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Oligopeptides Are the Main Source of Nitrogen for *Lactococcus lactis* during Growth in Milk

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The consumption of amino acids and peptides was monitored during growth in milk of proteinase-positive (Prt^+) and -negative (Prt^-) strains of *Lactococcus lactis*. The Prt^- strains showed monophasic exponential growth, while the Prt^+ strains grew in two phases. The first growth phases of the Prt^+ and Prt^- strains were the same, and no hydrolysis of casein was observed. Also, the levels of consumption of amino acids and peptides in the Prt^+ and Prt^- strains were similar. At the end of this growth phase, not all free amino acids and peptides were used, indicating that the remaining free amino acids and peptides were unable to sustain growth. The consumption of free amino acids was very low (about 5 mg/liter), suggesting that these nitrogen sources play only a minor role in growth. Oligopeptide transport-deficient strains (Opp^-) of *L. lactis* were unable to utilize oligopeptides and grew poorly in milk. However, a di- and tripeptide transport-deficient strain ($DtpT^-$) grew exactly like the wild type (Opp^+ $DtpT^+$) did. These observations indicate that oligopeptides represent the main nitrogen source for growth in milk during the first growth phase. In the second phase of growth of Prt^+ strains, milk proteins are hydrolyzed to peptides by the proteinase. Several of the oligopeptides formed are taken up and hydrolyzed internally by peptidases to amino acids, several of which are subsequently released into the medium (see also E. R. S. Kunji, A. Hagting, C. J. De Vries, V. Juillard, A. J. Haandrikman, B. Poolman, and W. N. Konings, J. Biol. Chem. 270:1569–1574, 1995). It is concluded that growth of *L. lactis* in milk depends on oligopeptides as nitrogen source for 98% of growth and that the oligopeptide transport system plays a crucial role in the utilization of these peptides.

Lactococci have numerous nutritional requirements for growth, especially nitrogen sources (25, 34). The amino acid requirement appears to be strain dependent, but most *Lactococcus lactis* strains need isoleucine, leucine, histidine, methionine, and valine for growth (2). Several distinct amino acid transport systems allow lactococci to utilize free amino acids as nitrogen sources (21). However, the concentrations of these essential amino acids, and especially of isoleucine and leucine, have been reported to be very low in milk (27). The use of amino acids and peptides in milk allows lactococci to grow to low cell densities. Peptide utilization requires the concerted action of a peptidase(s) and a transport system(s). Both biochemical and genetic approaches strongly suggest an intracellular location of all peptidases characterized to date (20, 39). Consequently, no extracellular degradation of milk peptides is expected, and translocation should occur prior to hydrolysis by internally located peptidases. Two distinct peptide transport systems have been evidenced in lactococci; they have been characterized physiologically (23, 37) and genetically (11, 44). Their respective roles in milk peptide utilization are not yet established, although attention has been devoted in the past to physiological implications of peptide transport in lactococci (for a review of the earliest studies, see reference 45).

The use of caseins strongly increases (4- to 10-fold) the extent of growth of lactococci in milk (42). The process of casein utilization by lactococci has been studied extensively over the last 15 years (for recent reviews, see references 30, 31, and 40). A complex proteolytic system, which includes a cell envelope-located proteinase, several peptidases, and the two peptide transport systems, is needed. It is now well established that the cell envelope-located proteinase is involved in the first step of casein degradation. The in vitro action of purified proteinases on caseins has been studied in great detail (17, 32, 33). The identified peptides derived from β-, κ-, and α_{S1}-caseins are systematically longer than 4 residues. As already stated for milk peptides, no further extracellular degradation of the proteinase-released peptides from casein is expected. Moreover, the oligopeptide transport system is essential for β-casein utilization and growth in milk (22, 44). Therefore, oligopeptide transport is very likely the next step in the proteolytic pathway and is followed by an internal degradation of the peptides by a variety of peptidases.

The degradation of casein by the cell envelope-located proteinase of *L. lactis* proceeds at a low rate, and the production of proteolytic products in milk is growth rate limiting (14). Overproduction of proteinase in *L. lactis* strains results in an increased growth rate in milk (1, 26). Consistent with these observations is that proteinase-negative (Prt^-) variants of *L. lactis* are unable to grow in milk in which a proteinase-positive strain (Prt^+) was grown previously, whereas the addition of amino acids to this precultured milk restores growth of the Prt^- variants (8, 17a). Several authors have reported an increased content of free amino acids and peptides in milk after growth of different Prt^+ strains of *L. lactis* (13, 18). This means that these accumulated products are not used for further growth of lactococci.

Unfortunately, very little information on the changes in amino acid and peptide contents of milk during growth of lactococci is available. One study reported amino acid limitation during growth of a Prt^+ strain of *L. lactis* subsp. *cremoris*

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TABLE 1. Bacterial strains used

L. lactis subsp. *lactis* strain	Phenotype[a]	Source or reference[b]
CNRZ 1076	Wild type	INRA
CNRZ 1075	Prt^- Lac^+ derivative of CNRZ 1076	INRA
NCDO 763	Wild type	NCDO
CNRZ 1259	Prt^- Lac^+ derivative of NCDO 763	INRA
MG1363	Prt^- Lac^- plasmid-free derivative of *L. lactis* NCDO 712	9
VS772	Em^r Prt^- Lac^- Opp^- derivative of MG 1363	44
AG300	Prt^- Lac^- $DtpT^-$ derivative of MG 1363	11
CV4	Em^r Prt^- Lac^- $DtpT^-$ Opp^- derivative of MG 1363	22

[a] Prt, ability to produce a functional cell-envelope located proteinase; Lac, ability to use lactose as energy source; Opp, ability to translocate oligopeptide; DtpT, ability to translocate di- and tripeptides; Em^r, resistance to erythromycin.
[b] INRA, Institut National de la Recherche Agronomique, Jouy-en-Josas, France; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom.

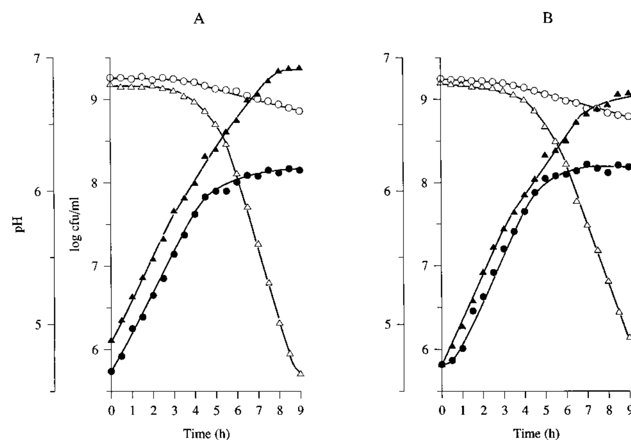

FIG. 1. Growth of *L. lactis* in milk. (A) *L. lactis* subsp. *lactis* CNRZ 1076 (Prt^+ strain) (▲) and its Prt^- variant, *L. lactis* subsp. *lactis* CNRZ 1075 (●). (B) *L. lactis* subsp. *lactis* NCDO 763 (Prt^+ strain) (▲) and its Prt^- variant, *L. lactis* subsp. *lactis* CNRZ 1259 (●). Open symbols correspond to the pH changes during growth of either Prt^+ strains (△) or Prt^- variants (○).

in milk (14). However, the fate of the amino acids and peptides initially present in milk during growth of lactococci remains unknown. The aim of this work was to analyze the utilization of free amino acids and peptides during growth of *L. lactis* in milk to evaluate their respective roles in growth of this microorganism.

MATERIALS AND METHODS

Strains and culture conditions. All strains used in this study belong to the subspecies *L. lactis* subsp. *lactis* (Table 1). *L. lactis* subsp. *lactis* MG1363 is a plasmid-free derivative strain of *L. lactis* subsp. *lactis* NCDO 712 (9), which has been shown to be very closely related to *L. lactis* subsp. *lactis* NCDO 763, also known as ML3 (4). The three well-defined peptide transport mutants *L. lactis* subsp. *lactis* AG300, VS772, and CV4 are derived from *L. lactis* subsp. *lactis* MG1363; either the di- and tripeptide transport gene (*dtpT*) has been deleted from the chromosome (11), the gene encoding the binding protein of the oligopeptide transport system (OppA) has been disrupted (44), or both manipulations have been performed (22), respectively. The two pairs of isogenic Prt^+ and Prt^- strains (CNRZ 1076 and CNRZ 1075; NCDO 763 and CNRZ 1259) were stored at −80°C in 10% (wt/vol) reconstituted sterile litmus milk supplemented with glucose and yeast extract (0.5% [wt/vol] each). *L. lactis* subsp. *lactis* MG1363 and its transport-negative mutants were stored at −80°C in M17 broth (41) containing glucose (0.5% [wt/vol]) and glycerol (10% [vol/vol]) as well as 5 μg of erythromycin per ml when necessary.

Cells were grown at 30°C in reconstituted skim milk (10% Nilac Low Heat milk powder; NIZO, Ede, The Netherlands). Given the good bacteriological quality of this milk (less than 10^2 bacteria per ml), no further heat treatment was required, so that its content of free amino acids and peptides was not further affected. In the case of lactose-negative (Lac^-) strains, milk was supplemented with glucose (1% [wt/vol]) and erythromycin (5 μg/ml) when needed. Milk was inoculated with 10^6 CFU of a preculture in the exponential stage of growth per ml. Lac^+ strains were precultured in milk. Lac^- strains were precultured in M17 broth (containing 5 μg of erythromycin per ml when required) and were washed twice in 50 mM KH_2PO_4-K_2HPO_4 (pH 6.5) prior to inoculation.

Sequential cultures of *L. lactis* were performed as already described (8, 17a). Milk was first inoculated with *L. lactis* subsp. *lactis* CV4 (Prt^- Opp^- $DtpT^-$) and incubated for 8 h. The pH of the milk was then adjusted to that of the noninoculated milk (i.e., 6.8) before pasteurization for 30 min at 63°C. A second culture of *L. lactis* was subsequently inoculated in this precultured milk. It has been shown previously that the behavior of the second culture was not affected by pH adjustment, pasteurization, or the presence of dead cells or lactate (17a).

Bacterial enumerations. Cell populations were estimated by spiral plating of appropriate milk dilutions on M17 medium. The accuracy and precision of this plating method have been assessed previously (12). The homogeneity of the Prt^+ population (possible presence of Prt^- variants) at the beginning and end of incubation was checked by use of Fast-Slow-Differential-Agar medium (15). The percentage of Prt^- variants at the end of culturing the Prt^+ strain was always less than 2% of the total population.

Cell lysis. Lysis of cells during growth was estimated from the release into the growth medium of the aminopeptidases PepN and/or PepC and of the X-prolyl-dipeptidyl-aminopeptidase PepX, which have all been characterized as intracellular enzymes (19, 39). The activities of PepN and PepC were checked with L-lysine-β-naphthylamide as a chromogenic substrate, and that of PepX was checked with L-phenylalanyl-L-proline-β-naphthylamide as a chromogenic substrate. When required, the pH of the milk was first decreased to 4.6 to precipitate casein. After removing cells and casein from the culture medium by centrifugation (3,000 × *g* for 10 min at 4°C) and adjusting the pH to 6.8, the supernatant was tested for the presence of peptidases by monitoring the liberation of β-naphthylamide from 0.25 mM chromogenic substrate after 2 h of incubation at 30°C by measuring the ΔA_{550}. The comparison of the activities of PepX-containing solutions diluted in milk or in buffer indicated that no interference by media constituents occurred in the assay. Aminopeptidase activities were compared with those of lysed cells. Lysis was achieved by incubating the cell suspension with mutanolysin (5 U/ml) and lysozyme (0.4 mg/ml) at 30°C, up to a stable final optical density value at 480 nm that was 10% of the initial one. The sensitivity threshold of this method was about 5×10^6 lysed cells per ml. Complete lysis of the cell suspension was probably not achieved by this procedure, which could lead to a slight overestimation of the number of lysed cells.

Amino acid analysis. Cells were removed by centrifugation (3,000 × *g* for 10 min at 4°C), and proteins and peptides were precipitated by 3% (vol/vol) 5-sulfosalicylic acid (10). After centrifugation, the supernatant was diluted with an equal volume of 0.12 M sodium citrate (pH 2.2) and filtered through a 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.). The amino acid content of this filtrate was determined with an LC 5000 amino acid analyzer (Biotronik, Munich, Germany), which was calibrated with an external standard containing 17 amino acids of known concentrations (Asn, Gln, and Trp were missing). Asparagine and glutamine could not be determined (coeluted with Thr and Ser).

Peptide analysis. Cells were removed by centrifugation (3,000 × *g* for 10 min at 4°C), and proteins were precipitated by 1% (vol/vol) trifluoroacetic acid. After removal of the proteins by centrifugation (3,000 × *g* for 15 min at 4°C), the supernatant was filtered through a 0.45-μm-pore-size filter (Millipore). The 1% trifluoroacetic acid-soluble peptides were separated at 40°C by high-performance liquid chromatography (HPLC) on a reverse-phase C_{18} column (Nucleosil, 10 μm, 4.6 mm [inside diameter] by 250 mm; SFCC, Eragny, France) as described previously (17). Solvent A was 0.11% (vol/vol) trifluoroacetic acid in MilliQ water, and peptides were eluted at 30°C with a linear gradient of solvent B (0.1% [vol/vol] trifluoroacetic acid, 60% [vol/vol] acetonitrile in MilliQ water).

RESULTS

Growth of *L. lactis* in milk. The proteinase-negative (Prt^-) *L. lactis* subsp. *lactis* strains CNRZ 1075 and CNRZ 1259 and the proteinase-positive (Prt^+) strains CNRZ 1076 and NCDO 763 (Table 1) were grown in milk (Fig. 1). The growth experiments were performed six times; the growth kinetic parameters are summarized in Table 2. The two Prt^- strains behaved identically and grew to low cell densities. The two Prt^+ strains displayed two exponential growth phases, and the maximal population level was markedly increased (about 10-fold). Remarkably, the shift in growth phase occurred at the same cell

TABLE 2. Growth characteristics of *L. lactis* subsp. *lactis* cultured in milk

L. lactis subsp. *lactis* strain	Growth rate (h^{-1})[a]		Cell density (CFU/ml)[a]	
	First phase	Second phase	End of first phase	Maximum
CNRZ 1076 (Prt^+)	1.67 ± 0.07	1.18 ± 0.07	$(10.8 \pm 3.2) \times 10^7$	$(2.3 \pm 0.4) \times 10^9$
NCDO 763 (Prt^+)	1.75 ± 0.06	1.17 ± 0.06	$(10.7 \pm 4.1) \times 10^7$	$(1.5 \pm 0.2) \times 10^9$
CNRZ 1075 (Prt^-)	1.63 ± 0.06	NO[b]	$(8.0 \pm 1.6) \times 10^7$	$(1.4 \pm 0.2) \times 10^8$
CNRZ 1259 (Prt^-)	1.67 ± 0.06	NO	$(7.9 \pm 2.1) \times 10^7$	$(1.6 \pm 0.2) \times 10^8$

[a] Values given are means of six repetitions ± confidence limits at *P* of 0.95.
[b] NO, not observed.

density at which the Prt^- variants ceased exponential growth. Moreover, the growth rate of the Prt^+ strains during the first exponential growth phase was identical to that of the Prt^- variants.

During growth of the Prt^- and Prt^+ strains in milk, no release of general aminopeptidases (PepN and/or PepC) or X-prolyl dipeptidyl-aminopeptidase (PepX) could be detected, indicating that cell lysis hardly occurred during the growth period.

Utilization of free amino acids during growth in milk. The utilization of the amino acids during growth in milk of the Prt^- strain *L. lactis* subsp. *lactis* CNRZ 1075 was analyzed (Table 3). The concentration of each amino acid just after inoculation was not significantly different ($P = 0.95$) from that in the non-inoculated milk. These concentrations were very close to those reported previously (27). The free amino acid pool of the milk was clearly not depleted at the end of growth. A significant decrease ($P = 0.95$) in concentration of only 5 amino acids (Thr, Ser, Ile, Leu, and Arg) could be evidenced. On the other hand, the concentration of only one amino acid (Met) increased during growth, presumably as a result of efflux from the cells. Similar results were obtained during growth of the Prt^- strain *L. lactis* subsp. *lactis* CNRZ 1259. Threonine and serine could no longer be detected in milk at the end of growth of this strain, whereas the concentrations of Ile, Leu, and Arg were lower than 1 mg/liter. The amino acid content of milk was also monitored during growth of the Prt^+ strain *L. lactis* subsp. *lactis* CNRZ 1076 (Table 3). A slight decrease of the amino acid pool was observed at the end of the first exponential growth phase. The small variations in amino acid concentrations were close to those observed during growth of the isogenic Prt^- variant CNRZ 1075. In the second exponential growth phase of the Prt^+ strain, the pool of free amino acids increased markedly. This increase resulted mainly from the accumulation of Glu and Pro, which accounted for about two-thirds of the total free amino acid content of the milk after growth. However, the concentrations of several amino acids remained low during this second growth phase. Similar results were obtained with *L. lactis* subsp. *lactis* NCDO 763.

TABLE 3. Free amino acid changes during growth of *L. lactis* subsp. *lactis* CNRZ 1075 (Prt^-) and CNRZ 1076 (Prt^+)

Amino acid	Concn (mg/liter)[a]			
	Initial	CNRZ 1075[b] (final)	CNRZ 1076[b]	
			End of first phase	Final
Asp	2.5 ± 0.8	3.0 ± 0.7	2.5 ± 0.9	10.8 ± 1.6
Thr	1.1 ± 0.3	0.4 ± 0.1	0.8 ± 0.3	0.7 ± 0.1
Ser	1.1 ± 0.2	0.3 ± 0.1	0.7 ± 0.3	0.5 ± 0.1
Glu	44.7 ± 4.1	43.5 ± 4.0	41.7 ± 3.5	97.6 ± 10.1
Pro	ND[c]	ND	1.5 ± 2.3[d]	71.4 ± 18.0
Gly	6.5 ± 0.5	5.7 ± 0.9	6.2 ± 0.4	ND
Ala	3.2 ± 0.3	2.4 ± 0.6	3.0 ± 0.2	1.3 ± 0.2
Cys	ND	ND	ND	ND
Val	2.1 ± 0.7	1.2 ± 0.7	2.3 ± 0.8	1.7 ± 0.9
Met	0.7 ± 0.2	1.7 ± 0.1	1.4 ± 0.8	1.9 ± 1.0
Ile	0.6 ± 0.2	ND	ND	ND
Leu	0.8 ± 0.1	ND	0.5 ± 0.2	1.3 ± 0.6
Tyr	2.5 ± 0.3	3.4 ± 0.7	3.0 ± 1.4	18.7 ± 2.0
Phe	2.6 ± 0.7	3.3 ± 0.1	1.8 ± 0.3	3.6 ± 1.3
His	4.8 ± 0.3	4.4 ± 0.1	4.1 ± 0.5	12.3 ± 3.0
Lys	5.8 ± 0.9	5.3 ± 0.9	5.0 ± 1.1	32.9 ± 8.8
Arg	4.9 ± 0.4	3.0 ± 0.9	4.4 ± 1.8	1.2 ± 0.4
Total	84.0 ± 4.8	77.6 ± 5.9	78.9 ± 6.9	255.9 ± 28.7

[a] Mean of four cultures ± confidence limits at *P* of 0.95.
[b] Cell density of CNRZ 1075 was $(1.5 \pm 0.3) \times 10^8$ CFU/ml at the stationary phase; cell densities of CNRZ 1076 were $(1.0 \pm 0.4) \times 10^8$ CFU/ml at the end of first growth phase and $(2.3 \pm 0.4) \times 10^9$ CFU/ml at the stationary phase.
[c] ND, not detected.
[d] Not detected in two of the four cultures.

Effect of the addition of free amino acids on the growth of *L. lactis* in milk. The results presented above revealed the consumption of certain amino acids during the growth of *L. lactis* subsp. *lactis* CNRZ 1076 (Prt^+ strain) and of its Prt^- variant, *L. lactis* subsp. *lactis* CNRZ 1075. To analyze the role of these amino acids on the growth of each strain, different mixtures of amino acids were added to the milk (Fig. 2).

The addition of a mixture of 20 common amino acids to milk at concentrations of 0.1 g of each per liter significantly stimulated the growth of the Prt^- *L. lactis* subsp. *lactis* CNRZ 1075. On the other hand, the growth of the Prt^+ strain CNRZ 1076 was not affected by the amino acid supplementation.

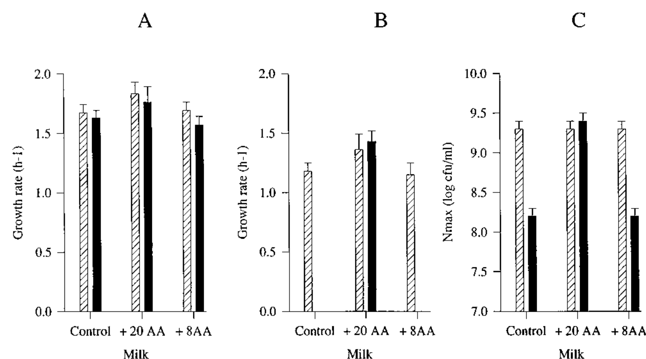

FIG. 2. Growth characteristics of *L. lactis* subsp. *lactis* CNRZ 1076 (▨) and its Prt^- variant (■) in milk supplemented with free amino acids. (A) Growth rate during the first exponential phase; (B) growth rate during the second exponential phase; (C) maximum cell density. +20 AA, milk supplemented with a mixture of the 20 usual amino acids (0.1 g of each per liter); +8 AA, milk supplemented with a mixture of Thr, Ser, Ala, Val, Ile, Leu, Arg, and Gly (0.1 g of each per liter).

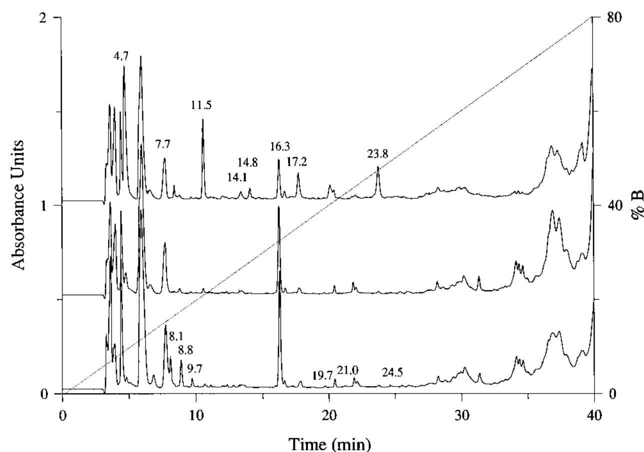

FIG. 3. Peptide chromatogram of milk during growth of *L. lactis* subsp. *lactis* CNRZ 1076 and its Prt$^-$ variant, *L. lactis* subsp. *lactis* CNRZ 1075. Traces: bottom, noninoculated milk; middle, after growth to stationary phase of the Prt$^-$ variant *L. lactis* subsp. *lactis* CNRZ 1075 (1.5×10^8 CFU/ml); top, after growth to stationary phase of the parental Prt$^+$ strain *L. lactis* subsp. *lactis* CNRZ 1076 (2×10^9 CFU/ml), respectively. Dotted line indicates the gradient. % B, percentage of solvent B in the gradient mixture.

The addition to milk of a mixture of eight amino acids, which decreased during growth of *L. lactis* subsp. *lactis* CNRZ 1076 and *L. lactis* subsp. *lactis* CNRZ 1075 and were present at concentrations below 1 mg/liter at the end of growth (i.e., Thr, Ser, Ala, Val, Ile, Leu, Arg, and Gly), did not affect the growth of the Prt$^+$ strain. Surprisingly, this addition also had no significant effect on the growth of the Prt$^-$ strain, suggesting that some other key nitrogen compounds were still missing.

Utilization of peptides during growth of *L. lactis* in milk. The content of 1% trifluoroacetic acid-soluble peptides in milk was determined by reverse-phase HPLC before and after culturing *L. lactis* (Fig. 3). The eluted peptides were not identified and are characterized by A_{214} and their retention times. The reverse-phase HPLC profiles of milk contained more than 50 peaks before culturing. The areas of some of them were too small to be determined accurately.

More than 40 peaks could still be detected at the end of growth of the Prt$^-$ strain CNRZ 1075, indicating that milk was also not completely depleted of peptides (Fig. 3). None of the individual peaks increased drastically, while 17 peaks decreased but to a generally low extent (Table 4). However, six of the small peaks were no longer detected after growth of the Prt$^-$ strain. The total decrease of the area of the 17 peaks during growth of *L. lactis* subsp. *lactis* CNRZ 1075 was less than 7% of the initial area of the chromatogram. The same analysis performed during the growth of *L. lactis* subsp. *lactis* CNRZ 1259 (Prt$^-$ strain) yielded similar results.

The variations in the peptide content of the milk during the first growth phase of the Prt$^+$ strain *L. lactis* subsp. *lactis* CNRZ 1076 were similar to those observed during growth of the isogenic Prt$^-$ variant *L. lactis* subsp. *lactis* CNRZ 1075. No increase in size of individual peaks could be evidenced. Moreover, all of the peaks which decreased during growth of the Prt$^-$ variant also decreased during growth of the Prt$^+$ strain (Table 4). Only some minor variations in intensities were noticed.

After the second growth phase of *L. lactis* subsp. *lactis* CNRZ 1076, the peptide content of milk was clearly more modified (Fig. 3). The total area of the chromatogram was slightly but significantly increased (110 ± 3 V/s versus 101 ± 3 V/s before culturing). This increase resulted from the appearance of new peaks and from the increase of peaks initially present in milk. On the other hand, some peaks which remained constant during the first growth phase, or during growth of the isogenic Prt$^-$ variant, decreased during the second growth phase, whereas others were definitely absent from the milk at the end of growth of the Prt$^+$ strain. The results

TABLE 4. Changes in the area of some peaks from HPLC analysis during growth of *L. lactis* subsp. *lactis* CNRZ 1075 (Prt$^-$) and CNRZ 1076 (Prt$^+$)

Retention time (min)	Peak area (mV/s)[a]			
	Initial	CNRZ 1075 (final)[b]	CNRZ 1076[b]	
			End of first phase	Final
4.8 ± 0.1	$(1.7 \pm 1.6) \times 10^2$	$(2.8 \pm 0.2) \times 10^2$	$(1.2 \pm 0.1) \times 10^2$	ND[c]
7.7 ± 0.1	$(5.5 \pm 0.1) \times 10^3$	$(4.4 \pm 0.3) \times 10^3$	$(4.1 \pm 0.3) \times 10^3$	$(3.4 \pm 0.1) \times 10^3$
8.1 ± 0.1	$(1.6 \pm 0.1) \times 10^2$	$(4.2 \pm 0.2) \times 10^1$	ND	ND
8.8 ± 0.1	$(1.3 \pm 0.1) \times 10^3$	$(2.1 \pm 0.4) \times 10^2$	$(1.1 \pm 0.7) \times 10^2$	$(1.6 \pm 1.5) \times 10^2$
9.7 ± 0.1	$(3.3 \pm 0.3) \times 10^2$	$(4.8 \pm 0.6) \times 10^1$	$(8.0 \pm 0.6) \times 10^1$	$(6.0 \pm 0.6) \times 10^1$
10.4 ± 0.1	$(1.9 \pm 1.0) \times 10^1$	ND	ND	ND
12.0 ± 0.1	$(6.0 \pm 1.9) \times 10^1$	$(1.8 \pm 1.9) \times 10^1$	$(1.9 \pm 0.3) \times 10^1$	$(2.0 \pm 0.1) \times 10^2$
12.1 ± 0.1	$(5.5 \pm 1.2) \times 10^1$	ND	ND	ND
12.3 ± 0.3	$(8.7 \pm 1.0) \times 10^1$	$(4.7 \pm 0.3) \times 10^1$	$(3.8 \pm 0.3) \times 10^1$	$(7.5 \pm 0.2) \times 10^1$
12.8 ± 0.1	$(8.4 \pm 0.3) \times 10^1$	$(4.7 \pm 0.8) \times 10^1$	ND	$(3.8 \pm 1.6) \times 10^1$
16.0 ± 0.1	$(8.5 \pm 7.9) \times 10^1$	ND	ND	$(3.9 \pm 0.2) \times 10^1$
16.3 ± 0.1	$(6.8 \pm 0.3) \times 10^3$	$(5.2 \pm 0.3) \times 10^3$	$(3.9 \pm 0.3) \times 10^3$	$(2.3 \pm 0.1) \times 10^3$
19.7 ± 0.1	$(7.5 \pm 1.4) \times 10^1$	$(5.4 \pm 0.4) \times 10^1$	$(3.4 \pm 0.6) \times 10^1$	$(5.3 \pm 0.8) \times 10^1$
21.0 ± 0.3	$(6.5 \pm 5.5) \times 10^1$	ND	ND	ND
24.5 ± 0.2	$(6.6 \pm 2.9) \times 10^1$	ND	ND	ND
27.5 ± 0.2	$(8.3 \pm 5.4) \times 10^1$	ND	ND	$(1.6 \pm 1.0) \times 10^2$
28.7 ± 0.1	$(1.1 \pm 0.2) \times 10^2$	$(7.4 \pm 0.4) \times 10^1$	$(3.3 \pm 1.6) \times 10^1$	$(5.1 \pm 0.4) \times 10^1$
35.0 ± 0.1	$(1.3 \pm 0.4) \times 10^3$	$(8.9 \pm 1.4) \times 10^1$	$(3.9 \pm 1.2) \times 10^1$	ND

[a] Mean of four cultures ± confidence limits at *P* of 0.95.
[b] Cell density of CNRZ 1075 was $(1.5 \pm 0.3) \times 10^8$ CFU/ml at the stationary phase; cell densities of CNRZ 1076 were $(1.0 \pm 0.4) \times 10^8$ CFU/ml at the end of first growth phase and $(2.3 \pm 0.4) \times 10^9$ CFU/ml at the stationary phase.
[c] ND, not detected.

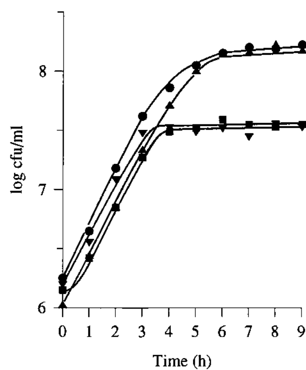

FIG. 4. Growth of peptide transport mutant strains of *L. lactis* in milk. Symbols: ▲, *L. lactis* subsp. *lactis* MG1363 (Prt^- Opp^+ $DtpT^+$); ●, *L. lactis* subsp. *lactis* AG300 (Prt^- Opp^+ $DtpT^-$); ▼, *L. lactis* subsp. *lactis* VS772 (Prt^- Opp^- $DtpT^+$); ■, *L. lactis* subsp. *lactis* CV4 (Prt^- Opp^- $DtpT^-$).

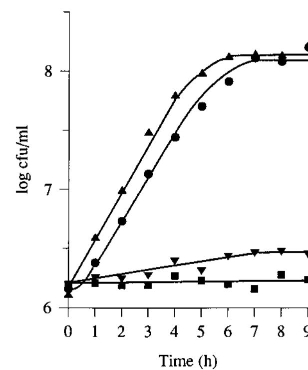

FIG. 5. Growth of peptide transport mutant strains of *L. lactis* in milk precultured with *L. lactis* subsp. *lactis* CV4. Symbols: ▲, *L. lactis* subsp. *lactis* MG1363 (Prt^- Opp^+ $DtpT^+$); ●, *L. lactis* subsp. *lactis* AG300 (Prt^- Opp^+ $DtpT^-$); ▼, *L. lactis* subsp. *lactis* VS772 (Prt^- Opp^- $DtpT^+$); ■, *L. lactis* subsp. *lactis* CV4 (Prt^- Opp^- $DtpT^-$).

obtained during growth of *L. lactis* subsp. *lactis* NCDO 763 were again very close to those obtained with CNRZ 1076.

Role of peptides initially present in milk for growth of *L. lactis*. The role of the peptides during growth was studied by growing different peptide transport mutants in milk. None of them was able to degrade casein (Prt^- strains) (Table 1), so their nitrogen requirements had to be obtained from the free amino acids and the peptides initially present in milk.

All peptide transport-defective strains were able to grow in milk (Fig. 4). However, the growth characteristics (determined from four replicates) depended strongly on the transport abilities of the strains. Inactivation of the di- and tripeptide transport system (*L. lactis* subsp. *lactis* AG300) did not affect growth. Interestingly, the peptide contents of milk after growth of *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *lactis* AG300 were identical and very close to those obtained after growth of *L. lactis* subsp. *lactis* CNRZ 1075 and CNRZ 1259 (data not shown). Inactivation of the oligopeptide transport system (*L. lactis* subsp. *lactis* VS772) strongly affected the extent of growth, with a fivefold decrease of the maximum density, but hardly affected the growth rate (1.33 ± 0.12 h^{-1} versus 1.49 ± 0.06 h^{-1} for the control strain MG1363). The strain lacking both functional di- and tripeptide and oligopeptide transport systems (*L. lactis* subsp. *lactis* CV4) had the same growth characteristics as the strain lacking only the oligopeptide transport *L. lactis* subsp. *lactis* VS772). The peptide content of the milk hardly changed during growth of the two oligopeptide transport mutant strains; only two peaks (retention times, 8.8 and 9.7 min) decreased (respective areas of 4×10^5 and 7×10^4 μV/s at the end of growth, compared with 1×10^6 and 3×10^5 μV/s before growth).

The growth of Prt^- Opp^- $DtpT^-$ *L. lactis* subsp. *lactis* CV4 suggests that the initial pool of free amino acids of the milk allows growth of lactococci to some extent. Therefore, growth of the peptide transport-deficient mutants was also studied in milk depleted of free amino acids (Fig. 5). The depletion was achieved by preculturing milk to the end of growth for 8 h with *L. lactis* subsp. *lactis* CV4. Reinoculated *L. lactis* subsp. *lactis* CV4 was unable to grow in precultured milk, indicating that the remaining free amino acid content of the precultured milk could not sustain growth. *L. lactis* subsp. *lactis* VS772 (Prt^- Opp^- $DtpT^+$) grew very poorly in precultured milk, with only one doubling of the cell density within the total incubation time. However, growth of *L. lactis* subsp. *lactis* MG1363 (Prt^- Opp^+ $DtpT^+$) and of *L. lactis* subsp. *lactis* AG300 (Prt^- Opp^+ $DtpT^-$) was hardly affected by the preculturing.

DISCUSSION

In this study, we have investigated the contribution of the various nitrogen compounds to the growth of *L. lactis* in milk. At the end of growth of nonproteolytic strains, the amino acid and peptide contents of milk are clearly not exhausted. The amino acids and peptides remaining in milk are thus unable to support growth. Apparently, some yet-unknown key nitrogen compounds are missing, since the subsequent addition of a complete mixture of amino acids to the milk strongly stimulates growth (28, 36). The total consumption of Ile and Leu, known to be essential for growth of lactococci (2), was not (only) responsible for the growth arrest, since the addition of a mixture of eight amino acids including Ile and Leu had no growth-promoting effect.

It has been calculated that the growth of *L. lactis* in milk to about 10^8 CFU/ml requires the synthesis of approximately 26 μg of bacterial protein per ml (27). The present study reveals the consumption of free amino acids during the growth of Prt^- strains of *L. lactis* (i.e., up to 1.5×10^8 CFU/ml) to be only 5 μg/ml. A slight underestimation of the amino acid consumption by the strains due to the export of amino acids from the cells cannot be excluded. However, these results strongly suggest that growth of Prt^- strains of *L. lactis* in milk rely mainly on peptides, despite the presence of various active amino acid transport systems (21). The growth patterns in milk of different peptide transport-negative strains of *L. lactis* and the HPLC analysis of the peptide content of the cultured milks are consistent with this notion. The inability to translocate peptides allows a Prt^- strain to reach a maximum cell density of only 3×10^7 CFU/ml. For this cell density, synthesis of approximately 5 μg of bacterial protein per ml is required. Interestingly, this corresponds to the amount of free amino acids that was consumed during growth of wild-type Prt^- strains. It therefore emphasizes the minor role of free amino acids during growth of Prt^- *L. lactis* subsp. *lactis* strains in milk.

The observation that inactivation of the di- and tripeptide transport system has no effect on the growth pattern (and on the peptide content of the milk) suggests strongly that the di- and tripeptides initially present in milk are apparently not essential for growth. On the other hand, the oligopeptides initially present in milk play a crucial role during growth of nonproteolytic strains of *L. lactis*. This contention is supported by (i) the poor extent of growth of an oligopeptide transport-defective (Opp^-) Prt^- strain, (ii) the complete inability of such a strain to grow in milk depleted of free amino acids, and (iii)

the disappearance of HPLC peaks evidenced only with Opp^+ strains. It is worthwhile noting that many of them could be the result of several coeluting peptides (17). Despite the fact that these peptides have not been identified yet, they obviously have to be translocated via the oligopeptide transport system.

Prt^+ strains display a biphasic exponential growth in milk (16, 43). The change to a lower growth rate was reported to be the consequence of a limiting proteolytic activity (14, 17a, 42), while an increase of the proteolytic activity of *L. lactis* results in an increased growth rate in milk (1, 26). The changes in the free amino acids and peptide content of the milk during the first growth phase of Prt^+ strains of *L. lactis* were very similar to those observed during culturing of the corresponding Prt^- variants. This suggests that during the first growth phase, the Prt^+ strains do not significantly degrade caseins, which is in full agreement with the reported absence of proteinase synthesis in the first stage of growth of *L. lactis* (24). It is possible that cell wall proteinase synthesis and activity are repressed by peptides which are present during the first growth phase (5, 6). During the second growth phase, the proteolytic system of the Prt^+ strains is clearly active and the content of free amino acids and peptides in milk increases markedly. Release of free amino acids by purified proteinase (except Phe) has not been observed during hydrolysis of β-, α_{S1}-, or κ-caseins (17, 32, 33). The increase of the extracellular pool of amino acids during growth of Prt^+ strains is therefore likely caused by the excretion of intracellular amino acids by the cells. Such a phenomenon has already been described (22). The very low concentration in milk of Arg, Gly, and Ala at the end of growth of Prt^+ strains correlates with the low frequency of these amino acids in the β-casein (35) and the preferential usage of this type of casein for growth of lactococci (7).

In contrast to our expectations, supplementation of milk with a mixture of eight amino acids that decreased significantly during growth of Prt^+ strains of *L. lactis* (i.e., Thr, Ser, Gly, Ala, Val, Ile, Leu, and Arg) had no stimulatory effect on growth. This observation is in apparent contradiction to the reported amino-acid-limited growth in milk of *L. lactis* subsp. *cremoris* strain HP (14). An explanation might be found in the nonoptimal composition of the amino acid mixture used in the present study. Both Ala and Gly, which were added to milk at a concentration of 1 mM each, are translocated into lactococci via the same transport system (21). The large difference in affinity of these two amino acids for the transport system (52 and 330 μM, respectively) might decrease the uptake of Gly. Since these two amino acids have to be translocated by *L. lactis* ML3 at the same rate (17.4 nmol/min/mg of protein) to meet a growth rate of 1.0 h^{-1} (29), the possible stimulative effect of the amino acid mixture may have been concealed.

Several peptides and amino acids accumulate in milk during growth of Prt^+ strains of *L. lactis*, despite functional transport systems. Prt^- variants are unable to grow in a milk in which Prt^+ strains have been cultured previously (8, 17a), which clearly indicates that these accumulated proteolytic products are unable to sustain the growth of lactococci. Since no peptidases are located extracellularly, the accumulated peptides are most likely not used because they are not transported. It might be the consequence of the size exclusion limits of the oligopeptide transporter, since peptides up to a length of 30 amino acids are released from casein by PrtP (17). Furthermore, certain peptides may not be transported because of the competition of the peptides for entry via a single oligopeptide transport system. Finally, the high hydrophobicity of many casein-derived peptides may also influence their utilization (3).

An estimation of the contribution of the different nitrogen sources to the growth process can now be made (Fig. 6). This

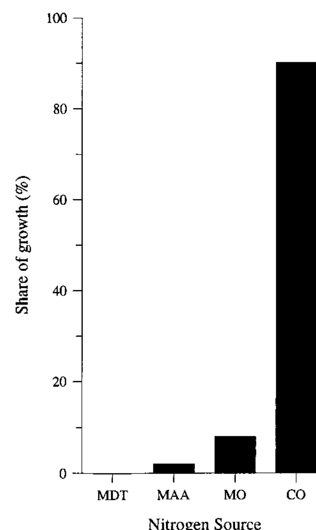

FIG. 6. Contribution of different nitrogen sources to growth of *L. lactis* in milk. Abbreviations: MDT, di- and tripeptides initially present in milk; MAA, free amino acids initially present in milk; MO, oligopeptides initially present in milk; CO, oligopeptides released from caseins by the action of the cell envelope-located proteinase.

estimation indicates that 90% of the growth of Prt^+ strains of *L. lactis* in milk is supported by casein-derived peptides. In a previous study, we have shown that the oligopeptide transport system (and not the di- and tripeptide transport system) is necessary for the utilization of peptides liberated from β-casein by the cell-envelope-located proteinase of *L. lactis* and for growth in milk (22). The present study shows that oligopeptides initially present in milk are necessary for early growth in milk. The oligopeptide transport system therefore plays a crucial role throughout the growth of *L. lactis* in milk. The oligopeptides, whatever their origin (i.e., initially present in milk or released from caseins by the action of PrtP), account for 98% of the growth of *L. lactis*. The role of the di- and tripeptide transport system (DtpT) in the utilization of the various nitrogen sources is not yet clear. It has been reported that mutants which were selected for resistance towards toxic di- and tripeptides were unable to use a mixture of different caseins for growth (38). In contrast, a genetically defined di- and tripeptide transport mutant grew equally well as the wild type in milk and in media containing β-casein plus His and Leu (22). Moreover, from the present study, we have concluded that DtpT is not essential for the use of peptides in milk. We therefore postulate that DtpT is involved in the utilization of milk proteins other than β-casein and that the amino acid deficiencies (most likely His or Leu) that are caused by mutation of DtpT can be relieved by the utilization of amino acids or oligopeptides present in milk. In the future, we aim to identify the peptides that are used for growth in milk to study the size restriction and substrate specificity of the oligopeptide transport system for its natural substrates.

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