JOURNAL OF BACTERIOLOGY, May 1989, p. 2795–2802
0021-9193/89/052795-08$02.00/0

Vol. 171, No. 5

A Maturation Protein Is Essential for Production of Active Forms of *Lactococcus lactis* SK11 Serine Proteinase Located in or Secreted from the Cell Envelope

PIETER VOS,* MARTIEN VAN ASSELDONK, FRANK VAN JEVEREN, ROLAND SIEZEN, GUUS SIMONS, AND WILLEM M. DE VOS

Molecular Genetics Group, Department of Biophysical Chemistry, Netherlands Institute for Dairy Research, P.O. Box 20, 6710 BA Ede, The Netherlands

Received 24 May 1988/Accepted 11 February 1989

The complete nucleotide sequence of a gene located immediately upstream of the *Lactococcus lactis* subsp. *cremoris* SK11 *prtP* gene encoding the cell envelope-attached proteinase was determined. This gene, designated *prtM*, was found to be transcribed from the same promoter region as was the proteinase gene but in the opposite direction. The *prtM* gene directed the expression in *Escherichia coli* of a protein with a size similar to the expected value of 33 kilodaltons, as deduced from the nucleotide sequence data. The derived amino acid sequence of the PrtM protein indicated the presence of a consensus lipoprotein signal sequence at the N terminus, which suggested that PrtM is a lipoprotein. Plasmids containing the *prtM* gene, the *prtP* gene, or both were constructed. Expression studies of *L. lactis* clones containing these plasmids showed that the *prtM* gene encodes a *trans*-acting activity involved in the maturation of cell envelope-located and -secreted forms of the SK11 proteinase.

Lactococci (*Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *diacetylactis*) are gram-positive bacteria used in the food industry for production of a variety of fermented milk products. During growth in milk, these bacteria produce proteolytic enzymes that break down milk proteins (25). The cell envelope-located proteinases of lactococci are key enzymes in the degradation of caseins, the major milk proteins. The cell envelope-located proteinases of a number of *Lactococcus* strains have been characterized and shown to be immunologically related proteins with similar molecular weights (14). On the basis of their cleavage specificity toward caseins, these proteinases can be divided into two major types, designated PI and PIII (28). The complete nucleotide sequences of the genes for the PI-type proteinase of *L. lactis* subsp. *cremoris* Wg2 (16) and the PIII-type proteinase of *L. lactis* subsp. *cremoris* SK11 (P. Vos, R. J. Siezen, G. Simons, and W. M. de Vos, submitted for publication) have been determined. Both genes encode very large proteins with a calculated molecular size of more than 200 kilodaltons (kDa). These proteinases are subtilisin-type serine proteinases synthesized as preproenzymes (16; Vos et al., submitted). The proteinases of these two *Lactococcus* strains, which have very different caseinolytic specificities, are 98% homologous (Vos et al., submitted). Both proteinase genes have been cloned and expressed in *L. lactis* (3, 17).

It has been well established that in most if not all *Lactococcus* strains, the proteinase genes are located on plasmids. The proteinase genes of a number of *Lactococcus* strains have been identified and characterized (4). As judged by their physical maps, these plasmids have very little homology except for the region encoding the cell envelope-located proteinase and the region immediately upstream of the proteinase gene. Kok et al. (16) have demonstrated that this region contains an open reading frame of at least 885 base pairs. Sequence data and in vitro expression studies suggest that this open reading frame is a functional gene transcribed from the same promoter region as is the cell wall proteinase gene (15, 16).

In this paper, we present the complete nucleotide sequence of the conserved region upstream of the proteinase gene. In addition, plasmids containing various parts of the proteinase region, were constructed and transformed to *L. lactis*. The results obtained show that immediately upstream of the proteinase gene another gene is located that encodes a *trans*-acting protein involved in processing of precursors of the proteinase into active enzymes. Similar results with the proteinase genes of *L. lactis* Wg2 have been obtained independently and are described in the accompanying paper (11).

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *L. lactis* subsp. *cremoris* SK112 is a single-strain derivative of the proteinase-producing strain *L. lactis* subsp. *cremoris* SK11 (5) that contains the proteinase plasmid pSK111. Strain *L. lactis* subsp. *lactis* MG1363, a plasmid-free, proteinase-deficient derivative of NCDO 4109 (9), was used as the recipient for all plasmid transformations. *L. lactis* strains were generally grown on M17 broth supplemented with 0.5% (wt/vol) glucose. Occasionally, *L. lactis* strains were grown on a whey-based medium containing 1.9% (wt/vol) β-glycerophosphate, 0.5% (wt/vol) glucose, and 0.1% (wt/vol) Casitone (Difco Laboratories, Detroit, Mich.). The ability of *L. lactis* cells to produce a functional proteinase was assayed by growth of these cells on 10% (wt/vol) reconstituted skimmed milk containing 0.5% (wt/vol) glucose. When necessary, media were supplemented with chloramphenicol (10 μg/ml), erythromycin (5 μg/ml), or both. Plasmid pNZ511 has been described previously (3). Plasmid pNZ521 is a derivative of pNZ511 containing the complete proteinase gene (W. M. de Vos, P. Vos, H. de Haard, and I. Boerrigler, submitted for publication; see Fig. 3). Plasmid pT7-5, a generous gift of

* Corresponding author.

Stanley Tabor, is a derivative of plasmid pT7-1 (23) containing a reversed ampicillin resistance gene. *Escherichia coli* NZ1012 is a derivative of *E. coli* MC1061 (2) carrying plasmid pGP1-2 (23), which contains the T7 RNA polymerase gene under control of the lambda p_L promoter and a temperature-sensitive *c*I repressor gene.

Molecular cloning. Isolation of plasmid DNA from *E. coli* was performed by the alkaline lysis method (1). Isolation of plasmid DNA from *L. lactis* was performed by a modified alkaline lysis method. Cells were incubated for 30 min at 37°C in 25% (wt/vol) sucrose–30 mM Tris hydrochloride (pH 8.3)–3 mM $MgCl_2$–1 mg of lysozyme per ml. Cells were chilled on ice, 2 volumes of 0.2 N NaOH–1% (wt/vol) sodium dodecyl sulfate was added, and the procedure was continued as for isolation of plasmid DNA from *E. coli*. All enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or New England BioLabs, Inc. (Beverly, Mass.), and used according to the instructions of the manufacturers. DNA was transformed to *L. lactis* subsp. MG1363 by electroporation, using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). *L. lactis* MG1363 cells were grown to a density of approximately 5×10^8 cells per ml in M17 broth containing 0.5% (wt/vol) glucose and 40 mM DL-threonine. Cells were collected by centrifugation and washed twice with equal volumes of electroporation buffer (25% [wt/vol] sucrose, 5 mM potassium phosphate [pH 7.0], 1 mM $MgCl_2$). Cells were resuspended in one-fifth volume of electroporation buffer and kept on ice for 15 min. DNA was added, and an electric pulse of 6,250 V/cm and 25 μF was applied. Cells were immediately chilled to 0°C for 10 min. Finally, 10 volumes of M17 broth containing 0.5% (wt/vol) glucose was added, and cells were incubated for 90 min at 30°C and plated on selective medium. Recombinant *L. lactis* clones were analyzed by restriction enzyme mapping, nucleotide sequence analysis, or both (10, 20). Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (New Brunswick Scientific Co., Inc., Norwalk, Conn.).

Nucleotide sequence analysis. A physical map of the region upstream of the proteinase gene was constructed (see Fig. 3). Two DNA fragments, a 1.0-kilobase (kb) *Cla*I-*Bgl*II fragment and a partially overlapping 0.5-kb *Hin*dIII-*Eco*RV fragment (24), were isolated and cloned into vectors M13mp18 and M13mp19 (33). Replicative-form DNA was prepared from recombinant M13 clones; single-stranded bacteriophage DNA was isolated from the clones containing the desired inserts (20). Sequence analysis of all clones was initiated with a universal M13 primer and extended by using specific oligonucleotides as primers for DNA synthesis.

Construction of plasmids. Plasmid pNZ511 (3; see Fig. 3) contains a 6.5-kb *Hin*dIII-*Sal*I fragment cloned in the *Sal*I site of lactococcal cloning vector pNZ122 by using a small double-stranded *Hin*dIII-*Pst*I-*Sal*I adapter fragment. This plasmid therefore contains almost the complete *prtM* gene and a truncated *prtP* gene. Plasmid pNZ511 was completely digested with *Hin*dIII and partially digested with *Cla*I. The resulting DNA fragments were filled in with the Klenow fragment of *E. coli* DNA polymerase I and circularized with T4 DNA ligase. The ligation mixture was transformed by electroporation to *L. lactis* MG1363; a recombinant *L. lactis* clone lacking the 0.9-kb *Hin*dIII-*Cla*I fragment (containing the *prtM* gene) was selected and designated pNZ516. A 3.1-kb *Bgl*II-*Xho*I fragment containing the complete C-terminal part of the proteinase gene was isolated from plasmid pPR310 (Vos et al., submitted) and ligated to plasmid pNZ516, which had been completely digested with *Bgl*II and partially digested with *Sal*I. The ligation mixture was transformed to *L. lactis* MG1363; a recombinant *L. lactis* clone in which the 1.3-kb *Bgl*II-*Sal*I fragment was exchanged for the 3.1-kb *Bgl*II-*Xho*I fragment was selected and designated pNZ526. Plasmid pNZ582 was constructed by insertion of a 1.4-kb *Pst*I fragment (containing the *prtM* gene, including its promoter) from plasmid pNZ511 in plasmid pIL253 (22). *L. lactis* clones containing both plasmids pNZ516 and pNZ582 or pNZ526 and pNZ582 were made by introducing both pairs of plasmids into *L. lactis* MG1363 and selecting recombinants by growth on M17 agar containing 0.5% (wt/vol) glucose and 5 μg of both chloramphenicol (selection for pNZ516 or pNZ526) and erythromycin (selection for pNZ582) per ml.

Proteinase expression studies. *L. lactis* cells were generally grown on a whey-based medium for expression studies. Cells were collected by centrifugation, and the supernatant was dialyzed against water. The dialyzed culture supernatant was freeze-dried and dissolved in 1/100 the original volume of water. Samples of the concentrated growth medium (20 μl) were analyzed on 10% polyacrylamide gels (18), and proteins were detected by staining with Coomassie brilliant blue. Cell wall release fractions were prepared as described previously (6). Samples of the release fractions (20 μl) were directly analyzed on 10% polyacrylamide gels, and proteins were detected by Western blotting (immunoblotting) (26), using a rabbit antiserum directed against the SK11 proteinase obtained by release from *L. lactis* cells (de Vos et al., submitted). Whole cells were also analyzed for the presence of products of the proteinase gene. For this purpose, cells were collected by centrifugation and suspended in 1/50 volume of 25% (wt/vol) sucrose–30 mM Tris hydrochloride (pH 8.3)–3 mM $MgCl_2$–1 mg of lysozyme per ml (wt/vol) and incubated for 30 min at 37°C to make protoplasts. An equal volume of 2-times-concentrated protein gel sample buffer was added to these protoplasts, and proteins were separated on a 10% polyacrylamide gel. Proteins were detected by Western blotting, using antiserum directed against the SK11 proteinase.

N-terminal amino acid analysis. *L. lactis* clones pNZ511 and pNZ516 were grown overnight in 50 ml of a whey-based medium. Cells were removed from the growth medium by centrifugation, and the supernatant was dialyzed against water for 16 h. The dialyzed growth medium was concentrated 100 times by freeze-drying and subsequent dissolving in 0.5 ml of protein gel sample buffer (18). The proteins of the concentrated growth medium were separated on a 10% polyacrylamide gel (18), which was subsequently blotted on polybrene-coated GFC paper (27). Protein bands were visualized by staining with fluorescamine, and small pieces of GFC paper containing the proteinases specified by pNZ511 and pNZ516 were directly analyzed in a gas-phase Sequenator (Applied Biosystems Inc.) (27).

Expression of the *prtM* gene in *E. coli*. A small double-stranded adapter sequence of 20 base pairs was synthesized that contained a *Sal*I site at the 5′ end, followed by the 15 nucleotides preceding the *prtM* gene (therefore including the ribosome-binding site) and, at the 3′ end, the *Cla*I site of the *prtM* gene. This double-stranded adapter was ligated together with the 1.0-kb *Cla*I-*Bgl*II fragment containing the entire coding sequence of the *prtM* gene to the T7 expression vector pT7-5. The ligation mixture was transformed to *E. coli* NZ1012, which contains a temperature-inducible T7 polymerase gene. Recombinants containing the desired fragments were selected, and one clone, designated pPQ32, was further analyzed by *E. coli* expression studies as described by Tabor and Richardson (23).

```
AACAAGATCGATAGCCCTTTCTTTTTCCTTTGCATCCAATATCCTCCACTTTCCAAAAGTTTTCATAACCAAATCAAAGCCTACGCTTGATGTAGTTAAGATTATATTATATAATAAT -241

TTATATACTATTATATTATATACTATTGAAGAACAAATTCAACGAAAATTTTAATCTTCTTATTAATTTTTTTATCTGTAAATTAACTTTTAGCGTTATTATTTAGCCGTTTACAGACTA -121

AGATGTTTTGAAATTTTTAGCACTTATTAGAAAATGAGTATTTGAATTGAAATTCTCAATTGCCATCGCTAGAATAAATACACAACGTTTACTGTAAGCATTTCAGAGGAGACCGAATCG -1

ATGAAGAAAAAAATGCGCCTTAAAGTATTATTGGCAAGTACCGCAACTGCTTTACTGCTGCTAAGCGGTTGTCAGTCAAATCAGACCGACCAAACAGTTGCGACCTATTCAGGTGGCAAG 120
 M  K  K  K  M  R  L  K  V  L  L  A  S  T  A  T  A  L  L  L  L  S  G  C  Q  S  N  Q  T  D  Q  T  V  A  T  Y  S  G  G  K

GTGACTGAAAGTAGCTTGTACAAGGAACTCAAACAGTCACCAACGACAAAGACCATGCTTGCTAACATGCTCATTTATCGTGCATTGAATCATGCCTATGGAAAATCGGTTAGCACTAAA 240
 V  T  E  S  S  L  Y  K  E  L  K  Q  S  P  T  T  K  T  M  L  A  N  M  L  I  Y  R  A  L  N  H  A  Y  G  K  S  V  S  T  K

ACAGTTAATGATGCCTATGATAGCTACAAACAACAATACGGCGAAAATTTCGATGCTTTCTTAAGTCAAAACGGTTTCAGTCGCAGTAGCTTCAAGGAAAGCCTACGAACCAACTTTTTA 360
 T  V  N  D  A  Y  D  S  Y  K  Q  Q  Y  G  E  N  F  D  A  F  L  S  Q  N  G  F  S  R  S  S  F  K  E  S  L  R  T  N  F  L

AGTGAAGTTGCACTGAAAAAGTTAAAAAAAGGTTTCTGAAAGCCAGCTCAAGGCCGCTTGGAAGACCTATCAGCCCAAAGTGACTGTCCAACATATTCTAACTAGCGACGAGGACACTGCT 480
 S  E  V  A  L  K  K  L  K  K  V  S  E  S  Q  L  K  A  A  W  K  T  Y  Q  P  K  V  T  V  Q  H  I  L  T  S  D  E  D  T  A

AAGCAAGTTATCAGTGATTTAGCAGCTGGCAAGGATTTTGCCATGCTTGCGAAAACTGATTCCATTGATACTGCGACTAAAGATAACGGCGGGAAGATTAGTTTTGAATTAAACAATAAA 600
 K  Q  V  I  S  D  L  A  A  G  K  D  F  A  M  L  A  K  T  D  S  I  D  T  A  T  K  D  N  G  G  K  I  S  F  E  L  N  N  K

ACGCTCGATGCCACATTTAAGGATGCTGCCTACAAATTAAAAAATGGTGACTACACGCAGACCCCAGTCAAAGTGACAGACGGGTATGAAGTTATTAAAATGATTAACCATCCCGCCAAA 720
 T  L  D  A  T  F  K  D  A  A  Y  K  L  K  N  G  D  Y  T  Q  T  P  V  K  V  T  D  G  Y  E  V  I  K  M  I  N  H  P  A  K

GGCACCTTTACTAGCAGTAAAAAGGCGCTAACTGCCAGCGTTTACGCTAAATGGTCTCGCGATTCAAGCATCATGCAACGCGTTATCAGTCAGGTATTGAAGAATCAGCATGTGACGATT 840
 G  T  F  T  S  S  K  K  A  L  T  A  S  V  Y  A  K  W  S  R  D  S  S  I  M  Q  R  V  I  S  Q  V  L  K  N  Q  H  V  T  I

AAAGACAAGGATCTTGCGGATGCGCTAGATAGTTATAAGAAGCTTGCGACAACGAATTAAGGCTTAACCGAGTAAGATGAAATAACAGCCAGTTCTTTTAGAAATTGGCTCTTCGTCAAG 960
 K  D  K  D  L  A  D  A  L  D  S  Y  K  K  L  A  T  T  N  *

TAGAGTTGATAGATCTGGTTCTGTTGCAAAGTTTTCCAAAAAATCTATTTTAGTGTAAAATTGAGAAAAAAGACAGAGAGGACAGAGTAATGAATCGTTTTAAAGGCAAACAATTCAAAA 1080
```

FIG. 1. Nucleotide sequence of the *prtM* gene and deduced amino acid sequence of the encoded protein. Nucleotide sequence from positions −1 to −360 has previously been determined (Vos et al., submitted) and contains promoter sequences for both the *prtM* and *prtP* genes. Possible −35 and −10 promoter sequences are underlined; ▭, putative ribosome-binding site.

RESULTS

Location of an oppositely directed gene immediately upstream of the cell wall proteinase gene in plasmid pSK111. Nucleotide sequencing (Fig. 1) revealed the presence of an open reading frame of 897 base pairs preceded by several consensus −35 and −10 promoter regions (12). The first ATG codon of the open reading frame is preceded by a consensus lactococcal ribosome-binding site (4) at a distance expected for a functional translation initiation codon (4, 21). These data suggest that this open reading frame is a functional gene that would encode a protein of 299 amino acids with a calculated molecular size of 33,132 Da.

A DNA fragment containing the complete putative coding sequence and ribosome-binding site was cloned into a T7 expression vector (23) to investigate whether this open reading frame is indeed a functional gene. Upon temperature induction, a protein with a molecular size close to the expected value of 33 kDa was synthesized in this clone (designated pPQ32) (Fig. 2). Synthesis of this protein was not affected by addition of rifampin to the growth medium, which indicated that the protein was translated from an mRNA transcribed by the T7 RNA polymerase. These data demonstrate that the open reading frame represents a functional gene, designated *prtM*.

The amino acid sequence of the 33-kDa PrtM protein as derived from the DNA sequence showed the presence of a consensus signal peptide sequence at the N-terminal end, i.e., a number of positively charged amino acids followed by a stretch of hydrophobic amino acids (29). Moreover, there is a consensus lipoprotein signal peptidase cleavage site between amino acids 23 and 24 of PrtM (Leu-Ser-Gly/Cys-Gln-Ser [31]), which suggests that the PrtM protein is a lipoprotein. This presumptive lipoprotein of 276 amino acids should have a molecular size of about 31 kDa (protein part) and is predicted to be highly charged, with a predominance of basic residues (40 basic versus 29 acidic residues). No significant sequence homology with any proteins in the NBRF/PIR (release 18.0), NBRF/NEW (release 36.0), and Swiss-Prot (release 9.0) data bases was detected.

Haandrikman et al. (11) have determined the nucleotide sequence of the corresponding *prtM* gene of *L. lactis* subsp. *cremoris* Wg2. In comparison with this sequence, the SK11

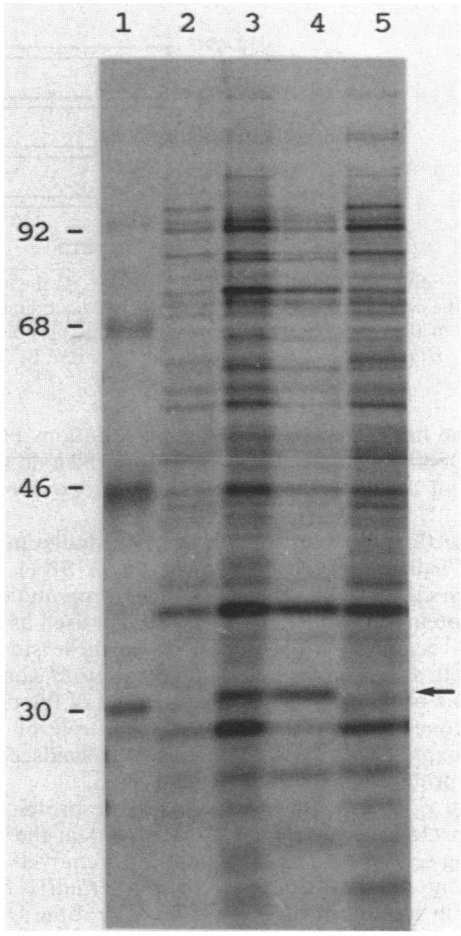

FIG. 2. *E. coli* expression studies (23) with clone pPQ32 containing the *prtM* gene. Lanes: 1, molecular size (in kilodaltons) markers (from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase); 2 and 5, noninduced cells; 3, induced cells; 4, induced cells to which rifampin was added. Arrow indicates position of the induced protein.

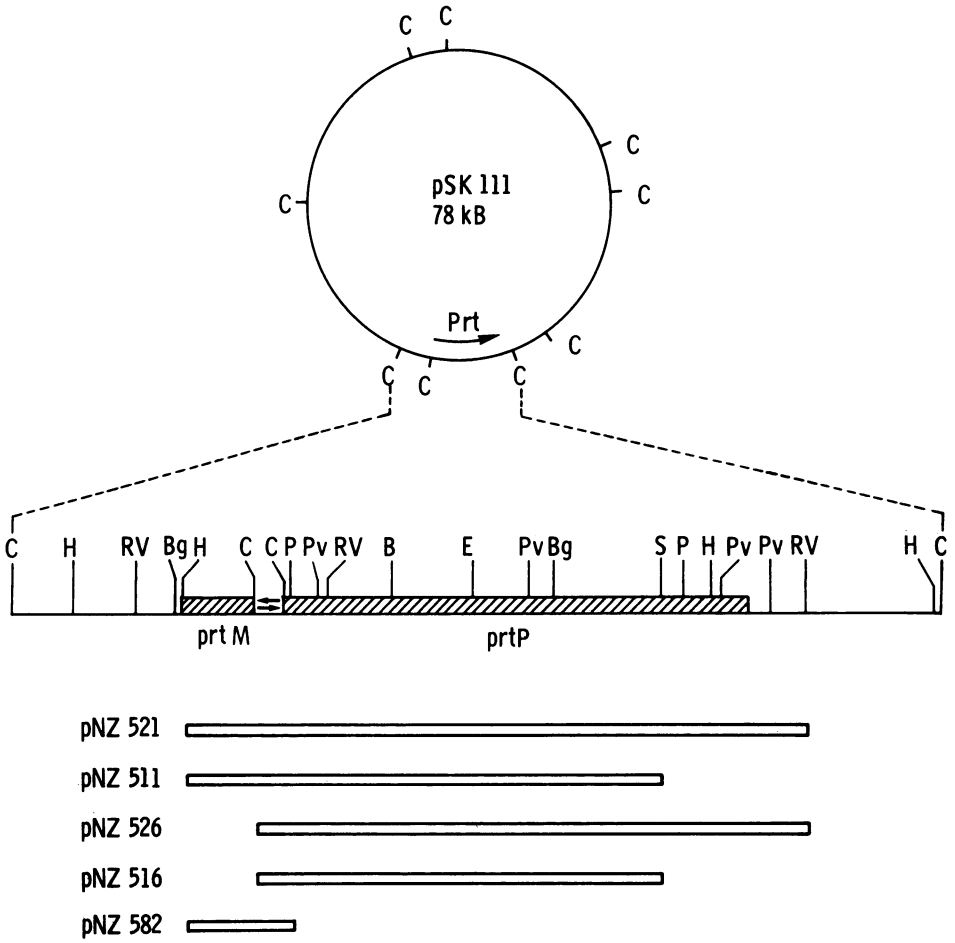

FIG. 3. Schematic representation of plasmids. At the top is shown the wild-type proteinase plasmid pSK111 along with the enlarged region encoding the *prtP* and *prtM* genes. Symbols: ▨, coding sequences; ⇄, promoters for both genes (Vos et al., submitted); ▭, cloned DNA fragments of the proteinase region in the various plasmids. Abbreviations for restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; RV, *Eco*RV; S, *Sal*I.

prtM gene has only two base pair substitutions (T to G at position 138 and C to T at position 825), which do not result in different amino acids. Therefore, the two proteins are identical.

Construction of plasmids for expression studies in *L. lactis*. The cell wall proteinase genes of *L. lactis* SK11 and Wg2 have been cloned and appear to restore proteolytic activity in the proteinase-deficient *L. lactis* strains used as hosts (3, 17). The recombinant plasmids used in these studies contained both the proteinase gene and the *prtM* gene; therefore, a role of the latter gene in expression of the proteinase gene is conceivable. To study a possible role of the *prtM* gene in expression of the proteinase, plasmids containing only the proteinase gene were constructed.

Plasmid pNZ521 contains the complete proteinase gene and the *prtM* gene except for 12 base pairs at the 3′ end of the coding sequence. The *prtM* gene was removed from this plasmid by deletion of a 0.9-kb *Cla*I-*Hin*dIII fragment, resulting in plasmid pNZ526 (Fig. 3). The same DNA fragment was removed from plasmid pNZ511, which is identical to plasmid pNZ521 but lacks the coding sequence for the C-terminal 403 amino acids of the cell wall proteinase, resulting in plasmid pNZ516. This C-terminal deletion in the proteinase gene results in a proteolytically active gene product which is no longer bound to the cell envelope but secreted into the growth medium (3). Plasmids pNZ516 and pNZ526 were transformed into the plasmid-free proteinase-deficient *L. lactis* strain MG1363 (9) and analyzed for the ability to grow in milk and therefore to break down caseins (Table 1). The recombinant *L. lactis* clones obtained showed the same growth characteristics in milk as did the plasmid-free strain, which indicated that normal growth in milk does not occur in the absence of the *prtM* gene.

***prtM* gene-encoded *trans*-acting function necessary for growth in milk.** On the proteinase plasmid pSK111 and on plasmids pNZ511 and pNZ521, the *prtM* and *prtP* genes are physically linked. We were interested in determining whether *L. lactis* cells containing both genes on separate plasmids would be able to grow well in milk. For this purpose, a plasmid containing only the *prtM* gene, denoted pNZ582, was constructed.

Plasmid pNZ582, which is compatible with plasmids pNZ516 and pNZ526, was transformed to *L. lactis* MG1363 carrying pNZ516 or pNZ526. The resulting *L. lactis* clones, harboring plasmid pNZ526 or pNZ516 as well as pNZ582, were analyzed for the ability to grow in milk (Table 1). *L. lactis* clones containing both genes on separate plasmids were able to grow well in milk, which demonstrated that the *prtM* gene encodes a *trans*-acting function which, in con-

TABLE 1. Phenotypes of *L. lactis* clones

Plasmid in *L. lactis* MG1363	Gene[a]	Prt[b]
pNZ511	*prtP'*, *prtM*	+
pNZ516	*prtP'*	−
pNZ521	*prtP*, *prtM*	+
pNZ526	*prtP*	−
pNZ582	*prtM*	−
pNZ516 − pNZ582[c]	*prtP'* − *prtM*	+
pNZ526 − pNZ582[c]	*prtP* − *prtM*	+
pNZ516 + pNZ582[d]	*prtP* + *prtM*	+

[a] *prtP*, presence of the complete proteinase gene; *prtP'*, presence of the truncated proteinase gene; *prtM*, presence of the complete *prtM* gene except for the four C-terminal amino acids.

[b] +, Phenotypically proteinase-positive clones (growth on milk to high cell densities accompanied by low final pH [<5.0] and clotting of the milk); −, phenotypically proteinase-negative clones (growth to moderate cell densities, resulting in a final pH of >5.5 and no clotting of the milk).

[c] Two plasmids in one cell.

[d] Coculturing experiments with the two plasmids present in different cells.

junction with the *prtP* gene product, is necessary for efficient breakdown of caseins.

We also investigated whether processing occurred when cells containing only the *prtP* gene were cocultured with cells containing only the *prtM* gene (Table 1). For this purpose, milk was inoculated with equal amounts of *L. lactis* clones pNZ516 and pNZ582. This mixture of cells was not able to grow well in milk, which indicated that active proteinase was not produced.

Involvement of the *prtM* gene product in maturation of the cell wall proteinase. The presence of the proteinase can easily be demonstrated in proteolytically active cells. In cells containing the naturally occurring proteinase plasmid pSK111 or plasmid pNZ521, a functional proteinase can be released from the cell wall by incubation in calcium-free buffer (cell wall release fraction) (6). Moreover, *L. lactis* cells harboring plasmid pNZ511, which specifies a proteinase that lacks the membrane-anchoring sequence located at the C terminus (Vos et al., submitted), secrete a proteolytically active gene product into the growth medium (3).

There are several ways in which the *prtM* gene might be involved in expression of the cell wall proteinase. Therefore, we first investigated whether a gene product of the proteinase gene could be detected in clones lacking the *prtM* gene. This was accomplished most easily with derivatives of pNZ511, because the proteinase specified by this plasmid can be recovered from the growth medium.

In the growth medium of *L. lactis* cells harboring plasmid pNZ516, a slowly migrating protein that reacted with antiserum directed against the SK11 proteinase could be detected (Fig. 4 and 5). This nonproteolytically active gene product was secreted into the growth medium in amounts similar to those of the protein produced in clone pNZ511. The apparent molecular size of this inactive proteinase was about 220 kDa, which does not agree very well with the molecular size of 165 kDa predicted for the primary translation product of clone pNZ516. The proteinase specified by clone pNZ516 migrated faster than did the complete *prtP* gene product synthesized in *E. coli* (Vos et al., submitted; Fig. 4). *L. lactis* cells containing both plasmids pNZ582 and pNZ516 produced a protein of the same size as the pNZ511-derived *prtP* gene product. These data indicate that the *prtM* gene product has no effect on the amount of proteinase synthesized but probably is involved in processing of the *prtP* gene product into a functional proteinase.

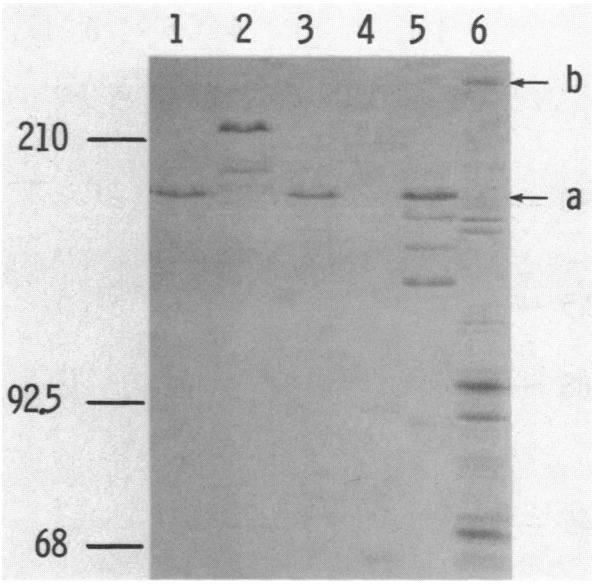

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins secreted into the growth medium of *L. lactis* clones pNZ511 (lane 1), pNZ516 (lane 2), and pNZ516-pNZ582 (lane 3). Other lanes: 4, molecular size (in kilodaltons, shown on left) markers (from top to bottom, myosin, phosphorylase *b*, and bovine serum albumin); 5, proteins released from the cell wall of *L. lactis* subsp. *cremoris* SK11 (arrow a indicates the released proteinase [6]); 6, total cellular proteins of *E. coli* clone pPR31 overproducing the proteinase (arrow b indicates the overexpressed protein).

Next we studied the expression of clone pNZ526, which contained the complete proteinase gene. This clone should produce a protein that, like the wild-type proteinase produced by clone pNZ521, is bound to the cell envelope. In the cell wall release fraction of clone pNZ526, no proteins that reacted with the proteinase antiserum could be detected (Fig. 5). In contrast, *L. lactis* cells containing plasmid pNZ521 or both plasmids pNZ582 and pNZ526 showed a

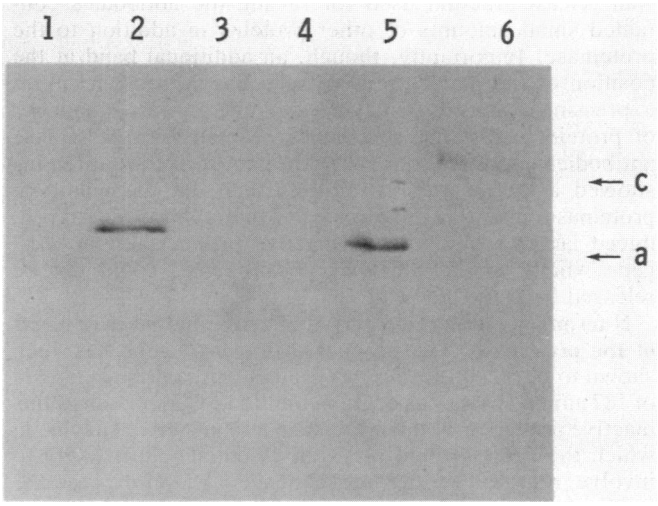

FIG. 5. Western blot analysis of cell wall release fractions of *L. lactis* subsp. *lactis* MG1363 (lane 1) and clones pNZ521, pNZ526, pNZ582, and pNZ526-pNZ582 (lanes 2 to 5, respectively). Arrows: a, position of the released proteinase; c, position of the secreted gene product of clone pNZ516 (lane 6).

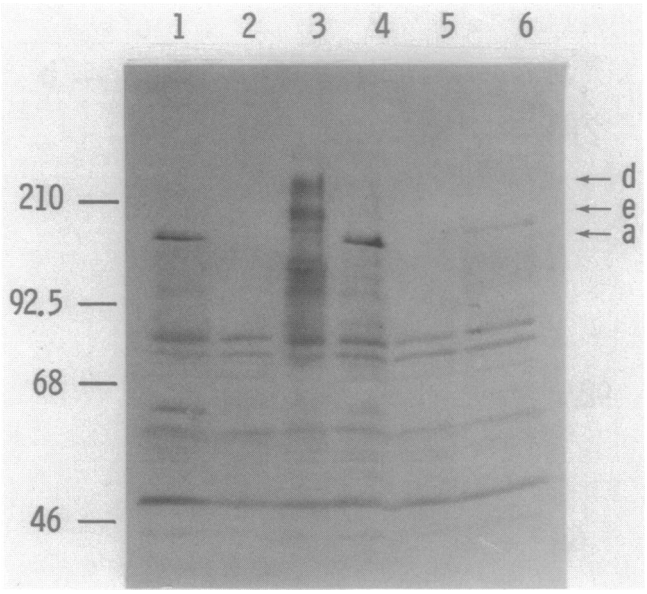

FIG. 6. Western blot analysis of total cellular proteins of clones pNZ521, pNZ582, pNZ526, and pNZ526/pNZ582 (lanes 1 to 4, respectively) and cellular proteins of *L. lactis* subsp. *lactis* MG1363 (lane 5) and *L. lactis* subsp. *cremoris* SK11 (lane 6). Numbers at the left indicate sizes (in kilodaltons) and positions of the marker proteins (from top to bottom) myosin, phosphorylase *b*, bovine serum albumin, and ovalbumin. Arrows: a, position of the cell wall-released proteinase; d and e, gene products of pNZ526 that specifically react with the proteinase antiserum.

protein released from the cell wall that corresponded to the mature proteinase. Since the presence of the proteinase in clone pNZ526 could not be demonstrated by release from the cell wall, whole cells were also investigated for the presence of the proteinase. Figure 6 shows a Western blot of whole-cell extracts of several *L. lactis* clones, performed by using an antiserum directed against the cell wall release fraction of *L. lactis* SK11. This antiserum also reacted with proteins of *L. lactis* MG1363 and *L. lactis* clone pNZ582, although these cells did not produce proteinase. It is probable that the cell wall release fraction used for raising the antibodies contained small amounts of other proteins in addition to the proteinase. Importantly, though, an additional band at the position of the mature proteinase could be seen in clones expressing a functional enzyme. In clone pNZ526, a number of protein bands that specifically reacted with proteinase antibodies could be seen. The two largest of these proteins showed a lower mobility on gels than did the wild-type proteinase. These results indicated that clone pNZ526 produced larger proteolytically inactive products of the *prtP* gene which, unlike the wild-type enzyme, could not be released from the cell wall.

N-terminal amino acid analysis of active and inactive forms of the proteinase. The product of the *prtP* gene has been shown to be a 206-kDa pre-proprotein with a pre-propeptide of 187 amino acids (Vos et al., submitted). Conversion of this inactive precursor of the proteinase into an active enzyme in which the PrtM protein plays an essential role is likely to involve removal of the prosequence. Therefore, the N-terminal amino acid sequence of the inactive enlarged proteinase produced by *L. lactis* lacking the *prtM* gene was determined and compared with the sequence of the mature enzyme.

The secreted proteinases produced by *L. lactis* clones pNZ511 and pNZ516 were isolated and subjected to N-terminal amino acid analysis (27). The sequence of the first five amino acids of the proteinase specified by pNZ511 was Asp-Ala-Lys-Ala-Asn, which is identical to the previously determined sequence of the proteinase released from the cell wall of *L. lactis* SK11 (Vos et al., submitted). The N-terminal amino acid sequence of the proteinase produced by clone pNZ516 could not be determined despite several attempts, which indicates that the N-terminus of this protein must be blocked.

DISCUSSION

In this paper, we present the nucleotide sequence of a region of 1.3 kb immediately upstream of the *L. lactis* SK11 proteinase (*prtP*) gene. This region contains a gene, designated *prtM*, that encodes a protein with a molecular size of 33 kDa. The product of the *prtM* gene is a *trans*-acting maturation protein (PrtM) that is involved in processing the translation product of the *prtP* gene into an active enzyme.

The molecular weights of the proteins synthesized in *L. lactis* clones pNZ516 and pNZ526 on the basis of migration in sodium dodecyl sulfate-polyacrylamide gels are higher than expected from the lengths of the primary translation products of the proteinase genes present in these clones. Glycosylation of the proteinase does not seem to occur in *L. lactis* (Fred Exterkate, personal communication). Aberrant migration of polypeptides in protein gels, especially of very large proteins, is not well understood and may be due to the relatively high proline content (4%) of the proteinase (7, 8, 13). Western blot analysis showed, in addition to the presence of the mature proteinase or proteinase precursors, the presence of proteinase-specific lower-molecular-weight polypeptides. These polypeptides probably represent degradation products of the proteinase generated by autolytic digestion or proteolysis by other proteinases present in the growth medium (F. A. Exterkate and G. J. C. M. de Veer, System. Appl. Microbiol., in press). These degradation products also occur in the case of proteinase precursors, which are unable to break down caseins.

The *L. lactis* SK11 proteinase is a serine proteinase which, like other serine proteinases (30, 32), is synthesized as a pre-proprotein; it contains a 187-amino-acid-long N-terminal pre-propeptide (Vos et al., submitted). Unlike most other serine proteinases, which are completely secreted into the growth medium, the lactococcal proteinase is associated with the cell envelope. For two other bacterial serine proteinases, from *Bacillus amyloliquefaciens* (19) and *Serratia marcescens* (32), it has been found that the inactive proenzymes are converted into active enzymes by autocatalytic removal of the propeptides after their transport across the cytoplasmic membrane. The results obtained here show that *L. lactis* containing only the *prtP* gene produces an inactive enlarged proteinase. It is possible that in such a protein the propeptide is still present, resulting in an inactive proteinase with an N-terminal extension of approximately 16 kDa. The amino terminus of the proteinase produced by *L. lactis* clone pNZ516 appeared to be blocked and could not be determined, in contrast to the situation with the proteinase specified by pNZ511. These N-terminal amino acid analyses do indicate that the active and inactive forms of the proteinase have different amino termini. In this case, removal of the propeptide would be triggered by the PrtM protein instead of being a direct autocatalytic cleavage, as is the case with the *B. amyloliquefaciens* and *S. marcescens* serine proteinases (19, 32).

The apparent size difference on protein gels (Fig. 4 and 6) between precursor and mature forms of the proteinase cannot easily be explained only by removal of the propeptide. Therefore, additional processing at the C terminus may explain the observed size reduction. This C-terminal processing may also explain the inability of *L. lactis* clones lacking the *prtM* gene to release the proteinase from the cell wall, since the proteinase has been shown to be bound to the cell envelope with its C terminus (Vos et al., submitted). We propose a model in which the proteinase precursor, after transport across the cell membrane, is processed at both the N and C termini in the presence of the PrtM protein. Under physiological conditions, the C-terminal part and the N-terminal part, from which the pre-pro-peptide is removed, remain associated, and therefore the proteinase stays attached to the cell envelope. Under certain conditions (e.g., incubation in calcium-free buffer or sodium dodecyl sulfate), however, the two parts may dissociate, resulting in a C-terminal part that stays attached to the cell envelope and an N-terminal part (the released proteinase) that contains the catalytic domain. This notion also explains why the protein found in the growth medium of *L. lactis* pNZ511 shows the same molecular weight on protein gels as does the protein released from the cell envelope.

The PrtM protein could be involved in activation of the proteinase in several ways. The protein could trigger the removal of the propeptide, thereby activating the proteinase, which in turn carries out the C-terminal processing. Alternatively, the PrtM protein may be involved in processing at the C terminus, which enables the proteinase to autocatalytically remove the pro-peptide, resulting in an active enzyme. Finally, the PrtM protein may be involved in processing of the proteinase at both the N and C termini.

The question arises as to where processing of the proteinase takes place. The amino acid sequence of the PrtM protein reveals the presence of a consensus lipoprotein signal sequence, which suggests that PrtM is a lipoprotein. Therefore, the PrtM protein may be located at the outside of the cell membrane, like the proteinase, and processing may occur at this site. This hypothesis is strengthened by the observation that processing does not occur when cells secreting an inactive proteinase (with plasmid pNZ516) are cocultured with cells expressing a functional maturation protein (with plasmid pNZ582).

The fact that two proteins, the maturation protein and the proteinase itself, are necessary for generation of an active proteinase is unexpected and suggests a mechanism of activation of the proteinase different from that of other procaryotic serine proteinases. Future investigations will be directed toward further unraveling of the mechanism of activation of the proteinase precursor and determining the exact cellular localization of these processing events.

ACKNOWLEDGMENTS

We thank Fred Exterkate for critical reading of the manuscript and Joop Mondria, Simon van der Laan, and Jacques Maessen for art work and photography.

This work was supported in part by the Biotechnology Action Program of the Commission of the European Communities under contract BAP-0011-NL.

LITERATURE CITED

1. **Birnboim, H., and J. Doly.** 1979. Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **143**:179–207.
3. **de Vos, W. M.** 1986. Genetic improvement of starter streptococci by the cloning and expression of the gene coding for a non-bitter proteinase, p. 465–472. *In* E. Magnien (ed.), Biomolecular engineering in the European community. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
4. **de Vos, W. M.** 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46**:281–295.
5. **de Vos, W. N., and F. L. Davies.** 1984. Plasmid DNA in lactic streptococci: bacteriophage resistance and proteinase plasmids in *Streptococcus cremoris* SK11, p. 201–206. *In* Third European Congress on Biotechnology, vol. 3. Verlag chemie, Munich.
6. **Exterkate, F. A., and G. J. C. M. de Veer.** 1985. Partial isolation of and degradation of caseins by cell wall proteinases of *Streptococcus cremoris* HP. Appl. Environ. Microbiol. **49**: 328–332.
7. **Franssen, H. J., J. P. Nap, T. Gloudemans, W. Stiekema, H. van Dam, F. Govers, J. Louwerse, A. van Kammen, and T. Bisseling.** 1987. Characterization of cDNA for nodulin-75 of soybean: a gene product involved in early stages of root nodule development. Proc. Natl. Acad. Sci. USA **84**:4495–4499.
8. **Freytag, J. W., M. E. Noelken, and B. G. Hudson.** 1979. Physical properties of collagen-SDS complexes. Biochemistry **18**:4761–4768.
9. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**:1–9.
10. **Guo, L. H., R. C. A. Yang, and R. Wu.** 1983. An improved strategy for rapid sequencing of both strands of long DNA molecules cloned in a plasmid. Nucleic Acids Res. **11**:5521–5539.
11. **Haandrikman, A. J., J. Kok, H. Laan, S. Soemitro, A. Ledeboer, W. N. Konings, and G. Venema.** 1989. Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. J. Bacteriol. **171**:2789–2794.
12. **Hawley, D. K., and W. K. McClure.** 1983. Compilation and analysis of *E. coli* promoter DNA sequences. Nucleic Acids Res. **11**:2237–2255.
13. **Hollingshead, S. K., V. A. Fishetti, and J. R. Scott.** 1986. Complete nucleotide sequence of typy 6M protein of the group A *Streptococcus*. J. Biol. Chem. **261**:1677–1686.
14. **Hugenholtz, J., F. A. Exterkate, and W. N. Konings.** 1984. The proteolytic systems of *Streptococcus cremoris*: an immunological analysis. Appl. Environ. Microbiol. **48**:1101–1110.
15. **Kok, J., D. Hill, A. J. Haandriksman, M. J. B. de Reuver, H. Laan, and G. Venema.** 1988. Deletion analysis of the proteinase gene of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. **54**:239–244.
16. **Kok, J., K. Leenhouts, A. J. Haandriksman, A. M. Ledeboer, and G. Venema.** 1988. Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. **54**:231–238.
17. **Kok, J., J. M. van Dijl, J. M. B. M. van der Vossen, and G. Venema.** 1985. Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50**:94–101.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
19. **Power, D. S., R. H. Adams, and J. A. Wells.** 1986. Secretion and autocatalytic maturation of subtilisin. Proc. Natl. Acad. Sci. USA **83**:3096–3100.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
21. **Shine, J., and L. Dalgarno.** 1974. The 3′ terminal sequence of *E. coli* 16S ribosomal RNA is complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**: 1342–1346.
22. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70**:559–566.

23. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
24. **Tautz, D., and M. Renz.** 1983. An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal. Biochem. **132:**14–19.
25. **Thomas, T. D., and G. G. Pritchard.** 1987. Proteolytic enzymes of dairy starter cultures. FEMS Microbiol. Rev. **46:**245–268.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
27. **Vandekerckhove, J., G. Bauw, M. Puype, J. van Damme, and M. van Montagu.** 1985. Protein blotting on polybrene-coated glass fiber sheets. Eur. J. Biochem. **152:**9–19.
28. **Visser, S., F. A. Exterkate, C. J. Slangen, and G. J. C. M. de Veer.** 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine αs1-, β-, and k-casein. Appl. Environ. Microbiol. **52:**1162–1166.
29. **von Heijne, G.** 1982. Signal sequences are not uniformly hydrophobic. J. Mol. Biol. **159:**537–541.
30. **Wells, J. A., E. Ferrari, D. J. Henner, D. A. Estell, and E. Y. Chen.** 1983. Cloning, sequencing and secretion of *Bacillus amyloliquifaciens* subtilisin in *Bacillus subtilis*. Nucleic Acids Res. **22:**7911–7925.
31. **Weyer, K. A., W. Schaefer, F. Lottspeich, and H. Michel.** 1987. The cytochrome subunit of the photosynthetic reaction center from *Rhodopseudomonas viridis* is a lipoprotein. Biochemistry **26:**2909–2914.
32. **Yanigida, N., T. Ouozomi, and T. Beppu.** 1986. Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli*. J. Bacteriol. **166:**937–944.
33. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.