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The Contribution of Caseins to the Amino Acid Supply for *Lactococcus lactis* Depends on the Type of Cell Envelope Proteinase

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The ability of caseins to fulfill the amino acid requirements of *Lactococcus lactis* for growth was studied as a function of the type of cell envelope proteinase (P_I versus P_{III} type). Two genetically engineered strains of *L. lactis* that differed only in the type of proteinase were grown in chemically defined media containing α_{s1}-, β-, and κ-caseins (alone or in combination) as the sources of amino acids. Casein utilization resulted in limitation of the growth rate, and the extent of this limitation depended on the type of casein and proteinase. Adding different mixtures of essential amino acids to the growth medium made it possible to identify the nature of the limitation. This procedure also made it possible to identify the amino acid deficiency which was growth rate limiting for *L. lactis* in milk (S. Helinck, J. Richard, and V. Juillard, Appl. Environ. Microbiol. 63:2124–2130, 1997) as a function of the type of proteinase. Our results were compared with results from previous in vitro experiments in which casein degradation by purified proteinases was examined. The results were in agreement only in the case of the P_I-type proteinase. Therefore, our results bring into question the validity of the in vitro approach to identification of casein-derived peptides released by a P_{III}-type proteinase.

Lactococci have numerous nutritional requirements for growth; in particular, nitrogen sources are required (13, 23, 33), because these organisms have a limited capacity to synthesize amino acids (3). Therefore, growth of *Lactococcus lactis* depends on the amino acids available in the culture medium. In milk, the concentrations of several essential amino acids, especially Ile, Leu, and Met, are very low (less than 1 mg/liter) (17, 26). On the other hand, only a small fraction of the peptides that are present in milk are utilized during growth (15, 17). In addition, the utilizable peptides are a poor source of Leu and Met (15). Consequently, caseins are the main source of amino acids and are responsible for about 90% of the growth of *L. lactis* in milk (17, 26). Casein utilization by *L. lactis* is mediated by a complex proteolytic system, which consists of a cell envelope proteinase, the oligopeptide transport system, and several intracellular peptidases (16, 19, 28). The proteinase is involved in the first step of casein degradation. Only some of the oligopeptides released by the proteinase are taken up by the oligopeptide transport system and subsequently cleaved into amino acids by intracellular peptidases (20, 21).

Two different types of proteinase (P_I and P_{III} types) have been identified in lactococci on the basis of their specificity for caseins (30, 31, 37). P_I-type proteinase cleaves β-casein preferentially, κ-casein to a lesser extent, and α_{s1}-casein insignificantly. In contrast, P_{III}-type proteinase cleaves β-, κ-, and α_{s1}-caseins equally well. The in vitro activity of purified proteinases on caseins has been studied extensively over the last few years (19). Most, if not all, of the peptides released by degradation of β-casein by purified P_I-type proteinase have been identified (18). Electrophoretic (37) and reverse-phase high-performance liquid chromatography (HPLC) (31) studies have shown that the two types of proteinase cleave β-casein at significantly different cleavage sites.

Surprisingly, very little is known about the consequences of these differences on the growth of *L. lactis* in milk or in casein-containing media. A previous study showed that optimal growth of *L. lactis* is related to β- and κ-casein degradation (6). On the other hand, Kunji and coworkers (21) reported that poor growth of *L. lactis* occurs in a culture medium containing β-casein as the sole source of amino acids. However, these results were obtained with P_I-type-proteinase-producing strains. No clear information concerning the ability of P_{III}-type-proteinase-producing strains to use the different caseins as sources of amino acids is available.

Two recent studies showed that the type of proteinase may influence the growth of *L. lactis* since (i) increasing the proteolytic activity of *L. lactis* cultures in milk by adding a purified lactococcal proteinase resulted in different effects, depending on the type of proteinase (11), and (ii) the associative growth of *L. lactis* in milk was influenced mainly by the type of proteinase produced by cocultured strains (7). The aim of the present study was to analyze the utilization of each type of casein (alone or in combination) as a source of essential amino acids for growth of *L. lactis* strains with different types of proteinase.

MATERIALS AND METHODS

Strains and culture conditions. Construction of the proteinase-negative (Prt^-), lactose-negative (Lac^-), plasmid-free strain *L. lactis* MG1363 and construction of the Prt^+ Lac^+ strains *L. lactis* MG611-1 (P_I-type proteinase) and SH5-1 (P_{III}-type proteinase) have been described elsewhere (2, 8, 11, 25). The two Prt^+ strains were derived from *L. lactis* MG1363; they differed only in the type of proteinase that they produced. Therefore, the three strains had identical nitrogen requirements; Gln, His, Met, Leu, Ile, and Val were essential amino acids. It was also determined that the three strains had identical peptidolytic and transport abilities, as previously described (1, 7). The strains were stored at −80°C in M17 broth (35) containing glycerol (10%, vol/vol) and 5 μg of erythromycin per ml (for MG611-1) or 5 μg of chloramphenicol per ml (for SH5-1).

Cells were grown at 30°C in reconstituted skim milk (10% [wt/wt] Nilac Low Heat milk powder; Netherlands Dairy Research Institute, Ede, The Netherlands) or in chemically defined medium (CDM) (29). When required (Lac^-

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strain), milk was supplemented with glucose (10 mg/ml). Pure caseins were added alone or in combination to the CDM as sources of amino acids at a final concentration of 2.4 g/liter. β-Casein and κ-casein were obtained from Sigma Chemical Co. (St. Louis, Mo.). The results obtained with these commercial caseins were confirmed by using β- and κ-caseins purified in our laboratory as previously described (9). α_{s1}-Casein was purified from skim milk by isoelectric precipitation, anion-exchange chromatography, and hydrophobic interaction chromatography as previously described (24). When added as a mixture, the α_{s1}-, β-, and κ-caseins were added at a ratio corresponding to the ratio in milk (i.e., 6:5:2). Culture media were inoculated with approximately 7×10^6 CFU of a preculture of the test strain in the exponential stage of growth in M17 broth per ml. Cells were washed twice in sterile 50 mM KH_2PO_4-K_2HPO_4 (pH 6.8) prior to inoculation.

Bacterial enumeration and statistical analysis. The chains of lactococci were first disrupted for 30 s with a mechanical blender (Ultra-Turrax model T25; Janke and Kunkel, Staufen, Germany). Cell populations were then estimated by plating appropriate dilutions of each culture on M17 agar with a spiral plater (Spiral System, Cincinnati, Ohio). The accuracy and precision of this plating method have been described previously (10). All growth experiments were repeated three times, unless otherwise stated. Growth rates (μ) were calculated from the slopes ($\log_{10}$ CFU per milliliter per hour) by using the following formula: $\mu = \text{slope}/\log_{10} 2$ (27). Confidence limits ($P = 0.95$) of the mean growth rates were calculated as described by Snedecor and Cochran (34), as follows: $(t \times \text{SD})/\sqrt{n}$, where t is obtained from the t distribution table ($t_{0.95} = 4.303$ in the case of three repetitions), SD is the standard deviation of the mean growth rate, and n is the number of repetitions.

Proteinase isolation. The Prt^+ *L. lactis* strains were grown in milk to the end of the exponential growth phase, removed from the culture medium by centrifugation ($10{,}000 \times g$ for 10 min at 4°C), and washed three times in sterile 50 mM Tris-HCl (pH 8) containing 30 mM $CaCl_2$. The proteinase was released from the cells by incubation for 30 min at 30°C in Ca^{2+}-free buffer (22) and was purified by ion-exchange chromatography as previously described (18). The proteolytic activity of the proteinase fractions was determined by using the chromogenic peptide methoxy-succinyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitroanilide (Chromogenix, Möldaln, Sweden) or fluorescein isothiocyanate-labeled casein (Sigma) as the substrate (4, 36).

Milk peptide analysis. Cells were removed by centrifugation ($10{,}000 \times g$ for 10 min at 4°C), and proteins were precipitated with 1% (vol/vol) trifluoroacetic acid (TFA). After the proteins were removed by centrifugation ($10{,}000 \times g$ for 10 min at 4°C), the supernatant was filtered through a 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.). The 1% TFA-soluble peptides were separated at 40°C by HPLC on a reverse-phase C_{18} column (Nucleosil; 4.6 by 250 mm; Shandon HPLC, Cheshire, United Kingdom). Solvents A and B were 0.11% (vol/vol) TFA in MilliQ-treated water (Millipore) and 0.1% (vol/vol) TFA–60% (vol/vol) acetonitrile in MilliQ-treated water, respectively. A 40-min linear 0 to 60% solvent B gradient with a flow rate of 1 ml/min was used. The eluted peptides were detected by on-line absorbance at 214 nm and fluorescence after postcolumn derivatization of the eluted peptides with *o*-phthalaldehyde, as previously described (12). UV detection was monitored prior to peptide derivatization. For detection of fluorescence the excitation and emission wavelengths were 340 and 425 nm, respectively.

RESULTS

Growth of *L. lactis* in milk. As reported previously for several lactococci (7, 11, 17), the genetically engineered strains *L. lactis* MG611-1 (P_I-type proteinase) and *L. lactis* SH5-1 (P_{III}-type proteinase) displayed biphasic exponential growth in milk, with the change to the lower growth rate corresponding to the utilization of caseins as an amino acid source (17). The concentrations of 1% TFA-soluble peptides in the milk before and after growth of the two strains were determined by reverse-phase HPLC. The peptide content of the milk changed drastically during growth and depended on the type of proteinase (Fig. 1). For instance, the peak eluting at 14.5 min was detected at the end of growth of *L. lactis* SH5-1, whereas no corresponding peptide(s) was present in the milk cultured with *L. lactis* MG611-1. Moreover, there were quantitative differences in the relative proportions of closely eluting peptides (for instance, peptides eluting at retention times of 18.4 and 18.8 min). As expected, these differences were detected only during the second phase of growth, as caseins were utilized as the source of amino acids during this phase (17).

Despite marked differences in the peptide contents of the milk following growth of *L. lactis* MG611-1 and SH5-1, no difference in the growth kinetics of the two strains was observed, as previously reported (7, 11). In particular, there was no significant difference ($P < 0.01$) between the growth rates of the two strains during the second exponential phase (0.75 ± 0.06 and 0.74 ± 0.07 h^{-1}, respectively).

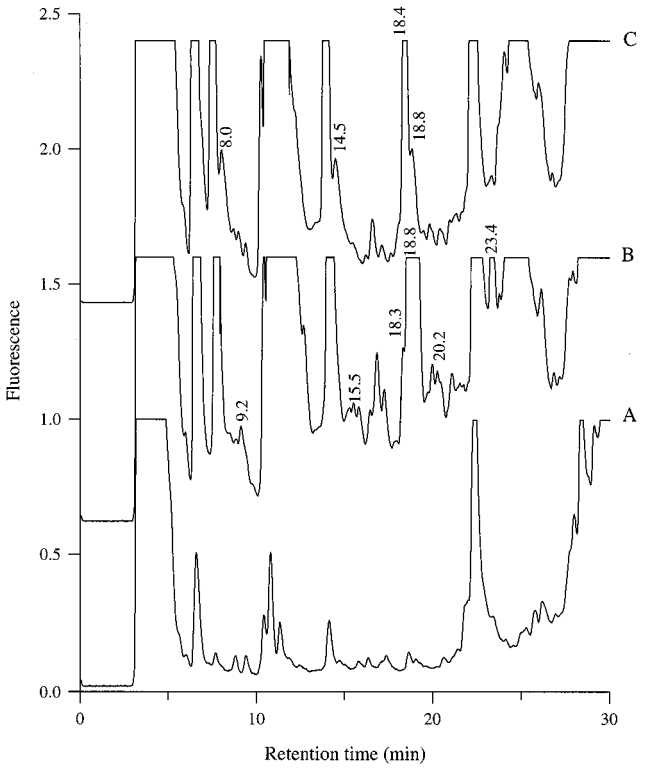

FIG. 1. (A) Peptide chromatogram for uninoculated milk. (B and C) Peptide chromatograms for milk cultures of *L. lactis* MG611-1 (P_I-type proteinase) (B) and SH5-1 (P_{III}-type proteinase) (C) grown to the stationary phase.

Hydrolysis of milk with purified proteinase. To analyze further the growth of *L. lactis* as a function of the type of proteinase, a complementary approach was used. Milk was incubated for 2 h with either P_I- or P_{III}-type purified proteinase prior to inoculation with the Prt^- strain *L. lactis* MG1363. Predigestion of milk caseins with purified proteinase stimulated the growth of *L. lactis* MG1363 (Fig. 2). However, the extent of the stimulation depended on the type of proteinase; the increases in the growth rate were 17% ± 6% and 37% ± 10% with the P_I- and P_{III}-type proteinases, respectively (means of three repetitions ± confidence limits; $P = 0.95$). Similarly, there were differences in the extent of the increases in the maximal populations of *L. lactis* MG1363.

As expected, the peptide content of the milk after incubation with purified proteinase depended on the type of enzyme added (Fig. 3). Interestingly, the six HPLC peaks produced only by the P_{III}-type proteinase (at retention times of 14.5, 14.7, 16.5, 16.7, 19.3, and 26.9 min) were not detected at the end of growth of the Prt^- strain. In contrast, only three of the peaks which were specifically produced by the P_I-type proteinase (at retention times of 19.1, 24.1, and 24.8 min) disappeared during growth of the proteinase-negative strain (data not shown).

Utilization of caseins as the sole source of amino acids. The results described above suggest that the amino acids obtained from caseins may be influenced by the type of proteinase. To confirm this, *L. lactis* MG611-1 (P_I-type proteinase) and SH5-1 (P_{III}-type proteinase) were cultured in CDM containing α_{s1}-,

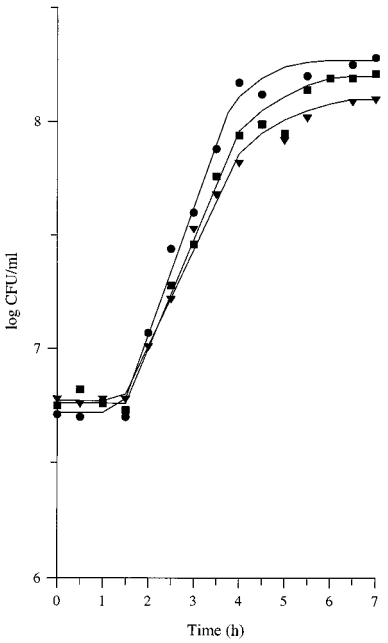

FIG. 2. Growth of *L. lactis* MG1363 (proteinase negative) in control milk (▼) and in milk previously incubated for 2 h with P_I-type purified proteinase (■) or P_{III}-type purified proteinase (●). P_I- and P_{III}-type proteinases were isolated from *L. lactis* MG611-1 and SH5-1, respectively, and were added to the milk at the same activity (4% of a 0.4% solution of fluorescein isothiocyanate-labeled casein was hydrolyzed within 1 h).

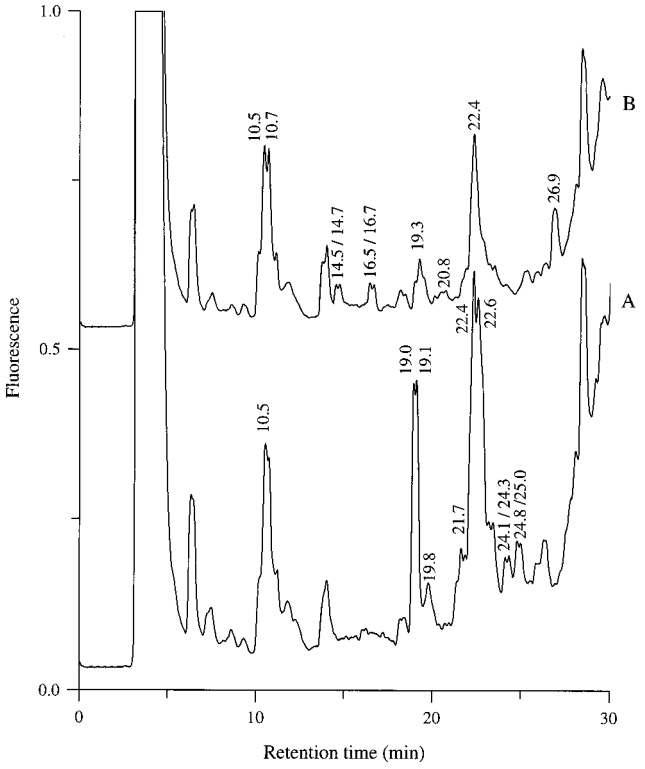

FIG. 3. Peptide chromatograms for milk following 2 h of incubation with P_I-type (A) or P_{III}-type (B) purified proteinase. P_I- and P_{III}-type proteinases were isolated from *L. lactis* MG611-1 and SH5-1, respectively.

β-, or κ-casein or CDM containing combinations of caseins as the sole sources of amino acids.

The ability of individual caseins to support growth of *L. lactis* did not depend on the type of proteinase, except for β-casein (Table 1). No growth was observed in the presence of α_{s1}-casein alone, indicating that at least one essential amino acid was not present in the casein-derived peptides that the strains were able to translocate. In contrast, all of the essential amino acids required by the strains were present in the peptides released from κ-casein, regardless of the type of proteinase. However, the growth rates were significantly lower than those in the presence of a mixture of 19 free amino acids, suggesting that they were limited by the rate at which amino acids were supplied.

Both *L. lactis* MG611-1 and SH5-1 grew to some extent in the presence of mixtures of caseins, but the growth rates were lower than the growth rates in the presence of free amino acids. Complementation between caseins as sources of amino acids was observed only with the P_I-type proteinase; the growth rate of *L. lactis* MG611-1 in the presence of a mixture of α_{s1}- and β-caseins or a mixture of β- and κ-caseins was higher than the growth rates obtained with individual caseins. In contrast, the growth rate of *L. lactis* in the presence of both α_{s1}- and κ-caseins was significantly lower ($P < 0.05$) than the growth rate in the presence of κ-casein alone, regardless of the type of proteinase. This suggests that the α_{s1}-casein-derived peptides had an inhibitory effect on the growth rate, which was partially overcome by adding β-casein to the mixture.

Nature of growth rate limitation. Experiments in which different combinations of five of six essential amino acids were added to CDM containing individual caseins made it possible to identify the amino acid deficiency which was responsible for the lack of growth of *L. lactis* when individual caseins were used as the sole sources of amino acids. For instance, the inability of β-casein to support growth of *L. lactis* MG611-1 was due to a lack of His-containing peptides (Fig. 4). α_{s1}-Casein was the poorest source of essential amino acids for *L. lactis*, regardless of the type of proteinase. Several essential amino acids were not provided. Surprisingly, the growth rate in the presence of α_{s1}-casein supplemented with the six essential amino acids was significantly ($P < 0.05$) lower than the growth rate in the presence of β- or κ-casein supplemented with the same mixture of amino acids, whatever strain was used. In addition, the growth rate in the presence of α_{s1}-casein and the

TABLE 1. Growth of *L. lactis* MG611-1 (P_I-type proteinase) and *L. lactis* SH5-1 (P_{III}-type proteinase) in CDM containing caseins as the sole sources of amino acids

Source of amino acids[a]	Growth rate (h^{-1})[b]	
	L. lactis MG611-1	*L. lactis* SH5-1
α_{s1}-Casein	NG[c]	NG
β-Casein	NG	0.09 ± 0.03
κ-Casein	0.77 ± 0.10	0.59 ± 0.08
α_{s1}-Casein + β-casein	0.30 ± 0.09	0.12 ± 0.04
α_{s1}-Casein + κ-casein	0.33 ± 0.03	0.40 ± 0.05
β-Casein + κ-casein	0.91 ± 0.13	0.50 ± 0.10
α_{s1}-Casein + β-casein + κ-casein	0.62 ± 0.04	0.53 ± 0.06
19 free amino acids	1.39 ± 0.06	1.35 ± 0.07

[a] Caseins were added to a final concentration of 2.4 g/liter. When a mixture was used, the ratio of α_{s1}-, β-, and κ-casein was the ratio in milk (i.e., 6:5:2). The amino acid concentration was the amino acid concentration established by Poolman and Konings (29).

[b] Mean of three determinations ± confidence limits at $P = 0.95$.

[c] NG, no growth.

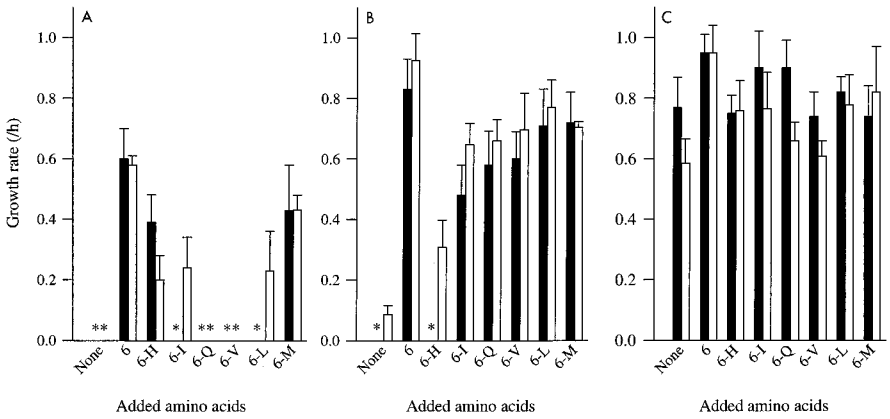

FIG. 4. Growth of *L. lactis* in CDM containing α_{s1}-casein (A), β-casein (B), or κ-casein (C) and supplemented with different mixtures of amino acids as nitrogen sources. Solid bars, *L. lactis* MG611-1 (P_I-type proteinase); open bars, *L. lactis* SH5-1 (P_{III}-type proteinase). 6, mixture of His (H), Ile (I), Gln (Q), Val (V), Leu (L), and Met (M); 6-X, mixture lacking amino acid X. The amino acid concentrations were the amino acid concentrations in CDM (27), and the casein concentration was 2.4 g/liter. Error bars indicate confidence limits at $P = 0.95$. Asterisks indicate that no growth occurred.

six essential amino acids was also lower than the growth rate observed during growth in the presence of only the essential amino acids (0.93 ± 0.07 h^{-1} for both strains). These results are consistent with the previously suggested hypothesis that the α_{s1}-casein-derived peptides inhibit the growth rate in some way.

It has been demonstrated previously that the rate of casein hydrolysis limits the growth rate of *L. lactis* in milk or in casein-containing media (11). Thus, a comparison of the growth rates in CDM containing individual caseins and the growth rates in CDM supplemented with different combinations of the essential amino acids should provide information concerning the rate of production of peptides containing each of the essential amino acids. For instance, the growth rate in CDM containing β-casein and a mixture of all of the essential amino acids except Ile depends on the rate of release of Ile-containing peptides from β-casein. In this medium, the growth rate of *L. lactis* MG611-1 was lower than the growth rate of *L. lactis* SH5-1, indicating that the amount of Ile-containing peptides released by the P_I-type proteinase limits the growth rate to a greater extent than the amount of Ile-containing peptides released by the P_{III}-type proteinase. Limitation of the growth rate in CDM containing β-casein was almost entirely overcome by adding His, Ile, and Gln, with the growth rate equivalent to 92 and 88% of the growth rates of *L. lactis* MG611-1 and SH5-1 in CDM containing β-casein and the six essential amino acids, respectively. Similarly, the availability of His, Val, and Met in peptides released from κ-casein limited the growth of *L. lactis* MG611-1 because (i) addition of a mixture lacking one of these amino acids did not increase the growth rate and (ii) addition of His, Val, and Met to the culture medium slightly increased the growth rate (0.83 ± 0.06 h^{-1}, compared with 0.77 ± 0.10 h^{-1} without amino acid addition). The limitation of the growth rate of *L. lactis* SH5-1 in CDM containing κ-casein was due to a deficiency of Val- and Gln-containing peptides; addition of these two amino acids resulted in a 30% increase in the growth rate of *L. lactis* SH5-1.

Quantitation of the limitation of the growth rate of *L. lactis* by individual caseins also made it possible to explain the previously observed complementation between caseins as the sole source of essential amino acids. For instance, hydrolysis of β-casein by *L. lactis* MG611-1 did not provide His-containing peptides but produced Ile-containing peptides. In contrast, hydrolysis of α_{s1}-casein by the same strain released His-containing peptides but no Ile-containing peptides. Therefore, complementation between these two types of caseins could be expected. Moreover, the limitation of the growth rate by Ile-containing peptides released from β-casein was less than the limitation of the growth rate by His-containing peptides derived from α_{s1}-casein (Fig. 4). Therefore, we expected that the growth rate of *L. lactis* MG611-1 in CDM containing both β- and α_{s1}-caseins as the sole source of amino acids would be limited by the amount of His-containing peptides. Consequently, this growth rate should be in the same range as the growth rate in CDM containing α_{s1}-casein and a mixture of all of the essential amino acids except His. The respective growth rates were 0.30 ± 0.09 and 0.39 ± 0.09 h^{-1}.

In contrast, no complementation between peptides released from β- and α_{s1}-caseins was expected with the P_{III}-type proteinase, although all of the amino acid deficiencies of the peptides released from α_{s1}-casein could be overcome by peptides derived from β-casein. Both β-casein and α_{s1}-casein released a growth-limiting amount of His-containing peptides. The growth rate in CDM containing α_{s1}-casein and a mixture of all of the essential amino acids except His was lower than the growth rate in CDM containing β-casein and the same mixture of amino acids (Fig. 4). Consequently, the growth rate of *L. lactis* SH5-1 in the presence of β- and α_{s1}-caseins as the sole source of amino acids should be in the same range as the growth rate in CDM containing only β-casein. This was observed, with growth rates of 0.12 ± 0.04 and 0.09 ± 0.03 h^{-1}, respectively.

DISCUSSION

The use of genetically engineered strains of *L. lactis* that differed only in the type of proteinase produced made it possible to study the contribution of caseins to the amino acid supply. Growth experiments showed that κ-casein is the best source of amino acids for growth and that a mixture of β- and κ-caseins results in high growth rates of *L. lactis* strains containing either P_I- or P_{III}-type proteinase. This is consistent with results reported previously for the P_I-type-proteinase-producing strain *L. lactis* subsp. *cremoris* HP (6) and suggests that a mixture of β- and κ-caseins fulfills the amino acid requirements of any *L. lactis* strain.

Biochemical and physiological analyses of casein hydrolysis led to opposite conclusions. Biochemical studies on the spec-

ificity of casein hydrolysis by purified proteinases indicated that (i) β-casein is the preferred substrate for P_I-type proteinases and (ii) α_{s1}-casein is not significantly cleaved by P_I-type proteinases (31, 37). From the present study, it is clear that β-casein is not the optimum source of amino acids for *L. lactis*. In addition, α_{s1}-casein provides His and Met to a P_I-type-proteinase-containing strain. The biochemical approach focuses mainly on the amount of substrate that is hydrolyzed; degradation of a small amount of caseins is considered insignificant, regardless of the nature of the peptides released. In contrast, the physiological approach focuses on only some of the released peptides (i.e., the peptides that can be translocated into the cell by the oligopeptide transport system). Because growth of *L. lactis* in milk up to the maximum yield (e.g., 2×10^9 CFU/ml) requires the synthesis of approximately 200 μg of bacterial proteins (14), hydrolysis of only 1% of the milk caseins should be sufficient to sustain maximum growth.

Growth of *L. lactis* in casein-containing media is limited by the rate of casein hydrolysis, regardless of the type of proteinase (11). Because translocated peptides are instantaneously cleaved to amino acids by intracellular peptidases (20), the amino acid deficiency responsible for the growth rate limitation depends on the ability of the strain to transport casein-derived peptides. Elucidation of the specificity of oligopeptide utilization by *L. lactis* MG1363 (15) makes it possible to compare our results with the results of in vitro analyses of peptides released from caseins by purified proteinases (18, 30–32). The ability of κ-casein to support growth is consistent with the observation that hydrolysis of κ-casein by purified P_I- and P_{III}-type-proteinase resulted in the early release of four and three different oligopeptides, respectively, which contained all of the amino acids required for growth of *L. lactis* MG1363 (30). In the present study, significant differences in the nature of growth limitation were observed for the two types of proteinases. The peptides initially released from κ-casein by a purified P_I-type proteinase are QILQWQVL, ARHPHPH LSFM, LSFM, and (to a lesser extent) KYIPIQYVL (30). Only one of these peptides, KYIPIQYVL, is expected to be translocated rapidly into cells by the oligopeptide transport system, because it is a basic peptide whose molecular weight ranges from 600 to 1,100 (15). This peptide does not provide His or Met to the cells, but it contains two Ile residues. In contrast, the three other peptides should be transported at lower rates, either because they are not basic or because their molecular weights are not in the optimum range (15). These three peptides contain few Val residues. Therefore, the growth rate of *L. lactis* MG611-1 in CDM containing κ-casein should be limited by the rate at which Met, Val, and His are supplied. This expectation is consistent with the data obtained from growth experiments. In contrast, the same approach suggests that the growth rate of *L. lactis* SH5-1 (P_{III}-type proteinase) in CDM containing κ-casein should be limited by the rate at which Met and Ile are supplied, because the peptides initially released by the purified proteinase are AVRSPAQILQWQVL (molecular weight, 1,609; pI 11.3), ARHPHPHLSFM (molecular weight, 1,329; pI 11.3), and TVQVTSTAV (molecular weight, 905; pI 6.1) (30). This is not consistent with our observation that growth was limited by the rate at which Val and Gln were produced. There are two hypotheses that can be used to explain this discrepancy. First, some other peptides which have not been detected and/or identified in previous studies, may be released early from κ-casein by purified proteinase. Consequently, the present estimates of amino acid supply may be incorrect. The other possible explanation is that the specificity of peptide bond cleavage with a purified P_{III}-type proteinase might differ significantly from the specificity of peptide bond cleavage with a native P_{III}-type proteinase (i.e., a proteinase bound to the cell envelope). It is worth noting that the P_{III}-type proteinase has been reported to cleave the 1-23 fragment of α_{s1}-casein in a different manner when it was used as a purified enzyme or bound to the cell envelope (5). Similarly, the identities of growth-limiting amino acids obtained from growth experiments performed with *L. lactis* MG611-1 in CDM containing β-casein were also consistent with the identities deduced from an analysis of the peptides released from β-casein by purified P_I-type proteinase (18, 31). In contrast, there was a discrepancy between the results of growth experiments performed in CDM containing α_{s1}- or β-casein and the results of an analysis of the products of hydrolysis of α_{s1}- and β-caseins by purified P_{III}-type proteinase (31, 32).

The contribution of caseins to the amino acid supply of *L. lactis* depends on the type of proteinase. As a result, *L. lactis* MG611-1 (P_I-type proteinase) grows at a higher rate than *L. lactis* SH5-1 (P_{III}-type proteinase) in CDM containing a mixture of caseins. However, the two strains grow at the same rate in milk. The growth rate in milk during the second phase (i.e., the phase corresponding to casein utilization) (17) is significantly higher than the growth rate in CDM containing caseins. Therefore, the data suggest that there is complementation between peptides released from caseins and the other sources of amino acids in milk, (i.e., the free amino acids and the peptides that are initially present in milk). Moreover, the complementation should be more efficient with the P_{III}-type proteinase than with the P_I-type proteinase. As *L. lactis* MG1363, MG611-1, and SH5-1 differ only in the proteinases that they produce, the causes of the growth arrest of *L. lactis* MG1363 (Prt^- variant) are similar to the causes of the change in the growth rates of the Prt^+ strains. The growth of *L. lactis* MG1363 in milk stops because the milk lacks sources of Met, Ile, Leu, and Val other than caseins (15). The growth of *L. lactis* MG611-1 (P_I-type proteinase) in CDM containing caseins as the sole source of amino acids is limited by the availability of His, Met, and Val. Therefore, the second growth phase of *L. lactis* MG611-1 in milk should be limited by the amount of Met and Val. Addition of these two amino acids to milk resulted in a 20% (± 4%) stimulation of the growth rate during the second exponential growth phase (mean of four repetitions ± confidence limits; $P = 0.95$). On the other hand, κ-casein was found to be the main source of Met and Val for *L. lactis* MG611-1. As the growth of *L. lactis* in milk is limited by the rate of casein hydrolysis (11), the growth rate in the second exponential growth phase of *L. lactis* MG611-1 should be limited by the rate of κ-casein hydrolysis. Consequently, it should be in the same range as the growth rate in CDM containing κ-casein and a mixture of essential amino acids without Met and/or Val (i.e., 0.74 h^{-1}). This is in full agreement with our observations; the growth rate of *L. lactis* MG611-1 during the second growth phase in milk was 0.75 ± 0.06 h^{-1}. Similarly, the growth rate of *L. lactis* SH5-1 (P_{III}-type proteinase) in CDM containing caseins was limited by the rate at which Val was supplied. Therefore, the second growth phase of this strain in milk should be limited by the amount of Val. Addition of this amino acid to the milk resulted in a 32% (± 5%) stimulation of the growth rate during the second exponential growth phase (mean of four repetitions ± confidence limits; $P = 0.95$). As the main source of Val for *L. lactis* SH5-1 is β-casein, the growth rate in milk during the second phase should be in the same range as the growth rate in CDM containing β-casein and a mixture of all of the essential amino acids except Val. Again, that is what was observed.

In conclusion, the contribution of bovine milk caseins to the amino acid supply for *L. lactis* depends on the type of cell

envelope proteinase. Because the growth rate of *L. lactis* in milk is limited by the rate of casein hydrolysis (11), the nature of the growth rate limitation in milk depends on the type of proteinase produced.

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