Reduced Lysis upon Growth of *Lactococcus lactis* on Galactose Is a Consequence of Decreased Binding of the Autolysin AcmA

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When *Lactococcus lactis* subsp. *lactis* IL1403 or *L. lactis* subsp. *cremoris* MG1363 is grown in a medium with galactose as the carbon source, the culture lyses to a lesser extent in stationary phase than when the bacteria are grown in a medium containing glucose. Expression of AcmA, the major autolysin of *L. lactis*, is not influenced by the carbon source. Binding studies with a fusion protein consisting of the MSA2 protein of *Plasmodium falciparum* and the C-terminal peptidoglycan-binding domain of AcmA revealed that cell walls of cells from both subspecies grown on galactose bind less AcmA than cell walls of cells grown on glucose. Cells grown on glucose or galactose and treated with trichloroacetic acid prior to AcmA binding bind similar amounts of AcmA. Analysis of the composition of the lipoteichoic acids (LTAs) of *L. lactis* IL1403 cells grown on glucose or galactose showed that the LTA composition is influenced by the carbon source: cells grown on galactose contain LTA with less galactose than cells grown on glucose. In conclusion, growth of *L. lactis* on galactose changes the LTA composition in the cell wall in such a way that less AcmA is able to bind to the peptidoglycan, resulting in a decrease in autolysis.

Gram-positive bacteria produce enzymes that hydrolyze peptidoglycan (PG), the major component of the cell wall (14). The model organism for lactic acid bacteria, *Lactococcus lactis*, produces three types of PG hydrolases: four *N*-acetyl-glucosaminidases (AcmA, AcmB, AcmC, and AcmD); two putative gamma-amidases (AcmB, Usp45, llmg_0904, and llmg_1890) with a C-terminal peptidoglycan-binding domain (21, 25). The genes for these PG hydrolases are present in the chromosomes of the *L. lactis* model organism for lactic acid bacteria, *L. lactis* subsp. *cremoris* MG1363 (4, 21). The enzyme binds to PG in the lactococcal cell wall in a way that less AcmA is able to bind to the peptidoglycan, resulting in a decrease in autolysis.

The PG hydrolases of *L. lactis* are modular enzymes that are composed of a signal sequence, an active-site domain, and a putative cell wall binding domain (21, 25). AcmA is the best-studied enzyme: the mature form comprises an N-terminal *N*-acetyl glucosaminidase active site (43) and a C-terminal domain that consists of three lysin motif (LysM) modules (8). The enzyme binds to PG in the lactococcal cell wall in a noncovalent manner via its LysM-containing domain (42, 44). Binding of AcmA has also been shown to occur intercellularly; that is, AcmA produced by and liberated from one cell can bind to and lyse another cell (8). To control the function of these potentially suicidal enzymes, both the expression and activity of cell wall hydrolases are well regulated. Expression of the genes is low and growth phase dependent (7, 25). The different enzymes have different pH dependencies: AcmD and YigB are active at a pH of around 4, AcmC is active below pH 7, and AcmA has been shown to be active below pH 10 (21, 45). AcmA is subject to proteolytic degradation by the extracellular lactococcal proteases PrtP and HtrA, resulting in reduced activity and/or reduced wall binding (6, 8, 34, 42, 43). Recently, it has been shown that modification of the PG may also be a manner to control the activity of AcmA. Veiga et al. (48) showed that increased O-acetylation of the PG of *L. lactis* results in resistance against AcmA, possibly by reduced binding of AcmA. De-N-acetylation of lactococcal PG did not affect the binding of AcmA (31). Secondary cell wall polymers such as S-layer proteins and lipoteichoic acids (LTAs) regulate the activity of AcmA by hindering its binding to PG (44). Although PG is present all over the lactococcal cell surface, AcmA binds only at specific sites on the lactococcal cell, predominantly around the septum and poles (44). When cells are boiled in trichloroacetic acid (TCA) and AcmA is subsequently added from the

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outside, AcmA binding occurs at the whole cell surface. A component that can be extracted from the cell wall by the TCA treatment, possibly LTA, is involved in hindering AcmA binding such that AcmA binds only to the mentioned sites (44). Indeed, for L. lactis strain SK110, it was shown that its LTAs are present in the sites in the cell wall where AcmA does not bind (44). LTAs are lipid-linked carbohydrates consisting of polyglycerolphosphate in L. lactis. They are involved in the control of autolysin activity (3, 15), in determining the electrochemical properties of the cell wall (33), in establishing a magnesium ion concentration (1, 19, 22, 25), and in determining the physicochemical properties of the cytoplasmic membrane (18). In the mid-1970s it was already shown that the teichoic acid moiety of the LTA of L. lactis contains 16 to 17 galactose and glucose differs considerably, which could explain the differences in autolysin binding.

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. lactis strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>L. lactis subsp. cremoris; Lac&lt;sup&gt;+&lt;/sup&gt; PrtP&lt;sup&gt;+&lt;/sup&gt;; plasmid-free derivative of NCD0712</td>
<td>16</td>
</tr>
<tr>
<td>MG1363 acmAΔ/</td>
<td>Derivative of MG1363 carrying a 701-bp SacI/Spel deletion in acmA</td>
<td>8</td>
</tr>
<tr>
<td>SK110</td>
<td>L. lactis subsp. cremoris; phage resistant; contains galactosyl in LTA</td>
<td>41</td>
</tr>
<tr>
<td>IL1403</td>
<td>L. lactis subsp. lactis; plasmid-free strain</td>
<td>13</td>
</tr>
<tr>
<td>IL1403 acmA::ISS/</td>
<td>acmA mutant of IL1403</td>
<td>This study</td>
</tr>
<tr>
<td>NZ9000 acmAΔ/</td>
<td>Derivative of NZ9000 carrying a 701-bp SacI/Spel deletion in acmA</td>
<td>45</td>
</tr>
<tr>
<td>NZ9700</td>
<td>Nisin-producing transconjugant of strain NZ9000 containing the nisin-sucrose transposon Tn5276</td>
<td>23</td>
</tr>
<tr>
<td><strong>E. coli</strong> EC101</td>
<td>E. coli JM101 with repA from pWV01 integrated in chromosome; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>26</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGh9::ISS/</td>
<td>Used in insertional mutagenesis; Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>28</td>
</tr>
<tr>
<td>pNG3041</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt;; pNZ8048 derivative containing a fusion of the pre-pro sequence of prtP to msa2 and the C-terminal domain of acmA under control of Pr&lt;sub&gt;nisA&lt;/sub&gt;</td>
<td>44</td>
</tr>
</tbody>
</table>

GM17 agar plates contained 1.5% (wt/vol) agar. For the selection of plasmid pNG3041 in L. lactis NZ9000 acmAΔ/, chloramphenicol (Sigma-Aldrich, St. Louis, MO) was added (5 µg/ml). Erythromycin (Boehringer GmbH, Mannheim, Germany) was added to a concentration of 5 µg/ml when L. lactis IL1403 (harboring the vector pGh9::ISS/) was grown at 30°C and to a concentration of 2 µg/ml when incubation was at 37°C. Escherichia coli was grown in tryptone yeast extract medium (Difco Laboratories) at 37°C with vigorous agititation or on tryptone yeast extract medium solidified with 1.5% (wt/vol) agar and containing 100 µg of erythromycin (Boehringer GmbH) per ml when required. For E. coli EC101, 40 µg/ml kanamycin (Boehringer GmbH) was used (26). All chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck (Darmstadt, Germany).

**General techniques and transformation.** Molecular cloning techniques were performed essentially as described by Sambrook et al. (40). Restriction enzymes and T4 DNA ligase were obtained from Boehringer GmbH and were used according to the instructions of the supplier. Genomic DNA of L. lactis was isolated as described by Buist et al. (5). Plasmid DNA was isolated at large scale using a Nucleobond Kit PC 100 (Machery-Nagel, Düren, Germany) as specified by the supplier. E. coli and L. lactis were transformed by electroporation using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) as described by Zabarovsky and Winberg (52) and Leenhouts and Venema (27), respectively.

**Screening for transposon mutants and generation of plasmid-free derivatives.** Mutagenesis of L. lactis IL1403 with pGh9::ISS/ and isolation of stable ISS/ insertion mutants by excision of integrated vector were performed as described previously (28). Cell wall-solubilizing activity was visualized as a halo around colonies grown for 36 to 48 h at 37°C on GM17 plates containing 0.2% (wt/vol) autoclaved, lyophilized Micrococcus lysodeikticus ATCC 4698 cells (Sigma Chemical Co., St. Louis, MO) (26). Plasmid-free derivatives of the pGh9::ISS/ insertion mutants of L. lactis IL1403 lacking a halo were generated from an overnight culture of the mutant diluted 10<sup>4</sup>-fold in fresh GM17 (without erythromycin) medium and grown for 18 h at 28°C. After growth the culture was diluted and plated onto GM17 plates to obtain single colonies. The removal of pGh9::ISS/ was verified by the absence of growth of the mutant on GM17 plates containing erythromycin.

**Southern transfer, DNA hybridization, and nucleotide sequencing.** To verify single-site integration of pGh9::ISS/ and to determine the restriction sites flanking the plasmid, chromosomal DNA of selected integrants of L. lactis IL1403 acmA::pGh9::ISS/ and isolation of stable ISS/ insertion mutants by excision of integrated vector were performed as described previously (28). Cell wall-solubilizing activity was visualized as a halo around colonies grown for 36 to 48 h at 37°C on GM17 plates containing 0.2% (wt/vol) autoclaved, lyophilized Micrococcus lysodeikticus ATCC 4698 cells (Sigma Chemical Co., St. Louis, MO) (26). Plasmid-free derivatives of the pGh9::ISS/ insertion mutants of L. lactis IL1403 lacking a halo were generated from an overnight culture of the mutant diluted 10<sup>4</sup>-fold in fresh GM17 (without erythromycin) medium and grown for 18 h at 28°C. After growth the culture was diluted and plated onto GM17 plates to obtain single colonies. The removal of pGh9::ISS/ was verified by the absence of growth of the mutant on GM17 plates containing erythromycin.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, growth media, growth conditions, and chemicals. Plasmids and bacterial strains used in this study are listed in Table 1. L. lactis was grown as standing cultures at 30°C or 37°C in M17 broth (Difco Laboratories, Detroit, MI) containing (in wt/vol for all sugars) the following: 0.5% glucose (GM17), 0.5% galactose, or a mixture of 0.25% glucose and 0.25% galactose.
hamshire, United Kingdom). Sample preparation and sequence reactions were performed with the DNA sequencing robot, the Vistra Systems DNA labstation 625, using an automated δSeq sequencing kit and the Texas-Red M13 forward primer of Amersham, according to the instructions of the supplier.

OD measurements, ninhydrin induction, enzyme assays, and microscopy. ODs of cultures were measured at 600 nm in a Novaspec II spectrophotometer (Pharmacia Biotech AB, Uppsala, Sweden). To produce MSA2cA protein, L. lactis NZ9000 (24) containing plasmid pNG3041 was grown in GM17 broth containing chloramphenicol at 30°C. The culture was grown until an OD600 of 0.5 was reached, after which it was induced with ninhydrin by the addition of 1,000 (vol/vol) of a supernatant of the culture of the ninhydrin-producing strain L. lactis NZ7970.

To measure the influence of the carbon source on cellular lysis, L. lactis MG1363, L. lactis MG1363 acmAΔ1, L. lactis IL1403, and L. lactis IL1403 acmAΔ1::IS1 were subsequently resuspended in 50 ml of the supernatants of the L. lactis MG1363 or L. lactis IL1403 cultures and incubated at 30°C for 96 h. Subsequently, as a measure of the extent of lysis, x-prolyl dipeptidyl aminopeptidase (PepX) was measured using the chromogenic substrate Ala-Pro-p-nitroanilid (Bachem Feinchemicalien AG, Bubendorf, Switzerland) as described earlier (6). Briefly, after 2 min of centrifugation in an Eppendorf microcentrifuge, 75 μl of a culture supernatant was added to 50 μl of substrate (2 mM) and 75 μl of HEPES buffer (pH 7.0). The mixture was pipetted into a microtiter plate well, and color development was monitored in a THERMOMax microtiter plate reader (Molecular Devices Corporation, Menlo Oaks, CA) at 405 nm for 20 min at 37°C.

Light microscopy pictures of L. lactis MG1363 acmAΔ1 and L. lactis IL1403 acmAΔ1::IS1 grown in M17 containing glucose or galactose were made by using a Zeiss microscope (Carl Zeiss, Thornwood, CA) and an Axiovison digital camera (Axion Technologies, Houston, TX). To determine the effect of AcmA on bacterial chain length, L. lactis MG1363 acmAΔ1 and L. lactis IL1403 acmAΔ1::IS1 cells (pellet of 1 ml of culture) were incubated with L. lactis MG1363 or L. lactis IL1403 supernatants (supernatant of 1 ml of culture) for 30 min at 30°C before microscopic examination. Electron microscopy was performed as described earlier (34).

Isolation and TCA treatment of cell walls and MSA2cA binding assay. Walls of cells grown in M17 medium with glucose or galactose were isolated and treated with TCA as described before (44). MSA2cA binding studies were performed with the MSA2cA protein, L. lactis MG1363 acmAΔ1::IS1 were subsequently resuspended in 50 ml of the supernatants of the L. lactis MG1363 or L. lactis IL1403 cultures and incubated at 30°C for 96 h. Subsequently, as a measure of the extent of lysis, x-prolyl dipeptidyl aminopeptidase (PepX) was measured using the chromogenic substrate Ala-Pro-p-nitroanilid (Bachem Feinchemicalien AG, Bubendorf, Switzerland) as described earlier (6). Briefly, after 2 min of centrifugation in an Eppendorf microcentrifuge, 75 μl of a culture supernatant was added to 50 μl of substrate (2 mM) and 75 μl of HEPES buffer (pH 7.0). The mixture was pipetted into a microtiter plate well, and color development was monitored in a THERMOMax microtiter plate reader (Molecular Devices Corporation, Menlo Oaks, CA) at 405 nm for 20 min at 37°C.

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Isolation and TCA treatment of cell walls and MSA2cA binding assay. Walls of cells grown in M17 medium with glucose or galactose were isolated and treated with TCA as described before (44). MSA2cA binding studies were performed with mixing equal amounts of L. lactis cells (the amount of cells present in 1 ml of culture with an OD600 of 1.0) or cell walls (1 mg) with 1 ml of supernatant of a nisin-induced L. lactis IL1403 culture supernatant (supernatant of 1 ml of culture) for 30 min at 30°C before microscopic examination. Electron microscopy was performed as described earlier (34).

RESULTS

Selection and isolation of an AcmA mutant of L. lactis subsp. lactis IL1403. In previous work we have shown that AcmA of L. lactis MG1363 is the major lactococcal PG hydrolase (8, 43). Comparison of the genome sequences of L. lactis subsp. cremoris MG1363 and L. lactis subsp. IL1403 showed that both strains possess the same chromosomally encoded PG hydrolases (50). To investigate and compare the effect of carbon sources on the activity of the major autolysin in both subspecies, we constructed an acmA mutant of L. lactis IL1403. As selection for an acmA deletion mutant on plates containing M. lysodeikticus cell wall fragments was previously shown to work well (26), this screening method was used to isolate an acmA mutant of L. lactis IL1403. A transposon mutant library of L. lactis IL1403 was generated using plasmid pGh9::ISS1 and screened for loss of extracellular PG hydrolase activity as described in Materials and Methods. Two out of 3,000 colonies were found to have completely lost halo-forming ability on plates containing M. lysodeikticus cell wall fragments. Hybridization of different digests of the chromosomal DNA of the mutants with a probe of plasmid pGh9::ISS1 revealed that the patterns for both mutants were identical (data not shown). To determine the site of integration in one of the mutants, L. lactis IL1403 acmA::pGh9::ISS1, the regions flanking pGh9::ISS1 were isolated using plasmid rescue. After DNA isolation and plasmid sequencing, pGh9::ISS1 was found to have integrated in the L. lactis IL1403 acmA gene immediately upstream of the codon for the active-site glutamine residue, resulting in loss of production of an active protein (Fig. 1).

AcmA of L. lactis subsp. lactis IL1403 is the strain's major autolysin and is involved in cell separation. After excision of integrated pGh9::ISS1 from the strain, the resulting mutant L. lactis IL1403 acmA::ISS1 was analyzed for the loss of AcmA activity by zymographic analysis at the natural temperature of 30°C. Hybridization of chromosomal DNA of the mutant with pGh9::ISS1 showed that the plasmid was lost and that a copy of the ISS1 was left behind in acmA (results not shown). As expected, no AcmA activity could be detected in cell and supernatant samples of the mutant using zymographic detection although AcmA activity was present in the samples of L. lactis IL1403 and L. lactis IL1403 acmA::ISS1 (results not shown). Comparison of cellular lysis during the stationary growth phase showed that L. lactis IL1403 wild-type cells lysed slightly more than cells of L. lactis MG1363 (Fig. 2). While lysis of the acmA mutant of L. lactis MG1363 is completely abolished, the L. lactis IL1403 acmA::ISS1 mutant still lyases to

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(32). Briefly, the disrupted cells were mixed with 60 ml of n-butanol (Merck, Darmstadt, Germany), stirred for 30 min at room temperature, and centrifuged at 13,000 × g for 40 min at room temperature. The centrifugation step was lengthened to obtain a higher yield of LTA. The aquatic phase was hylolyzed, filtered using a 0.22-μm-pore-size nitrocelullose membrane filter (Schleicher & Schuell, ‘s-Hertogenbosch, The Netherlands), resuspended in 5 ml of chromato-
some extent. As the numbers of bacterial genes encoding PG hydrolases are identical in both strains, this difference in lysis may be due to the difference in prophage-encoded lysis modules. The relative reduction of lysis of both the L. lactis IL1403 and L. lactis MG1363 acmA mutants to their respective parental strains is comparable.

After overnight growth in liquid medium, the sedimentation of cells and chain elongation of L. lactis IL1403 acmA::ISS1 were observed, as previously described for the acmA mutant of L. lactis MG1363 (8), indicating that the PG hydrolase AcmA of L. lactis IL1403 is also involved in autolysis and cell separation.

Growth of L. lactis on galactose results in lower stationary-phase lysis than when cells are grown on glucose. Riepe et al. (38) observed that when L. lactis subsp. cremoris strains CO and 2250 were grown on a medium containing galactose as the carbon source, these strains lysed to a lesser extent than when grown on a medium containing glucose, suggesting that the carbon source influences the autolysin activity. To further investigate the effect of galactose on autolysis, L. lactis strains MG1363 and IL1403 were grown in M17 medium supplemented with 0.5% glucose, 0.5% galactose, or a mixture of both sugars (0.25% of each sugar) for 48 h. The release of the intracellular enzyme PepX in the culture supernatant was taken as a marker for cellular lysis and was followed in time. Lower PepX activities were measured after 25 and 48 h in supernatants of cultures grown on galactose than in cultures grown on glucose (Fig. 3). When both sugars were added in equal amounts to the growth medium, intermediate levels of PepX activity were observed. PepX expression is not influenced by the carbon source since cell extracts of cells grown on glucose or galactose contained equal amounts of PepX activity (results not shown).

Similar amounts of AcmA were present in supernatants of cultures grown on glucose, galactose, or on the sugar combination, as was attested with a zymogram assay: no differences in clearing zones, resulting from AcmA activity, were observed between samples taken from cultures grown on glucose, galactose, or on the mixture of both sugars (results not shown). AcmA expression, therefore, is not influenced by the carbon source, and differences therein are thus not the cause of the observed reduced lysis of galactose-grown lactococcal cells.

Lactococcal cells grown on galactose are less susceptible to lysis by AcmA. To further examine whether growth on glucose or galactose influences AcmA-mediated cell lysis, L. lactis MG1363 acmAΔI and L. lactis IL1403 acmA::ISS1 were grown in the presence of glucose, after which the cells were collected. The cell pellets were resuspended in spent supernatants containing AcmA from L. lactis MG1363 cultures grown on glucose, galactose, or the sugar mixture. By using this approach, the influence of degradation of AcmA in the spent supernatant by proteinases that may be secreted from the mutant cells or of lysins possiblyexpressed from prophages in these cells (49) was excluded as the mutant cells do not lyse (L. lactis MG1363 acmAΔI) or lyse only very slightly (L. lactis IL1403 acmA::ISS1) (Fig. 2). Cellular lysis was determined by measuring the OD₆₀₀ decrease of the cell suspensions and by the PepX activity in the supernatants after 96 h of incubation at 30°C. Similar amounts of PepX were released in each case, independent of the supernatant used, confirming the zymographic data that similar amounts of AcmA are present in the supernatants of L. lactis MG1363 grown on glucose and/or galactose (Table 2). Subsequently, L. lactis MG1363 acmAΔI and L. lactis IL1403 acmA::ISS1 were grown on glucose, galactose, or a mixture of both sugars until stationary phase. Equal amounts of cells were collected and mixed with the supernatant of overnight cultures of L. lactis MG1363 grown on glucose, galactose, or the sugar mixture. L. lactis MG1363 acmAΔI and L. lactis IL1403 acmA::ISS1 grown on galactose released less PepX into the supernatant after incubation with AcmA than the strains grown on glucose, independent of the source of AcmA.
(Table 2; only the results of \(L. \text{lactis} \text{MG1363 acmA}\) are shown). Both mutant strains grown on the sugar mixture exhibited an intermediate extent of lysis. In conclusion, lactococcal cells grown on galactose are less susceptible to lysis by AcmA than lactococcal cells grown on glucose.

Cell separation is not affected by growth on galactose. AcmA is involved in cell separation of \(L. \text{lactis}\) (8; see also above). Microscopic analysis revealed that \(L. \text{lactis} \text{MG1363}\) and \(L. \text{lactis} \text{IL1403}\) grown on galactose form slightly longer chains (3 to 5 cells per chain) than when grown on glucose.

FIG. 2. Comparison of autolysis of \(L. \text{lactis}\) strains \(\text{MG1363 (•)}, \text{MG1363 acmAΔI (○)}, \text{IL1403 (■)}, \text{and IL1403 acmA::IS} \text{S1 (□)}\). Cells were grown in GM17 broth, and cellular lysis was subsequently followed by measuring PepX activity (in arbitrary units [AU]) (see the Materials and Methods section) released into the culture medium in time.

FIG. 3. Autolysis of \(L. \text{lactis}\) is carbon source dependent. \(L. \text{lactis} \text{MG1363 (A) and L. \text{lactis} IL1403 (B)}\) were grown in M17 medium containing (in wt/vol for each sugar) 0.5% glucose, 0.5% galactose, or a combination of 0.25% glucose plus 0.25% galactose, as indicated in the inset. Lysis was followed by measuring the PepX activity released into the culture supernatants in time. The outcome of a typical experiment is shown. Largely similar patterns were obtained in three independent experiments. AU, arbitrary units.
After prolonged incubation, however, the average chain length in all cultures is comparable (results not shown). Electron microscopy analysis revealed that cells of the acmA mutant of L. lactis MG1363 are connected via PG bridges with a density different from that of the cell wall itself (results not shown). These PG bridges seem to be hydrolyzed by AcmA during cell separation since separating wild-type L. lactis cells do not show this structure.

To study cell separation further, the two acmA mutant strains were grown on glucose or galactose, and when the cultures reached stationary phase, an AcmA-containing supernatant from an overnight culture of L. lactis MG1363 or L. lactis IL1403 was added to the L. lactis MG1363 acmAΔI or L. lactis IL1403 acmA::ISSI cells, respectively. The typical long chains of L. lactis MG1363 acmAΔI (8) and of L. lactis IL1403 acmA::ISSI, which are formed during growth on both glucose and galactose, were shortened up to the level of single cells after treatment with the AcmA-containing supernatant (Fig. 4). Thus, cell separation does not seem to be influenced by growth of L. lactis on galactose.

Binding of AcmA to cells grown on galactose is reduced. A deletion derivative of AcmA lacking the C-terminal PG-binding domain is not able to lyse L. lactis in vivo (44), suggesting that PG binding is important for activity of AcmA. To investigate whether binding of AcmA is involved in the decreased lysis of galactose-grown lactococcal cells, binding studies were performed with the C-terminal domain of AcmA. For this purpose, a fusion protein of the human malaria parasite Plasmodium falciparum antigen MSA2 (35) and the C-terminal PG-binding domain of AcmA of L. lactis MG136 was used. This protein, MSA2cA (44), was mixed with similar amounts (according to the OD600 of the cultures) of L. lactis MG1363 acmAΔI cells grown either on glucose or galactose. After in-

TABLE 2. Lysis of L. lactis MG1363 acmAΔI grown on glucose and/or galactose after incubation in L. lactis MG136 spent supernatants

<table>
<thead>
<tr>
<th>Carbon source ina:</th>
<th>Cells (MG1363 acmAΔI)</th>
<th>Supernatant (MG1363 culture)</th>
<th>% Lysisb</th>
<th>PepX release (AU)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Glucose</td>
<td>42 (0.2)</td>
<td>27 (0.2)</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>Galactose</td>
<td>42 (0.4)</td>
<td>30 (1.4)</td>
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</tr>
<tr>
<td>Glucose</td>
<td>Glucose-galactose</td>
<td>44 (1.7)</td>
<td>31 (2.2)</td>
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<td>Galactose</td>
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<td>24 (2.1)</td>
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<td>25 (2.9)</td>
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</tr>
<tr>
<td>Glucose-galactose</td>
<td>Glucose-galactose</td>
<td>32 (1.8)</td>
<td>27 (2.9)</td>
<td></td>
</tr>
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</table>

a Sugar concentrations (wt/vol) were as follows: glucose, 0.5%; galactose, 0.5%; glucose-galactose, 0.25% of each sugar.

b The percentage of lysis was calculated using the following formula: 100 × OD600 value at 96 h/maximum OD600 value. All samples were taken from two independent cultures, and each sample was measured twice to determine the OD600 and PepX activity. Standard deviations are given in parentheses. AU, arbitrary units.

FIG. 4. Growth of L. lactis on galactose does not affect cell separation. Typical pictures are shown for L. lactis MG1363 acmAΔI grown on 0.5% (wt/vol) glucose or 0.5% (wt/vol) galactose. Cells were mixed with the supernatant of an overnight culture of L. lactis MG1363 acmAΔI (−AcmA) or L. lactis MG1363 (+AcmA). The cells were incubated for 3 h and visualized using a Zeiss light microscope and a Zeiss digital camera. Magnification, ×1,000.
cubation for 5 min at room temperature, the cells were spun down, and the MSA2cA that fractionated with the cells was analyzed by Western hybridization using anti-MSA2 antibodies (35). As shown in Fig. 5A, less MSA2cA bound to cell walls grown on galactose than to cells grown on glucose. The same is true for cell walls isolated from these cells; cell walls from galactose-grown cells bind less MSA2cA (Fig. 5B). However, when the cell walls were first boiled in TCA, a treatment that increased binding of MSA2cA (44), the cell types bound equal amounts of MSA2cA (Fig. 5B).

The composition of the LTAs depends on the carbon source. In an earlier paper we have reported that LTAs might hinder the binding of AcmA to lactococcal cell walls (42, 44). The LTAs of _L. lactis_ strain SK110 were localized using fluorescent lectin and shown to be present at the sites where AcmA did not bind. The glycerolphosphates in LTA can be replaced with, e.g., alanine or galactose. To examine whether the carbon source during growth has an effect on the galactose or alanine substitutions in LTA, _L. lactis_ IL1403 was grown on glucose or galactose, its LTA was isolated, and the alanine and galactose substitutions were determined (Table 3). A lower percentage of the glycerolphosphates in LTA can be replaced with alanine than with galactose (11.8% ± 1.6%) than when they grow on glucose (46.0% ± 5.9% substitution rate). No significant differences in the percentages of glycerolphosphates replaced with alanines were observed between cells grown on glucose or galactose. Since only alanine and galactose substitutions in LTA were determined, it is possible that cells grown on galactose have increased amounts of other sugars substituted in their LTAs.

**DISCUSSION**

Lactococci express and secrete PG hydrolases with all kinds of functions in the cell wall and, thus, in growth and division of the cell. The lytic activity of PG hydrolases has to be controlled tightly since their activity is potentially lethal. Autolysin action is kept in check in various ways. In the case of the _L. lactis_ autolysin AcmA, proteolytic cleavage by the extracellular lactococcal proteinases PrtP and HtrA results in reduced activity and/or reduced wall binding (6, 8, 34, 42, 43). Also, the C-terminal LysM-containing domain of AcmA is involved in steering enzyme activity: the autolysin binds PG in the cell wall only at places where it is needed, i.e., around the poles of the cell (44). Here, we report that the carbon source of _L. lactis_ influences the binding of AcmA to PG: growth of both _L. lactis_ subspecies on galactose changes the carbohydrate composition or the amount of this component(s) in the cell wall such that binding of AcmA is decreased compared to growth of cells on glucose. The difference in binding of AcmA, effectuated by its cell wall-binding domain cA, is caused by a carbohydrate component on the cell wall that hinders binding but that is removable by TCA. It is not caused by a difference in the PG structure of cells grown on galactose.

The net result of these changes in AcmA binding is that cellular lysis is reduced. Decreased AcmA binding does not lead to diminished cell separation in stationary phase: cells grown on galactose still separate. Electron microscopy analysis revealed the presence of PG bridges between cells, which were present only in the _acmA_ mutant of _L. lactis_ MG1363 and are of a different density than the cell wall itself. The chemical composition of these PG bridges is apparently not influenced by growth on galactose, in contrast to the chemical composition of the cell wall itself.

From an earlier study it is known that LTAs are not present at the poles and septum of the lactococcal cells. As a consequence, AcmA is present only at the poles and septum of the cells (44). Growth on galactose could change the chemical composition of the lactococcal cell wall. _L. lactis_ uses the Leloir pathway to metabolize galactose (17, 36). Via the same Leloir pathway, UDP-glucose and UDP-galactose are formed from galactose and/or glucose. The UDP-sugars are, besides inter-

![FIG. 5. Western blot showing the effects of growth of _L. lactis_ on different carbon sources on the binding of AcmA via its C-terminal binding domain (cA). (A) MSA2cA binding to lactococcal cells grown on 0.5% (wt/vol) glucose or 0.5% (wt/vol) galactose. Equal amounts of lactococcal cells grown on glucose or galactose were mixed with equal amounts of MSA2cA protein, that is, the same amount of the supernatant of an _L. lactis_ culture secreting the MSA2cA protein, incubated at room temperature for 5 min, and pelleted. The pellets (cell-bound MSA2cA fraction) were analyzed by Western hybridization using anti-MSA2 antibodies. The binding experiments were performed in duplicate. d, degradation product of MSA2cA. (B) MSA2cA binding to cell walls isolated from cells grown on 0.5% (wt/vol) glucose or 0.5% (wt/vol) galactose. Equal amounts of cell walls were boiled in water (−TCA) or in 10% TCA (+TCA) and used for an MSA2cA binding assay as described in panel A. Only bound fractions are shown.](image-url)
mediates in the degradation of galactose to lactate, building blocks of cell wall sugars (e.g., in neutral polysaccharides and as substitutions in LTAs) (17). We presume that growth of *L. lactis* on galactose leads to differences in the amounts or ratios of UDP-glucose and UDP-galactose relative to growth on glucose. As a result, the amount or composition of the component that hinders the binding of AcmA to the PG, most likely LTA, may be changed such that less AcmA is able to bind. Since LTAs are not present at the septum and cell poles, cell separation is not influenced by growth on galactose. Interestingly, a long-chain phenotype was observed when the gene for UDP-glucose 4-epimerase (GalE) was interrupted in *L. lactis* MG1363 (17). Considering the above, this phenotype could be caused by reduced binding of AcmA to the galE mutant cells, which may contain LTAs with less galactose.

AcmA is a LysM domain-containing protein (8, 9). The LysM domain, in the C terminus of the protein (cA), constitutes a PG binding domain that has been used in a biotechnological application, namely, to bind antigens to certain gram-positive bacteria or cell walls for oral immunization purposes (5, 47). Hybrid proteins composed of this LysM-containing domain cA and an antigenic domain are produced and secreted from *L. lactis*. After isolation of the fusion proteins from the supernatant, they are loaded onto non-genetically modified gram-positive bacteria. The results from this study indicate that growth of the *L. lactis* production strain in medium containing galactose could enhance the yield of secreted fusion proteins.

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**REFERENCES**


