Genetic diversity in proteolytic enzymes and amino acid metabolism among *Lactobacillus helveticus* strains¹

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ABSTRACT

Lactobacillus helveticus CNRZ 32 is recognized for its ability to decrease bitterness and accelerate flavor development in cheese, and has also been shown to release bioactive peptides in milk. Similar capabilities have been documented in other strains of *Lb. helveticus*, but the ability of different strains to affect these characteristics can vary widely. Because these attributes are associated with enzymes involved in proteolysis or AA catabolism, we performed comparative genome hybridizations to a CNRZ 32 microarray to explore the distribution of genes encoding such enzymes across a bank of 38 *Lb. helveticus* strains, including 2 archival samples of CNRZ 32. Genes for peptidases and AA metabolism were highly conserved across the species, whereas those for cell envelope-associated proteinases varied widely. Some of the genetic differences that were detected may help explain the variability that has been noted among *Lb. helveticus* strains in regard to their functionality in cheese and fermented milk.

Key words: *Lactobacillus*, proteolysis, cheese flavor, bioactive peptide

INTRODUCTION

Lactobacillus helveticus is an obligately homofermentative lactic acid bacterium (**LAB**) that is widely used as a starter culture to manufacture yogurt and certain Swiss and Italian cheeses (Hassan and Frank, 2001; Gatti et al., 2004), and also as a flavor-enhancing adjunct culture for other cheese types (Pettersson

and Sjöström, 1975; Bartels et al., 1987a,b; Ardö and Pettersson, 1988; Drake et al., 1996). Moreover, milk fermented with certain *Lb. helveticus* strains has been shown to become enriched with antihypertensive and immunomodulatory bioactive peptides (Laffineur et al., 1996; LeBlanc et al., 2002; Hayes et al., 2007b; Gobetti et al., 2010). Although *Lb. helveticus* is commonly associated with milk environments, this species has also been recovered from whisky fermentations (Cachat and Priest 2005; Naser et al. 2006) and, more sporadically, from the human reproductive or gastrointestinal tracts (Anukam et al. 2006; Stoyancheva et al. 2006; Vitali et al. 2010).

Lactobacillus helveticus is auxotrophic for several AA (Kandler and Weiss, 1986; Christensen and Steele, 2003; Christiansen et al., 2008), so rapid growth in milk or other environments that contain low levels of free AA relies on a complex proteolytic enzyme system whose collective function involves the procurement of essential AA needed for growth (Christensen et al., 1999). Detailed analysis of the proteolytic enzyme system in dairy LAB, most notably *Lactococcus lactis* and *Lb. helveticus*, has shown that enzymes involved in the release of essential AA from large proteins such as CN can be loosely divided into 3 major categories: (1) an extracellular, cell envelope-associated proteinase (**CEP**) that hydrolyzes CN into oligopeptides; (2) specialized transport systems to take up those oligopeptides, as well as di- and tripeptides, and free AA that may be present in the medium; and (3) intracellular endopeptidases and exopeptidases, including many that are specific for proline-containing peptides, which degrade internalized peptides into di- and oligopeptides and free AA (Christensen et al., 1999; Savijoki et al., 2006).

Although the biological role of the proteolytic enzyme system is directed toward cellular growth needs, the actions of these enzymes in cheese and fermented foods has important practical consequences on the sensory, functional (stretch, melt), and bioactive attributes

Received December 6, 2010.

Accepted April 23, 2011.

¹This communication is approved as UAES Journal Paper Number 8273. Peggy Steele, a member of J. L. Steele's family, is employed by Danisco Inc. (Copenhagen, Denmark), a supplier of bacterial cultures to the food industry.

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of these products (Oommen et al., 2002; Broadbent and Steele, 2007; Hayes et al., 2007a). Moreover, subsequent conversion of some free AA into volatile and nonvolatile compounds by LAB in cheese is believed to represent the rate-limiting step in the development of mature flavor and aroma (Yvon and Rijnen, 2001; Ardö, 2006). For these reasons, the relationship between proteolytic enzyme activity and dairy foods quality has been a focal point of dairy research for decades.

Studies in the field have shown a variety of LAB may contribute to these reactions and properties (Fox, 1989; Gobetti et al., 2002; Azarnia et al., 2006; Cogan et al., 2007; Hayes et al., 2007b) and that the proteolytic enzymes found in these cells are, for the most part, distributed across all dairy LAB (Christensen et al., 1999; Savijoki et al., 2006; Liu et al., 2010). However, broad differences in specificity and relative activity of individual enzymes exist, with lactobacilli generally showing higher proteolytic activity than other types of LAB (Sasaki et al., 1995; Gilbert et al., 1997; Weimer et al., 1997; Laan et al., 1998). Lactobacilli also possess a greater number and diversity of genes for proteinases and peptidases. Only lactobacilli, for example, have been found to possess more than one gene for CEP, and these bacteria also show greater redundancy in the number of genes for di- and tripeptidases and for endopeptidase PepO (Pederson et al., 1999; Sridhar et al., 2005; Liu et al., 2010). Lactobacilli also have genes for several endo- and aminopeptidases, including oligoendopeptidases PepE and PepG, proline iminopeptidase PepI, prolyl aminopeptidase PepL, and prolinase PepR, which have not been found in *Lc. lactis* or *Streptococcus thermophilus* (Christensen et al., 1999; Savijoki et al., 2006; Liu et al., 2010).

Among lactobacilli, *Lb. helveticus* is especially recognized for its active protease and peptidase activities toward milk proteins (Gilbert et al., 1997; Christensen et al., 1999; Matar et al., 2001; Savijoki et al., 2006), and the most thoroughly characterized proteolytic enzyme system in *Lb. helveticus* belongs to strain CNRZ 32 (reviewed by Christensen et al., 1999; plus Pederson et al., 1999; Chen et al., 2003; Christensen and Steele 2003; Sridhar et al., 2005; Smeianov et al., 2007). *Lactobacillus helveticus* CNRZ 32 is an industrial bacterium that decreases bitterness and accelerates cheese flavor development, and has also been shown to be capable of producing anti-hypertensive peptides in milk (Bartels et al., 1987a,b; Christensen et al., 1999; Savijoki et al., 2006; Kilpi et al., 2007). Because these attributes are strain-specific (Laffineur et al., 1996; Gummalla and Broadbent 1999; Deutsch et al., 2000; Klein et al., 2001; Jensen et al., 2009; Jensen and Ardö, 2010), we performed comparative genome hybridizations (CGH) to a CNRZ 32 microarray to explore the distribution of

genes involved in proteolysis and AA catabolism among a bank of *Lb. helveticus* strains isolated from different sources.

MATERIALS AND METHODS

Comparative Genome Hybridizations

Lactobacillus helveticus strains used in this study for CGH were selected to provide broad representation of the ecological and industrial diversity in this species (Table 1). Industrial strains and whiskey isolates were obtained from culture collections at Utah State University, the University of Wisconsin-Madison, the University of Copenhagen, the Centre National de Recherche Zootechnique, and the Institut Rosell. The UPR natural starter strains were isolated at the University of Parma from Grana Padano, Provolone, or Parmigiano Reggiano cheese whey, or fresh Parmigiano Reggiano curd (Table 1), essentially as described by Gatti et al. (2003).

Stock cultures were maintained at -80°C in de Man, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) with 20% (vol/vol) glycerol. Working cultures were prepared from stock cultures by 2 successive transfers (1% inocula) in MRS broth at 37°C for 16 to 18 h. Comparative genome hybridization experiments were performed using an Affymetrix (Affymetrix Inc., Santa Clara, CA) custom microarray designed to include eleven 24-mer probes for each of 2,387 predicted coding sequences (CDS, including pseudogenes) in the *Lb. helveticus* CNRZ 32 genome, as well as all non-redundant CDS in the *Lactobacillus casei* ATCC 334 genome (Cai et al., 2009). The only CDS not included in the microarray design were duplicate copies of rRNA and transposase-encoding genes found in each species.

Genomic DNA for CGH was extracted from each strain using a MasterPure Gram-Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Five micrograms of genomic DNA was fragmented and labeled according to instructions for labeling mRNA for antisense prokaryotic arrays (Affymetrix Inc.). Reactions for hybridization contained 1 μg of labeled DNA and were performed using the fully automated components (Fluidics Station 450 and GeneChip Scanner 3000) of an Affymetrix GeneChip System operated by staff in the Utah State University Center for Integrated Biosystems (Logan, UT). Statistical analysis of microarray data was done using R software (<http://www.r-project.org>). To screen for artifacts that pose quality problems, the array images were reduced to intensity values for each probe and evaluated for quality using the AffyPLM program (Bolstad et al., 2005) for Bioconductor (<http://www.bioconductor.org>). Arrays that met acceptable quality control criteria established

Table 1. *Lactobacillus helveticus* strains used in comparative genome hybridizations

Strain	Origin (source or reference)
CNRZ 32	Industrial dairy starter culture (Centre National de Recherche Zootechnique, Jouy-en-Josas, France)
R0052	Industrial dairy starter culture (Naser et al., 2006)
ATCC 15009	Swiss cheese (type strain, also called DSM 20075; American Type Culture Collection, Manassas, VA)
CNRZ 32–1973	Archival CNRZ 32 sample stored at –80°C in 1973 (University of Copenhagen, Denmark)
CNRZ 32–1997	Archival CNRZ 32 sample stored at –80°C in 1997 (University of Copenhagen, Denmark)
CNRZ 303	Industrial dairy starter culture (Centre National de Recherche Zootechnique, Jouy-en-Josas, France)
LHC2	Industrial dairy starter culture (University of Wisconsin, Madison)
L10	Industrial dairy starter culture (Utah State University, Logan)
LH 212	Industrial dairy starter culture (Broadbent et al., 1997)
MI 2275	Industrial dairy starter culture (Jensen et al., 2009)
MI 2359	Kadett cheese (Jensen et al., 2009)
R150	Industrial dairy starter culture (Utah State University, Logan)
R211	Industrial strain used for production of bioactive dairy foods (Leclerc et al., 2002)
R220	Industrial dairy starter culture (Utah State University, Logan)
R389	Industrial strain used for production of bioactive dairy foods (Matar et al., 2001)
L89	Proteinase-deficient mutant of strain R389 (Matar et al., 2001)
UPR1	Natural whey starter, Grana Padano cheese (this study)
UPR2	Natural whey starter, Grana Padano cheese (this study)
UPR3	Natural whey starter, Grana Padano cheese (this study)
UPR4	Natural whey starter, Grana Padano cheese (this study)
UPR5	Natural whey starter, Parmigiano Reggiano cheese (this study)
UPR6	Fresh Parmigiano Reggiano curd (this study)
UPR8	Fresh Parmigiano Reggiano curd (this study)
UPR9	Fresh Parmigiano Reggiano curd (this study)
UPR10	Fresh Parmigiano Reggiano curd (this study)
UPR11	Natural whey starter, Parmigiano Reggiano cheese (this study)
UPR12	Natural whey starter, Parmigiano Reggiano cheese (this study)
UPR13	Natural whey starter, Provolone cheese (this study)
UPR14	Natural whey starter, Parmigiano Reggiano cheese (this study)
UPR15	Natural whey starter, Parmigiano Reggiano cheese (this study)
UPR16	Natural whey starter, Grana Padano cheese (this study)
UPR17	Natural whey starter, Grana Padano cheese (this study)
UPR18	Natural whey starter, Provolone cheese (this study)
UPR19	Natural whey starter, Provolone cheese (this study)
UPR20	Natural whey starter, Provolone cheese (this study)
LMG 22464	Japanese whiskey fermentation (Cachat and Priest 2005)
Y10	Japanese whiskey fermentation (Cachat and Priest 2005)
M4	Scottish whiskey fermentation (Cachat and Priest 2005)

by AffyPLM were used for further analysis. Array preprocessing (background correction, normalization, and summarization) was performed using the Robust Multi-array Average method (Bolstad et al., 2003) using Bioconductor. Cutoff values for presence/absence determination in *Lb. helveticus* strains were selected empirically based on hybridization signal intensity from positive (genomic DNA from *Lb. helveticus* CNRZ 32) and negative (hybridization of *Lb. casei* ATCC 334 genomic DNA) probe controls. Results were clustered and visualized by TIGR MultiExperiment Viewer (TMEV) 4.0 (Saeed et al., 2003), using Euclidean Distance as a distance metric. The Support Tree method of bootstrapping implemented in TMEV was used to test the reliability of the clustering patterns (1,000 bootstrap re-samplings).

Distribution of CEP Paralogs Among *Lb. helveticus* Strains

Multiplex PCR was used to evaluate the presence and distribution of CEP paralogs *prtH*, *prtH2*, *prtH3*,

and *prtH4* in 9 of the *Lb. helveticus* strains analyzed by CGH (CNRZ 32, R0052, ATCC 15009, R211, R389, L89, LMG 22464, Y10, and M4), 13 additional *Lb. helveticus* strains (ATCC 521, ITG LH1, ITG LH43, ITG LH44, ITG LH55, ITG LH56, ITG LH68, ITG LH77, R204, R234, R235, R247, and R338), 3 strains of *Lactobacillus acidophilus* (NCFM, R240, and R269), and in *Lactobacillus gallinarum* ATCC 53673.

The strains were propagated and DNA isolated essentially as described for CGH experiments, then 2 different multiplex PCR reactions were performed, one for *prtH* and *prtH3*, and the other for *prtH2* and *prtH4*. Both were carried out in 25-μL reactions that contained 1X polymerase buffer, 0.2 mM each of deoxyribonucleotide triphosphate (dNTP), 1 pmol/μL of each primer (Table 2), 100 ng of template DNA, and 0.05 U/μL of Taq DNA polymerase. One sample (negative control) contained water instead of template DNA. The PCR was initiated by template denaturation at 94°C for 5 min, followed by 30 cycles of heating to 94°C for 30 s, annealing at 54°C for 30 s, extension at

Table 2. Primers used in multiplex PCR reactions to detect cell envelope-associated proteinase paralogs in strains of *Lactobacillus helveticus*¹

Multiplex	Gene	Primer name	Primer sequence (5'-3')	Amplicon size (bp)
A	<i>prtH</i>	<i>prtHf</i>	CGGGAATCCTGATATGGAAACTTTAGGTAATC	1,332
		<i>prtHr</i>	ATTAAGACTAGCAAAGATTGGAAGATCTGAACCTTG	
	<i>prtH3</i>	<i>prtH3f</i>	GATGATCAAGCAGATGTAAACCGGCAGAAG	357
		<i>prtH3r</i>	ATTTACTGAAGAATTAGTCAAATGACCTGTTGTCTGG	
B	<i>prtH2</i>	<i>prtH2f</i>	AAGCAAAGGATGTTGTTCCAAGTAAGCCA	4,042
		<i>prtH2r</i>	CTCTCTTCCTTCTTACCAGTTGATGATTGAACT	
	<i>prtH4</i>	<i>prtH4f</i>	CTGAAGCAGCAACTAATGATCCTGG	3,386
		<i>prtH4r</i>	TGGATTAGGATCCGTTCTGGTTGTCTAG	

¹Primers were designed from previously described gene sequences for CNRZ 32 cell envelope-associated proteinase genes *prtH* (Pederson et al., 1999) and *prtH2* (Smeianov et al., 2007), as well as *prtH3* and *prtH4* (GenBank accession numbers HQ602769 and HQ602770, respectively).

65°C for 3 min, followed by 7 min at 65°C, and then samples were chilled to 4°C. The presence of amplicon in these reactions was confirmed by ethidium bromide staining of DNA after samples (5 µL) were separated by electrophoresis in 1% agarose gels.

Milk Acidifying Activity

The ability of some strains to acidify milk was investigated by inoculation (2% vol/vol) of fresh overnight culture into 10 mL of 10% (wt/vol) reconstituted skim milk powder (Oxoid Ltd., Basingstoke, UK) that had been adjusted to 6.8 pH with 0.1 M NaOH and sterilized by heating to 110°C for 30 min. The cells were incubated at 42°C overnight, then inoculated at 1% (vol/vol) into 50 mL of reconstituted skim milk and pH measured (Metrohm 654 pH meter; Metrohm Ltd., Herisau, Switzerland) immediately after inoculation and after 24 h of incubation at 42°C. Strains that failed to produce more than 1 U of decrease in the milk pH were designated as slow acid producers.

Growth in Cheese Whey

The contribution of PrtH in growth of CNRZ 32 in cheese whey was explored using 2 independently isolated *prtH* deletion mutants (Pederson et al., 1999). The wild-type strain and mutants were collected from -80°C freezer stocks, subcultured twice in 10 mL of MRS broth, then grown (1% inoculum) for 16 h in 10 mL of UHT-treated milk. Cells were then inoculated at 1% into duplicate 12-mL samples of filter-sterilized (0.2-µm filter; VWR International, Radnor, PA) cheese whey, and the absorbance at 600 nm measured at hourly intervals to 8h, then again after 18 h of growth at 37°C. At that point, a 1% inoculum of each culture was transferred into a fresh 12-mL whey sample and growth was monitored in the same manner. Additionally, 1-mL samples were collected from each tube at 0, 8, and 18 h on both days and plated on MRS agar. Colony enu-

meration was performed after 48 h of incubation in anaerobic jars at 37°C.

RESULTS AND DISCUSSION

Conservation of Peptidase Genes

The *Lb. helveticus* CNRZ 32 genome encodes numerous proteolytic enzymes involved in procurement of essential AA or housekeeping needs (e.g., protein processing and turnover; reviewed by Christensen et al., 1999; Pederson et al., 1999; Chen et al., 2003; Christensen and Steele 2003; Sridhar et al., 2005; Smeianov et al., 2007). As is shown in Table 3, the former group of enzymes includes 4 CEP paralogs, 7 oligoendopeptidases, 3 general aminopeptidases, 5 proline-specific peptidases, 8 di- or tri-peptidases, and at least 6 other peptidases. Remarkably, CGH experiments revealed that the peptidase genes were almost universally conserved among the disparate cheese, whey, whiskey malt, and commercial cultures selected for this research (Table 1). The only exceptions were 6 strains (UPR1, UPR2, UPR10, UPR11, UPR13, and UPR14) that lacked the oligoendopeptidase gene *pepO2*, and one strain (UPR15) that did not contain the *pepE2* gene.

Although peptidase genes are clearly highly conserved across *Lb. helveticus*, several studies have shown that peptidase activity in different strains, including 6 of those tested in this work, can vary considerably (Sasaki et al., 1995; Jensen and Ardö, 2010). Moreover, Soeryapranata et al. (2007) characterized the specificity of the 3 PepO paralogs from *Lb. helveticus* WSU 19, and demonstrated each of these enzymes was not identical to its counterpart in CNRZ 32. Furthermore, CGH does not provide information on whether a gene is functional or contains minor sequence polymorphisms that produce AA substitutions affecting enzyme activity. To explore the latter possibilities, we performed Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) homology searches

Table 3. Genes encoding cell envelope-associated proteinases and peptidases in *Lactobacillus helveticus* CNRZ 32

General enzyme category ¹	Gene name (GenBank accession number)
Cell envelope-associated proteinases (EC 3.4.21.96)	<i>prtH</i> (AAD50643) <i>prtH2</i> (DQ826130) <i>prtH3</i> (HQ602769) <i>prtH4</i> (HQ602770)
Cell envelope-associated maturation proteins (EC 5.2.1.8)	<i>prtM</i> (DQ826131) <i>prtM2</i> (DQ826132)
Oligoendopeptidases	
Thiol-dependent endopeptidase E (EC 3.4.22.–)	<i>pepE</i> (U77050) <i>pepE2</i> (AAQ72431)
Oligoendopeptidases F and O (EC 3.4.24.–)	<i>pepF</i> (AY365129) <i>pepO</i> (AF019410) <i>pepO2</i> (DQ826126) <i>pepO3</i> (AY365128)
O-sialoglycoprotein endopeptidase (EC 3.4.24.57)	<i>gcp</i> (DQ826107)
General aminopeptidases C (EC 3.4.22.40) and N (EC 3.4.11.15)	<i>pepCE</i> (JF811429) <i>pepC</i> (HQ602766) ² <i>pepN</i> (U08224)
Proline-specific peptidases	
X-prolyl diaminopeptidase (EC 3.4.14.11)	<i>pepX</i> (U22900)
Proline iminopeptidases (EC 3.4.11.5)	<i>pepI</i> (DQ826125) <i>pepR</i> (U05214)
Prolidase (EC 3.4.11.9)	<i>pepQ</i> (AF012084) <i>pepQ2</i> (DQ826127)
Di- and Tripeptidases	
Dipeptidase A (EC 3.4.13.–)	<i>pepD</i> (U34257) <i>pepD2</i> (DQ826122) <i>pepD3</i> (DQ826123) <i>pepD4</i> (DQ826124) <i>pepD5</i> (HQ602767) ²
Xaa-His dipeptidase (EC 3.4.13.3)	<i>pepV</i> (AF012085)
Tripeptidase T (EC 3.4.11.4)	<i>pepT</i> (DQ826128) <i>pepT2</i> (DQ826129)
Other enzymes	
Glutamyl aminopeptidase (EC 3.4.11.7)	<i>pepA</i> (DQ826138)
Methionine aminopeptidase (EC 3.4.11.8)	<i>map</i> (DQ826118)
Pyrrolidone carboxyl peptidase (EC 3.4.19.3)	<i>pcp</i> (DQ826121)
SprT-like metallopeptidase (EC 3.4.24.–)	<i>sprT</i> (HQ602765)
M16 family metallopeptidase (EC 3.4.–.–)	<i>hyp prt2</i> (DQ826112)
Membrane alanine aminopeptidase (EC3.4.11.2)	<i>pepM1</i> (HQ602768)

¹EC = Enzyme Commission [number].²Predicted pseudogene in *Lb. helveticus* CNRZ 32.

with the predicted protein sequences for 21 CNRZ 32 peptidases, plus 2 peptidases from DPC 4571 that occur as pseudogenes in CNRZ 32. As expected, AA sequence homology was generally very high over the full length of each protein, but perfect matches were uncommon and nonsense mutations (which created pseudogenes) were present in some strains (Table 4). As a whole, these reports show that even though the peptidase gene content of *Lb. helveticus* strains is identical, strain heterogeneity may be observed due to loss of gene function or sequence polymorphisms that affect the specificity or relative activity of individual peptidase enzymes.

Heterogeneity in the Distribution of CEP Paralogs

The 4 CEP genes found in *Lb. helveticus* CNRZ 32 are predicted to encode proteins that range in size from

1,642 to 1,843 AA for PrtH3 and PrtH, respectively, and share 29 to 70% AA identity over most of their length (Table 5). We tested a total of 51 *Lb. helveticus* for the presence of each CEP paralog by CGH (36 strains, plus 2 archival samples of CNRZ 32) or multiplex PCR (22 strains, including 9 also tested by CGH, plus 3 strains of *Lb. acidophilus* and one *Lb. gallinarum*). Data for strain DPC 4571 was derived from the published genome sequence for that strain (Callanan et al., 2008). Complete agreement between results was found for the 9 strains tested by both methods (Figure 1), and no PCR amplicons were detected after multiplex PCR with template DNA from *Lb. acidophilus* or *Lb. gallinarum* strains (Figure 2).

In contrast to the highly conserved peptidases, we found distribution of CEP genes differed widely among *Lb. helveticus* strains (Figures 1, and 2). Six of the

Table 4. Amino acid sequence homology among selected peptidases in different strains of *Lactobacillus helveticus*¹

CNRZ 32 enzyme (length) ²	DPC 4571	H10	ATCC 15009	WSU 19
Oligoendopeptidases				
PepE (438 residues)	437/438 (100)	436/436 (100)	438/438 (100)	436/438 (100)
PepE2 (437 residues)	434/435 (100)	433/434 (100)	437/437 (100)	NA ³
PepF (598 residues)	594/597 (100)	591/596 (100)	598/598 (100)	NA
PepO (647 residues)	643/644 (100)	Pseudogene	646/646 (100)	644/646 (100)
PepO2 (648 residues)	648/648 (100)	645/646 (100)	640/643 (100)	639/640 (100)
PepO3 (647 residues)	646/647 (100)	642/644 (100)	647/647 (100)	644/645 (100)
Gcp (372 residues)	372/372 (100)	352/353 (95)	371/371 (100)	NA
General aminopeptidases				
PepC (pseudogene) ⁴	(449 residues)	443/447 (100)	444/447 (100)	NA
PepCE (437 residues)	433/435 (100)	435/435 (100)	437/437 (100)	NA
PepN (844 residues)	840/843 (100)	834/838 (100)	844/844 (100)	837/841 (100)
Proline-specific peptidases				
PepX (793 residues)	787/789 (100)	766/780 (100)	789/791 (100)	NA
PepI (294 residues)	291/293 (100)	286/290 (100)	294/294 (100)	NA
PepR (304 residues)	303/304 (100)	304/304 (100)	304/304 (100)	NA
PepQ (368 residues)	365/367 (100)	366/367 (100)	368/368 (100)	NA
PepQ2 (369 residues)	368/368 (100)	366/368 (100)	368/368 (100)	NA
Di- and tripeptidases				
PepD (474 residues)	473/473 (100)	472/472 (100)	470/470 (100)	NA
PepD2 (473 residues)	473/473 (100)	473/473 (100)	473/473 (100)	NA
PepD3 (475 residues)	472/473 (100)	471/473 (100)	475/475 (100)	NA
PepD4 (407 residues)	406/406 (100)	403/404 (100)	407/407 (100)	NA
PepD5 (pseudogene) ^d	(332 residues)	Absent	Pseudogene	NA
PepV (467 residues)	442/444 (95)	442/444 (95)	467/467 (100)	NA
PepT (413 residues)	411/412 (100)	411/412 (100)	412/413 (100)	NA
PepT2 (428 residues)	426/427 (100)	428/428 (100)	ND ⁵	NA

¹Identical AA/similar AA (% query coverage).²Predicted from coding sequence.³Gene sequence not publicly available.⁴Predicted protein sequence from DPC 4571 ortholog used for homology comparisons.⁵Not detected in draft (incomplete) ATCC 15009 genome, but CGH data indicate the gene is present.

51 (12%) *Lb. helveticus* strains (including CNRZ 32) contained all 4 CEP paralogs. An additional 4 strains contained 3 paralogs, whereas 21 strains had genes for 2 CEP. Thus, over 60% of *Lb. helveticus* strains tested contained more than one gene encoding CEP. Only one strain, UPR13, lacked any detectable CEP gene (Figure 1). It is important to note, however, that the methods used in this study can only detect paralogs found in CNRZ 32, so the UPR13 genome could encode an additional and unrelated CEP. The work of Genay et al. (2009) provides evidence to support the latter

possibility, as those authors isolated DNA fragments from *Lb. helveticus* ROSELL 5088 that appear to encode a CEP enzyme whose sequence was similar to, but not identical with, PrtH. Very recently, the complete coding sequence for this new enzyme was identified in the genome sequence for *Lb. helveticus* H10 (Zhao et al., 2011). This new gene is predicted to encode a 2,011 residue-long pre-pro-cell-envelope proteinase with 67% identity and 80% AA similarity to PrtH, and includes a C-terminal anchor region with homology to S-layer proteins. However, the nucleotide sequence for

Table 5. Amino acid homology among the cell envelope-associated proteinase paralogs found in *Lactobacillus helveticus* CNRZ 32¹

Enzyme (length) ²	PrtH	PrtH2	PrtH3	La PrtP	Lj PrtP	Lu PrtP
PrtH (1,843 residues)	—	—	—	32/47 (57)	61/75 (90)	29/45 (57)
PrtH2 (1,786 residues)	34/50 (69)	—	—	61/75 (88)	29/47 (47)	55/70 (88)
PrtH3 (1,642 residues)	32/48 (60)	58/69 (100)	—	59/72 (98)	32/51 (50)	53/68 (98)
PrtH4 (1,658 residues)	30/45 (72)	57/70 (96)	53/68 (97)	52/68(99)	29/45 (57)	93/96 (100)

¹Percent AA identity/percent similarity (% query coverage). Cell envelope-associated proteinases from *Lactobacillus acidophilus* NCFM (La PrtP; 1,627 residues), *Lactobacillus johnsonii* NCC 533 (Lj PrtP; 2,209 residues), and *Lactobacillus ultunensis* DSM 16047 (Lu PrtP; 1,663 residues) are included for evolutionary reference. GenBank accession numbers for the latter enzymes are AAV43331, AAS09785, and EEJ72368, respectively. Bold font indicates closest taxonomic relative.

²Predicted number of AA in nascent pre-pro-protein.

Figure 1. Distribution of *Lactobacillus helveticus* CNRZ 32 genes associated with cell envelope-associated proteinases among other strains of *Lb. helveticus* as determined by comparative genome hybridization (CGH) or multiplex PCR. The presence (gray) or absence (white) of individual genes in different *Lb. helveticus* strains is indicated by the fill color. Strains tested by both CGH and multiplex PCR methods are highlighted in bold font.

the H10 proteinase lacks any measurable homology to the genome sequence data for strains CNRZ 32 and DPC 4571, or to the draft sequence for ATCC 15009. Because the ability of LAB to rapidly acidify milk requires CEP activity (for reviews, see Kunji et al., 1996; Siezen, 1999), we investigated this attribute in UPR13. Results showed that it reduced milk pH by only 0.9 pH unit after 24 h of incubation at 42°C, which indicates that UPR13 does not possess any functional gene for CEP.

The CGH and multiplex PCR approaches used in this work also cannot determine whether a gene is functional. Use of either technique with strain DPC 4571, for example, would be predicted to reveal this strain contains only *prtH3* (Figure 1). However, the genome sequence for DPC 4571 (Callanan et al., 2008) reveals the *prtH3* CDS contains a nonsense mutation, and the bacterium displays a proteinase-negative phenotype (P. Ross, Teagasc, Moorepark, Ireland, personal communication). Mutations are also likely to be present in the *prtH3* and *prtH4* CDS of strain L89, as this bacterium is also reported to show a proteinase-negative phenotype (Matar et al., 2001).

As was recently reported by Genay et al. (2009), the distribution of CEP paralogs among *Lb. helveticus* strains also was non-uniform. The most abundant paralog was *prtH3*, which was carried by over 80% of strains tested. Altogether, *prtH3* was detected in all but 2 of the strains that contained more than one gene for CEP, and in 13 of 19 strains found to carry only one CEP paralog (Figure 1). Genes for *prtH* and *prtH4* were also relatively common; each was detected in 21 of 49 distinct strains tested (total excluding the 2 archival samples of CNRZ 32), almost always in combination with other CEP genes. Interestingly, *prtH* was especially common among the 19 *Lb. helveticus* UPR strains isolated from Italian cheese whey or fresh curd (Table 1 and Figure 1). Fifteen (79%) of the UPR strains contained *prtH*, and this group represented 71% of all strains found to contain this paralog. Pederson and coworkers (1999) found *prtH* inactivation in CNRZ 32 did not affect its rates of acidification or growth in milk, but the disproportionate prevalence of *prtH* in *Lb. helveticus* collected from Italian cheese whey raised new questions about the contribution this enzyme may provide to cells growing in whey. To explore this question, we compared growth of CNRZ 32 wild-type cells to that for 2 independently isolated *prtH* deletion mutants (Pederson et al., 1999) in cheese whey. However, results showed *prtH* inactivation had no effect on growth rate of CNRZ 32 in cheese whey (data not shown).

Finally, *prtH2* was detected in less than 25% of *Lb. helveticus* strains tested, and like *prtH* and *prtH4*, it usually occurred in strains that carried other CEP

Figure 2. Agarose gel electrophoresis of amplicons derived from lactobacilli after multiplex PCR with *prtH* and *prtH3*- (panel A) or *prtH2* and *prtH4*-specific primers (panel B). Samples present in each gel include lane 1 to 22, *Lactobacillus helveticus* strains CNRZ 32, R0052, ATCC 15009, R211, R0389, L89, ITG LH44, ITG LH77, ITG LH43, ITG LH55, ITG LH56, ITG LH68, R204, R234, ITG LH1, R235, R247, ATCC 521, R338, LMG 22464, M4, and Y10, respectively; lanes 23 to 25, *Lactobacillus acidophilus* strains NCFM, R240, and R269, respectively; and lane 26, *Lactobacillus gallinarum* ATCC 53673. MW denotes a DNA fragment size standard run in the left lane of each gel, and W denotes the negative control that contained water instead of template DNA.

Figure 3. Organization of the *prtH3* locus in *Lactobacillus helveticus* CNRZ 32 and DPC 4571. The *prtH3* gene is indicated by black fill, and flanking genes common to both strains appear in gray directly above one another. The nonsense mutation present in DPC 4571 is reflected by the split *prtH3* coding sequence. Abbreviations for predicted gene functions: *agg*, aggregation-promoting protein; *hyp*, hypothetical protein; *tps*, transposase; *prtH3*, cell envelope-associated proteinase PrtH3; *abc*, ABC transporter, permease protein; *bdp*, tRNA binding domain protein; *trd*, thioredoxin; *tmt*, transfer RNA (tRNA) methyltransferase.

genes. Curiously, the only exceptions to this observation involved 3 strains, LMG 22464, M4, and Y10, which were originally isolated from whiskey fermentations (Table 1 and Figure 1). This observation suggests PrtH2 may contribute to growth of *Lb. helveticus* on plant-derived proteins, but additional experiments will be needed to test that hypothesis.

Our results related to the distribution of CEP paralogs among *Lb. helveticus* conflict with a recent report by Genay et al. (2009), who concluded *prtH2* was more prevalent than *prtH*. The CGH and multiplex PCR data presented here clearly show the most common CEP gene is *prtH3*, and *prtH2* is actually the least abundant paralog (Figures 1 and 2). The basis for this discrepancy is clear; Genay et al. (2009) did not have access to the *prtH3* or *prtH4* sequences, and opted to annotate the CEP pseudogene found in the sequenced bacterium DPC 4571 (and homologous sequences they detected by PCR in other strains) as a new *prtH2* allele rather than *prtH3*, despite the fact it showed only about 60% nucleotide and AA sequence identity to *prtH2*. However, alleles are partly defined by having a common location in the genome, whereas paralogous genes occur at different sites and may display a higher level of sequence divergence due to mutation or selection away from a common ancestral gene. The *prtH3* loci in CNRZ 32 and DPC 4571 are 99% identical at the nucleotide sequence level, and have shared flanking sequences (Figure 3). Thus, *prtH3* should be viewed as a paralog, and not an allele, of *prtH2* (which, of course, occurs at a different location in the CNRZ 32 genome).

The existence of 4 distinct CEP genes in CNRZ 32 poses interesting questions about the manner in which they may have been acquired. The BLAST homology searches against the protein and nucleotide databases reveal that PrtH is most similar to the CEP found in *Lactobacillus johnsonii*, an intestinal species (Table 5).

The PrtH2 and PrtH3 enzymes produced the highest homology scores against one another and with the PrtP enzyme from *Lb. acidophilus*, a species that also occurs in the intestinal tract. Although AA homology of both paralogs to PrtP was similar (Table 5), the alignment between PrtH3 and PrtP extended over 98% of the enzyme, whereas that for PrtH2 covered 88%. Finally, PrtH4 showed 92% identity at the nucleotide level, and 93% identity (as well as 96% similarity) at the AA level, over the entire length of the CEP found in *Lactobacillus ultunensis*, a species found in the human oral cavity and stomach. These findings, coupled with the relative distribution of each paralog in *Lb. helveticus* and the close phylogenetic relationship of this species with *Lb. acidophilus*, suggest that *prtH3* is the ancestral CEP in *Lb. helveticus*, and that *prtH2* likely arose from duplication of *prtH3*. Because PrtH and PrtH4 show highest homology to enzymes from other species of lactobacilli, it is likely that these enzymes were independently acquired through horizontal gene transfer.

In some species of LAB, CEP activation requires a maturation protein termed PrtM (Savijoki et al., 2006). Liu et al. (2010) recently surveyed genome sequence data for proteolytic enzymes in 19 species of LAB, and detected a *prtM* ortholog in only 3 species of lactobacilli: *Lb. johnsonii*, *Lb. casei*, and *Lactobacillus rhamnosus*. We previously identified 2 *prtM* paralogs, designated *prtM* and *prtM2*, in *Lb. helveticus* CNRZ 32 (Smeianov et al., 2007). These proteins are predicted to be 310 and 306 AA in length, respectively, and display 49% AA identity and 69% similarity to one another. The physical location of *prtM* in CNRZ 32 reflects the typical organization for this gene in LAB (Savijoki et al., 2006); it occurs immediately adjacent to, but in divergent orientation to, the 5' end of *prtH*. The *prtM2* gene shows similar basic organization to *prtH3*, except that the 2 CDS are separated by approximately 9 kb, with

several other CDS in between. As expected, protein homology searches showed that PrtM is most closely related (64% AA identity and 81% similarity) to its ortholog in *Lb. johnsonii*, and presence of this gene was almost exclusively restricted to strains that also contained *prtH* (Figure 1). The only exceptions were UPR 9 and UPR 13, which may indicate that these strains lost *prtH* through a deletion event (a possibility that is supported by the proteinase-negative phenotype of UPR 13) or, alternatively, that UPR9 contains a *prtH* paralog whose nucleotide sequence differs from that found in CNRZ 32. As has already been noted, evidence to support the latter possibility has been provided by Genay et al. (2009) and Zhao et al. (2011). Like *prtH* in CNRZ 32, the 5' end of the CEP gene in *Lb. helveticus* H10 is immediately adjacent to a *prsA* paralog (with 67% identity and 82% similarity to the CNRZ 32 PrtM maturase) that lies in divergent orientation. Interestingly, the coding sequences for the maturase also lacks any measurable nucleotide homology to the CNRZ 32 genome. The discovery of new *prtH* and *prtM* paralogs reinforces the view that the rich diversity of cell envelope proteinases in *Lb. helveticus* is unparalleled among other LAB, and supports our hypothesis that different proteinase combinations have a prominent role in the industrial functionality of this species.

In contrast to *prtM*, *prtM2* was detected by CGH in all *Lb. helveticus* strains tested, and BLAST searches revealed the presence of highly conserved (>90% AA identity) orthologs in *Lb. helveticus* DPC 4571, *Lb. acidophilus* NCFM, and *Lb. ultunensis* DSM 16047. In the latter strains, this gene is annotated as *prsA*, which encodes a lipoprotein foldase that (like PrtM) is a member of the peptidyl-prolyl *cis/trans* isomerase superfamily [Enzyme Commission (EC) number 5.2.1.8] involved in enzyme secretion and activation in gram-positive bacteria (Kontinen et al., 1991; Jacobs et al., 1993; Hermans et al., 2006). Three families of peptidyl-prolyl *cis/trans* isomerases, termed cyclophilins, FK506-binding proteins, and parvulins, are found in bacteria and higher cells (Rahfeld et al., 1994; Nagradova, 2007). In addition to *prtM* and *prtM2*, genes predicted to encode cyclophilin- and FKBP-type peptidyl-prolyl *cis/trans* isomerases are also present in CNRZ 32, and the latter genes were also detected by CGH in all *Lb. helveticus* strains tested.

Various orthologs of LAB PrtM proteins show 30 to 34% AA identity to the *Bacillus subtilis* PrsA protein, and both appear to belong to the parvulin family of peptidyl-prolyl *cis/trans* isomerases (Rahfeld et al., 1994). More importantly, Jacobs et al. (1993) showed PrsA had a crucial role in the proper folding of different exoproteins, including the serine proteinase subtilisin. Collectively, these findings strongly suggest that PrtM

is required for activation of PrtH, whereas PrtM2 could have a role in folding and activation of the other CEP paralogs found in *Lb. helveticus* and related species of lactobacilli.

Phylogenetic analysis of CEP proteins from CNRZ 32 and those from other LAB shows that enzymes from lactobacilli are distributed across 6 distinct clusters (Figure 4). One contains enzymes from the *Lactobacillus delbrueckii* group, a second holds *Lb. helveticus* PrtH and the *Lb. johnsonii* PrtP, the third includes PrtP enzymes of *Lb. casei* and *Lc. lactis*, the fourth includes CNRZ 32 PrtH2, PrtH3, and PrtH4 plus CEP from *Lb. acidophilus* NCFM and *Lb. ultunensis* DSM 16047, the fifth holds the *Lb. rhamnosus* PrtR enzyme, and the last includes PrtR-like enzymes from *Lb. casei*. Research with the highly related (98% AA sequence identity) CEP alleles found in *Lc. lactis* has shown that these enzymes may be differentiated by their relative affinity and specificity for individual CN (Kunji et al., 1996), and that these differences can impart significantly different effects on the peptide profiles and sensory quality of cheese (Broadbent et al., 2002, 2006; Pillidge et al., 2003). Enzymes from *Lb. helveticus* and other lactobacilli can also differ in their relative affinity and specificity toward CN (Kunji et al., 1996; Oberg et al., 2002; Jensen et al., 2009), and research suggests these differences can influence the functional properties of cheese (Oommen et al., 2002). Thus, the rich diversity of CEP enzymes found in *Lb. helveticus* has enormous implications for the applications of these cells in the manufacture of cheese and bioactive foods. For example, the basis for strain-to-strain variability in production of bioactive peptides by *Lb. helveticus* is unknown, but release of these peptides obviously requires hydrolysis of intact CN and CEP is the only proteolytic enzyme in LAB that efficiently catalyzes this reaction. It is, therefore, not surprising that CEP activity in *Lb. helveticus* has been implicated in the release of ACE inhibitory, immunomodulatory, and antimicrobial peptides from milk (Yamamoto et al., 1994; Matar et al., 2001; Minervini et al., 2003; de Moreno de LeBlanc et al., 2005). Results from this study indicate that CEP gene content may be a primary factor in determining the functionality of *Lb. helveticus* strains in cheese and bioactive milks.

Genes Associated with Amino Acid Metabolism

As a species, *Lb. helveticus* has more extensive AA requirements than most LAB (Kandler and Weiss, 1986), and previous analysis of the AA requirements for *Lb. helveticus* CNRZ 32 demonstrated this strain was auxotrophic for 14 AA (Christensen and Steele, 2003; Christiansen et al., 2008). Because AA breakdown by

Figure 4. Minimum evolution phylogenetic trees for lactocepin from 17 LAB strains. *Bacillus subtilis* is used as an outgroup. Bootstrap values on the bifurcating branches are based on 1,000 random bootstrap replicates for the consensus tree. Gene ID is given for strains with more than one homolog. Strain code: lac, *Lactobacillus acidophilus* NCFM; lbu, *Lactobacillus delbrueckii* ATCC BAA-365; lca, *Lactobacillus casei* ATCC 334; lcb, *Lb. casei* BL23; ldb, *Lb. delbrueckii* ATCC 11842; lhe_CN, *Lactobacillus helveticus* CNRZ32; lhe_H10, *Lb. helveticus* H10; ljo, *Lactobacillus johnsonii* NCC 533; lrh, *Lactobacillus rhamnosus* HN001; llc, *Lactococcus lactis* ssp. *cremoris* SK11; lul, *Lactobacillus ultunensis* DSM 16047; sag, *Streptococcus agalactiae* 2603 (serotype V); spn, *Streptococcus pneumoniae* TIGR4; spz, *Streptococcus pyogenes* MGAS5005 (serotype M1); spm, *Streptococcus pyogenes* MGAS8232 (serotype M18); ssv, *Streptococcus suis* 98HAH33; and ste, *Streptococcus thermophilus* LMD-9.

LAB often involves the reversible action of enzymes involved in anabolic pathways (Yvon and Rijnen, 2001; Ardö, 2006), we used CGH to investigate the distribution of CNRZ 32 genes for AA biosynthesis and metabolism among our bank of *Lb. helveticus* strains. Those experiments revealed that genes involved in AA metabolism were highly conserved across the species (Figure 5), and most of the observed differences would not be predicted to affect flavor or aroma production in cheese. Nonetheless, 2 noteworthy differences were detected. First, several of the Italian whey isolates did not contain cystathionine- β -lyase (EC 4.4.1.8), an en-

zyme that converts Met into methanethiol (MtSH), a volatile sulfur compound whose production has been associated with desirable cheese flavor and aroma (Dias and Weimer, 1998; Lee et al., 2007). Thus, *Lb. helveticus* strains lacking this enzyme may have a decreased capacity to produce sulfur-based flavor and aroma in cheese.

A second observation involved the absence of *serC*, which encodes phosphoserine transaminase (EC 2.6.1.52), in 8 strains, including all 3 whiskey isolates (Figure 5). In CNRZ 32, *serC* is adjacent to 3-phosphoglycerate dehydrogenase (*serA*; EC 1.1.1.95) and

Figure 5. Distribution of *Lactobacillus helveticus* CNRZ 32 genes associated with amino acid metabolism among other strains of *Lb. helveticus* as determined by comparative genome hybridization (CGH) alone. The presence (gray) or absence (white) of individual genes in different *Lb. helveticus* strains is indicated by the fill color.

phosphoglycerate mutase (*pgm*; EC 5.4.2.1), and all 3 genes are significantly upregulated during growth of this bacterium in milk (Smeianov et al., 2007). Because expression of other genes involved in Ser biosynthesis was not altered, those authors proposed that SerA, SerC, and Pgm allow *Lb. helveticus* to derive energy from the phosphoserine residues that are abundant in milk CN. The absence of *serC* in all of the whiskey isolates is consistent with the hypothesis that this pathway may reflect specialization of certain *Lb. helveticus* strains to a milk-based environment.

Even though most CNRZ 32 genes for AA biosynthesis and metabolism were conserved in all of the *Lb. helveticus* strains tested, previous investigators have shown the activity of these enzymes in different strains (including 6 tested in this study), as well as the levels of aroma compounds they produce, can vary (Klein et

al., 2001; Jensen and Ardö, 2010). To explore the molecular basis for this observation, we again performed BLAST homology searches with the predicted protein sequences for 33 CNRZ 32 enzymes involved in AA biosynthesis, as well as 5 enzymes from DPC 4571 or H10 that occur as pseudogenes in CNRZ 32, and 3 enzymes from related lactobacilli that were found to exist as pseudogenes in each of the sequenced strains of *Lb. helveticus* (Table 6). That analysis confirmed our conclusion from CGH data that some *Lb. helveticus* strains lack genes encoding cystathionine- β -lyase and phosphoserine transaminase. Moreover, it again revealed that although AA sequence homology was typically very high over the full length of each enzyme, perfect sequence matches were uncommon and nonsense mutations had occurred in some strains (Table 6). From these data, we conclude the heterogeneity that

Table 6. Amino acid sequence homology among AA biosynthetic enzymes in different strains of *Lactobacillus helveticus*¹

CNRZ 32 protein (length) ²	DPC 4571	H10	ATCC 15009
Aromatic aminotransferase (394 residues)	391/392 (100)	390/391 (100)	393/394 (100)
Aspartate aminotransferase (388 residues)	388/388 (100)	382/382 (100)	388/388 (100)
Branched-chain aminotransferase (341 residues)	338/340 (100)	338/341 (100)	341/341 (100)
Aminotransferase (392 residues)	392/392 (100)	Absent	391/391 (100)
Phosphoserine transaminase (334 residues)	325/325 (100)	Absent	ND ³
Ornithine carbamoyltransferase (pseudogene) ⁴	Pseudogene	Pseudogene	Pseudogene
Carbamate kinase (pseudogene) ⁴	Pseudogene	Pseudogene	Pseudogene
Arginine deiminase (pseudogene) ⁴	Pseudogene	Pseudogene	Pseudogene
Carbamoyl-phosphate synthase A (351 residues)	349/350 (100)	347/350 (100)	348/350 (100)
Carbamoyl-phosphate synthase A2 (361 residues)	354/357 (100)	355/358 (100)	361/361 (100)
Carbamoyl-phosphate synthase B (1,063 residues)	1054/1057 (100)	1052/1058 (100)	1054/1059 (100)
Carbamoyl-phosphate synthase B2 (1,062 residues)	1060/1060 (100)	968/1020 (100)	PG ⁵
Ornithine decarboxylase OdcI (701 residues)	697/699 (100)	691/694 (100)	ND
Ornithine decarboxylase OdcC (698 residues)	690/695 (100)	687/690 (100)	ND
Aspartate-ammonia ligase (337 residues)	333/336 (100)	327/334 (100)	336/336 (100)
Asparagine synthase (649 residues)	648/648 (100)	647/647 (100)	649/649 (100)
Asparaginase (334 residues)	331/333 (100)	321/323 (97)	332/334 (100)
Aspartate-semialdehyde dehydrogenase (352 residues)	351/352 (100)	348/349 (100)	352/352 (100)
Phosphoenolpyruvate carboxylase (pseudogene) ⁶	(912 residues)	892/902 (100)	873/886 (98)
Glutamate-ammonia ligase (445 residues)	445/445 (100)	443/444 (100)	445/445 (100)
O-Acetylserine (thiol) lyase (303 residues)	301/302 (100)	Absent	Pseudogene
Cystathionine-β-lyase (394 residues)	394/394 (100)	Absent	394/394 (100)
Serine acetyl-transferase (185 residues)	172/172 (92)	Absent	185/185 (100)
Homoserine-O-succinyl transferase (264 residues)	263/264 (100)	261/263 (100)	264/264 (100)
O-Acetylserine (thiol) lyase (pseudogene) ⁶	(309 residues)	305/307 (100)	307/309 (100)
5-Methyltetrahydropteroyl(Glu ₃)-homocysteine methyltransferase (761 residues)	761/761 (100)	755/760 (100)	761/761 (100)
Cystathionine-γ-lyase (pseudogene) ⁶	(385 residues)	381/383 (100)	Pseudogene
Diaminopimelate decarboxylase (438 residues)	434/435 (100)	433/434 (100)	438/438 (100)
2,3,4,5-Tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase (236 residues)	236/236 (100)	236/236 (100)	236/236 (100)
Succinyl-diaminopimelate desuccinylase (384 residues)	380/383 (100)	379/382 (100)	PG
Dihydrodipicolinate synthase (311 residues)	309/309 (100)	308/309 (100)	311/311 (100)
Dihydrodipicolinate reductase (259 residues)	257/257 (100)	256/256 (100)	259/259 (100)
Aspartate kinase (457 residues)	448/449 (100)	450/452 (100)	ND
Diaminopimelate epimerase (334 residues)	332/332 (100)	327/328 (100)	334/334 (100)
Acetolactate synthase (pseudogene) ⁷	Absent	(437 residues)	Pseudogene
Glycine hydroxymethyltransferase (411 residues)	409/410 (100)	405/408 (100)	411/411 (100)
Glycerate kinase (384 residues)	381/382 (100)	381/382 (100)	384/384 (100)
Phosphoglycerate dehydrogenase (388 residues)	383/384 (100)	Absent	ND
Phosphoserine phosphatase (293 residues)	289/292 (100)	290/292 (100)	293/293 (100)
L-Serine ammonia-lyase B (pseudogene) ⁶	(220 residues)	217/218 (100)	ND
L-Serine ammonia-lyase A (301 residues)	294/297 (100)	293/296 (100)	ND

¹Identical AA/similar AA (% query coverage).²Predicted from coding sequence.³Not detected in draft (incomplete) ATCC 15009 genome, but CGH data indicate the gene is present.⁴Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches for ornithine carbamoyltransferase, carbamate kinase, and arginine deiminase were performed using protein sequences from GenBank files ADZ07496, YP_004032111, and ADZ07497, respectively.⁵Partial or interrupted gene located near end of a sequence contig in the draft assembly. Full reading frame may or may not be intact.⁶Predicted protein sequence from DPC 4571 ortholog used for homology comparisons.⁷Predicted protein sequence from H10 ortholog used for homology comparisons.

has been noted among *Lb. helveticus* in regard to AA metabolism results from the combination of gene in-activation and sequence polymorphisms that alter the specificity or relative activity of individual enzymes.

CONCLUSIONS

Lactobacillus helveticus is recognized among LAB for its proteolytic activity and rich assortment of proteo-

lytic enzymes (Gilbert et al., 1997; Christensen et al., 1999; Matar et al., 2001; Savijoki et al., 2006). Interest in the proteolytic system of this bacterium is tied to its ability to decrease bitterness and accelerate flavor development in cheese (Bartels et al., 1987a,b; Ardö and Pettersson, 1988; Drake et al., 1996) and to release bioactive peptides in milk-based foods (Laffineur et al., 1996; LeBlanc et al., 2002; Hayes et al., 2007b; Gobetti et al., 2010). However, these attributes are strain

specific, and more detailed knowledge of the proteolytic enzyme systems and AA catabolism in *Lb. helveticus* is needed to better use these organisms for food and health. Results from this study suggest strain heterogeneity in peptidase activity or AA metabolism are not based on differences in gene content, but rather are more likely due to a combination of nonsense mutations plus sequence polymorphisms that affect the expression level, specificity, or activity of the individual enzymes involved in these reactions. In contrast, marked genetic differences were discovered in the distribution of CEP paralogs, and those differences are probably very important determinants of strain functionality in cheese and in production of bioactive peptides in fermented milks.

ACKNOWLEDGMENTS

The authors thank Julie Audy (Institut Rosell, Montreal, QC, Canada) for technical assistance with the multiplex PCR analysis, Marie Penderup Jensen (University of Copenhagen, Frederiksberg C, Denmark) for assistance with DNA extraction for CGH, Craig Brighton (Utah State University, Logan) for assistance with growth experiments in cheese whey, Theresa Walunas and Integrated Genomics (Mount Prospect, IL) for assistance in designing the *Lb. helveticus* CNRZ 32 microarray, the Utah State University (USU) Center for Integrated Biosystems for microarray hybridizations and scanning (Logan), and John R. Stevens of the USU Mathematics and Statistics Department (Logan) for help with R bioinformatics software. We also thank Sarah Highlander (Baylor College of Medicine, Houston, TX) and the Human Microbiome Project Reference Genome Sequencing Initiative, U54 HG003273, for assistance with access to the *Lb. helveticus* ATCC 15009 (DSM 20075) draft genome sequence. Portions of this research were supported by the United States Department of Agriculture, by Dairy Management Inc. [through the Center for Dairy Research (Madison, MI) and Western Dairy Center (Logan, UT)], and by the Utah Agricultural Experiment Station (Logan).

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