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Haandrikman, Alfred J.; Kok, Jan; Venema, Gerard

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Lactococcal Proteinase Maturation Protein PrtM Is a Lipoprotein

ALFRED J. HAANDRIKMAN,* JAN KOK, AND GERARD VENEMA

Department of Genetics, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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The production of enzymatically active proteinase by lactococci requires the joint presence of a proteinase gene, *prtP*, and a gene encoding a maturation protein, *prtM*. A 32-kDa protein produced by *Escherichia coli* upon expression of the *prtM* gene under the direction of the T7 RNA polymerase promoter was purified and used to obtain PrtM-specific antibodies. With these antibodies, immunogold labeling of lactococcal cells revealed that PrtM was associated with the lactococcal cell envelope. Western blot (immunoblot) analysis of whole lactococcal cells and isolated membrane vesicles indicated that PrtM was a membrane-associated protein. Radiolabeling of *Lactococcus lactis* with [^{3}H]palmitic acid showed that PrtM was a lipoprotein. Partial secretion of PrtM into the culture medium was observed after Cys-24, the target residue for lipid modification, was replaced by an Ala residue by means of site-directed mutagenesis. This mutation did not affect proteinase activity.

Lactococcus spp. are of considerable economic importance, because they are widely used in the production of cheese and other fermented milk products. In milk, these organisms critically depend on a proteolytic system for the breakdown of milk casein, because the concentration of free amino acids is too low to sustain fast growth (27). The key enzyme in the proteolytic system of lactococci is the cell envelope-associated serine proteinase. In all lactococcal strains examined so far, the gene encoding this proteinase has been located on a plasmid (8). The nucleotide sequences of the proteinase genes from *Lactococcus lactis* subsp. *cremoris* Wg2 (10) and SK11 (35) and *Lactococcus lactis* subsp. *lactis* NCDO763 have been determined (7). Although the proteinases produced by these strains differ considerably in both caseinolytic activities and immunological properties, the amino acid sequences deduced from the proteinase gene nucleotide sequences show surprisingly few differences (for a recent review, see reference 8).

The proteinase gene *prtP* encodes a serine proteinase of 1,902 amino acids in strains Wg2 and NCDO763 and one of 1,962 amino acids in strain SK11. All three enzymes are synthesized as pre-pro-proteins (7, 12, 35). The amino acid sequences of the lactococcal proteinases show considerable sequence similarity with the active-site regions of subtilisins, the serine proteinases produced by bacilli (10). The most striking difference between the proteinases from lactococci and bacilli concerns a long C-terminal extension present in the lactococcal proteinase. Deletion of up to 343 amino acids from the C-terminal part of the Wg2 proteinase did not affect the activity or the specificity of the truncated proteinase (9). Because of the presence of a C-terminal membrane anchor, C-terminal deletions removing this anchor result in the complete secretion of the proteinase (5, 7, 36).

On the proteinase plasmids of all three strains, an oppositely transcribed gene, *prtM*, is present directly upstream of the *prtP* gene. Both *prtP* and *prtM* are transcribed from regulatory sequences on a 0.32-kb DNA fragment located between the two genes. The *prtM* gene encodes a protein of 299 amino acids with an N-terminal sequence typical of a procaryotic prolipoprotein. The gene was overexpressed in *Escherichia coli* by use of the T7 RNA polymerase promoter and appeared to specify a protein of 31 to 32 kDa (5, 36). Deletion studies showed that the presence of both *prtP* and *prtM* is essential for proteolytic activity. In the absence of *prtM*, an inactive form of the proteinase was produced and subsequently translocated across the cytoplasmic membrane. The size of the secreted form of the lactococcal proteinase produced in the absence of *prtM* coincided with that of the inactive secreted form of the proteinase carrying an Asp-32→Asn-32 active-site mutation. Both inactive proteinases were larger than the active enzyme produced in the presence of PrtM. These findings led to the hypothesis that *prtM* effects the autoproteolytic removal of the N-terminal pro-sequence of the proteinase precursor, resulting in a fully active proteinase (5, 6, 36).

Here we report on the purification of a 32-kDa protein isolated from *E. coli* upon overexpression of the *prtM* gene and the isolation of PrtM-specific antisera. With these antisera, PrtM was identified as a lipoprotein associated with the lactococcal cell membrane.

MATERIALS AND METHODS

Plasmids, bacterial strains, and media. The bacterial strains and plasmids used in this study are listed in Table 1. A schematic representation of the different plasmids used in this study and their construction is shown in Fig. 1. *L. lactis* strains were grown in M17 broth (26) supplemented with 0.5% (wt/vol) glucose or in whey-based medium containing 5% (wt/vol) whey permeate, 2% (wt/vol) β-glycerophosphate, 0.5% (wt/vol) glucose, and 0.1% (wt/vol) Casitone (36). Erythromycin and chloramphenicol were used at a concentration of 5 μg/ml. *E. coli* strains were grown in TY broth (22). Ampicillin and chloramphenicol were added to concentrations of 100 and 25 μg/ml, respectively.

Molecular cloning and site-directed mutagenesis. Restriction enzymes were purchased from Boehringer GmbH (Mannheim, Germany) and used in accordance with the supplier's recommendations. General molecular cloning techniques were carried out as described by Maniatis et al. (17). *E. coli* competent cells were transformed as described before (16). DNA was transferred to *L. lactis* MG1363 by means of electrotransformation with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described by Leenhouts et al. (15). To change Cys-24 of PrtM to an Ala residue, we

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Source or reference
Strains		
E. coli		
BL21(DE3)	F⁻ *ompT* r_B^- m_B^- *int*; bacteriophage DE3 lysogen carrying the T7 RNA polymerase gene controlled by the *lacUV5* promoter	24
WK6	Δ(*lac-proAB*) *galE strA*/F′ *lacI*^{q} *ZΔM15 proAB*	38
WK6 *mutS*	Δ (*lac-proAB*) *galE strA mutS*::Tn*10*/F′ *lacI*^{q} *ZΔM15 proAB*	38
L. lactis subsp. *lactis*		
MG1363	Prt⁻; plasmid-free derivative of *L. lactis* subsp. *lactis* NCDO712	4
L. lactis subsp. *cremoris*		
Wg2 (Prt⁺)	Prt⁺; wild-type strain	20
Wg2 (Prt⁻)	Prt⁻ because of the loss of the 27-kb proteinase plasmid pWV05	20
Plasmids		
pT712	Amp^r	25
pSKH4	Amp^r; pT712 carrying the *prtM* gene	5
pMc5-8	Amp^s Cm^r	23
pMa5-8	Amp^r Cm^s	23
pSKH1	Amp^r; pUC19 carrying the *prtM* gene	5
pSKH6	Amp^s Cm^r; pMc5-8 carrying the *prtM* gene	This work
pSKH7	Amp^r Cm^s; pMa5-8 carrying the *prtM* gene with a Cys-24 → Ala-24 substitution	This work
pGKV2	Em^r Cm^r	31
pGKV500	Em^r; pGKV2 derivative containing *prtP* (lacking 130 3′ codons) and *prtM* (lacking 3 3′ codons)	11
pGKV507	Em^r; containing *prtP* (lacking 130 3′ codons)	9
pGKV552	Em^r; containing *prtM* (lacking 3 3′ codons) and the complete *prtP* gene	34a
pGKV1552	Em^r; pGKV552 specifying a proteinase carrying (6) an Asp-32 → Asn-32 active-site mutation	
pGKV550	Em^r; containing the complete *prtP* gene	5
pGKV573	Em^r; containing the complete *prtM* and *prtP* genes	This work
pGKV573C	Em^r Cm^r; pGKV573 with the Cm^r gene of pC194 inserted in *prtP*	This work
pGKV571	Em^r; pGKV573 with a Cys-24 → Ala-24 substitution in *prtM*	This work
pGKV571C	Em^r Cm^r; pGKV571 with the Cm^r gene of pC194 inserted in *prtP*	This work
pGKV574	Em^r; pGKV573 carrying a frameshift mutation in *prtM*	This work

inserted a 1-kb *Hin*dII-*Bgl*II fragment from pSKH1 (5) carrying the complete *prtM* gene into phasmid vector pMc5-8 (23) digested with *Bam*HI and *Sma*I. Single-stranded DNA of the resulting plasmid, pSKH6 (Fig. 1), was used to form a gapped duplex with denatured DNA from pMa5-8 (23) digested with *Bam*HI and *Eco*RI. Using this gapped duplex and a synthetic 33-mer (GGTCTGATTTGACTGTGCACCGCTTAGCAGCAG; nucleotide substitutions are underlined), we performed site-directed mutagenesis as described by Stanssens et al. (23) (Fig. 1, step 1). The resulting plasmid, pSKH7, carried an additional *Sno*I site because of the exchange of a TGT (Cys) codon for a GCA (Ala) codon at position 24 (see Fig. 7). *E. coli* WK6 and *E. coli* WK6 *mutS* (38) were used as host strains for pSKH6 and pSKH7. The incomplete *prtM* gene on pGKV552 lacks three 3′ codons and was reconstituted by exchanging the 1,260-bp *Cla*I-*Xba*I fragment from pGKV552 and the 975-bp *Cla*I-*Xba*I fragment from pSKH6, resulting in plasmid pGKV573 (Fig. 1, step 2). Similarly, the complete *prtM* gene carrying the Cys-24→Ala-24 mutation was cloned as a *Cla*I-*Xba*I fragment from pSKH7 in pGKV552, resulting in plasmid pGKV571 (Fig. 1, step 3). The unique *Sno*I site on pGKV571 was filled in with the Klenow fragment of DNA polymerase I (Fig. 1, step 4). The resulting plasmid, pGKV574, carried a frameshift mutation in the 24th codon of *prtM* (Fig. 1; see also Fig. 7). Plasmids pGKV571C and pGKV573C were constructed by inserting a 1.2-kb *Bam*HI fragment carrying the pC194 chloramphenicol resistance gene into the unique *Bam*HI site of pGKV571 and pGKV573, respectively. *L. lactis* MG1363 was used as a host for vectors carrying the proteinase gene *prtP*.

SDS-PAGE, Western blotting (immunoblotting), and immunodetection. Protein samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (14) with a Bio-Rad Protean II minigel system and stained with Coomassie brilliant blue. Proteins were transferred to BA85 nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) essentially as described by Towbin et al. (29). Antigens were detected with 5,000-fold-diluted PrtM-directed antisera and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Promega, Madison, Wis.) in accordance with the manufacturer's instructions.

Analytical methods. Protein concentrations were determined by the method of Bradford (1). Bovine serum albumin was used as a standard.

Lactate dehydrogenase activity was determined as described by Thomas et al. (28). The rate of NADH oxidation was monitored by measuring the decrease in the optical density at 450 nm with a Philips PU8720 UV-visible light scanning spectrophotometer after the addition of 25 or 50 μl of sample to 1,150 μl of 50 mM triethanolamine hydrochlo-

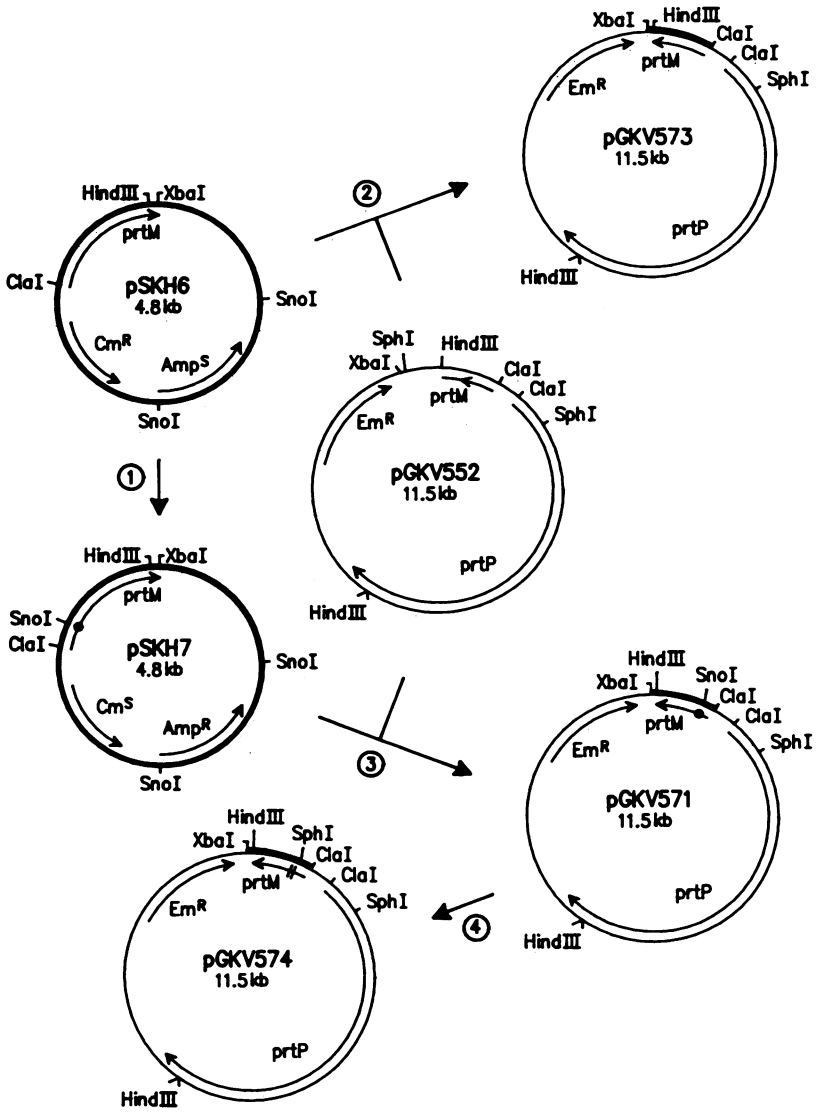

FIG. 1. Schematic representation of the construction of the plasmids used. Only restriction sites relevant for the construction are shown. The Cys-24→Ala-24 substitution in PrtM is indicated by a small open circle in pSKH7 and pGKV571. The position of the frameshift mutation in *prtM* in pGKV574 is indicated by double lines. For details, see the text.

ride (pH 6.9) containing 0.4 mM NADH, 10 mM sodium pyruvate, and 4 mM fructose 1,6-diphosphate.

Proteinase activities in overnight cultures grown in milk were determined by measuring the amount of hydrolyzed milk proteins with the *o*-phthalaldehyde (OPA) spectrophotometric assay (2). To 1.1 ml of 0.68 N trichloroacetic acid in a microcentrifuge tube was added 0.5 ml of an overnight culture. The mixture was left at room temperature for 10 min and centrifuged for 5 min. Fifteen microliters of the supernatant was mixed with 250 μl of OPA reagent in a well of a microtiter plate (Bioreba, Basel, Switzerland), and after 2 min the A_{340} was determined with a Titertek Multiskan MCC/340 P apparatus (Flow Laboratories, Rickmansworth, United Kingdom). OPA reagent consisted of 1 ml of OPA (40 mg/ml in methanol; Sigma Chemical Co., St. Louis, Mo.) added to 50 ml of 50 mM sodium tetraborate containing 1% (wt/vol) SDS and 0.2% (vol/vol) 2-mercaptoethanol.

PrtM purification. *E. coli* BL21(DE3)(pSKH4) was grown in 20 ml of TY broth to an optical density at 600 nm of 0.4. Isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 0.4 mM, and after incubation for 1 h at 37°C rifampin was added to a final concentration of 200 μg/ml. Following an additional 1 h of incubation at 37°C, cells were harvested by centrifugation (10 min, 20°C, 4,000 × *g*), resuspended in 3 ml of loading buffer (60 mM Tris-hydrochloride [pH 6.8], 2% [wt/vol] SDS, 5% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue, 2% [vol/vol] β-mercaptoethanol), and heated to 100°C for 5 min. Samples (0.8 ml) were subjected to 12.5% SDS-PAGE (gel, 3 by 150 by 250 mm). Prestained Rainbow marker (Amersham International, Buckinghamshire, United Kingdom) was used as a molecular weight standard. The band containing PrtM was excised from the gel, and PrtM was isolated from the polyacrylamide by electroelution in 25 mM Tris-hydrochloride (pH 8.4)–200 mM glycine–0.1% (wt/vol) SDS with a Bio-Trap (Schleicher & Schuell) in accordance with the manufacturer's instructions. The resulting protein isolate was dialyzed against several changes of 10 mM $NaHPO_4$ (pH 6.5)

at 10°C. From 50 ml of *E. coli* culture, approximately 1 mg of PrtM was isolated.

Isolation of antibodies. Two specific-pathogen-free New Zealand rabbits were injected subcutaneously with 1 ml of isolated PrtM in complete Freund adjuvant (approximately 100 μg/ml). After 2 and 3 weeks, intraperitonal booster injections were given. After 4 and 5 weeks, intramuscular injections of PrtM in incomplete Freund adjuvant were given. One week after the final injection, sera were collected and stored at −70°C.

Localization of PrtM. Whey-based medium-grown *L. lactis* cells were centrifuged in a microcentrifuge tube, resuspended in 1/10 of the original volume of loading buffer, and heated for 5 to 10 min at 100°C. Culture supernatants were collected, dialyzed against several changes of distilled water at 10°C, and lyophilized. The dry residue from 2 ml of culture supernatant was dissolved in 75 μl of loading buffer and heated for 5 min at 100°C. Samples of *L. lactis* cells corresponding to 100 μl of culture and culture supernatant corresponding to 175 μl of culture were subjected to 12.5% SDS-PAGE and Western blot analysis.

L. lactis membrane vesicles were isolated by the method of Otto et al. (21). For the analysis of total cell proteins, lactococcal cells were disrupted with glass beads by use of the Shake it, Baby cell disrupter (Biospec Products, Bartlesville, Okla.) essentially as described by van de Guchte (30).

Immunogold labeling. Cells of *L. lactis* subsp. *lactis* MG1363 carrying plasmid pGKV552 or pGKV2 were fixed in 3% glutaraldehyde (vol/vol) in 0.1 M cacodylate buffer (pH 7.2) and embedded in Lowicryl K4M. Immunogold labeling of PrtM was performed on ultrathin sections with PrtM-specific antisera and protein A-gold particles (32).

Radiolabeling of *L. lactis* with [^{3}H]palmitic acid. *L. lactis* MG1363 carrying pGKV571, pGKV1552, pGKV573, or pGKV574 was grown in whey-based medium to an optical density at 600 nm of 0.3. To 1 ml of culture was added 50 μCi of [9,10-*n*-^{3}H]palmitic acid (40 to 60 Ci/mmol; Amersham). Cells were grown for an additional 2 h at 30°C, corresponding to approximately two generations. Trichloroacetic acid was added to the culture to a final concentration of 10% (wt/vol), after which the cells were left on ice for 30 min. Cells were harvested by centrifugation, and cell pellets were rinsed with 100 μl of ice-cold acetone and then with 500 μl of lysis buffer (10 mM Tris-hydrochloride [pH 8.0], 25 mM $MgCl_2$, 200 mM NaCl) and resuspended in 100 μl of lysis buffer containing 5 mg of lysozyme (E. Merck AG, Darmstadt, Germany) and 15 U of mutanolysin (Sigma) per ml. After incubation at 37°C for 15 min and at 90°C for 2 min, 10 μl of 10% (wt/vol) SDS was added and the suspension was incubated for an additional 10 min at 90°C to complete lysis. Immunoprecipitation with PrtM-specific antibodies and protein A-Sepharose (Pharmacia, Uppsala, Sweden) was performed by the method of Edens et al. (3). Samples were subjected to 12.5% SDS-PAGE. Gels were incubated for 30 min in an isopropanol-water-acetic acid (25:65:10) mixture and then for 20 min in Amplify (Amersham), dried, and exposed at −80°C to preflashed Kodak X-Omat AR film for fluorography.

RESULTS

Isolation of PrtM. In a previous paper, we showed that *E. coli* BL21(DE3), carrying the *L. lactis* Wg2 *prtM* gene placed under the transcriptional control of the T7 RNA polymerase promoter on plasmid pSKH4, synthesized considerable amounts of a protein of approximately 32 kDa (Fig. 2) (5).

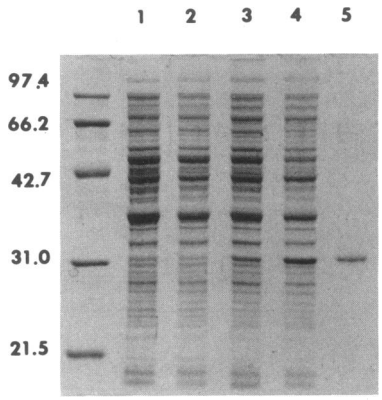

FIG. 2. Expression of *prtM* in *E. coli* BL21(DE3) and purification of PrtM. Samples of *E. coli* cells carrying either pT712 (lanes 1 and 2) or pSKH4 (lanes 3 and 4), both uninduced (lanes 1 and 3) and induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (lanes 2 and 4), were subjected to 12.5% SDS-PAGE. Lane 5 contains approximately 1 μg of purified PrtM protein. Standard molecular weight markers were phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (42,699), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400). Molecular weights (in thousands) of the proteins visible in the gel are shown on the left.

Using preparative SDS-PAGE and electroelution of the 32-kDa protein from the polyacrylamide, we were able to isolate approximately 1 mg of this protein from 50 ml of *E. coli* culture. When 1 μg of the isolated protein was subjected to analytical SDS-PAGE, a major band of 32 kDa and some minor bands in the 50- to 60-kDa region were present (Fig. 2).

Rabbits were immunized with the purified protein isolate to raise antibodies. The resulting antisera were used in Western blot analysis of lactococcal cells either with or without the *prtM* gene to ascertain that the protein isolated from *E. coli* was indeed PrtM. For this purpose, cells from 75-μl overnight cultures in whey-based medium were analyzed. Only in *L. lactis* MG1363 carrying the *prtM* gene (as on plasmid pGKV552) and in wild-type, proteinase-producing *L. lactis* Wg2 did a 32-kDa protein react with the antisera (Fig. 3, lanes 2 and 4). Minor protein bands of approximately

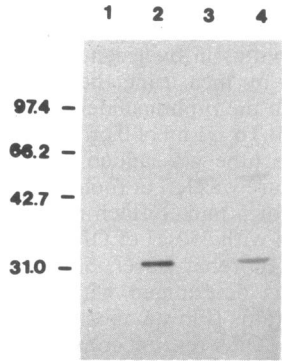

FIG. 3. Western blot analysis with PrtM-directed antibodies of whole cells of lactococcal strains. Lanes: 1, MG1363 carrying plasmid pGKV2; 2, MG1363 carrying plasmid pGKV552; 3, Wg2 (Prt^-); 4, Wg2 (Prt^+). Standard molecular weight markers were as in Fig. 2. Molecular weights (in thousands) are shown on the left.

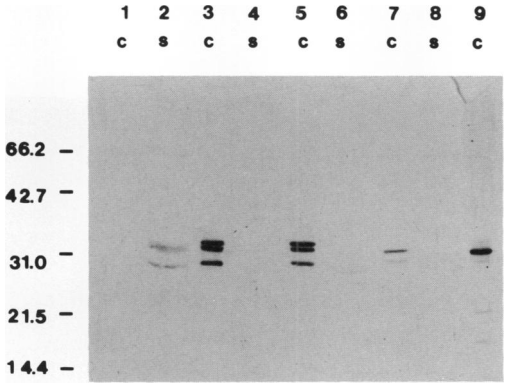

FIG. 4. Western blot analysis with PrtM-directed antibodies of whole cells (c) equivalent to 100 μl of culture and culture supernatant (s) equivalent to 175 μl of culture from *L. lactis* MG1363 with various plasmids. Lanes: 1, pGKV574; 2 and 3, pGKV571C; 4 and 5, pGKV571; 6 and 7, pGKV573C; 8 and 9, pGKV573. Standard molecular weight markers were as in Fig. 2. Molecular weights (in thousands) are shown on the left.

50 kDa were present in both active proteinase-producing strain Wg2 and its proteinase-negative, PrtM-deficient variant. Therefore, these bands were due to nonspecific binding. From these results, we concluded that the antibodies were specific for PrtM.

Cell association of PrtM. To determine whether PrtM is a secreted protein or a cell-associated protein, we analyzed both lactococcal cells and culture supernatants in Western blots. *L. lactis* MG1363 carrying the cloned *prtM* gene on plasmid pGKV573 was grown in whey-based medium. Samples of cells equivalent to 100 μl of culture and freeze-dried culture medium equivalent to 175 μl of culture were subjected to Western blot analysis. As shown in Fig. 4 (lanes 8 and 9), PrtM remained associated with the cells. PrtM could not be detected in the culture medium. Similar results were obtained when wild-type *L. lactis* Wg2 and *L. lactis* MG1363(pGKV500), which produces a fully secreted form of the active proteinase because of a deletion of the C-terminal membrane anchor, were analyzed (results not shown). These results indicated that PrtM is mainly cell associated and that the location of PrtM is independent of the location of the proteinase. As a consequence of the insertion of a Cm^r gene in the proteinase structural gene, no active proteinase was produced by *L. lactis* MG1363 carrying plasmid pGKV573C. Apart from the expected 32-kDa PrtM protein, a 29-kDa form of PrtM was produced and partly secreted into the culture medium by this strain (Fig. 4, lanes 6 and 7).

The subcellular location of PrtM was examined by immunogold labeling. For this purpose, ultrathin sections of whole lactococcal cells carrying the *prtM* gene and grown in whey-based medium were labeled with PrtM-specific antisera and protein A-gold particles (Fig. 5). In cells carrying the *prtM* gene, the gold particles were mainly located in the cell periphery. In cells not carrying the *prtM* gene, gold particles were scarce and randomly distributed throughout the preparation. From these results, we concluded that PrtM is associated with the cell envelope.

Membrane association of PrtM. A further indication of the subcellular location of PrtM was obtained by comparing total cell proteins with the proteins present in isolated membrane vesicles from *L. lactis* Wg2. As a measure of the purity of the isolated membrane vesicles, we assayed the presence of the cytoplasmic enzyme lactate dehydrogenase (28). Whereas lactate dehydrogenase activities of up to 56 and 49 U/mg of protein were measured in samples of total cell proteins from the Prt^+ and Prt^- variants of strain Wg2, less than 0.9 and 0.8 U/mg of protein, respectively, were associated with isolated membrane vesicles from both strains. Samples containing approximately 2 μg of protein of either total cell proteins or isolated membrane vesicles from the Prt^+ and Prt^- strains were subjected to Western blot analysis. As shown in Fig. 6, PrtM was coisolated with the membrane vesicles. These results indicated that PrtM is a membrane-associated protein.

PrtM is a lipoprotein. The deduced amino acid sequence of *prtM* suggested that PrtM is a lipoprotein and, consequently, should be associated with the cell membrane as the result of a lipid modification. Replacement of the codon for residue Cys-24, the most likely target residue for lipid modification, by an Ala residue should prevent lipid modification of the PrtM precursor (Fig. 7). To test this hypothesis, we attempted to label both PrtM and a form of PrtM in which the Cys-24 residue was replaced by an Ala residue with [^{3}H]palmitic acid. *L. lactis* MG1363 carrying pGKV571, pGKV1552, pGKV573, or pGKV574 was grown on whey-based medium in the presence of [^{3}H]palmitic acid for about two generations. Cells were lysed and subjected to immunoprecipitation with protein A-Sepharose and PrtM-specific antisera. As shown in Fig. 8 (lanes 2 and 3), a radiolabeled protein of 31.5 kDa was present when the cells carried *prtM* on plasmid pGKV573 or pGKV1552, indicating that PrtM indeed is a lipoprotein. As expected, labeling of PrtM was independent of the presence of active proteinase (Fig. 8, lane 2). No labeling with [^{3}H]palmitic acid was observed after the introduction of a frameshift mutation in *prtM*, as in plasmid pGKV574 (Fig. 8, lane 4). Also, no labeling with [^{3}H]palmitic acid was observed upon replacement of Cys-24 by an Ala residue, as in plasmid pGKV571 (Fig. 8, lane 1). These results indicated that PrtM residue Cys-24 is the target residue for lipid modification.

Effect of a Cys-24→Ala-24 mutation in PrtM on proteinase activity. Further elucidation of the PrtM-proteinase interaction might benefit from the availability of secreted forms of both the proteinase and PrtM. For this reason, we investigated whether preventing lipid modification of the PrtM precursor would result in a secreted form of PrtM. Replacement of the Cys-24 target residue for lipid modification by an Ala residue might result in the secretion of PrtM, as it leads to the introduction of a potential signal peptidase cleavage site. As shown in Table 2, lactococcal cells harboring pGKV552, pGKV573, or pGKV571 produced an active proteinase, enabling the strains to grow to a high cell density in milk. Apparently, the presence of the Cys-24→Ala-24 substitution in *prtM* did not affect the activity of the proteinase. The frameshift mutation in the 24th codon of the *prtM* gene present on pGKV574 resulted in the loss of proteolytic activity (Table 2).

Cells and culture medium of *L. lactis* MG1363 carrying the mutated *prtM* gene on plasmid pGKV571 were subjected to Western blot analysis. The results (Fig. 4) showed that three forms of PrtM of 29, 32, and 34 kDa were associated with the lactococcal cells (Fig. 4, lane 5). Only very small amounts of the 29- and 32-kDa forms of PrtM were present in the culture supernatant. Upon inactivation of the proteinase by insertion of a Cm^r gene in the coding sequence of *prtP*, as in pGKV571C, the amounts of the secreted forms of PrtM increased significantly (Fig. 4, lanes 2 and 4). These results suggested that preventing lipid modification of PrtM by the

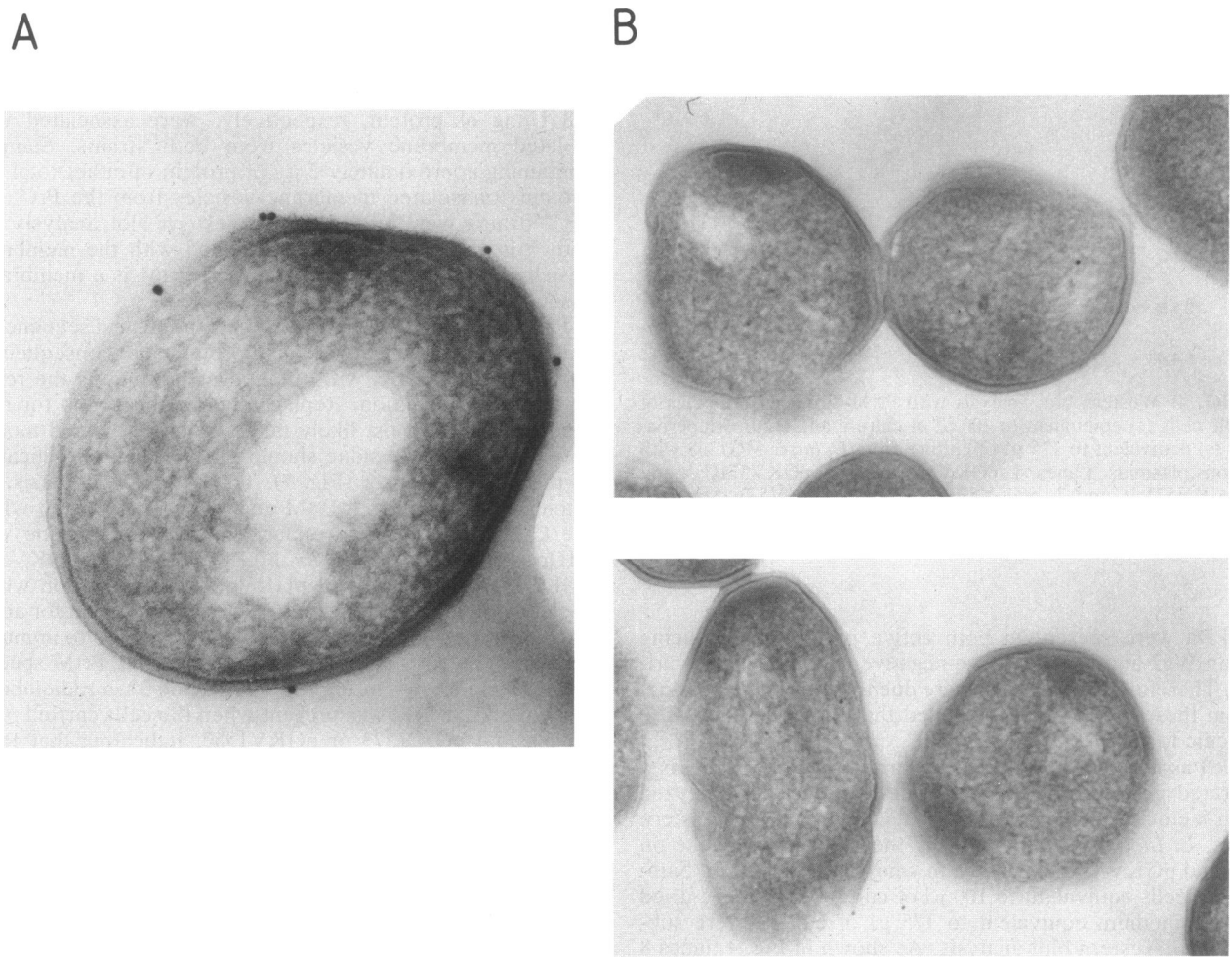

FIG. 5. Immunogold labeling of *L. lactis* MG1363 with (A) or without (B) the *prtM* gene by use of PrtM-specific antisera.

Cys-24→Ala-24 mutation only leads to detectable secretion of both 29- and 32-kDa forms of PrtM when the *prtP* gene is inactivated.

To determine whether the secreted forms of PrtM were capable of processing the inactive proteinase produced in the absence of *prtM*, we cocultured *L. lactis* MG1363 (pGKV507), producing inactive secreted proteinase, and *L. lactis* MG1363(pGKV550), producing an inactive cell-bound proteinase, with *L. lactis* MG1363(pGKV571C). As shown in Table 2, no proteolytic activity was detected in these mixed, overnight-grown milk cultures. The cell densities of the mixed cultures were similar to those of Prt^- strains carrying plasmid pGKV507, pGKV550, or pGKV571C alone. From these results, we concluded that the amount of PrtM secreted by cells carrying a *prtM* gene with a Cys-24→Ala-24 mutation is too low to facilitate proteinase activation or that proteinase maturation is restricted to the membrane surface.

DISCUSSION

The presence of two plasmid-encoded proteins, cell envelope-associated proteinase PrtP and maturation protein PrtM, is essential for proteolytic activity in lactococci (5, 36). In the present paper, we describe the purification of maturation protein PrtM and the isolation of PrtM-specific

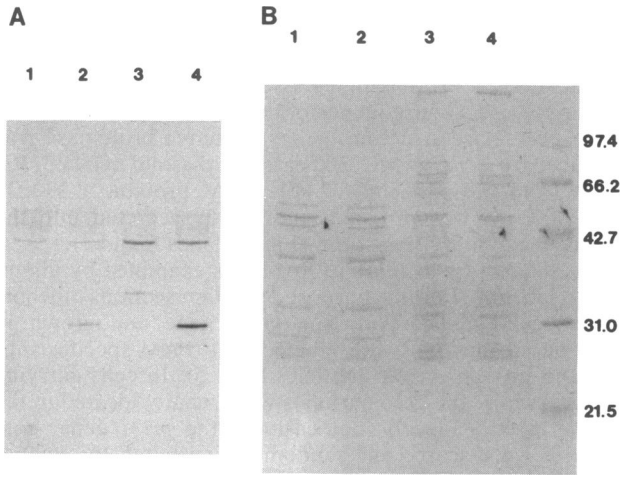

FIG. 6. Membrane association of PrtM. Shown are Western blot analysis (A) and 12.5% SDS-PAGE (B) of cells (lanes 1 and 2) and isolated membrane vesicles (lanes 3 and 4) from Wg2 (Prt^+) (lanes 2 and 4) and Wg2 (Prt^-) (lanes 1 and 3). Each sample contained approximately 2 μg of protein. Standard molecular weight markers were as in Fig. 2. Molecular weights (in thousands) are shown on the right.

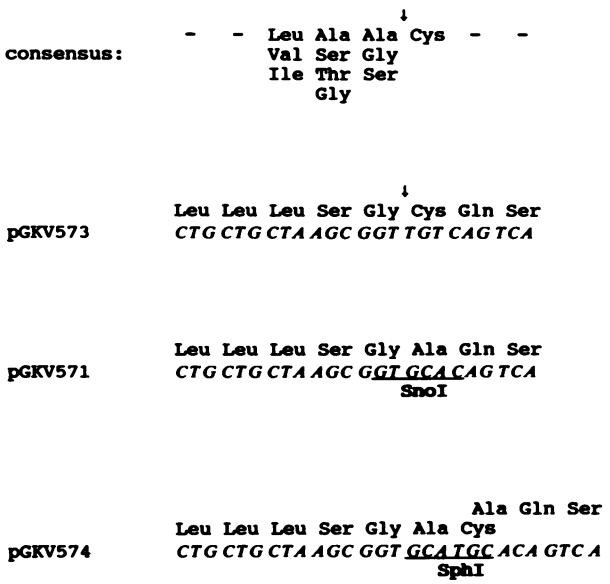

FIG. 7. Nucleotide sequence and deduced amino acid sequence of the *prtM* gene region encoding residues 20 to 27, as present on plasmids pGKV573, pGKV571, and pGKV574. The consensus sequence for lipid modification is also shown (34). Prolipoprotein signal peptidase cleavage sites are indicated by arrows. Filling in of the *Sno*I site in pGKV571 results in the formation of an *Sph*I site and a frameshift in pGKV574.

antisera, a prerequisite for the elucidation of the interaction between PrtM and the proteinase.

Overexpression of the *L. lactis* Wg2 *prtM* gene in *E. coli* gave rise to the production of a 32-kDa protein that was purified by preparative SDS-PAGE. Antisera directed against the purified protein were tested on PrtM-containing and PrtM-deficient strains of lactococci and proved to be highly specific for PrtM. As shown in Fig. 3, only lactococcal strains carrying either the cloned or the wild-type *prtM* gene gave rise to the formation of a 32-kDa protein band in Western blot analysis. This size of the protein produced by

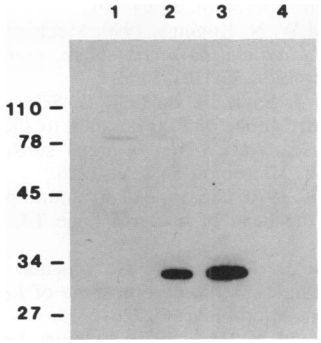

FIG. 8. ^{3}H-labeled proteins from *L. lactis* MG1363 cells carrying plasmid pGKV571 (lane 1), pGKV1552 (lane 2), pGKV573 (lane 3), or pGKV574 (lane 4) and grown in the presence of [^{3}H]palmitic acid. Cells were lysed and subjected to immunoprecipitation with PrtM-specific antisera. The precipitated proteins were subjected to 12.5% SDS-PAGE. Standard molecular weight markers (apparent molecular weights [in thousands]) are shown on the left: phosphorylase *b* (110), bovine serum albumin (78), ovalbumin (45), carbonic anhydrase (34), and soybean trypsin inhibitor (27).

TABLE 2. Proteolysis of milk proteins and cell densities in milk-grown cultures of *L. lactis* MG1363 carrying various plasmids

Plasmid	Proteolysis (A_{340})[a]	Density (CFU/ml)[b]
None (milk blank)	0.133	
pGKV507	0.109	3.2×10^8
pGKV550	0.110	3.1×10^8
pGKV552	0.487	4.1×10^9
pGKV573	0.521	4.1×10^9
pGKV571	0.467	3.1×10^9
pGKV574	0.108	2.7×10^8
pGKV573C	0.106	4.3×10^8
pGKV571C	0.104	1.5×10^8
pGKV571C + pGKV507	0.114	2.6×10^8
pGKV571C + pGKV550	0.112	3.9×10^8
pGKV573C + pGKV507	0.112	2.9×10^8
pGKV573C + pGKV550	0.113	4.3×10^8

[a] Measured by the OPA spectrophotometric assay (2) as described in Materials and Methods.

[b] Determined in cultures grown overnight at 30°C in 10% (wt/vol) reconsituted skim milk.

lactococci corresponded to the size of the protein isolated from *E. coli*.

As a first step in clarifying the role of PrtM in the activation of the proteinase, the subcellular location of PrtM was investigated. PrtM was found to be mainly associated with lactococcal cells. In Western blot analysis, only limited amounts of PrtM antigen could be detected in the culture medium, and only so in the absence of *prtP*-specified proteolytic activity. Immunogold labeling of ultrathin sections of lactococcal cells with PrtM-specific antisera revealed that PrtM was located in the cell envelope. This conclusion was corroborated by the finding that PrtM was coisolated with lactococcal membrane vesicles. Contamination of membrane vesicles with cytoplasmic proteins could be excluded because of the low activity of the cytoplasmic enzyme lactate dehydrogenase in the membrane fractions.

On the basis of the amino acid sequence deduced from the nucleotide sequence, it was proposed that *prtM* encodes a prolipoprotein (5, 36). The N terminus of the *prtM*-encoded protein contains charged residues followed by a stretch of hydrophobic residues. As shown in Fig. 7, the amino acid sequence preceding the Cys-24 residue perfectly matches the consensus sequence of a prolipoprotein signal sequence cleavage site proposed by Von Heijne (34). Only after glyceride-fatty acid modification of the Cys residue will prolipoprotein signal peptidase cleave the peptide bond preceding that Cys residue. The resulting processed lipoprotein will thus carry an N-terminally modified Cys residue (37). In recent literature, a number of methods for identifying a lipoprotein have been described, among which are (i) radiolabeling of the protein with either [^{3}H]glycerol or [^{3}H]palmitic acid and (ii) inhibition of prolipoprotein signal peptidase by globomycin (37). In a lysate of lactococcal cells grown in the presence of [^{3}H]palmitic acid, a 31.5-kDa ^{3}H-labeled protein precipitated with the PrtM-specific antibodies; therefore, we concluded that PrtM is indeed a lipoprotein. In the deduced amino acid sequence of PrtM, Cys-24 is the only Cys residue present. Replacement of the codon for Cys-24 in *prtM* by an Ala codon by site-directed mutagenesis prevented the labeling of PrtM by [^{3}H]palmitic acid. Consequently, residue Cys-24 is the target for lipid modification of the PrtM precursor. Therefore, the lipid-

modified Cys-24 residue must be the N-terminal residue of mature PrtM. The interaction of the lipid moiety at the PrtM N terminus with the cytoplasmic membrane is in complete accordance with the membrane association of PrtM.

A 29-kDa form of PrtM was found both associated with the cells and in the culture medium, but only so in the absence of proteolytic activity. This 29-kDa protein probably resulted from alternative and, apparently, inefficient processing of the PrtM precursor by signal peptidase. Cleavage of the lipoprotein precursor by both signal peptidases and prolipoprotein signal peptidase has also been invoked to explain two mature forms of the lipoprotein penicillinase from *Bacillus licheniformis* and the lipoprotein β-lactamase III from *Bacillus cereus*. These proteins are present in both secreted and membrane-bound forms (18, 19).

Lipid modification of the PrtM precursor could be prevented by removal of the target Cys residue for this modification. Interestingly, *L. lactis* carrying the Cys-24→Ala-24-mutated *prtM* gene produced three forms of PrtM, with sizes of 29, 32, and 34 kDa. The size of the 34-kDa protein, which was found to be associated with the cells, matched well with the calculated size of the PrtM precursor, 33.1 kDa. As discussed above, the 29-kDa form of PrtM may have arisen from cleavage by signal peptidase. The presence of the partly secreted 32-kDa form of PrtM may be explained by assuming that, upon introduction of the Cys-24→Ala-24 mutation, an additional signal peptidase cleavage site was formed. In fact, this amino acid substitution results in an amino acid sequence with a high probability of processing, according to the rules of Von Heijne (33). The fact that the 29- and 32-kDa forms of mutated PrtM were both associated with the cells and secreted in the culture medium suggests that PrtM dissociated very inefficiently from the lactococcal cell envelope. The 29- and 32-kDa secreted forms of PrtM were only detectable in the absence of *prtP*-specified proteinase activity. Apparently, PrtM is degraded by the proteinase.

In the coculture experiments, we were unable to detect activation of inactive secreted proteinase by cells producing the secreted forms of PrtM, whereas cells carrying the mutated *prtM* gene retained their proteolytic activity. These observations indicate that the PrtM precursor which, like mature, wild-type PrtM, may be located at the surface of the cytoplasmic membrane, is still capable of proteinase activation. Upon deletion of the 130 C-terminal amino acids, the normally cell envelope-associated proteinase is secreted into the medium without the loss of proteolytic activity. In a previous paper, we hypothesized that PrtM effects the autoproteolytic removal of the N-terminal pro-sequence of the proteinase precursor, resulting in an active, smaller form of the secreted proteinase (5, 6). In the present paper, we show that PrtM remains associated with the lactococcal cells, both in strains producing a cell envelope-associated form of the proteinase (*L. lactis* Wg2 and *L. lactis* MG1363 carrying plasmid pGKV573) and in a strain producing a secreted form of the proteinase (*L. lactis* MG1363 carrying plasmid pGKV500). These results indicate that PrtM is involved in proteinase maturation in a transient step, taking place during or directly upon membrane translocation of the proteinase precursor. The exact nature of the PrtM-proteinase interaction remains unclear. The availability of both proteinase-specific monoclonal antibodies (13) and the PrtM-specific antisera described in this paper will be helpful in further studies of the unique role of PrtM in the activation of the lactococcal proteinase.

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