Characterization of the Highly Autolytic Lactococcus lactis
subsp. cremoris Strains CO and 2250†

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Two highly autolytic Lactococcus lactis subsp. cremoris strains (CO and 2250) were selected and analyzed for their autolytic properties. Both strains showed maximum lysis when grown in M17 broth containing a limiting concentration of glucose (0.4 to 0.5%) as the carbohydrate source. Lysis did not vary greatly with pH or temperature but was reduced when strains were grown on lactose or galactose. Growth in M17 containing excess glucose (1%) prevented autolysis, although rapid lysis of L. lactis subsp. cremoris CO did occur in the presence of 1% glucose if sodium fluoride (an inhibitor of glycolysis) was added to the medium. Maximum cell lysis in a buffer system was observed early in the stationary phase, and for CO, two pH optima were observed for log-phase and stationary-phase cells (6.5 and 8.5, respectively). Autolysins were extracted from the cell wall fraction of each strain by using either 4% sodium dodecyl sulfate (SDS), 6 M guanidine hydrochloride, or 4 M lithium chloride, and their activities were analyzed by renaturing SDS-polyacrylamide gel electrophoresis on gels containing Micrococcus luteus or L. lactis subsp. cremoris CO cells as the substrate. More than one lytic band was observed on each substrate, with the major band having an apparent molecular mass of 48 kDa for CO. Each lytic band was present throughout growth and lysis. These results suggest that at least two different autolytic enzymes are present in the autolytic L. lactis subsp. cremoris strains. The presence of the lactococcal cell wall hydrolase gene, acmA (G. Buist, J. Kok, K. J. Leenhouts, M. Dabrowska, G. Venema, and A. J. Haandrikman, J. Bacteriol. 177:1554–1563, 1995), in strains 2250 and CO was confirmed by Southern hybridization. Analysis of an acmA deletion mutant of 2250 confirmed that the gene was involved in cell separation and had a role in cell lysis.

Autolysis of Lactococcus lactis strains used as starters in cheese manufacture plays a key role in the cheese-ripening process. Rapid autolysis of lactococcal starter strains, which causes faster release of intracellular enzymes into the cheese matrix, could possibly reduce cheese-ripening time and decrease bitterness (7, 8, 35). Conversely, excessive starter autolysis could contribute to insufficient intact starter cells being present in the curd, causing excessive residual lactose to remain, with concomitant growth of undesirable nonstarter lactic acid bacteria (8).

The autolytic system of bacteria comprises one or more endogenous peptidoglycan hydrolases that eventually can autolyse the cell (29). Autolysins are most commonly classified as belonging to one of four categories on the basis of their cleavage specificities for the cell wall peptidoglycan: there are two types of glycosidases (β-N-acetylmuramidases [lysozyme] and β-N-acetylglucosaminidases), an N-acetylmuramyl-l-alanine amidase, and peptidases of various specificities (28). Peptidoglycan hydrolases which are unable to induce lysis are not classified as autolysins (28).

A number of surveys comparing autolysis in lactococcal strains have been conducted, and more recently, the autolytic systems of several strains have been characterized (4, 5, 23), including the cloning and sequencing of the major lactococcal autolysin N-acetylMuramidase (AcmA) by Buist et al. (3).

However, there is little detailed information on industrial lactococcal strains which exhibit extensive autolysis. In this study, 31 industrial lactococcal starter strains and L. lactis subsp. cremoris CO were screened for their rates of autolysis in Tris-HCl and phosphate buffers. Most strains did not lyse or lysed only slightly; however, two strains (CO and 2250) were identified as being highly autolytic and were chosen for further study. An understanding of autolysis in these autolytic lactococci might facilitate the selection or manipulation of starter strains for improved and/or controlled cheese flavor development.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Lactococcal starter strains were obtained from the Food Science Department, University of Minnesota, and the New Zealand Dairy Research Institute culture collections. Lactococcal strains were grown by using a 1% inoculum at 30°C in M17 broth (31) (Difco Laboratories, Detroit, Mich.) containing 0.5% glucose or 0.5% lactose, in Elliker broth (10) containing 0.5% glucose, or on M17 agar plates supplemented with 1% glucose. L. lactis subsp. cremoris CO and 2250 were grown in M17 broth containing limited glucose (0.4%) to ensure lysis or containing excess glucose (1%) to prevent lysis. Growth and autolysis for CO and 2250 were determined by measuring absorbance at 650 or 600 nm with a DU-70 spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.). Initially, a Bio Kinetics Reader EL340 (Bio-Tek Instruments Inc., Winoski, Vt.) at a dual absorbance wavelength (490 and 630 nm) was used to measure growth and autolysis of CO. Lysophilized Micrococcus luteus (ATCC 4698) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Escherichia coli strains were grown by using a 1% inoculum in Luria-Bertani broth (26) at 37°C with shaking. Erythromycin was used at final concentrations of 5 μg/ml for lactococci and 100 μg/ml for E. coli.

Effect of glucose concentration and pH on autolysis in M17 broth. Cultures were grown in a vessel with a 500-ml working capacity (Series III Fermentor; LH Engineering, Stoke Poges, England). The temperature was maintained at 30°C, and the pH was adjusted by automatic addition of 0.1 N NaOH or 0.1 N HCl. The glucose concentration in culture supernatants was determined by using Peridochrom reagent (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the manufacturer’s instructions.
Autolysis of whole cells in buffer systems. Cells were washed in cold distilled water and suspended in various buffers to an optical density at 600 nm (OD600) of between 0.35 and 0.45 under the conditions described for each experiment. Autolysis was monitored by the decrease in OD600 of the suspended cells. Two parameters were used to characterize autolysis: the extent of autolytic activity (expressed as the percentage decrease in OD600, as indicated in the experiment), and the rate of autolysis, expressed as the decrease in OD600 per minute during the first 60 min. OD was measured in a spectrophotometer with 1-cm cuvettes.

Isolation of autolysin-containing cell walls. L. lactis subsp. cremoris cells grown in M17 containing 1% glucose were harvested (10,000 × g, 10 min, 4°C) after reaching the stationary phase. The cell pellet was washed twice in 50 mM Tris-HCl (pH 7.0) containing 10 mM MgCl2, and washed cells were suspended as a 2% (wt/vol) cell suspension in sterile distilled water containing 0.2% sodium azide and stored at 4°C. For sodium dodecyl sulfate (SDS)-treated cell wall substrates, washed cells were lyophilized and suspended as a 2% (wt/vol) cell suspension in 50 mM Tris-HCl (pH 7.0) containing 10 mM MgCl2 and passed twice through a French press at 120 MPa. Cell walls were recovered for 90 min at 37°C, followed by 10 min at 100°C. Cell walls were washed with distilled water to remove SDS and then lyophilized. Lyophilized cell walls were suspended in twice their wet volume in 50 mM Tris-HCl (pH 7.0) containing 10 mM MgCl2, and passed twice through a French press at 120 MPa. Alternatively, cells were broken with a homogenizer (Braun, Melsungen, Federal Republic of Germany). The digested cells were treated with DNase (100 µg/ml) and centrifuged (3,000 × g, 10 min, 4°C) to remove intact cells, and the cell wall fraction was recovered by centrifugation at 20,000 × g (20 min, 4°C). Cell wall extracts were resuspended in 50 mM Tris-HCl (pH 7.0) containing 10 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), and either 4% SDS, 6 M guanidine hydrochloride, or 4 M LiCl. The SDS-cell wall mixture was shaken on a rotary shaker at 150 rpm for 90 min at room temperature and then sonicated on ice for 2 min with a Vibra cell (Pierce Chemical Company, Rockford, Ill.) at the maximum power setting. The LiCl and guanidine hydrochloride cell wall mixtures were centrifuged at 20,000 × g (20 min, 4°C), resuspended in sterile distilled water, and hophosphlated. Lyophilized cell walls were extracted and treated with three different agents, i.e., SDS, guanidine hydrochloride, and lithium chloride. Cell walls (100 mg) were resuspended in 8 ml of 50 mM Tris-HCl (pH 7.0) containing 10 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), and either 4% SDS, 6 M guanidine hydrochloride, or 4 M LiCl. The SDS-cell wall mixture was shaken on a rotary shaker at 150 rpm for 90 min at room temperature and then sonicated on ice for 2 min with a Vibra cell (Pierce Chemical Company, Rockford, Ill.) at the maximum power setting. The LiCl and guanidine hydrochloride cell wall mixtures were incubated with constant agitation at 4°C for 30 min. Cell walls were removed from each suspension by centrifugation (20,000 × g, 5 min). Cell wall extracts were dialyzed overnight against 1,000 volumes of 50 mM Tris-HCl (pH 7.0) containing 10 mM MgCl2, and 0.5 mM phenylmethylsulfonyl fluoride. Either SDS, 40% polyacrylamide gels (10 or 12% [wt/vol] polyacrylamide gels (10 or 12% [wt/vol] polyacrylamide gels) containing 0.2% (wt/vol) lyophilized M. lactis or lactococcus cells as the substrate for the detection of lytic activity. Upon completion of electrophoresis, the gels were soaked for 45 min in 250 ml of distilled water at room temperature with gentle agitation. Gels were then transferred to 250 ml of renaturation buffer [25 mM Tris-HCl (pH 7.5) containing 0.1% Triton X-100 and 10 mM MgCl2] and incubated with gentle rotation at 37°C for 16 h. Bands with lytic activity were observed as clear bands in the opaque gel. To enhance detection of lytic bands, gels were stained for 3 h in 0.1% methylene blue in 0.01% KOH and destained in distilled water. For both SDS-PAGE and zymograms, samples were mixed 1:1 (vol/vol) with sample buffer at final concentrations of 1% SDS, 10% glycerol, 1 mM EDTA, 0.025% (wt/vol) bromophenol blue, and 0.002% (wt/vol) bromophenol blue. The gels were electrophoresed at 200 V for 3 or 30 min at 100°C and applied to the gel, using equal amounts of protein (20 µg). Total protein was determined by using bicinchoninic acid according to the directions of the manufacturer (Pierce). Apparent molecular masses were determined by comparison to rainbow standards (14.3- to 220-kDa Rainbow markers; Amersham Corporation, Arlington Heights, Ill.). For zymograms, standards were run on the same gel, removed, and stained separately. Some variation in the migration of lower-molecular-mass standards (less than 69 kDa) was observed, particularly with cells that were pretreated with SDS. Total DNA was isolated by the alkaline lysis procedure (26). Total DNA was digested with EcoRI and BamHI (26) and cloned into pBR322. Total DNA probe was obtained by labeling with [32P]dCTP by random priming (26). Probes were used to detect presence of amide genes, Degenerate primers for PCR were designed based on the amide protein sequences from the following bacterial species or phages (databases and accession numbers are shown in parentheses): Streptococcus pneumoniae (Pfam A25634 and A24925); S. pneumoniae phage h3 (Pfam A21515), Bacillus subtilis (GenBank M59232 and SwissProt Q01214), and Bacillus licheniformis (SwissProt Q91125 and P36550 and PIR S23572). Nondegenerate primers were also designed from the L. lactis phage u3 amide (GenBank M86423). Primers were as follows (redundancies are shown in International Union of Pure and Applied Chemistry format): TWGSWNTGARATNTGYKW and CCRTRRTYTDTDCDCYRTTRTC (Bacillus amidas); CARAAARGCRCGNGAYCAYACA and CAYYTNCTNG CDAYTTTTGTTCC (first pair) and CAYGARCTCGYGNACAAANC and AYYTTYCRTAAYTTCTYCT (second pair) (S. pneumoniae amidas), and GTGCTTCTGACGGACATTTG and CGATAGTGCTTGTGCGCA (L. lactis phage u3 amide). PCR conditions were as described by Duwat et al. (9), except that the annealing temperature was set to 50°C instead of 55°C.

RESULTS

Effect of growth conditions on lysis. L. lactis subsp. cremoris CO and 2250 were selected for further study based on previous results (unpublished), which showed that they were unusually autolytic compared to most industrial L. lactis cheese-making strains. When grown in sodium β-glycerophosphate-buffered M17 broth containing 0.4 to 0.5% glucose (M17G) at 30°C, L. lactis subsp. cremoris CO and 2250 lysed extensively after reaching stationary phase, compared to other L. lactis strains such as C2 and HP (Fig. 1). Once maximum growth was reached, CO and 2250 cells lysed more rapidly in glucose than in lactose or galactose when limiting concentrations of carbohydrate (0.4 to 0.5%) were used. For example, 8 h after reaching maximum growth, CO underwent 62, 18, and 4% lysis in M17 when glucose, lactose, or galactose, respectively, was added as the carbon source (Fig. 1A). Similar results were observed for L. lactis subsp. cremoris 2250 (Fig. 1B). Lysis was not observed when excess carbohydrate (1%) was present when cells were grown in Elliker broth (data not shown).

Depletion of the carbon source from the medium appeared to trigger lysis when the pH was >5.5. When L. lactis subsp. cremoris CO was grown in M17 containing limited glucose, growth was observed while glucose was available. Upon consumption of the carbon source, lysis occurred (Fig. 2), at which point the final culture pH was 5.5. When β-glycerophosphate was omitted from M17, however, residual glucose remained in the medium, the final culture pH was 4.4, and lysis did not occur (data not shown). In contrast, exhaustion of glucose did not trigger lysis of L. lactis subsp. cremoris C2 when grown in M17, even though the pH also reached 5.5. Another L. lactis subsp. cremoris strain, SK11, showed a slight lytic response even though residual glucose remained in the medium (data not shown).

Lerer et al. (18) observed that B. subtilis, Neisseria subflava, and a streptococcal species exhibited extensive lysis after growth in media containing the tripeptide glycylglycine. Although L. lactis subsp. cremoris CO did not undergo autolysis when grown in the presence of 1% glucose, we also observed that if NaF was added to a growing culture of CO in M17 broth, autolysis was induced even in the presence of 1% glucose (Fig. 3).
To determine the extent of autolysis of CO and 2250 at a pH typical for ripening of Cheddar cheese, cells were grown in a fermentor containing M17G and the pH was lowered to 5.2 after 8 h. Although this caused an apparent decrease in autolysis for both CO and 2250 (to 27 and 36%, respectively), the levels of autolysis were still well above those observed for most other lactococcal strains (0 to 2%). Growth at lower temperatures also did not prevent lysis of \textit{L. lactis} subsp. \textit{cremoris} CO. When CO was grown in M17 at 35, 30, 22, and 17°C, rapid lysis occurred in each case once maximum growth was reached (data not shown). The final extent of lysis appeared to be greater at the lower temperatures.

In cheese making, starter strains usually require a fast milk coagulation phenotype. Unlike \textit{L. lactis} subsp. \textit{cremoris} 2250, CO has a slow milk coagulation phenotype due to a lack of proteinase activity (unpublished results). Restoration of proteinase activity and hence of the fast milk coagulation phenotype in CO by introduction of the proteinase plasmid pGKV500 (15) via electroporation reduced the level of autolysis of CO in M17 broth containing 0.4% glucose from 85 to 29% after 22 h (data not shown).

Autolysis in buffer systems. The extents of autolytic activity for \textit{L. lactis} subsp. \textit{cremoris} CO and 2250 varied in relation to their growth phase in M17G (Fig. 4). When cells from CO were harvested by centrifugation and resuspended in 0.1 M Tris-HCl (pH 7.0), maximum autolysis (45% after 3 h) occurred for cells harvested at early stationary phase, just after autolysis in M17G had begun. This lysis was greatest at 30°C in the presence of 1 mM Mg\textsuperscript{2+}. For both mid-exponential- and early-stationary-phase CO cells resuspended in different buffers, two pH optima for autolysis were observed (pH 6.5 and 8.5) (Fig. 5). Like the case for strain CO, lysis of strain 2250 was greatest (36%) when early-stationary-phase cells were resuspended in buffer (0.1 M sodium phosphate buffer, pH 6.0) (Fig. 4, bottom). However, unlike CO, there was no lysis of 2250 in 0.1 M Tris-HCl.

Identification of autolysins by using zymograms. Chaotropic agents (guanidine hydrochloride and LiCl) and SDS were more effective than mechanical disruption for extracting autolysins from CO and 2250 cell walls, and of these, SDS was the most effective extraction agent. Following renaturation of cell wall proteins extracted from \textit{L. lactis} subsp. \textit{cremoris} CO by using 4% SDS in a zymogram containing \textit{M. luteus} as the substrate, two lytic bands were observed (Fig. 6, top gel). The first band, band A, which was visible in the gel but not in the photograph, was narrow and nontransparent; its apparent molecular mass was 56 kDa. Band A was observed only in the SDS-treated cell wall extract; however, its appearance was inconsistent from one SDS extraction to another. The second band, band B, had an apparent molecular mass of about 48 kDa. Autolysins from \textit{L. lactis} subsp. \textit{cremoris} 2250 had a similar clearance activity when run on gels with \textit{M. luteus} as the substrate, except that a single major band having an apparent molecular mass of 42 kDa was observed, along with a series of minor bands (also see Fig. 7).

When autolysins extracted from \textit{L. lactis} subsp. \textit{cremoris} CO were run on gels containing autoclaved whole CO cells as the substrate (Fig. 6, middle gel), two narrow lytic bands, A’ and B’, which had apparent molecular masses of about 48 and 45 kDa, respectively, were consistently observed. The two bands were identical in intensity; however, the top lytic band was visible after renaturation for 1 h, while overnight renaturation was required to visualize the bottom lytic band. A similar lytic profile was observed when the cell wall fraction from strain CO was used as a substrate, except the intensity of the bottom lytic band was greater than that of the top lytic band (Fig. 6, bottom)
In addition, a diffuse lytic band having an apparent molecular mass of 30 kDa (band C) was observed. The bands observed in the lytic doublet on the CO substrate differ in molecular mass by 3 kDa, while the two lytic bands seen with M. luteus as the substrate differ by 6 kDa. It is believed that the two lytic bands observed with CO as the substrate are equivalent to the two lytic bands observed with M. luteus as the substrate. The disagreement in apparent molecular masses could be due to variance in electrophoretic mobility caused by the two different substrates, and discrepancies in intensities of lytic bands could be due to differences in substrate preference.

The effect of pH on lytic activity of the protein bands shown in Fig. 6 was examined by renaturing identical gel slices in an appropriate buffer at different pHs. When L. lactis subsp. cremoris CO cells were used as the substrate, the lytic bands (A’, B’, and C) varied in intensity depending on the pH (data not shown). In contrast, only one lytic band (B) of similar intensity was observed when M. luteus was used as the substrate. In particular, renaturation of gel slices at pH 5 to 8 did not affect the intensity of lytic band B or A’, whereas the intensity of band B’ decreased under acidic conditions (pH of <5). In contrast, lytic band C was more intense when gels were renatured under acidic conditions.

The influence of growth phase on the presence of the activity bands was examined for L. lactis subsp. cremoris CO. Cells were harvested during mid-exponential, late exponential, early stationary, and stationary phases of growth; their autolysins were extracted as before and run on zymograms containing M. luteus or L. lactis subsp. cremoris CO whole cells (data not shown). The two activity bands A and B, identified on gels containing M. luteus, were present at each stage of growth examined. However, the intensity of lytic band A was greater for cells harvested during early stationary phase. Similarly, on gels with L. lactis subsp. cremoris CO as the substrate, the three lytic bands A’, B’, and C showed maximum intensity when cells were harvested during the early stationary phase.

Presence of acmA. The presence of the lactococcal cell wall hydrolase enzyme (an N-acetylmuramidase), encoded by the gene acmA, was confirmed for L. lactis subsp. cremoris CO and 2250 by PCR with the primers palA-4 and palA-14 (3) and by Southern hybridization (data not shown). Total DNA digests from both strains, along with DNA from L. lactis subsp. cremoris MG1363, were probed with the acmA PCR product. The acmA gene was found to reside on a 4.14-kb Sau3A fragment in strains CO and 2250, as previously shown for L. lactis subsp. cremoris MG1363 (3), and on a 2.47-kb EcoRV fragment. PvuII digestion, which cuts the acmA gene at a single site, yielded two DNA fragments that hybridized to the probe; however, in this case there was a difference in band sizes between MG1363 (5.9 and 3.48 kb) and CO and 2250 (both strains had 3.48- and 1.42-kb bands) (data not shown).

Construction and analysis of an acmA deletion mutant. An acmA deletion mutant of L. lactis subsp. cremoris 2250 was constructed by gene replacement with the integration vector pINTAA, as previously described for L. lactis subsp. cremoris MG1363 (3). The deletion mutant was shown by PCR and Southern hybridization analysis to have the expected 487-bp internal deletion in the acmA gene (data not shown). As with the MG1363 deletion mutant (3), in M17G broth the strain 2250 deletion mutant formed long entangled chains of cells which settled to the bottom of the tube. Deletion of the acmA gene in 2250 resulted in complete loss of lysis, even after prolonged incubation in M17 containing a limiting amount of glucose.

Zymogram analysis of L. lactis subsp. cremoris MG1363 and 2250 cell wall fractions with 6 M guanidine hydrochloride and M. luteus autoclaved cells as the substrate showed a 42-kDa lytic band that was present in wild-type cell wall extracts of MG1363 and 2250 but not present in extracts from the deletion mutant of 2250 (Fig. 7). Minor lytic bands were present in cell wall extracts from both the wild-type L. lactis subsp. cremoris 2250 and its deletion mutant but not in those from MG1363. A...
prominent low-molecular-mass band was present in all of these cell wall extracts. This band is possibly a lysozyme-like enzyme, based on its clearance activity and apparent molecular mass of 14.3 kDa.

Screening of strains for amidase genes. In conjunction with the lactococcal cell wall muramidase (AcmA), it seemed possible that a different autolysin might be present in L. lactis subsp. cremoris CO and 2250, resulting in cell lysis. One common class of autolysins distinct from the muramidase is the amidase, which is present in several gram-positive and gram-negative bacteria and their phages. We used degenerate PCR to screen strains CO and 2250 for amidase genes. Based on computer database sequence data, three distinct groups of amidases were identified as belonging to species most closely related to L. lactis and were aligned by using the PILEUP program (Genetics Computer Group, University of Wisconsin). The three distinct groupings were (i) Bacillus amidases, (ii) S. pneumoniae and S. pneumoniae phage amidases, and (iii) the L. lactis phage us3 amidase (24). Primers based on these sequences were designed as described in Materials and Methods, and PCRs were carried out. For all sets of primers, however, negative results were obtained for both L. lactis subsp. cremoris CO and 2250 (data not shown).

DISCUSSION

L. lactis subsp. cremoris CO and 2250 exhibited elevated levels of autolysis in buffer systems and in M17 medium, in contrast to most other lactococcal strains (reference 17 and unpublished observations). Our results suggest that autolysis in CO and 2250 could be due to more than one autolytic enzyme. Zymogram analysis of proteins extracted from the cell wall fractions of strains CO and 2250 revealed multiple lytic bands which differed in pH sensitivity, substrate specificity, and apparent molecular mass. The autolytic profile of CO with M. luteus as a substrate, although similar in some respects to those of the nonautolytic L. lactis subsp. cremoris MG1363 (3) and other lactococcal strains (23), had an additional, lower-molecular-mass lytic band (Fig. 6, bottom gel, band C) which was observed only when gels were renatured under acidic conditions. It is likely that this 30-kDa band in CO represents a distinct enzyme.

When L. lactis subsp. cremoris CO whole cells were used as the substrate, the bottom band of the lytic doublet (band B') showed reduced intensity (in contrast to the 30-kDa band) when the pH dropped below 7.0. This sensitivity to pH has been observed in some lactococcal strains (4) but not in L. lactis subsp. cremoris MG1363 (3). Whether the lytic doublet represents distinct enzymes or, alternatively, the same autolysin attached to variable numbers of cell wall units, as observed for other cell wall surface proteins (27), is currently unknown. The same lytic doublet was observed when the cell wall fraction of L. lactis subsp. cremoris was used as the substrate (Fig. 5).
of CO was used as the substrate, except that the overall intensities of the lytic bands were lower, indicating that whole cells are a better substrate for detection, as was similarly observed by Østlie et al. (23). Other lytic bands having lower molecular masses were also present, and some of these could have been due to proteolysis of autolysins. In this regard, Foster (12) presented findings suggesting that multiple autolysins in a strain of CO were the product of a single Staphylococcus aureus. The unusually high level of autolysis observed when L. lactis subsp. cremoris CO was used as the substrate and analyzed by renaturing SDS–10% PAGE. The gels contained 0.2% (wt/vol) lyophilized M. luteus (top gel), L. lactis subsp. cremoris CO whole cells (middle gel), or CO SDS-extracted cell walls (bottom gel). After electrophoresis, gels were washed in distilled water and then in 25 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂ and 0.1% Triton X-100. Lane 1, 4% SDS-extracted cell walls; lane 3, 6 M guanidine hydrochloride cell wall extract; lane 5, 4 M LiCl cell wall extract. Lanes 2, 4, and 6 correspond to the same extracts as in lanes 1, 3, and 5, respectively, but after boiling for 30 min. The positions of the lytic bands are indicated by arrows on the right. Numbers on the left are molecular masses in kilodaltons.

Attempts to obtain an AcmA deletion mutant of L. lactis subsp. cremoris CO were unsuccessful; however, an AcmA deletion mutant was constructed for L. lactis subsp. cremoris 2250. This was used to show that autolysis in 2250 required the presence of AcmA. AcmA autolysin was identifiable in L. lactis subsp. cremoris MG1363 and 2250 on zymograms containing M. luteus cells; however, additional, lower-molecular-mass lytic bands unrelated to AcmA were also present in both the wild-type and mutant 2250 strains, but not in MG1363 (Fig. 7). This suggests that autolysis in strain 2250, while requiring AcmA, may be due to the activity of different autolytic enzymes.

The unusually high level of autolysis observed when L. lactis subsp. cremoris CO and 2250 were grown in M17 occurred at low (≤0.5%) glucose, lactose, or galactose concentrations and over a wide range of temperatures (17 to 35°C) and pHs. Furthermore, initiation of lysis for CO directly correlated with exhaustion of glucose from the medium, suggesting that lysis was related to depletion of the energy source. Moustafa and Collins (21) observed similar rapid lysis in liquid media for L. lactis subsp. biocytus diacyetylactis DRC1; however, the strain was resistant to lysis when grown at a low temperature or in a medium with a carbon source other than glucose. Strains CO and 2250 also exhibited decreased lysis on carbon sources other than glucose, as well as during growth at low pH (<5.2), and complete absence of lysis when excess (1%) carbohydrate was added to the medium.

Although the mechanism of induction of autolysis in L. lactis subsp. cremoris CO and 2250 remains to be elucidated, it is possible that autolysis could occur as a result of dissipation of the proton motive force. This was first proposed by Jolliffe et al. (14) in relation to the control of autolysis in B. subtilis. Among the experimental observations supporting their premise were the fact that addition of agents dissipating either the electrical or the pH gradient across the membrane resulted in rapid lysis of cells and the fact that starved cells lysed in dilute buffers or in media devoid of an oxidizable carbon source (14). Since lactococci are fermentative organisms, lack a cytochrome system, and obtain energy for growth by glycolysis (32), the exhaustion of glucose from the medium or the addition of NaF, an inhibitor of glycolysis in lactococci (19), could similarly induce autolysis by disrupting the proton motive force. Alternatively, the negative correlation between increased glucose concentrations and onset of lysis in CO may be due to regulation of autolysis, either by catabolite repression or by a two-component regulatory system similar to that in S. aureus (2).

Autolytic activity in relationship to the growth phase was examined for L. lactis subsp. cremoris CO and 2250, both in buffer systems and by zymogram analysis of cell extracts. For strain CO, zymogram analysis showed that while each lytic band was present throughout the growth, maximum clearance activities occurred for early-stationary-phase cells. These results differ from those obtained with Lactobacillus helveticus (33), where the major autolysin was essentially absent once stationary phase was reached.

Bacteria are capable of autolysis when cells are harvested and resuspended in Tris-HCl or phosphate buffers. Typically, for other gram-positive bacteria the autolytic activity is greater
during exponential growth, where autolysins are thought to play one or more roles in bacterial growth and division (6, 36). However, in *L. lactis* subsp. *cremoris* CO and 2250, maximum autolysis was observed in buffer after the cells reached stationary phase. Suggesting that autolytic activity may be regulated differently in these strains. This differs from results reported for other lactococcal strains, where autolytic activity was greater in exponential-phase cells (20, 22, 23). The optimal temperature for autolysis of CO was 30°C (data not shown), which is similar to that for many other lactococcal strains (23, 34), although temperature optima of 5 and 10°C have been reported for *L. helveticus* (33). The autolysis of strain CO was stimulated by low concentrations of Mg²⁺, as was reported for other lactococcal strains (21). Unlike the case for other lactococcal strains (19, 23), two pH optima were observed for the autolysis of CO cells in buffer, one at 6.5 and the other at 8.5. Each pH optimum could reflect the activity of a different autolysin.

It has been suggested that in *B. subtilis*, autolytic activity is partially regulated by proteolytic degradation (13). Because lysis of *L. lactis* subsp. *cremoris* CO was considerably decreased in a proteinase-positive strain of CO, it might be possible that the lack of proteinase activity in *L. lactis* subsp. *cremoris* contributed to the presence of the major autolysin during stationary phase.

In conjunction with the zymogram analysis, these results support the suggestion that at least two different autolysins are present in *L. lactis* subsp. *cremoris* CO and 2250 during the exponential and stationary growth phases. The roles of these additional autolysins, which presumably act in conjunction with the major cell wall hydrolase, AcmA, to cause lysis in 2250 and CO, and their distribution in other lactococcal strains are yet to be determined.

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