Deletion Analysis of the Proteinase Gene of Streptococcus cremoris Wg2

Kok, Jan; Hill, Dymphna; Haandrikman, Alfred J.; Reuver, Marjon J.B. de; Laan, Harm; Venema, Gerhardus

Published in:
Applied and environmental microbiology

Publication date:
1988

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1988, p. 239–244
0099-2240/88/010239-06$02.00/0

Vol. 54, No. 1

Deletion Analysis of the Proteinase Gene of *Streptococcus cremoris* Wg2

JAN KOK,[1]* DYMPHNA HILL,[1] ALFRED J. HAANDRIKMAN,[1] MARJON J. B. DE REUVER,[1] HARRY LAAN,[2] AND GERARD VENEMA[1]

Institute of Genetics[1] and Department of Microbiology,[2] University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Received 26 June 1987/Accepted 22 October 1987

The *Streptococcus cremoris* Wg2 proteinase gene, cloned in *S. lactis*, specified a proteinase which exhibited the same specificity toward casein as did the proteinase isolated from the original host. Although the cloned gene lacked the last 130 codons, the proteinase still specifically degraded β-casein. Deletion of the C-terminal 343 amino acids from the proteinase did not influence this specificity. Cell-free transcription-translation studies of plasmids carrying deletion derivatives of the proteinase gene showed that the 100-kilodalton C-terminally truncated proteinase still exhibited proteolytic activity. Crossed immunoelectrophoresis revealed that proteins A and B identified in the proteolytic system of *S. cremoris* Wg2 are both encoded by the proteinase gene. A working model based on integration of available genetic, immunological, and biochemical data is presented to explain this result.

Recently, the complexity of the proteolytic systems of lactic acid streptococci has received much attention (for reviews, see references 12 and 18). Several studies on the same set of *Streptococcus cremoris* strains have resulted in their classification on the basis of three different characteristics of their proteolytic systems. The earliest attempt to classify the strains was based on their proteinase activities as measured on whole cells (4). Three activities were distinguished: two acid proteolytic activities that differ in optimum temperature, PI and PIII (40 and 30°C, respectively), and a third activity at 30°C that was optimal at neutral pH (PII). On the basis of the presence of combinations of these activities, the *S. cremoris* strains were divided into five groups. The difficulty in assigning these activities to separate enzymes led Hugenholtz et al. (7) to raise antibodies against partially purified proteolytic systems of representatives of all five activity groups and to characterize the proteolytic systems immunologically. This work resulted in classification of the strains into four groups according to strain-specific precipitation peaks in crossed immunoelectrophoresis (CIE). However, inconsistencies between the results of the two approaches exist. A third classification used differences in the specificities of partially purified proteolytic systems to distinguish among *S. cremoris* strains (20). Depending on the specific breakdown products produced from α_{s1}-, β-, and κ-casein, the strains were classified into three groups: one each exemplified by *S. cremoris* HP (HP type) and *S. cremoris* AM1 (AM1 type) and a third group containing strains that exhibit both specificities.

In the same report, Visser et al. (20) mentioned that the PII activity reported earlier (4) could be ascribed to a difference in the stability of PI under the reaction conditions used to define PI and PII. Furthermore, they showed that it was difficult to isolate all components of the proteolytic system from some strains in a reproducible way, an observation also made in the immunological analysis of the proteinases (7, 8). In *S. cremoris* AC1, Geis et al. (6) observed a dual reaction in proteinase activity tests. Whole cells possessed a proteolytic activity optimal at acid pH and at a temperature of 30°C (PIII), whereas the purified proteinase had an optimum temperature of 40°C (PI). A property further hampering the biochemical characterization of the proteinases is their inherent instability. Visser et al. (20) used only partially purified proteolytic systems because the more purified enzyme preparations "lost their activity rather quickly." Hugenholtz et al. (8) showed that care has to be taken to ascribe a single band on a sodium dodecyl sulfate (SDS)-polyacrylamide gel to a single proteinase because both proteinases A and B of the *S. cremoris* Wg2 proteolytic system differed only slightly in size and could not be separated by conventional column chromatographic methods. Part of the observed instability of proteolytic activity and the variable properties of the isolates may be ascribed to autoproteolysis. Geis et al. (6) added 20 mM $CaCl_2$ to the purified enzyme of *S. cremoris* AC1 to prevent self-digestion. Several proteolytically active components with molecular weights as low as 60,000 could be isolated from *S. cremoris* Wg2 (J. Erkelens, personal communication). The same was observed during purification of the proteolytic activity from *S. cremoris* HP (4a).

These complications in the purification of proteolytic systems and the characterization of their biochemical properties prompted us to investigate the proteinase complexity in lactic streptococci in a different way. In a previous paper we reported on the cloning of a DNA fragment that specifies proteins A and B of the *S. cremoris* Wg2 proteolytic system (10). In subsequent work we showed that two incomplete open reading frames (ORFs) were present on the DNA fragment, one of which specified a serine proteinase (8a). Here we present data on the specificity of the proteinase cloned in the heterologous host *S. lactis* and on in vitro deletion experiments meant to establish the relationship between proteins A and B of the proteolytic system of *S. cremoris* Wg2.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Bacillus subtilis*, *S. cremoris*, and *S. lactis* were grown and maintained as previously described (10). Derivatives of plasmid pGKV500 were constructed in *B. subtilis* PSL1 (16). *S. cremoris* and *S.*

* Corresponding author.

lactis cells used for the proteinase specificity test were grown in 10% (wt/vol) reconstituted skim milk.

Molecular cloning. Plasmids were isolated as described previously (10). Restriction enzymes, Klenow fragment of *Escherichia coli* DNA polymerase I (Klenow polymerase), and T4 DNA ligase were used as instructed by the supplier (Boehringer Mannheim Biochemicals) as was *dam* methylase (New England BioLabs, Inc.). General procedures for DNA manipulations and cloning were essentially as described by Maniatis et al. (13). Transformation of *B. subtilis* protoplasts was done by the method of Chang and Cohen (1). Protoplasts of *S. lactis* were transformed by using the transformation protocol with liposomes developed by van der Vossen et al. (FEMS Microbiol. Lett., in press).

In vitro deletions of the proteinase gene. Figure 1 shows the construction, in *B. subtilis*, of a number of deletion derivatives of pGKV500 (10). pGKV500 contains a *Hin*dIII insert carrying ORF1 with 295 codons and the major part (1,772 codons) of the proteinase gene of *S. cremoris* Wg2 (8a). The large *Hin*dIII-*Sal*I fragment of pGKV500 was recloned in pGKV21, a pWV01 derivative (19). This removed the C-terminal 343 amino acids from the proteinase (pGKV502). From pGKV502, the *Sma*I-*Nar*I fragment was removed, and after treatment of the vector fragment with Klenow polymerase this resulted in pGKV512, deleting the C-terminal 771 amino acids from the proteinase. pGKV513 was constructed by treatment of the *Eco*RV-*Sma*I vector fragment of pGKV502 with Kle-now polymerase. The deleted proteinase gene present in pGKV513 contains 164 codons and can specify a protein of approximately 17 kilodaltons (kDa). ORF1 was deleted as shown in Fig. 1. pGKV500 was treated with *E. coli dam* methylase, thereby methylating the 3′ *Cla*I site which is part of the sequence ATCGATC. This protects the site from *Cla*I digestion. Subsequent treatment of *dam*-methylated pGK V500 with *Cla*I and filling in of the recessed ends of the 5′ *Cla*I site with Klenow polymerase converted this *Cla*I site to an *Nru*I site (in pGKV506). ORF1 was deleted from pGKV506 by removal of the 781-base-pair *Nru*I fragment, giving pGKV507. The unique *Cla*I site in pGKV507 was cut with *Cla*I and changed into an *Nru*I site by Klenow polymerase treatment. This resulted in the introduction of two bases in codon 9 of the proteinase gene, thus shifting the reading frame of the gene in pGKV511. The 345-base-pair *Cla*I fragment of pGKV500 was deleted from pGKV500 by cutting with *Cla*I and religation of the large fragment, resulting in plasmid pGKV501.

In vitro transcription and translation. The Amersham procaryotic DNA-directed cell-free coupled transcription-translation system derived from *E. coli* was used throughout (Amersham International, Buckinghamshire, England). $CsCl_2$-purified covalently closed circular DNA (0.5 to 1.0 μg) was used in the reaction. One-third of the reaction mixture was analyzed on a 10% SDS-polyacrylamide gel (11). After electrophoresis, the gels were processed for fluorography as described by Skinner and Griswold (17).

Proteinase isolation and specificity test. To isolate proteinases, *S. cremoris* and *S. lactis* cells were passaged for at least two transfers in 10% reconstituted skim milk supplemented with 0.5% glucose and, for strains carrying pGKV 500 derivatives, 5 μg of erythromycin per ml. This preculture was diluted 30-fold in unautoclaved supplemented 10% reconstituted skim milk (freshly prepared in sterile distilled water, buffered with 75 mM β-glycerophosphate, and centrifuged at 120 × *g* for 5 min).

The cultures were grown for 3 to 4 h at 30°C and placed on ice for 5 min. Subsequently, the cells were spun out at 12,000 × *g* (1 min), washed with 50 mM Tris hydrochloride (pH 7.5)–25 mM $CaCl_2$ (4°C), and suspended in 1/1,000 the original volume of 100 mM sodium phosphate buffer (pH 7.0). The supernatants of cells subjected to two successive 30-min incubation periods at 30°C were pooled and used for the specificity test. The pooled fractions were mixed 1:1 with either whole casein (20 mg/ml), or pure β-casein (Sigma Chemical Co.; stock solution of 5 mg/ml). The caseins were

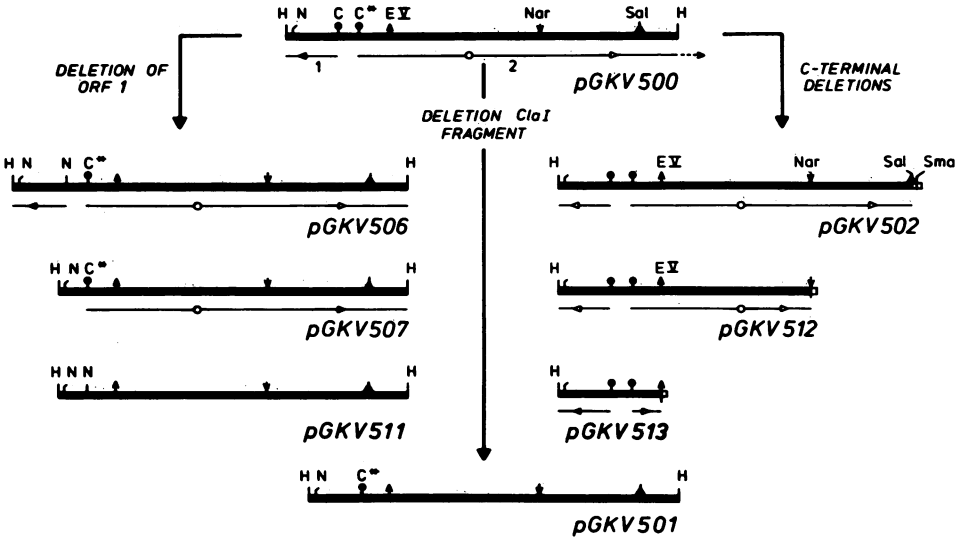

FIG. 1. Construction of deletion derivatives of pGKV500. For reasons of clarity, only the *S. cremoris* Wg2-derived *Hin*dIII fragment present in pGKV500 (10) is shown (bold line). The gene products of ORF1 (1) and ORF2 (2) carried by the various deletion derivatives are drawn as thin lines. Ser-620 of the active site of the proteinase specified by ORF2 (8a) is indicated (○). Cloning of the *Hin*dIII-*Sal*I fragment of pGKV500 in pGKV21 (19) introduced a multiple cloning site at the *Sal*I side of the fragment in pGKV502, of which only the *Sma*I site is shown. Sites: C, *Cla*I; H, *Hin*dIII; N, *Nru*I; EV, *Eco*RV; Nar, *Nar*I; Sal, *Sal*I; ▬, *Sma*I-*Nar*I fusion; ▬, *Sma*I-*Eco*RV fusion; C*, *dam* methylatable *Cla*I site. For details, see the text.

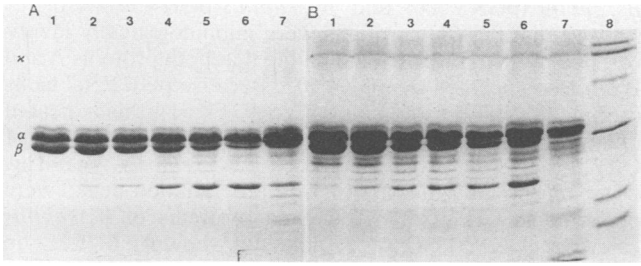

FIG. 2. Substrate specificity of the proteinase activity released from intact cells of *S. cremoris* Wg2 (Prt$^+$) (panel A) and *S. lactis*(pGKV500) (panel B). Lanes: 1 to 7, 0, 1, 2, 4, 6, 8, and 24 h of incubation, respectively; 8, molecular weight standards of 12,300 (cytochrome *c*), 17,200 (myoglobin), 30,000 (carbonic anhydrase), 45,000 (ovalbumin), 66,250 (bovine serum albumin), and 76,000 to 78,000 (ovotransferrin). α-, β-, and κ-casein bands are indicated. Reaction products were analyzed on a 15% SDS-polyacrylamide gel.

dissolved in 100 mM sodium phosphate buffer (pH 7.0). β-Casein was prepared immediately before use, and whole casein was stirred overnight. The samples were incubated at 30°C, and 25-μl samples were taken at various time intervals. After being mixed 1:1 with protein solvent (125 mM Tris hydrochloride, 10 mM EDTA, 4% SDS, 25% glycerol, 5% β-mercaptoethanol, 0.07% bromophenol blue), the samples were analyzed on 15% SDS-polyacrylamide gels as described by Laemmli and Faure (11). The gels were stained with Coomassie brilliant blue. The molecular weight standard (LKB Instruments, Inc.) ranged from 12,300 to 78,000.

CIE. Overnight cultures of *B. subtilis* carrying pGKV500 or one of its derivatives were diluted 100-fold in tryptone yeast broth and grown to mid-log phase (optical density at 660 nm, about 0.6). Cells were removed by centrifugation, and ammonium sulfate precipitation was performed on the supernatant of the culture (ammonium sulfate was added to 60% saturation). The precipitate of 250 ml of culture was collected by centrifugation (30 min, 50,000 × *g*, 4°C) and dissolved in 200 μl of 25 mM Tris hydrochloride (pH 7.0)–25 mM NaCl. After dialysis against several changes of distilled water, the samples were stored at −20°C until used for CIE. CIE was done as described previously (7, 10), with 10 to 15 μl of the proteinase preparation and 50 to 75 μl of antibodies (28 mg/ml) specific for the purified proteolytic system of *S. cremoris* Wg2. Antibodies were kindly provided by J. Hugenholtz.

RESULTS

Specificity of cloned *S. cremoris* Wg2 proteinase. pGKV500 (Fig. 1) carries a 6.5-kilobase *Hin*dIII fragment from the proteinase plasmid of *S. cremoris* Wg2 (10). DNA sequence analysis revealed two incomplete ORFs on the DNA fragment (8a). The largest of the two ORFs specifies a serine proteinase but lacks the last 130 codons at the 3′ end. Complementation analysis showed that the proteinase gene was still able to confer a Prt$^+$ phenotype on the host (10). The partially purified proteinase of *S. cremoris* Wg2 specifically breaks down β-casein (20). To investigate whether the introduction of the proteinase gene in the heterologous host *S. lactis* or the absence of the C-terminal 130 amino acids affected the specificity of the proteinase, its action on different caseins was examined. The proteolytic activity was released from milk-grown whole cells of *S. lactis*(pGKV500) by incubation in Ca^{2+}-free buffer (15). The specificity of whole-casein breakdown was compared with that of the proteinase(s) released from *S. cremoris* Wg2 cells. Both extracts specifically degraded β-casein from the mixture of α-, β-, and κ-casein (Fig. 2). pGKV502, lacking the terminal 343 codons of the proteinase gene (Fig. 1), was introduced into *S. lactis* (Prt$^-$) and still enabled the cells to grow rapidly in milk. The leakage fraction obtained from these cells also specifically degraded β-casein (data not shown). The hydrolysis of pure β-casein was investigated by using the leakage fractions from *S. lactis*(pGKV502) and *S. cremoris* Wg2. Both extracts degraded β-casein into three major fragments (Fig. 3). This breakdown was very rapid and occurred within minutes. This result indicated that the specificity of the proteinase, lacking 130 amino acids (in pGKV500), or 343 amino acids (in pGKV502) at its C terminus, was not altered.

In vitro transcription and translation. All of the derivatives of pGKV500 depicted in Fig. 1 were analyzed in an *E. coli*-derived cell-free transcription-translation system. pGKV500 is pGKV2 containing a 6.5-kilobase *Hin*dIII insert (10). From a comparison of lanes 2 and 7 in Fig. 4, it is clear that the *Hin*dIII fragment specified a number of bands. A high-molecular-weight band of approximately 180,000 can be seen in the pGKV500 sample, as well as a multitude of smaller protein bands. A marked band at about 30,000 is the protein specified by ORF1 (295 codons; 8a), because in the sample derived from pGKV507, lacking ORF1 (Fig. 1), this band is missing (Fig. 4, lane 5). All bands disappeared when the 345-base-pair *Cla*I fragment was removed from pGKV500 (data not shown), indicating that this fragment promoted the expression of both genes on the *Hin*dIII fragment. A frame shift introduced in codon 9 of the proteinase gene resulted in the loss of all bands above 30,000 (Fig. 4, compare lanes 5 [pGKV507] and 6 [pGKV511]). Evidently, the putative transcription and translation signals as defined earlier are functional in vitro, and the translation start is most probably at the ATG just upstream of the 3′ *Cla*I site (8a). The effect of deletions made at the 3′ end of the proteinase gene on the banding pattern is shown in Fig. 4, lanes 2 to 4. Whereas pGKV500 (lane 2) gave a band at approximately 180,000, pGKV502 and pGKV512 gave the highest-molecular-weight bands at approximately 170,000 and 100,000, respectively. This is in agreement with what was expected from the length of the proteinase gene remaining in the various deletion derivatives (8a). The cell wall-bound *S. cremoris* proteinases are inhibited by phenylmethylsulfonyl fluoride (PMSF) (4a, 6, 7) and by Ca^{2+} ions at concentrations of 10 mM and greater (6). At least six bands in the sample derived from pGKV500 seem to be self-digestion products of the high-

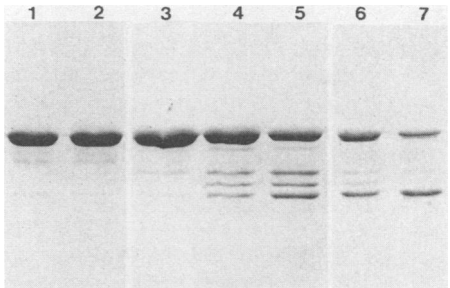

FIG. 3. Breakdown of β-casein by proteinase activity released from intact cells of *S. cremoris* Wg2 (Prt$^-$) (lanes 1 and 2), *S. cremoris* Wg2 (Prt$^+$) (lanes 3 to 5), and *S. lactis*(pGKV502) (lanes 6 and 7). Times of incubation (lanes): 1, 2 h; 2, 4 h; 3, 0 min; 4 and 6, 20 min; 5 and 7, 40 min.

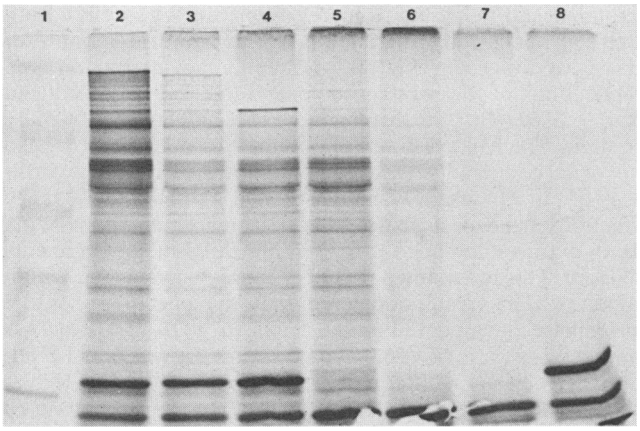

FIG. 4. In vitro transcription-translation products derived from (lanes): 2, pGKV500; 3, pGKV502; 4, pGKV512; 5, pGKV507; 6, pGKV511; 7, pGKV2; 8, pGKV513. Lane 1, ^{14}C-methylated size reference: 200 kDa (myosin), 92.5 kDa (phosphorylase *b*), 69 kDa (bovine serum albumin), 46 kDa (ovalbumin), and 30 kDa (carbonic anhydrase). The reaction products were separated on a 10% SDS-polyacrylamide gel.

molecular-weight proteinase initially formed. This is indicated by the fact that their appearance was inhibited by the addition of 10 mM $CaCl_2$ or PMSF (at 0.5 or 5 mM) to the reaction mixture (Fig. 5). Moreover, a concomitant increase in intensity of the 180-kDa band was observed. The internal control, the 30-kDa band specified by ORF1 of pGKV500, showed that the transcription-translation system was not affected by these additions. Since the same bands were also present in the pGKV512 sample, it can be concluded that the 100-kDa C-terminally truncated protein specified by pGKV512 still exhibited proteinase activity (Fig. 4, lane 4).

CIE. Proteins present in the supernatant of logarithmically grown *B. subtilis* cells carrying the various pGKV500 derivatives were isolated as described in Materials and Methods and subjected to CIE. pGKV500 specified proteins A and B from the *S. cremoris* Wg2 proteolytic system (7, 10; Fig. 6A). From the supernatant of *B. subtilis*(pGKV507) two proteins could also be isolated that reacted with the antibodies to give two peaks in CIE (Fig. 6C). Tandem CIE of *B. subtilis*(pGKV500) and *B. subtilis*(pGKV507) isolates showed that the four proteins were immunologically identical, two by two (Fig. 6E), indicating that both proteins A and B are also specified by pGKV507. Because pGKV507 lacks the small ORF1 present on pGKV500 (Fig. 1), this indicated that proteins A and B both resulted from expression of ORF2, the serine proteinase gene of *S. cremoris* Wg2. This was confirmed by the fact that no precipitation peaks were observed in CIE with extracellular proteins of *B. subtilis* carrying pGKV513 (Fig. 1; data not shown). In CIE on extracellular proteins of *B. subtilis* carrying pGKV511 (Fig. 1), no protein-antibody precipitation lines could be detected (data not shown). These results show that either protein A or B is a product of the other. Whereas *B. subtilis*(pGKV502) still produced proteins A and B (Fig. 6B), in CIE only one precipitation line was obtained from the strain carrying pGKV512 (Fig. 5D). Since the pGKV512 peak fused with protein B of the pGKV500 sample in tandem CIE (Fig. 5F), the epitopes for protein A are either completely removed by the deletion in pGKV512 or masked in the truncated protein specified by this plasmid.

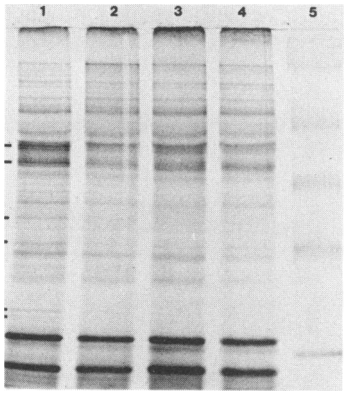

FIG. 5. Products of in vitro transcription-translation directed by pGKV500. Additions made to the standard reaction mixture (lanes): 2, 10 mM $CaCl_2$; 3, 0.5 mM PMSF; 4, 5 mM PMSF. Lane 5, Size references (same as in Fig. 4.). The bands that disappeared in the presence of Ca^{2+} and PMSF are indicated.

DISCUSSION

From the results presented here, it is clear that the serine proteinase gene originating from *S. cremoris* Wg2 and cloned in *S. lactis* specifies a functional proteinase. The data obtained from the cell-free transcription-translation system show that both ORFs identified on the *Hin*dIII fragment in pGKV500 are preceded by functional signals to promote their expression. Apparently, the small *Cla*I fragment located between both ORFs contains these signals. Introduction of a 2-base-pair frame shift close to the start of the proteinase gene resulted in the disappearance of all of the bands specified by this gene. This result indicates that these bands are not the result of translational reinitiation but are products of autolysis of the original 180-kDa proteinase, are caused by premature interruption of transcription-translation, or both. Moreover, the results obtained with the frameshift mutation show that the putative ribosome-binding site and translation start were, most probably, correctly

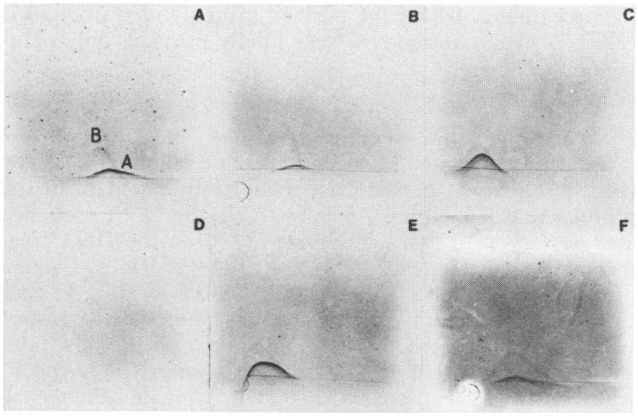

FIG. 6. CIE of extracellular proteins of *B. subtilis* carrying pGKV500 (A), pGKV502 (B), pGKV507 (C), and pGKV512 (D). Tandem CIE of extracellular proteins of *B. subtilis*(pGKV500) and (pGKV507) (E), and *B. subtilis*(pGKV500) and (pGKV512) (F). In both panels E and F, the pGKV500 extract was applied in the lower sample hole. In gel A, the peaks corresponding to proteins A and B are indicated.

identified (8a). Not only did the proteinase gene restore proteolytic activity in the proteinase-deficient *S. lactis* strain to which it was transferred (10), but the proteinase also exhibited the same specificity in this new host. Like the proteinase isolated from *S. cremoris* Wg2, it degraded only β-casein. This specificity was not altered, although the gene was partially cloned and encoded only the N-terminal 85% of the proteinase. From the DNA sequencing data, it was known that all three regions with homology to the subtilisins are located between residues 200 and 700 and, most probably, all of the features important for enzyme activity are contained within the first 700 amino acids of the proteinase (8a). This seems to be confirmed by the cell-free transcription-translation experiments showing that the proteinase gene lacking the terminal 771 codons (in pGKV512) specified a truncated protein of 100 kDa that still exhibited proteinase activity resulting in autoproteolysis. Apparently, a large part of the C terminus of the proteinase can be removed without impairment of proteolytic activity. This conclusion is in agreement with proteinase purification data, indicating that proteolytically active autolysis products as small as 60 kDa can be isolated (Erkelens, personal communication). Moreover, the casein breakdown tests showed that at least part of the C-terminal end can be deleted without loss of proteinase specificity.

The present paper shows that both proteins A and B of the proteolytic system are specified by the proteinase gene of *S. cremoris* Wg2. Because nucleotide sequence data excluded the possibility of overlapping genes (8a), this implies that either protein A or B is derived from the other. Removal of the last 771 codons from the proteinase gene resulted in loss of the protein A peak in CIE, whereas a peak immunologically identical to peak B was still present. The fact that proteins A and B never fuse in CIE (7) implies that the antigenic determinants of A and B are different. Therefore, a change of one proteinase from conformation A to conformation B has to be postulated in such a way that the antigenic determinants of one conformation of the protein completely mask the antigenic determinants of the other conformation.

From DNA sequence analysis, it is known that the proteinase gene contains 1,902 codons and can, therefore, specify a protein of 200 kDa. The largest enzyme forms isolated are, however, approximately 140 to 145 kDa, and smaller autoproteolytic products are often observed during the purification of lactic streptococcal proteinases (4a; H. Laan, unpublished data; Erkelens and Geis, personal communication). To fit these data, we postulated a self-digestion step during incubation of the cells in Ca^{2+}-free buffer, which may be necessary to release proteinase from the cell wall (8a). In Fig. 7, a working model is presented which integrates genetic, immunological, and biochemical data and assumptions which may account for a number of characteristics of the proteolytic system of *S. cremoris* Wg2 and, probably, other lactic streptococci.

In this model, we envisage that a large cell wall-bound proteinase is released from the cell wall by a self-digestion step (activated by incubation of the cells in a Ca^{2+}-free or low-Ca^{2+} buffer). As pointed out earlier, it may well be that the mature cell wall-bound proteinase is actually about 180 kDa as the result of an activation step that removes a putative N-terminal prepro sequence from the initial 200-kDa proteinase. The hydrolysis postulated to detach the mature proteinase from the cell wall may take place at a putative self-digestion site present in the C terminus of the proteinase, resulting in formation of a 140-kDa truncated proteinase (8a). In analogy to the situation in the cloned *S. cremoris* SK11 proteinase, the C terminus of the *S. cremoris* Wg2 proteinase may be involved in cell wall binding (2). The released proteinase exposes antigenic determinants for conformation A. As the result of further self-digestion, removing an amino acid sequence stabilizing conformation A, the proteinase may change to conformation B, exposing a different set of epitopes. Because the molecular weight difference between proteins A and B is very small (8), this amino acid sequence has to be of limited size, at most, some tenths of residues. Alternatively, the lack of Ca^{2+} ions, which are known to stabilize several proteinases (3, 5, 14), may cause destabilization and, consequently, a transition of the proteinase from one conformation to the other. Both possibilities are depicted in Fig. 7. Whether caused by self-digestion, the absence of Ca^{2+} ions, or a combination of both possibilities, the difference between conformations A and B is likely to be of limited extent. This small difference can only be reconciled with the immunological data by assuming that the antibody preparation used to differentiate between proteins A and B contained only a limited number of antibodies specific for each protein. In the 100-kDa C-terminally truncated proteinase specified by pGKV512, either all of the determinants for conformation A were removed or the presumptive amino acid sequence that stabilizes conformation A was removed. Both would result in an irreversible

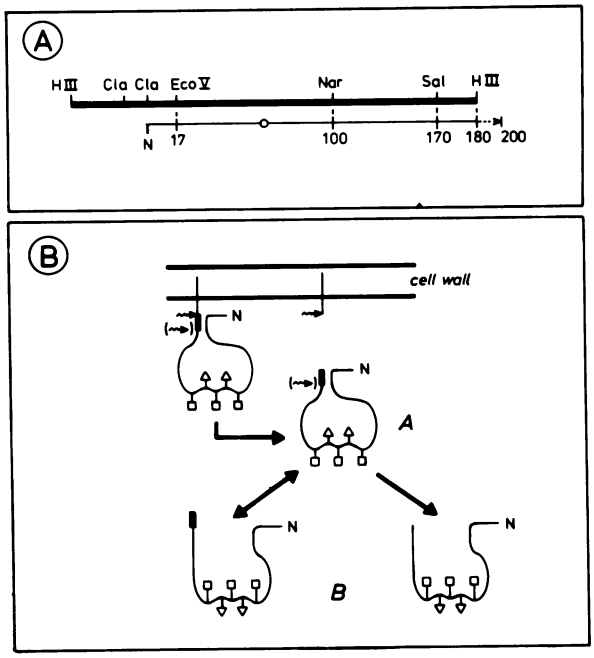

FIG. 7. Proteinase degradation model. Panel A shows the *Hin*dIII fragment of pGKV500 with restriction enzyme sites of interest (bold line) and the *S. cremoris* Wg2 proteinase it encodes. Numbers refer to sizes of proteinase gene products (in kilodaltons) of the whole gene and of the various deletion derivatives. Ser-620 is indicated (○). In panel B, the proteinase is shown, bound to the cell wall by its C terminus. The wavy arrows indicate putative self-digestion sites. One site is hydrolyzed to yield a soluble truncated proteinase in conformation A. Conformation A is changed to conformation B by either self-digestion (single-headed arrow) or limited destabilization due to the absence of Ca^{2+} ions (double-headed arrow). For details, see the text. Sites: Cla, *Cla*I; HIII, *Hin*dIII; EcoV, *Eco*RV; Nar, *Nar*I; Sal, *Sal*I. N, N terminus; ▬, putative amino acid sequence that stabilizes conformation A; ▭, antigenic determinant in conformation A; ◁, antigenic determinant in conformation B.

change to conformation B. It is easy to envisage that proteinase B is degraded further, which may ultimately lead to isolation of an active 60-kDa autoproteolysis product. Whether the protein C observed in some strains of *S. cremoris* (7) is such an autoproteolytic fragment remains to be established. The presence of self-digestion sites in the proteinase amino acid sequence and the accessibility of such putative sites may explain the observed reproducible differences between the CIE precipitation patterns of the strains of *S. cremoris* tested (7). Proteinase A is immunologically indistinguishable in all strains (7). Therefore, the differences among the proteinases of the various strains with regard to casein breakdown have to be ascribed to differences in the amino acid sequences of the enzymes which are not detectable in CIE. The proteinase gene of *S. cremoris* SK11, a strain that exhibits AM1-type specificity (20), has recently been cloned (2). The elucidation of the nucleotide sequence of this proteinase gene and comparison of this sequence with that of the *S. cremoris* Wg2 proteinase gene, which specifies an HP-type enzyme, should reveal the difference between the two proteinases at the amino acid level and explain their different specificities.

ACKNOWLEDGMENTS

This work was supported by the Programme Committee on Biotechnology of the Netherlands and the Biomolecular Engineering Programme of the Commission of the European Communities.

We thank Beike Leegte for typing the manuscript and Henk Mulder for photography and preparation of the figures.

LITERATURE CITED

1. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168:**111–115.
2. **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. Final report. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
3. **Diermayer, P., S. Kroll, and H. Klostermeyer.** 1987. Mechanisms of heat inactivation of a proteinase from *Pseudomonas fluorescens* biotype I*. J. Dairy Res. **54:**51–60.
4. **Exterkate, F. A.** 1976. Comparison of strains of *Streptococcus cremoris* for proteolytic activities associated with the cell wall. Neth. Milk Dairy J. **30:**95–105.

4a. **Exterkate, F. A., and G. J. C. M. de Veer.** 1987. Complexity of the native cell wall of *Lactococcus lactis* subsp. *cremoris* Hp and purification of the enzyme. Syst. Appl. Microbiol **9:**183–191.

5. **Feder, J., L. R. Garret, and B. S. Wildi.** 1971. Studies on the role of calcium in thermolysin. Biochemistry **10:**4552–4555.
6. **Geis, A., W. Bockelmann, and M. Teuber.** 1985. Simultaneous extraction and purification of a cell wall-associated peptidase and β-casein specific protease from *Streptococcus cremoris* AC1. Appl. Microbiol. Biotechnol. **23:**79–84.
7. **Hugenholtz, J., F. Exterkate, and W. N. Konings.** 1984. The proteolytic systems of *Streptococcus cremoris*: an immunological analysis. Appl. Environ. Microbiol. **48:**1105–1110.
8. **Hugenholtz, J., D. van Sinderen, J. Kok, and W. N. Konings.** 1987. The cell wall-associated proteases of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. **53:**853–859.

8a. **Kok, J., K. J. Leenhouts, A. J. Haandrikman, A. M. Ledeboer, and G. Venema.** 1988. Nucleotide sequence of the gene for cell wall proteinase of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. **54:**231–238.

9. **Kok, J., J. M. B. M. van der Vossen, and G. Venema.** 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. **48:**726–731.
10. **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.
11. **Laemmli, U. K., and K. Faure.** 1983. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80:**575–599.
12. **Law, B. A., and J. Kolstad.** 1983. Proteolytic systems in lactic acid bacteria. Antonie van Leeuwenhoek J. Microbiol. Serol. **49:**225–245.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Matsubara, H., B. Hagihara, M. Nakai, T. Komaki, T. Yonetani, and K. Okunuki.** 1958. Crystalline bacterial proteinase. II. General properties of crystalline proteinase of *Bacillus subtilis* N′. J. Biochem. **45:**251–255.
15. **Mills, O. E., and T. D. Thomas.** 1978. Release of cell wall-associated proteinase(s) from lactic streptococci. N.Z. J. Dairy Sci. Technol. **13:**209–215.
16. **Ostroff, G. R., and J. J. Pène.** 1983. Molecular cloning with bifunctional plasmid vectors in *Bacillus subtilis*: isolation of a spontaneous mutant of *Bacillus subtilis* with enhanced transformability for *Escherichia coli*-propagated chimeric plasmid DNA. J. Bacteriol. **156:**934–936.
17. **Skinner, M. K., and M. D. Griswold.** 1983. Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem. J. **209:**281–284.
18. **Thomas, T. D., and O. E. Mills.** 1981. Proteolytic enzymes of starter bacteria. Neth. Milk Dairy J. **35:**255–273.
19. **van der Vossen, J. M. B. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50:**540–542.
20. **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 α_{s1}-, β-, and κ-casein. Appl. Environ. Microbiol. **52:**1162–1166.