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

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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, Feitag and McKay (11) mutagenised *L. lactis* C2 and obtained thermolysin 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 10⁶ 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 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 (OD₆₀₀) of 0.5. Oligonucleotide pALA-26 (5′-CGCCAGCAAATTTTGCGC AGGATGTTG)-3′ was synthesized with a 381A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.), was used for primer extension.
structured by subcloning of the 1,943-bp SspI (SacI) fragment of pEG10. After 10 min of incubation at 42°C, an excess of cold dATP was added, and the reaction was synthesized with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). The reaction mixture containing dCTP, dGTP, dTTP, and [γ-32P]ATP was treated with Klenow enzyme, ligated, and used to electrotransform E. coli MC1000 unless stated otherwise. The ligation mixtures were used to transform L. lactis 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 SacI fragment originating from the SspI fragment of phage RV-1. All cloning steps for the construction of pAL12 (Fig. 1) were performed with E. coli MC1000 unless stated otherwise. The SacI site present in the multiple-cloning site of pAL01 was removed by cutting with EcoRI and SmaI. 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 SacI site of pAL08 a 2,716-bp SacI fragment originating from the SspI fragment of Bacillus subtilis (42). This resulted in pAL10.

One of the two SacI sites present in pIR12 (25) was deleted by replacing the 2,750-bp SalI-HindIII fragment of pAL10 containing the 1,804-bp SalI-HindIII fragment of pAL10 with a 1,785-bp SalI-HindIII fragment of pAL10 carrying a unique SspI site. The resulting plasmid, pAL11, was digested with SacI 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 OD600 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 OD600 values were measured in a Philips PU8720 UV/VIS spectrophotometer (Pye Unicam Ltd., Cambridge, United Kingdom).

RESULTS

AcmA is required for autolysis of L. lactis during stationary phase. Overnight cultures of L. lactis MG1363 and its acmA deletion mutant MG1363acmAΔI (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 MG1363acmAΔI, 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 ODmax of both cultures decreased and remained stable for approximately 1 week of incubation (results not shown). The average maximal percent ODmax reduction, [ODmax − ODmin]/ODmax × 100%, was 63% for the wild type and 14% for the mutant (mean of results from three independent experiments). Apparently, the major autolysis of L. lactis is not only required for cell separation (3) but is also responsible for cell lysis upon prolonged incubation.
Complementation of acmAΔl and localization of the acmA promoter. A putative −35 hexanucleotide and a −10 sequence preceded by the start codon of acmA (Fig. 1). The sequence 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 SspI–ScaI fragment carrying this sequence, whereas pGKAL2 does not (see Fig. 1). L. lactis MG1363(pGK13) and L. lactis MG1363acmAΔl 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 SspI–ScaI 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 MG1363acmAΔl con-
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 OD600 of MG1363 acmA D1 (pGKAL1) was much higher than that of MG1363 (pGK13). As expected, during the same period, nearly no reduction in OD600 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 D1, 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 OD600 during the prolonged stationary phase of the mixed culture is nearly equal to that of the cultures of MG1363 and MG1363 pepT, while the chains were very long in the acmA D1 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 D1. The supernatant of the mixed culture contained 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 D1 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 shown).

\[\text{VOL. 63, 1997 INDUCIBLE LYSIS OF LACTOCOCCUS LACTIS 2725}\]
All strains used for this experiment grew with the same (19) to ensure efficient replication of pWV01-derived vectors. The acmA gene on the chromosome of MG1363 strain (results not shown). MG1363acmAΔΔl 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 MG1363acmAΔΔl, were liberated in the mixed culture (Fig. 4B and C). This must have been caused by AcmA, produced and released from MG1363pepT, degrading the cell walls of the MG1363acmAΔΔl 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). 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 MG1363acmAΔΔl, were liberated in the mixed culture (Fig. 4B and C). This must have been caused by AcmA, produced and released from MG1363pepT, degrading the cell walls of the MG1363acmAΔΔl 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.

The acmA gene on the chromosome of 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 OD600 reached was 1.6, whereas LL302 (pIR12) did not reach this OD600 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).
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 peptides PepT and PepO. The reduction in OD$_{600}$ of MG1363acmAΔI 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 MG1363acmAΔI(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-t-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-t-alanine amidase (cwIB) 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* bta, the gene encoding N-acetylmuramoyl-t-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 Tn917-lacZ insertion mutagenesis (21). The strains lacked the endo-β-N-acetylglicosaminidase (51-kDa) and N-acetylmuramyl-t-alanine amidase (62-kDa) activities, which Sugai et al. (36) later showed were involved in the separation of daughter cells.

Vegard 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 65%, 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 adjacent 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 mytomycin, 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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