Simple Tests for Predicting the Lytic Behavior and Proteolytic Activity of Lactococcal Strains in Cheese

R. BOUTROU,* A. SEPULCHRE,† J. C. GRIPON,* and V. MONNET*,[1]

*Unité de Biochimie et Structure des Protéines,
Institut National de la Recherche Agronomique,
Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France
†Rhône-Poulenc-TEXEL, Zone d'activités de Buxières,
BP 10, 86220 Dangé-Saint-Romain, France

ABSTRACT

The variations of the autolytic and proteolytic potential of lactococci need to be taken into account because these variations probably influence the development of organoleptic properties during cheese ripening. To predict lytic capacity, proteolytic potential, and specificity of cell surface-associated proteinase, we developed simple tests that were applied to 26 industrial strains and a few reference strains of *Lactococcus lactis*. The tests allowed us to measure the autolytic capacity of lactococci in a buffer or in a pseudo curd under conditions that were close to those of cheese ripening and to evaluate global peptidase activity, proteinase activity, and specificity using casein or casein hydrolysate as a substrate. We confirmed the variability of the autolytic capacity of lactococci and classified the strains into three groups by low, moderate, or high lytic capacity according to their behavior in the buffer and in pseudo curd tests. Validation of the latter was obtained by the observation of similar lytic behavior in the two reference strains of *Lactococcus lactis*, AM2 and NCDO763, which were highly autolytic and poorly autolytic, respectively, both in our tests and in previously reported cheese experiments. The global activities of peptidases and proteinase varied from 1 to 6 among the strains. Most of the proteinases that were isolated from the highly proteolytic industrial strains hydrolyzed β-casein preferentially and, consequently, are more like the PI-type proteinase than the PIII-type. We used simple, rapid methods to test a large number of strains to predict their lytic behavior and proteolytic activity in cheese.

(**Key words**: *Lactococcus lactis*, proteolysis, autolysis, predictive tests)

INTRODUCTION

Numerous types of fermented foods are manufactured using lactic acid bacteria. Improved knowledge of the metabolic pathways of these industrial bacteria has economic benefits for the dairy industry, including improved selection of starter strains, shorter cheese ripening process, better quality control, and increased sensory properties. Consequently, the amount of research on lactic acid bacteria has been increasing. Research areas being investigated include carbohydrate catabolism, bacteriophages, genome organization, proteolytic system, and casein utilization.

Proteolysis is one of the main phenomena occurring during cheese ripening that can have either negative or positive consequences on the development of flavor, depending on its intensity. Rennet and, to a lesser extent, the cell-wall proteinase from starter bacteria are responsible for the development of the bitterness defect. The latter is attributed to the accumulation of bitter peptides that contain a large proportion of hydrophobic amino acids (16) and can be suppressed by peptidases from lactic acid bacteria. These peptidases further hydrolyze large and bitter peptides into free amino acids, which are precursors of aroma compounds. The proteolytic system of lactococci, which is important in the development of the sensory properties of cheese, is now well described and has been recently reviewed (1, 13, 22). An extracellular cell-wall proteinase is able to hydrolyze caseins with a specificity that varies among strains. Using α_{s1}-CN, β-CN, and κ-CN, Visser et al. (29) have defined two types of cell-wall specificities: PI, which preferentially hydrolyzes β-CN, and the PIII, which acts on α_{s1}-CN, β-CN and κ-CN. More recently, Exterkate et al. (9) have proposed a more detailed classification indicating seven types of proteinase specificities. Regardless of its specificity, the cell-wall proteinase generates oligopeptides that are further hydrolyzed by several peptidases. Twelve peptidases with different specifici-

Received July 28, 1997.
Accepted May 1, 1998.
[1]Corresponding author.

ties have been characterized in lactococci (17, 23), and all have been localized intracellularly (14, 26). Consequently, these peptidases could only be released into the curd after bacterial lysis. There they are potentially active and in contact with more substrates. Bacterial lysis should also be considered as an essential event during cheese ripening because lysis allows higher proteolysis in cheese. Moreover, Chapot-Chartier et al. (5) have observed for *Lactococcus lactis* strains AM2 and NCDO763 that bacterial lysis in cheese varied between strains. Lysis results either from the action of prophage lysins or from the action of bacterial enzymes, the autolysins, which are able to hydrolyze peptidoglycan, the main component of the cell wall. Biochemical and genetic studies on autolysins are recent and increasingly more important [review (4)], mainly because the control of lysis is considered to be a promising way to accelerate ripening (11).

The lysis capacity and the proteolytic potential, therefore, have to be taken into account during the selection of industrial strains of lactic acid bacteria, as well as the resistance to bacteriophages, the capacity to produce biomass, and the resistance to conservation processes. In this context, the aim of our work was to establish simple and reliable tests to evaluate 1) lytic behavior, 2) global peptidase activity, and 3) proteinase activity and specificity. These tests, which were applied to several industrial strains and a few reference strains, allowed us to predict the lytic behavior and proteolytic activity of lactococci in semi-hard cheeses. Validation of the tests in cheese-making experiments (Saint-Paulin type cheese) has already been obtained for the *L. lactis* NCDO763 and AM2 (5) and for several industrial strains (2).

MATERIALS AND METHODS

Strains, Growth Conditions, and Harvesting

The 26 industrial lactococci, numbered from RD226 to RD245 and RD247 to RD252, were obtained from the Rhône-Poulenc-TEXEL (Dangé-St-Romain, France) culture collection. The *L. lactis* reference strains AM2, HP, NCDO763, and SK11 were obtained from the UBSP culture collection (Institut National de la Recherche Agronomique, Jouy-en-Josas, France). The strains were stored at –80°C in 100 g/L of reconstituted skim milk (Elle & Vire, Compagnie Laitière Food Service, Le Pecq, France) supplemented with yeast extract (3 g/L), glucose (3 g/L), and 12% (vol/vol) glycerol. Cells were grown at 30°C in 20 ml of reconstituted and autoclaved (110°C for 12 min) milk buffered at pH 7.0 by the addition of 2.5% (vol/vol) 3 *M* sodium β-glycerophosphate. Cell growth was assessed by absorbance measurements at 480 nm after a 10-fold dilution in 2 g/L of EDTA, pH 12.0, as described by Thomas and Turner (27). Bacteria were harvested at the end of the exponential growth phase (i.e., absorbance at 480 nm increased by 5 units) after centrifugation (8000 $\times$ *g* for 15 min at 4°C). The pellet was then washed twice in 50 m*M* sodium β-glycerophosphate buffer at pH 7.0.

Lysis of Lactococci in the Buffer

The pellets of 10-ml milk cultures were resuspended in 8 ml of 50 m*M* sodium citrate buffer, pH 5.0, containing 15 g/L of NaCl and were incubated at 13°C for 30 d. Cell lysis was followed by absorbance measurement of the suspension at 650 nm with a spectrophotometer (Uvikon 931; Kontron Instruments S.p.A., Milano, Italy). Cell autolysis was characterized using two parameters: the global speed of cell lysis and the percentage of cell lysis, which was defined as follows: $(A_0 - A_t) \times 100/A_0$ where A_0 = initial absorbance, and A_t = absorbance measured after t days of incubation.

Lysis of Lactococci in the Pseudo Curd

A solution of reconstituted native phosphocaseinate (100 g/L) (21) was autoclaved at 115°C for 15 min. Then, 6 g/L of $CaCl_2$ were added, and the mixture was artificially acidified to approximately pH 5.0 with 15 g/L of glucono-Δ-lactone. Simultaneously, the bacterial pellet from a 10-ml culture was added to 10 ml of the phosphocaseinate solution and distributed in 1-ml vials. Within 1 h, pseudo curds were formed and then stored at 13°C for 30 d. Every 4 or 5 d, a 1-ml sample of pseudo curd was homogenized (Ultra-Turrax disperser; Janke and Kungel GmbH, Staufen, Germany) for 30 s at 13,000 rpm and used to estimate cell viability by the two following measurements: first, bacterial numerations on M17 lactose agar plates and, second, measurement of the activity of the intracellular X-prolyl dipeptidyl-aminopeptidase PepX (EC 3.4.14.5) using Phe-Pro β-naphtylamide as a substrate. In the latter assay, 50 μl of homogenized pseudo curd were incubated at 37°C with 0.66 m*M* substrate for 30 min. The reaction was stopped by the addition of 50 μl of Fast-Garnet reagent [1 g/L of Fast-Garnet, 10% (vol/vol) Triton X-100, and 1 *M* sodium acetate, pH 4.0]. After incubation for 25 min at 30°C, the samples were centrifuged at 17,000 $\times$ *g* for 10 min to remove the precipitated proteins, and the absorbance at 550 nm was meas-

ured using a microplate spectrophotometer (Titertek Multiskan Plus, Eflab, Finland). The unit of activity was nanokatals per milliliter (i.e., the quantity of enzyme releasing 1 nmol of β-naphtlylamine per second and per milliliter of pseudo curd; the extinction coefficient of β-naphtlylamine at 550 nm is 15,520 $M^{-1} \times cm^{-1}$).

Preparation of Cell-Free Extracts

The cells were resuspended in 1 ml of 50 m*M* triethanolamine buffer, pH 7.0, in the presence of 0.6 g of fine glass beads (diameter ≤106 μm) and then disrupted (Mini-Beadbeater 8™ cell disrupter; Biospec Products, Bartlesville, IL) for three cycles with 1 min of disruption and 1 min of cooling. The suspension obtained was centrifuged (20,000 × *g* for 10 min at 4°C) to remove cell debris, and the supernatant corresponding to the cell-free extract was stored at –20°C.

Total Protein Determination

Protein concentration was determined using the Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Determination of Global Peptidase Activity

The global peptidase activity contained in the cell-free extracts was measured with a casein hydrolysate as a substrate prepared as follows: 10 g/L of whole casein (in 0.05 *M* sodium citrate solution, pH 5.4, containing 0.2 g/L of sodium azide) was heated at 85°C for 10 min, cooled at 30°C in a water bath, and incubated at 30°C for 24 h with 0.09% (vol/vol) rennet containing 520 mg/L of chymosin. Chymosin activity was stopped by heat treatment (85°C for 10 min), and the pH was adjusted to 6.0 by dilution (vol/vol) with 0.05 *M* sodium citrate, pH 7.0. The resulting substrate was filtered at 0.2 μm and stored at –20°C.

A volume of cell-free extract containing 100 μg of total proteins was incubated with 100 μl of casein hydrolysate for 23 h at 13°C. Global peptidase activity was determined by measuring free amino groups with trinitrobenzenesulfonic acid. After precipitation in 1% trifluoroacetic acid, 10 μl of the supernatant were added to 100 μl of 1 *M* potassium borate, pH 9.2, and 40 μl of 1.2 g/L of trinitrobenzenesulfonic acid and the sample was incubated for 1 h at 37°C. The absorbance at 405 nm was measured using a microplate spectrophotometer, and free amino groups were quantified with glycine as the standard.

Proteinase Assays

After growth on milk, lactococci were collected by centrifugation, washed twice with 50 m*M* β-glycerophosphate, pH 7.0, and resuspended in 1/20 of the volume of the culture of 50 m*M* bis-Tris buffer, pH 6.5. The bacterial concentration was determined by measuring the absorbance at 650 nm. The concentration of bacterial suspension was standardized to 0.6 absorbance, and 0.31 ml of each suspension was incubated at 35°C with 0.31 ml of 10 g/L of methyl-^{14}C-labeled whole casein. The proteinase activity was estimated by measuring the initial release over a 10-min period of 6% trichloroacetic acid-soluble radioactive products (Beckman LS 1701; Beckman Instruments, Fullerton, CA). Under these conditions, bacterial lysis is negligible, and only the proteinase activity associated with the cell surface was measured.

Proteinase specificities were estimated on extracted proteinases according to a modification of the method of Juillard et al. (12). After growth on milk, the harvested cells were washed twice in 50 m*M* β-glycerophosphate, pH 7.0, containing 30 m*M* $CaCl_2$. The proteinase was then released from the cell wall by two successive incubations of the washed cells in 1/20 of the volume of the culture of 50 m*M* bis-Tris, pH 6.5, containing 15 m*M* EDTA at 30°C. The supernatants containing proteinase were then filtered at 0.45 μm. Because PI-type proteinases act preferentially on β-CN and PIII-type proteinases act on both α_{s1}-CN and β-CN, proteinase specificity was determined by measuring the hydrolysis of ^{14}C-labeled α_{s1}-CN and β-CN as described previously for whole casein. The results are expressed as the ratio of the percentage of α_{s1}-CN hydrolysis to the percentage of β-CN hydrolysis.

RESULTS

Lytic Capacity of Lactococcal Strains

The autolysis of 27 lactococcal strains incubated at 13°C in citrate buffer (pH 5.0) was followed by absorbance measurement during 30 d. Lysis (Figure 1a) differed among the strains that were arbitrarily classified into three groups according to the rapidity and intensity of lysis. The group of low lytic capacity was composed of strains that lysed poorly during the first 10 d (<10%) and had a negative percentage of lysis at the end of the incubation, probably because of a modification of bacterial chain length (e.g., strains RD228 and NCDO763). The group of moderate lytic capacity was composed of strains that lysed poorly

during the first 10 d and had a percentage of lysis at the end of the incubation <15% (e.g., strains RD233 and RD241), and the group of high lytic capacity was composed of strains that lysed very quickly and had >15% of lysis at the end of the incubation (e.g., strains AM2 and RD242; Figure 1a). We noticed that a 2-wk incubation period was sufficient to enable us to predict the lytic capacity. Consequently, the incubation time of this test could be shortened.

The autolysis of the strains was also monitored in a pseudo curd test. Before this test was implemented, we determined the concentration of glucono-Δ-lactone that was necessary to coagulate rapidly the solution of phosphocaseinate (about 40 min as during manufacture of Saint-Paulin-type cheese) and to obtain a pseudo curd with a pH close to those of cheese (about 5.2). Addition of NaCl, which enhanced cell lysis in the buffer test, has also been tested in the pseudo curd. Regardless of the concentration of glucono-Δ-lactone (up to 40 g/L), addition of NaCl (from 5 to 15 g/L) prevented the coagulation of calcium phosphocaseinate. Thus, we decided not to add NaCl in pseudo curd.

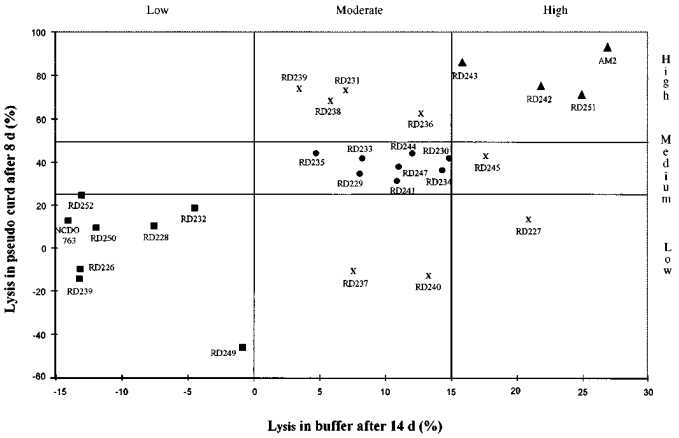

Figure 2. Classification of the strains by lytic capacity, measured using buffer or the pseudo curd test. The strains classified in the low (■), moderate (●), or high (▲) lysis group by both tests are distinguishable from other strains (x).

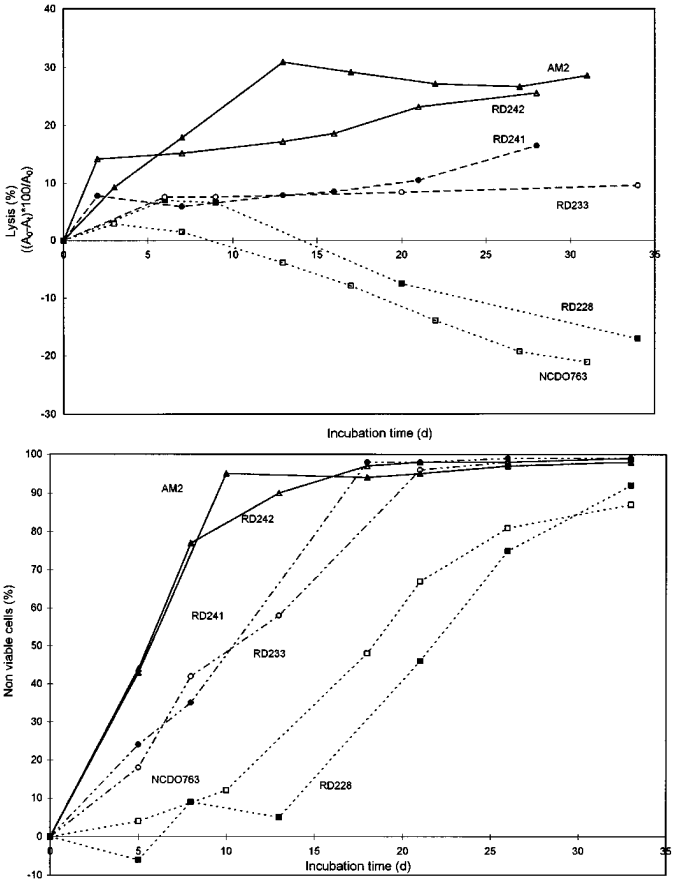

Figure 1. Evaluation of the ability of cells to lyse at 13°C a) in the buffer (50 m*M* Na citrate pH 5.0, containing 15 g of NaCl/L) and b) in the pseudo curd (100 g of phosphocaseinate/L containing 6 g of $CaCl_2$ and acidified at pH 5.0 with 15 g of glucono-Δ-lactone); strains are from groups with low (■, □), moderate (●, o), and high (▲, △) lytic capacities.

The autolysis of the 27 lactococcal strains was monitored for about 30 d by bacterial counts (Figure 1b) and measurements of aminopeptidase PepX activity (data not shown). As observed in the buffer, cell lysis differed among lactococci. The results obtained after 8 d were used to classify the strains into one of the three lysis groups as follows: the group of low lytic capacity was composed of strains that were up to 25% nonviable and had PepX activity from 0 to 1.5 nkatal/ml, the group of moderate lytic capacity was composed of strains that were from 25 to 50% nonviable and had PepX activity from 1.5 to 10 nkatal/ml, and the group of high lytic capacity was composed of strains that were from 50 to 100% nonviable and had PepX activity from 10 to 40 nkatal/ml. Of the 27 tested strains, 70% were classified as being in the same lysis group according to the cell counts and the activity of PepX. Other strains were classified in one of the three lysis groups mainly by considering the evolution of enumerations along the incubation time. Data obtained after a 21-d incubation period confirmed those data obtained after a 8-d incubation period.

The classification of all strains tested by both methods (i.e., in buffer and in pseudo curd) is reported in Figure 2; 67% of the strains tested were classified in the same lysis group by both tests. The remaining 23% of strains are classified in neighboring groups, except for the strain RD227, which exhibited different lytic behaviors in the two tests. The methods

used to evaluate cell lysis differed between the two tests (absorbance measurements and numerations). However, the relationship between the two methods was checked for the two strains NCDO763 and AM2, which belong to the groups of low lytic and high lytic capacities, respectively. Their bacterial viability was followed during a 28-d-period both by absorbance measurements and bacterial numerations. Correlation between the two methods used was satisfactory (Figure 3). The experiment demonstrated that the viability of lactococci was lower in buffer than in the pseudo curd (10^5 compared with 10^8 cfu/ml after a 28-d incubation period for the strain NCDO763 and 10^4 compared with 10^8 cfu/ml after a 7-d incubation period for the strain AM2). The extent of lysis observed in the pseudo curd (5 and 88% of viable cells after 10 d for reference strains AM2 and NCDO763, respectively) correlated well with those already observed in semi-hard cheese for the 2 reference strains (5) and for 4 industrial strains (2).

Global Peptidase Activity

Global peptidase activities in cell-free extracts of lactococcal strains were measured using a casein hydrolysate as a substrate. The results showed that the global peptidase activity that was measured after 23 h of incubation at 13°C was not equivalent for all of the 28 strains tested (Figure 4). Activities varied gradually from 0.6 to 4.1 m*M* equivalent glycine; thus, lactococcal strains were available that possessed different global peptidase activities.

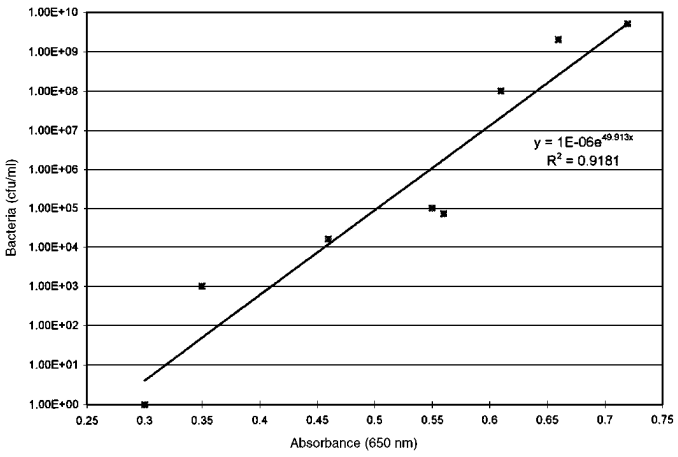

Figure 3. Correlation between absorbance at 650 nm and bacterial counts used to follow bacterial viability in strains NCDO763 and AM2 during a 28-d period.

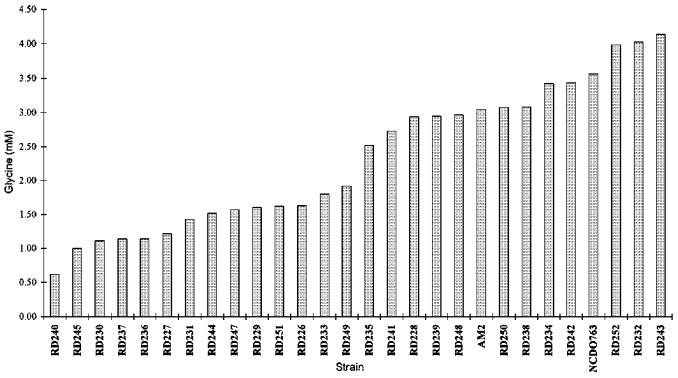

Figure 4. Global peptidase activities estimated by incubation of the casein hydrolysate with cell-free extracts for 23 h at 13°C.

Proteinase Activity and Specificity

The activity of proteinase, the first enzyme of the lactococcal proteolytic system acting on casein, was determined on whole cells resuspended in buffer. Results of activity for the 30 tested strains are presented in Figure 5. The percentages of whole casein hydrolysis after 10 min of incubation varied from 1- to 6-fold (1.7 to 9.7% casein hydrolysis); the range of values was similar to that observed for global peptidase activity.

We determined the specificity of the cell-wall proteinases by comparing the hydrolysis rate of α_{s1}-CN and β-CN by cell-wall extracts. Both HP and SK11 strains were used as reference strains for the PI-type proteinase that preferentially hydrolyzes β-CN and the PIII-type proteinase that hydrolyzes α_{s1}-CN and β-CN, respectively. The HP and SK11 strains were effectively located at either end of the histogram (Figure 5). In addition to two distinct groups of proteinase specificity (PI and PIII), there was a continuum of different specificities included between the extreme PI- and PIII-types. In agreement with the results of Bruinenberg and Limsowtin (3), we observed that the majority of the industrial strains resembled the PI-type and hydrolyzed β-CN in particular.

DISCUSSION

Evaluation of Lytic Capacity

Spontaneous cell lysis of lactococci in buffer or in M17 medium under optimal lysis conditions has been observed by several research groups (15, 18, 19, 20, 28). More complex tests have also been performed

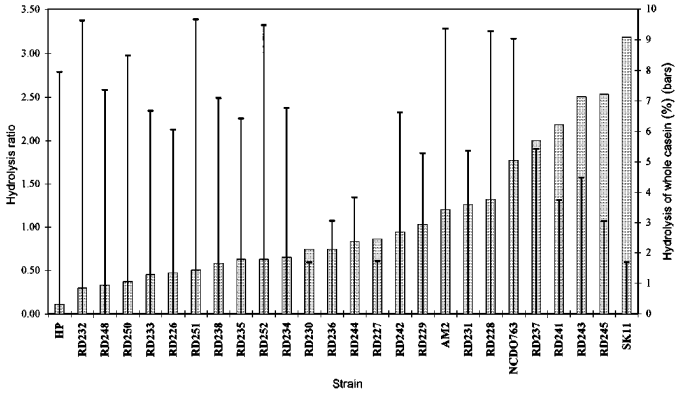

Figure 5. Proteinase activities estimated by the hydrolysis of whole radioactively labeled casein after 10 min of incubation at 35°C (histogram), and proteinase specificities estimated by the ratio of the percentage of α_{s1}-CN hydrolysis to the percentage of β-CN hydrolysis after 10 min of incubation at 35°C (black bar).

using cheese slurries or pseudo curds (10, 25), which are heavy and more difficult to manipulate. In addition, measurements of the diameter of clear zones around lactococcal colonies in M17 agar plates containing *Micrococcus luteus* have also been used to evaluate the lactococcal lytic activity (20). All of these methods indicate that the lytic capacity of lactococcal strains varies from 1- to 6-fold.

We chose to evaluate lactococcal lysis by simple tests under conditions similar to those used to make semi-hard cheese. First, we followed the lysis of lactococci grown in milk and resuspended in a buffer under typical conditions of cheese manufacture (i.e., at pH, temperature, and salt concentrations similar to those found in Saint-Paulin-type cheese) rather than under optimal conditions for cell lysis. The buffer test did not allow the measurement of maximal lysis, but, applied to 27 strains, confirmed the difference of lytic capacities among lactococci. The main advantage of the buffer test is that the user can easily modify the environmental conditions of cell lysis. However, this simple environment differs significantly from that of the curd, a complex and changing environment for cells, which led to our interest in the pseudo curd for use as a test to study the cell lytic capacity. In this test, the extent of cell lysis, up to 100% after 8 d, was similar to that already observed in cheese in which lysis can reach 90% after 1 wk (5). By contrast, the extent of cell lysis, although measured using a different method, remained weaker in the buffer test. Despite this difference, the classification of lactococci by their lytic capacity evaluated either in buffer or in pseudo curd tests differed little. We therefore implemented two simple tests to evaluate the lytic behavior of the lactococcal strains: the buffer test using absorbance measurements, and the pseudo curd test, using cell viability counts. The buffer test, which would be preferred by users because of its simplicity, was validated by the correlation of strains that were classified in the same lysis group according to the buffer test and the pseudo curd test. Moreover, the behaviors of reference strains AM2 and NCDO763 in our tests was in agreement with their behaviors in cheese (5), and with behavior of 4 industrial strains (2). This observation helped to validate our tests.

Screening of Peptidase Activity

To estimate the potential global peptidase activity in cheese curd following cell lysis, we developed a test conducted in conditions close to those of cheese ripening. The test was performed for 1 d at ripening temperature and pH; whole casein that was partially hydrolyzed by rennet was used as a substrate. To reproduce the in situ conditions more precisely, the casein hydrolysate should have been further hydrolyzed by the proteinase action of the corresponding tested strain, which would have required the preparation of proteinase from each tested strain. The protocol would have thus been too complicated for routine use. The test allowed us to measure global peptidase activity, which varied from 1- to 6-fold among the strains tested. Our results can be compared with those of others who measured several specific peptidase activities (PepN, PepC, PepA, and PepX) in optimal conditions for the enzymes contained in the free-cell extracts. Those researchers used either chromophoric peptide substrates (3, 7) or peptides (1) and concluded that lactococci significantly differed in peptidase activities. Bruinenberg and Limsowtin (3) have estimated that the sum of PepN, PepA, and PepX activities presented a 9-fold variation among 23 strains of lactococci. We already knew that the activity of peptidases was severely reduced under cheese-making conditions. For example, the activity of the purified aminopeptidase PepX, which is optimal at pH 8.5, is reduced by 50% at pH 5.5, and the enzyme is two times less active at 30°C than at the optimal temperature between 40 to 45°C (30). Because all of the characterized peptidases are optimally active at pH ≥6 and at temperature ≥30°C (12, 17), their activities in cheese curd are lower than under optimal incubation conditions, which confirms the advantage of our test to evaluate the peptidases activities under conditions of cheese manufacture.

Cell Surface-Associated Proteinase of the Lactococcal Strains

We have determined the cell surface-associated proteinase activity of each lactococcal strain using ^{14}C-labeled whole casein as substrate. To simplify this test and to remain as close as possible to cheese manufacturing conditions, we measured the proteinase activity with whole cells. The active proteinases are attached to the cell, which may influence the accessibility of active site and result in a selection of oligopeptides likely to be hydrolyzed by the proteinase action. Results showed that proteinase activity varied from 1.7 to 9.7% (×5.7) among lactococcal strains. Using fluorescent β-CN, Coolbear et al. (6) have estimated that, when it is clearly present, the cell surface-associated proteinase activity of 53 lactococcal strains varied by a factor of 8, which is similar to our results.

The method we used to identify the type of cell surface-associated proteinase was based on the ability of the proteinase to hydrolyze α_{s1}-CN and β-CN (29) and is different from the methods already reported (3, 9, 24). As indicated (3), the majority (73%) of the industrially used strains that were selected for their high acidifying capacity possess a proteinase that is more similar to the PI-type proteinases than to the PIII-type proteinases. There might be different explanations for this result. First, PI-type proteinases are more prevalent, and, thus, more strains possessing a PI-type are selected. Second, the selection of industrially used strains, based on the capacity to acidify quickly, is associated with a PI-type proteinase (8, 9). We also have noticed that PI-type proteinases hydrolyze whole casein more rapidly, or more intensively, or both than do PIII-type proteinases: 75% of PI-type proteinases (ratio of hydrolysis of α_{s1}-CN to β-CN <1) hydrolyzed over 6% whole casein in 10 min, but only 3 of the 9 PIII-type enzymes (ratio of hydrolysis of α_{s1}-CN to β-CN >1) reached this percentage of hydrolysis. These results tend to confirm that the selection of industrially used strains according to their fast growth coincides with the PI-type proteinase of these strains. Because the PI-type strains preferentially hydrolyze β-CN, they are suspected to produce more bitter peptides than the PIII-type strains (3).

The variations of proteinase specificity and activity among lactococcal strains may largely influence the nature and the quantity, respectively, of the peptides that are generated from casein. These peptides can enter the cell via specific transporters or serve as substrates for peptidases that are potentially liberated after cell lysis.

We observed important variations in the four parameters considered: lytic capacity, peptidase activity, proteinase activity, and proteinase specificity, which indicated the presence of a vast number of strains that differ in the rate and the manner in which they hydrolyze proteins in cheese.

We have developed and applied easy tests to evaluate the lysing ability of lactococcal cells and their global peptidase activity. Because of the ease and the rapidity of the tests, they could be routinely employed in the dairy industry for the selection of lactococcal strains. Cheese-making experiments demonstrated that the validation of our tests observed with reference and industrial strains can indeed be extended to all strains.

ACKNOWLEDGMENTS

This work has been done as a part of Bioavenir program supported by Rhône-Poulenc with the participation of the French Ministry of Research and French Ministry of Industry.

REFERENCES

1 Bockelmann, W. 1995. The proteolytic system of starter and non-starter bacteria: components and their importance for cheese ripening. Int. Dairy J. 5:977–994.
2 Boutrou, R., A. Sepulchre, G. Pitel, C. Durier, L. Vassal, J. C. Gripon, and V. Monnet. Lactococcal lysis and curd proteolysis: two predictable events important for the development of cheese flavor. Int. Dairy J. (accepted).
3 Bruinenberg, P. G., and G.K.Y. Limsowtin. 1995. Diversity of proteolytic enzymes among lactococci. Aust. J. Dairy Technol. 50:47–50.
4 Chapot-Chartier, M. P. 1996. Les autolysines des bactéries lactiques. Lait 76:91–109.
5 Chapot-Chartier, M. P., C. Deniel, M. Rousseau., L. Vassal, and J. C. Gripon. 1994. Autolysis of two strains of *Lactococcus lactis* during cheese ripening. Int. Dairy J. 4:251–269.
6 Coolbear, T., C. J. Pillidge, and V. L. Crow. 1994. The diversity of potential cheese ripening characteristics of lactic acid starter bacteria: 1. Resistance to cell lysis and levels and cellular distribution of proteinase activities. Int. Dairy J. 4:697–721.
7 Crow, V. L., R. Holland, G. G. Pritchard, and T. Coolbear. 1994. The diversity of potential cheese ripening characteristics of lactic acid starter bacteria: 2. The level and subcellular distributions of peptidase and esterase activities. Int. Dairy J. 4: 723–742.
8 Exterkate, F. A. 1976. Comparison of strains of *Streptococcus cremoris* for proteolytic activities associated with the cell wall. Neth. Milk Dairy J. 30:95–105.
9 Exterkate, F. A., A. C. Alting, and P. G. Bruinenberg. 1993. Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. Appl. Environ. Microbiol. 59: 3640–3647.
10 Farkye, N. A., S. A. Madkor, and H. G. Atkins. 1995. Proteolytic abilities of some lactic acid bacteria in a model cheese system. Int. Dairy J. 5:715–725.
11 Fox, P. F., J. M. Wallace, S. Morgan, C. M. Lynch, E. J. Niland, and J. Tobin. 1996. Acceleration of cheese ripening. Antonie van Leeuwenhoek 70:271–297.

12 Juillard, V., H. Laan, E.R.S. Kunji, C. M. Jeronimus-Stratingh, A. P. Bruins, and W. N. Konings. 1995. The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes β-casein into more than hundred different oligopeptides. J. Bacteriol. 177: 3472–3478.
13 Kunji, E.R.S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic system of lactic acid bacteria. Antonie van Leeuwenhoek 70:187–221.
14 Laan, H., R. E. Haverkort, L. de Leij, and W. N. Konings. 1996. Detection and localization of peptidases in *Lactococcus lactis* with monoclonal antibodies. J. Dairy Res. 63:245–256.
15 Langsrud, T., A. Landaas, and H. B. Castberg. 1987. Autolytic properties of different strains of group N streptococci. Milchwissenschaft 42:556–560.
16 Lemieux, L., and R. E. Simard. 1991. Bitter flavour in dairy products. I. A review of the factors likely to influence its development, mainly in cheese manufacture. Lait 71:599–636.
17 Monnet, V., M. P. Chapot-Chartier, and J. C. Gripon. 1993. Les peptidases des lactocoques. Lait 73:97–108.
18 Mou, L., J. J. Sullivan, and G. R. Jago. 1976. Autolysis of *Streptococcus cremoris*. J. Dairy Res. 43:275–282.
19 Niskasaari, K. 1989. Characteristics of the autolysis of variants of *Lactococcus lactis* subsp. *cremoris*. J. Dairy Res. 56:639–649.
20 Ostlie, H. M., G. Vegarud, and T. Langsrud. 1995. Autolysis of lactococci: detection of lytic enzymes by polyacrylamide gel electrophoresis and characterization in buffer systems. Appl. Environ. Microbiol. 61:3598–3603.
21 Pierre, A., J. Fauquant, Y. Le Graet, M. Piot, and J. L. Maubois. 1992. Préparation de phosphocaséinate natif par microfiltration sur membrane. Lait 72:461–474.
22 Poolman, B., E.R.S. Kunji, A. Hagting, V. Juillard, and W. N. Konings. 1995. The proteolytic pathway of *Lactococcus lactis*. J. Appl. Bacteriol. Symp. Suppl. 79:65S–75S.
23 Pritchard, G. G., and T. Coolbear. 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. 12:179–206.
24 Reid, J. R., T. Coolbear, C. J. Pillidge, and G. G. Pritchard. 1994. Specificity of hydrolysis of bovine κ-casein by cell envelope-associated proteinases from *Lactococcus lactis* strains. Appl. Environ. Microbiol. 60:801–806.
25 Roberts, M., C. Wijesundera, P. G. Bruinenberg, and G.K.Y. Limsowtin. 1995. Development of an aseptic cheese curd slurry system for cheese ripening studies. Aust. J. Dairy Technol. 50: 66–69.
26 Tan, P.S.T., M. P. Chapot-Chartier, K. M. Pos, M. Rousseau, C. Y. Boquien., J. C. Gripon, and W. N. Konings. 1992. Localization of peptidases in lactococci. Appl. Environ. Microbiol. 58: 285–292.
27 Thomas, T. D, and K. W. Turner. 1977. Preparation of skim milk to allow harvesting of starter cells from milk cultures. N.Z. J. Dairy Sci. Technol. 12:15–21.
28 Vegarud, G., H. B. Castberg, and T. Langsrud. 1983. Autolysis of group N streptococci. Effects of media composition modifications and temperature. J. Dairy Sci. 66:2294–2302.
29 Visser, S., F. A. Exterkate, C. J. Slangen, and J.C.M. de Veer. 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α_{s1}-, β- and κ-casein. Appl. Environ. Microbiol. 52:1162–1166.
30 Zevaco, C., V. Monnet, and J. C. Gripon. 1990. Intracellular X-prolyl dipeptidyl peptidase from *Lactococcus lactis* spp. *lactis*: purification and properties. J. Appl. Bacteriol. 68:357–366.