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Genetic Characterization of a Cell Envelope-Associated Proteinase from *Lactobacillus helveticus* CNRZ32

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A cell envelope-associated proteinase gene (*prtH*) was identified in *Lactobacillus helveticus* CNRZ32. The *prtH* gene encodes a protein of 1,849 amino acids and with a predicted molecular mass of 204 kDa. The deduced amino acid sequence of the *prtH* product has significant identity (45%) to that of the lactococcal PrtP proteinases. Southern blot analysis indicates that *prtH* is not broadly distributed within *L. helveticus*. A *prtH* deletion mutant of CNRZ32 was constructed to evaluate the physiological role of PrtH. PrtH is not required for rapid growth or fast acid production in milk by CNRZ32. Cell surface proteinase activity and specificity were determined by hydrolysis of α_{s1}-casein fragment 1-23 by whole cells. A comparison of CNRZ32 and its *prtH* deletion mutant indicates that CNRZ32 has at least two cell surface proteinases that differ in substrate specificity.

Lactic acid bacteria (LAB) are essential for the manufacture of a variety of dairy products, such as cheese and yogurt. Because they are auxotrophic for a number of amino acids, LAB depend upon a complex proteolytic system to obtain essential amino acids from caseins during growth in milk (23). This proteolytic system also plays an important role in cheese flavor development (20). The hydrolysis of casein into amino acids for use by LAB is initiated by a cell envelope proteinase (CEP) which hydrolyzes casein into oligopeptides (23). Oligopeptides are then transported into the bacterial cell via an oligopeptide transport system (Opp) (17, 42). Once the oligopeptides are inside the cell, intracellular peptidases hydrolyze them to free amino acids (25, 27).

Several CEPs (or PrtP proteinases [PrtPs]) from various lactococcal strains have been characterized both biochemically and genetically (23). PrtP is synthesized as a pre-pro-protein of approximately 200 kDa. Autocatalytic cleavage of the proregion results in a mature, active protein with a molecular mass of approximately 180 to 190 kDa (23). The genes encoding PrtPs have been sequenced from a number of different *Lactococcus lactis* strains (8, 18, 22, 24, 44, 46). The lactococcal PrtPs are more than 98% identical at the amino acid level (21). Despite this high degree of sequence identity, PrtPs can be classified into at least eight different groups based on substrate specificity by use of α_{s1}-casein fragment 1-23 [α_{s1}-CN (f1-23)] (4, 10, 11). Protein engineering studies have shown that a small number of amino acid substitutions can result in changes in substrate specificity (5, 35, 36, 43).

Much less is known about the CEPs of lactobacilli. The genes encoding CEPs have been cloned from *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (13, 15). The deduced CEP amino acid sequences are 95 and 27% identical, respectively, to those of the lactococcal PrtPs. Comparisons of different lactobacilli have indicated heterogeneity of cell surface proteinase activity within the genus *Lactobacillus* (14, 19). Recent studies have indicated that *Lactobacillus helveticus* may contain two proteinases with different substrate specificities (14). In addition, a zinc-dependent cell surface proteinase has been purified from *L. delbrueckii* subsp. *bulgaricus* (39). This paper describes the genetic and physiological characterization of a CEP from *L. helveticus* CNRZ32.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. All cultures were maintained at −80°C in 11% nonfat dry milk–10% glycerol. *Escherichia coli* DH5α (Gibco-BRL Life Technologies Inc., Gaithersburg, Md.) was grown in Luria-Bertani medium (32). *L. helveticus* CNRZ32 was propagated in MRS medium (Difco Laboratories, Detroit, Mich.) without shaking at 42°C. Strains for Southern hybridization were obtained from the American Type Culture Collection (Rockville, Md.). *L. helveticus* L89 was kindly provided by Fred A. Exterkate from The Netherlands Institute for Dairy Research collection. Growth studies with milk were performed by use of twice-steamed, pasteurized skim milk (pasteurized skim milk was steamed for 20 min, kept at 42°C for 2 h, and then steamed for another 20 min) as described previously (6).

Molecular cloning techniques. Recombinant DNA techniques were essentially those described by Sambrook et al. (32). Restriction enzymes and T4 DNA ligase were purchased from Gibco-BRL Life Technologies and were used according to the manufacturer's instructions. *E. coli* transformation was performed with a Gene Pulser by following the instructions recommended by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Transformation of *L. helveticus* was performed essentially as described previously (6). All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

PCR. All primers were synthesized by Gibco-BRL Custom Primers (Grand Island, N.Y.). PCR amplifications were performed with a Perkin-Elmer (Norwalk, Conn.) model 480 thermal cycler. Two primers were designed from an alignment of the conserved regions surrounding the active-site residues of the proteinase genes (Asn_{196} and Ser_{433}; numbering is that of *L. lactis* subsp. *cremoris* SK11 CEP) from various LAB. The sequences of the primers were as follows: Jp1, 5′GTTATCTCTGCTGGGAAC3′; and Jp2, 5′GTGAAGCCATT GAAGTCC3′. An inverse PCR strategy was used to identify adjacent DNA regions (32). CNRZ32 chromosomal DNA (1 to 5 μg) was digested with an appropriate restriction enzyme. The digested DNA was incubated at 65°C for 20 min to inactivate the restriction enzyme, precipitated in ethanol, and resuspended in 15 μl of deionized H_2O. The digested DNA was self-ligated overnight at 15°C in a 20-μl reaction mixture. A 1-μl sample of the overnight ligation mixture was used as a template for PCR. As the known sequenced progressed, new primers were designed accordingly.

The *L. helveticus* L89 *prt* gene was amplified by PCR with primers Jp6 (5′TG GCAGAACCTGTGCCTA3′) (Fig. 1, nucleotides 1522 to 1505) and Jp17 (5′C GATGATAATCCTAGCGAGC3′) (Fig. 1, nucleotides 903 to 922).

DNA sequence analysis. PCR products were purified with a Qiagen Inc. (Hilden, Germany) PCR purification kit. DNA sequencing reactions were performed with a Perkin-Elmer model 480 thermal cycler and a Prism Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc.,

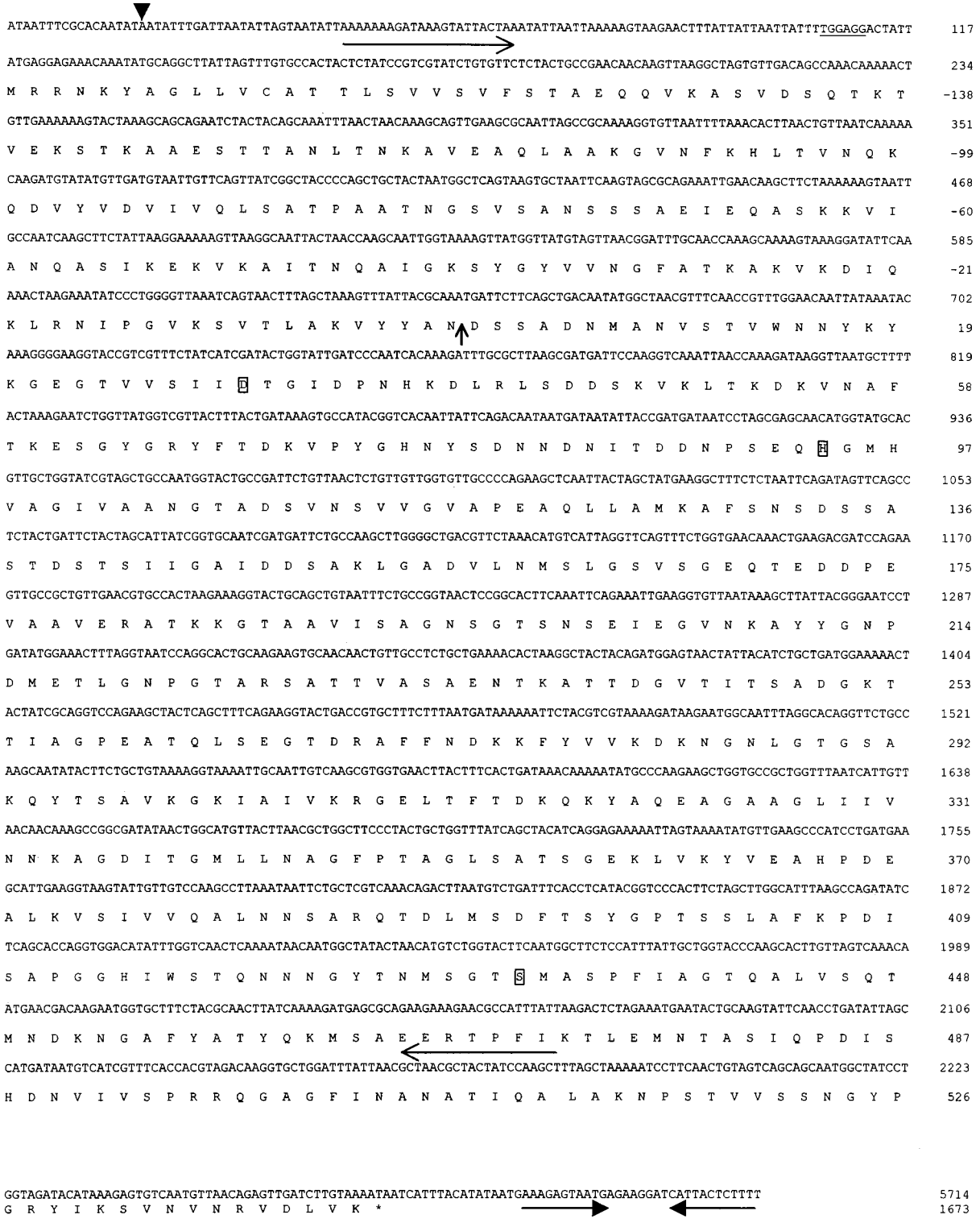

FIG. 1. Partial nucleotide and deduced amino acid sequence of the *L. helveticus* CNRZ32 *prtH* gene. The arrowhead indicates the start of transcription. A putative Shine-Delgarno sequence is underlined. The putative cleavage site for the pre-pro-region is marked with a vertical arrow. The putative active-site residues are boxed. Long horizontal arrows indicate primers used for DNA probe synthesis. The stop codon is indicated with an asterisk. Short horizontal arrows indicate the putative transcriptional terminator.

Foster City, Calif.). The DNA sequence determination was conducted at the Nucleic Acid and Protein Facility of the University of Wisconsin—Madison Biotechnology Center with an ABI Prism model 377XL DNA automated sequencer. Sequences were analyzed with the Genetics Computer Group (Madison, Wis.) sequence analysis program. Protein homology searches were performed by use of the BLAST network service (1). All reported DNA sequence data was confirmed by sequencing both DNA strands from at least two independent PCR products.

TABLE 1. Sequence alignment of the substrate binding regions of PrtH and other members of the subtilase family

Protein	Sequence of substrate binding site and adjacent residues				
	137–139[a]	166[a]	156[b]	217[b]	747–748[a]
PrtH	FSNSDSS**AST**DSTSII	SLGS**V**S	SAGN**S**	**M**SGTS	SLS**AT**KTYYN
PrtP (SK11)	FSNSTDS**AKT**GSATVV	SLGS**N**S	SAGN**S**	**M**SGTS	STN**RK**KTYYN
PrtP (Wg2)	FTNSDTS**ATT**GSSTLV	SLGS**D**S	SAGN**S**	**M**SGTS	STN**LT**KTYYN
PrtB	FSNNAKN**SGA**YDDDII	SLGS**V**S	SAGN**S**	**M**SGTS	GKE**GT**KDYYS
Thermitase[c]	LDN---S**GSG**TWTAVA	SLGG**T**V	AAGN**A**	**L**SGTS	
Subtilisin BPN′[c]	LGA---D**GSG**QYSWII	SLGG**P**S	AAGN**E**	**Y**NGTS	
	↑ ↑↑↑↑↑ ↑	↑↑↑↑↑↑	↑ ↑↑↑	↑↑↑↑↑	

[a] Numbering corresponds to that of *L. lactis* subsp. *cremoris* SK11 proteinase.
[b] Numbering corresponds to that of subtilisin BPN′.
[c] Alignment is from reference 37, and arrows indicate subtilisin BPN′ and thermitase substrate binding residues.

RNA methods. Total RNA was isolated by use of an SV Total RNA Isolation System (Promega Corporation, Madison, Wis.). Mapping of the 5′ end of the *prtH* transcript was conducted by use of a 5′ rapid amplification of cDNA ends (5′ RACE) kit (version 2.0; Gibco-BRL). The nucleotide sequences of the three *prtH*-specific primers were 5′ATGATAGAAACGACGGTACC3′ (Jp16), 5′AACGGTTGAAACGTTAGC3′ (Jp43), and 5′GCTTGGTTAGTAATTGCC3′ (Jp45).

Southern blot analysis. Chromosomal DNA isolation and Southern hybridization procedures were performed as described previously (9). Probe synthesis was performed as described for a Genius kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) with a 2.0-kb internal fragment from the catalytic domain of *prtH*. The template used for probe synthesis was made by PCR amplification with primers Jp22 (5′CTCTATCCGTCGTATCTGTG3′) and Jp23 (5′GCTTGGATAGTAGCGTTAGC3′). Hybridizations were carried out at 42°C. Low-stringency conditions were achieved by use of 10% formamide in the hybridization buffer.

Construction of a *prtH* deletion mutant of CNRZ32. Primers Jp22bam (primer Jp22 with a *Bam*HI extension at the 5′ end) and Jp23bam (primer Jp23 with a *Bam*HI extension at the 5′ end) were synthesized. The Jp22bam-Jp23bam PCR product (described above) was digested with *Bam*HI and ligated into pTRKL2. The resulting plasmid was used as a template for reverse PCR with primers Jp25 (5′GGTGAACAAACTGAAGACG3′) (nucleotides 1144 to 1162) and Jp26 (5′ATTGTGACCGTATGGCACT3′) (nucleotides 876 to 858). The PCR product was self-ligated and transformed into *E. coli* DH5α. The construct that was created contained a 270-bp internal in-frame deletion subcloned into pTRKL2. An integration vector was constructed by subcloning the deletion fragment into pSA3. The deletion was confirmed by PCR and DNA sequencing. The resulting construct was used to construct a *prtH* deletion derivative of CNRZ32 by gene replacement as described previously (2) with modifications described by Christensen and Steele (7). Transformation of CNRZ32 was performed at 37°C. Six transformants were chosen at random for spread plating on MRS medium plates containing 50 ng of erythromycin per ml at 44°C (nonpermissive temperature for pSA3). Integrants were grown in MRS broth at 37°C without erythromycin, and erythromycin-sensitive colonies were selected for further characterization.

Proteinase activity. Cells were grown from frozen stocks in MRS broth to the logarithmic growth phase and then were transferred to citrate-milk medium that had been steamed for 30 min. Cells were grown to an optical density of 0.7 absorbance unit at 590 nm, harvested by centrifugation, washed twice with 50 mM Na_2PO_4 buffer (pH 6.8), and resuspended in Jennes-Koops buffer (16). Isolation of α_{s1}-CN (f1-23) was performed as described by Exterkate and Alting (12). *L. helveticus* whole cells were incubated in Jennes-Koops buffer with the α_{s1}-CN (f1-23) fragment at 30°C for 5 min, 15 min, 30 min, or 1 h at pH 6.5 (16). Samples from the reaction mixture were analyzed by high-performance liquid chromatography (HPLC) as described previously (4). Several hydrolysis products [α_{s1}-CN (f1-6), α_{s1}-CN (f18-23), α_{s1}-CN (f9-23), and α_{s1}-CN (f10-23)] were identified by mass spectrometry at the Nucleic Acid and Protein Facility of the University of Wisconsin—Madison Biotechnology Center. All other peptides were identified by use of standards as described previously (4).

Nucleotide sequence accession number. The GenBank accession no. for the nucleotide sequence reported in this paper is AF133727.

RESULTS

Identification and sequencing of a cell envelope-associated serine proteinase gene (*prtH*) from *L. helveticus* CNRZ32. PCR amplification was used to identify a CEP gene from *L. helveticus* CNRZ32. A single 700-bp PCR product was obtained and sequenced (data not shown). New primers were designed based on the sequence of this product, and inverse PCR was used to identify the adjacent DNA regions.

By sequencing 6.2 kb of contiguous DNA, an open reading frame that has significant DNA sequence similarity to known lactococcal *prtP* genes was identified. This *L. helveticus* cell envelope-associated proteinase is referred to as PrtH, and its corresponding gene is referred to as *prtH*. *prtH* encodes a putative protein of 1,849 amino acids and with a deduced molecular mass of 204 kDa (Fig. 1). The start of transcription, as determined by 5′ RACE, is 90 nucleotides upstream of the start codon. A putative ribosome-binding site (TGGAGG) is found at position −7 from the start codon. Downstream of *prtH* is a putative *rho*-independent transcriptional terminator (AAAGAGTAATGAGAAGATCATTACTCTTT) with a change in free energy of −14.4 kcal/mol (41).

Comparison with other cell surface proteinases. A comparison of the N-terminal region of PrtH with those of other CEPs indicates that PrtH may be synthesized as a pre-pro-protein. The N-terminal segment of the deduced PrtH is positively charged and is followed by a putative membrane-spanning domain. This region closely resembles the signal peptide sequence for gram-positive bacteria (33, 38). The predicted cleavage site for the signal peptide is at Ala_{-152}-Glu_{-151} (29). Adjacent to the signal peptide is a region that has 51% amino acid identity with the pro-region of the PrtPs. This finding suggests that PrtH will be processed similarly. Such processing would result in a mature PrtH of 1,673 amino acids and having 45% identity with the lactococcal PrtPs.

The N-terminal region of the mature proteinase (~500 amino acids) is referred to as the catalytic domain. This region has similarity to the subtilisin-like serine proteinases (subtilases); therefore, PrtH can be classified within this family (34, 36). The subtilase family of proteins is characterized by a catalytic triad, Asp-His-Ser. The putative catalytic residues of PrtH are at positions Asp_{30}, His_{94}, and Ser_{432} (Fig. 1). The catalytic sites, as well as adjacent residues, are very well conserved among PrtH, PrtB, PrtP, and other members of the subtilase family (data not shown).

A number of residues in the lactococcal PrtPs have been implicated in substrate specificity by homology modeling, sequence alignments, and protein engineering studies (5, 35, 36, 43). The substrate binding residues are divergent among PrtH, PrtP, PrtB, subtilisin BPN′, and thermitase (Table 1). *L. lactis* subsp. *cremoris* SK11 PrtP residues 137 to 139, 166, and 748 have been demonstrated to effect substrate specificity (35). PrtH has a unique amino acid substitution at position 138 (Ser) compared to all other CEPs from LAB. Position 166 is occupied by Val in both PrtH and PrtB, while Asn and Asp are found at this position in PrtP from SK11 and Wg2, respectively. Thr occupies position 748 in PrtH, PrtP from Wg2, and PrtB. Because of the unique combination of amino acids at residues

thought to be involved in substrate specificity, PrtH cannot be classified in any of the previously described groups of CEPs. Therefore, PrtH is classified as a new group, designated group I.

The C-terminal region of PrtP has a conserved LPxTG motif, which is found in many cell surface proteins (28). The LPxTG motif functions as an anchor to the cell membrane. This motif is not found in PrtH, although PrtH is most likely located on the cell surface. A 101-amino-acid region at the C terminus of PrtH (amino acid residues 1538 to 1639 of the mature proteinase) has 32% identity with the C terminus of the surface-layer (S-layer) protein (amino acid residues 314 to 415) from *Lactobacillus acidophilus* (data not shown) (3).

Distribution of *prtH* within *L. helveticus*. Southern blot analysis was used to determine the distribution of *prtH* among various strains of *L. helveticus* (CNRZ32, ATCC 15009, ATCC 10797, ATCC 12046, ATCC 8018, ATCC 15807, ATCC 10386, and L89). Under low-stringency conditions (10% formamide and 42°C), a *prtH* DNA probe hybridized only to a 4.1-kb DNA fragment from *L. helveticus* CNRZ32 and L89 (data not shown). Because the hybridization patterns were identical for CNRZ32 and L89, we compared the DNA sequences of the substrate binding regions for these two proteinases. The L89 substrate binding region was amplified by PCR with primers specific for the CNRZ32 *prtH* gene. Sequence analysis revealed that the L89 subtilase-like substrate binding region is 100% identical at the nucleotide level to *prtH* (data not shown). Although *L. helveticus* ATCC 15009, ATCC 10797, ATCC 12046, ATCC 8018, ATCC 15807, and ATCC 10386 have cell surface proteinase activity, as measured by the hydrolysis of α_{s1}-CN (f1-23) (data not shown), Southern blot analysis indicates that they do not contain a *prtH*-like gene.

Physiological role of PrtH. To determine the physiological role of PrtH, an in-frame deletion was constructed in *prtH*. A 1.7-kb DNA fragment internal to *prtH* was constructed to contain a deletion of 270 bp. This deletion removed the active-site residue, His_{94}, and the subtilase-like substrate binding region. The 1.7-kb *prtH* deletion construct was subcloned into the plasmid vector pSA3 and used to create a *prtH* deletion mutant of CNRZ32 (data not shown). Growth in milk of CNRZ32 and the *prtH* deletion mutant was examined. No difference in acidification rate or maximum specific growth rate was observed between CNRZ32 and the *prtH* deletion mutant (data not shown).

Characterization of cell surface proteinase activity and specificity with α_{s1}-CN (f1-23) as a substrate. To determine the cell surface proteinase activity and specificity of CNRZ32 whole cells, α_{s1}-CN (f1-23) was used as a substrate for hydrolysis. The hydrolysis products were analyzed by reverse-phase HPLC (Fig. 2A). Brief incubations (5 to 15 min) with CNRZ32 whole cells results in the formation of eight peptides: α_{s1}-CN (f1-9), α_{s1}-CN (f1-6), α_{s1}-CN (f1-17), α_{s1}-CN (f1-16), α_{s1}-CN (f17-23), α_{s1}-CN (f18-23), α_{s1}-CN (f9-23), and α_{s1}-CN (f10-23). This finding indicates that several bonds are preferentially hydrolyzed. Hydrolysis of the Leu_{16}—Asn_{17} and Asn_{17}—Glu_{18} bonds results in the formation of four peptides: α_{s1}-CN (f1-16), α_{s1}-CN (f17-23), α_{s1}-CN (f1-17), and α_{s1}-CN (f18-23). Hydrolysis of the Gln_9—Gly_{10} bond results in the formation of two peptides: α_{s1}-CN (f1-9) and α_{s1}-CN (f10-23). Other bonds that appear to be hydrolyzed are the Ile_6—Lys_7 and His_8—Gln_9 bonds.

The cell surface proteinase activity of the *prtH* deletion mutant was also analyzed (Fig. 2B). Incubation of α_{s1}-CN (f1-23) with whole cells resulted in a pattern of hydrolysis different from that of the wild type. The α_{s1}-CN (f1-9), α_{s1}-CN (f1-6), α_{s1}-CN (f9-23), and α_{s1}-CN (f10-23) peptides are still formed at approximately the same rates. However, the α_{s1}-CN (f1-16), α_{s1}-CN (f17-23), α_{s1}-CN (f1-17), and α_{s1}-CN (f18-23) peptides are not detected.

DISCUSSION

A CEP that has 45% identity to the lactococcal PrtPs was identified in *L. helveticus* CNRZ32. The highest sequence identity (65%) is within the N-terminal catalytic domain. The substrate binding region of PrtH is distinct from those of all previously identified CEPs; thus, PrtH is classified as a new group, designated group I (Table 1).

Much is known concerning structure-function relationships in the subtilase family (30). An alignment of subtilisin BPN′, thermitase, and the CEPs from LAB reveal regions that are highly conserved (Table 1). Gln_{156} and Tyr_{217} have been shown to effect substrate specificity in subtilisins (45). All identified CEPs from LAB contain Ser and Met at the corresponding positions. Interestingly, amino acid substitutions at position 156 in the subtilisins can alter the pH profile by affecting the pK_a of the active-site His (31). Comparisons such as this can lead to protein engineering strategies to change the substrate specificity and pH profile for CEPs.

These studies reveal significant differences in the proteolytic systems of CNRZ32 and lactococci. First, CNRZ32 appears to have at least two proteinases present at the cell surface. A *prtH* deletion mutant of CNRZ32 is indistinguishable from wild-type CNRZ32 in growth rate and acid production in milk. This finding is in contrast to the requirement of PrtP for rapid growth and fast acid production in lactococci. The most probable explanation is the presence in CNRZ32 of a second proteinase that is sufficient for rapid growth and fast acid production in milk. Recent studies support the hypothesis of at least two proteinases present at the cell surface of some lactobacilli (14, 40). In addition to a serine proteinase, *L. delbrueckii* subsp. *bulgaricus* ACA DC235 has a zinc-dependent cell surface proteinase (39).

Characterization of cell surface proteinase activity further supports the hypothesis that CNRZ32 has at least two cell surface proteinases. It appears that PrtH hydrolyzes the Leu_{16}—Asn_{17} and Asn_{17}—Glu_{18} bonds of α_{s1}-CN (f1-23), resulting in the formation of peptides α_{s1}-CN (f1-16), α_{s1}-CN (f17-23), α_{s1}-CN (f1-17), and α_{s1}-CN (f18-23). These peptides are not detected from hydrolysis of α_{s1}-CN (f1-23) in the *prtH* deletion mutant of CNRZ32. However, peptides α_{s1}-CN (f1-9), α_{s1}-CN (f10-23), α_{s1}-CN (f1-6), and α_{s1}-CN (f9-23) are detected in approximately equal quantities in both wild-type CNRZ32 and the *prtH* deletion mutant. Therefore, a second proteinase on the CNRZ32 cell surface is likely responsible for the formation of these peptides. These findings demonstrate that CNRZ32 has at least two cell surface proteinases that differ in substrate specificity.

Cell surface proteinase activity was detected in all *L. helveticus* strains tested (data not shown). However, Southern blot analysis indicates that *prtH* is not broadly distributed within the species. A *prtH* DNA probe hybridized to only *L. helveticus* CNRZ32 and L89. Sequence analysis of the L89 CEP substrate binding region revealed 100% identity at the nucleotide level to *prtH*. The L89 proteinase has been purified, and its substrate specificity has been characterized (26). Like PrtH, many CEPs, including the L89 CEP, are able to hydrolyze the Leu_{16}—Asn_{17} and Asn_{17}—Glu_{18} bonds (23). Although we expect PrtH and the L89 CEP to have identical substrate specificities, further comparisons are not possible because PrtH has not yet been purified and CNRZ32 has at least two cell surface proteinases. The proteinase activity detected in the other *L. helveticus*

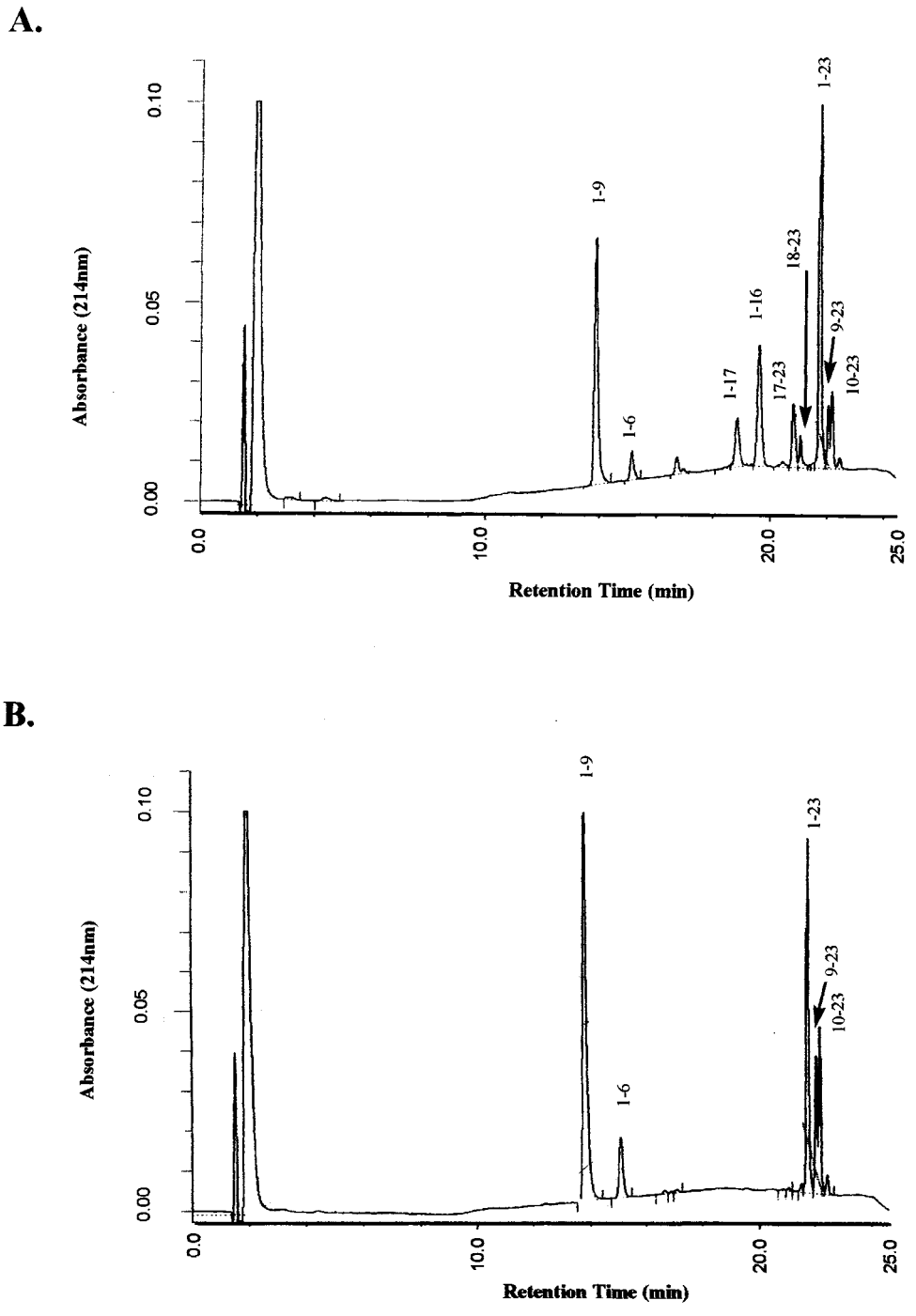

FIG. 2. Reverse-phase HPLC patterns of the hydrolysis products from α_{s1}-CN (f1-23) after 15 min of incubation with wild-type CNRZ32 whole cells (A) and CNRZ32 *prtH* deletion mutant whole cells (B).

strains examined is most likely due to an unknown cell surface proteinase that does not have significant sequence similarity to PrtH.

Neither PrtH nor PrtB has the cell membrane anchor motif LPxTG, which has been found in many cell surface proteins, including the lactococcal PrtPs (28). However, both PrtH and PrtB have C-terminal regions similar to those of S-layer proteins from lactobacilli (3). The C-terminal region of PrtB (amino acid residues 1743 to 1938) has up to 25% identity to the C-terminal region of the S-layer protein from *L. acidophilus* (3). These results suggest that PrtH and PrtB are anchored to the cell envelope in a manner similar to that of S-layer proteins.

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