Cloning and partial sequencing of the proteinase gene complex from *Lactococcus lactis* subsp. *lactis* UC317

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The proteinase genes from *Lactococcus lactis* subsp. *lactis* UC317 were identified on a plasmid, pCI310, which is a deletion derivative of a cointegrate between pCI301, the 75 kb Lac Prt plasmid from UC317 and the 38.5 kb cryptic plasmid from that strain. The *prt* genes were cloned using a replacement cloning strategy whereby fragments from pCI310 were exchanged with the equivalent fragments in pNZ521, which contains the cloned proteinase genes from *L. lactis* subsp. *lactis* SK112. This generated two plasmids which encoded a cell-envelope-associated and a secreted proteinase, respectively. Specific regions of the UC317 structural *prtP* gene known to encode seven of the amino acids essential for substrate cleavage specificity were sequenced and compared with the known sequences of *prt* genes from *L. lactis* strains SK112, Wg2 and NCDO763. In spite of various differences that were detected in the nucleotide sequence of this region, it appears that these seven amino acids in strains UC317 and NCDO763 are identical, and represent a combination of three of the amino acids from SK112 and four from Wg2. These results indicate that the UC317 proteinase is a natural hybrid of the SK112 and Wg2 proteinases.

Introduction

Members of the genus *Lactococcus* are used as starter bacteria in the manufacture of a range of fermented milk products and consequently are of considerable commercial importance (Daly, 1983; Sandine, 1988). Lactococci are nutritionally fastidious and have complex amino acid requirements (Thomas & Pritchard, 1987). Since free amino acids are not present at sufficient levels in milk to support optimal growth of these bacteria, the concerted action of the cell-envelope-associated proteinase and a range of peptidases is required to release peptides and amino acids from the milk protein, casein. Thus in fermented milks a functional proteolytic system is essential for growth and reliable acid production, while

in ripened products proteolysis is also necessary for the development of unique and fine flavour characteristics (Law & Kolstad, 1983; Thomas & Pritchard, 1987; Laan *et al.*, 1989). Genetic studies have established that, like other important traits in these bacteria, proteinase production is invariably linked to plasmid DNA (Otto *et al.*, 1982; McKay, 1985; Kok, 1990); and in several strains this trait and the *lac* genes have been shown to be encoded on single lactose/proteinase (Lac Prt) plasmids (McKay & Baldwin, 1974; Gasson *et al.*, 1987; Hayes *et al.*, 1990).

Biochemical analysis of the cell-envelope-associated proteinases has indicated that they are serine proteinases of molecular mass approximately 145 kDa, they have a pH optimum between 5.5 and 6.5 and they require calcium for stable attachment to the cell envelope (Exterkate & de Veer, 1987a; Geis *et al.*, 1985; Hugenholtz *et al.*, 1987; Monnet *et al.*, 1987). Based on their casein degradation specificities, a simplified classification of proteinase enzymes from lactococci has been proposed (Visser *et al.*, 1986). PI-type proteinases primarily degrade β -casein, with only very slow rates of hydrolysis of α - and κ -casein, whereas the PIII type degrade α - and κ -casein in addition to β -casein.

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The nucleotide sequence data in this paper have been submitted to GenBank and have been assigned the accession number M85288.

Considerable interest has been focused on the physical characterization of proteinase genes, and those from *L. lactis* subsp. *cremoris* strains Wg2 (PI type; Kok *et al.*, 1985, 1988), SK112 (PIII type; de Vos *et al.*, 1989; Vos *et al.*, 1989a, b) and *L. lactis* subsp. *lactis* NCDO763 (Kiwaki *et al.*, 1989) have been extensively studied. In addition, parts of the proteinase genes from *L. lactis* subsp. *cremoris* E8 (Kok, 1990), ML1 (P. Vos and others, unpublished results) and H2 (Xu *et al.*, 1990), as well as that from *L. lactis* subsp. *lactis* 712 (Gasson *et al.*, 1987), have been cloned and expressed in *L. lactis* or *Escherichia coli*. Deletion, subcloning and nucleotide sequence analyses of cloned proteinase genes from *L. lactis* subsp. *cremoris* Wg2 and SK112 have revealed the presence of two essential, adjacent but divergently transcribed genes designated *prtP* and *prtM*. The *prtP* gene encodes a 200 kDa pre-pro-proteinase (Kok *et al.*, 1988; Kiwaki *et al.*, 1989; Vos *et al.*, 1989a) while the *prtM* gene specifies a 30 kDa lipoprotein which is implicated in the conversion of the inactive *prtP* product to an active form (Vos *et al.*, 1989b; Haandrikman *et al.* 1989).

Preliminary studies on the purified proteinase from *L. lactis* subsp. *lactis* UC317 indicated that it degrades α - and β -casein in a manner which distinguishes it from the PI-type proteinase of Wg2 and the PIII type of SK112 (J. Law and others, unpublished data). These differences suggested that this enzyme possesses a novel activity,

which warranted molecular analysis of the *prtP* gene of strain UC317. The cloning and analysis of this gene are described here. Previous results relating to the characterization of the *prtP* gene of Wg2 and SK112 allowed specific regions of the UC317 determinant to be targeted for comparative analysis, particularly the domains responsible for substrate cleavage specificity (Vos *et al.*, 1991).

Methods

Bacterial strains and plasmids. These are described in Table 1.

Media. *E. coli* strains were grown in Luria-Bertani medium (Maniatis *et al.*, 1982) at 37 °C. Wild-type *L. lactis* strains were grown at 30 °C in M17 medium (Terzaghi & Sandine, 1975). *L. lactis* MG1363Sm (Gasson, 1983), the plasmid-free derivative of strain 712, was used as host in lactococcal transformations and was maintained in M17 medium in which glucose (0.5%) replaced lactose (GM17). For solid media, agar (1.5%, w/v, Oxoid no. 3) was added to broth before autoclaving. Chloramphenicol (5 and 20 µg ml⁻¹ for *L. lactis* and *E. coli*, respectively) was included in media when required. To assess the ability of selected lactococcal strains to utilize milk proteins, reactions on fast slow differential agar (FSDA; Huggins & Sandine, 1984) and in steamed reconstituted non-fat milk (supplemented with 0.5% glucose and/or 0.5% tryptone when required) were examined.

Chemical reagents were obtained from Sigma.

Plasmid preparation, analysis and transformation. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Birnboim & Doly

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Characteristics	Reference or source
<i>E. coli</i>		
MC1061	<i>hsdR araD139 Δ(ara-leu)7697 rpsL lacZ74 galK</i>	Casadaban & Cohen (1980)
TG1	<i>Δ(lac-pro) supE thi hsdD5/F' traD36 proA⁺B⁺ lac^R lacZΔM15</i>	Clark (1987)
<i>L. lactis</i>		
subsp. <i>lactis</i>		
MG1363Sm	Lac ⁻ Prt ⁻ Sm ^R plasmid-free derivative of <i>L. lactis</i> NCDO712	Gasson (1983)
UC317	Lac ⁺ Prt ⁺ multiplasmid wild-type strain	Hayes <i>et al.</i> (1990)
NCDO763	Lac ⁺ Prt ⁺ multiplasmid wild-type strain	NCDO culture collection
FH041	Lac ⁺ Prt ⁻ derivative of UC317	F. Hayes (pers. comm.)
FH048	Lac ⁺ Prt ⁺ Sm ^R MG1363Sm harbouring pCI301	F. Hayes (pers. comm.)
FH088	Lac ⁻ Prt ⁺ Sm ^R MG1363Sm harbouring pCI310	Hayes <i>et al.</i> (1990)
J3601	Lac ⁻ Prt ⁺ Sm ^R MG1363Sm harbouring pCI3601	This work
J3602	Lac ⁻ Prt ⁺ Sm ^R MG1363Sm harbouring pCI3602	This work
<i>L. lactis</i>		
subsp. <i>cremoris</i>		
SK112	Lac ⁺ Prt ⁺ multiplasmid wild-type strain	de Vos <i>et al.</i> (1984)
Wg2	Lac ⁺ Prt ⁺ multiplasmid wild-type strain	NIZO culture collection
Plasmids		
pNZ122	Cm ^R Km ^R , pSH71 replicon (2.8 kb)	de Vos (1986)
pNZ123	pNZ122 with multiple cloning site	P. Vos <i>et al.</i> (unpublished)
pNZ521	Cm ^R Km ^R , pSH71 replicon, Prt ⁺ (10.5 kb)	de Vos <i>et al.</i> (1989)
pCI301	75 kb Lac ⁺ Prt ⁺ plasmid from UC317	Hayes <i>et al.</i> (1990)
pCI303	38 kb cryptic plasmid from UC317	Hayes <i>et al.</i> (1990)
pCI310	22.5 kb Prt ⁺ : deletion derivative of a cointegrate between pCI301 and pCI303	Hayes <i>et al.</i> (1990)
pCI3601	3.0 kb <i>Bst</i> EII- <i>Sac</i> I fragment from pCI3603 subcloned into <i>Bst</i> EII/ <i>Sac</i> I-cleaved pCI3602 (10.5 kb)	This work
pCI3602	6.5 kb <i>Hind</i> III fragment of pCI310 subcloned in <i>Hind</i> III-cleaved pNZ521 (10.5 kb)	This work
pCI3603	3.5 kb <i>Bgl</i> II- <i>Xba</i> I fragment of pCI310 cloned into pNZ123 (6.3 kb)	This work

(1979) and from *L. lactis* by a modification of this method (de Vos *et al.*, 1984). DNA was then prepared for analysis essentially as described by Hayes *et al.* (1990). Restriction endonucleases and T4 DNA ligase were supplied by Pharmacia and were used according to the supplier's instructions. Restriction endonuclease digestions were analysed by electrophoresis on 0.7% horizontal agarose gels containing 0.5 µg ethidium bromide ml⁻¹ and run at 100 V cm⁻¹ for 3 h in Tris/acetate (pH 7.6) buffer. DNA fragments were recovered from agarose gels using the 'Gene Clean' kit (Bio 101). *E. coli* was transformed by the method of Mandel & Higa (1970); *L. lactis* was transformed by electroporation using a Gene Pulser apparatus (Bio-Rad), as follows. *L. lactis* MG1363Sm cells were grown in M17 medium containing 0.5% (w/v) glucose and 40 mM-DL-threonine to an OD₆₀₀ of 0.5. Cells were harvested by centrifugation, washed three times in ice-cold sterile distilled water, and resuspended in 1/50 the original volume of 15% (v/v) ice-cold glycerol. DNA was added to 400 µl of cells in 2 mm electroporation cuvettes and subjected to an electric pulse at settings of 2.5 kV, 1000 Ω and 25 µF. Cells were immediately added to 10 ml GM17 and incubated at 30 °C for 90 min, then plated on selective media.

Proteinase specificity studies. Lactococcal cells were grown on a whey-based medium containing 1.9% (w/v) β-glycerophosphate, 0.5% (w/v) glucose and 0.1% (w/v) casitone (Vos *et al.*, 1989b). Cell-envelope-associated proteinases were liberated by incubation in release buffer (50 mM-sodium acetate/phosphate, pH 6.5) at 30 °C as described by Exterkate & de Veer (1987b) while non-cell-envelope-associated proteinases were isolated by the method of Vos *et al.* (1989b). Fractions were analysed on 0.1% (w/v) SDS, 10% (w/v) polyacrylamide gels (Laemmli, 1970) and proteins were detected by staining with Coomassie brilliant blue R-250. These fractions were also incubated with α- and β-casein (2 mg ml⁻¹ dissolved in 50 mM-sodium acetate/phosphate buffer, pH 6.5, a generous gift from Dr S. Visser, NIZO, Ede, The Netherlands) for various time intervals and the degradation products were subsequently detected on 0.1% SDS, 20% polyacrylamide gels stained with Coomassie brilliant blue. The low-molecular-mass standards ranged from 14.3 to 66 kDa and were supplied by Sigma.

Nucleotide sequence analysis. All DNA fragments used for cloning into vectors M13mp10, M13mp11, M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985) were isolated from agarose gels. Replicative-form and single-stranded phage DNA was isolated from recombinant M13 clones by the method of te Riele *et al.* (1986). Sequencing by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) was performed on both single-stranded M13 DNA and double-stranded plasmid DNA and was initiated using the M13 universal primer or with primers synthesized on a Biosearch Cyclone DNA synthesizer (New Brunswick Scientific). All sequences were determined in both directions.

Growth rate studies. Growth rates of lactococcal cultures in milk-based medium were determined by inoculating (2%, v/v) the test strain into 10% (w/v) reconstituted skim milk supplemented with 0.5% (w/v) glucose and incubating at 30 °C. The percentage of lactic acid developed was monitored at specific time intervals and the growth rates and doubling times calculated.

Results

Cloning of the *prt* gene complex from *L. lactis* subsp. *lactis* UC317

Plasmid pCI310 (Fig. 1a) is a 22.5 kb spontaneous deletion derivative of a cointegrate between the *L. lactis*

Fig. 1. Replacement strategy for cloning the *L. lactis* subsp. *lactis* UC317 proteinase genes. (a) The proteinase-encoding region of pCI310; (b) pCI3602, in which the native 6.5 kb *Hind*III fragment of pNZ521 is replaced with the equivalent fragment from pCI310; (c) pCI3603; (d) pCI3601, containing the intact proteinase genes from pCI310.

UC317 plasmids pCI301 (75 kb, Lac Prt) and pCI303 (38 kb, cryptic) (Hayes *et al.*, 1990). Based on hybridization analysis, detailed restriction mapping and the Prt⁺ phenotype conferred upon pCI310-containing hosts, it has been shown that this plasmid encodes the entire proteinase complex from UC317 (Hayes *et al.*, 1990). The *prt* genes of UC317 were cloned using a replacement cloning strategy whereby a 6.5 kb *Hind*III fragment from pNZ521, which contains the cloned *prtP* and *prtM* genes from SK112, was exchanged with the equivalent 6.5 kb *Hind*III fragment from pCI310, resulting in the 10.5 kb plasmid pCI3602 (Fig. 1a, b).

It was found that unlike the cell-envelope-associated proteinases produced by MG1363Sm containing either pNZ521 or pCI310, the proteinase activity of the same strain harbouring pCI3602 could not be isolated from the cell envelope (Fig. 2, lane 1) but was found exclusively in the growth medium, indicating that the enzyme was secreted (Fig. 2, lane 2). This extracellular location can

Fig. 2. Polyacrylamide gels (10%, w/v) showing production of proteinase from MG1363Sm harbouring either pCI3601 or pCI3602. Lanes: 1, release fraction of MG1363Sm containing pCI3602; 2, growth medium fraction of MG1363Sm containing pCI3602; 3, release fraction of MG1363Sm containing pCI3601; 4, growth medium fraction of MG1363Sm containing pCI3601. S, molecular mass standards (myosin from rabbit muscle, β -galactosidase, phosphorylase b, bovine albumin and egg albumin) on the left.

be attributed to the deletion of a 16 bp *Hind*III fragment in pCI3602, which results in a truncated proteinase lacking the C-terminal membrane anchor, as has been described previously (Haandrikman *et al.*, 1989; Kiwaki *et al.*, 1989; Vos *et al.*, 1989a). To clone the intact and fully functional C-terminus of the structural pCI310-derived *prtP* gene, a 3.4 kb *Xba*I–*Bgl*II fragment from pCI310 (Fig. 1a) was inserted into the high-copy-number vector pNZ123 (de Vos *et al.*, 1989), resulting in pCI3603 (Fig. 1c). A 3.0 kb *Sac*I–*Bst*EII fragment from pCI3603 was subsequently cloned into the equivalent sites on pCI3602, resulting in a 10.4 kb plasmid, pCI3601 (Fig. 1d). As predicted, pCI3601 encoded a fully functional cell-envelope-associated proteinase in that the enzyme was located in the release fraction of the MG1363Sm host (Fig. 2, lane 3) while no enzyme was detected in the medium fraction (lane 4).

Nucleotide sequence analysis of *prtP* from UC317

Specific regions of the *prtP* gene from SK112 which encode amino acids involved in cleavage specificity determination have been identified (Vos *et al.*, 1991; de Vos *et al.*, 1991). The nucleotide sequence of the equivalent regions of the UC317 structural *prtP* gene was determined and found to differ from that of the other known lactococcal sequences (Fig. 3). These specific

Table 2. Summary of seven of the amino acids present in the proteinases of lactococcal strains UC317, SK112, Wg2 and NCDO763 at positions important in substrate cleavage specificity

Amino acid position	UC317/NCDO763	SK112	Wg2
131	Thr	Ser	Thr
138	Thr	Lys	Thr
142	Ala	Ala	Ser
144	Leu	Val	Leu
166	Asp	Asn	Asp
747	Arg	Arg	Leu
748	Lys	Lys	Thr

amino acids and those in corresponding positions in the proteinases of SK112, Wg2 and NCDO763 are summarized in Table 2. Analysis of the sequence shows that the UC317 proteinase enzyme contains alanine, arginine and lysine at positions 142, 747 and 748, respectively, which are also present in the SK112 proteinase, but absent in that of Wg2. However, at positions 131, 138, 144 and 166 the amino acids in the UC317 and Wg2 proteinases are identical, but differ from equivalently positioned amino acids in SK112. All seven amino acids at the positions listed in Table 2 are identical in the UC317 and NCDO763 proteinases. However, when the sequence presented in Fig. 3 is examined, it is clear that the UC317 *prtP* gene has at least four unique nucleotides, resulting in three unique amino acids at positions 691, 694 and 712 not present in the proteinases of NCDO763 or of the other two strains.

Caseinolytic cleavage specificities

The cleavage specificities of proteinases from *L. lactis* strains UC317, NCDO763, Wg2 and SK112 as well as the enzymes from MG1363Sm containing either pNZ521 or pCI3601 are illustrated for α - and β -casein in Figs 4(a) and 4(b), respectively. The cleavage patterns on either α - or β -casein generated by the proteinases isolated from the wild-type UC317 host or from MG1363Sm harbouring the recombinant proteinase plasmid pCI3601 (strain J3601) were identical (Fig. 4a, b, lanes 2 and 3, respectively), substantiating the previous observation by de Vos *et al.* (1989) that the caseinolytic cleavage specificity is determined by the *prtP* gene and not by the host. The cleavage patterns of the two proteinases from UC317 and NCDO763 were also identical (Fig. 4a, b, lanes 2 and 6). This was expected since these proteinases share the same seven amino acids involved in determining cleavage specificity (see Table 2). The Wg2-derived proteinase cleaved β -casein in an

identical manner to UC317 and NCDO763 (Fig. 4b, lanes 2, 6, 7) but did not degrade α -casein (Fig. 4a, lane 7). The α - and β -caseinolytic cleavage specificities of the SK112-derived proteinase differed from those of UC317. Although the UC317 and SK112 enzymes yielded several cleavage products of apparently similar size from either α - or β -casein, the relative amounts of these products differed. For example the 20 kDa product (Fig. 4a, lane 4) of the SK112 proteinase was present in greater concentration than the equivalent-sized protein generated by the UC317 enzyme (lane 2).

Enzyme production and growth studies

When the relative amounts of proteinase enzyme produced by UC317 and by MG1363Sm harbouring either the native Lac P_{rt} plasmid from UC317 (strain FH048) or the cloned *p_{rt}* genes (strain J3601) were compared, it was clear that significantly higher levels were produced by the strains containing the cloned genes (Fig. 5, lanes 2–5). Similarly, higher levels of proteinase were produced from MG1363Sm harbouring pNZ521, which contains the cloned *p_{rt}* genes from SK112, than from the wild-type SK112 strain (Fig. 5, lanes 6 and 7), confirming the previous observations of de Vos *et al.* (1989). However, as expected, no proteinase production was detected in FH041, the P_{rt}[–] derivative of UC317 (Fig. 5, lane 1).

The higher levels of enzyme produced by J3601 compared to FH048 also resulted in more rapid degradation of β -casein (data not shown; following 40 min incubation, complete degradation of β -casein by J3601 had occurred while significant amounts of undegraded substrate remained in the case of the FH048-derived enzyme). However, it is noteworthy that no significant differences were observed between the growth rates, measured in 10% (w/v) reconstituted skim milk supplemented with 0.5% (w/v) glucose, of FH048 ($\mu = 0.439 \text{ h}^{-1}$), J3601 ($\mu = 0.41 \text{ h}^{-1}$) or J3602, the isolate producing the secreted proteinase ($\mu = 0.403 \text{ h}^{-1}$).

Discussion

The proteinase gene complex originally identified on the Lac P_{rt} plasmid pCI301 of *L. lactis* subsp. *lactis* UC317 was cloned from a derivative plasmid pCI310 using a replacement cloning strategy. This approach was necessary since other more direct cloning strategies were unsuccessful. It is interesting that one of the constructs, pCI3602, encoded a secreted enzyme in contrast to the typical cell-envelope-associated enzyme generally ob-

served in the lactococci (Kok, 1990), which was attributable to removal of a 16 bp fragment during the cloning procedure used to generate this recombinant. It appears likely that the 16 bp deletion resulted in transcription termination and consequently a truncated protein which did not possess the membrane-anchoring region (Vos *et al.*, 1989a; Haandrikman *et al.*, 1989).

When the proteinases were isolated from strains J3601 and J3602 and examined by polyacrylamide gel electrophoresis, several bands were observed in addition to the expected 145 kDa mature proteinase (e.g. Fig. 2, lane 3). These are likely to represent autodigestion products since it has been indicated that lactococcal proteinases are subject to self-degradation at the C-terminus (Laan & Konings, 1989).

Nucleotide sequence analysis of regions of the UC317 cloned proteinase proved fruitful in comparing its *p_{rtP}* gene with the well-characterized genes of SK112, Wg2 and NCDO763. Like other lactococcal proteinases, the proteinase from UC317 is a serine proteinase displaying a high degree of homology with the subtilisins, all of which have an active-site triad of aspartic acid, histidine and serine. Included in the regions of the UC317 proteinase which were sequenced (Fig. 3) is a histidine at position 94 which is also seen in the active sites of the SK112, Wg2 and NCDO763 proteinases (Kok, 1990). Also within this sequence is a region including seven amino acids (Table 2) that are involved in substrate binding specificity and showing homology to the substrate-binding region of the serine proteases of the subtilisin family (Vos *et al.*, 1991). Sequence analysis indicated differences between these amino acids in the proteinases from UC317 and from SK112 and Wg2. It appears that the UC317 proteinase, like that from NCDO763, represents a new class of naturally occurring hybrid which exhibits some of the properties of both the PI- and PIII-type proteinases produced by strains Wg2 and SK112, respectively. It is tempting to speculate that a natural recombination event between genes encoding these types of activities could have been responsible for producing the UC317-type proteinase with mixed specificity. Further screening of proteinase activity in other lactococcal strains may reveal additional hybrid-type enzymes with novel caseinolytic specificities.

It is notable that enzymes with novel specificities have been engineered *in vitro* by interchanging regions involved in substrate cleavage specificity between the *p_{rtP}* genes of SK112 and Wg2 (Vos *et al.*, 1991). Nucleotide sequence analysis revealed that the seven amino acids which determined substrate specificity are identical in UC317 and NCDO763 and, as expected, the caseinolytic cleavage specificity of UC317 and NCDO763 was also identical on α - and β -casein. However, despite the similarity between the UC317 and

																						NCD0763
																						Wg2
																						SK112
TAT	TTT	AAT	TCA	AAA	GTG	CCA	TAT	GGG	TTC	AAT	TAC	GCT	GAT	AAT	AAC	GAC	ACC	ATT	ACA	819	UC317	
Y	F	N	S	K	V	P	Y	G	F	N	Y	A	D	N	N	D	T	I	T	86		
																						NCD0763
																						Wg2
																						SK112
GAT	GAT	AAG	GTT	GAC	GAA	CAA	CAC	GGC	ATG	CAT	GTT	GCT	GGG	ATC	ATC	GGT	GCT	AAC	GGG	879	UC317	
D	D	K	V	D	E	Q	H	G	M	H	V	A	G	I	I	G	A	N	G			
																						NCD0763
																						Wg2
																						SK112
ACA	GGT	GAC	GAT	CCA	GCC	AAG	TCT	GTT	GTC	GGA	GTT	GCG	CCA	GAA	GCA	CAG	CTA	CTG	GCA	939	UC317	
T	G	D	D	P	A	K	S	V	V	G	V	A	P	E	A	G	L	L	A	126		
																						NCD0763
																						Wg2
																						SK112
ATG	AAA	GTT	TTC	ACC	AAC	TCT	GAC	ACT	TCT	GCA	ACA	ACC	GGG	TCA	GCT	ACC	TTG	GTT	TCT	999	UC317	
M	K	V	F	(T) S	N	S	D	T	S	A	(T) K	T	G	S	(A) S	T	(L) V	V	S			
																						NCD0763
																						Wg2
																						SK112
GCC	ATT	TAA	GAC	TCG	GCA	AAA	ATC	GGT	GCC	GAT	GTC	CTC	AAC	ATG	TCC	TTA	GGA	TCT	GAT	1059	UC317	
A	I	E	D	S	A	K	I	G	A	D	V	L	N	M	S	L	G	S	(D) N	166		
																						NCD0763
																						Wg2
																						SK112
TCA	GGC	AAT	CAA	ACC	TTG	GAG	GAT	CCA	GAA	CTT	GCT	GCG	GTG	CAA	AAT	GCT	AAC	GAA	TCA	1119	UC317	
S	G	N	Q	T	L	E	D	P	E	L	A	A	V	Q	N	A	N	E	S			
																						NCD0763
																						Wg2
																						SK112
GGA	ACA	GCC	GCC	GTT	ATT	TCT	GCT	GGG	AAC	TCA	GGA	ACA	TCC	GGT	TCA	GCA	ACT	GAA	GGC	1179	UC317	
G	T	A	A	V	I	S	A	G	N	S	G	T	S	G	S	A	T	E	G	206		
																						NCD0763
																						Wg2
																						SK112
GTC	AAC	AAA	GAT	TAT	TAC	GGT	TTG	CAA	GAC	AAT	GAA	ATG	GTG	GGA	ACG	CCA	GGG	ACA	TCA	1239	UC317	
V	N	K	D	Y	Y	G	L	Q	D	N	E	M	V	G	T	P	G	T	S			
																						NCD0763
																						Wg2
																						SK112
CGA	GGA	GCG	ACC	ACA	GTT	GCT	TCC	GCT	TAA	AAC	ACG	GAT	GTC	ATC	ACT	CAG	GCA	GTG	ACC	1299	UC317	
R	G	A	T	T	V	A	S	A	E	N	T	D	V	I	T	Q	A	V	T	246		
																						NCD0763
																						Wg2
																						SK112
ATT	ACA	GAT	GGT	ACA	GGT	TTA	CAG	CTT	GGA	CCG	GAA	ACC	ATT	CAG	CTT	TCA	AGC	AAC	GAT	1359	UC317	
I	T	D	G	T	G	L	Q	L	G	P	E	T	I	Q	L	S	S	N	D			
																						NCD0763
																						Wg2
																						SK112
TTC	ACT	GGT	AGC	TTT	GAC	CAA	AAG	AAG	TTT	TAT	GTT	GTT	AAA	GAT	GCT	AGT	GGC	AAC	CTC	1419	UC317	
F	T	G	S	F	D	Q	K	K	F	Y	V	V	K	D	A	S	G	N	L	286		

Fig. 3. Nucleotide sequence of two distinct regions of pCI3601 encompassing seven amino acids (each in parentheses) known to be important in substrate cleavage specificity. Nucleotide and amino acid numbers on this sequence correspond to those on the published SK112 sequence (Vos *et al.*, 1989a), while nucleotide differences between UC317 and the SK112, Wg2 and NCDO763 sequences, respectively, are indicated in ascending order directly above this sequence.

Fig. 4. Gel electrophoretograms [0.1% (w/v) SDS, 20% (w/v) polyacrylamide] of digestion products produced following incubation of the proteinase enzymes from UC317 (lane 2), J3601 (lane 3), SK112 (lane 4), MG1363Sm harbouring pNZ521 (lane 5), NCDO763 (lane 6) and Wg2 (lane 7) with α -casein (a) or β -casein (b) for 2 h at 30 °C. Undigested casein is shown in lanes 1 of both panels. S, molecular mass standards (bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor and α -lactalbumin).

Fig. 5. Gel electrophoretograms [0.1% (w/v) SDS, 10% (w/v) polyacrylamide] of the proteinases isolated from the following strains: lane 1, FH041; lane 2, J3601; lane 3, FH048; lane 4, J3601; lane 5, UC317; lane 6, MG1363Sm(pNZ521); lane 7, SK112. S, molecular mass standards (myosin from rabbit muscle, β -galactosidase and phosphorylase b).

NCDO763 proteins at these positions, these enzymes are not identical since UC317 has at least three unique amino acids at positions 691, 694 and 712, within the regions sequenced, not seen in NCDO763 or the other two sequenced proteinases. However, these differences had no effect on cleavage specificity. The cleavage patterns of the cloned proteinase enzymes in MG1363Sm were identical to those generated by the wild-type strains, indicating that specificity is independent of the host strain. These results also confirm the validity of the classification of Visser *et al.* (1986), who showed that PI-type proteinases degrade β - and not α -casein while the PIII type degrade both α - and β -casein. However, this and other studies indicate that it may be necessary to broaden this classification scheme to include natural hybrid enzymes with the specificities of UC317 and NCDO763 which degrade β -casein in a PI-type manner but which also degrade α -casein like PIII-type proteinases.

Cloning of proteinase genes in a high-copy-number vector clearly resulted in higher amounts of enzyme production. This is consistent with the findings of de Vos *et al.* (1989). However, the greater levels of proteinase in J3601 and J3602 did not result in a higher growth rate in milk. This may indicate that the wild-type strain is able to produce the required levels of peptides for optimum growth and that their more rapid production by cloned enzymes would therefore have no stimulatory effect.

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