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A New Cell Surface Proteinase: Sequencing and Analysis of the *prtB* Gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Investigation of the chromosomal region downstream of the *lacZ* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* revealed the presence of a gene (*prtB*) encoding a proteinase of 1,946 residues with a predicted molecular mass of 212 kDa. The deduced amino acid sequence showed that PrtB proteinase displays significant homology with the N termini and catalytic domains of lactococcal PrtP cell surface proteinases and is probably synthesized as a preproprotein. However, the presence of a cysteine near the histidine of the PrtB active site suggests that PrtB belongs to the subfamily of cysteine subtilisins. The C-terminal region strongly differs from those of PrtP proteinases by having a high lysine content, an imperfect duplication of 41 residues, and a degenerated sequence compared with the consensus sequence for proteins anchoring in the cell walls of gram-positive bacteria. Finally, the product of the truncated *prtM*-like gene located immediately upstream of the *prtB* gene seems too short to be involved in the maturation of PrtB.

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the lactic acid bacteria used in industrial milk fermentation. The proteolytic system is essential to ensure rapid growth in milk and supply auxotrophic lactic acid bacteria with amino acids from caseins, the major milk proteins. This system is complex and has been extensively studied in lactococci (21, 35). The first step in milk casein degradation is performed by a cell surface proteinase, PrtP. Two types of PrtP enzymes have been distinguished on the basis of their substrate specificities. PI-type proteinases preferentially cleave β-casein, whereas PIII-type enzymes degrade α, β, and κ caseins (10). Lactococcal PrtPs are serine proteases and show extensive homology with subtilisins secreted by bacilli. Lactococcal proteinases are synthesized as inactive preproproteins. An N-terminal signal peptide of 33 residues is removed during translocation through the cytoplasmic membrane, and the C terminus remains anchored in the cell envelope. Then, a maturation process mediated by a membrane-located lipoprotein, PrtM, leads to the removal of the pro region (154 residues). The 33-kDa PrtM protein is encoded by a gene located immediately upstream and in the opposite direction of *prtP* (13, 49). Incubation of lactococci cells in a calcium-free buffer promotes self-digestion of the carboxy terminus of PrtP and its release into the extracellular medium.

Lactobacilli have been investigated to a lesser extent but display a high cell surface proteinase activity with substrate specificity differing from that of lactococci (4). Cell surface proteinases have been purified from *Lactobacillus paracasei* subsp. *paracasei* NCDO151, *L. helveticus* CNRZ303, and L89, and *L. delbrueckii* subsp. *bulgaricus* CNRZ397 (24, 27, 33, 52). The sequence of the gene encoding proteinase from *L. paracasei* subsp. *paracasei* was determined; the deduced amino acid sequence shows 1,902 residues and a high degree of identity (96%) with lactococcal PrtP (15). The presence of a *prtM* gene suggests a maturation process similar to lactococcal PrtP maturation. In contrast, proteinases from thermophilic lactobacilli appear to be different from those of lactococci. The *L. helveticus* CNRZ303 proteinase is characterized by a unique specificity toward α_{s1} casein (1-23 fragment). The proteinases of *L. delbrueckii* subsp. *bulgaricus* CNRZ397 and *L. helveticus* L89 cannot be released from the cell wall by a procedure using calcium-free buffer. Moreover, the *L. delbrueckii* subsp. *bulgaricus* enzyme is sensitive to serine and cysteine protease inhibitors.

Spontaneous lactose-negative mutants of *L. delbrueckii* subsp. *bulgaricus* NCDO1489 were characterized by large chromosomal deletions of several kilobases including the *lacS* and *lacZ* genes, coding for a permease and β-galactosidase, respectively (11, 32). We took advantage of the availability of some mutants with a proteinase-deficient phenotype to identify and localize the structural gene (*prtB*) coding for the cell surface proteinase. A comparison with PrtP proteinases highlighted new characteristics of the *L. delbrueckii* subsp. *bulgaricus* cell envelope proteinase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *L. delbrueckii* subsp. *bulgaricus* NCDO1489 and mutants N310 and LFi5.F6 (spontaneous *lacZ* derivatives from NCDO1489) were from the Nestec collection (32), and strain CNRZ 397 was obtained from the Institut National de la Recherche Agronomique (Jouy en Josas, France). *Lactococcus lactis* subsp. *lactis* NCDO763 was a gift of A. Hirashima (20). *Escherichia coli* DH5α [*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was purchased from Gibco BRL (Eragny, France). Plasmids pUC18 and pCU19 (51) were used for cloning PCR fragments and DNA sequencing.

L. delbrueckii subsp. *bulgaricus* strains were routinely cultured at 40°C in MRS broth (7), and *Lactococcus lactis* subsp. *lactis* were grown at 30°C in M17 broth supplemented with 0.5% glucose (45). Reconstituted 10% Gamma milk from skim milk powder (Prolait, Niort, France) was centrifuged (15,000 × *g*, 15 min, 6°C) and immediately used. *E. coli* was grown with shaking at 37°C in L broth (30). *E. coli* transformants were selected in the presence of ampicillin (100 μg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 0.1 mM) to detect β-galactosidase activity.

Analysis of casein hydrolysis. Bacterial cells were collected in exponential phase as described previously (2) and treated by the procedure of Hill and Gasson (14) as modified (24). Peptides resulting from casein degradation were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23) on

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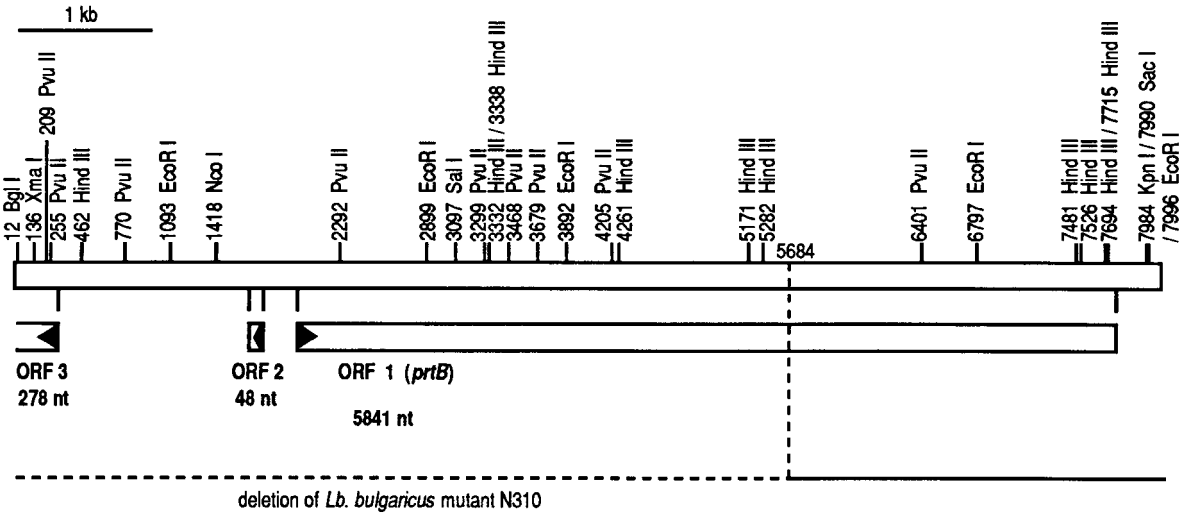

FIG. 1. Restriction map of a chromosomal DNA sequence of 8,071 nucleotides (nt) from *L. delbrueckii* subsp. *bulgaricus* NCDO1489. Directions of transcription of three ORFs are indicated by arrows. Deletion of mutant N310 within the *prtB* gene is shown by dashed lines.

12% acrylamide gels and stained with Coomassie blue as described previously (24).

Molecular techniques and transformation. Genomic DNA of *L. delbrueckii* subsp. *bulgaricus* was purified as described previously (6). Reverse PCR was performed from self-ligated *Eco*RI fragments of chromosomal DNA as described previously (11). PCR-amplified DNA fragments were blunt ended with T4 polymerase and cloned in plasmid pUC18 or pUC19. Plasmid extraction, restriction enzyme digestion, ligation, and *E. coli* transformation were performed according to standard procedures (37). Stringent conditions were used for Southern blot hybridizations (44). Primers were made by Schmiheini (Windisch, Switzerland) and the Molecular and Cellular Genetics Centre of the University Lyon I (Villeurbanne, France).

DNA sequencing and analysis. DNA sequences of plasmid inserts were determined by the dideoxy-chain termination method with the T7 and DeazaT7 sequencing kits (Pharmacia) and universal and reverse primers (38). Alternatively, the Fmol DNA sequencing system (Promega Corp., Madison, Wis.) was used for direct sequence determination of some DNA fragments.

Nucleotide and amino acid sequences data were analyzed by using the Bisance databases (8), and the sequence alignments were performed with the FASTA program (26).

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence reported in this paper is L48487.

RESULTS AND DISCUSSION

***L. delbrueckii* subsp. *bulgaricus* mutant with a cell wall-bound proteinase deficiency.** Mutant N310, with a large chromosomal deletion (12 kb) from the *lacZ* gene (11), was unable to develop normally in milk supplemented with glucose; rapid growth was restored by addition of a protein digest (proteose peptone) (data not shown). Kinetics of β-casein hydrolysis by whole cells of *L. delbrueckii* subsp. *bulgaricus* CNRZ397, NCDO1489, and LFi5.F6 (a mutant carrying downstream of the *lacZ* gene a deletion shorter than that detected in mutant N310) (11) showed the same major products (our unpublished results). β-Casein was not hydrolyzed by mutant N310. Consequently, this mutant is deficient in a cell surface proteinase activity, and genes involved in biosynthesis or biosynthesis regulation or in maturation of the proteinase were suspected to be located near the *lac* region. The β-casein hydrolysis pattern obtained with *Lactococcus lactis* subsp. *lactis* NCDO763 showed additional peptides. This result suggests that the cleavage specificity of cell surface proteinases from *L. delbrueckii* subsp. *bulgaricus* and *L. lactis* subsp. *lactis* are different.

Identification, DNA sequencing, and analysis of the *prtB* region. The DNA region located downstream of the deletion in the *lacZ* gene from mutant N310 was isolated by reverse PCR, and its DNA sequence was determined (Fig. 1). Search for protein homology in protein libraries revealed an identity of 27.8% between the deduced amino acid sequence and the sequence of PrtP from *L. paracasei* subsp. *paracasei* NCDO 151. The region downstream of *lacZ* and including the entire *prtP*-like gene was further isolated and sequenced in a cascade PCR strategy using synthetic primers as the known sequence progressed. A restriction map and partial sequence of 8,071 identified nucleotides are presented in Fig. 1 and 2 and showed three relevant open reading frames (ORFs).

The largest ORF (ORF1) displays homology with lactococcal *prtP* genes and displays three putative start ATG codons in frame at positions 1862, 1886, and 1940. However, the protein alignment with PrtP and the presence of a Shine-Dalgarno sequence (at position 1930) suggest the ATG at position 1940 as a valid start codon. We will refer to the *L. delbrueckii* subsp. *bulgaricus* proteinase as PrtB and its gene as *prtB*. The putative *prtB* is 5,841 nucleotides long and is preceded by promoter-like sequences; −35 (TTCAGA) and −10 (TTTGAT) boxes with a spacing of 16 nucleotides are in accordance with the promoter consensus sequences of *E. coli* (TTGACA and TATAAT, respectively). A UP element, characterized by oligo(A) and T stretches, is detected 5 nucleotides upstream the −35 box. Such an element is known to enhance transcription in bacteria (36) and has been found upstream of promoters of lactococcal *prtP* genes and several peptidase genes of *L. lactis* (22, 28). The stop codon (TAA) at position 7778 is followed by a dyad symmetry. A hairpin, detected between nucleotides 7784 and 7818, is consistent with a terminator structure and displays a stem of 11 bp (with two mismatches) and a loop of 13 nucleotides ($\Delta G = -18.1$ kcal [ca. −75.7 kJ]/mol). The GC content of the *prtB* gene is 47.6%, which is very similar to the values calculated for the *prtP* genes of *L. paracasei* subsp. *paracasei* and *Lactococcus lactis* subsp. *lactis* NCDO763 (47.5 and 47.2%, respectively). Consequently, the GC contents of proteinase genes differ significantly from the GC contents of their respective genomes: 54% for *L. delbrueckii* subsp. *bulgaricus* and 38% for *L. lactis* subsp. *lactis* (19). Other genes (*lacZ*, *lacS*, and *pepIP*) from *L. delbrueckii* subsp. *bulgaricus* show GC contents within the expected ranges (52.5, 53.1, and 56.9%, respectively) (1, 25).

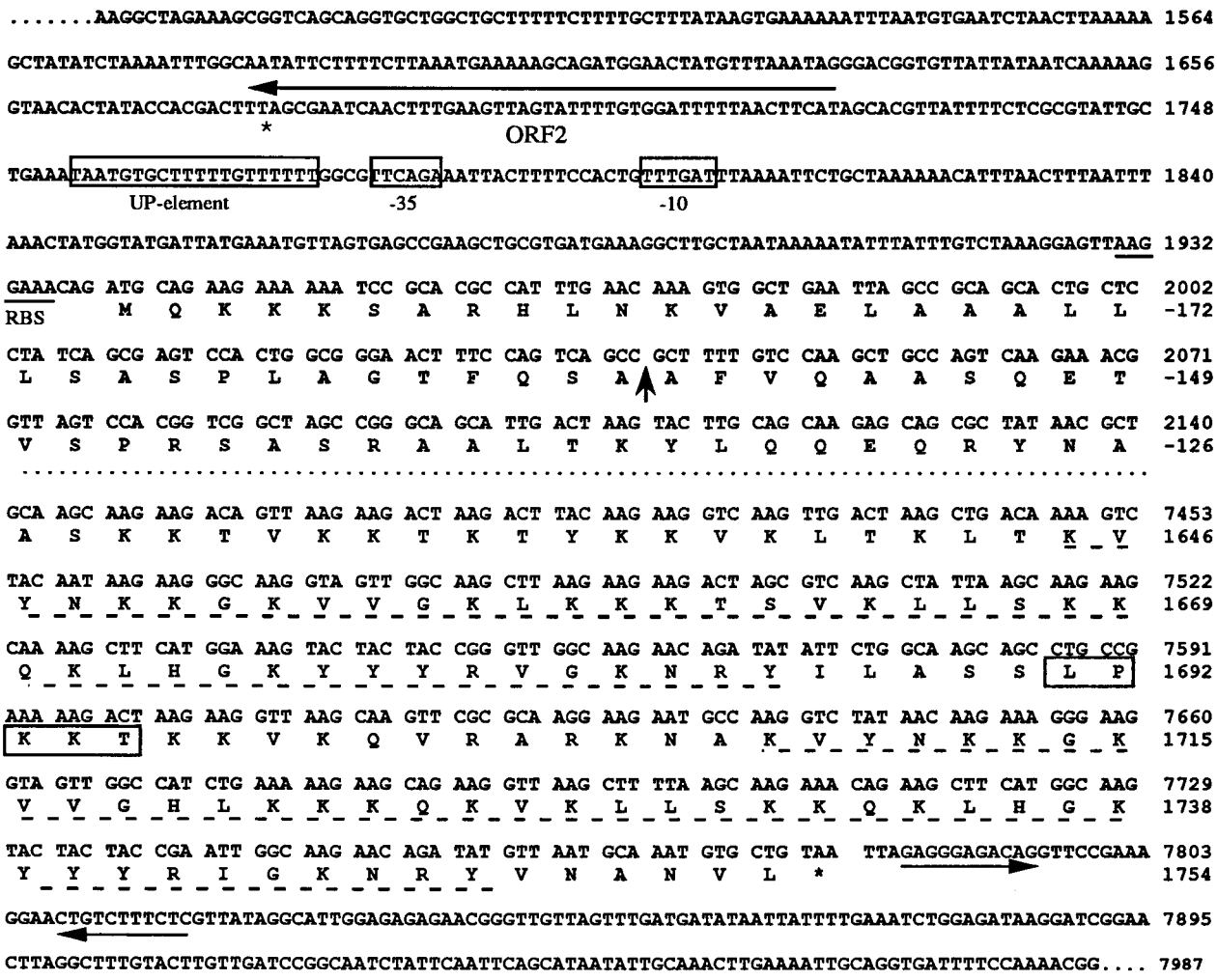

FIG. 2. Partial nucleotide and deduced amino acid sequences of the *L. delbrueckii* subsp. *bulgaricus prtB* gene and its flanking regions. The predicted −35 and −10 regions of the putative promoter and the UP element of the *prtB* gene are boxed. The proposed ribosome binding site (RBS) is underlined. The dyad symmetry of the terminator structure is indicated by convergent arrows. The amino acid sequence deduced from the *prtB* gene is numbered from the first amino acid of the putative mature proteinase. The putative cleavage site of the signal sequence is shown by a vertical arrow. The duplicated C-terminal stretches are underlined with dotted lines, and the LPKKT motif is boxed. ORF2 is shown by an arrow above the sequence indicating the direction of transcription. Stop codons are indicated with asterisks.

A DNA region (1711 to 1750) located upstream of the *prtB* gene displays 73.8% identity with the *L. paracasei* subsp. *paracasei prtM* gene. ORF2 is divergent from the *prtB* gene, partially overlaps this region homologous to *prtM*, and spans positions 1723 (ATG) to 1678 (TAA). The resulting polypeptide (MKLKIHKILTSRLIR) is very too short to act as a functional chaperone. No sequences homologous to *prtM* or ORF2 were found downstream of *prtB*.

A truncated ORF (ORF3) is located 1,663 nucleotides upstream and in the opposite direction of *prtB*. The start codon (ATG) is at position 278, and the deduced polypeptide shows no homology with a known protein and is devoid of signal peptide at the N terminus.

Amino acid content of PrtB and comparison with PrtP and other lactobacilli proteins. The putative PrtB proteinase consists of 1,946 amino acids and is characterized by a predicted M_r of 212,271. This length is consistent with those of previously described PrtP proteinases: 1,902 residues in *L. paracasei* subsp. *paracasei*, *Lactococcus lactis* subsp. *cremoris* Wg2, and *L. lactis* subsp. *lactis* NCDO763 and 1,962 residues in *Lactococcus lactis* subsp. *cremoris* SK11, associated with a duplication of 60 amino acids at the C-terminal end (15, 20, 22, 48). PrtB shows 27% identity with PrtP of *L. paracasei* subsp. *paracasei* over the first 1,806 residues and up to 39.5% when only the first 820 residues are compared. The C terminal part of PrtB shows no homology with PrtP proteinases.

The major amino acid of PrtB is lysine (12%), compared with leucine for other *L. delbrueckii* subsp. *bulgaricus* enzymes

TABLE 1. Comparison of domains surrounding the cleavage sites of pro regions from cell surface proteinases

Surface proteinase	Cleavage site of the pro region[a]
PrtB from *L. delbrueckii* subsp. *bulgaricus*	VKNVTPVKVYHPTDESADQMAQVQDVW
PrtPs[b]	VKTVTLAKVYYPTDAKANSMANVQAVW ↑

[a] The cleavage site of PrtPs is indicated by an arrow.

[b] PrtP sequences are related to those of *L. paracasei* subsp. *paracasei* NCDO151, *Lactococcus lactis* subsp. *cremoris* Wg2 and SK11, and *Lactococcus lactis* subsp. *lactis* NCDO763.

TABLE 2. Comparison of C termini of *L. delbrueckii* subsp. *bulgaricus* PrtB, PrtPs and some proteins harboring the anchoring signal LPxTG, protein A from *S. aureus*, and protein M6 from *Streptococcus pyogenes*

Protein	C terminus[a]
L. delbrueckii subsp. *bulgaricus* PrtB	**LPKKT**KKVKQ**V**RARKSAKVYNKKGKVVGHL-**KKK**QK**VK**KLLSK..[b]
L. paracasei subsp. *paracasei* NCDO151 PrtP	**LPKTA**ETTER**P**AFGFLGVIVVSLMGVLGL--**KRK**QR**EE**
Lactococcus lactis subsp. *cremoris*	↓
Wg2 PrtP	**LPKTG**ETTER**P**AFGFLGVIVVILMGVLGL--**KRK**QR**EE**
SK11 PrtP	**LPKTG**ETTER**P**AFGFLGVIVVSLMGVLGL--**KRK**QR**EE**
Lactococcus lactis subsp. *lactis* NCDO763 PrtP	**LPKTG**ETTER**P**AFGFLGVIVVSLMGVLGL--**KRK**QR**EE**
S. aureus protein A	**LPETG**E--EN**P**LIGTTVFGGLSLAGAALLAG**RRRE**L
Streptococcus pyogenes protein M6	**LPSTG**ETA-N**P**FFTAAALTVMATAGVAAVV-**KRKEE**N

[a] LPxTG sorting signal, the proline (P) preceding the α-helix, and the charged tail are indicated in boldface. A vertical arrow shows the cleavage site between threonine (T) and glycine (G).
[b] An extension of 23 residues is present without any E.

(LacS, β-galactosidase, and PepIP) or threonine for other lactococcal and *L. paracasei* subsp. *paracasei* proteinases and S-layer protein (SlpA) of *L. brevis* (47). Codon usage is similar to that of other *L. delbrueckii* subsp. *bulgaricus* enzymes for lysine and glycine, but serine, aspartic acid, and valine are preferentially encoded as in lactococci (data not shown).

PrtB and cell envelope location. The sequence homology between N-terminal regions of PrtB with PrtP proteinases suggests that PrtB is probably synthesized as a preproprotein. PrtB exhibits a positively charged N extremity followed by a predicted membrane-spanning helix with a high content in leucine and alanine. This domain closely resembles those of signal peptides of exported proteins in gram-positive bacteria (42). The putative cleavage site compared with those that of other PrtP proteinases (Fig. 2) is most probably located between Ala-159 and Ala-158. A second cleavage site between Ala-154 and Ala-153 cannot be ruled out. The consensus recognition sequence is Ala-X-Ala, with a cleavage after the carboxy-terminal alanine, but a cleavage between two alanines was also reported (neutral proteases from *Bacillus amyloliquefaciens* and *B. subtilis*) (42). Consequently, the expected signal peptide might be 34 (or 39) residues long, which is in agreement with the lengths of the signal peptides from gram-positive bacteria. The signal sequence is followed by a region homologous to pro regions of lactococcal and *L. paracasei* subsp. *paracasei* PrtP proteinases. The cleavage site of pro regions of lactococcal PrtPs was determined between residues 186 (T) and 187 (D) (Table 1). A domain with high identity was found in *L. delbrueckii* subsp. *bulgaricus* PrtB, and we assume that a cleavage may occur between residues 192 (T) and 193 (D at position 1). The resulting mature PrtB protein would contain 1,754 residues.

The C extremity of PrtB is quite different from the corresponding domains described for PrtP proteinases. The PrtB C terminus is characterized by a high lysine content and displays an imperfect duplication of a stretch of 41 amino acids, residues 1645 to 1685 and 1708 to 1748, with different amino acids at four positions (Fig. 2). The carboxy end of PrtP proteinases shows a consensus sequence detected in several cell envelope-associated proteins of gram-positive bacteria, e.g., protein A from *Staphylococcus aureus* and protein M6 from *Streptococcus pyogenes* (16, 46, 48) (Table 2). A predicted hydrophobic α helix of 18 to 20 amino acids is preceded by a proline residue and followed by a charged tail. Recent data showed that a conserved motif, LPxTG, located 10 amino acids upstream of the proline, acts as a sorting signal anchoring the protein in the cell wall (40). A mechanism was proposed for all surface proteins harboring this sorting signal. A specific cleavage occurs between the threonine and glycine, and a peptide bond would occur between the liberated carboxy terminus of the threonine and the N terminus of a residue from the peptidoglycan cross bridge (34). PrtB displays a degenerated sorting signal (LPKKT, 1691 to 1695) immediately followed by a charged region; this signal is located between both duplicated stretches of 41 residues. In contrast to PrtP, the region preceding the sorting motif shows a high lysine content. These data suggest that PrtB is attached to the cell envelope differently from the other proteinases.

TABLE 3. Comparison of sequences surrounding the amino acids involved in the active site of cell surface proteinases and other subtilisin family proteases

Protein	Sequence[a]			
	D-30,[b] D-30[c]	H-94, H-94	N-189, N-196	S-425, S-433
Surface proteinase				
L. delbrueckii subsp. *bulgaricus*	VISII**D**TGIDS	G**C**GEM**H**GQHVA	VISAG**N**SGVAG	MSGT**S**MASP
L. paracasei subsp. *paracasei* NCDO151	VVSVI**D**TGIDP	TVDEQ**H**GMHVA	VISAG**N**SGTSG	MSGT**S**MASP
Lactococcus lactis subsp. *cremoris*				
Wg2	VVSVI**D**SGIDP	TVDEQ**H**GMHVA	VISAG**N**SGTSG	MSGT**S**MASP
SK11	VVSVI**D**SGIDP	KVDEQ**H**GMHVA	VISAG**N**SGTSG	MSGT**S**MASP
Lactococcus lactis subsp. *lactis* NCDO763	VVSVI**D**SGIDP	TVDEQ**H**GMHVA	VISAG**N**SGTSG	MSGT**S**MASP
B. subtilis Carlsberg subtilisin	KVAVL**D**TGIQA	TDGNS**H**GTHVA	VAAAG**N**SGNSG	LNGT**S**MASP
Saccharomyces cerevisiae Protease B	TSYVI**D**TGVNI	LDGNG**H**GTH**C**A	AVAAG**N**ENQDA	LSGT**S**MASP
Tritirachium album Proteinase K	CVYVI**D**TGIEA	RDGNG**H**GTHCA	AVAAG**N**NNADA	ISGT**S**MATP
T. vulgaris Thermitase	KIAIV**D**TGVQS	QNGNG**H**GTHCA	VAAAG**N**AGNTA	LSGT**S**MATP

[a] Cysteines and amino acids involved in the catalysis and stabilization of the oxyanion hole are shown in boldface.
[b] Numbering corresponds to the *L. delbrueckii* subsp. *bulgaricus* mature PrtB.
[c] Numbering corresponds to the mature PrtPs of *L. paracasei* subsp. *paracasei* NCDO 151 and *Lactococcus lactis* subsp. *cremoris* SK11.

TABLE 4. Comparison of sequences surrounding several substrate-binding sites in cell surface proteinases and other subtilisin family proteases

Protein	Sequence at indicated site[a]					
	124[b] 131[c]	131 138	135–137 142–144	159 166	215 222	728–729 747–748
Surface proteinase						
L. delbrueckii subsp. *bulgaricus*	AMKVFS**N**NAKNSGAYDDDIISA			MSLGSVSSDVG	ELSTVGTPGT	GVGKEGTKDYYS
L. paracasei subsp. *paracasei* NCDO151	AMKVFT**N**SDTSATTGSATLVSA			MSLGSDSGNQT	DNEMVGTPGT	SSSTNQTKTYYD
Lactococcus lactis subsp. *cremoris*						
Wg2	AMKVF**T**NSDTSA**T**TGS**S**T**L**VSA			MSLGS**D**SGNQT	DNEMVG**T**PGT	SSSTN**LT**KTYYN
SK11	AMKVF**S**NSDTSA**K**TGS**A**T**V**VSA			MSLGS**N**SGNQT	DNEMVG**S**PGT	SSSTN**RK**KTYYN
Lc. lactis NCDO763	AMKVF**T**NSDTSA**T**TGS**A**T**L**VSA			MSLGS**D**SGNQT	DNEMVG**T**PGT	SSSTN**RK**KTYYN
B. subtilis Carlsberg subtilisin	AVKVLNSSGSGS---YSGIVSG			MSLGGPSGS--	-----GYPAK	
T. vulgaris thermitase	AVRVLDNSGSGT---WTAVANG			LSLGGTVGN--	-----NYPAY	

[a] Amino acids different between PI and PIII lactococcal proteinases are indicated in boldface.
[b] Numbering corresponds to the *L. delbrueckii* subsp. *bulgaricus* mature PrtB.
[c] Numbering corresponds to the mature PrtPs of *L. paracasei* subsp. *paracasei* NCDO151 and *Lactococcus lactis* subsp. *cremoris* SK11.

Putative active site of PrtB. Comparison of the PrtB amino acid sequence with those of other PrtP proteinases shows a strong homology of the stretches surrounding the three amino acids (Asp-30, His-94, and Ser-425) involved in the catalytic site and the asparagine (N-189) providing electrostatic stabilization of the oxyanion hole (50) (Table 3). The known PrtPs and subtilisins are devoid of cysteine residues (43). In contrast, a single cysteine has been found at position 90 of PrtB, i.e., closely upstream of the histidine of the active site. Interestingly, PrtB is known to be sensitive to cysteine and serine protease inhibitors (24). These characteristics prompted us to propose PrtB as a member of the cysteine-containing subgroup of subtilisins, exhibiting a cysteine residue four amino acids downstream of the histidine. The identified enzymes of this subgroup are the eukaryotic proteinase K (*Tritirachium album*) and protease B (*Saccharomyces cerevisiae*) and thermitase, an extracellular proteinase from *Thermoactinomyces vulgaris* (18, 29, 31). Thermitase displays 44% identity with subtilisins, and the most striking homologies are observed in the regions involving the active site; the overall tertiary structure of its active site is not thought to be modified by the presence of a cysteine residue.

Putative substrate binding regions of PrtB. Two regions of lactococcal PrtP proteinases are known to play a role in the specific hydrolysis of caseins (41). The lactococcal region from 130 to 225 is homologous to the substrate-binding region of subtilisins. In a three-dimensional structure, lactococcal residues 138 and 166 are located on either side of the substrate-binding cleft. They correspond to threonine (T) or lysine (K) (at position 138) and aspartic acid (D) or asparagine (N) (at position 166) in *Lactococcus lactis* subsp. *cremoris* Wg2 or SK11, respectively (Table 4). In *L. delbrueckii* subsp. *bulgaricus*, a glycine (G) corresponds to the lactococcal residue 138 and a valine (V) to residue 166. Furthermore, the PrtB domain corresponding to lactococcal residues 133 to 145 is quite different, and the substitutions of residues 137 to 139 in *Lactococcus lactis* subsp. *cremoris* SK11 are known to modify the substrate specificity and activity of PrtP (41).

The second substrate-binding region of PrtP proteinases is located in the remote C extension (with no equivalent in subtilisin) and characterized by the adjacent residues 747 and 748: RK in *Lactococcus lactis* subsp. *cremoris* SK11 and *Lactococcus lactis* subsp. *lactis* NCDO763, LT in *Lactococcus lactis* subsp. *cremoris* Wg2, and QT in *L. paracasei* subsp. *paracasei*. The PrtP domain including residues 747 and 748 shows homology with a region of PrtB, but the amino acids corresponding to lactococcal residues 747 and 748 are different: glycine (G-728) and threonine (T-729) (Table 4).

These results are in accordance with the biochemical data of casein degradation and strengthen the hypothesis of different

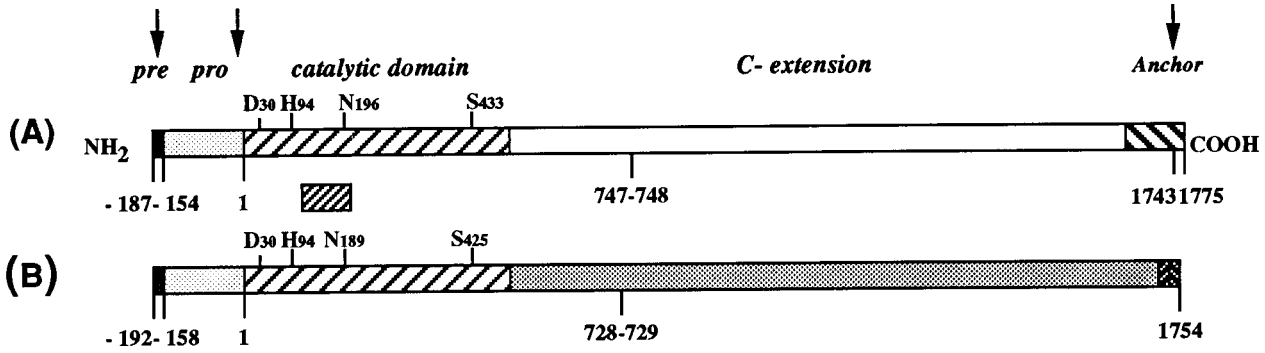

FIG. 3. Schematic comparison of preproproteinases from *Lactococcus lactis* subsp. *cremoris* SK11 (PrtP) (A) and *L. delbrueckii* subsp. *bulgaricus* NCDO1489 (PrtB) (B). The various domains are indicated, and arrows correspond to the cleavage sites identified in PrtP. The active-site triad (aspartic acid [D], histidine [H], and serine [S]) and asparagine (N) necessary for oxyanion binding are noted. The region from 130 to 225 (▨) is located in the catalytic domain and is homologous to the substrate-binding sites of subtilisins. Residues 747 and 748 of PrtP are also thought to take part in this substrate-binding site and are located in the long C extension. This latter region, and especially the C extremity, is significantly different in the two proteinases: PrtP is characterized by a 60-amino-acid duplication followed by a consensus sequence containing the sorting signal LPxTG; this motif is probably cleaved (at position 1743), causing the cell wall anchoring of PrtP. With regard to PrtB, a duplication of 41 residues is identified but the LPxTG motif is degenerated.

substrate specificities for proteinases from lactobacilli and lactococci.

In conclusion, the cell surface proteinase PrtB of *L. delbrueckii* subsp. *bulgaricus* is probably synthesized as a preproprotein of 1,946 amino acids but strongly differs from PrtP proteinases (Fig. 3). The absence of a *prtM*-like gene located immediately upstream or downstream of the *prtB* gene and expressing a functional chaperone gives rise to two hypotheseses. The maturation and export of PrtB could be processed by a PrtM-like chaperone encoded by a gene located elsewhere on the chromosome. However, it cannot be ruled out that a general chaperone could act on different extracytoplasmic proteins, PrtB included, as does the PrsA protein involved in the maturation and export of subtilisins and α-amylase of *B. subtilis* (17).

In contrast to the PrtP proteinases, PrtB is not recovered in the culture medium and consequently is not able to remove its C extremity by self-digestion. The difference of amino acid sequence in this domain suggests an absence of essential cleavage sites or a three-dimensional structure not consistent with the recognition of these sites. Assuming that the LPxTG signal and the closed α helix are essential to the anchorage of PrtPs in the cell wall, the attachment of PrtB to the envelope likely depends on a different mechanism.

The peptides released from β-casein by PrtB action are different from those resulting from the hydrolysis by PrtP. Other steps of the proteolytic system of *L. delbrueckii* subsp. *bulgaricus* are also suspected to be different. For instance, *L. delbrueckii* subsp. *bulgaricus* exhibits high peptidase activities toward substrates containing proline (5, 12, 39).

Comparison of PrtB and PrtP proteinases showed that these serine proteases probably evolved from two closely related enzyme families, subtilisin and cysteine subtilisin, respectively. A divergence is also observed with the *lac* system, essential to the growth of lactic acid bacteria in milk. In most mesophilic lactococci, the lactose degradation system (phosphoenolpyruvate-dependent phosphotransferase system and phospho-β-galactosidase) (9) and the *prtP* and *prtM* genes are often harbored on different plasmids. It is interesting that although the two systems are different in *L. delbrueckii* subsp. *bulgaricus*, they are located in the same chromosomal region.

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