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Interaction between Proteolytic Strains of *Lactococcus lactis* Influenced by Different Types of Proteinase during Growth in Milk

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The influence of the type of cell envelope-located proteinase (P_I versus P_{III}) on the associative growth of *Lactococcus lactis* in milk was studied. Two genetically engineered strains, differing only by the type of proteinase, were first used as a model study. An interaction occurred during the second exponential growth phase of the mixed culture and resulted in a decrease in growth rate of the P_I-type proteinase strain, whereas that of the P_{III}-type proteinase strain remained unaffected. The reduction in proteolytic activity of the P_I-type proteinase strain (presumably resulting from an inhibition of the synthesis of the enzyme) due to the peptides released by the P_{III}-type proteinase was found to be partly responsible for this interaction. Extension of the study to wild-type proteinase-positive *L. lactis* strains showed a systematic imbalance of the mixture of the two strains in favor of the P_{III}-type proteinase strain.

Starter cultures used in the cheese industry are formed mainly of *Lactococcus lactis* strains. Starter culture bacteria have four important functions during cheese manufacture: (i) production of lactic acid from lactose, (ii) degradation of caseins, (iii) formation of aroma compounds, and (iv) inhibition of undesired microorganisms (due to the acidification of milk and possible production of bacteriocins). Altogether, these physiological properties largely contribute to the development of the desired texture and flavor of cheese. To efficiently play these parts, *L. lactis* has to reach high levels of population in milk (about 10^9 CFU/ml).

L. lactis strains have numerous nutritional requirements especially of nitrogen sources (19, 30), for growth. They have limited capacities to synthesize amino acids (2) and therefore depend on nitrogen sources for growth. The use of free amino acids and peptides of the milk allows lactococci to grow to only low cell densities (11, 24). As a result, the caseins represent the main source of amino acids for *L. lactis* during growth in milk.

The process of casein utilization has been extensively studied over the last decade (for recent reviews, see references 9, 15, and 25). The cell envelope-located proteinase PrtP is involved in the first step of casein utilization. Two types of proteinase have been identified among lactococci on the basis of their specificity towards caseins. Both types act preferentially on β-casein, but α_{s1}- and κ-caseins are better substrates for the P_{III} type than for the P_I type. In addition, β-casein is cleaved in a different manner by the two types of proteinases. Although both types of PrtP release exclusively oligopeptides from caseins, the proteolysis products are different (12, 27–29). The second step is the transport of peptides into the cells. Three different peptide transport systems have been identified among lactococci (4, 17). However, only the oligopeptide transport system has been shown to play a significant role in the casein utilization process (16). Finally, the internalized peptides are cleaved into free amino acids by a large set of peptidases, with overlapping specificities (for recent reviews, see references 23 and 25).

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Casein degradation by the proteolytic system provides all the amino acids that are needed for growth of *L. lactis* up to high cell densities. However, the growth rate of *L. lactis* in milk is limited by the rate of oligopeptide production (8). As a result, *L. lactis* displays two exponential growth phases in milk (7). The first one relies on the use of the free amino acids and utilizable oligopeptides originally present in milk; the second relies on the use of caseins (11).

The type of proteinase has been shown to play a crucial role in the interaction between proteolytic (Prt^+) and nonproteolytic (Prt^-) *L. lactis* strains associatively cultured in milk (10). Unfortunately, no experimental data are available concerning the incidence that the type of proteinase may have on the interaction between Prt^+ strains of *L. lactis* during their associative growth in milk. Since the production of bitter peptides from casein was found to be dependent on the type of proteinase of *L. lactis* (32, 33), a stimulation of one of the two types during mixed cultures of Prt^+ strains in milk should be of great ecological and technological importance. The aim of the present study was therefore to analyze the interaction between *L. lactis* strains associatively grown in milk, with respect to their type of proteinase.

MATERIALS AND METHODS

Strains and culture conditions. The *L. lactis* strains used in the present study are listed in Table 1. The proteinase specificities of the strains (P_I or P_{III} type) have been assessed according to the methods of Exterkate (3). *L. lactis* MG611 and the plasmid pNZ521 were generous gifts from J. Kok (University of Groningen, Groningen, The Netherlands) and W. de Vos (Netherlands Dairy Research Institute, Ede, The Netherlands), respectively. Constructions of *L. lactis* MG611 and *L. lactis* SH5 and their lactose-positive derivatives, *L. lactis* MG611-1 and SH5-1, respectively, have been described elsewhere (1, 8, 20). Both strains derived from the plasmid-free strain *L. lactis* MG1363 (6) and therefore had similar nitrogen requirements and peptidolytic and transport abilities. All strains were stored at −80°C in sterile litmus milk supplemented with yeast extract (0.5%, wt/vol) and glucose (0.5%, wt/vol), supplemented with erythromycin (5 μg/ml) in the case of *L. lactis* MG611-1.

Cells were grown at 30°C in reconstituted skim milk (10% [wt/vol] Nilac Low Heat milk powder; Netherlands Dairy Research Institute). Given the good bacteriological quality of this milk (less than 10^2 bacteria per ml), no further heat treatment was required. Milk was inoculated with *L. lactis* cells (approx. 7×10^6 CFU/ml) precultured in milk up to the end of the second exponential phase of growth.

TABLE 1. Bacterial strains used in this study

L. lactis strain	Relevant properties[a]	Source or reference[b]
MG1363	Prt^- Lac^-; plasmid free	6
MG611-1	Em^r Prt^+ (P_I type) Lac^+; MG1363 carrying 8 to 9 copies of the proteinase plasmid pKLG610 and harboring the lactose plasmid pIL9	8, 20
SH5-1	Cm^r Km^r Prt^+ (P_{III} type) Lac^+; MG1363 harboring the proteinase plasmid pNZ521 and the lactose plasmid pIL9	1, 8
Wg2	Wild type; Prt^+ (P_I type) Lac^+	NIZO
E8	Wild type; Prt^+ (P_I type) Lac^+	NZDRI
SK11	Wild type; Prt^+ (P_{III} type) Lac^+	NZDRI
AM1	Wild type; Prt^+ (P_{III} type) Lac^+	NZDRI
CNRZ 1259	Wild type; Prt^- Lac^+	INRA

[a] Prt, ability to produce a functional cell envelope-located proteinase; Lac, ability to use lactose as energy source; Em^r, Cm^r, and Km^r, resistance to erythromycin, chloramphenicol, and kanamycin, respectively.

[b] NIZO, Netherlands Dairy Research Institute; NZDRI: New Zealand Dairy Research Institute, Palmerston North, New Zealand; INRA, Institut National de la Recherche Agronomique, Jouy-en-Josas, France.

In the case of mixed cultures, the strains were precultured separately. The size of the inoculum (i.e., total cell number) was kept constant; only the proportion of each strain varied in the inoculum.

Sequential cultures were performed as already described (11, 14). Briefly, milk was first cultured for 8 h with the Prt^- strain *L. lactis* subsp. *lactis* CNRZ 1259. After adjustment of the pH to that of the noninoculated milk (i.e., pH 6.8), milk was heated for 30 min at 63°C and inoculated with a culture of a Prt^+ strain.

Bacterial enumeration. The chains of lactococci were first disrupted for 30 s with a mechanical blender (Ultra-Turrax model T25; Janke and Kunkle, Staufen, Germany). Cell populations were then estimated by plating appropriate milk dilutions on M17 medium (31) by using a spiral plater (Spiral System, Cincinnati, Ohio). The accuracy and precision of this plating method have been previously assessed (7).

Differential enumeration of the Prt^+ strains in mixed culture was achieved by plating milk dilutions on selective media. The nature of the selective media depended on the composition of the mixed culture. When *L. lactis* MG611-1 was used, selective enumeration of this strain was obtained by plating the culture samples on M17 medium supplemented with erythromycin (5 μg/ml). A preliminary study confirmed that the presence of erythromycin in the enumeration medium did not significantly ($P = 0.001$) affect cell counts. In other mixed cultures involving *L. lactis* SH5-1, selective enumeration of this strain was achieved by plating milk samples on chemically defined medium (26) deprived of Phe and Pro, since *L. lactis* SH5-1 was the only strain able to grow in this medium. It has also been checked previously that no significant difference ($P = 0.001$) was observed between cell counts of *L. lactis* SH5-1 in chemically defined medium deprived of Phe and Pro and in M17. Statistical analysis of the results was performed as previously described (10).

Proteinase isolation. *L. lactis* was grown in milk to the end of the second exponential growth phase. The cells were collected by centrifugation (10,000 × *g*, 10 min, 4°C) and washed three times with 50 mM Tris HCl, pH 8, containing 30 mM $CaCl_2$. The proteinase was released from the cell envelope by incubation for 30 min at 30°C in 50 mM Tris HCl, pH 6.5, containing 15 mM EDTA (18).

Proteinase activity. Activity of the proteinase (either in solution or bound to the cell) was determined by using the chromogenic peptide methoxy-L-succinyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitro-anilide as a substrate, according to the methods of Exterkate (3), or by measuring the rate of β-casein hydrolysis as previously described (10).

Peptide isolation. Peptides were isolated from milk cultured by *L. lactis* to the end of the second exponential growth phase according to the methods of Helinck et al. (8). Cells were first removed by centrifugation (10,000 × *g*, 10 min, 4°C), and caseins were precipitated by 1% (vol/vol) trifluoroacetic acid. After the proteins separated by centrifugation were discarded, the supernatant was ultrafiltered through a 3,000-Da-pore-size membrane (PBLC membrane; Millipore Corp., Bedford, Mass.). Peptide purification was achieved by solid-phase extraction (Sep-Pak C_{18} cartridge; Waters, Milford, Mass.). The pool of peptides was eluted with 30% acetonitrile in MilliQ water and freeze-dried in a Speed-Vac concentrator (Savant Instruments Inc., Framingdale, Col.). Peptide concentration was estimated by the methods of Lowry et al. (21), using bovine serum albumin as the standard.

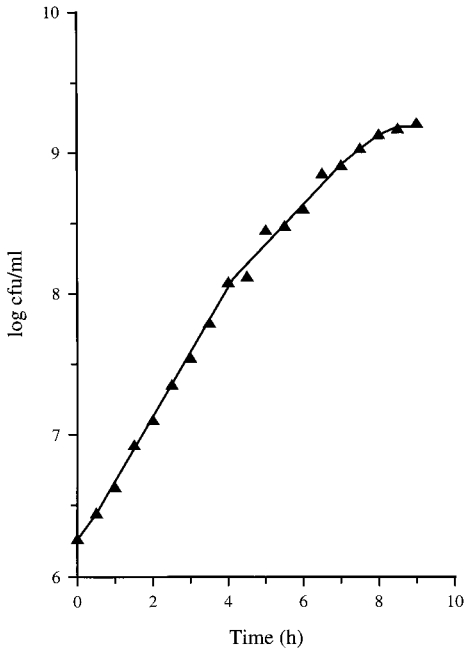

FIG. 1. Growth of *L. lactis* SH5-1 as pure culture in milk.

RESULTS

Pure cultures. All strains were first grown as pure cultures to determine their growth parameters in milk. They all displayed two distinct exponential growth phases, as exemplified in Fig. 1. The growth parameters of each strain were calculated on the basis of four independent experiments (Table 2). The growth rates in the first phase were in the same range for all strains (less than 5% variation between mean values). Larger variations (24%) were observed between the growth rates of the strains during the second phase. This suggests differences between strains in their ability to use caseins as a nitrogen source, but no relationship could be drawn between the growth rate of the strain and the type of proteinase.

The two genetically engineered strains, *L. lactis* MG611-1 (P_I-type proteinase) and SH5-1 (P_{III}-type proteinase), behaved similarly in milk. In particular, no significant differences ($P < 0.01$) could be evidenced between the growth rates in the second phase, indicating that the type of proteinase did not influence this growth parameter.

Mixed cultures of the two genetically engineered reference strains. The two genetically engineered strains *L. lactis* MG611-1

TABLE 2. Growth rates of *L. lactis* strains grown as pure cultures in milk

L. lactis strain	Type of proteinase[a]	Growth rate (h^{-1})[b]	
		First phase	Second phase
MG611-1[c]	I	1.39 ± 0.13	0.75 ± 0.06
SH5-1[c]	III	1.53 ± 0.03	0.74 ± 0.07
Wg2	I	1.46 ± 0.07	1.10 ± 0.07
E8	I	1.36 ± 0.33	0.53 ± 0.23
SK11	III	1.53 ± 0.10	0.93 ± 0.10
AM1	III	1.46 ± 0.07	0.76 ± 0.10

[a] Determined according to the methods of Exterkate (3).

[b] Means of four determinations ± confidence limits, $P = 0.95$.

[c] Genetically engineered reference strains (cf. Table 1).

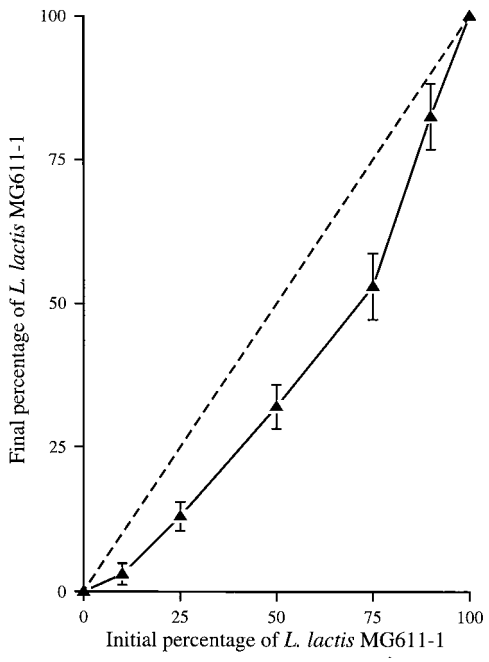

FIG. 2. Changes in population ratios during mixed cultures in milk of the two genetically engineered strains, *L. lactis* MG611-1 (P_I-type proteinase) and *L. lactis* SH5-1 (P_{III}-type proteinase). Values are means of four independent determinations (error bars, confidence limits, $P = 0.95$). Dashed line, expected composition of the mixture at the end of the culture if no interaction occurred between the two strains.

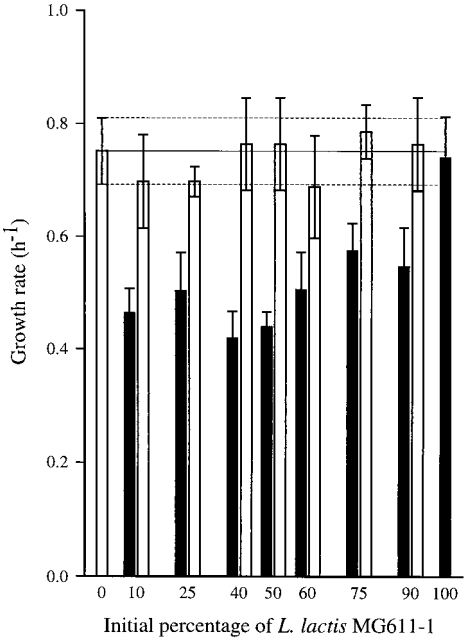

FIG. 3. Growth rate of *L. lactis* MG611-1 (P_I-type proteinase) (■) and *L. lactis* SH5-1 (P_{III}-type proteinase) (□) during the second growth phase of the mixed culture as a function of the composition of the inoculum. Values are means of four independent determinations (error bars, confidence limits, $P = 0.95$).

(P_I-type proteinase) and *L. lactis* SH5-1 (P_{III}-type proteinase) were associatively grown in milk, with varying proportions of each strain in the inoculum. A change in the proportion of the two strains systematically occurred in favor of the P_{III}-type proteinase strain at the end of coculturing (Fig. 2). Since there is no significant difference between the growth rates of the two strains during pure cultures (Table 2), absence of interactions in the mixed culture should have resulted in an unchanged ratio of the two strains at the end of growth. These results undoubtedly demonstrate the existence of interactions between the two strains during their associative growth in milk.

The growth rates of the two cocultured strains have been studied as a function of the initial composition of the mixed culture. The initial percentage of the two strains did not affect their growth rates during the first growth phase, which were not significantly different ($P < 0.01$) from those of the pure cultures (data not shown). On the other hand, during the second growth phase, the growth rate of the P_I-type proteinase strain was clearly affected by the presence of the P_{III}-type proteinase strain in the culture medium, whereas that of the P_{III}-type proteinase strain remained unaffected (Fig. 3). In particular, a 40% decrease in growth rate was observed when the inoculum contained 50% *L. lactis* SH5-1 cells.

Extension of the study to wild-type strains. Similar experiments were conducted by replacing one of the two genetically engineered strains with a wild-type strain having the same type of proteinase. The study was limited to an initial percentage of the P_I-type proteinase strain to 50 to 60%. *L. lactis* MG611-1 (P_I-type proteinase) was cocultured with either *L. lactis* SK11 or *L. lactis* AM1, both of which produce a P_{III}-type proteinase, whereas *L. lactis* SH5-1 (P_{III}-type proteinase) was cocultured with either *L. lactis* Wg2 or E8, both of which produce a P_I-type proteinase.

As the cocultured strains differed in growth rate in pure cultures (Table 2), a modification in the mixture composition was expected to occur during the mixed cultures, even if no interaction took place between the two strains. Thus, the theoretical final percentages of the P_I-type proteinase strain were calculated in the absence of any interaction and compared to the experimental values (Table 3). At the end of the first phase of growth, a slight imbalance of the mixture was observed, but no relationship between the promoted strain and the type of proteinase could be drawn. In contrast, the observed final percentages of the P_I-type proteinase strain were always lower than those deduced from the calculation. This deviation clearly revealed the existence of interactions between the two cocul-

TABLE 3. Percentages of P_I-type proteinase *L. lactis* strains cocultured with P_{III}-type proteinase *L. lactis* strains

L. lactis strain (proteinase type)	P_I-type proteinase strain (%)			
	At end of first phase		Final	
	Expected[a]	Observed[b]	Expected[a]	Observed[b]
MG611-1 (P_I) cocultured with[c]:				
SK11 (P_{III})	45	38 (2.8)	32	23 (3.5)
AM1 (P_{III})	47	42 (0.7)	47	19 (2.1)
SH5-1 (P_{III}) cocultured with[d]:				
Wg2 (P_I)	38	37 (2.8)	60	20 (5.6)
E8 (P_I)	33	34 (1.4)	21	15 (0.7)

[a] Values predicted from the growth rates of the strains in pure cultures, assuming no interaction occurred between the strains when cocultured.
[b] Mean of two repetitions (standard deviation given in parentheses).
[c] P_I strain was initially 50%.
[d] P_I strains were initially 40%.

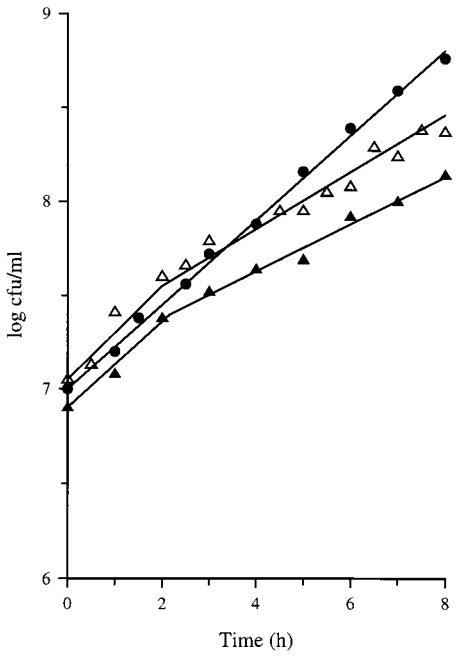

FIG. 4. Growth of *L. lactis* MG611-1 (P_I-type proteinase) in depleted milk (●) or in depleted milk supplemented with peptides isolated from a milk culture of *L. lactis* SH5-1 (P_{III}-type proteinase) grown as pure (▲) or mixed culture (△). Mixed culture consisted of *L. lactis* MG611-1 and SH5-1. Depletion of the milk was achieved by preculturing *L. lactis* CNRZ 1259 for 8 h. Peptides were isolated from the milk culture by solid-phase extraction and were added to the depleted milk at a final concentration of 50 μg/ml.

tured strains, systematically in favor of the P_{III}-type proteinase strain during the second phase of growth.

Causes for interaction. The proteolysis products released by one strain were assumed to affect the growth of the paired strain. To demonstrate this hypothesis, *L. lactis* MG611-1 and SH5-1 were grown as pure or mixed cultures in milk. Growth was interrupted at the end of the second exponential growth phase (i.e., pH 6.0), and milk peptides (about 50 μg/ml) were isolated by solid-phase extraction. The effect of the isolated peptides was studied in a milk sample previously deprived of assimilable nutrients, since the interaction between the strains took place during the second growth phase (i.e., corresponding to the utilization of casein as the nitrogen source). Depletion was achieved by preculturing the Prt^- strain *L. lactis* CNRZ 1259 for 8 h at 30°C.

The growth rate of *L. lactis* MG611-1 in depleted milk was significantly decreased in the presence of peptides (50 μg/ml) isolated from a mixed culture of the two strains (Fig. 4). This inhibitory effect was even greater with the addition of the same amount of P_{III}-type peptides (i.e., released during growth of the P_{III}-type proteinase strain *L. lactis* SH5-1), with a 47% ± 1% decrease in growth rate (mean of six determinations ± confidence limit, $P = 0.95$). In contrast, the addition of P_I-type peptides (50 μg/ml) did not significantly affect ($P < 0.01$) the growth rate of the P_{III}-type proteinase strain *L. lactis* SH5-1 (data not shown). Increasing the peptide concentration 10-fold did not produce any effect. At least, the presence of P_{III}-type peptides did not affect the growth rate of *L. lactis* SH5-1, whereas that of *L. lactis* MG611-1 was only slightly decreased in the presence of P_I-type peptides (85% of the control). Similar trends were observed with the wild-type strains *L. lactis* Wg2 and SK11 in place of the corresponding genetically engineered strains, *L. lactis* MG611-1 and SH5-1, respectively (data not shown).

Furthermore, the cell-bound proteolytic activity of *L. lactis* MG611-1 cells was slightly (12%) but significantly ($P < 0.01$) reduced when the strain was grown in the presence of peptides isolated from an *L. lactis* SH5-1 milk culture. A similar trend was observed when the strain was grown in the presence of peptides isolated from a mixed culture of *L. lactis* MG611-1 and SH5-1. However, the presence of these peptides (isolated from either the pure or the mixed culture) did not affect the rate of hydrolysis of a control substrate (either β-casein or the chromogenic substrate) by the P_I-type proteinase purified from *L. lactis* MG611-1 (data not shown). One can therefore assume that the reduction in proteolytic activity of the *L. lactis* MG611-1 population resulted from the inhibition to some extent of the proteinase synthesis.

DISCUSSION

The experiments described in this study represent the first demonstration of an interaction between strains of *L. lactis* with different types of cell envelope-located proteinase. Mixed cultures of two proteinase-positive strains of *L. lactis* with different types of proteinase resulted in a partial growth inhibition of the P_I-type proteinase strain at the end of growth, regardless of the nitrogen requirements, the transport abilities, and the peptidase content of the paired strains. The imbalance of a mixed culture in milk in favor of the P_{III}-type proteinase strain therefore seems to be a common feature of such cocultures. The enrichment in the P_{III}-type proteinase strain was systematic only during the second phase of growth, which is in perfect agreement with the fact that casein is not utilized as a source of amino acids during the first growth phase (11). The imbalance in population at the end of the first phase was the result of factors, other than proteinase activity, which either partly counterbalance or increase the P_{III}-type proteinase strain dominance at the end of growth. Nevertheless, the share of the cell envelope-located proteinase in the interaction is not surprising, since growth of *L. lactis* in milk has been shown to be limited by the rate of casein hydrolysis (8).

The use of a genetically engineered pair of *L. lactis* strains made it possible to analyze the role of the proteinase during the interaction, since only this component of the proteolytic system varied between strains. As expected, the interaction occurred between the paired strains only during the second exponential growth phase. The negative effect the P_{III}-type proteinase strain had on the P_I-type proteinase strain was due to the action of peptides released by the P_{III}-type proteinase. These peptides induced a 12% reduction in proteolytic activity expressed by the P_I-type proteinase population, presumably due to an inhibition of the synthesis of the proteinase. The behavior of the culture should therefore be comparable to that of a mixed culture containing 88% Prt^+ cells and 12% isogenic Prt^- cells. The growth rate of a Prt^+ strain during the second phase of such a mixed culture was shown to be only 5% reduced, compared to the pure Prt^+ culture (13). Consequently, the up-to-40% reduction in growth rate during the second growth phase of the P_I-type proteinase strain when cocultured with the isogenic P_{III}-type proteinase strain cannot be exclusively attributed to the reduction in proteolytic activity expressed by the P_I-type proteinase strains. The reduction in growth rate of the P_I-type proteinase strain might also result from a competition between peptides released by the two types of proteinase for the single binding protein of the oligopeptide transport system.

In conclusion, mixed cultures of proteolytic strains of *L.*

lactis with different types of proteinase represent a complex ecological system. According to Meers (22), an amensalistic interaction is defined as a relationship in which the growth of one population is repressed by a compound produced by another population. Therefore, the interaction presented in this work might be defined as an amensalism on the part of the P_{III}-type proteinase strain towards the P_I-type proteinase strain, presumably combined with a competition between the two strains for peptide translocation. The paired strains interact in more than one way, which is reported to be a common phenomenon among binary microbial interactions (5). At least, these results emphasize the large ecological impact of differences prevailing between the two types of proteinase, although the encoding genes have 98% homology (15).

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