JOURNAL OF BACTERIOLOGY, Jan. 2005, p. 114–124 Vol. 187, No. 1
0021-9193/05/$08.00+0 doi:10.1128/JB.187.1.114–124.2005

Autolysis of *Lactococcus lactis* Is Increased upon D-Alanine Depletion of Peptidoglycan and Lipoteichoic Acids

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Received 12 May 2004/Accepted 27 September 2004

Mutations in the genes encoding enzymes responsible for the incorporation of D-Ala into the cell wall of *Lactococcus lactis* affect autolysis. An *L. lactis* alanine racemase (*alr*) mutant is strictly dependent on an external supply of D-Ala to be able to synthesize peptidoglycan and to incorporate D-Ala in the lipoteichoic acids (LTA). The mutant lyses rapidly when D-Ala is removed at mid-exponential growth. AcmA, the major lactococcal autolysin, is partially involved in the increased lysis since an *alr acmA* double mutant still lyses, albeit to a lesser extent. To investigate the role of D-Ala on LTA in the increased cell lysis, a *dltD* mutant of *L. lactis* was investigated, since this mutant is only affected in the D-alanylation of LTA and not the synthesis of peptidoglycan. Mutation of *dltD* results in increased lysis, showing that D-alanylation of LTA also influences autolysis. Since a *dltD acmA* double mutant does not lyse, the lysis of the *dltD* mutant is totally AcmA dependent. Zymographic analysis shows that no degradation of AcmA takes place in the *dltD* mutant, whereas AcmA is degraded by the extracellular protease HtrA in the wild-type strain. In *L. lactis*, LTA has been shown to be involved in controlled (directed) binding of AcmA. LTA lacking D-Ala has been reported in other bacterial species to have an improved capacity for autolysin binding. Mutation of *dltD* in *L. lactis*, however, does not affect peptidoglycan binding of AcmA; neither the amount of AcmA binding to the cells nor the binding to specific loci is altered. In conclusion, D-Ala depletion of the cell wall causes lysis by two distinct mechanisms. First, it results in an altered peptidoglycan that is more susceptible to lysis by AcmA and also by other factors, e.g., one or more of the other (putative) cell wall hydrolases expressed by *L. lactis*. Second, reduced amounts of D-Ala on LTA result in decreased degradation of AcmA by HtrA, which results in increased lytic activity.

AcmA, the major autolysin of *Lactococcus lactis* MG1363, is responsible for stationary phase cellular lysis and is involved in cell separation of this organism (9). The enzyme consists of two domains: the N-terminal region contains an N-acetyl-glucosaminidase active site domain (9; A. Steen, G. Buist, G. Horsburgh, S. J. Foster, O. P. Kuipers, and J. Kok, unpublished data) while the C-terminal region contains three so-called LysM domains, with which it specifically binds to peptidoglycan of *L. lactis* and of other gram-positive bacteria (49). Peptidoglycan, the major cell wall component in bacteria and the substrate of AcmA, consists of glycan strands cross-linked by peptide side chains. The peptide chain contains alternating L- and D-amino acids. D-Alanine (D-Ala) is incorporated into the peptidoglycan peptide moiety as a D-Ala–D-Ala dipeptide, where it is involved in cross-linking of adjacent peptidoglycan strands. In many bacteria alanine racemase is responsible for the synthesis of D-Ala from L-Ala, the naturally occurring alanine isomer (53). *Bacillus subtilis* expresses at least one alanine racemase: Dal (14). A *dal* mutant is dependent on D-Ala supplementation to be able to grow in a rich medium; cells start to lyse in the absence of D-Ala (4, 14, 20). In minimal medium the mutant is D-Ala dependent when L-Ala is supplemented, suggesting that a second, L-Ala-repressible racemase is present (4, 14). *Lactobacillus plantarum* probably expresses only one alanine racemase, as an *alr* mutant is totally dependent on D-Ala for growth (22). D-Ala deprivation of an *alr* mutant of *L. plantarum* resulted in growth arrest, a rapid loss of cell viability, and an aberrant cell morphology (43). Electron microscopy analyses showed that mainly the cell septum is affected in this mutant. Like *L. plantarum alr, L. lactis alr* is totally dependent on the addition of D-Ala to the growth medium (23); when D-Ala was removed from the growth medium when the cells were in exponential growth phase, *L. lactis alr* growth was impaired and the culture started to lyse (17). The *alr* gene was used as a food-grade plasmid selection marker in the *alr* mutants of *L. plantarum* and *L. lactis*, complementing the D-Ala auxotrophy (7). Moreover, the *alr* mutants of *L. plantarum* and *L. lactis* were used in a mucosal vaccination study, in which these two mutants were shown to enhance the mucosal delivery of the tetanus toxin fragment C model antigen in mice (17).

Although peptidoglycan covers the whole surface of *L. lactis*, AcmA binds to lactococcal cells at specific loci, namely around the poles and septum of the cell, exactly those places where cell lysis has been shown to start (35, 49). Trichloroacetic acid treatment of cells causes binding of AcmA over the whole cell surface. Lipoteichoic acid (LTA) is a candidate-hindering component that is removed by this treatment, as it seems to be present in *L. lactis* at those positions where AcmA is not able to bind (49). LTA is a secondary cell wall polymer suggested to be involved in the control of autolysin activity (5, 15), in determining the electrochemical properties of the cell wall (42), in establishing a magnesium ion concentration (2, 21, 26, 31),

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
L. lactis strains		
MG1363	Plasmid-free and prophage-cured derivative of NCDO712	16
NZ3900	MG1363 derivative, used as wild-type control (Δ*lacF pepN*::*nisR-nisK*)	12
PH3960	NZ3900 derivative carrying a deletion in *alr* (Δ*alr*)	This study
GB3960	PH3960 derivative carrying a deletion in *acmA* (Δ*alr acmA*Δ*1*)	This study
MG1363*acmA*Δ*1*	MG1363 derivative carrying a deletion in *acmA* (*acmA*Δ*1*)	9
MG1363 (*dltD*)	MG1363 derivative carrying an IS*1* insertion in *dltD* (*dltD*::IS*1*)	13
MG1363 (*dltD acmA*Δ*1*)	MG1363 (*dltD*) derivative carrying a deletion in *acmA* (*dltD*::IS*1 acmA*Δ*1*)	This study
NZ9000	MG1363 *pepN*::*nisRK*	30
NZ9700	Nisin producing transconjugant of NZ9000 containing the nisin-sucrose transposon Tn*5276*	29
Plasmids		
pINTAA	Integration plasmid used for the introduction of a 701-bp deletion in *acmA*; Em^r	9
pGIP011	PJDC9 derivative containing an internal fragment of *alr* from MG1363; Em^r	23
pGIP016	PGIP011 derivative containing an in frame deletion of *alr* from MG1363; Em^r	This study
pNG3041	Plasmid used for the nisin induced overexpression of MSA2cA; Cm^r	Laboratory collection

and in determining the physicochemical properties of the cytoplasmic membrane (18). LTA can be modified by various compounds, such as glycosyl residues (15) and D-Ala esters (1).

In gram-positive bacteria, the products of the *dlt* operon are involved in D-alanylation of LTA. The operon comprises four genes: *dltA*, encoding D-alanine–D-alanine carrier protein ligase (Dcl), the product of which is an activated D-Ala; *dltC*, specifying the D-alanyl-carrier protein (Dcp); *dltB*, which encodes a putative transmembrane protein involved in the secretion of the activated D-alanine; and *dltD*, encoding a protein that facilitates the binding of Dcp and Dcl for ligation with D-Ala and has thioesterase activity for mischarged D-alanyl-acyl carrier proteins (11, 28, 38). A link between D-alanylated LTA and autolysin activity has been reported earlier: autolysis of *B. subtilis dltA*, *dltB*, *dltC*, or *dltD* mutants was enhanced, and the bacteria were more susceptible to methicillin, which resulted in accelerated cell wall lysis, a faster loss of cell viability, and a slower recovery of the cells in the post-antibiotic phase (52). Furthermore, the absence of D-Ala in the LTA of a *dltB* as well as a *dltD* mutant of *B. subtilis* causes an increase in the net negative charge of the cell wall, resulting in an increase in the rate of posttranslational folding of some exported proteins (27). In the absence of D-alanylation, the yield of secreted recombinant anthrax protective antigen was increased 2.5-fold (50). *B. subtilis* cell growth, basic metabolism, cellular content of phosphorus-containing compounds, cell separation, and surface charge were not altered (52). Insertional mutagenesis in the *dlt* operon of *Staphylococcus aureus* resulted in methicillin resistance and an increased autolysis (36). In *L. lactis* subsp. *lactis* IL1403, the *dlt* operon comprises four genes: *dltA*, *dltB*, *dltC*, and *dltD* (6). An *L. lactis dltD* mutant was obtained by random insertion mutagenesis and screening for UV-sensitive mutants (13). Apart from its UV sensitivity, the *dltD* mutant was characterized by having a lower plasmid transfer rate during conjugation, and it was possible to make the mutant electrocompetent without the addition of glycine to the growth medium (13). Insertion mutagenesis of *dltA* of *L. lactis* resulted in secretion defects of the staphylococcal nuclease, which was used as a reporter for secretion. The secretion defect is most probably caused by an entrapment of the reporter protein in the cell wall, which could be the result of the interaction of the positively charged nuclease with the expected negatively charged cell wall of the *dltA* mutant (41).

In this paper we show that mutations in the *alr* and *dltD* genes of *L. lactis* differentially affect autolysis, and we investigate the role of the major autolysin AcmA therein.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown at 30°C in M17 broth (Difco Laboratories, Detroit, Mich.) or on twofold diluted M17 (1/2M17) medium containing 0.5% glucose (GM17 or G1/2M17, respectively). When appropriate D-alanine was added to reach a 2 mM end concentration. For plasmid selection in *L. lactis*, chloramphenicol or erythromycin (Sigma-Aldrich, St. Louis, Mo.) was added (each to a concentration of 5 μg/ml). For plasmid selection in *Escherichia coli*, erythromycin or ampicillin (Sigma-Aldrich) was used at a concentration of 100 μg/ml.

Chemicals and enzymes. All chemicals used were of analytical grade and, unless indicated otherwise, were obtained from Merck (Darmstadt, Germany). Enzymes for molecular biology were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and used according to the suppliers' instructions.

DNA manipulations and transformation. Molecular cloning techniques were performed essentially as described by Sambrook et al. (47). Electrotransformation of *E. coli* and *L. lactis* was performed by using a gene pulser (Bio-Rad Laboratories, Richmond, Calif.) as described by Zabarovsky and Winberg (54) and Leenhouts and Venema (32), respectively. Mini-preparations of plasmid DNA from *E. coli* and *L. lactis* were obtained by the alkaline lysis method as described by Sambrook et al. (47) and Seegers et al. (48), respectively. PCR products were purified by using a High Pure PCR purification kit (Roche Molecular Biochemicals).

Construction of an alanine racemase-deficient mutant and *acmA* knockouts in *L. lactis*. The suicide plasmid used to generate the *alr* disruption was constructed as follows. A 0.68-kb PCR product was amplified from MG1363 chromosomal DNA by using primers derived from the *L. lactis* IL1403 genome sequence (LLALR3, 5′-CCTGTCGAAAATATTTATAAAGCTG, and LLALR4, 5′-CG A*GGATCC*CCAAATTTCCGCATTAGGGTGAATATG [BamHI site in italics]). This DNA fragment was restricted with PstI and BamHI and cloned into the corresponding sites of the pBLUESCRIPT SK^+ (Stratagene GmbH, Heidelberg, Germany) plasmid. The DNA fragment was next recovered as a SalI-BamHI fragment and cloned into the corresponding sites of the suicide plasmid pGIP011 (described in reference 23) to generate plasmid pGIP016. The resulting plasmid contains an in-frame deletion of the *alr* gene (30 nucleotides), resulting in the removal of the pyridoxal-P binding site of the Alr enzyme. The stable *alr* mutant was constructed by two successive crossover events. First-step integrant candidates of strain NZ3900 (isogenic to MG1363) resulting from a single crossover recombination of the pGIP016 suicide vector at the *alr* locus were selected on GM17 plates containing both erythromycin and D-Ala. Three D-Ala auxotroph candidates were obtained and were grown in GM17 (plus D-Ala) without erythromycin during 120 generations in order to allow excision of the plasmid through intrachromosomal recombination. A total of 1,500 colonies (GM17 plus

D-Ala) were examined by replica plating on GM17 containing both D-Ala and erythromycin or on GM17 without D-Ala. Twenty-seven erythromycin-sensitive candidates were obtained, one of which was a D-Ala auxotroph. This mutant strain (named PH3960) was retained for detailed phenotypic analysis following further validation by PCR amplification and Southern blotting (data not shown).

The *acmA* deletion derivatives of *L. lactis* MG1363 (*dltD*) and *L. lactis* PH3960 (Δ*alr*) were obtained by replacement recombination with plasmid pINTAA, replacing *acmA* with the *acmAΔ1* gene which has an internal deletion, as described by Buist et al. (9).

Enzyme assays and optical density measurements. AcmA activity was visualized on G1/2M17 agar plates containing 0.2% autoclaved lyophilized *Micrococcus lysodeikticus* 4698 cells (Sigma-Aldrich) as halos around *L. lactis* colonies after overnight growth at 30°C.

X-prolyl dipeptidyl aminopeptidase (PepX) was measured as described by Buist et al. (10). Similar patterns were obtained in three independent experiments. Optical densities at 600 nm (OD_{600}) of cell cultures were measured in a Novaspec II spectrophotometer (Pharmacia, Uppsala, Sweden).

To study lysis caused by AcmA, 1 ml of an overnight culture of *L. lactis* MG1363*acmAΔ1* or *L. lactis* MG1363 (*dltD acmAΔ1*) was mixed with 1 ml of AcmA-containing supernatant of an overnight *L. lactis* MG1363 culture. After overnight incubation at 37°C, the amount of PepX released from the cells was measured as described above.

Lactate dehydrogenase activity in the supernatant was determined by using pyruvate as a substrate as previously reported (23). Aliquots (1 ml) of the culture were withdrawn at various time points, cells were removed by centrifugation at 12,000 × *g* for 5 min, and the supernatant was stored immediately at −20°C until enzyme assays were performed. One unit of activity corresponds to the oxidation of 1 μmol of NADH per min.

Total protein concentration was measured by the Bio-Rad protein assay method.

Triton X-100 induced lysis under nongrowing conditions. The procedure of Triton X-100-induced lysis was performed as described previously (46) with the following modifications. Cells were grown in GM17 for 16 h at 30°C, harvested by centrifugation (12,000 × *g* for 5 min at room temperature), washed three times with an equal volume of a potassium-sodium phosphate ($K\text{-}NaPO_4$) buffer (10 mM; pH 6.5) and resuspended in $K\text{-}NaPO_4$ buffer (200 mM; pH 6.5) containing 0.05% Triton X-100 (vol/vol). The cell suspension was incubated at 37°C under agitation, and autolysis was monitored by the decrease in OD_{600} in time.

SDS-PAGE, AcmA zymograms, and Western hybridization. *L. lactis* cell and supernatant samples were prepared as described previously (10). AcmA activity was detected by a zymogram staining technique by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5 or 17.5% polyacrylamide gels containing 0.15% autoclaved, lyophilized *M. lysodeikticus* ATCC 4698 cells as described before (9). The standard low-range and prestained low- and high-range SDS-PAGE molecular weight markers of Bio-Rad were used as references. Proteins were transferred from SDS–10% polyacrylamide gels to polyvinylidene difluoride membranes (Roche Molecular Biologicals) as described by Towbin et al. (51). MSA2 antigen was detected with 10,000-fold diluted rabbit polyclonal anti-MSA2 antiserum (45), and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Pharmacia) by using an ECL chemiluminescence detection system and protocol (Amersham, Piscataway, N.J.).

Binding of MSA2cA and AcmA to *L. lactis* cells. Binding of the MSA2cA fusion protein was studied by growing *L. lactis* NZ9000 (pNG3041) at 30°C until an OD_{600} of 0.4 was reached. *L. lactis* NZ9000 (pNG3041) was induced with nisin by adding a 1/1000 volume of the supernatant of the nisin producer *L. lactis* NZ9700 and incubated at 30°C for 2 h. The cells of 1 ml of *L. lactis* MG1363*acmAΔ1* or *L. lactis* MG1363 (*dltD acmAΔ1*) culture were resuspended in 1 ml of supernatant of the induced *L. lactis* NZ9000 (pNG3041) culture. The suspensions were incubated for 5 min at room temperature and spun down, after which the cells were washed with 1 ml of fresh 1/2M17 medium and resuspended in 100 μl of denaturation buffer (3). After boiling for 5 min, the samples were subjected to SDS–10% PAGE, followed by Western hybridization by using anti-MSA2 antibodies. The binding of AcmA to lactococcal cells was studied by mixing the cells of 1 ml of an overnight culture of *L. lactis* MG1363*acmAΔ1* or *L. lactis* MG1363 (*dltD acmAΔ1*) with 1 ml of AcmA-containing supernatant of an *L. lactis* MG1363 culture. The suspensions were incubated for 5 min at room temperature and centrifuged. The supernatant (0.5 ml) was mixed with 0.5 ml of an *M. lysodeikticus* suspension (0.2% in 100 mM potassium phosphate buffer, pH 5.4). The OD_{600} of the suspensions were measured at time zero and at 30 min. To determine the maximal amount of AcmA activity present in the MG1363 supernatant, 0.5 ml of *L. lactis* MG1363 supernatant was mixed with the *M. lysodeikticus* cells (control suspension). The ΔOD_{600} of the *M. lysodeikticus* suspensions with MG1363 supernatant preincubated with wild-type or mutant cells was calculated as a percentage of the ΔOD_{600} of the control suspension, resulting in the percentage of AcmA that did not bind to the cells. The unbound percentage was then subtracted from 100% to give the bound percentage.

Immunofluorescence microscopy, electron microscopy, and measurement of cell wall and septum thickness. Immunofluorescence studies to localize MSA2cA on the cell surface of *L. lactis* were performed as described before (49) by using anti-MSA2 antibodies and Oregon green-labeled secondary antibody (Molecular Probes, Oreg.).

Samples for transmission electron microscopy were prepared as follows. Cells (mid-exponential or stationary phase) were harvested by low-speed centrifugation (3,000 × *g* for 5 min). The cells were washed twice in sodium cacodylate buffer (200 mM; pH 7.3), prefixed in 2.5% (wt/vol) glutaraldehyde and fixed with 1% (wt/vol) OsO_4. The samples were embedded in Epon resin (Fluka Chemie, Buchs, Switzerland), and thin sections were prepared by using a Reichert-Jung Ultracut microtome (Milton Keynes, United Kingdom). The sections were stained with 4% (wt/vol) uranyl acetate and then with 0.4% (wt/vol) lead citrate and were examined with a JEOL JEE 1200 EXII electron microscope at 100 kV. Cell wall and septum thickness was measured by using the software program XL Docu version 3.0 (Soft Imaging System GmbH, Munster, Germany).

RESULTS

Mutation of *alr* in *L. lactis* increases autolysis. The stable isogenic *L. lactis* NZ3900 *alr* deletion mutant *L. lactis* PH3960 (Δ*alr*) is only able to grow when D-Ala is present in the growth medium (17, 23). When D-Ala is removed from the growth medium in exponential phase by washing the culture with fresh medium and resuspending the culture in medium without D-Ala, the growth of *L. lactis* PH3960 (Δ*alr*) is impaired and cell lysis starts (reference 17 and Fig. 1A). Depletion of D-Ala did not affect *L. lactis* NZ3900 with respect to growth and autolysis. *L. lactis* PH3960 (Δ*alr*) behaved like the control strain *L. lactis* NZ3900 in the presence of D-Ala (Fig. 1A).

When glycine is added to the growth medium, it will be incorporated in the peptidoglycan in place of D-Ala, affecting the peptidoglycan cross-linking and resulting in a destabilized cell wall (19). Indeed, when glycine was added to the growth medium, more lysis was observed than when no glycine was added, both for the wild-type *L. lactis* NZ3900 and for strain PH3960 (Δ*alr*) (Table 2). However, strain PH3960 (Δ*alr*) is more sensitive to glycine and lyses to a greater extent than strain NZ3900, even when grown in the presence of D-Ala (Table 2). When D-Ala is depleted, the addition of glycine (1 as well as 2%) increases the lysis of strain PH3960 (Δ*alr*); lysis of strain PH3960 (Δ*alr*) under these conditions was almost complete after 22 h, as evidenced by a very low OD_{600} (Table 2). To examine whether osmotic stabilizers could reduce lysis of *L. lactis* PH3960 (Δ*alr*), sodium chloride or sucrose was added at a concentration of 0.5 M during D-Ala starvation. An effect on lysis was observed, as the OD_{600} of the cultures grown in the presence of the osmotic stabilizers were higher than when no osmotic stabilizers were present (Fig. 1B). Protoplast formation in the presence of 0.5 M NaCl after D-Ala starvation was observed by using a light microscope (data not shown). The morphology of strain PH3960 (Δ*alr*) during D-Ala starvation was studied by using transmission electron microscopy; the septum and cell wall were thinner than those of the wild-type (Fig. 2, compare A and B), and occasionally holes were observed in the septal region (Fig. 2C).

The major autolysin AcmA of *L. lactis* MG1363 binds around the septal region of the cell (49). To study the possible involvement of AcmA in the lysis of *L. lactis* PH3960 (Δ*alr*), an isogenic *acmA alr* double mutant was constructed. In the absence of D-Ala, *L. lactis* GB3960 (Δ*alr acmAΔ1*) still lyses and

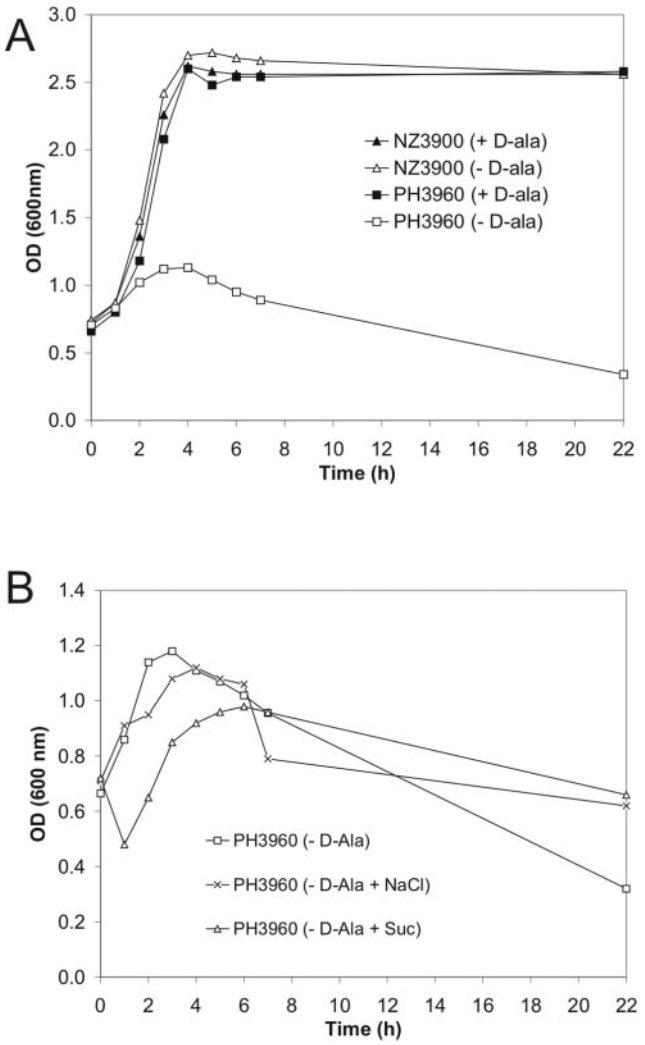

FIG. 1. Effect of D-Ala depletion on growth and lysis of *L. lactis* PH3960. (A) Growth and lysis, as measured by following the OD_{600}, of *L. lactis* strains NZ3900 (control) and PH3960 (Δ*alr*) in the presence and absence of D-Ala. The strains were grown in GM17 supplemented with 2 mM D-Ala until an OD_{600} of 0.6 was reached. Subsequently, the cultures were split: one half was grown in GM17 with 2 mM D-Ala while the other half was grown without D-Ala. (B) Growth and lysis of PH3960 (Δ*alr*) after D-Ala depletion and in the presence of 0.5 M sucrose or 0.5 M NaCl. Similar results were obtained in independent experiments.

releases intracellular proteins into its medium, albeit to a much lesser extent than strain PH3960 (Δ*alr*) (Fig. 3). After 20 h of D-Ala starvation, the OD_{600} of strain GB3960 (Δ*alr acmA*Δ*1*) was approximately two times higher (Fig. 3) than that of strain PH3960 (Δ*alr*). Total protein release from strain GB3960 (Δ*alr acmA*Δ*1*) is two times lower than that from strain PH3960 (Δ*alr*) upon D-Ala starvation (Fig. 3). In an *acmA*Δ*1* mutant of *L. lactis* MG1363, the isogenic parent of all strains used in this study, neither lysis nor a decrease in the OD_{600} value was observed (Fig. 4A) (9).

The thickness of the septum and cell wall (excluding septum) of the mutant strains was measured. Global cell wall thickness of strains PH3960 (Δ*alr*) and GB3960 (Δ*alr acmA*Δ*1*) are not affected by the absence of D-Ala. The septum of strain PH3960 (Δ*alr*) is a bit thinner than that of strain NZ3900. Strain GB3960 (Δ*alr acmA*Δ*1*) has a thinner septum than strain PH3960 (Δ*alr*), which is an effect of the absence of AcmA (Table 3 and see below).

A mutation in *dltD* affects growth and autolysis of *L. lactis*. A mutation in *alr* affects both peptidoglycan synthesis and LTA decoration with D-Ala, while a mutation in *dltD* only affects decoration of LTA with D-Ala (37). In *L. lactis* MG1363 the organization of *dltA*, *dltB*, *dltC*, and *dltD* and their surrounding genes is identical to that in *L. lactis* IL1403. *L. lactis* MG1363 *dltD* was obtained by insertion of IS*S1* in the *dltD* gene (13), and this mutant was used to investigate the effect of changes in LTA on AcmA activity. Growth and lysis of *L. lactis* MG1363 (*dltD*) were followed in time (Fig. 4A). *L. lactis* MG1363 (*dltD*) grows more slowly and lyses to a greater extent than strain MG1363, releasing more intracellular protein in the culture supernatant when stationary phase is reached, as attested in the supernatant by an increase in activity of the cytoplasmic enzyme lactate dehydrogenase (Fig. 4A).

As the mutation in *dltD* clearly affects autolysis, the role of AcmA in this phenomenon was investigated by constructing an isogenic *dltD acmA* double mutant. Growth and lysis of *L. lactis* strains MG1363*acmA*Δ*1* and MG1363 (*dltD acmA*Δ*1*) are similar, and no proteins are released (Fig. 4A), which is a clear indication that AcmA is responsible for the increased lysis of MG1363 (*dltD*). *L. lactis* MG1363 (*dltD*) is more sensitive to incubation with Triton X-100 (a lysis inducer) than *L. lactis* MG1363 (*dltD acmA*Δ*1*) (Fig. 4B). The OD_{600} of *L. lactis* MG1363 (*dltD*) drops dramatically in the first 30 min of incubation compared to the value in the control strain *L. lactis* NZ3900, and more proteins are released into the supernatant. Only limited lysis is observed for strains MG1363 (*dltD acmA*Δ*1*) and MG1363*acmA*Δ*1*, with small amounts of proteins being released under these circumstances.

The mutation of *dltD* does not affect septum and cell wall thickness (Table 3) and cell shape (data not shown). By contrast, the septa of *L. lactis* MG1363*acmA*Δ*1* and *L. lactis* MG1363 (*dltD acmA*Δ*1*) are thinner than that of *L. lactis* NZ3900. Apparently, AcmA is involved in the synthesis of the septum.

AcmA binding to *dltD* mutant cells is not affected. AcmA has to bind to the peptidoglycan of the lactococcal cell wall to be able to lyse the cell (49). It is an enzyme with a high pI of approximately 10 and is, therefore, positively charged at the pH value of the medium (pH 6.8). During growth of *L. lactis*

TABLE 2. Effect of glycine on lysis of *L. lactis* NZ3900 (control) and *L. lactis* PH3960 (Δ*alr*) in the presence or absence of D-Ala[a]

Strain	% Glycine added	% of maximum OD_{600}	
		With D-Ala	Without D-Ala
NZ3900	0	98	94
NZ3900	1	94	97
NZ3900	2	89	84
PH3960 (Δ*alr*)	0	99	30
PH3960 (Δ*alr*)	1	83	5.7
PH3960 (Δ*alr*)	2	60	5.9

[a] Strains were grown until an OD_{600} of 0.5 was reached, after which the cultures were split in two, centrifuged, washed once with an equal volume of fresh medium, and resuspended in fresh medium. D-Ala (2 mM) and/or glycine (1 or 2%) were added and growth was followed during 22 h.

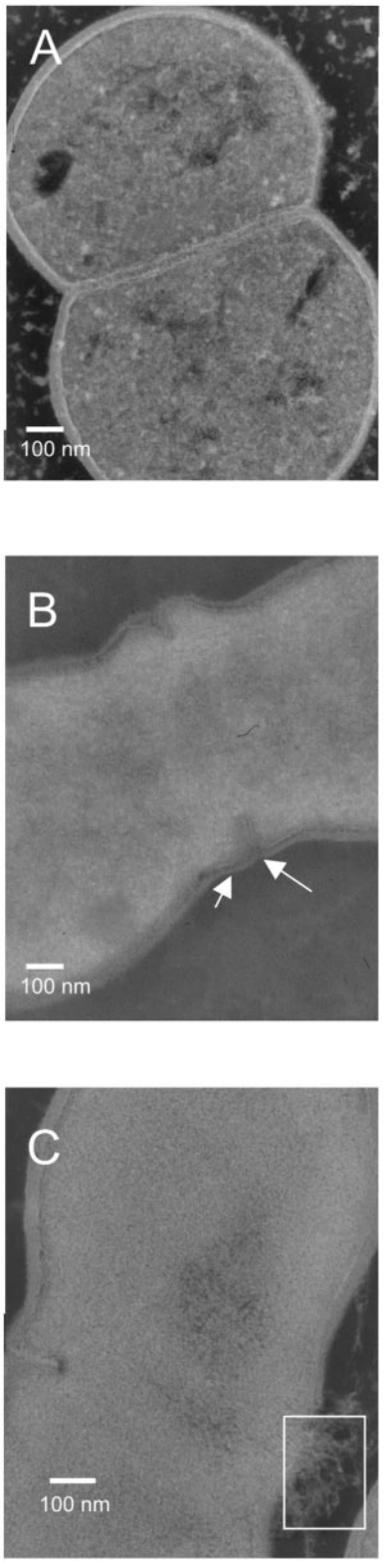

FIG. 2. Effect of D-Ala depletion on the cell integrity. (A) Electron microscopy image of *L. lactis* NZ3900 in stationary phase. (B) Electron microscopy image of *L. lactis* PH3960 (Δ*alr*) after 5 h of D-Ala depletion. Arrows show the thinner cell wall around the septum. (C) Electron microscopy image of *L. lactis* PH3960 (Δ*alr*) after 2 h of D-Ala depletion. The white rectangle shows where release of cytoplasmic material occurs.

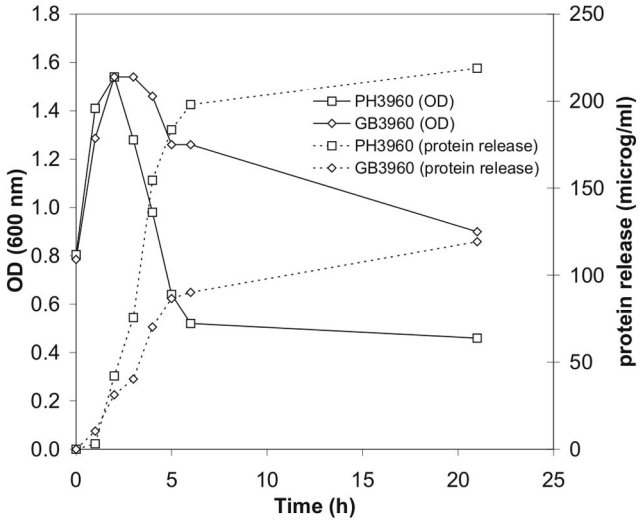

FIG. 3. Growth (OD_{600}, solid lines) and release of cytoplasmic proteins (dashed lines) by *L. lactis* PH3960 (Δ*alr*) and *L. lactis* GB3960 (Δ*alr acmA*Δ*1*). The two strains were grown in GM17 medium with 2 mM D-Ala until exponential phase, after which D-Ala was removed from the growth medium by centrifugation and cells were resuspended in fresh GM17 broth without D-Ala.

the pH value drops due to the production of lactate. *L. lactis* MG1363 (*dltD*) contains a fivefold reduced amount of D-Ala on its LTA compared to MG1363 (D-Ala to GroP ratio of 5.8% in *L. lactis* MG1363 [*dltD*], compared to D-Ala to GroP ratio of 28.5% in *L. lactis* MG1363; N. Kramer, S. Morath, T. Hartung, E. J. Smid, E. Breukink, J. Kok, and O. P. Kuipers, unpublished data).

The LTA of D-Ala-depleted *L. lactis* MG1363 (*dltD*) is expected to have an increased net negative charge, which could possibly allow this mutant to bind, via electrostatic interactions, more AcmA at the pH value of the medium than the wild-type strain. To investigate whether an increase in the binding of AcmA could explain the increase in lysis of *L. lactis* MG1363 (*dltD*), equal amounts of exponential phase cells of *L. lactis* MG1363*acmA*Δ*1* and *L. lactis* MG1363 (*dltD acmA*Δ*1*) were mixed with an AcmA-containing supernatant of an *L. lactis* MG1363 culture. After mixing, the cell-AcmA suspensions were centrifuged and the supernatants, containing nonbound AcmA, were mixed with *M. lysodeikticus* autoclaved cells. The resulting decrease in the OD_{600} value was taken as a measure of the amount of AcmA present in the supernatants with the original AcmA-containing supernatant set at 100%. Equal amounts of AcmA bound to the cells of *L. lactis* MG1363 (*dltD acmA*Δ*1*) and *L. lactis* MG1363*acmA*Δ*1* (Table 4), and lysis of both strains, as measured by the release of the intracellular marker enzyme PepX, was the same (data not shown).

AcmA binds with its C-terminal domain (cA) to specific loci on the lactococcal cell surface, namely, around the septum and at the poles of the cell (49). To examine whether a mutation in *dltD* affects the localization of AcmA and, consequently, influences its activity, immunofluorescence studies were performed. The MSA2cA protein, which is a fusion of the reporter protein MSA2 of the malaria parasite *Plasmodium falciparum* and the cA domain of AcmA (49), bound to the same loci on the cell surface of strains MG1363 (*dltD acmA*Δ*1*) and

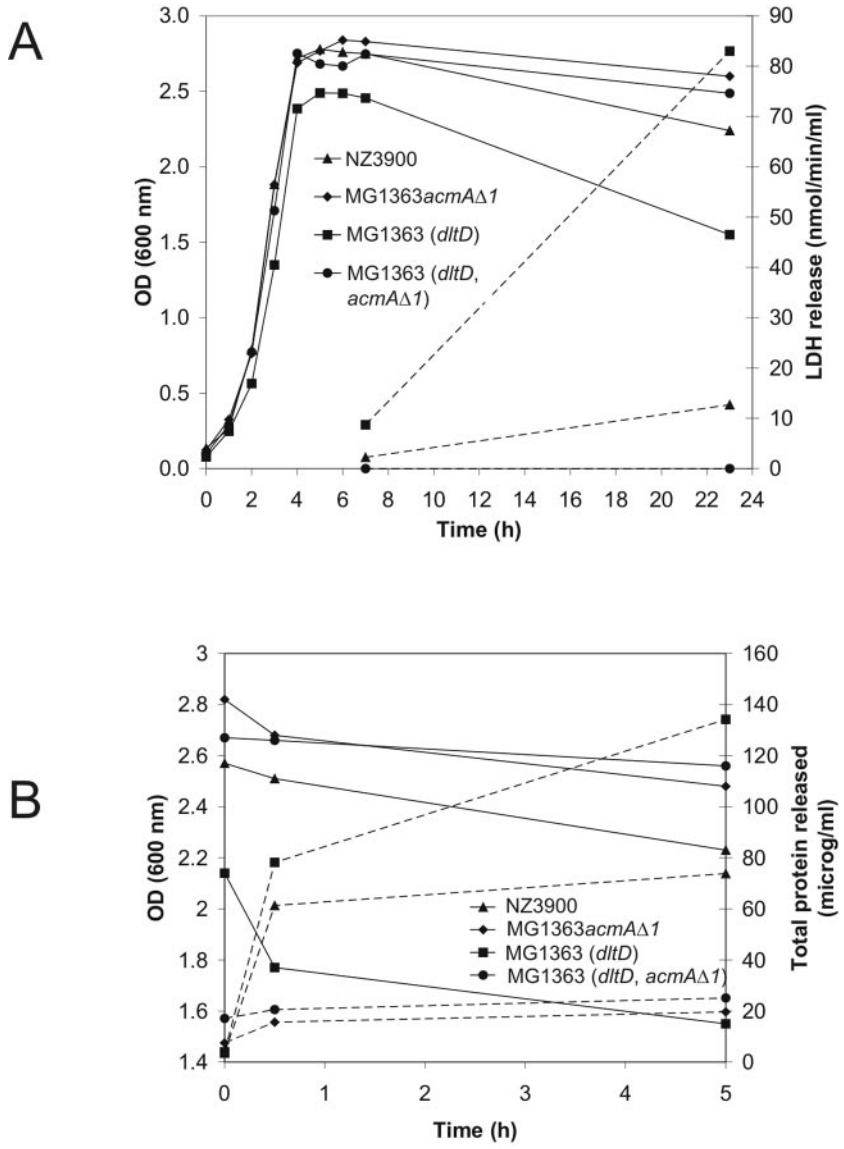

FIG. 4. (A) Effect of AcmA on growth and lysis of *L. lactis* NZ3900, MG1363*acmAΔ1*, MG1363 (*dltD*), and MG1363 (*dltD acmAΔ1*). The OD_{600} of GM17 cultures of *L. lactis* was followed for 24 h (solid lines). Lactate dehydrogenase release in culture supernatant of the same strains was measured at time (t) = 7 and t = 22 h (dashed lines). The data are from a single experiment that was representative of two independent analyses in which similar results were obtained. (B) Effect of Triton X-100 on lysis of cell suspensions of *L. lactis* NZ3900 (control), MG1363*acmAΔ1*, MG1363 (*dltD*), and MG1363 (*dltD acmAΔ1*). Cells were harvested, washed, resuspended in phosphate buffer supplemented with 0.05% Triton X-100, and incubated at 37°C. The OD_{600} of the cell suspensions was followed for 5 h (solid lines). Total protein release in the supernatant of the cell suspensions was measured at t = 0, t = 0.5, and t = 5 h (dashed lines). The data are from a single experiment that was representative of two independent analyses in which similar results were obtained.

MG1363*acmAΔ1* (Fig. 5), indicating that the distribution of AcmA over the cell surface is not altered by the inactivation of *dltD*. Western hybridization analysis with MSA2-specific antibodies showed that equal amounts of MSA2cA were bound to cells of *L. lactis* MG1363*acmAΔ1* and *L. lactis* MG1363 (*dltD acmAΔ1*) (Fig. 5A).

A *dltD* mutation reduces the breakdown of AcmA. From studies in *B. subtilis*, it is known that D-alanylation of LTA affects the stability and breakdown of several heterologous proteins (27). To examine whether the stability of AcmA of *L. lactis* is affected by a mutation in *dltD*, supernatants of stationary phase cultures of *L. lactis* strains MG1363 and MG1363 (*dltD*) were analyzed on a zymogram containing *M. lysodeikticus* cell wall fragments (Fig. 6). Next to mature AcmA, several smaller bands of activity of breakdown products of AcmA were observed in the supernatant of *L. lactis* MG1363, as shown previously (9). In the supernatant of *L. lactis* MG1363 (*dltD*) only full-size AcmA was detectable.

DISCUSSION

D-Ala is essential for *L. lactis*. An *L. lactis alr* mutant, which does not synthesize D-Ala from L-Ala, starts to lyse upon D-Ala depletion. This mutant is expected to be defective in cross-

TABLE 3. Cell wall and septum thickness of the strains used in this study[a]

Strain	Thickness (nm)	
	Cell wall	Septum
MG1363	39.9 ± 8.5	51.9 ± 3.8
MG1363*acmAΔ1*	34.8 ± 11.6	33.2 ± 4.5
MG1363 (*dltD*)	29.4 ± 3.9	55.4 ± 3.0
MG1363 (*dltD acmAΔ1*)	29.0 ± 2.0	37.8 ± 3.1
PH3960 (Δ*alr*)	32.6 ± 8.9	43.8 ± 2.9
GB3960 (Δ*alr acmAΔ1*)	33.9 ± 6.5	33.3 ± 0.8

[a] Measurements on *L. lactis* NZ3900 (control), *L. lactis* MG1363*acmAΔ1*, *L. lactis* MG1363 (*dltD*), and *L. lactis* MG1363 (*dltD acmAΔ1*) were performed on exponentially growing cells. *L. lactis* PH3960 (Δ*alr*) and *L. lactis* GB3960 (Δ*alr acmAΔ1*) were depleted for D-Ala for 5 h before measurements were performed. For measurement the standard deviations are given.

TABLE 4. Quantification of AcmA-binding to *L. lactis* MG1363*acmAΔ1* and *L. lactis* MG1363 (*dltD acmAΔ1*)[a]

Cells added	OD_{600} reduction	Bound AcmA activity (%)
No cells	0.053	0
MG1363*acmAΔ1*	0.032	40
MG1363 (*dltD acmAΔ1*)	0.031	42

[a] The percentage of AcmA activity from 1-ml supernatant samples that bound to cells from 1-ml culture samples was determined as described in Materials and Methods. Samples containing AcmA were incubated with *M. lysodeikticus* cells for 30 min. Similar results were obtained in independent experiments.

linking of the glycan strands, which is necessary for the rigid structure of peptidoglycan, since D-Ala in the disaccharide pentapeptide precursor of peptidoglycan plays an important role in the cross-linking process. In addition, D-alanylation of the LTA in this mutant will be affected since this process also depends on the formation of D-Ala from L-Ala. In an analysis of the lysis of the *alr* mutant, we show that cell lysis increases when glycine is added to the growth medium, even in the presence of D-Ala, showing that glycine and D-Ala compete; it has been shown that during the biosynthesis of the peptidoglycan precursor (disaccharide pentapeptide), glycine can replace D-Ala in, e.g., *L. plantarum* and *Staphylococcus aureus* (19). While *L. lactis* Alr^+ is able to synthesize D-Ala from L-Ala, the *L. lactis alr* mutant depends on the D-Ala that is added to the culture growth medium. When the D-Ala concentration becomes limiting for the *alr* mutant, the glycine incorporation into the peptidoglycan will increase. The *alr* mutant of *L. lactis* is therefore more sensitive to the addition of glycine than *L. lactis* Alr^+.

Analysis of *L. lactis* cell morphology by electron microscopy confirms that mutation of *alr* affects cell wall synthesis; cell wall defects are observed, mainly in the septal region of the cell.

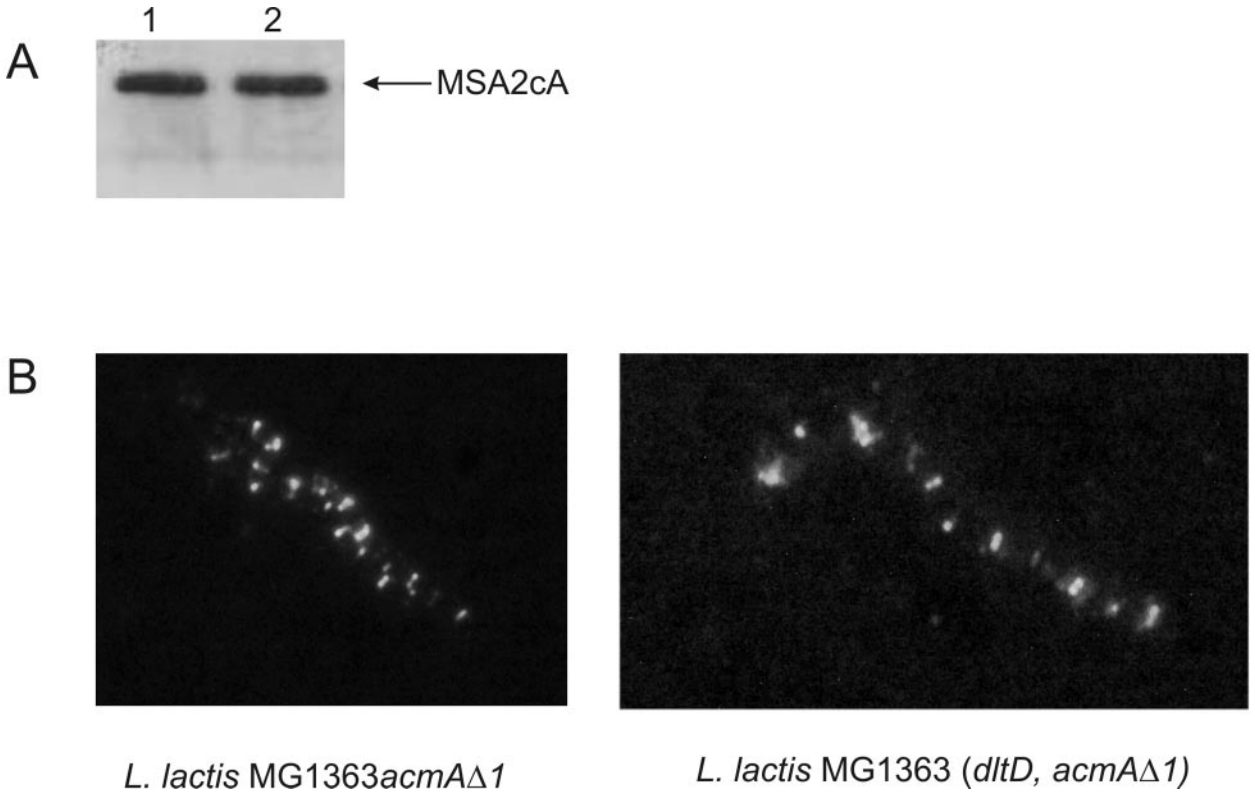

FIG. 5. (A) MSA2cA binding to cells. Equal amounts of cells of overnight cultures of *L. lactis* strains MG1363*acmAΔ1* and MG1363 (*dltD acmAΔ1*) were mixed with MSA2cA, a fusion protein consisting of the *Plasmodium falciparum* protein MSA2 and the C-terminal cell wall binding domain of AcmA. The suspensions were incubated for 5 min and centrifuged. The cells were washed once and cell-bound protein was visualized by Western hybridization with anti-MSA2 antibodies. Lane 1, MSA2cA bound to *L. lactis* MG1363 (*dltD acmAΔ1*); lane 2, MSA2cA bound to *L. lactis* MG1363*acmAΔ1*. (B) Localization of MSA2cA on the cell surface of *L. lactis*. MSA2cA was visualized on cells of *L. lactis* strains MG1363*acmAΔ1* and MG1363 (*dltD acmAΔ1*) by immunofluorescence microscopy by using anti-MSA2 antibodies and a fluorescently labeled secondary antibody.

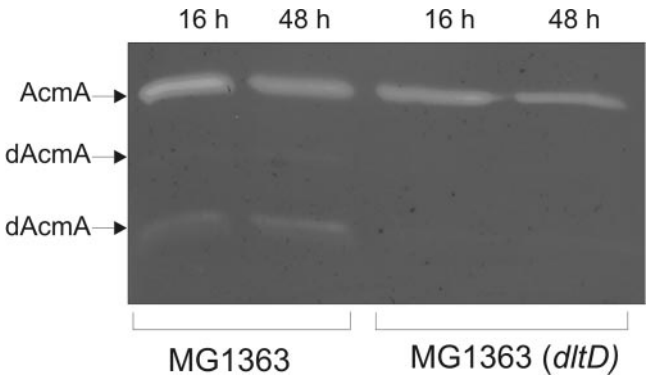

FIG. 6. Zymographic analysis of AcmA activity in the supernatant of *L. lactis* MG1363 and *L. lactis* MG1363 (*dltD*) after culturing for 16 h or 48 h in G1/2M17 at 30°C. dAcmA, breakdown products of AcmA generated by the protease HtrA.

The major autolysin, AcmA, is involved in cell separation and has been shown to bind and act in this region (49). We show here that, indeed, AcmA is involved in the increased lysis of *L. lactis* (*alr*). To our knowledge, this is the first time that an autolysin is shown to be directly involved in the lysis of mutants defective in peptidoglycan precursor biosynthesis.

The lysis observed in the *alr* mutant is not fully AcmA mediated. The *L. lactis alr acmA* double mutant still lyses, albeit to a lesser extent than *L. lactis alr*. Other factors, e.g., the activity of one or more other cell wall hydrolases, could be involved. Indeed, genes for three homologues of AcmA (AcmB, AcmC, and AcmD) and a putative lytic endopeptidase (YjgB) are present in the genome of *L. lactis* (6), and recently activity of these peptidoglycan hydrolases has been shown in vitro (24). AcmB has autolytic activity, since an *acmB* mutant of MG1363 lyses to a lesser extent than its parent (25). Lytic activity of AcmB, however, depends on the presence of AcmA as MG1363 (*acmAΔ1 acmB*) lyses to the same extent as MG1363*acmAΔ1* (25). AcmB is, therefore, not expected to be involved in the lysis of *L. lactis* GB3960 (Δ*alr*, *acmAΔ1*) observed in this study.

Since mutating *alr* will not only affect peptidoglycan synthesis but also the D-alanylation of LTA, we also investigated whether a reduction of the D-alanyl substitution level of LTA could stimulate autolysis. A *dltD* mutant of *L. lactis* was examined with respect to lysis behavior, since a mutation in the *dlt* operon is expected to affect only the D-alanylation of LTA and not the peptidoglycan cross-linking (37).

D-Ala substitution in LTA is strongly reduced in the *dltD* mutant of *L. lactis*; a fivefold reduced amount of D-Ala in its LTA compared to levels in the wild-type *L. lactis* MG1363 is observed (D-Ala to GroP ratio of 5.8% for the *dltD* mutant compared to D-Ala to GroP ratio of 28.5% for the wild-type strain; N. Kramer et. al., unpublished data). We show here that this strong reduction in D-Ala substitution in LTA results in increased lysis of *L. lactis*. A slower growth rate of the mutant is also observed. The same phenomena have been observed in *B. subtilis* (52). A *dltD acmA* double mutant of *L. lactis* does not lyse more, and growth is completely restored; the increased lysis and the effect on growth of *L. lactis* MG1363 (*dltD*) are, therefore, dependent on the presence of AcmA. LTA lacking D-Ala substitutions is expected to be more negatively charged. Fischer et al. (15) have reported in *S. aureus* that acidified LTA, due to a lack of D-Ala, has an improved capacity for binding of the *S. aureus* autolysin. The possibility that the increased lysis of *L. lactis* (*dltD*) was the result of increased binding of AcmA to the cell wall was investigated, since autolysins, including Atl of *S. aureus* and AcmA, are positively charged enzymes. Mutation of *dltD* in *L. lactis*, however, did not influence the binding of AcmA; similar amounts of AcmA bound to cells of *L. lactis* MG1363*acmAΔ1* and *L. lactis* MG1363 (*dltD acmAΔ1*), and the binding was at the same loci in both strains. Also lysis of both strains upon the external addition of AcmA was identical, indicating that the lysis activity of AcmA remained unchanged and is not influenced by D-alanylation of LTA. In a previous study (49) it has been shown that LTA is likely to be involved in hindering of AcmA binding to cells. Since D-Ala in LTA has no effect on AcmA binding, the present study supports the conclusion that D-Ala in LTA is not involved in hindering AcmA binding.

Although the quantity and location of AcmA binding are not affected in *L. lactis* (*dltD*), the strain lyses to a higher extent than its parent. The thickness of the cell wall and septum is not affected by the *dltD* mutation, precluding the possibility that the lysis increase of *L. lactis* (*dltD*) is a consequence of a thinner cell wall. On the other hand, a direct correlation between the mutation of AcmA and a thinner septum was observed, indicating that AcmA is important for the development of a normal septum.

The cell wall of *L. lactis* MG1363 (*dltD*) does seem to be weaker than that of the wild-type strain. When lysis is induced with Triton X-100, *L. lactis* MG1363 (*dltD*) lyses to a greater extent than its parent. Triton X-100-induced lysis of *L. lactis* MG1363 (*dltD acmAΔ1*) and *L. lactis* MG1363*acmAΔ1* is of the same low level. Incubation of stationary phase cells of *L. lactis* MG1363*acmAΔ1* or *L. lactis* MG1363 (*dltD acmAΔ1*) with AcmA does not result in increased lysis of the double mutant, which indicates that AcmA, only when expressed in *L. lactis* MG1363 (*dltD*) during growth, results in a weaker cell wall.

D-Alanylation of LTA has been shown previously to be an important factor in the stability of secreted proteins. A *dlt* mutant of *B. subtilis* showed an increase in the rate of posttranslational folding of exported proteins, especially those proteins that are susceptible to proteolytic degradation (27). In the absence of D-alanylation, the yield of secreted recombinant anthrax protective antigen was increased 2.5-fold (50). The protease that is involved in this phenomenon has not been identified. Here we show that in *L. lactis* a mutation in the *dltD* gene also stabilizes a secreted protein, i.e., AcmA. Zymographic analysis of AcmA activity in supernatants of *L. lactis* MG1363 and its *dltD* mutant revealed that the HtrA-mediated breakdown of AcmA (44), which can be seen in MG1363, is not detectable in the mutant.

L. lactis HtrA cleaves AcmA in the C-terminal peptidoglycan-binding domain (8, 44). The AcmA breakdown products are still active (Fig. 6) but will bind less strongly to the lactococcal cell wall, and, as a consequence, they have a lower lytic ability (A. Steen et al., unpublished data, and reference 8). HtrA degradation of AcmA could be a way for the cell to modulate the activity of this potentially lethal enzyme. This control mechanism on AcmA activity is absent in *L. lactis*

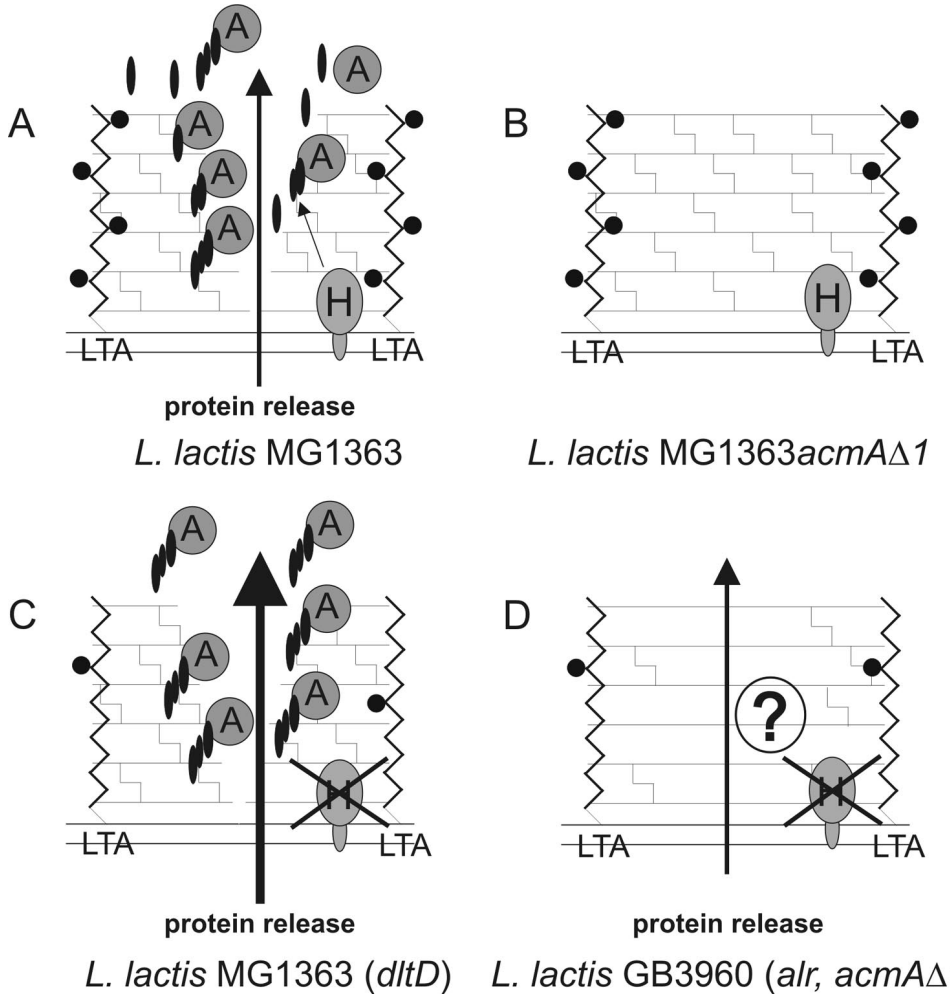

FIG. 7. Schematic representation of the main conclusions of this paper. (A) In *L. lactis* MG1363 (and, for that matter, *L. lactis* NZ3900) AcmA hydrolyses peptidoglycan, resulting in cell lysis and subsequent protein release. AcmA is degraded by HtrA in the C-terminal domain which contains the LysM motifs (small black ovals). AcmA degradation results in a less active enzyme. (B) Since no AcmA is present in the *acmA* mutant of *L. lactis* MG1363, no lysis and protein release are observed for this strain. (C) In the *dltD* mutant of *L. lactis*, the reduction of D-alanylation of LTA results in a strong reduction of degradation of AcmA by HtrA. As a result, increased cellular lysis and protein release are observed. When AcmA is deleted in this *dltD* mutant, no lysis is observed (not shown in this figure, but compare with panel B). (D) An *alr acmA* double mutant, however, lyses, although AcmA is not present in this strain. The lysis of this strain is caused by an unknown factor. The high cellular lysis of the *alr* single mutant is most likely a combination of the reduced degradation of AcmA by HtrA, as shown in panel C and the AcmA-independent lysis as shown in panel D. A, AcmA; H, HtrA.

(*dltD*) and could result in the weakening of the peptidoglycan sacculus by the hydrolytic action of AcmA, resulting in increased lysis.

The mutation in *dltD* could influence the stability of AcmA in two ways. First, HtrA activity could be decreased by the altered physicochemical properties of the cell wall. Second, since folded proteins are expected to be less prone to degradation by HtrA, the folding of AcmA could be faster, due to the proposed increase of cation concentration in the cell envelope (26), a factor known to influence the folding rate of secreted proteins (27, 50). The exact mechanism in the case of AcmA is unknown.

Interestingly, *B. subtilis* expresses autolysins which contain peptidoglycan binding domains homologous to that of AcmA (33, 34), as well as homologues of HtrA (39, 40). It would be interesting to investigate the role of HtrA in the stability of autolysins and other secreted proteins in *dlt* mutants of *B. subtilis*, as it is known that mutation in the *dlt* operon increases cell lysis in *B. subtilis* (52).

In conclusion, whereas in the *dltD* mutant the increased lysis is caused by an effect of reduced D-alanylation levels of LTA on HtrA activity, cell lysis of the *alr* mutant of *L. lactis* is likely a combined effect of a defective peptidoglycan synthesis and a reduced D-alanylation level of LTA (Fig. 7). Since a *dltD acmA* double mutant of *L. lactis* does not lyse, D-alanylation of LTA only affects cell lysis when AcmA is expressed; D-alanylation of LTA is therefore not involved in the observed lysis of the *alr acmA* double mutant. The lysis of this double mutant is most likely caused by the defective peptidoglycan synthesis, which makes the cell wall more sensitive to other factors, including, e.g., one or more of the (putative) cell wall hydrolases other than AcmA.

ACKNOWLEDGMENTS

This research has been carried out with financial support from the Commission of the European Communities, specific RTD projects LABVAC (BIO4-CT96-0542), LABDEL (QLK3-2000-00340) and FAIR (CT98-4396). The content of this article does not necessarily reflect the Commission's views and in no way anticipates the its future policy in this area.

E.P. holds a doctoral fellowship from FRIA. P.H. holds a scientific collaborator fellowship from FNRS.

We are grateful to K. Schanck for skillful help in the construction and characterization of the stable *alr* mutant. We thank E. Maguin for providing strain MG1362 (*dltD*) and P. Renault for sequence information on the *alr* gene from *L. lactis* IL1403 preceding publication. We thank Naomi Kramer, Siegfried Morath, Thomas Hartung, Eddy J. Smid, Eefjan Breukink, Jan Kok, and Oscar P. Kuipers for sharing the results on the D-Ala determination prior to publication.

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