Comparison of cell surface proteinase activities within the *Lactobacillus* genus

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Summary. Whole cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 (*Lb. bulgaricus* CNRZ 397) are able to hydrolyse α- and β-caseins. We have isolated a mutant of *Lb. bulgaricus* altered for growth in milk and unable to hydrolyse α- or β-casein. Normal growth was restored by adding amino acids or tryptone to milk. No significant difference between the peptidase activities of parent and mutant strains was observed. The cell surface caseinolytic activities of three lactobacilli species and *Lactococcus lactis* subsp. *lactis* (*Lc. lactis*) were compared. As expected, the characteristics of the cell surface proteinase activity of *Lb. casei* were similar to those of *Lc. lactis*. We showed that the cleavage specificities of the cell surface proteinase activities from lactobacilli were species-dependent and at least three types of activity were distinguished. The regulation of the biosynthesis of cell surface proteinase activities was medium-dependent and different within the *Lactobacillus* genus and even within the *Lb. delbrueckii* species. In contrast to *Lb. bulgaricus*, the cell surface proteinase activity of *Lb. lactis* was totally inhibited in a medium rich in peptides or amino acids. In contrast, the cell surface of *Lb. helveticus* probably displayed two proteinases with different cleavage specificities and with a biosynthesis regulation sensitive to different medium components.

Lactic acid bacteria are used in a variety of food fermentation processes, especially in the dairy industry. The low concentrations of free amino acids in milk are not sufficient to ensure the growth of these auxotrophic bacteria to high cellular densities. Their complex proteolytic system breaks down milk proteins (mainly caseins) into small peptides and free amino acids, fulfilling their nutritional requirements and contributing to the final flavour of fermented milks and cheeses (Law & Mulholland, 1995; Steele, 1995). Many components of the *Lactococcus* (*Lc.*) proteolytic system have been purified and characterized and most corresponding genes cloned and sequenced (for reviews, see Pritchard & Coolbear, 1993; Poolman *et al.* 1995; Juillard *et al.* 1996). The initial step of casein degradation is performed by a cell surface proteinase (PrtP) and a large part of the resulting peptides is taken up via the oligopeptide transport system. Further degradation to amino acids is mediated by a set of peptidases. The genes encoding several PrtP from different

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lactococci strains have been sequenced and the amino acid sequences deduced show > 98% identity. The maturation of PrtP requires the action of PrtM, a lipoprotein which plays a role of chaperone. Proteinase biosynthesis has been shown to be dependent on the composition of the culture medium (Marrug *et al.* 1995). In most *Lactococcus* strains, the *prtP* and *prtM* genes are located on a plasmid and this has made it possible to isolate spontaneous mutants deficient in the cell surface proteinase activity (Otto *et al.* 1982; Kamaly & Marth, 1988).

The proteolytic system of lactobacilli has been recently investigated. Similar *prtP* and *prtM* genes have been identified on the chromosomes of two *Lactobacillus* (*Lb.*) species, *Lb. casei* HN14 and *Lb. paracasei* subsp. *paracasei* NCDO 151 (Holck & Næs, 1991; Kojic *et al.* 1991). The peptide sequence of *Lb. paracasei* cell surface proteinase appears to be highly identical (96%) with those of lactococcal PrtP. Hence, the hypothesis of a similar caseinolytic pathway in lactococci and lactobacilli has prevailed during recent years.

In *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 397 (*Lb. bulgaricus* CNRZ 397), a proteinase active towards caseins was purified from cell wall extracts (Laloi *et al.* 1991). In contrast to PrtP, this proteinase is not released from cells incubated in a Ca^{2+}-free buffer. No plasmid was detected in most *Lb. bulgaricus* strains and the chromosomal gene encoding this cell surface proteinase has been recently sequenced (Gilbert *et al.* 1996). Comparison of DNA sequences showed little genetic homology between genes coding for cell surface proteinases of lactococci and *Lb. bulgaricus*. This hypothesis might be extended to other lactobacilli because no immune response was found between extracts of several lactobacilli (*Lb. bulgaricus*, *Lb. helveticus*, *Lb. buchneri*) and monoclonal antibodies raised against PrtP from *Lc. cremoris* Wg2 or SK11 (Sasaki *et al.* 1995*b*).

Different peptidases have been identified in *Lb. bulgaricus* CNRZ 397: X-prolyl-dipeptidyl aminopeptidase (PepX) (Atlan *et al.* 1990), a lysyl aminopeptidase (general aminopeptidase PepN) (Atlan *et al.* 1989), which are enzymes widespread among lactic acid bacteria, and a proline iminopeptidase (PepIP) (Atlan *et al.* 1994; Gilbert *et al.* 1994) corresponding to a major activity in thermophilic lactobacilli in contrast to lactococci. *Lb. bulgaricus* mutants deficient in PepX or PepN will grow in milk medium, which leads us to propose that these enzymes are not essential for caseinolytic catabolism.

The purpose of the present study was to investigate the cell surface proteinase activities of thermophilic lactobacilli. With this in mind, the hydrolytic patterns of β-casein by different whole cells of lactobacilli were compared and medium parameters affecting the cell surface proteinase activities were studied. In addition, we also report the isolation and partial characterization of a mutant of *Lb. bulgaricus* unable to grow in milk and exhibiting a total deficiency in the cell surface proteinase activity.

Materials and methods

Bacterial strains, plasmids and culture conditions

The following strains of *Lactobacillus* and *Lactococcus* species were used. *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 397 and NCDO 1489 (*Lb. bulgaricus*), *Lb. delbrueckii* subsp. *lactis* CNRZ 250 and 1183 (*Lb. lactis*) and *Lb. helveticus* ATCC 15009 were obtained from the Centre National de Recherches Zootechniques (INRA, F-78352 Jouy-en-Josas, France). *Lb. helveticus* NCDO 766 was obtained from the Institute of Food Research (Reading RG6 6BZ, UK). *Lb. casei* ATCC 4646 and *Lc.*

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lactis subsp. *lactis* NCDO 763 (*Lc. lactis*) were gifts from Dr Posno (TNO Nutrition and Food Institute, NL-2288 GJ Rijswijk, The Netherlands) and Dr Hirashima (Yakult Central Institute for Microbiological Research, Kunitachi-shi, Tokyo 186, Japan) respectively.

Routinely, lactobacilli were grown without shaking in the peptide-rich medium MRS (Difco Laboratories, Detroit, MI 48232-7058, USA; De Man *et al.* 1960) at 40 °C for thermophilic strains (*Lb. bulgaricus*, *Lb. helveticus* and *Lb. lactis*) and 30 °C for *Lb. casei*. M17 broth supplemented with 5 g glucose/l was used for the culture of *Lc. lactis* at 30 °C (Terzaghi & Sandine, 1975). Reconstituted Gamma milk (100 g/l) was made by adding sterile water to skim milk powder sterilized by γ radiation (Prolait, F-79001 Niort, France). Synthetic medium was prepared according to a modification of the procedure described by Ledesma *et al.* (1977). Amino acid concentrations were increased to 3-fold, except for leucine (0·36 g/l), lysine (1·40 g/l) and valine (0·12 g/l). No purine or pyrimidine was added, and only the following five vitamins were required: cyanocobalamin (10 μg/l), calcium pantothenate (1 mg/l), nicotinic acid (1 mg/l), pyridoxal (5 mg/l) and riboflavin (0·5 mg/l). Ledesma casein medium contained whole casein (2 g/l) and no free amino acids.

Growth was estimated by measuring the increase in turbidity of cell suspensions. One unit of absorbance at 600 nm measured with a spectrophotometer (model SP320, Jouan, F-44608 Saint-Nazaire, France) corresponded to 250 μg bacterial dry weight/ml or 10^8 cells/ml.

Mutagenesis

Lb. bulgaricus CNRZ 397 was subjected to mutagenesis with nitrosoguanidine (50 μg/ml, 60 min; Serva, D-69042 Heidelberg 1, Germany), as described by Atlan *et al.* (1989). Under these conditions, 50% of the cells survived.

Total cellular extracts

Cells grown in 100 ml MRS broth were harvested by centrifugation (10000 ***g***, 6 °C, 10 min), washed with 10 ml 0·2 M-Tris-HCl, pH 7·0 and disrupted with a French pressure cell (138 MPa) at 6 °C. Unbroken cells and cell debris were removed by centrifugation (25000 ***g***, 6 °C, 20 min).

Enzyme assays

Aminopeptidase activities were assayed using the following substrate stock solutions in distilled water: 3 mM-lysyl-*p*-nitroanilide, 3·5 mM-prolyl-*p*-nitroanilide and 6 mM-alanyl-prolyl-*p*-nitroanilide as described previously (Atlan *et al.* 1989; Gilbert *et al.* 1994). β-Galactosidase activity was determined by a standard method using *o*-nitrophenyl-β-D-galactopyranoside as the substrate (Miller, 1972).

The Api 50CHL kit test (BioMérieux, F-69280 Marcy l'Etoile, France) was used to test the fermentation of 49 sugars. Strains were grown in Api 50CHL broth with different sugars as the carbon source. A yellow colour resulting from acid production developed when sugar was fermented.

Caseinolytic activity was assayed using fluorescein isothiocyanate-labelled casein as the substrate. Substrate preparation and enzyme reaction were performed by a modification of the procedure described by Twining (1984), as described by Atlan *et al.* (1989). One relative fluorescent unit corresponded to the fluorescence at 450 nm of 48 μM-quinine sulphate solution under an excitation wavelength of 350 nm.

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Electrophoretic analysis of casein hydrolysis

The method used was based on the procedure of Hill & Gasson (1986). Whole bacterial cells in late exponential phase were incubated with dephosphorylated α- or β-casein as described by Laloi *et al.* (1991). β-Casein (13 μg) was incubated with 6×10^7 bacterial cells grown in MRS. After growth in Gamma milk, bacterial densities used for kinetic studies were reduced to 4×10^7 cells for *Lb. lactis*, *Lb. casei* and *Lc. lactis*, and 9×10^6 cells for *Lb. bulgaricus* and *Lb. helveticus*.

Caseins and hydrolysed products were separated by SDS-PAGE as described by Laemmli (1970) with a Protean II slab cell system (Bio-Rad Laboratories, Richmond, CA 94547, USA). Acrylamide slab gels (150 g/kg) were run at 10 °C and 38 mA constant current.

Before electrophoresis, samples were boiled for 3 min in 0·1 M-2-mercaptoethanol in the presence of 10 g SDS/l. After electrophoresis, gels were stained with Coomassie brilliant blue R250 (10 g/l; Prolabo, F-75011 Paris, France) in methanol–acetic acid–water (50:10:40 by vol.) at 50 °C, and diffusion destained in successive baths of methanol–acetic acid–water (5:10:85 by vol.) at 37 °C.

SeeBlue™ prestained standard solution (Novex, San Diego, CA 92121, USA) containing a mixture of proteins was used for molecular mass markers.

RESULTS

Isolation and characterization of a mutant deficient in cell surface proteinase activity

The parent strain, *Lb. bulgaricus* CNRZ 397, was subjected to mutagenesis with nitrosoguanidine. After growth on MRS agar plates, colonies were analysed on three different solid media, Gamma milk, Ledesma and Ledesma casein. Of the 20000 colonies tested, 75 were altered for growth on Gamma milk and Ledesma casein. These mutants were able to grow on MRS or Ledesma (which contains amino acids). It should be noted that all these mutants were able to use lactose or glucose as energy sources, so their growth deficiencies were consequences of defects in a component of the proteolytic system (biosynthesis or activity of a proteolytic enzyme or uptake system).

In order to identify a mutant altered for the first step of casein degradation, the 75 mutant strains were tested for their ability to hydrolyse α- or β-casein. After growth in MRS broth, whole cells were incubated with α- or β-casein. Under these conditions, cellular lysis was $< 2\%$. Hydrolysed products from caseins were separated by SDS-PAGE. Only mutant strain 1159 was unable to hydrolyse α- and β-casein, even after 4 h incubation; similar results were obtained with cells in late stationary phase.

Physiological characterization of mutant 1159. Growth kinetics in MRS medium of mutant 1159 and its parent strain, *Lb. bulgaricus* CNRZ 397, were similar and characterized by doubling times of 45 and 51 min respectively (Fig. 1). With growth in Gamma milk, a short lag period (1 h) was observed with both strains, and the parent strain exhibited a shorter exponential phase with a doubling time similar to that in MRS. On the other hand, the growth of mutant 1159 was very limited: a doubling time of 2 h was calculated and the biomass reached in stationary phase was only 0·22 that of the parent strain. This weak growth may reflect the presence of low concentrations of peptides resulting from the degradation of milk caseins by intracellular proteinases released from autolysed cells.

The effects of 10 g casamino acids/l (complete acid-hydrolysed casein, Difco) or

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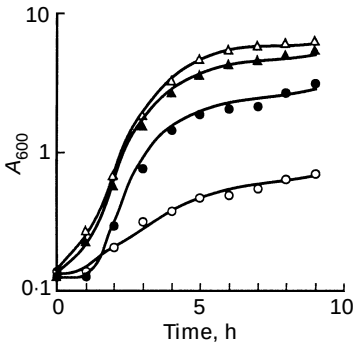


Fig. 1. Growth kinetics of ▲, ●, *Lactobacillus bulgaricus* CNRZ 397 and △, ○, its derivative mutant 1159 in ▲, △, MRS and ●, ○, Gamma milk (see text) media.

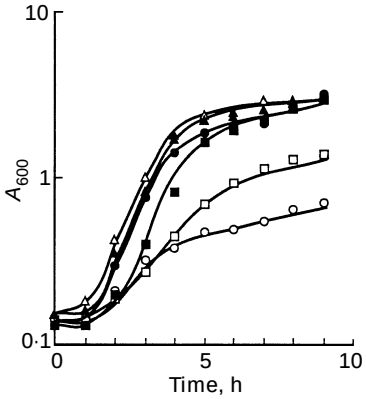


Fig. 2. Growth kinetics of ▲, ●, ■, *Lactobacillus bulgaricus* CNRZ 397 and △, ○, □, its derivative mutant 1159 in ●, ○, Gamma milk (see text) and Gamma milk supplemented with ▲, △, 10 g tryptone/l (casein hydrolysate) and ■, □, 10 g casamino acids/l (acid-hydrolysed casein).

10 g tryptone/l (casein hydrolysate, Difco) in Gamma milk were tested (Fig. 2). Optimal growth of strain 1159 in Gamma milk medium was completely restored when tryptone was added, but addition of casamino acids only partly restored growth. The high content (200 g/l) of NaCl in the casamino acid preparation was responsible for the slight reduction of the growth of the parent (results not shown).

Biochemical characterization of mutant 1159. The ability of this mutant to degrade 49 sugars was tested using the Api 50CHL test. Mutant 1159 and its parent strain were able to utilize four sugars: glucose, fructose, mannose and lactose.

Total cellular extracts were prepared from the parent strain and mutant 1159 after growth in MRS and aminopeptidase and caseinolytic activities were assayed. Total caseinolytic activity measured in mutant 1159 was one-third that of the parent strain, and similar activities were found for the three major peptidases PepN, PepIP and PepX. We concluded that mutant 1159 differed from its parent strain only by a deficiency in cell surface proteinase activity.

Effect of the composition of the culture medium on proteolytic activities

Total cellular extracts from different species were assayed for peptidase and caseinolytic activities (Table 1). A large variation in activity was found, depending on species and culture media.

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Table 1. *Proteolytic activities of* Lactobacillus *and* Lactococcus *strains after growth in different media*

Strain	Culture medium	Specific activity: PepN† (Lys-*p*NA)§	PepX† (Ala-Pro-*p*NA)	Caseinolytic‡ (FITC-labelled casein)
Lactobacillus delbrueckii subsp. *bulgaricus* CNRZ 397	MRS	481	3220	4800
	Milk	566	3480	16620
	Milk and 1 g tryptone/l	611	3886	6810
	Milk and 10 g tryptone/l	617	3233	4480
Lb. delbrueckii subsp. *lactis* CNRZ 250	MRS	145	799	1080
	Milk	255	1546	22870
	Milk and 1 g tryptone/l	255	1072	1280
Lb. helveticus NCDO 766	MRS	1827	3044	2430
	Milk	1351	2269	4880
Lb. casei ATCC 4646	MRS	188	245	1074
	Milk	250	510	10780
Lactococcus lactis subsp. *lactis* NCDO 763	MRS	14	1629	1440
	Milk	164	3970	11610

PepN, general aminopeptidase; PepX, X-prolyl-dipeptidyl aminopeptidase; Lys-*p*NA, lysyl-*p*-nitroanilide; Ala-Pro-*p*NA, alanyl-prolyl-*p*-nitroanilide; FITC, fluorescein isothiocyanate.

† Specific activity is expressed as μmol *p*-nitroanaline released/min per mg protein.

‡ Specific activity is expressed as relative fluorescent units per mg protein.

§ Substrates are given in parentheses.

The highest total caseinolytic activities were exhibited by *Lb. bulgaricus* and *Lb. lactis* after growth in milk. Whatever the species, total caseinolytic activities were higher in cells grown in milk rather than MRS and the ratio of activities ranged from 2 (*Lb. helveticus*) to 21 (*Lb. lactis*). The addition of 1 g tryptone/l to milk resulted in a specific activity similar to that found with cells grown in MRS. Compared with *Lb. lactis*, the decrease in caseinolytic activity of *Lb. bulgaricus* was less marked, even with 10 g tryptone/l.

Lb. helveticus had by far the highest PepN activity and *Lc. lactis* the lowest. Of the strains tested, a significant effect of the culture medium on the PepN activity was detected only with *Lc. lactis*. *Lactobacillus* and *Lactococcus* strains were associated with a high PepX activity, except for *Lb. casei*. This species was characterized by lower peptidases and caseinolytic activities than those of the three thermophilic lactobacilli (*Lb. bulgaricus*, *Lb. lactis* and *Lb. helveticus*).

Cell surface proteinase activities of lactobacilli and lactococci grown in MRS

In order to compare the cell surface proteinase activities of different bacterial strains, whole cells of lactococci and lactobacilli were incubated with β-casein. The hydrolytic patterns of β-casein degradation with both strains of *Lb. bulgaricus* (CNRZ 397 and NCDO 1489) were similar and gave nine products (lanes 1–6, Fig. 3*a*). During the first 15 min, the major product was peptide 2 (lanes 2, 3 and 5, Fig. 3*a*) and then up to 60 min it was peptide 4 (lanes 4 and 6, Fig. 3*a*). The rate of β-casein hydrolysis was significantly lower with *Lb. helveticus*, *Lb. casei* and *Lc. lactis* strains, and no proteinase activity was detected with *Lb. lactis* CNRZ 250 (lanes 7 and 8, Fig. 3*a*) and 1183 (results not shown).

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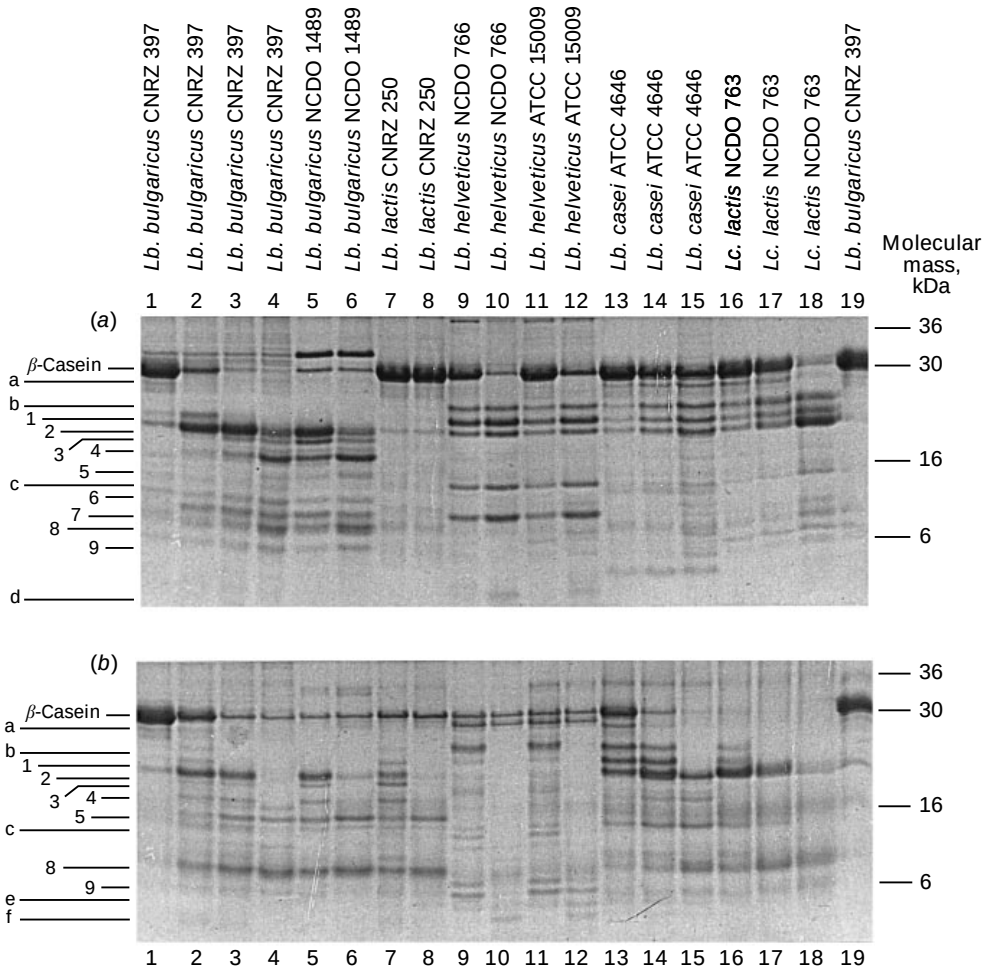


Fig. 3. SDS-PAGE analysis of β-casein hydrolysis by whole bacterial cells after growth in (*a*) MRS or (*b*) milk media: *Lactobacillus bulgaricus* CNRZ 397 (lanes 1 and 19, 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 1 h); *Lactobacillus bulgaricus* NCDO 1489 (lane 5, 15 min; lane 6, 1 h); *Lactobacillus lactis* CNRZ 250 (lane 7, 15 min; lane 8, 1 h); *Lactobacillus helveticus* NCDO 766 (lane 9, 15 min; lane 10, 1 h); *Lactobacillus helveticus* ATCC 15009 (lane 11, 15 min; lane 12, 1 h); *Lactobacillus casei* ATCC 4646 (lane 13, 5 min; lane 14, 15 min; lane 15, 1 h); *Lactococcus lactis* NCDO 763 (lane 16, 5 min; lane 17, 15 min; lane 18, 1 h). The numbering of peptides resulting from β-casein hydrolysis refers to the action of *Lb. bulgaricus* cells. The letters depict peptides not detected in the *Lb. bulgaricus* patterns (see text for details of organisms).

The caseinolytic patterns obtained with *Lb. helveticus* NCDO 766 and ATCC 15009 (lanes 9–12, Fig. 3*a*), *Lb. casei* ATCC 4646 (lanes 13–15, Fig. 3*a*) and *Lc. lactis* NCDO 763 (lanes 16–18, Fig. 3*a*) were quite different from those found with *Lb. bulgaricus* strains. Apart from the hydrolysis rates, the caseinolytic patterns of *Lb. casei* and *Lc. lactis* were similar. Major products not observed with *Lb. bulgaricus* strains were identified: peptide a characterized by a molecular mass close to that of β-casein (with *Lb. casei* and *Lc. lactis*), peptide b (with *Lb. helveticus*, *Lb. casei* and *Lc. lactis*), peptides c and d (with *Lb. helveticus*).

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Cell surface proteinase activities of lactobacilli and lactococci grown in Gamma milk

Hydrolytic patterns of both *Lb. bulgaricus* strains revealed some differences from those obtained after growth in MRS: peptides 2 and 8 were major products, whereas peptide 4 was rapidly hydrolysed (lanes 1–6, Fig. 3*b*). The same patterns were observed with both strains of *Lb. lactis* CNRZ 250 (lanes 7–8, Fig. 3*b*) and 1183 (results not shown).

The levels of cell surface proteinase activities of *Lb. helveticus* strains (lanes 9–12, Fig. 3*b*) were as high as those of *Lb. bulgaricus* strains, but their hydrolytic patterns appeared different both from those observed after growth in MRS and from those obtained with all the other species studied. However, major products were peptides a and b, already detected with *Lb. casei* and *Lc. lactis*. It is noteworthy that peptide b was further preferentially degraded and that the products with the lowest molecular mass were detected with *Lb. helveticus* strains.

The cell surface proteinase activities of *Lb. casei* and *Lc. lactis* were similar but somewhat higher than those of cells grown in MRS. Thus there was rapid hydrolysis of major peptides a, b and 1, and products with lower molecular masses appeared (c and 8).

The addition of 1 g tryptone/l in Gamma milk resulted in the total inhibition of β-casein degradation with cells of *Lb. lactis* and 10 g tryptone/l was inhibitory for biosynthesis in *Lb. casei*. The presence of 10 g casamino acids/l in Gamma milk also had a marked effect on the proteinase biosynthesis of both these *Lactobacillus* species: only weak degradation of β-casein was observed after 60 or 15 min incubation with *Lb. lactis* or *Lb. casei* cells respectively (results not shown).

The cell surface proteinase activities of *Lb. bulgaricus* and *Lb. helveticus* were not appreciably modified after growth in Gamma milk supplemented with tryptone or casamino acids (both 1 g/l), or 2 mM-prolyl-leucine.

DISCUSSION

Mutants of *Lb. bulgaricus* CNRZ 397 unable to develop in milk and growing in media rich in peptides or amino acids have been isolated. They may be altered in different essential steps of the caseinolytic pathway. The deficiency of the cell surface proteolytic activity of mutant 1159 could be due to a mutation within the active site or a defect in biosynthesis, export or maturation of a single cell surface proteinase. The similarity of β-casein hydrolytic patterns obtained with the purified cell surface proteinase, cell wall extracts and whole cells (Laloi *et al.* 1991) rules out the hypothesis that a second proteinase is present at the cell surface of *Lb. bulgaricus* CNRZ 397 whatever the culture medium or the growth phase. In contrast, strain ACA DC235 was reported to synthesize two cell surface proteinases, a calcium-dependent serine proteinase and a heat-labile zinc-dependent metalloproteinase (Stefanitsi *et al.* 1995).

It has been reported that proteinase-negative lactose-negative (Prt^-Lac^-) derivatives of *Lc. lactis* and *Lc. cremoris* devoid of their resident plasmid (Kamaly & Marth, 1988) exhibit a residual proteinase activity corresponding to 40% of that of the parent strain, as did mutant 1159 of *Lb. bulgaricus*. In contrast to mutant 1159, lactococcal $PrtP^-$ derivatives have higher lysyl and leucyl aminopeptidase activities than parent strains. We have previously shown that *Lb. bulgaricus* mutants deficient in PepN or PepX activity displayed an increased cell wall proteinase activity (Atlan *et al.* 1989, 1990). The properties of the different mutants from *Lactococcus* spp. and

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Lb. bulgaricus strongly suggest, on the one hand, that common regulatory mechanisms may control the biosynthesis of the cell wall proteinase and of some peptidases and, on the other hand, that these regulatory mechanisms are different in lactococci and lactobacilli.

The growth of mutant 1159 could be restored by adding peptides or amino acids to milk, confirming that the cell surface proteinase of *Lb. bulgaricus* was essential for growth in milk and catalysed the first step of casein degradation.

The use of chromogenic substrates allowed Sasaki *et al.* (1995*a*) to reveal the existence of at least three different proteinases or endopeptidases in lactobacilli. However, these enzyme assays were carried out with total cellular extracts and did not reflect the characteristics of cell surface proteinases. Our investigation of casein degradation with whole cells of different lactobacilli has demonstrated the existence of at least three types of cell surface proteinase activities with a species-dependent cleavage specificity. Type B proteinases belonged to *Lb. delbrueckii* (subspp. *bulgaricus* and *lactis*). Type P proteinases (PrtP) were present in *Lb. casei* and *Lc. lactis*. These results are consistent with the presence of *prtP* genes in both strains coding for closely homologous cell surface proteinases (Holck & Næs, 1991). The third type (H) corresponded to proteinases of *Lb. helveticus* strains. It is important to stress that the cleavage specificities of proteinases of types B and H markedly differed from those of the well known PrtP.

The biosynthesis of lactococcal PrtP is known to be dependent on the peptide content of the culture medium and to be repressed by specific dipeptides such as prolyl-leucine (Marrug *et al.* 1995). In contrast to peptidase activities, the production of the cell surface proteinases of lactobacilli was also significantly higher after growth in milk rather than MRS. It must be kept in mind that the enzyme:substrate ratio was not the same for each species, specially for *Lb. bulgaricus* and *Lb. helveticus* that displayed by far the highest cell surface caseinolytic activities. Hence this could affect the subsequent hydrolysis of peptides resulting from β-casein hydrolysis. The study of the effect of the composition of culture medium on the cell surface caseinolytic activities has also revealed the intricacy and differences of the regulatory mechanisms controlling the biosynthesis of three types of proteinase activities within the *Lactobacillus* genus. For instance, the cell surface proteinases activities from *Lb. lactis* and *Lb. bulgaricus* belonged to the same type B, but their biosynthesis lacked the same sensitivity to the concentration of peptides and amino acids present in the culture medium. Consequently, in contrast to *Lb. bulgaricus*, the biosynthesis regulation of *Lb. lactis* proteinase resembles that of the proteinase PrtP (*Lb. casei* and *Lc. lactis*), except for the sensitivity to the dipeptide prolyl-leucine.

Moreover, our results suggested that peptides resulting from casein degradation are probably more potent inhibitors of the proteinase biosynthesis of *Lb. lactis* than the amino acids. It is tempting to speculate that products of first steps of casein catabolism may control directly or indirectly proteinase biosynthesis via a feedback mechanism.

With *Lb. helveticus* cells, the addition of peptides or amino acids to milk did not restore a hydrolytic pattern similar to that observed with cells grown in peptide-rich medium. Thus these results gave support to the hypothesis of the biosynthesis of two cell surface proteinases with different cleavage specificities and medium-dependent regulation. The identification of two cell surface proteinases with different molecular masses from two strains of *Lb. helveticus* is consistent with this speculation (Yamamoto *et al.* 1993; Martín-Hernández *et al.* 1994). This hypothesis implies that a milk component (caseins or derivatives) had an inhibitory effect on the biosynthesis

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of the proteinase synthesized during growth in MRS. Further biochemical and genetic studies will be necessary to confirm this hypothesis.

The cell surface proteinases of lactobacilli and lactococci are a limiting step in the caseinolytic pathway. Strains with high cell surface proteinase activity such as *Lb. bulgaricus* and *Lb. helveticus* are of interest for cheesemaking. A better understanding of the regulatory mechanisms will provide us with the information to alleviate the repression of the expression of genes encoding proteinases and to construct high producing strains.

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