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Transcriptional Pattern of Genes Coding for the Proteolytic System of *Lactococcus lactis* and Evidence for Coordinated Regulation of Key Enzymes by Peptide Supply

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The transcription of 16 genes encoding 12 peptidases (*pepC, pepN, pepX, pepP, pepA, pepF2, pepDA1, pepDA2, pepQ, pepT, pepM,* and *pepO1*), P_I and P_{III} proteinases (*prtP1* and *prtP3*), and three transport systems (*dtpT, dtpP,* and *opp-pepO1*) of *Lactococcus lactis* MG1363 was analyzed in response to different environmental factors. Promoter fusions with luciferase reporter genes and/or mRNA analysis were used to study the effects of sugar sources, growth at 37°C, and peptide supply on the transcription of these genes. Only transcription of the *pepP* gene is modulated by the source of sugar. The presence of potential catabolite-responsive element (CRE) boxes in its promoter region suggests that expression of this gene is directly controlled by catabolic repression. Elevated temperature had no significant effect on the level of transcription of these genes. *prtP1, prtP3, pepC, pepN, pepX,* and the *opp-pepO1* operon are the most highly expressed genes in chemically defined medium, and their expression is repressed 5- to 150-fold by addition of peptide sources such as Casitone in the medium. Moreover, the transcription of *prtP1, prtP3, pepC, pepN*, and the *opp-pepO1* operon is repressed two- to eight-fold by the dipeptides leucylproline and prolylleucine. The transcription of *pepDA2* might also be repressed by the peptide sources, but this effect is not observed on the regulation of *dtpT, pepP, pepA, pepF2, pepDA1, pepQ, pepT, pepM*, and the *dtpP* operon. The significance of these results with respect to the functions of different components of the proteolytic system in *L. lactis* are discussed.

Proteolysis in bacteria plays a central role in turnover, maturation, and regulation of proteins and in assimilation of extracellular proteins and peptides (17). Lactic acid bacteria that are isolated from many dairy products, such as cheeses and yogurts, generally possess and efficient proteolytic system to break down caseins, the main proteins in milk, into the amino acids necessary for their growth (26). This function sometimes limits the growth of lactic acid bacteria in milk since these bacteria have multiple amino acid auxotrophies (5, 44). Casein breakdown products (peptides, amino acids and derivatives of amino acids) also contribute to the formation of flavor and texture of the fermented milk products. The proteolytic system of lactococci is one of the best documented. The biological and genetic properties of the majority of the enzymes involved in this system have been recently reviewed (6, 29). This system is composed of (i) an extracellular proteinase, (ii) peptide transport systems, and (iii) intracellular peptidases (Fig. 1). The degradation of milk proteins is initiated by an extracellular proteinase, PrtP, that is bound to the cell wall. Two types of proteinase, P_I and P_{III}, have been characterized in *Lactococcus lactis* subsp. *cremoris* on the basis of the casein degradation pattern (25, 57). In *L. lactis*, peptides produced by the proteinase are internalized by three transporters. The Opp system takes up oligopeptides of 4 to 18 residues, while DtpT and DtpP transport hydrophilic and hydrophobic di- and tripeptides, respectively (12, 29). Internalized peptides are further hydrolyzed by several intracellular peptidases that are classified depending on their cleavage specificity. Six aminopeptidases (PepN, PepC, PepP, PepX, PepA, and Pcp) generate dipeptides and free amino acids by cleaving the N-terminal end of oligopeptides. Endopeptidases such as PepO1, PepO2, PepF1, and PepF2 cleave internal peptide bonds of oligopeptides, and several other peptidases, such as PepV, PepQ, and PepT, cleave di- or tripeptides (29, 40, 42, 46). PepN and PepC aminopeptidases, PepV dipeptidase, and PepT tripeptidase display a low substrate specificity (4, 19, 40, 55), while PepA glutamyl aminopeptidase liberates N-terminal Glu and Asp residues (31). PepQ prolidase, PepP aminopeptidase P, PepX X-prolyl-dipeptidyl aminopeptidase, and PepI proline iminopeptidase are found in hydrolyzing peptides containing proline residues (1, 3, 38, 45). The pyrrolidone carboxylyl peptidase (Pcp) specifically cleaves N-terminal pyrrolidone carboxylyl residues of peptides (11). Last, we have found in the genome of *L. lactis* IL 1403 genes for potential peptidases such as PepDA1 and PepDA2 that share sequence similarity with the PepD dipeptidase of *Lactobacillus helveticus* and PepM, showing sequence similarity with the methionyl peptidase of *Escherichia coli* (2).

Although functional analysis of peptidase genes has been systematically carried out, regulation of expression of the various components of the proteolytic pathway is still poorly documented. The regulation of the plasmid-encoded cell wall proteinase PrtP is the best known among the components of this system. Early experiments showed that in several strains, the synthesis of the cell wall proteinase is reduced during growth in rich media compared to milk medium (23). Moreover, proteinase activity in *L. lactis* subsp. *cremoris* AM1 is repressed after

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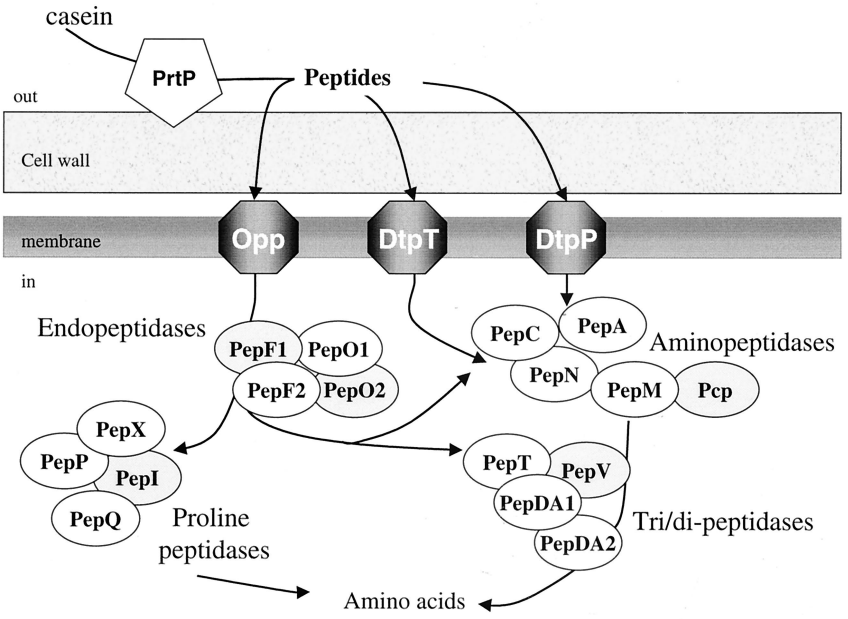

FIG. 1. Schematic representation of the *L. lactis* proteolytic system. The cell wall proteinase (pentagon), three transport systems (hexagon), and 18 intracellular peptidases (oval) are represented in their relative locations in the cell. Peptidases are classified on the basis of their cleavage specificity. White and grey ovals represent peptidases that were included and not included, respectively, in this study.

the addition of peptides to the growth medium and is increased in the stationary phase (10). Transcription of the extracellular proteinase gene of *L. lactis* SK11 has been analyzed by *prtP-gusA* gene fusion (36). A 10-fold repression of initiation of transcription was observed by adding a complex peptide mixture to the medium. Moreover, peptide-dependent regulation was examined by adding specific peptides to the growth medium. Out of 12 di- and tripeptides tested, only leucylproline (LP) and prolylleucine (PL) repressed the transcription of the *prtP-gusA* fusion (36). Finally, the activities of PepN and PepX and the expression of three transport systems are greater when cells grow in chemically defined medium (CDM) compared to media containing complex peptide sources (8, 12, 18, 39). Similarly to PrtP, PepN and PepX activities are repressed in the presence of the dipeptide PL (39).

Here we describe a systematic study of the transcription of 16 genes involved in the proteolytic system of *L. lactis*. mRNA analysis showed that the transcription of several genes was regulated by the peptide supply. The activities of 15 promoter regions were measured during growth in several media by using luciferase fusion, enabling direct comparison of promoter strengths. We report that the transcription of eight promoters is regulated by the peptide content of the medium; of these, five promoters are repressed by specific dipeptides. On the other hand, *pepP* transcription is regulated by the carbon source.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. *E. coli* TG1 was used for plasmid propagation (14). *E. coli* was grown at 37°C in Luria-Bertani medium (35). *L. lactis* strains were grown at 30°C on M17 glucose medium (M17) (53) or on CDM (51). GalCDM is CDM with glucose replaced by galactose (0.5% [wt/vol]). CDM was supplemented with Casitone (CDM Casitone; Sigma-Aldrich) at 1.5% (wt/vol) or at different concentrations (0.1 to 2% [wt/vol]) where specified, with Casamino Acids (CDM CAA; Difco Laboratories, Detroit, Mich.) at 1.5% (wt/vol) or the dipeptide PL (Sigma) or LP (Sigma) at 1 mM. When needed, erythromycin (5 μg/ml for *L. lactis*; 100 μg/ml for *E. coli*), tetracycline (5 μg/ml for *L. lactis*), or ampicillin (100 μg/ml for *E. coli*) was added to the culture medium.

DNA manipulation procedures. Plasmids and total DNA were prepared as previously described (32, 35, 50). Procedures for DNA manipulations, transformation of *E. coli* cells, and cloning were essentially as described by Maniatis et al. (35). Electrotransformation of *L. lactis* was performed as described by Holo and Nes (20). All enzymes for DNA technology were used according to the manufacturer's specifications. Oligonucleotides were synthesized on an Oligo 1000M DNA synthesizer system (Beckman). Standard procedures were used for Southern hybridization analysis (48). Digested chromosomal DNA (2 μg) was transferred to a nylon membrane (Hybond N+) and hybridized with DNA probes that were labeled with the ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection system. Hybridization and detection were performed according to the Amersham ECL protocol. Sequence analysis of double-stranded DNA was carried out according to the Applied Biosystems protocol accompanying the 370A DNA sequencer. DNA was used in dideoxynucleotide chain termination sequencing reactions with a Big Dye terminator kit (Applied Biosystems) and was sequenced on both strands.

Construction of *lux* transcriptional fusions. PCR fragment products containing different promoters were cloned in *E. coli*, and their sequences verified. For this purpose, chromosomal DNA from *L. lactis* MG1363 was used as the template to generate 500- to 900-bp fragments containing the promoters and the putative start codons of *pepA, pepC, pepDA1, pepF2, pepM, pepN, pepP, pepT, pepQ, pepX*, and the *opp-pepO1* operon. The two *pepF2* promoters were designated P*pepF21* and P*pepF22*, and the two *opp-pepO1* promoters were designated P*oppD* and P*oppA* (Fig. 2). P*oppD* is located upstream of the *opp-pepO1* operon, and P*oppA* is between the *oppC* and *oppA* genes (46, 54). Plasmid DNA from *L. lactis* SK11 and Wg2 was used to amplify fragments carrying promoter regions of *prtP1* and *prtP3*, encoding the P_I and P_{III} proteinases, respectively. The specific oligonucleotide pairs used in this work are described in Table 1. PCR fragments containing promoter regions of *pepC, pepN, pepX*, and *pepF2* (P*pepF22*) were cut by *Eco*RI and *Bam*HI present in the primers and cloned in pBluescript KS(+) (pBSSK$^+$) cut by the same enzymes. PCR fragments containing promoter regions of *prtp1, prtP3, pepA, pepDA1, pepF2* (P*pepF21*), *pepM, pepP, pepQ, pepT*, and the *opp-pepO1* operon (P*oppD* and P*oppA*) were cloned directly in pGEM-T Easy vectors (Promega).

The integrative plasmids carrying the *lux* transcriptional fusions were con-

TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or oligonucleotide	Relevant markers and characteristics	Reference or origin
Strains		
E. coli TG1	*supE* Δ*thi(lac-proAB) hsdD5 (*F′$^+$ *traD36 proAB lacI*^{q}*ZDM15)*	14
L. lactis		
MG1363	Plasmid-free derivative of *L. lactis* subsp. *cremoris* NCDO712, Lac$^-$ Prt$^-$	13
Wg2	Wild-type *L. lactis* subsp. *cremoris* strain harboring pWV05, *prtP1*	27
SK11	Wild-type *L. lactis* subsp. *cremoris* strain harboring pSK111, *prtP3*	9
Plasmids		
pBSSK$^+$	Amp^r, M13*ori* pBR322*ori*	Stratagene
pGEM-T Easy	Amp^r, M13*ori* pBR322*ori*, linear T-overhang vector	Promega
pGhost 8	Tet^r, thermosensitive replicative plasmid in *L. lactis*	34
pJIM2366	Em^r, promoter probe vector containing *luxAB* genes	47
pJIM2374	Em^r, integrative promoter probe vector containing *luxAB* genes	7
Integrative plasmids carrying *lux* fusion		
pJIM3029	P*pepC-lux, Xba*I fusion of pJIM2374 and pBSSK$^+$ containing P*pepC* on a 654-bp fragment	This work
pJIM3030	P*pepN-lux, Xba*I fusion of pJIM2374 and pBSSK$^+$ containing P*pepN* on a 767-bp fragment	This work
pJIM3031	P*pepX-lux, Xba*I fusion of pJIM2374 and pBSSK$^+$ containing P*pepX* on a 687-bp fragment	This work
pJIM3032	P*pepF22-lux, Xba*I fusion of pJIM2374 and pBSSK$^+$ containing P*pepF22* on a 654-bp fragment	This work
pJIM3063	P*pepF21-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*pepF21* on a 772-bp fragment	This work
pJIM3066	P*pepA-lux, Bam*HI fusion of pJIM2374 and pGEM-T Easy containing P*pepA* on a 575-bp fragment	This work
pJIM3064	P*oppD-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*oppD* on a 587-bp fragment	This work
pJIM3065	P*oppA-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*oppA* on a 688-bp fragment	This work
pJIM3080	P*pepDA1-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*pepDA1* on a 763-bp fragment	This work
pJIM3081	P*pepM-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*pepM* on a 913-bp fragment	This work
pJIM3082	P*pepP-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*pepP* on a 778-bp fragment	This work
pJIM3106	P*pepQ-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*pepQ* on a 760-bp fragment	This work
pJIM3118	P*pepT-lux, Spe*I fusion of pJIM2374 and pGEM-T Easy containing P*pepT* on a 866-bp fragment	This work
Replicative plasmids carrying *lux* fusion		
PJIM3120	P*prtP1-lux*, pJIM2366 carrying P*prtP1* on a 1,051-bp *Spe*I-*Not*I fragment from strain Wg2	This work
pJIM3119	P*prtP3-lux*, pJIM2366 carrying P*prtP3* on a 1,056-bp *Spe*I-*Not*I fragment from strain SK11	This work
Oligonucleotide pairs used for amplification or promoter and probe fragments		
P*pepA*	GGGAATTCTAAAGTTTTGGCATT/CGGGATCCATAAATCCGACCTCAT	X81089
P*pepC*	GGGAATTCCTTTGATTATTCATC/CGGGATCCAGATTGTTTTTGATTC	M86245
P*pepDA1*	TGTAGCAGACCTTGTATCAGC/GACATCGCAACATTAGCACTA	AE005176
P*pepF21*	GGGAATTCCAATTGAGCGAACTA/CGGGATCCTTGAGACGCTTAATA	X99710
P*pepF22*	GGGAATTCTAGTTTTATTCGTCA/CGGGATCCTTGATACTTTGCTTCTC	X99710
P*pepM*	ACCTTTCCAGATGACCCGTTA/GTGCCTACTTTGGCTTGTTCA	AE005176
P*pepN*	GGGAATTCTTGTTTTAAATGATGG/CGGGATCCTTCTTCTTCATCAAAT	M87840
P*pepP*	TGGTTCGTCGTGAAGCACCAT/TCATCAGCAATTGCACAGGCT	Y08842
P*pepT*	GCTATTGAGCCTTTTACT/GTTGTTCCATCAGTATGA	L27596
P*pepQ*	TCTCCATCGATTGTTCCGACT/TCATCTGCTGACTTAATCAAA	AE005176
P*pepX*	GGGAATTCCAAACATCGCACCTA/CGGGATCCCAATCTAATTCTATA	M35865
P*oppD*	GGGAATTCTGCTTTTATTATTTCCT/CGGGATCCTACTTGTTCTAAAA	L18760
P*oppA*	GGGAATTCTTTGGGAACAATGATAA/CGGGATCCGTTACTTCTGAACCA	L18760
P*prtP*	CGGAATTCCTTGTACAAGCTACT/CGGAATTCAGCTTATTGTAATCA	M26310
pepQ	ATCCGACAACTCTAAATTATCT/TAGCAGCGCGAGCTCCTGATA	AE005176
oppA	AGCAGCTACACTCCTAAGTGCT/CGGGATCCGTTACTTCTGAACCA	L18760
oppD	AGTCTTAAACGTGGTGAAGT/CGGGATCCTACTTGTTCTAAAA	L18760
P*pepDA2*	TGAGGTTTCTGTCGCAGTCAT/CGGTCAGCAATGGTTACCTTA	AE005176
dtpT	TGACTCACGTCGTGACACTGGAT/TCCGTTCAAGAGACCTGGAAGT	U05215
dtpP	ACAGCACTCTATGCAAGGGTCATA/AGTCGCTGGATCAACCGCACGTT	AE005176

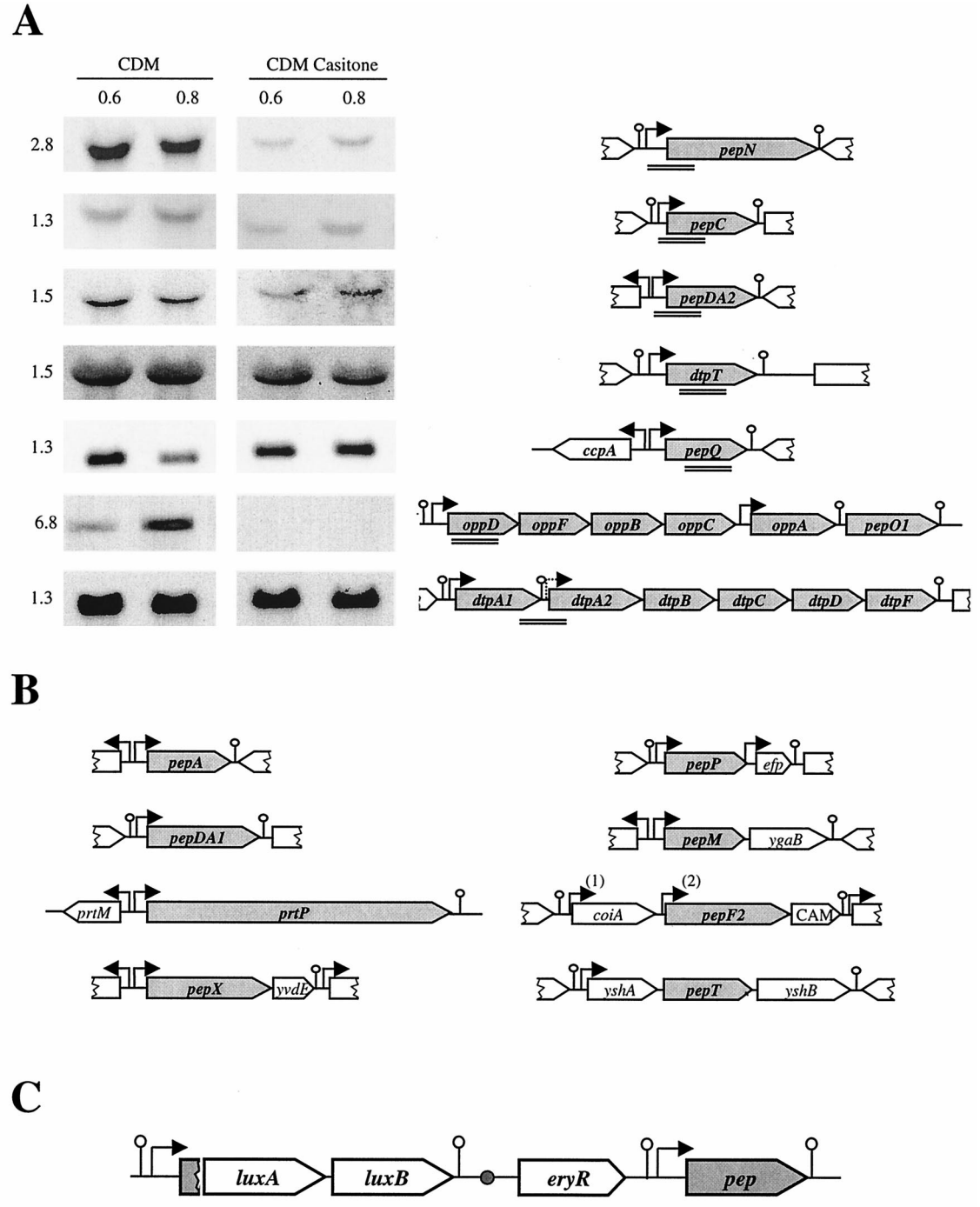

FIG. 2. Genetic organization of genes encoding the proteolytic system of *L. lactis*. Schematic representation of the genetic organization of genes was deduced from published papers and completed by data of the *L. lactis* IL1403 genome sequence (AE005176) as mentioned in the text. (A) Northern blot analysis of the *pepN, pepC, pepDA2, pepQ*, and *dtpT* genes and the *opp-pepOI* and *dtpP* operons. Total RNA was extracted from *L. lactis* MG1363 at an OD_{600} of 0.6 and 0.8 in CDM and CDM Casitone. Total RNA was hybridized with PCR fragments used as probes denoted by double lines. The size of the messenger in kilobases is indicated on the left. Arrows and lollipops present the putative promoters and terminators, respectively. (B) Schematic representation of the genetic organization of *prtP, pepA, pepDA1, pepM, pepX, pepP, pepF2*, and *pepT*. Promoters were deduced only from DNA sequence analysis, except for the *prtP* promoter. The two *pepF2* promoters, P*pepF21* and P*pepF22*, are designated by "(1)" and "(2)", respectively. *prtM, efp*, and *cam* (CAM on the diagram) encode the proteinase maturase, elongation factor P, and a potential methyltransferase, respectively. *coiA* is the counterpart of a gene whose product is involved in competence development in *Streptococcus pneumoniae*. *ygaB, yshA*, and *yshB* encode proteins sharing homology with membrane proteins and transporters, and *yvdE* encodes a protein similar to proteins of unknown function. Terminators localized downstream of *yvdE, efp*, and *yshB* and upstream of *yshA* and putative promoters localized upstream *efp* and *yshA* were deduced, in this work, from the sequence of the IL1403 genome (AE005176). (C) General scheme of the luciferase fusion integrated on the chromosome by single crossover. Upon insertion, the promoter is duplicated and drives expression of the *luxAB* genes and of the functional gene denoted in grey. The small circle represents the origin of replication of the integrative plasmid that is inactive after the helper plasmid is removed.

structed by fusing pGEM-T Easy or pBSSK$^+$ containing promoter regions with the *L. lactis* intergrative vector pJIM2374 as described in Table 1. These plasmids were integrated at the promoter locus in the chromosome of *L. lactis* MG1363 by single crossover with pGhost 8 as a helper as described by Godon et al. (16). Strains were screened by PCR amplification with specific primers for monocopy integration of plasmid and further verified by Southern blotting. The resulting strains contained the *lux* genes downstream of the cloned promoter region followed by a copy of the intact gene (Fig. 2C). The *lux* transcriptional fusions with *prtP1* and *prtP3* promoters were obtained after cloning fragments carrying promoters from pGEM-T Easy into pJIM2366, an *L. lactis* replicative vector, as described in Table 1. These plasmids were transformed into *L. lactis* MG1363.

Determination of luciferase activity in *L. lactis* and growth rate of culture. Luciferase assays were carried out on a Bertold Lumat LB9501 apparatus. One milliliter of *L. lactis* culture was mixed with 5 μl of nonaldehyde, and the light emission was immediately measured. The value of the peak obtained was standardized to the optical density at 600 nm (OD_{600}) of the culture. Luciferase activity was measured throughout the growth of the culture. Values reported in Fig. 3 and Table 2 were measured at OD_{600} of 0.4. Luciferase assays were determined on *L. lactis* culture grown in several media where the growth rate differs significantly depending on the nitrogen and carbon sources (generation times are 190 min in GalCDM, and 60 min in CDM, with or without dipeptides and CAA, and 50 min in M17 or CDM Casitone). In this study, we considered that a 2- to 3.5-fold modulation in luciferase measurement was not significant if the growth rate was different. Indeed, variation in the growth rate might significantly affect the balance between synthesis, degradation, and dilution of the reporter gene during growth.

Northern blot analysis. RNA was isolated from *L. lactis* MG1363 grown in CDM and in CDM Casitone (1.5% [wt/vol]; Sigma) at different times of growth corresponding to an OD_{600} of 0.6 and 0.8. Total RNA was prepared as previously described for *Bacillus subtilis* (15). After extraction and treatment with phenol-chloroform, RNA was precipitated with ethanol. Then 25 μg of glyoxalated RNA was subjected to electrophoresis through a 1% agarose gel. Transfers and hybridizations were performed as described by Maniatis et al. (35). Hybridization was performed with PCR fragments generated with oligonucleotides presented in Table 1 and summarized in Fig. 2A. Hybridization data were collected on a Storm instrument and quantified by the ImageQuant image analysis software package (Molecular Dynamics).

RESULTS

Transcriptional organization of the genes involved in peptide utilization in *L. lactis*. The genetic organization of proteolytic genes studied here is presented Fig. 2A and B. Potential promoters were deduced from DNA sequence analysis, except for the transcription initiation site of *prtP* (37, 56). Only sizes of *pepN, pepV*, and *pepF1* transcripts have been determined (19, 46, 52). To experimentally confirm the functionality of the promoters presented here, we checked that PCR fragments containing the promoter regions of *oppD, oppA, pepN, pepC, pepX, pepM, pepT, pepP, pepQ, pepDA1, pepF21, pepF22*, and *pepA* were able to drive luciferase activity when cloned in pJIM2374 maintained under its replicative form in *L. lactis* (not shown). To confirm the structural organization of several genes of the proteolytic system and assess the effect of adding a rich peptide source to CDM, we carried out Northern blotting on total RNA extracted during the exponential growth phase from MG1363 cells grown in CDM with and without Casitone. RNA was hybridized with fragments covering part of *pepQ, oppD, dtpT, pepN, pepC, pepDA2, dtpPA1*, and *dtpA2* as shown in Fig. 2A.

In CDM, *pepN, pepC, pepDA2, dtpT*, and *pepQ* produced single transcripts of 2.8, 1.3, 1.5, 1.5, and 1.3 kb, respectively (Fig. 2A). These transcriptional patterns were in agreement with the sizes of the genes and confirmed their monocistronic organization. The *oppD* probe revealed a single 6.8-kb band, which should end within *pepO1* and thus be a 3′-end degradation of the expected 8-kb transcript, confirming the polycistronic organization of the *opp* genes (Fig. 2A). The full-size 8-kb transcripts covering *dtpP* is barely detectable (data not shown) compared to the clear short transcript covering the 3′ end of *dtpPA1*, suggesting that expression of the second part of the *dtpP* operon is weak. Except for *opp-pepO1* and *pepQ* transcripts, all transcripts had constant relative amounts during exponential growth.

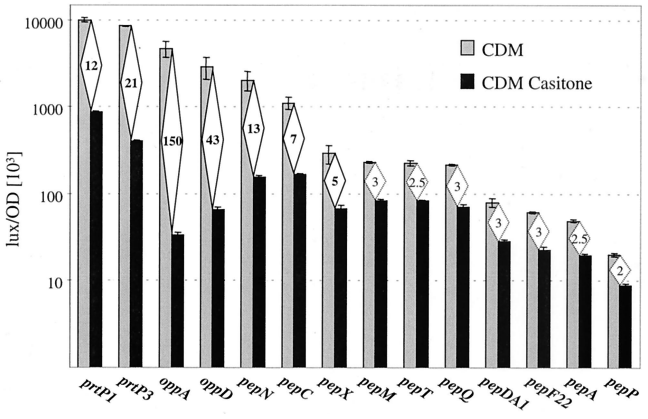

FIG. 3. Histogram of luciferase activities obtained from *lux* fusions with 14 promoters. *L. lactis* MG1363 strains carrying the fusions were grown in CDM and CDM Casitone. The values reported correspond to those obtained at an OD_{600} of 0.4. Error bars indicate standard deviations. Diamonds contain the strength of repression corresponding to the ratio of luciferase activities obtained in CDM and in CDM Casitone.

pepDA2, pepC, pepN, and *opp-pepO1* transcripts were 3-, 3-, 15-, and 20-fold respectively, more abundant in CDM than in CDM Casitone, while the transcription of *dtpT, pepQ*, and *dtpPA1* was not affected more than 1.5-fold by Casitone (Fig. 2A). These results suggested that transcription of the *pepDA2, pepC*, and *pepN* genes and the *opp-pepO1* operon is negatively controlled by Casitone.

Effect of peptide supply on transcription of the proteolytic system. To verify the results obtained by RNA analysis, we constructed 15 transcriptional *luxAB* gene fusions with promoters P*prtP1*, P*prtP3*, P*oppD*, P*oppA*, P*pepN*, P*pepC*, P*pepX*, P*pepM*, P*pepT*, P*pepP*, P*pepQ*, P*pepDA1*, P*pepF21*, P*pepF22*, and P*pepA*. The resulting fusions were inserted in single copy by homologous recombination at their loci in *L. lactis* MG1363 (Fig. 2C). The fusions with P*prtP1* and P*prtP3* were carried on multicopy plasmids, as *prtP* genes are in multicopy in natural strains. To test the effects of peptide sources on promoter expression, the activities of the *lux* fusions were compared in CDM, CDM Casitone (Fig. 3), and M17 (data not shown). CDM contains all amino acids necessary for *L. lactis* growth (51), Casitone is an enzymatic casein hydrolysate that contains 80% peptides and 20% amino acids (36), and M17 contains a complex nitrogen source, including rapidly assimilated peptides (53). Luciferase activities were reduced in CDM Casitone and M17 compared to CDM for all promoters except P*pepF21*, for which no significant activity was detected (0.2 lux/OD unit [10^3]). However, the difference depended markedly on the promoters. The strength of P*pepP*, P*pepA*, P*pepF22*, P*pepDA1*, P*pepQ*, P*pepT*, and P*pepM* was only 2- to 3-fold lower in Casitone, whereas that of P*pepX*, P*pepC*, P*pepN*, P*prtP1*, P*prtP3*,

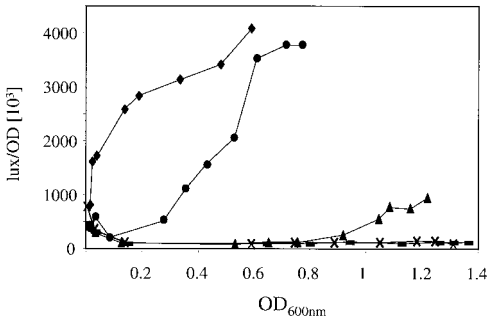

FIG. 4. Luciferase activities of P*oppA-lux* fusion promoter during batch culture of *L. lactis* MG1363 in CDM (◆) and in CDM containing 0.1% (●), 0.5% (▲), 1% (×), and 2% (▬) Casitone.

P*oppD*, and P*oppA* was 5- to 150-fold lower (Fig. 3). The decrease of transcription was similar in CDM Casitone compared to M17 for all promoters except P*prtP1*, which was repressed twofold more in M17 (480 ± 10 versus 875 ± 25 lux/OD unit [10^3]). These results suggested that Casitone and M17 contain components that significantly repress the transcription of at least seven promoters, P*pepX*, P*pepC*, P*pepN*, P*prtP1*, P*prtP3*, P*oppD*, and P*oppA*. In addition, *prtP1* transcription might be repressed by specific components in M17. These results were in agreement with RNA analysis and indicated that repression by the regulatory components of Casitone and M17 occurs at the level of the initiation of transcription.

Repression depends on the Casitone concentration. We studied the effect of the Casitone concentration on P*oppA* expression. Luciferase activities were more than 100-fold higher in CDM than in CDM supplemented with 1 or 2% Casitone (Fig. 4). At these concentrations, the repression of *opp-pepO1* transcription was constant throughout cell growth. Interestingly, with 0.5 and 0.1% Casitone, expression was also repressed until the OD_{600} reached 0.8 and 0.3, respectively. The expression determined thereafter increased significantly (Fig. 4). These results indicated that the components which repressed *opp-pepO1* transcription can be degraded or assimilated by *L. lactis*.

Dipeptides are involved in the transcriptional repression of peptidases. Since M17 and Casitone contain complex nitrogen sources, we decided to better define the elements affecting negatively peptidase expression. We tested the effects of different supplementary nitrogen sources on the activity of all *lux* fusions, such as CAA (acid casein hydrolysate containing 20% peptides and 80% amino acids) and dipeptides. All promoters repressed by Casitone except P*pepX* were also repressed by CAA. The rate of repression by CAA corresponding to the ratio of values obtained in CDM compared to those obtained in CDM with CAA is 2- to 12-fold for P*prtP3*, P*prtP1*, P*pepC*, P*pepN*, P*oppD*, and P*oppA* (data not shown). The rate of repression in CDM CAA is less than 1.5-fold for P*pepX*, P*pepP*, P*pepA*, P*pepDA1*, P*pepQ*, P*pepT*, and P*pepM* (data not shown). Although the nitrogen content of CAA is close to that of Casitone (two casein hydrolysates), the repression was approximately 5- to 10-fold lower in the medium with CAA, suggesting that the element-repressing peptidase genes are less abundant or less efficient in CAA. Effects of dipeptides LP and PL, known to regulate expression of the *prtP3* gene (37), were tested on the promoters regulated by Casitone (Table 2). PL or LP repressed two- to eightfold the expression of luciferase genes under control of P*prtP3*, P*pepC*, P*pepN*, P*oppD*, and P*oppA*. These results indicated that at least five promoters out of seven previously shown regulated by Casitone were repressed by specific dipeptides, although at a lower level than by Casitone.

Effect of heat shock and catabolic repression on transcription of the proteolytic system. It was reported earlier that a heat shock response might be involved in regulation of some peptidases (17). We therefore tested the effect of temperature on expression of the genes of the proteolytic system by monitoring the luciferase activity of *lux* fusions at 38°C. No significant difference of expression was observed at elevated temperature compared to 30°C, the usual temperature of growth for *L. lactis* (data not shown).

Since it was reported earlier that catabolic repression might be involved in the regulation of some peptidases (49), the activities of all *lux* fusions were compared in CDM containing as a carbon source glucose or galactose, in CDM, and in Gal-CDM. Galactose is assimilated slowly, which limits the growth rate (150 min, versus 60 min in the presence of glucose) and does not cause catabolic repression in *L. lactis* (33). The luciferase activity of the P*pepP* fusion was 8.5-fold higher in Gal-CDM than in CDM (170 ± 10 versus 20 ± 1 lux/OD unit [10^3]), whereas those of the other promoters did not increase significantly (<3.5-fold [data not shown; see Materials and Methods]). We searched for catabolite-responsive element (CRE) boxes, mediating catabolic repression in gram-positive

TABLE 2. Expression of proteolytic system gene promoter-*lux* fusions in CDM supplemented with dipeptides LP and PL at 1 mM

Promoter	CDM (lux/OD unit [10^3]; mean ± SD)	CDM + LP (lux/OD unit [10^3]; mean ± SD)	Strength of repression by LP[a]	CDM + PL (lux/OD unit [10^3]; mean ± SD)	Strength of repression by PL[b]
P*prtP3*[c]	8,540 ± 95	3,800 ± 100	2	4,300 ± 300	2
P*oppA*	5,040 ± 300	760 ± 30	6.5	635 ± 1	8
P*oppD*	2,820 ± 160	750 ± 50	4	620 ± 65	4.5
P*pepN*	2,020 ± 140	830 ± 50	2.5	900 ± 70	2
P*pepC*	1,180 ± 130	630 ± 20	2	790 ± 10	1.5
P*pepX*	320 ± 30	ND[d]	ND	305 ± 20	1
P*pepQ*	215 ± 4	ND	ND	210 ± 10	1

[a] Ratio between the values obtained in CDM and CDM + LP.
[b] Ratio between the values obtained in CDM and CDM + PL.
[c] P*prtP3-lux* fusion is carried on a high-copy-number plasmid.
[d] ND, not determined.

TABLE 3. Potential CRE boxes present in promoter regions of peptidase genes

Gene	Distance (bp) to[a]:		No. of mismatches	Sequences of box[b]
	Start codon	−10 box		
pepM	+23	+68	2	TGAAATCGA**G**CAA**A**
pepP	−81	−4	2	AGGAAA**C**GTTAAC**T**
	−65	−20	2	TGTTAGCGTTTTT**G**
	+99	+154	1	TGGAACCGC**G**GGCA
	+122	+177	2	TGACAGCGA**A**GC**G**A
pepX	−265	−221	2	**GT**TCAACGTTAGCA
dtpT	+78	+83	2	TGGGAGCGTTTTT**C**
Consensus				TGNNANCGNTNNCA

[a] Relative position of potential CRE site upstream (−) or downstream (+) of the first codon and the −10 promoter box.
[b] Mismatches to the CRE box consensus sequence are shown in bold.

bacteria (22), near the 15 promoters studied in *lux* fusions. Two mismatches from the consensus sequence TGNNANCGNT NNCA were allowed at any position except the central CG motif that is conserved in all known CRE boxes (Table 3) (22). Possible CRE boxes were found in the vicinity of *pepM, pepP, pepX*, and *dtpT* promoters (Table 3). None were detected within 250 bp from the −10 box of promoters of the other proteolytic genes. The absence of CRE boxes in most promoter regions was in agreement with the lack of significant variation of luciferase activities as a function of the carbon source. These results indicated that the expression of these genes were not under the control of catabolic repression. Absence of variation in luciferase activities of *pepX* and *pepM* fusions suggested that the potential CRE boxes found near their promoters were not active. On the other hand, the presence of four potential CRE sites and the differential expression of *pepP* depending on the carbon source strongly suggested that its transcription is controlled by catabolic repression.

DISCUSSION

L. lactis possesses a number of genes involved in the utilization of proteins present in the medium such as extracellular protease, peptide transport systems, and intracellular peptidases (Fig. 1). Here, we conducted a systematic study to determine some parameters regulating their expression. The sequences of many of these genes have been previously characterized (for recent reviews, see references 6 and 29), but promoter identification and transcriptional data were available only for *prtP* (37, 56) and for *pepN, pepV*, and *pepF1* (19, 46, 52). We also included four genes and one operon revealed by the complete sequence of the *L. lactis* IL1403 genome (*pepDA1, pepDA2, pepM, pepQ*, and *dtpP* [2]). A schematic representation of the genetic organization of these genes, presented in Fig. 2, is deduced from published papers and completed by sequence analysis from the *L. lactis* IL1403 genome sequence (AE005176). By Northern blot analysis, we confirmed the sizes of *pepN, pepC, pepDA2, dtpT*, and *pepQ* predicted from nucleotide sequence analysis. We demonstrated that these genes had a monocistronic organization and that *opp* genes are in an operon. In this study, the use of *lux* as a gene reporter enabled us (i) to detect the presence of a promoter in cloned regions, (ii) to analyze the level of transcription as a function of growth in different environmental conditions, and (iii) to perform an initial comparison of the strengths of the 15 different promoters.

Catabolic repression might control *pepP* transcription only. In *Lactobacillus delbrueckii*, CcpA, the regulator for catabolic repression in gram-positive bacteria, was proposed to regulate the transcription of *pepQ* and probably *pepI* and *pepX* (49). However, although *pepQ* is also divergently transcribed from *ccpA* in *L. lactis* (Fig. 2), we have shown that its transcription is not regulated by carbon source. This result is in agreement with the lack of CRE sites at less than 300 bp from the most probable transcriptional start of *pepQ* and strongly suggests that *pepQ* transcription is not under the control of catabolic repression. Analysis of the other *lux* fusions revealed that only the transcription of *pepP* was 8.5-fold more expressed in CDM with galactose. Analysis of the promoter region of *pepP* allowed us to find four potential CRE boxes present 4 and 20 bp upstream and 151 and 177 bp downstream of the −10 box of the promoter. Since these potential CRE boxes are located at a distance suitable to enable the repression of transcription of the P*pepP*, it is likely that *pepP* is effectively regulated by CcpA. This aminopeptidase cleaves off any N-terminal amino acid linked with proline, and it was proposed that PepP of *E. coli* is probably involved in the maturation of the N-terminal ends of proteins (38). Its coexpression with an elongation factor in many gram-positive bacteria argues for such a function (unpublished data). It is thus not surprising that *pepP* is regulated by factors other than those related to peptide supply such as carbon or energy metabolism.

Casitone-regulated genes encode key enzymes for proteolysis, while Casitone-independent genes would have another role. The repression of transcription by nitrogen sources such as Casitone was particularly significant (5- to 150-fold) for *prtP1, prtP3, pepX, pepN, pepC*, and *opp-pepO1* (Fig. 3). Transcriptional repression of *pepN, pepC*, and *opp-pepO1* was confirmed by mRNA analysis. Furthermore, *pepDA2*, but not *pepQ*, *dtpP*, and *dtpT*, might also be regulated by Casitone (Fig. 2A). Interestingly, Casitone-regulated genes are those that have the highest expression level in CDM. Their promoter strength is similar to that of highly expressed lactococcal genes such as those encoding glycolytic enzymes (E. Jammet, personal communication). Moreover, functional studies have suggested that they play a significant role in protein utilization. The PrtP proteinase and Opp transport systems have been shown to be essential for growth in milk since they are involved in the first step of casein degradation and in the uptake of the resulting peptides, respectively (25, 26, 54). Moreover, although the inactivation of single peptidase genes does not generally lead to a drastic effect on growth in milk, the inactivation of *pepN, pepC*, and *pepO1* leads to 25, 10, and 9% decreases, respectively, in growth rate in milk (6, 41). Combinations of these mutations have a drastic effect on growth, suggesting their crucial role in peptide or nutritional metabolism in the cell (30, 41). Last, the growth of the *pepX* mutant is clearly affected in medium containing casein as the sole peptide source (40 to 25% longer generation time) and in milk (15% longer generation time) (6, 30, 38). PepN, PepC, and PepO1 were showed to be the most important intracellular enzymes for the degra-

dation of oligopeptides provided by the casein breakdown and PepX for peptides containing proline (6).

Most genes encoding these key enzymes in peptide utilization are either transcribed as single genes, such as the genes encoding PrtP, PepN, and PepC, or cotranscribed, such as the genes encoding oligopeptidase O and the oligopeptide transport system in the *opp-pepO1* operon. By contrast, *pepF2* (46), *pepM* (2), *pepP* (38), and *pepT* (40) appear to be linked to genes that are not involved in peptidolysis (Fig. 2). These genes as well as *pepDA1, pepA*, and *pepQ* are expressed at a low level and are not modulated by the peptide source. These results suggested they may not be involved in external peptide source utilization but have a role in other cellular processes. Indeed, as mentioned above, PepP might be involved in protein maturation (38). PepM, a methionine-specific aminopeptidase which removes N-terminal methionine residues from proteins, is essential and might also be involved in protein maturation in *Salmonella enterica* serovar Typhimurium (43). PepDA1 might result from the duplication of PepDA2 and have evolved to fulfill a particular role in the cell. The chromosomal *pepF2* gene was also found duplicated on plasmids in several lactococcal strains. The loss of either copy results in a decrease in the growth rate in minimal media, suggesting a role of oligopeptidase PepF in protein turnover (46). Moreover, *pepF2* is in an operon with a gene homologous to a gene induced during competence in *Streptococcus pneumoniae*, and P*pepF21* displays a sequence signature similar to that present in the streptococcal regulated promoters involved in cellular competence (2).

Specific peptides control Casitone-regulated genes. The transcription of *prtP1, prtP3, pepN, pepC, pepX, pepDA2*, and the *opp-pepO1* operon, encoding the main components of the proteolytic system of *L. lactis*, is controlled by the complex nitrogen source contained in M17 and Casitone. Since Casitone is a proteolytic hydrolysate of casein, this result suggests that the signal for regulation is a peptide or a mixture of peptides. In addition to Casitone, CAA repressed the transcription of *prtP1, prtP3, pepN, pepC*, and *opp-pepO1*, although to a 10-fold-lower extent (data not shown). Moreover, we showed that addition of dipeptides LP and PL in the medium decreased the level of transcription of *prtP3, pepN, pepC*, and *opp-pepO1* to the same extent as CAA (Table 3). These dipeptides have been previously reported to repress *prtP3* transcription approximately 10-fold and decrease PepN and PepX enzymatic activities 1.7- and 1.5-fold, respectively (36, 39). These results suggest that PepX expression could be regulated at a level other than transcription since in our conditions, *lux* fusion showed that *pepX* expression was regulated by Casitone but not by dipeptides LP and PL.

The nature of the signal-repressing proteolytic gene is probably complex. Addition of CAA or specific dipeptides such as LP and PL has not the full repressing effect obtained with Casitone, and the efficiency of dipeptide repression is not increased when fivefold-higher concentrations are used (data not shown), suggesting that other peptides from Casitone might be more active or that a mix of specific peptides is required. However, the growth rate is higher in CDM Casitone than in CDM with CAA or dipeptides, indicating that peptides present in Casitone provide a better nitrogen source than CAA or dipeptides. Interestingly, the repressing factor(s) present in Casitone is used by *L. lactis*, since the duration of the repression is correlated with the amount of Casitone added in the medium (Fig. 4). A simple explanation would be that the repression requires transport and/or assimilation of certain peptides by the cell. Indeed, it has been shown that peptides are in competition to enter the cell (28) and that certain peptides are assimilated well whereas others accumulate in the medium during casein utilization (24).

Addition of peptide sources to CDM, a medium containing the 18 free amino acids (all amino acids except aspartic acid and glutamic acid [51]) required for rapid growth, causes repression of some proteolytic genes. However, even in an excess of free amino acids in CDM, their availability inside the cell could be limiting due to a low rate of uptake and lead to a partial starvation. This starvation could be overcome by addition of peptides efficiently taken up. Nevertheless, the major factor involved in this regulation is not due to a severe amino acid starvation, since addition of CAA or dipeptides LP and PL to CDM induces significant repression without improving the growth rate of *L. lactis*. Coordinate regulation of the proteolysis genes in *L. lactis* would thus depend on the content of the peptide source, presumably by a signal sensing the nutritional state of the cell for amino acid supply.

This study provided a set of data on the transcription of 16 genes potentially involved in protein utilization. Analysis of *lux* fusion data, including assessment of the relative level of transcription and regulation by environmental factors, provided new insight into the probable roles of the different components of the proteolytic system. Genes expressed at a low level are not regulated by the peptide source and probably encode enzymes involved in cellular functions other than peptide utilization. Moreover, the most highly expressed genes are repressed by the peptide source and encode the enzymes most important for proteolysis. Their transcription is repressed by dipeptides LP and PL, and more dipeptides should be tested to better define the signal-repressing proteolytic genes. Identification of the cellular factors that are involved in this repression mechanism will provide new understanding of the control of peptide or nutritional metabolism in *L. lactis*.

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