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The Effects of Adding Lactococcal Proteinase on the Growth Rate of *Lactococcus lactis* in Milk Depend on the Type of Enzyme

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Increasing the proteolytic activity of *Lactococcus lactis* cultures in milk by adding the corresponding proteinase resulted in a stimulation of the growth rate regardless of the strain and the type of proteinase, demonstrating that the rate of casein degradation was responsible for the growth rate limitation of *L. lactis* in milk. However, the stimulation was only transient, and the reduction in growth rate in the poststimulation phase depended on the type of cell envelope proteinase. When a P_I-type proteinase was added, three causes were involved in the subsequent reduction in growth rate: degradation of the added proteinase, repression of the proteolytic activity expressed by the cells, and competition for peptide uptake. When a P_{III}-type proteinase was added, the cessation of stimulation was due to the autoproteolysis of the added enzyme only.

Lactococci have specific nutritional requirements for growth. The number and the nature of essential amino acids are strain dependent, but most *Lactococcus lactis* strains need at least isoleucine, leucine, histidine, methionine, and valine (2). The concentrations of these essential amino acids in milk are very low, especially those of isoleucine, leucine, and methionine (<1 mg/liter) (12, 27). Consequently, the pool of free amino acids initially present in milk supports only 2% of the growth of *L. lactis* (12). Because only a small fraction of the peptides that are present in milk (mainly oligopeptides) are utilized during growth, caseins represent the main source of amino acids, accounting for about 90% of the growth of *L. lactis* in milk (12, 27).

Casein utilization by *L. lactis* is mediated by a complex proteolytic system which consists of a cell envelope-located proteinase (PrtP), three different peptide transport systems, and a large number of intracellular peptidases. Most of the components of the proteolytic system have been biochemically and genetically characterized (15, 30). In particular, two different types of proteinase (P_I and P_{III}; only one type per strain) have been distinguished among lactococci on the basis of their specificity toward caseins (32, 38). Casein is first extracellularly hydrolyzed by PrtP into different oligopeptides (13, 32). Only some of them are taken up by the oligopeptide transport system (Opp) and subsequently cleaved into amino acids by intracellular peptidases (15–17). Inactivation of individual peptidases, as well as inactivation of the di- and tripeptide transport systems, has either little or no effect on the growth of *L. lactis* in milk (7, 16, 26). In contrast, inactivation of either the proteinase or the Opp system or both strongly affects the organism's growth in milk (12, 16). Therefore, both PrtP and Opp appear to play a central role in the proteolytic pathway of lactococci when grown in milk.

Several lines of evidence indicate that casein utilization by *L. lactis* does not proceed fast enough to ensure maximal growth rates: (i) all proteinase-positive strains so far studied display a biphasic exponential growth in milk, with a change to a lower rate corresponding to the utilization of casein as an amino acid source (12, 37); (ii) higher growth rates are obtained in rich laboratory media than in milk (10, 11); and (iii) the growth rate in milk is systematically stimulated upon addition of a mixture of amino acids or peptides (11, 27, 34). However, it is unclear whether the rate of casein hydrolysis by PrtP or the peptide uptake via the Opp system is responsible for the limitation. The effect of PrtP overproduction on the growth of *L. lactis* in milk has been studied (1, 21, 24), but the conclusions appear to be somewhat conflicting, presumably because different strains were used. Leenhouts and coworkers observed that a 50% increase in proteolytic activity did not influence the growth of *L. lactis* in a major way (21). Bruinenberg and coworkers (1) suggested limitation of the growth rate by the caseinolytic activity of the proteinase, since a threefold overproduction of the proteinase resulted in a 20% stimulation in growth rate of *L. lactis*. In contrast, McGarry et al. (24) suggested that the transport of peptides rather than their production rate was the limiting factor, since a threefold increase in proteolytic activity did not stimulate the growth rate of *L. lactis.*

To definitely determine which step of casein utilization by *L. lactis* is growth rate limiting, the effect of increasing the proteolytic activity in several milk cultures of *L. lactis* by adding the corresponding purified proteinase was analyzed. The results clearly showed that the rate of casein hydrolysis limits the growth rate of *L. lactis*, regardless of the strain and the type of proteinase. However, adding proteinase to the milk rapidly resulted in a subsequent limitation of the growth rate, which depended on the type of proteinase.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *L. lactis* strains and plasmids used in this study are listed in Table 1. *L. lactis* MG611 and pNZ521 were generous gifts from J. Kok (University of Groningen, Groningen, The Netherlands) and W. de Vos (Netherlands Institute for Dairy Research, Ede, The Netherlands), respectively. Plasmid pIL9 was isolated from *L. lactis* IL2662.1 according to established procedures (33). *L. lactis* MG611 was constructed previously by integration of the plasmid-encoded proteinase genes *prtP* and *prtM* of *L. lactis* Wg2 into the *L. lactis* MG1363 chromosome (21). To ensure growth in milk, plasmid pIL9 (encoding lactose utilization) was introduced into *L. lactis* MG611 by transformation according to the method of Dornan and Collins (3), generating *L. lactis* MG611-1. *L. lactis* SH5 was constructed as described previously (1) and consisted of *L. lactis* MG1363 into which the recombinant plasmid pNZ521 was introduced by transformation. The presence of plasmids in the transformed strains was checked by agarose gel electrophoresis.

The wild-type strains were stored at −80°C in sterile litmus milk supplemented with yeast extract (0.5%, wt/vol) and glucose (0.5%, wt/vol). *L. lactis* MG1363 and genetically engineered strains MG611-1 and SH5 were stored at −80°C in

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties[a]	Source or reference[b]
L. lactis strains		
NCDO 712	Wild type, Prt^+ (P_I type) Lac^+	NCDO
MG1363	Prt^- Lac^- plasmid-free derivative of NCDO 712	8
MG611	Em^r Prt^+ (P_I type) MG1363 carrying 8–9 copies of pKLG610 in the chromosome	21
MG611-1	Em^r Prt^+ (P_I type) Lac^+ MG611 harboring the lactose plasmid pIL9	This work
SH5	Cm^r Km^r Prt^+ (P_{III} type) MG1363 harboring the multicopy plasmid pNZ521	This work
CNRZ 1259	Prt^- Lac^- derivative of ML3	INRA
Wg2	Wild type, Prt^+ (P_I type) Lac^+	NIZO
CNRZ 1076	Wild type, Prt^+ (P_I type) Lac^+	INRA
SK11	Wild type, Prt^+ (P_{III} type) Lac^+	NZDRI
AM1	Wild type, Prt^+ (P_{III} type) Lac^+	NZDRI
IL 2661-2	Prt^- Lac^+, harboring the lactose plasmid pIL9	INRA
Plasmids		
pKLG610	Em^r Prt^+ (P_I type), carrying the complete *prtP* and *prtM* genes of *L. lactis* Wg2	21
pNZ521	Cm^r Km^r Prt^+ (P_{III} type), carrying the complete *prtP* and *prtM* genes of SK11	1
pIL9	Lac^+, carrying the lactose genes of IL 2661-2	INRA

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

[b] NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; NZDRI, New Zealand Dairy Research Institute, Palmerston North, New Zealand; INRA, Institut National de la Recherche Agronomique, Jouy-en-Josas, France; NIZO, Netherland Dairy Research Institute, Ede, The Netherlands.

M17 broth (36) containing glycerol (10%, vol/vol) and the appropriate antibiotics (5 μg/ml). Plasmids were stored at −20°C in ethanol (70%, vol/vol).

Culture conditions and bacterial enumeration. Cells were grown at 30°C in reconstituted skim milk (10% [vol/vol] Nilac Low Heat milk powder; NIZO, Ede, The Netherlands) or in chemically defined medium (CDM) (29) containing β-casein (2.5 g/liter; Sigma), histidine (2 mM), and leucine (2 mM) as the sole sources of amino acids (16). When required, cells were grown on milk depleted of utilizable free amino acids and peptides to ensure that growth of the strains would rely only on caseins. Depletion was achieved as previously described (12, 14) by culturing a Prt^- strain (*L. lactis* CNRZ 1259) to the end of growth, i.e., 1.6×10^8 CFU/ml. The pH of the milk was then readjusted to that of the noninoculated milk (i.e., 6.8) before pasteurization for 30 min at 63°C. It has been previously shown that the behavior of subsequent cultures was not affected by pH readjustment, pasteurization, or presence of dead cells or lactate (12, 14).

Chains of lactococcal cells were disrupted for 30 s at 20,000 rpm in a mechanical blender (Ultra-Turrax model T25; Janke & Kunkel, Staufen, Germany) as described by Hassan et al. (9). Cell populations were then estimated by spiral plating appropriate dilutions of the cultures on M17 agar.

Proteolytic activity of the cells. Cells were removed from the culture medium by centrifugation (10,000 × *g* for 10 min at 4°C), washed twice in 50 mM Tris-HCl (pH 8) containing 30 mM $CaCl_2$, and resuspended in 50 mM Tris-HCl (pH 6.5) containing 30 mM $CaCl_2$ to a final optical density at 650 nm (OD_{650}) of 1 (i.e., corresponding to 200 μg of protein per ml and ca. 10^9 CFU/ml). Cell-bound proteinase activity was determined by using the chromogenic peptide methoxy-succinyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitroanilide (MeO-Suc-Arg-Pro-Tyr-*p*NA; Chromogenix, Mölndal, Sweden) as a substrate (4).

Proteinase isolation. Six Prt^+ *L. lactis* strains (MG611-1, Wg2, CNRZ 1076, SH5, SK11, and AM1) were grown in M17 medium to the stationary phase, and their proteinase was released from the cell envelope by incubation for 30 min at 30°C in Ca^{2+}-free buffer (18). The proteolytic activity of the proteinase fractions was determined by using the chromogenic peptide MeO-Suc-Arg-Pro-Tyr-*p*NA as the substrate (4) and expressed as the amount of *p*-nitroanilide (*p*NA) released per minute per milliliter. Further purification of PrtP isolated from *L. lactis* MG611-1 was achieved by anion-exchange chromatography (13). Absence of peptidase activity in the proteinase solutions was checked by using substrates specific for different peptidases (i.e., Lys-*p*NA, Glu-*p*NA, Gly-Pro-*p*NA, and bradykinin) as previously described (13).

Peptide isolation. *L. lactis* MG611-1 and SH5 were grown in depleted milk. After 8 h of culturing, cells were removed by centrifugation (10,000 × *g* for 10 min at 4°C), and the proteins were precipitated by 1% (vol/vol) trifluoroacetic acid. After removal of the proteins by centrifugation, the supernatant was ultrafiltered through a 3,000-Da-cutoff membrane (Amicon Corp., Beverly, 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., Farmingdale, Colo.). Peptide concentration was estimated by the method of Lowry et al. (22), using bovine serum albumin as the standard.

Statistical analyses. All growth experiments were repeated four times unless otherwise stated. Linear regression analysis was performed on experimental data, and standard deviations of the slopes were calculated as described by Snedecor and Cochran (35). Linearity of growth curves was assumed when standard deviations of the slopes were below 0.07 $\log_{10}$ CFU ml^{-1} with no sign of systematic deviation of experimental data from the regression line (9). Hourly growth rates (μ) were calculated from the slopes ($\log_{10}$ CFU per milliliter per hour) by using the following formula: $\mu = \text{slope}/\log_{10}2$ (28). Confidence limits ($P = 0.95$) of the mean growth rates were calculated as described by Snedecor and Cochran (35): $[t \times \text{Sd}]/\sqrt{n}$, where t is given by the t distribution table ($t_{0.95} = 3.182$ with four repetitions) and n is the number of repetitions.

Transport assays. Transport experiments were adapted from previous reports (6, 16). Cells were grown to mid-OD_{650} (approximately 0.8) in chemically defined medium containing 17 free amino acids as the nitrogen source (29). Prior to transport assays, cells were deenergized for 30 min at 30°C with 10 mM 2-deoxy-D-glucose. For transport assays, cells (OD_{650} of approximately 1) were preincubated for 5 min in the presence of 25 mM glucose, and then 0.05 mM Leu-enkephalin (YGGFL) was added. Uptake of Leu-enkephalin was monitored by determining the intracellular concentration of free amino acids after derivatization with dansyl chloride, using reversed-phase analysis. The dansylated amino acids were separated at 40°C by high-performance liquid chromatography on a reverse-phase C_{18} column (Nucleosil, 5 μ [granulometry], 4.6 mm [inside diameter] by 150 mm; Shandon HPLC, Cheshire, United Kingdom). Solvent A was 10 mM sodium citrate (pH 6.2), and dansylated amino acids were eluted with a linear gradient (25 to 75%) of solvent B (60% [vol/vol] acetonitrile in sodium citrate) within 25 min. Fluorescence was monitored by a Waters 474 scanning fluorescence detector. Excitation and emission wavelengths were 340 and 530 nm, respectively. A specific internal volume of 3.6 μl/mg of protein was used to calculate the intracellular concentrations (31).

RESULTS

Growth of *L. lactis* in milk. The two genetically engineered strains *L. lactis* MG611-1 and SH5 as well as the four wild-type strains (Wg2, CNRZ 1076, SK11, and AM1) were grown in control and depleted milk. Figure 1 illustrates a typical growth curve of *L. lactis* MG611-1. The growth experiments were repeated four times, to generate mean growth parameters with confidence limits ($P = 0.95$). As shown in Table 2, all strains displayed two exponential growth phases in control milk. In contrast, they all displayed a single phase in depleted milk. The addition of peptides and free amino acids previously isolated from a noninoculated milk to the depleted milk restored the two exponential growth phases of *L. lactis* MG611-1 (data not shown). This result again confirms that the disappearance of the first growth phase in depleted milk was due only to the consumption of the initial pool of free amino acids and peptides of the milk during the preculture, as previously stated (14). In addition, the growth rate of each *L. lactis* strain in depleted milk was not significantly different ($P < 0.05$) from that observed during the second growth phase in control milk, which corresponds to the utilization of caseins as the source of amino acids (12).

Since the two genetically engineered strains were derived from the same parental strain, they grew at the same rate during the first phase of growth in control milk, which corresponds to the use of the free amino acids and assimilable oligopeptides of the milk as the amino acid source (12). Also, no significant difference ($P < 0.05$) was observed between the growth rate of these two strains in depleted milk or during the second growth phase in control milk, although it corresponds to the use of casein-derived oligopeptides (12). It is worth mentioning that the two genetically engineered strains exhib-

TABLE 2. Growth parameters of *L. lactis* strains cultured in control and depleted milk

L. lactis strain	PrtP type	Growth rate (h^{-1})[a]		
		Control milk		Depleted milk[b]
		1st phase	2nd phase	
MG611-1[c]	I	1.56 ± 0.13	0.90 ± 0.13	0.91 ± 0.10
Wg2	I	1.46 ± 0.07	1.10 ± 0.10	1.15 ± 0.14
CNRZ 1076	I	1.67 ± 0.07	1.18 ± 0.07	1.19 ± 0.19
SH5[c]	III	1.66 ± 0.17	1.03 ± 0.10	0.95 ± 0.08
SK11	III	1.53 ± 0.10	0.93 ± 0.07	1.06 ± 0.07
AM1	III	1.58 ± 0.08	0.82 ± 0.09	0.87 ± 0.04

[a] Average of four independent experiments with confidence limits at $P = 0.95$.
[b] Depletion of utilizable free amino acids and peptides was achieved by culturing milk with a Prt^- strain (*L. lactis* CNRZ 1259) to the stationary phase of growth (1.6×10^8 CFU/ml).
[c] Genetically engineered strain (see Table 1).

ited the same extent of proteolytic activity during growth in milk (data not shown).

Addition of proteinase to the growth medium. To determine whether the rate of casein hydrolysis was responsible for the reduction in growth rate in control milk, *L. lactis* MG611-1 and SH5 were grown in depleted milk supplemented with the corresponding proteinases (i.e., originating from *L. lactis* MG611-1 and SH5, respectively). The proteinases were released from a 10^{10}-CFU/ml cell suspension, yielding activities of 65 and 50 pmol of *p*NA released min^{-1} ml^{-1}, respectively. Addition of proteinase solution to the depleted milk (1% [vol/vol], i.e., corresponding to the activity expressed by 10^8 CFU/ml) rapidly resulted in a significant stimulation of the growth rate of both strains (Fig. 2). Surprisingly, the stimulation was only transient. The poststimulation phase was characterized by a marked decrease in growth rate. The extent of the decrease depended on the strain. With *L. lactis* MG611-1, the growth rate of the poststimulation phase was consistently lower ($P < 0.05$) than that obtained in depleted milk without addition of proteinase. In contrast, the growth rate of the poststimulation phase of *L. lactis* SH5 was not different from that observed in depleted milk without proteinase enrichment. Addition of proteinase to the depleted milk did not change the final population of the two strains (about 1.5×10^9 CFU/ml) but significantly affected the time needed to reach the maximal population (approximately 3 h later and 1.5 h earlier than in depleted milk in the cases of *L. lactis* MG611-1 and SH5, respectively).

Similar patterns were observed when *L. lactis* MG611-1 was cultured in CDM containing β-casein, His, and Leu as the sole sources of amino acids or when proteinase purified to homogeneity was added instead of a crude proteinase-containing solution. Increasing 10-fold the amount of added proteinase did not change the extent of initial stimulation and subsequent reduction in growth rate. Adding a P_I-type proteinase to *L. lactis* SH5 and vice versa also resulted in a transient stimulation of the growth rate. The extents of the stimulation were 66% (±38%) and 45% (±29%), respectively (means of three determinations, with confidence limits at $P = 0.95$). The growth rates of the poststimulation phase of *L. lactis* SH5 and *L. lactis* MG611-1 represented 115% (±30%) and 78% (±5%), respectively, of those observed without addition of proteinase.

Extension to wild-type *L. lactis* strains. To determine whether all *L. lactis* strains behave the same in depleted milk enriched with corresponding proteinase, we conducted a similar investigation using the four wild-type strains. In the presence of added proteinase, the wild-type strains were first stimulated, the extent of the stimulation depending on the type of proteinase (Table 3). Lower stimulation was observed with the P_I-type strains than with the P_{III}-type strains. The stimulative effect of proteinase addition on the growth rate was of short duration, regardless of the type of proteinase. Here again, differences in growth rates during the poststimulation phase were evident, depending on the type of proteinase. In the presence of P_{III}-type proteinase, the poststimulation growth rates of the corresponding strains were in the same range as in control depleted milk. In contrast, for P_I-type proteinases, the poststimulation growth rates of the corresponding strains were significantly lower ($P < 0.05$) than in control depleted milk.

Addition of a P_{III}-type proteinase (originating from *L. lactis* SK11) to *L. lactis* Wg2 or of a P_I-type proteinase (isolated from *L. lactis* Wg2) to *L. lactis* SK11 also resulted in a transient stimulation of the growth rate (of about 30% in both cases). The growth rate of *L. lactis* SK11 in the poststimulation phase was in the same range as in control depleted milk, whereas that of *L. lactis* Wg2 represented 68% (±13%) of the control level.

Causes of the ending of stimulation. Addition of a mixture of amino acids during the poststimulation phase of *L. lactis* MG611-1 allowed the strain to resume growth at a rate similar to that of the initial stimulation (Fig. 3). Similar behavior was obtained with *L. lactis* SH5 (data not shown). Thus, the reduction in growth rate after 3 to 4 h of stimulation can be undoubtly attributed to a deficiency in the amino acid supply for the strains.

At the end of the stimulation phase, or during the poststimulation phase of *L. lactis* MG611-1, no more proteolytic activity could be detected in the growth medium, indicating autoproteolysis of the added proteinase (19). Additional supplementation with proteinase in the poststimulation phase again stimulated the growth rate of *L. lactis* MG611-1. However, the new growth rate (0.7 ± 0.1 h^{-1}) remained significantly lower than that of the initial addition (1.1 ± 0.2 h^{-1}). Therefore, the

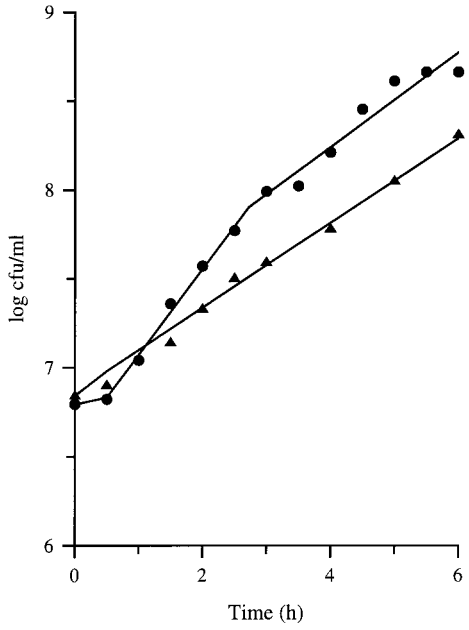

FIG. 1. Growth of *L. lactis* MG611-1 in control (●) and depleted milk (▲). Depletion of utilizable free amino acids and peptides was achieved by culturing milk with a Prt^- strain (*L. lactis* CNRZ 1259) to the stationary phase of growth (1.6×10^8 CFU/ml).

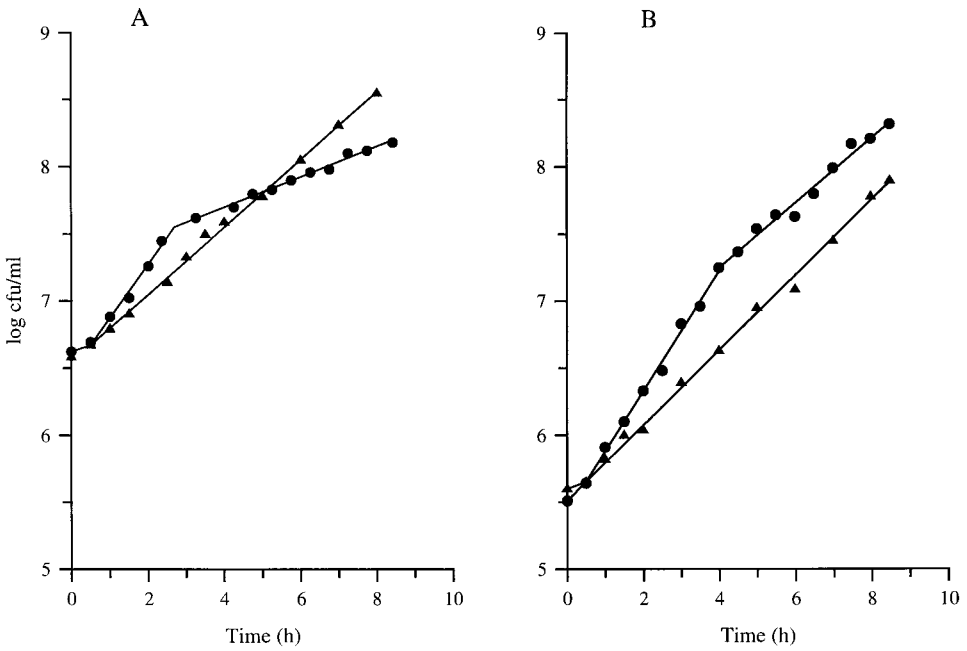

FIG. 2. Growth of *L. lactis* MG611-1 (P_I-type proteinase) (A) and *L. lactis* SH5 (P_{III}-type proteinase) (B) in depleted milk supplemented (●) or not (▲) with corresponding proteinase solutions. The proteinases were added at final activities of 6.4 and 5.0 pmol of *p*NA min^{-1} ml^{-1} released from MeO-Suc-Arg-Pro-Tyr-*p*NA, respectively.

reduction in growth rate in the poststimulation phase was not due only to the self-digestion of the free P_I-type proteinase. In contrast, a second addition of proteinase in the poststimulation phase of *L. lactis* SH5 restored the initial stimulation of the growth rate (0.9 ± 0.2 h^{-1}, compared to 1.0 ± 0.2 h^{-1}). This result strongly suggests that the ending of stimulation in depleted milk supplemented with a P_{III}-type proteinase was mainly due to a self-digestion of the free proteinase.

Causes of the decrease in growth rate of *L. lactis* during the poststimulation phase. In an attempt to identify possible other reasons for the decrease in growth rate during the poststimulation phase of *L. lactis* MG611-1, some complementary experiments were carried out. The peptides which accumulated in milk during the poststimulation phase were isolated by solid-phase extraction and added to a new batch of depleted milk. Addition of peptides resulted in a significant decrease in growth rate of *L. lactis* MG611-1 (Fig. 4). Interestingly, the new growth rate was not significantly different ($P < 0.05$) from that obtained during the poststimulation phase (0.6 ± 0.1 h^{-1}, compared with 0.5 ± 0.1 h^{-1}). In contrast, the same experiment carried out with *L. lactis* SH5 showed that the corresponding accumulated peptides did not affect the growth rate of the strain (Fig. 4). Moreover, the growth rate of *L. lactis*

TABLE 3. Growth parameters of *L. lactis* strains grown in depleted milk supplemented with corresponding proteinase

L. lactis strain	Added PrtP		Growth rate[b]	
	Type	Activity[a]	Stimulation phase	Poststimulation phase
MG611-1	P_I	6.4	131 ± 6	33 ± 9
Wg2	P_I	4.5	123 ± 7	18 ± 4
CNRZ 1076	P_I	6.2	139 ± 6	22 ± 10
SH5	P_{III}	5.0	160 ± 13	96 ± 16
SK11	P_{III}	8.0	152 ± 5	92 ± 45[c]
AM1	P_{III}	4.7	164 ± 6	97 ± 20

[a] PrtP was released from each strain and added to the depleted milk. Activity was determined from the hydrolysis of MeO-Suc-Arg-Pro-Tyr-*p*NA and expressed as picomoles of *p*NA released per minute per milliliter.

[b] Percentage of the growth rate in depleted milk (see Table 2); average of four experiments, with confidence limits at $P = 0.95$ except as noted otherwise.

[c] Average ± confidence limits at $P = 0.95$ of three experiments.

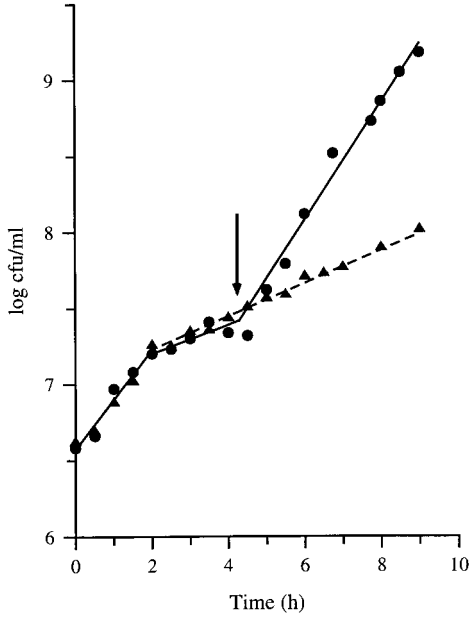

FIG. 3. Effect of amino acid supplementation on the growth of *L. lactis* MG611-1 in depleted milk supplemented with proteinase. The proteinase (P_I type) was added at a final activity of 7.2 pmol of *p*NA min^{-1} ml^{-1} released from MeO-Suc-Arg-Pro-Tyr-*p*NA. The composition of the added amino acid mixture was that of the chemically defined medium (29). ↓, addition of amino acids.

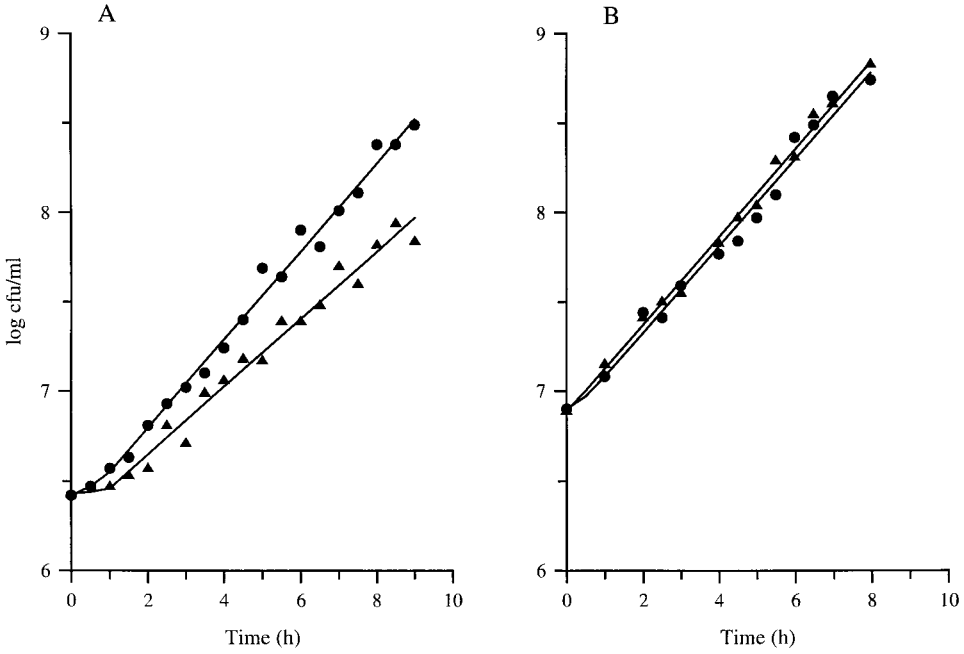

FIG. 4. Growth of *L. lactis* MG611-1 (A) and *L. lactis* SH5 (B) in depleted milk supplemented (▲) or not (●) with peptides isolated from a previous culture of the same strain in depleted milk supplemented with proteinase. The peptide solution was added at a final concentration of 54 μg/ml, i.e., corresponding to that of the depleted milk cultured for 8 h with *L. lactis* MG611-1 in the presence of added proteinase. Peptides were isolated by solid-phase extraction as indicated in Materials and Methods.

MG611-1 was also reduced in the presence of peptides generated by the P_{III}-type proteinase (0.50 h^{-1}, compared with 0.90 h^{-1} in the absence of peptides), whereas the presence of peptides generated by a P_I-type proteinase did not significantly affect ($P < 0.05$) the growth of a strain producing a P_{III}-type proteinase.

These results strongly suggest that the reduction in growth rate in the poststimulation phase of *L. lactis* MG611-1 was due to some inhibitory effect of peptides released by the proteinase. The inhibitory effect of the peptides released by the P_I-type proteinase was analyzed further. First, the proteolytic activity of the *L. lactis* MG611-1 cells was estimated in the poststimulation phase and compared to that of cells grown in depleted milk without proteinase supplementation. The addition of the P_I-type proteinase resulted in a 50% reduction in cell-bound proteinase activity. Similar trends were obtained in proteinase-enriched CDM containing β-casein, His, and Leu as the sole sources of amino acids, with a 75% reduction in cell-located activity. In contrast, the proteolytic activity expressed by *L. lactis* SH5 cells remained unaffected when the P_{III}-type proteinase was added to the depleted milk, with at least 90% of the proteolytic activity of the control.

The effect that peptides which accumulated in milk during the poststimulation phase of *L. lactis* MG611-1 following addition of the corresponding proteinase could have on the transport of oligopeptides was also investigated. Transport of the control peptide (Leu-enkephalin) by *L. lactis* MG611-1 was monitored by measuring the increase in concentration of intracellular leucine. To ensure that the accumulation of leucine into the cells originated from uptake of Leu-enkephalin only, the pool of peptides isolated from milk was deprived of assimilable leucine-containing peptides prior to transport experiments. Deprivation was achieved by culturing *L. lactis* MG611-1 in CDM containing the peptides which accumulated in milk during the poststimulation phase as the sole source of leucine, all other amino acids being in excess in the free form. The stationary phase of growth of *L. lactis* MG611-1 occurred at about 10^8 CFU/ml, indicating the consumption of all leucine-containing assimilable peptides. The residual peptides were isolated from CDM by solid-phase extraction and used for transport assays at a final concentration of 80 μg/ml (i.e., approximately three times the concentration of Leu-enkephalin). No accumulation of intracellular leucine was observed when Leu-enkephalin was omitted, confirming that no more leucine-containing transportable peptides were present in the pool of residual peptides. The uptake of Leu-enkephalin was inhibited (competitively) by a threefold excess of residual peptides (Fig. 5).

DISCUSSION

This report demonstrates that the rate of casein hydrolysis is responsible for the growth rate limitation of *L. lactis* in milk, since addition of lactococcal proteinase to the growth medium primarily stimulates the growth rate of *L. lactis*, regardless of the strain and the type of proteinase. The extent of the stimulation was two times higher when a P_{III}-type proteinase was added than when a P_I-type proteinase was added, suggesting that the action of the P_{III}-type proteinase on caseins results in the liberation of a larger amount of utilizable peptides than the P_I-type and/or in the liberation of peptides which are more easily translocated into the cells than the peptides released by a P_I-type proteinase.

Nevertheless, addition of lactococcal proteinase to the milk rapidly resulted in a new limitation of the growth rate of *L. lactis*. Some casein-derived peptides are known to exhibit antibacterial activity (39). However, no antibacterial activity was produced in the present case, since the addition of a mixture of amino acids overcame the reduction in growth rate. The reasons for this new limitation depend on the type of proteinase.

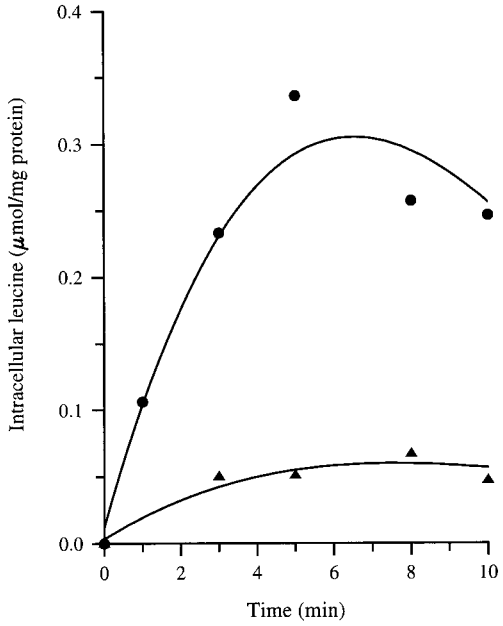

FIG. 5. Uptake of Leu-enkephalin by *L. lactis* MG611-1 in the presence (▲) or absence (●) of peptides isolated from a culture of the same strain in depleted milk supplemented with proteinase. Peptides were isolated from cultured milk by solid-phase extraction, deprived of Leu-containing peptides as indicated in the text, and used at a final concentration of 80 μg/ml. Uptake was initiated by the addition of Leu-enkephalin (0.05 mM).

P_I-type proteinase. Lactococcal proteinases are known to autoproteolyze (19). However, the reduction in growth rate observed when a P_I-type proteinase was added to the milk cultured with a strain producing a P_I-type proteinase was not due only to the self-digestion of the enzyme, since it was mimicked by the addition of the peptides which accumulated during the poststimulation phase. A significant repression of the proteolytic activity expressed by the cells was observed during growth in milk supplemented with proteinase. Peptides released from caseins by a P_I-type proteinase have a high content of Pro (13, 15). Since *L. lactis* synthesizes a large number of proline-specific peptidases (15, 30), the intracellular cleavage of translocated peptides should result in a large amount of proline-containing dipeptides, which are known to regulate the synthesis of the P_I-type proteinase (19).

The repression of the proteolytic activity expressed by the cells cannot completely explain the reduction in growth rate, since a second addition of proteinase to the milk did not restore the initial growth rate. The peptides which accumulate in milk during the poststimulation phase (competitively) inhibited the uptake of a control oligopeptide. Since the growth of *L. lactis* in milk is known to depend on the utilization of oligopeptides only (12, 16), the reduction in growth rate in the poststimulation phase was also presumably due to a competition between utilizable and nonutilizable oligopeptides for the single binding protein (OppA) of the oligopeptide transport system. However, the relative contributions of these two inhibitory effects on the growth rate of *L. lactis* in milk following addition of proteinase remain to be determined. These inhibitory effects might also explain why the extent of the initial stimulation was lower with a P_I-type proteinase.

P_{III}-type proteinase. When a P_{III}-type proteinase was added to the milk, the reduction in growth rate of a strain producing a P_{III}-type proteinase was due only to a self-digestion of the enzyme, since a second addition of proteinase completely restored the initial stimulation. No repression of the proteolytic activity expressed by the cells was detected. The synthesis of the P_{III}-type proteinase is also regulated by proline-containing dipeptides (23, 25), but the P_{III}-type proteinase releases a lower number of proline-containing peptides from caseins than the P_I-type proteinase (15, 32). On the other hand, the peptides released from caseins by a P_{III}-type proteinase were not responsible for a competition between utilizable and nonutilizable peptides for the translocation, since their addition to the milk did not affect the growth rate of a P_{III}-type-proteinase-producing *L. lactis* strain.

These results identify differences between the effects of P_I- and P_{III}-type proteinases on the growth of corresponding strains. Addition of a P_I-type proteinase to a strain producing a P_{III}-type proteinase, and vice-versa, emphasizes these differences. The inhibition of the growth rate of the P_I-type proteinase-producing strain in the poststimulation phase following the addition of a P_{III}-type proteinase is in perfect agreement with the fact that during mixed cultures of proteinase-positive strains, the P_I-type-proteinase-producing strain was always inhibited (5). Peptides generated by one type of proteinase did not result in the same effects when added to an *L. lactis* strain containing either a P_I- or a P_{III}-type proteinase, which strongly suggests that the two types of proteinase are not regulated in the same way. Moreover, the fact that one strain did not react the same when grown in the presence of peptides generated by either a P_I- or a P_{III}-type proteinase indicates that the two types of proteinase do not generate the same peptides from caseins.

In conclusion, the growth rate of *L. lactis* in milk is primarily limited by the rate of casein degradation by the cell envelope-located proteinase, regardless of the type of the proteinase of the strain. In contrast, the subsequent limitation of the growth rate observed when proteinase was added to the milk depended on the type of the enzyme. The opposite effects of the two types of proteinase in the poststimulation phase probably explain the conflicting reports on the effects of proteinase overproduction on the growth of *L. lactis* (1, 21, 24). Overproduction of a P_{III}-type proteinase should result in a continuous stimulation of the growth rate. In contrast, the negative effects observed in the present study upon addition of a P_I-type proteinase (repression of the proteolytic activity and competition for oligopeptide transport) are expected to occur during culturing of a P_I-type-proteinase-overproducing strain, which would counterbalance the initial stimulation of the growth rate.

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