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., Winooski, Vt.) at a dual absorbance wavelength (490 and 630 nm) was used to measure growth and autolysis of CO. Lyophilized *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 Fermenter; 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.

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Autolysis of whole cells in buffer systems. Cells were washed once in cold distilled water and suspended in various buffers to an optical density at 450 nm (OD_{450}) of between 0.35 and 0.45 under the conditions described for each experiment. Autolysis was monitored by the decrease in OD_{650} of the suspended cells. Two parameters were used to characterize autolysis: the extent of autolytic activity, expressed as the percent decrease in OD_{650} at 30°C after 3 h (or the time indicated in the experiment), and the rate of autolysis, expressed as the decrease in OD_{650} per minute during the first 60 min. OD was measured in a spectrophotometer with 1-cm cuvettes.

Preparation of whole-cell and SDS-treated cell wall substrates. *L. lactis* subsp. *cremoris* cells grown in M17 containing 1% glucose were harvested ($10,000 \times g$, 5 min, 4°C) after reaching the stationary phase. The cell pellet was washed twice in 50 mM Tris-HCl (pH 7.0) and suspended in distilled water. For whole-cell substrates, washed cells were lyophilized and suspended as a 2% (wt/vol) cell suspension in distilled water. The cell suspension was autoclaved and stored at 4°C. For sodium dodecyl sulfate (SDS)-treated cell wall substrates, washed cells were passed twice through a French press at 120 MPa. Cell walls were recovered by using centrifugation ($20,000 \times g$, 20 min, 4°C) and were treated with 4% SDS 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 to 2% (wt/vol) in sterile distilled water containing 0.02% sodium azide and stored at 4°C.

Isolation of autolysin-containing fractions. Cells were harvested after reaching stationary phase (unless otherwise indicated) and washed in 50 mM Tris-HCl (pH 7.0) containing 10 mM $MgCl_2$. Washed cells were suspended in twice their wet volume in 50 mM Tris-HCl (pH 7.0) containing 10 mM $MgCl_2$ and passed twice through a French press at 120 MPa. Alternatively, cells were broken with a homogenizer (Braun, Melsungen, Federal Republic of Germany). The disintegrated cells were treated with DNase (100 µg/ml) and centrifuged ($3,000 \times g$, 10 min, 4°C) to remove intact cells, and the cell wall fraction was recovered by centrifugation at $20,000 \times g$ (20 min, 4°C). The cell wall pellet was washed twice with 0.9% NaCl and then with 50 mM Tris-HCl (pH 7.0) containing 10 mM $MgCl_2$; each time the cell wall fraction was recovered by centrifugation ($20,000 \times g$, 20 min, 4°C), resuspended in sterile distilled water, and lyophilized.

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 $MgCl_2$, 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 ($20,000 \times g$, 5 min). Cell wall extracts were dialyzed overnight against 1,000 volumes of 50 mM Tris-HCl (pH 7.0) containing 10 mM $MgCl_2$ and 0.5 mM phenylmethylsulfonyl fluoride. After dialysis, extracts were centrifuged ($10,000 \times g$, 15 min) to remove insoluble proteins and concentrated with Centriprep-3 concentrators (Amicon, Inc., Beverly, Mass.) according to the manufacturer's instructions.

Renaturing SDS-PAGE (zymograms). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Laemmli (16) with 10 or 12% (wt/vol) polyacrylamide separating gels. Following electrophoresis (9 mA at constant current for 16 to 18 h or 150 V for 5 to 7 h) at room temperature, gels (18 by 16 by 0.1 cm) were stained with either Coomassie brilliant blue 250 (Sigma) or silver stain (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions. Renaturing SDS-PAGE (zymogram) was conducted essentially as described by Foster (11). SDS-polyacrylamide gel (10 or 12% [wt/vol] acrylamide) containing 0.2% (wt/vol) lyophilized *M. luteus* or lactococcal cells as the substrate were used for the detection of lytic activity. Upon completion of electrophoresis, 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 $MgCl_2$) 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, 0.0025% (wt/vol) bromophenol blue, 5% β-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8). Samples were heated 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 when cells were present in the gels; hence, under these conditions, calculated molecular masses are approximate.

DNA manipulations. Total and plasmid DNAs were isolated from lactococcal strains by the method of Anderson and McKay (1), and plasmid DNA from *E. coli* was isolated by the alkaline lysis procedure (26). Total DNA was diluted 1:100 for PCR or, after digestion with the appropriate restriction enzyme

(Boehringer Mannheim), electrophoresed in a 0.8% agarose gel and blotted onto Hybond-N nylon membranes (Amersham) by the Southern method as described by Sambrook et al. (26). A 1.1-kb DNA fragment was generated from the *acmA* gene in *L. lactis* subsp. *cremoris* MG1363 by PCR with the primers palA-4 and palA-14, as described by Buist et al. (3), and this fragment was used to probe total genomic DNA from strains by using the ECL chemiluminescent gene detection system (Amersham) according to the manufacturer's instructions. An *acmA* deletion mutant of *L. lactis* subsp. *cremoris* 2250 was constructed by using pINTAA, as described previously (3). For electroporation, cells of strain 2250 were grown in M17 broth containing DL-threonine (30), chilled on ice, and then harvested and resuspended in ice-cold buffer (0.5 M sucrose, 5 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.4). Electroporations were performed with a Bio-Rad Gene Pulser apparatus under the conditions described by Powell et al. (25).

Screening of strains for the presence of amidase genes. Degenerate primers for PCR were designed based on the amidase protein sequences from the following bacterial species or phages (databases and accession numbers are shown in parentheses): *Streptococcus pneumoniae* (PIR A25634 and A42935), *S. pneumoniae* phage hb3 (PIR S16016), *Bacillus subtilis* (GenBank M59232 and SwissProt Q02114), and *Bacillus licheniformis* (SwissProt Q99125 and P36550 and PIR S23572). Nondegenerate primers were also designed from the *L. lactis* phage us3 amidase (GenBank M90423). Primers were as follows (redundancies are shown in International Union of Pure and Applied Chemistry format): TWGSWGTNGARATNTGYKW and CCRTTRTTYTTDGCDCCYTTRTC (*Bacillus* amidases), CARAAYGARGCNGAYTAYCA and CCAYTTNTCNG CDATYTTYTTCC (first pair) and CAYGARTAYTGYACNAAYAAYC and AYYTTYTCRAAYTTRTCYT (second pair) (*S. pneumoniae* amidases), and TTGCTTCTGACGGACGATTG and CGATAGTCCATTGGTCGGCA (*L. lactis* phage us3 amidase). 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 or 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).

Lesher et al. (18) observed that *B. subtilis*, *Neisseria subflava*, and a streptococcal species exhibited extensive lysis after growth in media containing sodium fluoride, an inhibitor of glycolysis. 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).
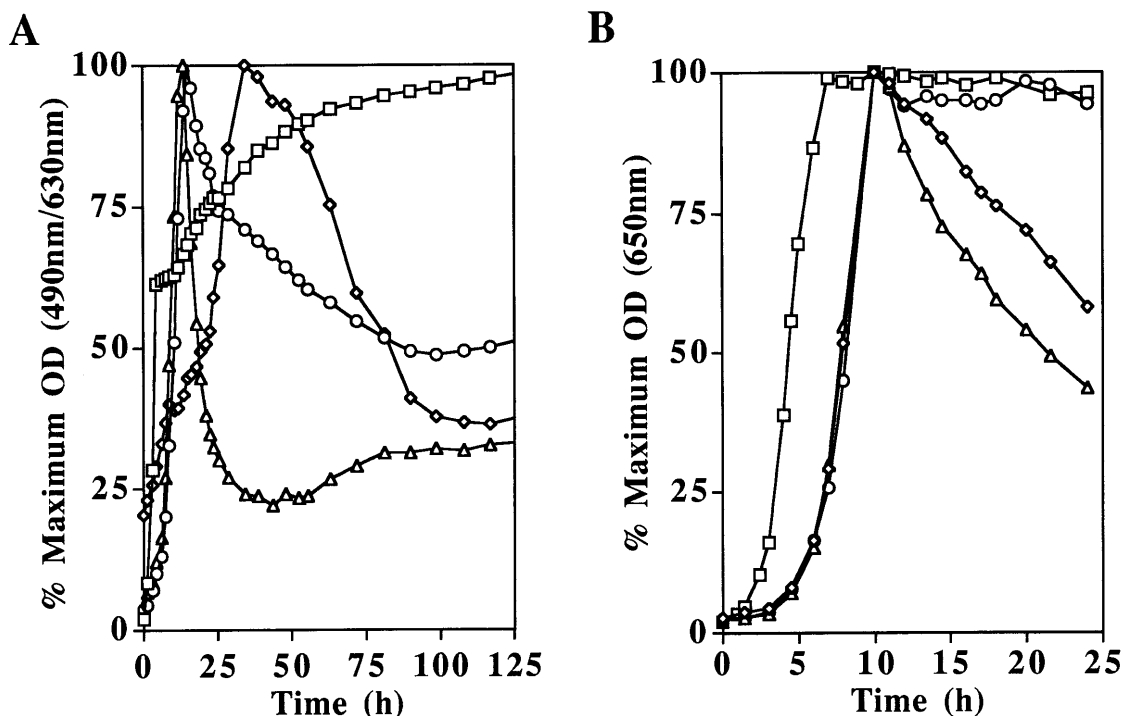
FIG. 1. Influence of carbohydrate source on growth and lysis of lactococcal strains in M17 at 30°C. (A) *L. lactis* subsp. *cremoris* CO grown with 0.5% glucose (triangles), 0.5% lactose (diamonds), or 0.5% galactose (circles) and *L. lactis* subsp. *cremoris* C2 grown with 0.5% glucose (squares). (B) *L. lactis* subsp. *cremoris* 2250 grown with 0.4% glucose (triangles), 0.4% lactose (diamonds), or 1.0% glucose (circles) and *L. lactis* subsp. *cremoris* HP grown with 0.5% glucose (squares). Percent maximum OD = 100 × (minimum OD/maximum OD).

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 *L. lactis* subsp. *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 *L. lactis* subsp. *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 *L. lactis* subsp. *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^{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 *L. lactis* subsp. *cremoris* CO by using 4% SDS in a zymogram containing *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 *L. lactis* subsp. *cremoris* 2250 had a similar clearance activity when run on gels with *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 *L. lactis* subsp. *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
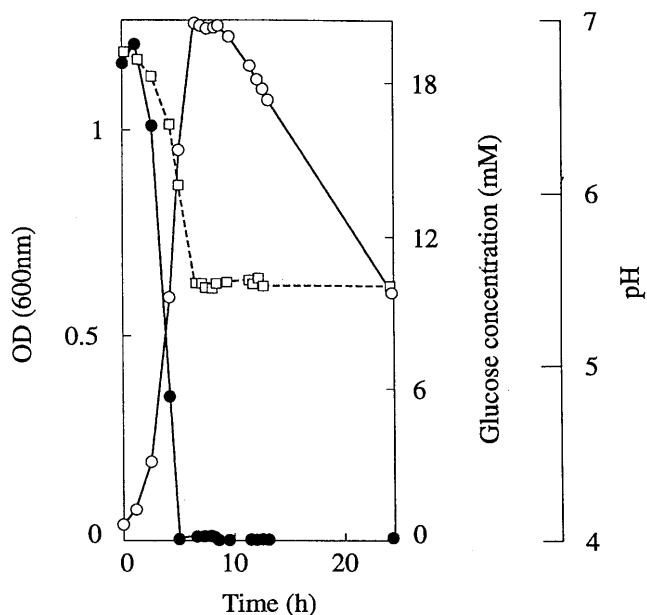
FIG. 2. Depletion of glucose (closed circles) and change in pH (open squares) corresponding to growth and lysis (open circles) of *L. lactis* subsp. *cremoris* CO at 30°C in M17 containing 0.35% glucose.

gel). 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 *Sau*3A fragment in strains CO and 2250, as previously shown for *L. lactis* subsp. *cremoris* MG1363 (3), and on a 2.47-kb *Eco*RV fragment. *Pvu*II 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
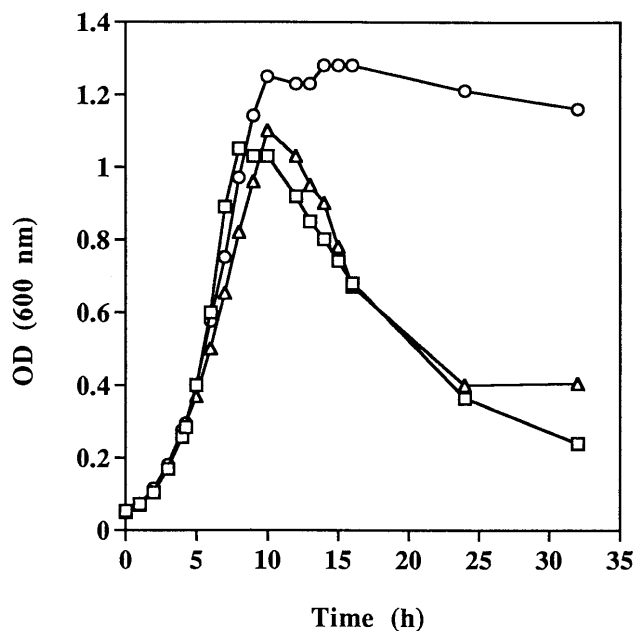

FIG. 3. Effect of NaF on lysis of *L. lactis* subsp. *cremoris* CO. *L. lactis* subsp. *cremoris* CO was grown in M17 broth with 0.4% glucose (squares), 1% glucose (circles), or 1% glucose with 7.5 mM NaF added to the medium after the OD reached about 0.3 (triangles).
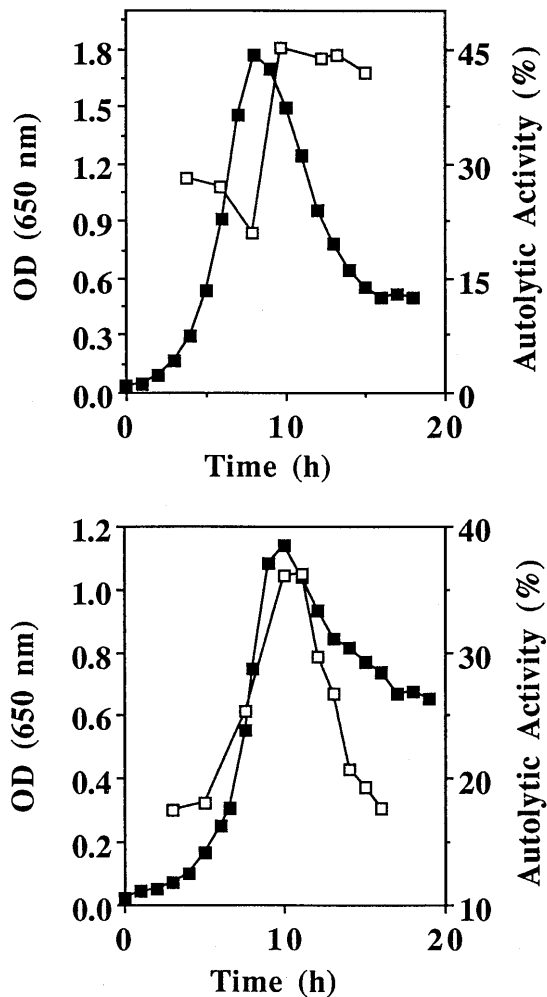
FIG. 4. Changes in autolytic activity of cells in buffer (open squares) during growth and lysis of *L. lactis* subsp. *cremoris* CO and 2250 in M17G at 30°C (closed squares). Cells were harvested at different stages of growth, washed once in distilled water, and suspended in 0.1 M Tris-HCl buffer (pH 7.0) for strain CO (top graph) or 0.1 M potassium phosphate buffer (pH 6.0) for strain 2250 (bottom graph). Autolytic activity was defined as the percent decrease in OD_{650} (compared to the maximum OD) after 3 h at 30°C.

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
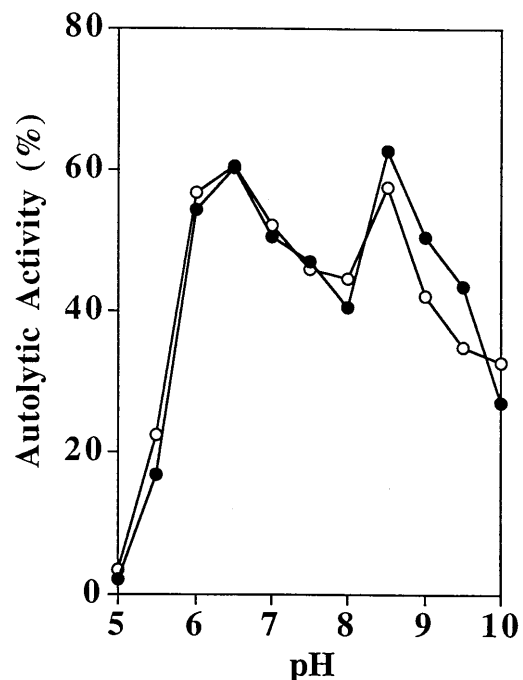

FIG. 5. Influence of pH on autolytic activity of *L. lactis* subsp. *cremoris* CO. Cells from exponential-phase (closed circles) and stationary-phase (open circles) growth at 30°C in M17 containing 0.4% glucose were harvested, washed, and suspended in 50 mM acetate buffer (pH 5.5), 50 mM sodium phosphate buffer (pH 6.0 to 8.5), and 50 mM glycine-sodium hydroxide buffer (pH 9.0 to 10.0). Autolytic activity was defined as the percent decrease in OD_{450} after 5 h at 30°C.
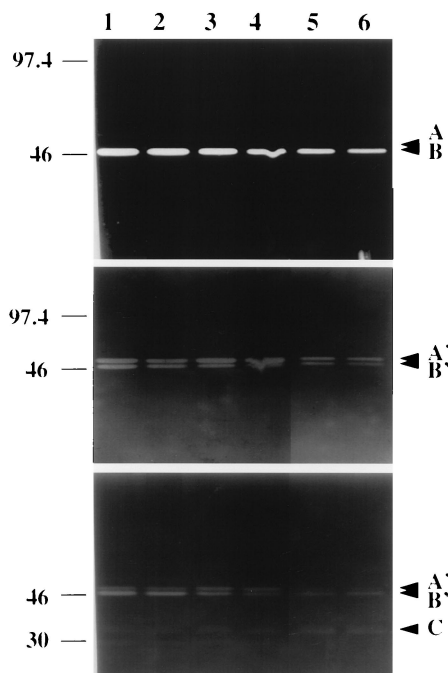
FIG. 6. Autolytic profile of proteins extracted from the cell wall fraction of *L. lactis* subsp. *cremoris* CO by various agents and analyzed by renaturing SDS–10% PAGE. The gels contained 0.2% (wt/vol) lyophilized *M. luteus* (top gel), *L. lactis* subsp. *cremoris* CO whole cells (middle gel), or 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 MgCl$_2$ 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.

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 *Staphylococcus aureus* were the product of a single gene and that protein processing was responsible for the multiple bands visualized by renaturing gel electrophoresis. The two lytic bands (46 and 41 kDa) detected in MG1363 were identified by Buist et al. (3) as the precursor and mature forms, respectively, of the major lactococcal cell wall hydrolase, AcmA, previously cloned from the nonautolytic strain MG1363 (3).

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. *lactis* biovar *diacetylactis* 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
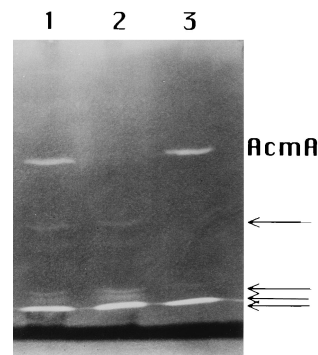

FIG. 7. Autolytic profile of *L. lactis* subsp. *cremoris* 2250 (lane 1), its deletion mutant 2250*acmAD* (lane 2), and *L. lactis* subsp. *cremoris* MG1363 (lane 3). Proteins were extracted from the cell wall fraction with 6 M guanidine hydrochloride and analyzed by renaturing SDS–10% PAGE. The gel contained 0.2% (wt/vol) autoclaved *M. luteus* cells. The 42-kDa AcmA band is absent from the 2250*acmAD* deletion mutant, and the other minor lytic bands present in both wild-type strain 2250 and 2250*acmAD* are indicated by the arrows.

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 Mg^{2+}, 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* CO 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.

ACKNOWLEDGMENTS

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