The peptide transport system Opt is involved in both nutrition and environmental sensing during growth of *Lactococcus lactis* in milk

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Lactococcus lactis is known to take up extracellular peptides via at least three distinct peptide transporters. The well-described oligopeptide transporter Opp alone is able to ensure the growth of *L. lactis* in milk, while the di- and tripeptide transporter DtpT is involved in a peptide-dependent signalling mechanism. The oligopeptide Opt transporter displays two peptide-binding proteins, OptA and OptS. We previously demonstrated that OptA-dependent transport is dedicated to nutritional peptides, as an *optABCDF* mutant (of a strain devoid of Opp) has an impaired capacity to grow in milk. Using isogenic peptide transport mutants, this study shows that biosynthesis of the Opt transporter is much less sensitive to downregulation that is dependent on extracellular peptides taken up by DtpT than is Opp biosynthesis; this peptide-dependent regulation relies on the transcriptional repressor CodY. We demonstrate the dual function of the Opt system; while OptA contributes to the bacterial nutrition during growth in milk, OptS is involved in the transport of signalling peptides derived from milk and controlling *opp* expression. So, these results shed new light on the peptide-dependent regulation relying on two peptide transporters with different specificities: DtpT and Opt (via OptS).

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INTRODUCTION

Peptide transport plays a major role in bacteria, and more particularly in nutrition and signalling. Extracellular signalling peptides allow bacteria to sense modifications to their environment, such as population density, and adapt to their ecological niche. Some peptides directly interact extracellularly with a two-component membrane receptor, while others require internalization via peptide transporters to control biological functions such as competence, sporulation and conjugation (reviewed by Monnet, 2003; Lazazzera, 2001). *Lactococcus lactis* is a non-pathogenic bacterium used in the fermentation of many dairy products. *L. lactis* growth in milk relies on a complex proteolytic system that provides the bacterium with essential amino acids derived from milk caseins. Casein is first hydrolysed by the cell-surface proteinase PrtP into peptides of various lengths. Some of them are taken up by oligopeptide transporters, before being further hydrolysed by a large set of intracellular peptidases (Kunji *et al.*, 1995). Three distinct transport systems have been described in *L. lactis*: the di- and tripeptide transporter DtpT and two

oligopeptide transporters Opp and Opt. DtpT belongs to the PTR family (proton motive-force driven system); it is a 12-transmembrane domain protein encoded by a single gene. Opp is an ABC transporter encoded by the *oppDFBCA* operon, and is composed of a membrane lipoprotein OppA that binds extracellular peptides, two transmembrane proteins OppB and OppC, and two cytoplasmic ATPases OppD and OppF (Tynkkynen *et al.*, 1993). Opt also belongs to the ABC transporter family but differs from Opp by the presence of two peptide-binding proteins OptS and OptA (Lamarque *et al.*, 2004). While the *optS* gene is transcribed independently (M. Lamarque and D. Atlan, unpublished results), *optA* is part of the *optABCDF* operon. In *L. lactis*, expression of many genes of the proteolytic system was shown to be peptide-dependent (Guédon *et al.*, 2001). Transport of extracellular peptides containing branched-chain amino acids (BCAAs; Ile, Leu, Val) results in a rapid increase in the intracellular BCAA pool. BCAAs are direct co-factors of CodY, a global regulator of Gram-positive bacteria, and stimulate its binding to specific DNA sequences (CodY boxes) located in gene promoter regions (Guédon *et al.*, 2001; den Hengst *et al.*, 2005; Sonenshein, 2005). Consequently, expression of genes located immediately downstream of the CodY

Abbreviations: βGlu, β-glucuronidase; BCAA, branched-chain amino acids; PNPU, *p*-nitrophenyl β-D-glucuronide.

boxes is repressed in the presence of BCAAs. The specificity of the DtpT transporter is limited to di- and tripeptides and has so far been considered as the major pathway for the entry of peptides involved in gene regulation in *L. lactis* (Kunji *et al.*, 1993; Doeven *et al.*, 2005). For instance, Leu-Pro and Pro-Leu dipeptides, internalized via DtpT, were shown to downregulate the expression of the gene encoding the PrtP proteinase of *L. lactis* SK11 (Marugg *et al.*, 1995). In contrast with di- and tripeptides, longer oligopeptides were considered to be dedicated to bacterial nutrition. Transport of nutritional oligopeptides was first described to be solely carried out by the Opp transporter (Tynkkynen *et al.*, 1993; Kunji *et al.*, 1995). However, the Opt oligopeptide transporter is also present in most lactococcal strains and is able to replace Opp for bacterial growth in milk (Lamarque *et al.*, 2004).

The objectives of this study were to unravel the respective roles of the Opt binding proteins (OptS and OptA) and to determine the importance of the Opt system during *L. lactis* growth in milk compared with the oligopeptide transport system Opp involved in the nutrition and the DtpT peptide-signalling pathway.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* K-12 strains were grown with aeration at 37 °C in Luria-Bertani (LB) broth (Miller, 1992). *L. lactis* strains constructed in the present study all derived from *L. lactis* IL1403, a plasmid-free strain with a $\text{Prt}^- \text{Lac}^- \text{Opp}^-$ phenotype (Bolotin *et al.*, 2001). *L. lactis* cultures were incubated at 30 °C without shaking in M17 broth (Terzaghi & Sandine, 1975) or in chemically defined medium (CDM; Poolman & Konings, 1988) supplemented with 0.5 % (w/v) glucose or in reconstituted 10 % (w/v) G milk (Standa Industrie). When necessary, erythromycin (5 µg ml⁻¹ for *L. lactis* and 25 µg ml⁻¹ for *E. coli*), chloramphenicol (5 µg ml⁻¹ for *L. lactis* and 20 µg ml⁻¹ for *E. coli*) or ampicillin (100 µg ml⁻¹) was added to the medium.

Peptide transport. Peptide transport assays were performed at 22 °C as described previously (Charbonnel *et al.*, 2003). Briefly, cells were grown in CDM (OD₆₅₀ 0.8), collected by centrifugation and washed twice in 50 mM KH₂PO₄-K₂HPO₄ pH 7.5, and the intracellular pool of amino acids was exhausted by incubating cells in the presence of 1 mM 2-deoxy-glucose. Prior to transport experiments, cells were energized with 25 mM glucose. Energized cells (OD₆₅₀ 1.0) were incubated at 22 °C in the presence of 50 µM peptide, unless otherwise stated. Peptide transport was determined by measuring the internal accumulation of the amino acids constituting the peptide under study. Amino acids were detected by RP-HPLC after pre-column derivatization with o-phthalaldehyde.

General DNA techniques. Lactococcal genomic DNA was extracted according to a rapid method described previously (Charbonnel *et al.*, 2003). Plasmid DNA was purified from 2 ml overnight cultures according to the method described by Birnboim & Doly (1979) with an additional cell treatment with lysozyme (10 g l⁻¹) for 30 min at 37 °C before lysis. Digested plasmid DNA and PCR fragments were purified with the GeneClean turbo kit (Q-BIOgene) and QIAquick PCR purification kit (Qiagen), respectively.

Transformation of *E. coli* K-12 and *L. lactis* IL1403 and derivatives. Electrocompetent *E. coli* cells were prepared according

to the method described by Dower *et al.* (1988), except that the electric shock was performed at 2000 V. *L. lactis* IL1403 cells were treated according to the method described by Wells *et al.* (1993) and used immediately for electroporation, as described previously (Lamarque *et al.*, 2004).

Construction of IL1403-derived mutants. Partial deletion (~700 bp) of each gene of interest was obtained by double-crossover homologous integration using the thermosensitive plasmid pGh5:ISS1 (Biswas *et al.*, 1993). 5' and 3' homologous regions were amplified by PCR using DyNAzymeTMEXT polymerase (Finnzymes), *L. lactis* IL1403 DNA as template and the specific primers listed in Table 1. The resulting PCR fragments were subcloned into PCR-Script Cam SK⁺ vector (Stratagene) by using HindIII-EcoRI and EcoRI-PstI restriction sites, respectively. The partially deleted genes were further subcloned into HindIII- and PstI-digested pGh9:ISS1 (which also deletes ISS1 located between HindIII and PstI sites). Following *L. lactis* IL1403 electroporation with modified pGh9:ISS1, clones carrying a deleted version of the gene of interest were selected according to the procedure described by Biswas *et al.* (1993). Mutants of *optS*, *optA*, *dtpT* and *codY* were verified by PCR amplification and sequencing.

Construction of transcriptional fusions and genomic-site-specific integration. DNA regions of 200 bp upstream of the SK11 *oppDFBCA* operon and 231 bp upstream of the IL1403 *optABCD* operon, respectively, were amplified by PCR using the primers listed in Table 1. The PCR fragments were independently cloned between the XbaI and PstI sites of plasmid pLB85 upstream of the promoterless *uidA* gene, encoding the *E. coli* β-glucuronidase (βGlu) as a reporter (Brøndsted & Hammer, 1999). The recombinant vectors were subsequently checked by appropriate restriction analysis and sequencing prior to electroporation into *L. lactis* strains. The generation of a new *attL* site and the removal of the former *attB* site following chromosomal integration were further confirmed by PCR using the primers listed in Table 1.

Enzyme and protein assays from bacterial extracts. *L. lactis* cells grown in 100 ml CDM medium (or milk) up to OD₆₀₀ ~0.7–0.8 were harvested by centrifugation, washed with 40 ml GUS buffer (50 mM NaHPO₄, pH 7.0) and resuspended in 4 ml of the same buffer. After cell breakage by a French press at 20 000 p.s.i. and centrifugation, the supernatants were used as soluble cellular extracts for βGlu assays. Enzymic reactions were prepared as follows: 100 µl extract, 790 µl GUS buffer, 100 µl 10 mM *p*-nitrophenyl β-D-glucuronide (PNPU) and 10 µl 1 M β-mercaptoethanol. Time-course release of *p*-nitrophenol from PNPU was followed at 415 nm. PepN activity was assayed with 0.3 mM lysyl-*p*-nitroanilide as described previously (Atlan *et al.*, 1989). The protein content was determined as described by Bradford (1976) using the Bio-Rad protein assay (Bio-Rad) with BSA as the standard.

Immunoblotting of OptA and OptS. Immunodetection of OptA and OptS was carried out as described previously (Lamarque *et al.*, 2004). The antibody dilutions used were as follows: rabbit anti-OptA, 1:8000; rabbit anti-OptS, 1:2000; anti-rabbit immunoglobulin G-peroxidase conjugate, 1:4000 (Sigma-Aldrich). Detection was performed using Lumi-LightPlus Western blotting substrate (Roche Diagnostics).

Calculations and statistical analyses. All data are expressed as mean ± SEM of values for three reactions, unless otherwise stated. The effect of treatments (gene inactivation, addition of peptides) on βGlu activity was assessed using ANOVA for repeated measurements. When ANOVA indicated significant differences, treatments were compared using the Student-Newman-Keuls multiple comparison test. Statistical significance was set at *P* < 0.05. Calculations were performed using the SigmaStat software package (Systat Software).

Table 1. Primers used in this study

Restriction sites are underlined.

Gene	Primer	Primer sequence (5'–3')
Construction of deletion mutants		
<i>ΔoptA</i>	optAmut3	AACTGCAGAGCATGCGGCAATAGCG
	optArev3	GCTCTAGAGCCAATAATCGTCATTCTTCACG
	optAmut4	GGAATTCATTGAGCGAAATTGGCGA
<i>ΔoptS</i>	optA mut6	CCCAAGCTTTTATTATTTGAGGTAAGCTGTTTGAAGTCAT
	optS3Hind	CCCAAGCTTAAAAACAAGTGAGCAAAAAAACATC
	optSrdelta	CGGAATTCCTCCCATCAAATAGGCATAT
	optsensdelta	CGGAATTCCTGTCATTTAAAACTTGTTCC
	optSrev1	AACTGCAGTTATTTAACATAAGCCGATTTTAGG
<i>ΔdtpT</i>	dtpTsens1	AACTGCAGTCGGTCAACCTCGCGGC
	dtpTmut1	GGAATTCCTGGAGCAATCAGAGAACCCATGTT
	dtpTmut2	GGAATTCCTCGCTCTTCATCGTTCTCTTGTCAC
	dtpTrev1	CCCAAGCTTGACAATAAGGAGGATAATTCCCACG
<i>ΔcodY</i>	codYsens1	AACTGCAGGCAAGATGGAGTGACCG
	codYrev1	GGAATTCACGTACTGCCGCACGAAC
	codYsens2	GGAATTCGACACCATGGCAACTATGGC
	codYrev2	CCCAAGCTTCCTGGCTTTAGAAATTACG
Promoter regions		
<i>optA</i>	PoptA1	GCTCTAGAAAAACACGAACCAGTCAGAGCC
	PoptA3	AAAAGCTGCAGTTCATGAATTGTGTCTGACTTCGC
<i>oppD</i>	PoppDSKsens	GCTCTAGAGCAATTCGGGTATAATAAAGC
	PoppDSKrev	AACTGCAGTGAAATCTTTCTAAATAGACTTAATAA
Detection of <i>attB</i> and <i>attL</i> sites*		
	pattBL	CTACTGCTTCACCAG
	gusArev	GTCGAGTTTTTTGATTTACGGG
	BI-POB1inv	GTATGCAGCGATGTCGTTACCC

*The presence of the chromosomal *attB* site was detected by PCR with the primer pair pattBL/BI-POB1inv. Insertion of a fusion at *attB* generated a new site (*attL*) detected by PCR with the primers pattBL/gusArev.

RESULTS

Extracellular peptides affect *opt* expression

In *L. lactis*, dipeptides have been shown to affect the transcriptional pattern of genes encoding proteins of the proteolytic system. These peptides are hydrophobic, contain at least one branched-chain amino acid and are internalized into the bacteria via the di- and tripeptide transporter DtpT (Guédon *et al.*, 2001, 2005). To investigate whether Opt expression was sensitive to peptide-dependent regulation, we chose a transcriptional fusion strategy in isogenic peptide-transport mutants. We took advantage of the IL1403 strain background that did not express *opp* genes to focus our study on the effect of peptides solely taken up by Opt and DtpT.

The promoter regions of *optS* (PoptS) and the *optABCDF* operon (PoptA) from *L. lactis* IL1403, or *oppDFBCA* (PoppD) from *L. lactis* SK11 (PoppD from *L. lactis* IL1403 is not functional) (Lamarque *et al.*, 2004) were cloned in the pLB85 vector, upstream of the promoterless *uidA* gene encoding the *E. coli* βGlu enzyme as a reporter. Then, the resulting constructs were integrated at the *attB*

chromosomal site of *L. lactis* IL1403 or in derived strains deleted for either *optA*, *optS*, *dtpT* or *codY* genes (Brøndsted & Hammer, 1999). In order to assess the effect of peptides originating from a pancreatic digest of casein (tryptone), bacteria were cultured in CDM or CDM supplemented with 1.5 % tryptone (CDM_{try}) (Fig. 1). βGlu activities were measured using PNPU as the substrate. In the parental strain IL1403, no βGlu activity driven by PoppD could be detected in the presence of a pancreatic digest of caseins in the culture medium, which reflects a full repression of the PoppD–*uidA* fusion (Fig. 1a). Repression was abolished in a *codY* and single or multiple *dtpT* mutants. Deletions of *optA* or *optS* did not affect βGlu activities, suggesting that the Opt transporter was not involved in the transport of peptides affecting the transcriptional regulation of PoppD–*uidA*. These results confirm that inhibitory peptides are taken up by DtpT and that this signalling pathway resulted in the activation of the transcriptional repressor CodY (Fig. 1a).

Analysis of PoptA–*uidA* expression revealed that the βGlu activity was decreased 3.6-fold in bacteria grown in CDM_{try} compared with CDM, and that this inhibition was less severe

Fig. 1. Peptide- and CodY-dependent transcriptional repression of the *opt* system. IL1403 or derived strains were grown in CDM (solid bars) or CDM_{tryp} (CDM supplemented with 1.5 % tryptone; hatched or dotted bars) and assayed for βGlu activities driven by (a) *PoppD* or (b) *PoptA*. Data are the means ± SEM of three independent experiments. Statistically significant differences (*t*-test) are highlighted by an asterisk: **P* ≤ 0.05, ***P* ≤ 0.01. (c) Peptidase PepN activity was used as a control for peptide-dependent regulation. 1, IL1403; 2, Δ*codY*; 3, Δ*optA*; 4, Δ*optS*; 5, Δ*optS-optA*; 6, Δ*dtpT*; 7, Δ*dtpT-optA*; 8, Δ*dtpT-optS*; 9, Δ*dtpT-optS-optA*. (d) Immunodetection of peptide-binding proteins OptA and OptS in IL1403 or derived strains using rabbit polyclonal antibodies. Equivalent amounts of *L. lactis* cells were loaded in each lane.

in *codY* or *dtpT* mutants (Fig. 1b). This significant repression effect (*P* < 0.05) closely resembled that observed for PepN peptidase activity used as a control of the peptide-dependent regulation of the proteolytic system (Fig. 1c). To further verify that the transcriptional repression observed with the promoter-*uidA* fusion constructs reflected genuine protein level changes, immunodetection of peptide-binding proteins OptA and OptS was carried out after growth of IL1403 in CDM or CDM_{tryp}. The OptA protein level perfectly matched *PoptA* transcriptional regulation, i.e. there was a significant decrease of OptA biosynthesis in the presence of peptides and suppression of this effect in a *codY* mutant but not in an *optS* mutant (Fig. 1d). Interestingly, immunodetection of OptS revealed that the biosynthesis of this protein was much more sensitive to the added peptides than OptA biosynthesis. The fact that OptS downregulation in CDM_{tryp} cultures was abolished in a *codY* mutant suggests CodY-dependent regulation. Nevertheless, this hypothesis could not be confirmed at the transcriptional level by *PoptS-uidA* fusion, as no enzyme activity driven by *PoptS* could be detected under our conditions.

Thus, these results showed that biosynthesis of both *L. lactis* oligopeptide transporters (Opp and Opt) was down-regulated by peptides originating from a pancreatic digest of casein and taken up by DtpT.

Opt and DtpT are entry pathways for signalling peptides from milk

Previous work on peptide-dependent regulation of proteolytic genes has been carried out using synthetic or commercial peptide mixtures (Guédon *et al.*, 2001; Marugg *et al.*, 1995). Nevertheless, the effect of either endogenous

milk peptides or peptides resulting from casein proteolysis by the lactococcal cell-surface proteinase PrtP has not yet been addressed. As the strain IL1403 used in this study is devoid of the plasmid *prtP* and *lac* systems essential for optimal growth in milk (de Vos *et al.*, 1989), we restored the capacity of bacterial growth in milk supplemented with glucose by transferring pNZ521, which encodes the P_{III}-type proteinase from *L. lactis* SK11; the resulting recombinants are hereafter indicated with “-P”. As expected, the IL1403-P strain exhibited a biphasic exponential growth in milk supplemented with glucose, with the first exponential phase corresponding to the utilization of free amino acids and peptides from milk, while the second phase reflects utilization of casein-derived peptides generated by PrtP (Fig. 2a) (Juillard *et al.*, 1995). The *optA*-P mutant was severely impaired for growth in milk, while deletion of *optS* or *dtpT* had no significant effect. These observations confirmed previous results suggesting that OptA was involved in supplying *L. lactis* with nutritional peptides (Lamarque *et al.*, 2004).

The plasmid pNZ521 was also transferred into isogenic peptide transport mutants harbouring transcriptional *PoppD-uidA* or *PoptA-uidA* fusions. βGlu activities were measured in the middle of the second exponential growth phase corresponding to PrtP-dependent casein hydrolysis. Surprisingly, βGlu activity driven by *PoppD-uidA* was not detected after growth of IL1403-P in milk (Fig. 2b). However, this transcriptional fusion is functional, as enzymic activity was detected after growth of the same strain in CDM. Thus, we can conclude that *oppD* expression was totally repressed in the IL1403-P strain grown in milk. In contrast, βGlu activity was detected in

Fig. 2. DtpT and OptS are entry pathways for signalling peptides from milk. (a) Growth in milk of *L. lactis* IL1403 and isogenic peptide transport mutants harbouring a plasmid with the *prtP* gene encoding the cell-surface PrtP proteinase (-P strains): IL1403 (□), IL1403-P (■), $\Delta optS$ -P (◇, dotted line), $\Delta dtpT$ -P (●, dotted line) and $\Delta optA$ -P (◆, dotted line). (b, c) βGlu activities of *PoppD-uidA* (b) or *PoptA-uidA* (c) fusion after growth of IL1403 or derived strains in milk. Cells were collected at OD₆₀₀ ~0.7–0.8, corresponding to the second exponential growth phase. βGlu activity was measured using PNPu as the substrate. Statistically significant differences (*t*-test) are highlighted by an asterisk: **P* ≤ 0.05, ***P* ≤ 0.01.

strains carrying deletions of the *optS* or *dtpT* genes. This result clearly shows that OptS and DtpT are responsible for the uptake of repressive peptides originating from milk casein or resulting from caseinolysis by PrtP.

Analysis of *PoptA-uidA* expression revealed that cultures grown in milk displayed a similar βGlu activity to that measured in CDM (Figs 1b and 2c). While the *optS* deletion did not significantly affect the level of this activity, deletion of *dtpT* led to a twofold decrease in βGlu activity compared with the parent IL1403-P (*P* < 0.05). This result

suggests that DtpT, so far known as a transporter of repressing peptides, might also be responsible for the uptake of activating di- and tripeptides from milk (or milk hydrolysis).

Taken together, these results expand the role of DtpT as the major transporter of signalling peptides and further validate our genetic approach. Consequently, these data show for the first time, to our knowledge, that OptS is also involved in environmental sensing by taking up repressive peptides from milk.

OptS and OptA display unique peptide specificities

Our results indicate that OptS and DtpT are main entry pathways for regulatory peptides while OptA would be involved in the uptake of nutritional peptides. We had previously analysed the transport of three peptides by *optS* or *optABCD* interrupted mutants (Lamarque *et al.*, 2004). Here, we took advantage of the new isogenic *optA* and *optS* single and double peptide transport mutants to further define the respective OptS- and OptA-dependent transport specificities. The strains were tested for their transport capabilities towards 17 peptides containing 2–10 amino acids. The parental IL1403 strain was able to take up peptides containing up to six residues but not longer peptides (Table 2). In contrast, an *optA* mutant was totally impaired in its transport capacity of tri- to hexapeptides. This tripeptide uptake ability was not expected, as so far OptA was thought to be restricted to oligopeptide transport. Absence of OptS did not affect transport rates of tri- to hexapeptides or of hydrophobic FL and MM dipeptides, which correlates with the reported DtpT specificity and highest affinity towards peptides containing at least one hydrophobic residue (Fang *et al.*, 2000). However, the *optS* mutant exhibited a significant fourfold decrease in the transport rate of the EK dipeptide, suggesting that OptS might display preferences for charged dipeptides.

Taken together, these data highlight specificity differences between both peptide-binding proteins OptS and OptA, and clearly demonstrate the originality of Opt transport specificity compared with those of Opp and DtpT.

DISCUSSION

Previous studies have demonstrated the nutritional function of the oligopeptide transport system Opp in *L. lactis* (Kunji *et al.*, 1995; Juillard *et al.*, 1995). Characterization of a second oligopeptide transport system Opt raised the question of the respective role of each system in *L. lactis*. Our data clearly show that Opt supports a dual function, in both nutrition and signalling. Indeed, we provide evidence with IL1403 isogenic peptide transport mutants that Opt is able, via OptA, to sustain *L. lactis* growth in milk. In contrast, neither OptS nor DtpT was required for optimal

Table 2. Initial rate of peptide transport by *L. lactis* IL1403 and derived strains

Peptide concentration used for transport assay was 50 μM , except for VGDE (300 μM). Values are the mean of two independent experiments.

Length (aa)	Peptide	Initial transport rate [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$]			
	Amino acid sequence	IL1403	ΔoptS	ΔoptA	$\Delta\text{optS-optA}$
1	L	16.4	17.2	20.8	18.0
2	FL	20.7	32.3	30.4	22.2
2	MM	22.3	27.9	22.0	22.0
2	EY	0	0	0	0
2	EK	10.2	2.5	8.0	1.5
3	EGH	18.1	14.8	0	0
3	KGE	42.4	33.2	0	0
3	GFL	46.7	39.5	0	0
3	LLL	60.9	49.2	0	0
4	MRFA	34.4	42.9	0	0
4	VGDE	4.0	2.3	0	0
5	YGGFL	40.7	34.9	0	0
6	DMPIQA	45.6	34.2	0	0
7	YFPGPPI	0	0	0	0
9	RPPGFSPFR	0	0	0	0
10	SFPWMESDVT	0	0	0	0

growth of IL1403-P in milk. This marked difference in nutritional function is also reflected in the difference in the substrate specificity of OptA- and OptS-dependent transport. Performing transport assays with a large panel of

synthetic peptides revealed that OptA was responsible for the uptake of tri- to hexapeptides, while OptS was restricted to di-peptides at least under our experimental conditions, thereby confirming that OptA is an oligopep-

Fig. 3. Model of peptide-dependent nutrition and regulation involving the peptide transporters (Opp, Opt and DtpT) during growth of *L. lactis* in milk. (a) The nutritional function. During exponential growth in milk, the *opt* and *opp* systems are expressed. As a consequence, Opp and Opt (via OptA) transporters sustain bacterial growth with the free oligopeptides present in milk (first exponential bacterial growth phase) or those released upon casein hydrolysis by the cell envelope proteinase (PrtP) (second exponential growth phase). (b) Environmental sensing. Casein degradation results in an increased concentration of regulatory casein-derived (di-tri)peptides, which are taken up via OptS and DtpT, and leads to a progressive repression of *opp* expression. This mechanism is controlled by the transcriptional repressor CodY that senses the pool of BCAAs, and probably by another regulatory protein(s) not yet identified. Simultaneously, some peptides only internalized by DtpT might activate *optABCD* expression by a mechanism that remains to be elucidated.

tide-binding protein. It is worth mentioning that determination of the upper size limit for peptide transport is very challenging, as model peptides used for transport studies do not necessarily have a high enough affinity for the binding protein to be transported. In fact, we had previously shown that *L. lactis* SK11 deprived of the Opp transporter was able to transport peptides of seven or nine residues purified from milk (Lamarque *et al.*, 2004). However, it might be possible that OptA displays a broader specificity towards natural milk peptides than to the synthetic ones used in this study. What is clear is that OptA specificity appears much more limited than that of OppA, which can bind peptides from between 4 and 35 residues (Detmers *et al.*, 2000).

Biosynthesis of two oligopeptide transporters has been described in other bacteria. In *Bacillus subtilis*, for instance, Opp and App oligopeptide transporters display overlapping specificities that improve peptide transport efficiency according to environmental changes (Koide *et al.*, 1999). The presence of multiple peptide-binding proteins encoded by genes under the control of different promoters is an advantageous strategy to broaden substrate specificity and improve adaptation capacities, as was described in *Lactobacillus delbrueckii* subsp. *bulgaricus* (Peltoniemi *et al.*, 2002). Similarly, our results showed that during bacterial growth in CDM, OptA and OptS biosynthesis was repressed in a CodY-dependent manner by signalling peptides taken up by DtpT, but not to the same extent, since the OptS biosynthesis was much more sensitive to peptides present in the tryptone added to the culture medium. Besides, the moderate repression of the *PoptA-uidA* fusion, compared with the full repression of *PoppD-uidA*, could be explained by differences in the CodY box sequences located in these promoter regions (den Hengst *et al.*, 2005). Actually, the CodY box located in the *optA* promoter differs from the consensus sequence by two nucleotides, whereas a unique substitution was observed in the CodY box of the *oppD* promoter from *L. lactis* SK11.

Importantly, we showed for the first time that signalling peptides, originating from milk and possibly generated by the cell-surface proteinase PrtP, are delivered into the bacteria via OptS and downregulate *opp* expression. Hence, the Opt transporter would support a dual function, one of nutrition via OptA and a second in environmental sensing via OptS (Fig. 3). Based on the observations that Opp biosynthesis was much more sensitive to repressive peptides than Opt and that *PoppD* promoter activity was not detected during the second exponential growth phase in milk, it could be supposed that Opt might be the major nutrient transporter in wild-type *Lactococcus* strains harbouring both transport systems, at least during certain bacterial growth phases in milk.

The peptide-dependent regulation during *L. lactis* growth in milk involves two peptide transporters with different specificities: DtpT and Opt (via OptS) (Fig. 3b). DtpT is able to take up di- and tripeptides from milk and those

resulting from casein degradation by the *Lactococcus* proteinase PrtP (or a pancreatic extract such as tryptone); in contrast, di-(tri)peptides internalized by OptS are more specifically associated with *Lactococcus* growth in milk and seem dedicated to the downregulation of Opp. However, it is also possible that other unidentified regulation mechanisms could be set up by the bacteria to finely regulate and coordinate the expression of genes encoding different components of the proteolytic system. In support of this hypothesis, our results tend to suggest the existence of an activating mechanism of *optA* expression depending on di- and tripeptides that would be taken up by DtpT (Fig. 3b). Future work will be necessary to fully understand the complexity of peptide transport regulation and specificity in dairy milk bacteria.

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