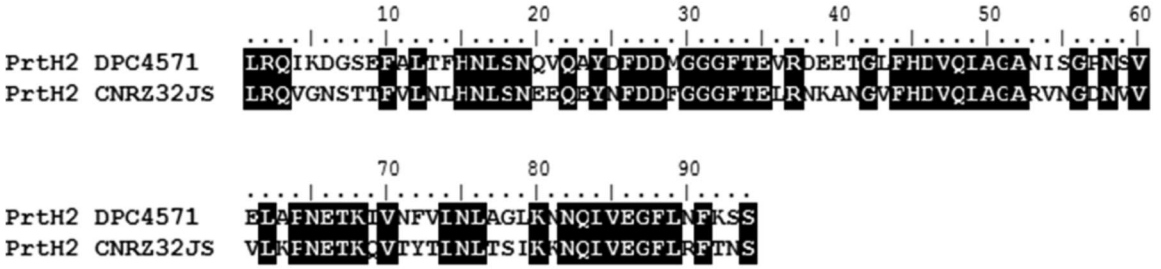

FIG. 4. Alignment of the deduced amino acid sequences of part of PrtH2 from the sequenced region of the *prtH2* genes of DPC4571 and CNRZ32JS. The alignment was made with the BioEdit software. Black boxes indicate residues that are similar between the two sequences, and white boxes indicate residues that differ. A well-conserved region in the A domain is overlined (29).

acids levels. Figure 4 shows an alignment of the partial PrtH2 protein sequences deduced from the nucleotide sequences of two representative strains, DPC4571 and CNRZ32JS. Some regions are well conserved, including one described as the first region involved in the recognition of the substrate in the A domain of subtilisin-like serine proteinases (29).

Thus, all of the strains tested possess a *prtH2* gene, as shown by PCR and Southern blotting experiments, but sequencing of an internal fragment allowed us to distinguish two groups with only 60% identity in the sequenced part. The emerging question is if both forms detected correspond to two alleles of *prtH2* or if the group including ROSELL 5088, ROSELL 5089, CIP 103146T, CP615, DPC4571, and ITG LH77 does not, in fact, have the *prtH2* gene first described in CNRZ32 but another homologue such as, for example, *prtH3* or *prtH5* (22, 31).

The *prtH* gene sequences obtained for ITG LH1, CNRZ32, CNRZ32JS, CNRZ32A, CNRZ32C, and CNRZ241 showed 99% identity with the published sequence of *prtH* of CNRZ32 (accession no. AF133727). One GC-to-CG inversion at positions 1935 and 1936 compared to the published sequence was detected that caused a Pro-to-Ala modification at position 646 of the PrtH protein of CNRZ32 (accession no. AAD50543). The sequence of the mutant CNRZ32B showed two substitutions compared to the strains listed above, a T-to-C substitution at position 1887 of the *prtH* sequence (accession no. AF133727) and a G-to-A modification at position 1917. The first would have no impact at the protein level (Asn at position 629 is unchanged), while the second would change Met to Ile at position 639 of the sequence with accession no. AAD50543.

The ROSELL 5088 strain differed from the others because the sequenced region showed only 72% identity at the nucleotide level (Fig. 5A) and 74% identity at the amino acid level (Fig. 5B) with the sequences of the six strains listed above. For the ROSELL 5089 strain, whose PCR product was about 800 bp longer than those of the other strains, the region sequenced with the PrtH-for-1 primer showed 100% identity with that of *prtH* of the ROSELL 5088 strain in the 5′ part but not in the 3′ part (Fig. 6A) and the region sequenced with the PrtH-rev-1 primer showed 100% identity with that of *prtH* of the ROSELL 5088 strain in the 3′ part but not in the 5′ part (Fig. 6B). A homology search carried out with the BLAST program revealed that the 3′ part of "prtH for RSL89" and the 5′ part of "prtH rev RSL89" showed 99% identity with the mobile genetic element ISL2 (accession no. X77332), showing that this element was inserted into the *prtH* gene of the ROSELL 5089 strain. As this element consists of 858 bp, it explains why the PCR product from ROSELL 5089 was about 800 bp longer than those of the other strains. When this element was deleted from the sequence of ROSELL 5089 in silico, 99% identity with the sequence of the gene of ROSELL 5088 was observed, the difference consisting of an insertion of 3 nt (ATT), the mark of ISL2 insertion (39).

In conclusion, sequence analyses confirmed the results of our PCR and Southern blotting experiments, as ITG LH1, CNRZ32, CNRZ32JS, CNRZ32A, CNRZ32B, CNRZ32C, CNRZ241, ROSELL 5088, and ROSELL 5089 had two different genes showing homology with both the *prtH* and *prtH2* genes whereas the seven remaining strains possessed only one gene showing the highest homology with the *prtH2* gene. Interestingly, the ROSELL strains showed a *prtH* allele different from those of the others and the ROSELL 5089 strain could not express an active PrtH protein because of the presence of the mobile genetic element ISL2 inserted in its *prtH* gene. Sequencing also revealed different alleles. For *prtH2*, 6 strains showed an allele similar to that of DPC4571 and the other 10 strains showed an allele similar to that of CNRZ32 (31). The CEP-encoding gene of DPC4571 was annotated as a pseudogene (3). Alignment of lhv_1641 (from nt 1614455 to nt 1619425) and *prtH2* of CNRZ32 (accession no. DQ826130) revealed 56% nucleotide identity and 59% amino acid identity. However, the CEP of DPC4571 could be truncated (3), as a STOP codon was present in the region corresponding to the B domain, compared with PrtH2 of CNRZ32 (ABI13574), probably leading to the synthesis of a 1,286-aa protein lacking the H and W domains. As strains CP615, CIP 103146T, ITG LH77, ROSELL 5088, and ROSELL 5089 showed a sequenced product similar to that of DPC4571, their gene could also contain a STOP codon and therefore produce a nonfunctional proteinase.

Detection of *prtH* and *prtH2* transcripts in dairy matrices. The presence of two genes that encode CEPs in *L. helveticus* raised the question of their functionality. In order to determine if both genes are transcribed, the total RNAs of the 16 *L. helveticus* strains were extracted from UHT milk cultures at pH 5.6, i.e., in the exponential phase of growth. The total RNAs were converted into cDNA, and PCRs were achieved with primers PrtH-for-1/PrtH-rev-1 for *prtH* detection and PrtH2-for-3/PrtH2-rev-2 for *prtH2* detection. A transcript was detected in all of the strains with the PrtH2-for-3/PrtH2-rev-2 primers, whereas a *prtH* transcript was observed in the nine strains with the *prtH* gene in their genomes (data not shown).

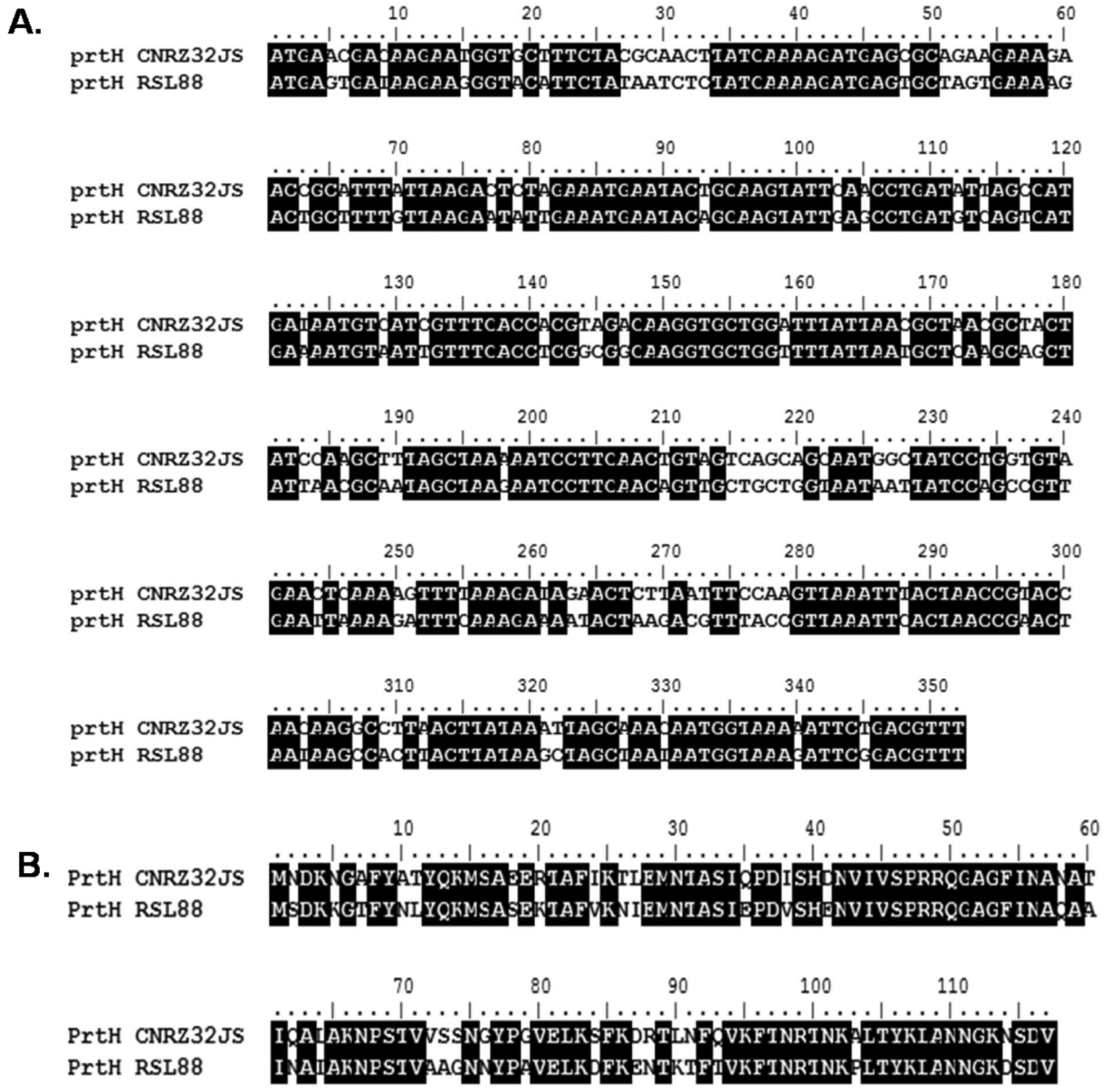

FIG. 5. Alignment of the sequenced region of the *prtH* genes (A) and deduced amino acid sequences (B) of CNRZ32JS and ROSELL 5088 (RSL88). The alignment was made with the BioEdit software. Black boxes indicate residues that are similar between the two sequences, and white boxes indicate residues that differ.

For the ROSELL 5089 strain, as observed with genomic DNA, a band of 1.3 kb was observed (data not shown).

So, in all of the strains studied, the presence of a CEP-encoding gene in the *L. helveticus* genome leads to the detection of a transcript. The emerging question was if both genes are or are not transcribed at the same stages of growth. To verify this, we extracted the total RNAs of two strains, CNRZ32 and ITG LH77, during growth in MRS broth and in UHT milk. In MRS broth, both genes were expressed at different growth stages (1, 4, 6, 8, 10, and 12 h of culture; data not shown). In UHT milk, total RNAs were extracted during the lag phase (i.e., at pH 6.3), during the early and late exponential growth phases (i.e., at pHs 5.9 and 5.1), and during the stationary phase (i.e., at pH 4.6). RNA was converted into cDNA, and PCRs were achieved as described above. With the PrtH2-for-3/PrtH2-rev-2 primers, a transcript of about 430 bp was observed at each point for the ITG LH77 strain (Fig. 7A, left), indicating that the corresponding gene was transcribed at all stages of growth in UHT milk. No *prtH* transcript was observed, as expected since this strain was devoid of this gene (Fig. 7A, right). For the CNRZ32 strain, both transcripts were detected at all stages of growth (Fig. 7A).

Finally, in order to know if these genes are actually transcribed during cheese making, total RNAs were extracted during the manufacture of two mini-Emmental cheeses containing

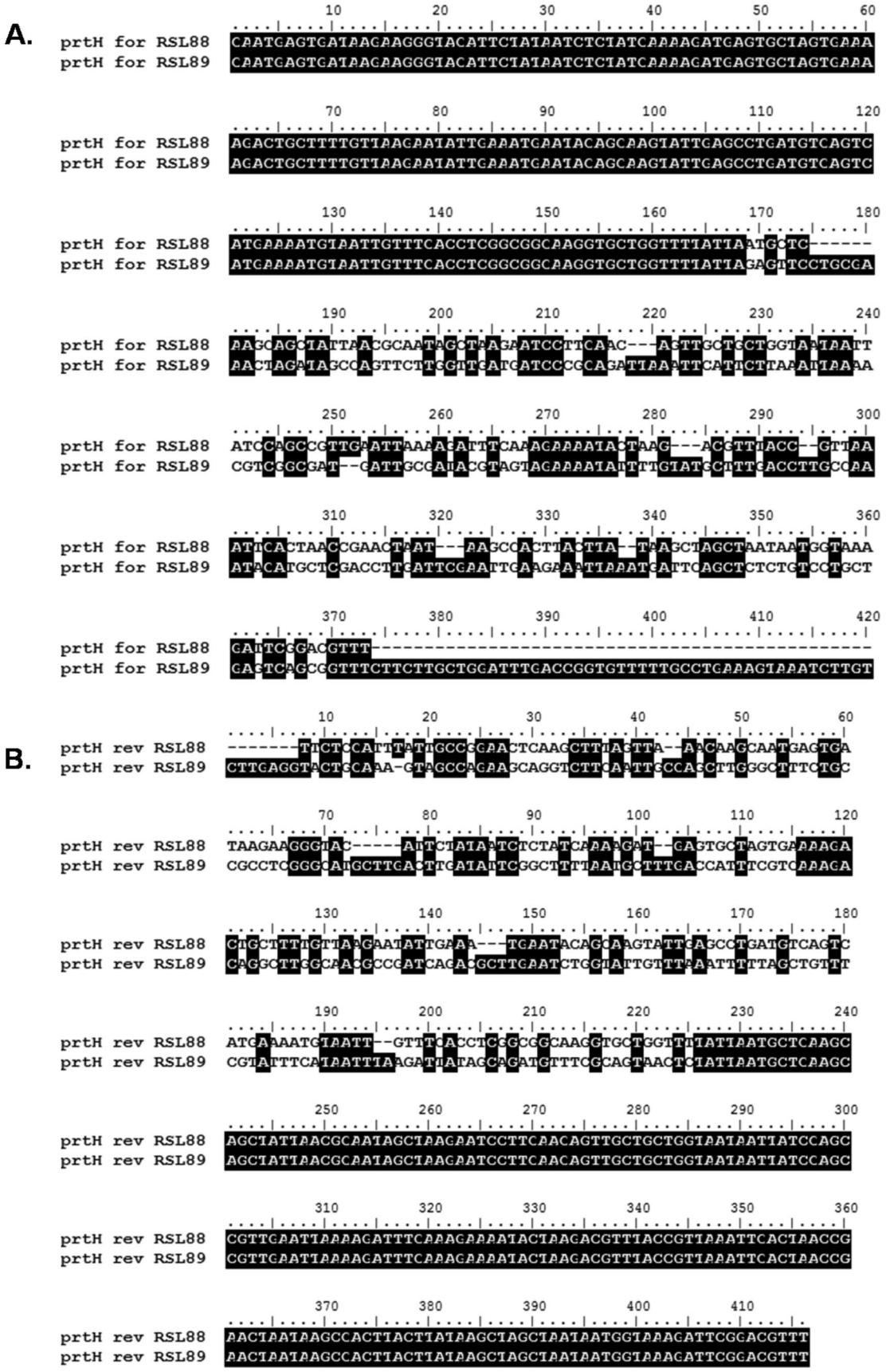

FIG. 6. Sequence alignment obtained for ROSELL 5088 and ROSELL 5089 with the PrtH-for-1 primer (A; prtH for RSL88 versus prtH for RSL89) and the PrtH-rev-1 primer (B; prtH rev RSL88 versus prtH rev RSL89). The alignment was made with the BioEdit software. Black boxes indicate residues that are similar between the two sequences, and white boxes indicate residues that differ.

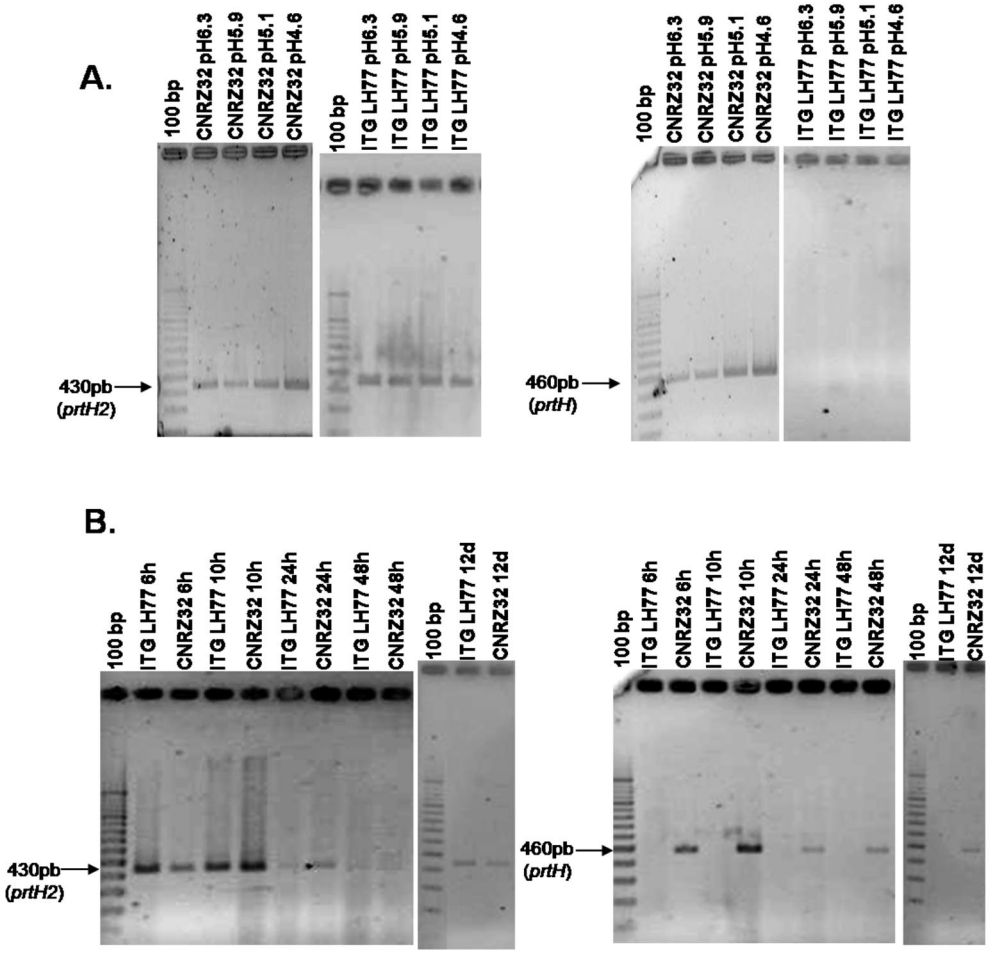

FIG. 7. Results of amplification of *prtH2* (left) and *prtH* (right) with cDNAs obtained from total RNA extraction at different stages of growth of *L. helveticus* strains CNRZ32 and ITG LH77 in UHT milk (A) or mini-Swiss-type cheese (B). PCR products were obtained with the PrtH2-for-3/PrtH2-rev-2 (on the left) and PrtH-for-1/PrtH-rev-1 (on the right) primers and separated on a 2% (wt/vol) agarose gel in TBE 1x at 100 V for 45 min. The O'RangeRuler 100-bp DNA ladder (lane 100 bp) was used as a molecular weight marker.

as starters the same strains used in milk experiments (CNRZ32 and ITG LH77). Ten-gram samples of cheese were collected after 6 h (i.e., during molding), 10 h (i.e., when the pH reached 5.3), 24 h, 48 h (i.e., after salting and the beginning of ripening), and 12 days (i.e., during ripening in a cold room), and total RNAs were extracted. With PrtH2-for-3/PrtH2-rev-2, a transcript was detected for both strains at each of the five time points tested (Fig. 7B, left). No *prtH* transcript was detected in cheese containing the ITG LH77 strain, whereas it was detected at each time in CNRZ32 (Fig. 7B, right).

DISCUSSION

The intraspecific diversity of CEP-encoding genes in *L. helveticus* was highlighted and further explored in this work by screening a collection of 29 strains from various origins. Two CEPs were previously described in *L. helveticus*, PrtH2 and PrtH, both belonging to the subtilisin-like serine proteinase family, as suggested by conserved domains, but exhibiting only 22% amino acid sequence identity. *prtH2* was shown here to be a ubiquitous gene in *L. helveticus* (100% of the strains tested), whereas the presence of *prtH* was shown to be strain dependent (in 18 of the 29 strains). Previously published Southern blotting analysis revealed that only two of eight strains of *L. helveticus* tested showed a band corresponding to the *prtH* gene (22), in accordance with our observations. All of the strains of *L. helveticus* thus possessed at least one gene that encodes a CEP, with the highest homology with *prtH2*. For some strains, Southern blotting experiments revealed the presence of two or even more CEP-encoding genes, a result which was not widely obtained before, even if the existence of *prtH3* and *prtH5*, two potential genes induced during growth in milk, has been suggested (31). *L. helveticus* is described as one of the most proteolytic species of LAB, and the presence of several genes constituting its proteolytic system could explain its efficiency.

The transcriptome assays described here for 16 strains of *L. helveticus* grown in milk revealed that the *prtH2* and *prtH* genes, when present, systematically led to the corresponding transcripts in the exponential growth phase. In a more detailed kinetic analysis of two strains grown in MRS broth, in milk, and in mini-Swiss-type cheeses, *prtH* and *prtH2* were shown to be transcribed at all growth phases. The absence of repression by

free peptides or final amino acids (like branched-chain amino acids) is different from what was observed previously in *L. lactis*, where CEP expression is under the control of the repressor CodY. One explanation can be that *prtH* and *prtH2* are not under the control of CodY in *L. helveticus* as we did not find any homologues of *codY* in the whole genome of *L. helveticus* strain DPC 4571 (4). Quantitative RT-PCR experiments could highlight different levels of expression of the genes according to the growth phase or environmental conditions. Microarray analyses revealed that transcription of *prtH* and *prtH2* was induced, respectively, 5.81- and 4.34-fold in milk compared to that in MRS medium (31). It has also been proposed that both CEPs of *L. helveticus* show a specific medium-dependent regulation (10) whose mechanisms remain to be elucidated.

prtH and *prtH2* both encode CEPs of the subtilisin family with little identity, resembling, respectively, the proteinase of *L. johnsonii* and that of *L. acidophilus*. This raises the question of what advantage is conferred by the occurrence of two different CEPs. They could have different substrate and cleavage specificities, or they could be redundant. One hypothesis would be that strains containing only one CEP-encoding gene had lost additional redundant genes such as, for example, *prtH*. The other hypothesis is that CEPs have distinct and complementary properties and that some strains have acquired an additional CEP-encoding gene that was maintained because it provides an adaptive advantage, in particular regarding milk protein hydrolysis. The facts that a *prtH* deletion mutant of the CNRZ32 strain was indistinguishable from the wild type in growth rate and showed a casein hydrolysis pattern different from that of the wild type (22) support the second hypothesis. Moreover, previous studies have shown that a large phenotypic variability exists among *L. helveticus* strains regarding proteolytic activity, with varied casein breakdown abilities (9). These phenotypic differences could be explained by the number of genes that encode CEPs or by their sequence variability, as detected in this work. Indeed, the partial sequencing of PCR products obtained for 16 strains revealed the existence of different alleles for both the *prtH2* and *prtH* genes. For *prtH2*, six strains showed an allele similar to that of sequenced strain DPC 4571 and the other 10 strains showed an allele similar to that of CNRZ32 (31). These two alleles showed only 60% identity in the sequenced region. For *prtH*, seven strains showed an allele similar to that of CNRZ32 (22) while the two ROSELL strains showed a specific allele. Interestingly, the PrtH proteinase of ROSELL 5089 might not be functional because of the presence in the gene of the mobile genetic element ISL2. The complete genome sequence of DPC 4571 reveals the presence of 213 IS elements of 21 different types and in particular the presence of the mobile genetic element ISL2 18 times in the genome (3). This is consistent with what had been reported previously (39); the ISL2 element, which is specific to the *L. helveticus* species of LAB, is present 4 to 21 times, depending on the strain. It is therefore not surprising to find it inserted in CEP-encoding genes in some strains, as is the case for ROSELL 5089.

As proteolysis is important in the manufacture of cheese, by influencing texture and flavor, as well as the level of some bioactive peptides, improvement of the selection of *L. helveticus* starter strains would be highly valuable. Further work on phenotypic characteristics, and in particular the peptides released from caseins, by the 16 strains well characterized in this work would help in clarifying the relationship between genomic CEP content and strain activity on milk caseins.

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REFERENCES

1. **Blanc, B., P. Laloi, D. Atlan, C. Gilbert, and R. Portalier.** 1993. Two cell-wall-associated aminopeptidases from *Lactobacillus helveticus* and the purification and characterization of APII from strain ITGL1. J. Gen. Microbiol. **139:**1441–1448.
2. **Bütikofer, U., J. Meyer, R. Sieber, B. Walther, and D. Wechsler.** 2008. Occurrence of the angiotensin-converting enzyme inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in different cheese varieties of Swiss origin. J. Dairy Sci. **91:**29–38.
3. **Callanan, M., P. Kaleta, J. O'Callaghan, O. O'Sullivan, K. Jordan, O. McAuliffe, A. Sangrador-Vegas, L. Slattery, G. F. Fitzgerald, T. Beresford, and R. P. Ross.** 2008. Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. J. Bacteriol. **190:**727–735.
4. **Callanan, M. J., R. P. Ross, and T. P. Beresford.** 2007. Insertion sequence elements as mediators of strain diversity in *Lactobacillus helveticus*. Int. J. Food Microbiol. **120:**120–123.
5. **Deutsch, S. M., D. Mollé, V. Gagnaire, M. Piot, D. Atlan, and S. Lortal.** 2000. Hydrolysis of sequenced β-casein peptides by peptidases from thermophilic lactic acid bacteria highlights the intrinsic resistance of phosphopeptides. Appl. Environ. Microbiol. **66:**5360–5367.
6. **Deutsch, S. M., A. Neveu, S. Guezenec, P. Ritzenthaler, and S. Lortal.** 2003. Early lysis of *Lactobacillus helveticus* CNRZ 303 in Swiss cheese is not prophage-related. Int. J. Food Microbiol. **81:**147–157.
7. **Fernandez-Espla, M. D., P. Garault, V. Monnet, and F. Rul.** 2000. *Streptococcus thermophilus* cell wall-anchored proteinase: release, purification and biochemical and genetic characterization. Appl. Environ. Microbiol. **66:**4772–4778.
8. **Fischer, G., B. Decaris, and P. Leblond.** 1997. Occurrence of deletions, associated with genetic instability in *Streptomyces ambofaciens*, is independent of the linearity of the chromosomal DNA. J. Bacteriol. **179:**4553–4558.
9. **Fortina, M. G., G. Nicastro, D. Carminati, E. Neviani, and P. L. Manachini.** 1998. *Lactobacillus helveticus* heterogeneity in natural cheese starters: the diversity in phenotypic characteristics. J. Appl. Microbiol. **84:**72–80.
10. **Gilbert, C., B. Blanc, J. Frot-Coutaz, R. Portalier, and D. Atlan.** 1997. Comparison of cell surface proteinase activities within the *Lactobacillus* genus. J. Dairy Res. **64:**561–571.
11. **Klaenhammer, T., E. Altermann, F. Arigoni, A. Bolotin, F. Breidt, J. Broadbent, R. Cano, S. Chaillou, J. Deutscher, M. Gasson, M. van de Guchte, J. Guzzo, A. Hartke, T. Hawkins, P. Hols, R. Hutkins, M. Kleerebezem, J. Kok, O. Kuipers, M. Lubbers, E. Maguin, L. McKay, D. Mills, A. Nauta, R. Overbeek, H. Pel, D. Pridmore, M. Saier, D. van Sinderen, A. Sorokin, J. Steele, D. O'Sullivan, W. de Vos, B. Weimer, M. Zagorec, and R. Siezen.** 2002. Discovering lactic acid bacteria by genomics. Antonie van Leeuwenhoek **82:**29–58.
12. **Kok, J., and W. M. De Vos.** 1994. The proteolytic system of lactic acid bacteria, p. 169–210. *In* M. J. Gasson and W. M. De Vos (ed.), Genetics and biotechnology of lactic acid bacteria. Blackie Academic & Professional, Glasgow, United Kingdom.
13. **Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings.** 1996. The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek **70:**187–221.
14. **LeBlanc, J. G., C. Matar, J. C. Valdez, J. Leblanc, and G. Perdigon.** 2002. Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. J. Dairy Sci. **85:**2733–2742.
15. **Maeno, M., N. Yamamoto, and T. Takano.** 1996. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. J. Dairy Sci. **79:**1316–1321.
16. **Martin-Hernandez, M. C., A. C. Alting, and F. A. Exterkate.** 1994. Purification and characterization of the mature, membrane-associated cell-envelope proteinase of *Lactobacillus helveticus* L89. Appl. Microbiol. Biotechnol. **40:** 828–834.

17. **Matar, C., S. S. Nadathur, A. T. Bakalinsky, and J. Goulet.** 1997. Antimutagenic effects of milk fermented by *Lactobacillus helveticus* L89 and a protease-deficient derivative. J. Dairy Sci. **80:**1965–1970.
18. **Oberg, C. J., J. R. Broadbent, M. Strickland, and D. J. McMahon.** 2002. Diversity in specificity of the extracellular proteinases in *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Lett. Appl. Microbiol. **34:**455–460.
19. **Ong, L., and N. P. Shah.** 2008. Influence of probiotic *Lactobacillus acidophilus* and *L. helveticus* on proteolysis, organic acid profiles, and ACE-inhibitory activity of cheddar cheeses ripened at 4, 8, and 12°C. J. Food Sci. **73:**M111–M120.
20. **Ono, H., N. Yamamoto, M. Maeno, T. Takano, and H. Momose.** 1997. Purification and characterization of a cell-wall associated proteinase of *Lactobacillus helveticus* CP53. Milchwissenschaft **52:**373–377.
21. **Pastar, I., I. Tonic, N. Golic, M. Kojic, R. van Kranenburg, M. Kleerebezem, L. Topisirovic, and G. Jovanovic.** 2003. Identification and genetic characterization of a novel proteinase, PrtR, from the human isolate *Lactobacillus rhamnosus* BGT10. Appl. Environ. Microbiol. **69:**5802–5811.
22. **Pederson, J. A., G. J. Mileski, B. C. Weimer, and J. L. Steele.** 1999. Genetic characterization of a cell envelope-associated proteinase from *Lactobacillus helveticus* CNRZ32. J. Bacteriol. **181:**4592–4597.
23. **Pritchard, G. G., and T. Coolbear.** 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. **12:**179–206.
24. **Richoux, R., L. Aubert, G. Roset, and J.-R. Kerjean.** 2008. Impact of the proteolysis due to lactobacilli on the stretchability of Swiss-type cheese. Dairy Sci. Technol. **89:**31–41.
25. **Sasaki, M., B. W. Bosman, and P. S. T. Tan.** 1995. Comparison of proteolytic activities in various lactobacilli. J. Dairy Res. **62:**601–610.
26. **Savijoki, K., H. Ingmer, and P. Varmanen.** 2006. Proteolytic systems of lactic acid bacteria. Appl. Microbiol. Biotechnol. **71:**394–406.
27. **Scolari, G., M. Vescovo, C. Zacconi, and F. Vescovi.** 2006. Extraction and partial characterization of proteolytic activities from the cell surface of *Lactobacillus helveticus* Zuc2. J. Dairy Sci. **89:**3800–3809.
28. **Seppo, L., T. Jauhiainen, T. Poussa, and R. Korpela.** 2003. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. Am. J. Clin. Nutr. **77:**326–330.
29. **Siezen, R. J.** 1999. Multi-domain, cell-envelope proteinases of lactic acid bacteria. Antonie van Leeuwenhoek **76:**139–155.
30. **Siezen, R. J., and J. A. Leunissen.** 1997. Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci. **6:**501–523.
31. **Smeianov, V. V., P. Wechter, J. R. Broadbent, J. E. Hughes, B. T. Rodriguez, T. K. Christensen, Y. Ardo, and J. L. Steele.** 2007. Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. Appl. Environ. Microbiol. **73:**2661–2672.
32. **Smid, E. J., B. Poolman, and W. N. Konings.** 1991. Casein utilization by lactococci. Appl. Environ. Microbiol. **57:**2447–2452.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
34. **Stefanitsi, D., G. Sakellaris, and J.-R. Garel.** 1995. The presence of two proteinases associated with the cell wall of *Lactobacillus bulgaricus*. FEMS Microbiol. Lett. **128:**53–58.
35. **Ulvé, V. M., C. Monnet, F. Valence, J. Fauquant, H. Falentin, and S. Lortal.** 2008. RNA extraction from cheese for analysis of *in situ* gene expression of *Lactococcus lactis*. J. Appl. Microbiol. **105:**1327–1333.
36. **Valence, F., and S. Lortal.** 1995. Zymogram and preliminary characterization of *Lactobacillus helveticus* autolysins. Appl. Environ. Microbiol. **61:**3391–3399.
37. **Vinderola, G., C. Matar, and G. Perdigon.** 2007. Milk fermented by *Lactobacillus helveticus* R389 and its non-bacterial fraction confer enhanced protection against *Salmonella enteritidis* serovar *Typhimurium* infection in mice. Immunobiology **212:**107–118.
38. **Yamamoto, N., A. Akino, and T. Takano.** 1994. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP 790. J. Dairy Sci. **77:**917–922.
39. **Zwahlen, M. C., and B. Mollet.** 1994. ISL2, a new mobile genetic element in *Lactobacillus helveticus*. Mol. Gen. Genet. **245:**334–338.