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Autolysis of *Lactococcus lactis* Caused by Induced Overproduction of Its Major Autolysin, AcmA

GIRBE BUIST, HARMA KARSENS, ARJEN NAUTA, DOUWE VAN SINDEREN,† GERARD VENEMA,* AND JAN KOK

Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute,
University of Groningen, 9751 NN Haren, The Netherlands

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The optical density of a culture of *Lactococcus lactis* MG1363 was reduced more than 60% during prolonged stationary phase. Reduction in optical density (autolysis) was almost absent in a culture of an isogenic mutant containing a deletion in the major autolysin gene, *acmA*. An *acmA* mutant carrying multiple copies of a plasmid encoding AcmA lysed to a greater extent than the wild-type strain did. Intercellular action of AcmA was shown by mixing end-exponential-phase cultures of an *acmA* deletion mutant and a tripeptidase (*pepT*) deletion mutant. PepT, produced by the *acmA* mutant, was detected in the supernatant of the mixed culture, but no PepT was present in the culture supernatant of the *acmA* mutant. A plasmid was constructed in which *acmA*, lacking its own promoter, was placed downstream of the inducible promoter/operator region of the temperate lactococcal bacteriophage r1t. After mitomycin induction of an exponential-phase culture of *L. lactis* LL302 carrying this plasmid, the cells became subject to autolysis, resulting in the release of intracellular proteins.

The action of some of the bacterial peptidoglycan hydrolases (proteins degrading the peptidoglycan of bacterial cell walls) can result in cell lysis (30). Therefore, the potentially lethal enzymes causing this phenomenon can be referred to as autolysins. In the only paper to date on the genetics of autolysis of *Lactococcus lactis*, we have described the cloning of the major autolysin gene, *acmA*, of *L. lactis* subsp. *cremoris* MG1363 (3). AcmA is a lysozyme-like enzyme (muramidase) that hydrolyzes the *N*-acetylmuramyl-1,4-β-*N*-acetylglucosamine bonds in the peptidoglycan.

Autolysis and the subsequent release of intracellular substances from the cells of a number of lactococcal strains have been shown during growth in liquid media (2, 17, 28, 29, 44) as well as during cheese production (5–7, 18, 46). Various factors such as pH, temperature, carbon source, and salt concentration appear to be important for the autolytic process. The degree of autolysis is strain dependent, and the process starts after exponential growth has ceased. The proteolytic activities of lactococci are involved in ripening and in flavor development in fermented milk products, such as cheese (27, 45). Lactococci contain more than 10 different intracellular peptidases (14) whose action leads to the production of small peptides and free amino acids which are flavors and flavor precursors. The degree and rate of release of these peptidases into the cheese matrix after lysis of the cells is of great importance for cheese maturation and flavor development (5, 7, 45, 46). Cheese maturation is a slow and therefore costly process and may be accelerated by enhanced lysis of cells with concomitant quick release of intracellular peptidases.

In a first attempt to construct starters with enhanced autolytic properties, Feirtag and McKay (11) mutagenised *L. lactis* C2 and obtained thermolytic variants which lysed at 38 to 40°C but grew normally at 32°C. Lysis was evidenced by the reduction in optical density of the culture and by the release of the intracellular enzyme phospho-β-galactosidase. Shearman et al. (35) have constructed a lactococcal strain containing the ØvML3 lysin gene under the control of its own promoter. After growth in milk at 30°C and subsequent storage at 12°C, the number of viable cells dropped to zero within 28 days, whereas the control strain still contained more than 10^6 viable cells per ml. Apparently, the lysin caused enhanced lysis of lactococcal cells, although this was not documented by showing a release of intracellular components.

In this study, we proved that AcmA is an autolysin involved in stationary-phase lysis of *L. lactis* and used this information to construct a system for *L. lactis* with which enhanced autolysis and release of intracellular proteins was obtained. This system is based on the recently characterized promoter/operator region of the temperate lactococcal bacteriophage r1t (25, 43).

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown at 30°C in 0.5× M17 broth (Difco, West Molesey, United Kingdom) containing 1.9% β-glycerophosphate (Sigma Chemical Co., St. Louis, Mo.), or in M17 when indicated. M17 agar plates contained 1.5% agar. All of these media were supplemented with 0.5% glucose. When needed, 5 μg of erythromycin (Boehringer GmbH, Mannheim, Germany) per ml was added. *Escherichia coli* was grown in TY (Difco Laboratories, Detroit, Mich.) medium at 37°C with vigorous agitation or on TY agar plates containing 1.5% agar. Ampicillin (Sigma) and erythromycin were used at final concentrations of 100 μg/ml.

General DNA techniques and transformation. Molecular cloning techniques were performed essentially as described by Sambrook et al. (31). Restriction enzymes, Klenow enzyme, T4 DNA polymerase and T4 DNA ligase were obtained from Boehringer and used as specified by the supplier. Deoxynucleotides were obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. *E. coli* and *L. lactis* were transformed by electroporation with a gene pulser (Bio-Rad Laboratories, Richmond, Calif.), as described by Zabarovsky and Winberg (47) and Leenhouts and Venema (20), respectively. Plasmid DNA was isolated from *E. coli* and *L. lactis* by the method of Birnboim and Doly, with minor modifications for *L. lactis* (34).

Primer extension analysis. RNA was isolated as previously described (39) from an exponentially growing *L. lactis* culture at an optical density at 600 nm (OD_{600}) of 0.5. Oligonucleotide pALA-26 (5′-CGCCAGCAAATTTTGTGGCTGGTTTATAAAAAGCGAGTGG), synthesized with a 381A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.), was used for primer extension

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* Corresponding author. Mailing address: Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31-50-3632093. Fax: 31-50-3632348. E-mail: VenemaG@BIOL.RUG.NL.

† Present address: Department of Microbiology, University College Cork, Cork, Ireland.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Source or reference
Strains		
L. lactis subsp. *cremoris*		
MG1363	Plasmid-free strain	12
MG1363*acmA*Δ*1*	MG1363 derivative carrying a deletion in the *acmA* gene	3
MG1363*pepT*	MG1363 derivative containing deletion in the *pepT* gene	22
LL302	MG1363 carrying the pWV01 *repA* gene on the chromosome	19
E. coli		
NM522	*supE thi* (*lac-proAB*) *hsd-5* (r_K^- m_K^-) [F′ *proAB lacI*^{q}*Z*Δ*M15*]	Stratagene, La Jolla, Calif.
MC1000	*araD139 lacX74* (*ara*, *leu*) *7697 galU galK strA*	4
Plasmids		
pAL01	Ap^r, pUC19 containing 4,137-bp lactococcal chromosomal DNA insert carrying the *acmA* gene	3
pAL08	Ap^r, pAL01 with *Sma*I-*Eco*RI deletion	This work
pAL10	Ap^r, pAL08 containing 2,716-bp *Sac*I fragment of ORF1 of the *srfA* operon of *B. subtilis*	This work
pAL11	Em^r, pIR1EF containing 4,520-bp *Sca*I-*Bam*HI fragment of pAL10	This work
pAL12	Em^r, pIR12 containing *acmA* under control of the regulatory region of phage r1t	This work
pIR12	Em^r, pWV01 derivative carrying the regulatory region of phage r1t	25
pEF+	Ap^r, SK+ containing a 1,740-bp *Eco*RI fragment of phage r1t	43
pIR12EF	Em^r, pIR12 containing a 1,785-bp *Sal*I-*Bam*HI fragment of pEF+	This work
pIR1EF	Em^r, pIR12EF in which *Sac*I site was removed	This work
pGK13	Em^r Cm^r, pWV01-based lactococcal plasmid	13
pGKAL1	Em^r Cm^r, pGK13 containing 1,942-bp *Ssp*I-*Bam*HI fragment of pAL01	This work
pGKAL2	Em^r Cm^r, pGK13 containing 1,804-bp *Sca*I-*Bam*HI fragment of pAL01	This work

reactions. Nucleotide sequence reactions were done on plasmid pAL01 by the dideoxy chain termination method (33) with the T7 sequencing kit and protocol (Pharmacia). A 25-ng portion of primer was added to 3.5 μg of RNA in a reaction mixture containing dCTP, dGTP, dTTP, and [α-S^{35}]dATP, and cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (Boehringer). After 10 min of incubation at 42°C, an excess of cold dATP was added, and incubation was continued for another 10 min at 42°C. The products were analyzed on a 6% polyacrylamide (PAA) sequencing gel.

Plasmid constructions. Plasmids pGKAL1 and pGKAL2 (Fig. 1) were constructed by subcloning of the 1,943-bp *Ssp*I-*Bam*HI or the 1,804-bp *Sca*I-*Bam*HI fragment of pAL01 (3), respectively, into the *Eco*RV-*Bam*HI sites of lactococcal plasmid pGK13. Plasmid pAL01 is a pUC19 derivative containing a 4,137-bp chromosomal DNA fragment from *L. lactis* MG1363 encompassing *acmA*. The ligation mixtures were used to transform *L. lactis* MG1363*acmA*Δ*1*.

All cloning steps for the construction of pAL12 (Fig. 1) were performed with *E. coli* MC1000 unless stated otherwise. The *Sac*I site present in the multiple-cloning site of pAL01 was removed by cutting with *Eco*RI and *Sma*I. The plasmid was treated with Klenow enzyme, ligated, and used to electrotransform *E. coli* NM522, resulting in plasmid pAL08. Because *E. coli* grows very poorly when it carries an intact *acmA* gene (3), *acmA* was disrupted by cloning into the unique *Sac*I site of pAL08 a 2,716-bp *Sac*I fragment originating from the *srfA* operon of *Bacillus subtilis* (42). This resulted in pAL10.

One of the two *Sac*I sites present in pIR12 (25) was deleted by replacing the 2,750-bp *Sal*I-*Xho*II fragment by a 1,785-bp *Sal*I-*Bam*HI fragment, taken from pEF+ (43). The remaining *Sac*I site in the resulting plasmid, pIR12EF, was removed by digestion with *Sac*I and treatment with T4 DNA polymerase. After self-ligation, pIR1EF was obtained. The 1,764-bp *Eco*RV-*Xho*II fragment of pIR1EF was replaced by the 4,520-bp *Sca*I-*Bam*HI fragment of pAL10 containing the interrupted *acmA* gene. The resulting plasmid, pAL11, was digested with *Sac*I to remove the DNA fragment interrupting *acmA*. After self-ligation, the mixture was used to transform *L. lactis* LL302 and plasmid pAL12 was obtained.

Mitomycin induction. An overnight culture of *L. lactis* was diluted 100-fold in GM17 and grown to an OD$_{600}$ of 0.2. The culture was divided into two portions, and mitomycin (Sigma) was added to one of them to a final concentration of 1 μg/ml. Incubation was continued at 30°C. The OD$_{600}$ values were measured in a Philips PU8720 UV/VIS spectrophotometer (Pye Unicam Ltd., Cambridge, United Kingdom).

Sample preparation, SDS-PAGE, and detection of lytic activity. For the analysis of the intercellular action of AcmA, 2-ml samples of culture were subjected to centrifugation. A 1-ml volume of the supernatant fraction was dialyzed against several changes of demineralized water, lyophilized, and dissolved in 0.5 ml of denaturation buffer (1). The cell pellet was resuspended in 1 ml of denaturation buffer, and cell extracts were prepared as described by van de Guchte et al. (41). The samples were boiled for 2 min and centrifuged, and 30 μl of the mid-exponential-phase samples and 15 μl of the other samples were loaded onto sodium dodecyl sulfate (SDS)-PAA gels.

For the analysis of (induced) lysis, 1-ml samples were treated as described above and the supernatant and cell fractions were dissolved in 0.2 ml of denaturation buffer. The amount of sample loaded was equalized according to the measured optical density. SDS-PAGE was carried out by the method of Laemmli (16) with the Protean II minigel system (Bio-Rad). The standard low-range and prestained low- and high-range SDS-PAGE molecular weight markers of Bio-Rad were used as references. SDS-PAA gels were stained with Coomassie brilliant blue (Bio-Rad).

Lytic activity was detected in situ by using SDS-12.5% PAA gels containing 0.15% autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma) as described previously (3). Protein renaturation was performed at room temperature for 14 h.

Western blotting and immunodetection. After SDS-PAGE, the proteins were transferred to BA85 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) as described by Towbin et al. (38). Endopeptidase and tripeptidase antigens were detected with 1:8,000-diluted polyclonal anti-endopeptidase antibodies (23) and 1:4,000-diluted polyclonal anti-tripeptidase antibodies (22), respectively, and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega Corp., Madison, Wis.) by using the Western-light chemiluminescent detection system and protocol (TROPIX Inc., Bedford, Mass.).

RESULTS

AcmA is required for autolysis of *L. lactis* during stationary phase. Overnight cultures of *L. lactis* MG1363 and its *acmA* deletion mutant MG1363*acmA*Δ*1* (3) were diluted 200-fold in fresh prewarmed M17 broth. During the first 9 h of growth and hourly sampling, the cultures were gently shaken at 30°C to prevent settling of MG1363*acmA*Δ*1*, which grows as long filaments due to improper cell separation (3). Further incubation was carried out without shaking, but the cultures were briefly shaken before sampling. The doubling time of the wild-type and mutant strains was 33 min. During stationary phase, the OD$_{600}$ of both cultures decreased and remained stable after approximately 1 week of incubation (results not shown). The average maximal percent OD$_{600}$ reduction, $[(OD_{max} - OD_{7days})/OD_{max}] \times 100\%$, was 63% for the wild type and 14% for the mutant (mean of results from three independent experiments). Apparently, the major autolysin of *L. lactis* is not only required for cell separation (3) but is also responsible for cell lysis upon prolonged incubation.

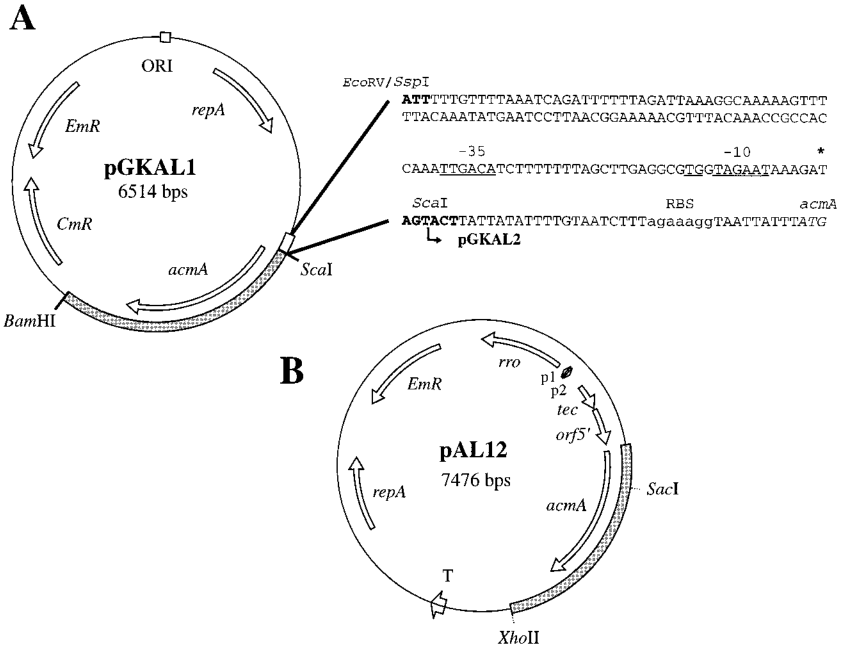

FIG. 1. Plasmid maps and nucleotide sequence of the *acmA* promoter region. (A) Map of pGKAL1. The putative ribosome binding site (RBS and lowercase letters), −10 and −35 sequences (underlined), start codon (italic), transcriptional start point (asterisk), and the *Ssp*I (only half) and *Sca*I restriction enzyme sites are indicated. *EmR* and *CmR*, erythromycin and chloramphenicol resistance genes, respectively; ORI (open square), origin of replication of the lactococcal plasmid pWV01; *repA*, gene encoding the replication initiation protein of pWV01; *acmA*, *N*-acetylmuramidase gene of *L. lactis* MG1363. The fragment from pAL01 (3) containing *acmA* is indicated with a grey bar. The cloning vector (thin line) is pGK13. In pGKAL2, the *Sca*I site was fused to the *Eco*RV site of pGK13. (B) Map of pAL12. The abbreviations are the same as in panel A. P1 and P2 (◁ ▷), promoters from bacteriophage r1t; T, transcription terminator of the lactococcal proteinase gene *prtP*; *rro*, r1t repressor gene; *tec*, topological equivalent of lambda *cro*; *ORF5'*, 5'-end of ORF5 of phage r1t. Only relevant restriction enzyme sites are shown for both plasmids.

Complementation of *acmA*Δ*1* and localization of the *acmA* promoter. A putative −35 hexanucleotide and a −10 sequence preceded by the sequence TGN, found in more than 40% of the lactococcal promoters analyzed so far (10), is present upstream of the start codon of *acmA* (Fig. 1). The spacing between the two consensus sequences (23 nucleotides) is exceptionally large. To examine whether this sequence is functional, pGKAL1 and pGKAL2 were constructed. pGKAL1 contains a 138-bp *Ssp*I-*Sca*I fragment carrying this sequence, whereas pGKAL2 does not (see Fig. 1). *L. lactis* MG1363(pGK13) and *L. lactis* MG1363*acmA*Δ*1* containing either pGK13, pGKAL1, or pGKAL2 were patched onto a GM17 plate containing 0.15% autoclaved *M. lysodeikticus* cells, and the plate was incubated for 36 h at 30°C. The results are presented in the inset in Fig. 2 and show that no halo had formed around the colony of cells containing pGKAL2 but that a large halo was present around the cells containing pGKAL1. The halo was even larger than that formed by *L. lactis* MG1363. Apparently, *L. lactis* can cope with multiple copies of *acmA* and with the increased amount of the deleterious enzyme AcmA. This result also indicates that the 138-bp *Ssp*I-*Sca*I fragment is required for *acmA* expression. This fragment, when cloned upstream of the promoterless *E. coli lacZ* gene in plasmid pORI13 (32), drove β-galactosidase expression in *E. coli* but not in *L. lactis* (results not shown). Primer extension analysis performed on RNA isolated from MG1363 cells revealed that the *acmA* mRNA starts at the T residue 6 bases downstream of the −10 hexanucleotide (result not shown). Whereas the same RNA sample gave normal primer extension products of the transcripts of two other genes, an exposure of 1 week was needed to visualize a faint band of the extension product, indicating that the promoter is only very weakly expressed. This is in agreement with the fact that we were unable to identify a protein band in a 200-fold-concentrated sample of culture supernatant of *L. lactis* MG1363 run on a PAA gel and stained with Coomassie brilliant blue which would correspond to the position of AcmA clearing bands in an activity gel.

Increased production of AcmA leads to more lysis. Overnight cultures of MG1363(pGK13) and MG1363*acmA*Δ*1* con-

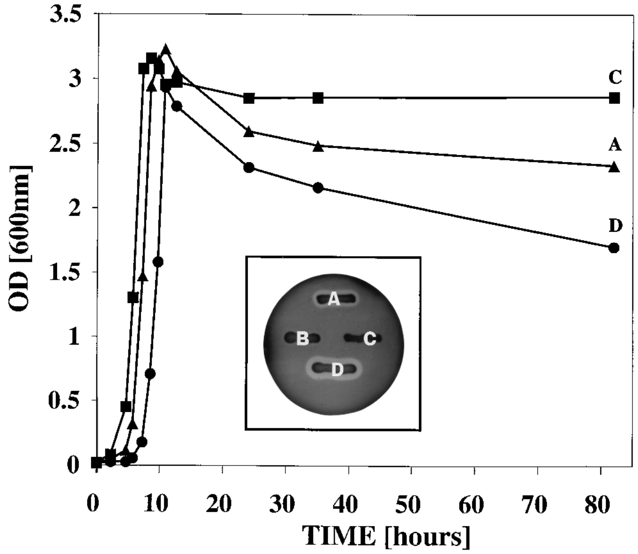

FIG. 2. GM17 growth curves and halo formation, on a GM17 plate containing 0.15% autoclaved *M. lysodeikticus* cells (inset), of *L. lactis* MG1363(pGK13) (▲, A), and *L. lactis* MG1363*acmA*Δ*1* containing pGK13 (■, C), pGKAL1 (●, D), or pGKAL2 (B).

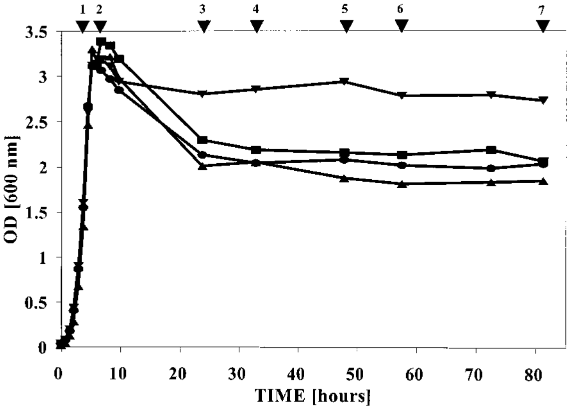

FIG. 3. GM17 growth curves of *L. lactis* MG1363 (●), *L. lactis* MG1363*pepT* (▲), *L. lactis* MG1363*acmA*Δ*1* (▼), and a coculture of *L. lactis* MG1363*pepT* and *L. lactis* MG1363*acmA*Δ*1* mixed 1:1 at the end of the exponential phase (■). The numbered arrowheads at the top of the figure indicate the points at which samples were taken for further analysis (see Fig. 4).

taining pGK13 or pGKAL1 were diluted 100-fold in fresh medium (0.5× M17), and the OD_{600} was monitored (Fig. 2). During the exponential growth phase the strains grew equally fast. During the following 70 h of incubation, the reduction in the OD_{600} of MG1363*acmA*Δ*1*(pGKAL1) was much higher than that of MG1363(pGK13). As expected, during the same period, nearly no reduction in OD_{600} was observed with the deletion mutant containing pGK13. Apparently, increased production of AcmA from pGKAL1 (see the inset in Fig. 2) results in a higher reduction of the OD compared to the wild-type situation.

AcmA acts intercellularly. Overnight cultures of MG1363, MG1363*acmA*Δ*1*, and MG1363*pepT* were diluted 100-fold in fresh 0.5× M17 medium, and their growth was monitored (Fig. 3). At the end of the exponential phase of growth, equal amounts of the cultures of the *acmA* and *pepT* deletion mutants were mixed. The presence of AcmA activity (Fig. 4A), the release of proteins into the culture medium (Fig. 4B), and the presence of PepT in the supernatant fractions (Fig. 4C) of all four cultures were monitored during 80 h of incubation at 30°C. The reduction of OD_{600} during the prolonged stationary phase of the mixed culture is nearly equal to that of the cultures of MG1363 and the *pepT* deletion mutant (Fig. 3). The average chain length in the mixed culture was equal to that of the chains of MG1363 and MG1363*pepT*, while the chains were very long in the *acmA*Δ*1* culture (reference 3 and results not shown). As expected, AcmA activity was seen in the supernatants of MG1363 and MG1363*pepT* but was absent in MG1363*acmA*Δ*1*. The supernatant of the mixed culture contains AcmA produced by the *pepT* cells (Fig. 4A). Clearly, the activity in the mixture is lower than that in the *pepT* culture, due to the presence of equal amounts of nonexpressing MG1363*acmA*Δ*1* cells. AcmA produces the typical banding pattern due to proteolytic degradation (3). Autolysis results in the release of proteins into the culture medium of the AcmA-producing strains MG1363 and MG1363*pepT* (Fig. 4B). As the protein banding pattern was the same as that of a cell extract of *L. lactis* (results not shown, but compare with Fig. 7A, lane 5), intracellular proteins are liberated. This was confirmed (Fig. 4C) by the presence of the intracellular peptidase PepT (22) among the proteins released from MG1363. Of course, no PepT antigen was present in MG1363*pepT* in the supernatant fraction of this strain (Fig. 4C) or in the cell extract (results not

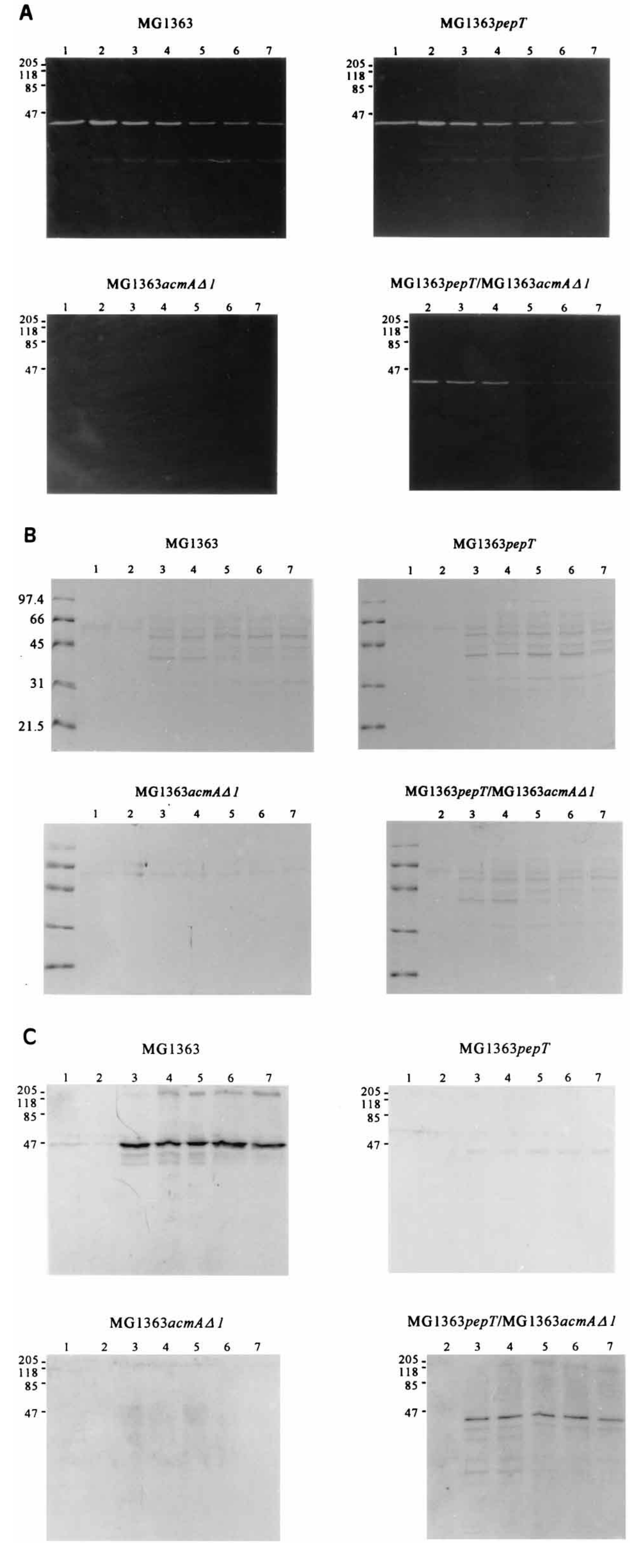

FIG. 4. (A) Detection of AcmA activity in renaturing SDS–12.5% PAA gels containing 0.15% autoclaved *M. lysodeikticus* cells. (B) Analysis by SDS–12.5% PAGE of proteins present in culture supernatants. The gels were stained with Coomassie brilliant blue. (C) Detection of PepT among the proteins in the supernatant fractions with PepT-specific antibodies. Only the results for the supernatant fractions of the samples taken as indicated in Fig. 3 are shown. Lanes 1 to 7 correspond to the time points indicated in Fig. 3. The equivalent of 60 μl (lane 1) or 30 μl (all other lanes) of supernatant was applied to the gels. Molecular masses (in kilodaltons) of standard proteins are shown in the left margins.

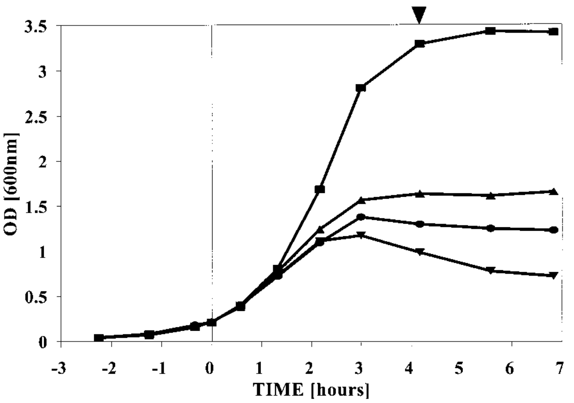

FIG. 5. Effect of mitomycin (1 μg/ml) addition at time zero on OD_{600} of *L. lactis* MG1363(pGK13) (▲) and *L. lactis* LL302 containing pIR12 (●) or pAL12 (▼). A control culture of *L. lactis* LL302(pAL12) which was not induced by mitomycin is also included (■). The arrowhead (▼) at the top of the figure indicates the time point at which 1-ml samples were taken and processed for the analysis of AcmA activity (Fig. 6) and protein and peptidase antigen (Fig. 7).

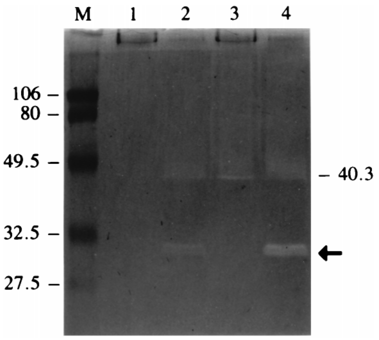

FIG. 6. Renaturing SDS-PAGE analysis of the autolysin activity of *L. lactis* LL302 containing pIR12 (lanes 1 and 2) and pAL12 (lanes 3 and 4). Samples of 1 ml were taken 4 h after the addition of mitomycin to exponentially growing cells (arrowhead in Fig. 5). Cell extracts (lanes 1 and 3) and supernatant fractions (lanes 2 and 4) were loaded on a 12.5% PAA gel containing 0.15% autoclaved *M. lysodeikticus* cells. Molecular masses of standard proteins (lane M) are shown on the left, and the clearing bands due to mature AcmA (40.3 kDa) activity are indicated on the right, (all in kilodaltons). Clearing bands caused by degradation products of AcmA (3) are indicated by an arrow.

shown). MG1363*acmA*Δ*1* does not autolyze (Fig. 3) and consequently does not release intracellular proteins (Fig. 4B). Although PepT antigen was not found in the supernatant of this culture, it was clearly present in the cell extract of this strain (results not shown). Intracellular proteins, including PepT antigen present in the cells of MG1363*acmA*Δ*1*, were liberated in the mixed culture (Fig. 4B and C). This must have been caused by AcmA, produced and released from MG1363*pepT*, degrading the cell walls of the MG1363*acmA*Δ*1* cells. Both the total amount of released proteins and the AcmA activity decreased over time (Fig. 4), probably due to the action of released intracellular proteolytic enzymes.

Induced expression of AcmA. The *acmA* gene lacking its native promoter but retaining its own ribosome binding site was taken from pAL01 and inserted into pIR12 (25). In the resulting plasmid, pAL12 (Fig. 1), expression of *acmA* is repressed by the repressor Rro and is induced by mitomycin (25). Plasmid pAL12 was used to transform *L. lactis* LL302 which contains a copy of the pWV01 *repA* gene on the chromosome (19) to ensure efficient replication of pWV01-derived vectors. All strains used for this experiment grew with the same μ_{max} and reached similar final OD values in the absence of mitomycin. After 2 to 3 h after mitomycin addition, the OD_{600} of LL302(pAL12) decreased gradually and steadily (Fig. 5). MG1363(pGK13) and LL302(pIR12), the latter of which produces *E. coli* β-galactosidase from the r1t promoter/operator cassette (25), did not show detectable lysis in this assay. Clearly, the addition of 1 μg of mitomycin per ml resulted in a reduction in the growth rates of all cultures. In MG1363 (pGK13) the maximal OD_{600} reached was 1.6, whereas LL302 (pIR12) did not reach this OD_{600} and appeared to lyse slightly.

To examine the level to which AcmA was induced, samples were taken 4 h after mitomycin addition and inspected by renaturing SDS-PAGE (Fig. 6). Increased activity of AcmA was present in an *L. lactis* LL302(pAL12) cell extract compared to the amount in cell extracts of *L. lactis* LL302(pIR12). Qualitatively, the same was true for the supernatant fractions of the strains: only *L. lactis* LL302(pAL12) produced enhanced clearing bands at a position corresponding to proteins of approximately 30 kDa, which have previously been shown to be active degradation products of AcmA (3).

Although the level of AcmA production was clearly increased, the OD measurements did not conclusively show that it resulted in cell lysis. To examine this in a more direct way, the supernatant fractions of cultures induced for 4 h were assayed for the presence of intracellular proteins by SDS-PAGE. The results (Fig. 7A) show that only one protein is detectable in uninduced cultures. Most probably, this protein is the previously described major secreted protein, Usp45, of *L. lactis* (40). Upon induction, proteins normally present in the cell extracts only are, to a considerable extent, extruded into the culture medium in the case of *L. lactis*(pAL12) (Fig. 7A, lane 4). To a lesser extent, *L. lactis*(pIR12) released proteins into the supernatant. To ascertain that cytoplasmic proteins were indeed present in the culture medium after mitomycin induction, immunoblots were performed on supernatants of cells carrying the various plasmids. The results in Fig. 7B show that antibodies raised against the cytoplasmic lactococcal peptidase PepO (23) gave a strong signal with the supernatant of *L. lactis*(pAL12) and only a weak one with that fraction of *L. lactis*(pIR12). The reacting band at a position of around 40 kDa is caused by an impurity in the antibody preparation (23).

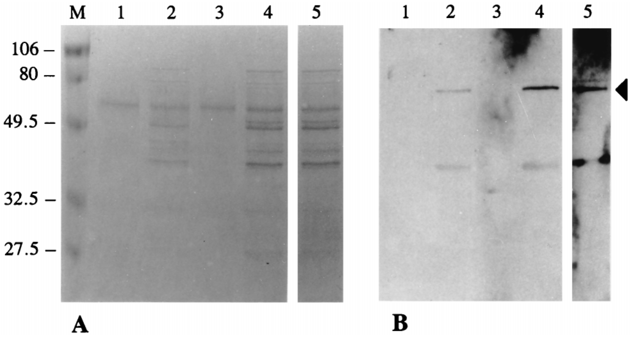

FIG. 7. (A) Analysis by SDS–12.5% PAGE of proteins present in the culture supernatants of induced (lanes 2 and 4) and noninduced (lanes 1 and 3) *L. lactis* LL302 containing pIR12 (lanes 1 and 2) or pAL12 (lanes 3 and 4). The samples were taken at the time point indicated in Fig. 5. Lane 5 contains a cell extract of noninduced *L. lactis* LL302(pIR12). The gel was stained with Coomassie brilliant blue. Molecular masses (in kilodaltons) of standard proteins (M) are shown on the left. (B) Western blot analysis of the gel shown in panel A with polyclonal antibodies raised against the lactococcal intracellular endopeptidase PepO (23). The arrowhead indicates the position of the endopeptidase.

DISCUSSION

In this work we have clearly shown that AcmA of *L. lactis* is required for autolysis of this organism during stationary phase. Deletion of the *acmA* gene resulted in complete loss of the autolytic behaviour. Autolysis resulted in the release of intracellular proteins, including the intracellular peptidases PepT and PepO. The reduction in OD_{600} of MG1363*acmA*Δ*1* was at most 15% during stationary phase. This decrease occurred immediately after the culture had reached its maximum OD_{600}. Thereafter, the OD_{600} of the culture remained constant for at least 7 days. The OD reduction was not accompanied by a release of intracellular proteins (Fig. 4B and C), indicating that it is not caused by (auto)lysis. In other words, in *L. lactis* MG1363, AcmA is the only enzyme responsible for autolysis. The initial steep drop in the OD_{600} of approximately 15% after reaching stationary phase was observed in all the strains examined. Since the viable count of MG1363*acmA*Δ*1*(pGK13) did not change from the point of maximum OD_{600} to 10 h thereafter (unpublished data), the initial OD_{600} reduction has to be explained by general changes in cell morphology and/or intracellular components influencing light scattering and thus reducing OD_{600}.

Although Mou et al. (24) and Niskasaari (26) detected only muramidase activity in two strains of *L. lactis*, Østlie et al. (28) have recently shown that three other *L. lactis* strains contained a glucosidase and an *N*-acetylmuramoyl-L-alanine amidase or endopeptidase activity. Also, Crow et al. (6) suggested the presence of more than one autolytic enzyme in lactococci on the basis of activity profiles in renaturing SDS-PAGE activity assays. From the literature, it is clear that autolytic behavior is different among lactococcal strains, and it will be interesting to determine the actual contribution of each of these (putative) enzymatic activities to autolysis. Based on the data presented here and our unpublished results that an active copy of *acmA* is present in more than 15 different (industrial) strains of *L. lactis*, we postulate that AcmA is the only or major enzyme involved in stationary phase autolysis in many, if not all, lactococci.

Loss of autolysis was also seen in other gram-positive bacteria when expression of peptidoglycan hydrolases was prevented. Insertional inactivation of the gene encoding the major autolysin *N*-acetylmuramoyl-L-alanine amidase (*cwlB*) of *B. subtilis* led to loss of approximately 90% of the total cell wall hydrolytic activity of stationary-phase cells. The mutant strain was extremely resistant to cell lysis but did not grow in filaments (15). Interruption of *Streptococcus pneumoniae lytA*, the gene encoding *N*-acetylmuramoyl-L-alanine amidase, resulted in loss of autolysis during stationary phase. No significant difference in chain formation was observed between the wild-type and mutant strains (37). Two mutants of *Staphylococcus aureus* showing negligible autolysis during a prolonged stationary phase were created by Tn*917-lacZ* insertion mutagenesis (21). The strains lacked the endo-β-*N*-acetylglucosaminidase (51-kDa) and *N*-acetylmuramyl-L-alanine amidase (62-kDa) activities, which Sugai et al. (36) later showed were involved in the separation of daughter cells.

Vegarud et al. (44) have shown that changes in the composition of M17 leading to a reduction in maximal OD generally resulted in a reduction of autolysis. This is in agreement with observations made in this study. As detailed in Results, autolysis of MG1363 grown in M17 medium was estimated to be 63%, which is similar to that measured by Østlie et al. for two lactococcal strains grown under the same conditions (29). When MG1363 was grown in 0.5× M17 (Fig. 3), the decrease in OD was only 35%. The difference in autolysis cannot be explained by a difference in final culture medium pH, an important factor for AcmA activity (reference 24 and unpublished results), because the pHs reached in both media used in this study were 5.2.

AcmA was shown to also act intercellularly, releasing the cellular content of an AcmA-nonproducing strain. Although AcmA is normally attached to the cell wall through its C-terminal repeat domain (reference 3 and unpublished results), the enzyme is apparently not covalently linked. It can be released and can subsequently recognize, bind, and hydrolyze the wall of another cell. This observation opens the possibility of using *L. lactis* for the controlled overexpression of AcmA and adding such a strain to the mixture of strains present in a cheese starter culture. Induction of the *acmA* gene in the adjunct strain could lead to the enhanced lysis of all strains in the starter. Among the proteins released would be flavor-enhancing enzymes. To have such an inducible lysis system at one's disposal could be of great industrial interest. As a first step toward an inducible system for *L. lactis*, *acmA* lacking its own promoter was cloned downstream of the promoter/operator region of the temperate lactococcal bacteriophage r1t. Expression of AcmA from this construct was inducible by the addition of mitomycin. Increased expression of AcmA was observed 4 h after induction of the lactococcal strain containing pAL12. Mitomycin did not induce expression of the chromosomal copy of *acmA*. AcmA induction was much lower than β-galactosidase induction (25) when the same genetic element was used. Among other possibilities, this may be due to factors needed for (extracellular) AcmA activity that become limiting. In this respect, it is interesting that part of an operon which is involved in the secretion of the strongly homologous muramidase-2 of *Enterococcus hirae* was recently cloned and sequenced (9).

A decrease in OD_{600} with the release of intracellular proteins was seen in cultures of the strain overexpressing AcmA, but limited lysis of cells was also observed in the strain overexpressing β-galactosidase. The slow decrease in the OD_{600} of the latter strain may be caused by the production of deleterious quantities of β-galactosidase only or in combination with the presence of mytomicin, a substance which clearly inhibits cell growth.

Although we have successfully overproduced AcmA with concomitant cell lysis, it is clear that the system is not yet optimal and cannot be used for industrial fermentations. Research is currently focused on the isolation of a temperature-sensitive mutant of the repressor (Rro), which would allow us to lyse cells and release important proteins and enzymes in cibo in a food-grade way.

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