Specificity of Peptide Transport Systems in Lactococcus lactis
Foucaud, Catherine; Kunji, Edmund R.S.; Hagting, Anja; Richard, Jean; Konings, Wil N.; Desmazeaud, Michel; Poolman, Bert

Published in:
Journal of Bacteriology

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
A proton motive force-driven di-tripeptide carrier protein (DtpT) and an ATP-dependent oligopeptide transport system (Opp) have been described for Lactococcus lactis MG1363. Using genetically well-defined mutants in which dtpT and/or opp were inactivated, we have now established the presence of a third peptide transport system (DtpP) in L. lactis. The specificity of DtpP partially overlaps that of DtpT. DtpP transports preferentially di- and tripeptides that are composed of hydrophobic (branched-chain amino acid) residues, whereas DtpT has a higher specificity for more-hydrophilic and charged peptides. The toxic dipeptide 1-phe

nylalanyl-b-chloro-i-alanine has been used to select for a di-tripeptide transport-negative mutant with the ΔdtpT strain as a genetic background. This mutant is unable to transport di- and tripeptides but still shows uptake of amino acids and oligopeptides. The DtpP system is induced in the presence of di- and tripeptides containing branched-chain amino acids. The use of ionophores and metabolic inhibitors suggests that, similar to Opp, DtpP-mediated peptide transport is driven by ATP or a related energy-rich phosphorylated intermediate.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and growth media. L. lactis subsp. lactis MG1363 wild-type and isogenic mutants, i.e., the di- and tripeptide transport mutant AG300, the oligopeptide transport mutant VS72, and the peptide transport double mutant CV4, have been described previously (6, 11, 29) and are presented in Table 1. Strains were grown at 30°C in M17 broth (27) or in a CDM (19) at pH 6.8 supplemented with 0.5% (wt/vol) glucose or 0.5% (wt/vol) maltose in combination with 25 mM arginine to induce the arginine deiminase pathway (17). Recombinant strains carrying replicating plasmids were grown in the presence of erythromycin (5 mg/liter). The strains were maintained in CDM containing 10% glycerol and stored at −8°C. Growth of L. lactis on peptides was tested in CDM containing all amino acids except for one essential amino acid, which was supplied in the form of a di- or a tripeptide. The influence of growth conditions on peptide uptake was tested in CDM in which Leu was supplied in the form of Leu (3.6 mM), Leu-Leu (1.8 mM), or Leu-Leu-Leu (1.2 mM).

Isolation of mutants resistant to toxic peptide analogs. Toxic-peptide-resistant mutants were isolated from L. lactis AG300 according to the method of Smit et al. (26). Approximately 10⁶ CFU of an exponentially growing CDM culture were spread on a 1.2% (wt/vol) agar plate containing a complete amino acid mixture.

Growth inhibition was observed after 48 h of incubation at 30°C. Spontaneous FCA-resistant mutants were isolated from the inhibition zones and maintained in CDM plus 0.25 mM FCA.

The peptidease patterns of the AG300 FCA⁻ mutants were compared with those of L. lactis MG1363 and AG300 by using enzymatic test strips containing different substrates (API 1 to II; Bio-Mérieux, Marcy l’Etoile, France).

Transport assays. Cells were harvested by centrifugation in the exponential phase of growth (A₆₅₀ of approximately 0.6), washed twice, and resuspended to a final A₆₅₀ of approximately 25 in 50 mM potassium phosphate (pH 6.5)–2 mM MgSO₄. Cells were de-energized with 10 mM 2-deoxy-o-glucose for 30 min at 30°C (28), which results in the depletion of intracellular amino acid pools. De-energized cells were washed twice with 50 mM potassium phosphate (pH 6.5)–2 mM MgSO₄. In experiments in which ionophores and metabolic inhibitors were used, the buffer was either 50 mM potassium phosphate (pH 6.5 or 7.5)–2 mM MgSO₄ or 50 mM sodium piperezine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.5)–2 mM MgSO₄, as specified in the text. The pH dependency of uptake was determined in 20 mM potassium succinate–20 mM potassium phosphate–20 mM Tris–2 mM MgSO₄. The buffers were adjusted to the desired pHs with KOH. All transport assays were performed at 30°C. The uptake of peptides was monitored by determining the intracellular concentrations of the corresponding amino acids by means of reversed-phase high performance liquid chromatography (HPLC) analysis as described previously (11). The dansylated amino acids were separated by HPLC on a C₁₈ column (Novapak C₁₈, 3.9 × 150
TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>L. lactis subsp.</th>
<th>Relevant characteristic(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1363 Lac^− Prt^− Opp^+ Dtp^+ Dtp^−, plasmid-free derivative of NCD0712</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>AG300 MG1363 OPP^+</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>VS772 MG1363oppA::pLS19A, Em^r</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>CV4 MG1363 OPP^+ oppA::pLS19A, Em^r</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>CF310 AG300, FCA</td>
<td>This work</td>
<td></td>
</tr>
</tbody>
</table>

^a Em^r, resistance to erythromycin.

RESULTS

Utilization of peptides by L. lactis strains during growth.

The utilization of essential amino acids containing di- and tripeptides was analyzed for L. lactis MG1363 and peptide transport mutant strains growing in CDM (Table 2). As expected L. lactis MG1363 and VS772 (Opp^−) can satisfy their requirements for essential amino acids by utilization of all di- and tripeptides except for Ile-*Arg (26) and Gly-*His-Gly. Surprisingly, L. lactis AG300, which lacks the di- and tripeptide transport system Dtp^+, can utilize the same set of peptides except for Gly-Leu. This pattern of growth was also observed with the double mutant CV4. These results suggest the presence of at least one other peptide transport system which facilitates the transport of dipeptides such as Leu-Leu, Val-Leu, Leu-Met, Phe-Val, His-Leu, Met-Met and Ile-Ile and of tripeptides such as Leu-Leu-Leu, His-Pro-Val, His-*Gly-Gly, Ala-His-Ala, and Gly-Leu-Tyr. The external breakdown of these di- and tripeptides, as a result of cell lysis, and the subsequent transport of the corresponding amino acids are unlikely, since Ile-*Arg, Gly-Leu, and Gly-*His-Gly were not hydrolyzed. These nontransported peptides as well as others are rapidly hydrolyzed upon permeabilization of the cells (results not shown).

Evidence for a second di- and tripeptide transport system.

The presence of a second, previously undiscovered, di- and tripeptide transport system was studied further in glycolyzing L. lactis CV4 cells (Fig. 1). The uptake of Leu-Val, Met-Met, and His-*Gly-Gly was observed for L. lactis CV4, provided that an energy source such as glucose was present. The uptake of these peptides was inhibited (competitively) by a fivefold excess of one of the other peptides; the inhibiting peptide itself accumulated (as amino acids) inside the cell. The pattern of inhibition suggests that the affinity of the transporter for Leu-Val exceeds that of Met-Met and His-*Gly-Gly. Free amino acids such as Leu and Gly and the oligopeptide Tyr-*Gly-Gly-Phe-Leu did not affect the transport of the di- and tripeptides (data not shown). Free peptides could not be detected in the cell, which indicates that peptide uptake is accompanied by rapid intracellular hydrolysis (data not shown). Assuming that the intracellular peptidase activity does not limit the peptide utilization rate, the uptake rate of a given di- or tripeptide can be estimated from the accumulation rates of the amino acids and the frequency of a particular amino acid residue in the peptide. The rates of uptake of Met-Met^*, Leu-Val^*, and His-*Gly-Gly^* were 11, 19, and 21 nmol/min/mg of protein, respectively (the amino acid considered is marked with an asterisk). No amino acids could be detected in the external medium, which confirms that the external breakdown of peptides followed by transport of the amino acids does not occur.

Isolation and characterization of FCA-resistant mutants.

The toxicity of the peptide analog L-alanyl-β-chloro-L-ala-lanine has previously been used to isolate peptide transport mutants with Dtp^+ phenotypes (26). Since the putative second di- and tripeptide transport system has a specificity for more-hydrophobic peptides, we tested the toxicity of the peptide analog FCA on confluently plated L. lactis AG300 (Dtp^+) cultures as described in Materials and Methods. Spontaneous FCA-resistant mutants (Table 1, AG300, FCA^2) were isolated with a mutation frequency of about 5 × 10^-6. As the resistance towards the dipeptide can be the result of an inactive peptide transport system(s) and/or intracellular peptidase(s), the peptide and transport activities in L. lactis MG1363, AG300, and one of the FCA-resistant mutants (designated CF310) were compared. The peptidase pattern of L. lactis CF310 was similar to those of the wild-type L. lactis MG1363 and L. lactis AG300 (data not shown). Furthermore, the uptake of Tyr-*Gly-Gly-Phe-Leu and Ala were the same for L. lactis CF310, MG1363, and AG300. On the contrary, the uptake of Leu-Leu-Leu, Met-Met, Met-Leu, Phe-Ala, Leu-Val, Val-Leu, and His-*Gly-Gly was not detectable in L. lactis CF310 (Table 3), indicating that a di- and tripeptide transport system was specifically inactivated in this mutant. The inactivation of the di- and tripeptide transport system (designated Dtp^P) in L. lactis CF310 was confirmed by growth experiments, since CF310 could not use any of these di- and tripeptides as a source of essential amino acids (data not shown). It was not possible to isolate FCA^2 mutants with MG1363 or VS772 as
the parent strain, most likely because FCA is transported by DtpT and DtpP.

**Substrate specificity.** The uptake of peptides by *L. lactis* MG1363 was compared with those by the transport mutants AG300, CV4, and VS772 (Table 3). The dipeptide Pro-Gly and the pentapeptide Tyr-Gly-Gly-Phe-Leu have previously been shown to be specific for DtpT and Opp, respectively (6, 10). Indeed, Pro-Gly requires a functional DtpT, whereas Opp activity is needed for the uptake of Tyr-Gly-Gly-Phe-Leu (Table 3). Peptides containing acidic amino acids, i.e., Glu-Val, Gly-Asp, or Met-Asp, or those containing basic amino acids, i.e., Lys-Leu, Arg-Gly, or His-Gly, were taken up by *L. lactis* MG1363. The uptake of both types of peptides was severely reduced or could not be detected in the double mutant *L. lactis* CV4. The position of the acidic residues in the dipeptide was not crucial. Comparable findings were made with neutral glycine-containing peptides, i.e., X-Gly and Gly-X. Since the oligopeptide transport system is specific for peptides containing at least four residues (29), the defect in the uptake of charged and Gly-containing neutral dipeptides in the double mutant must be the result of the dtpT mutation. On the other hand, Phe-containing peptides were transported by *L. lactis* CV4, albeit at reduced rates compared with that for the wild-type strain MG1363. Also the hydrophobic peptides (containing Met, Leu, and/or Val) were taken up by *L. lactis* CV4. The transport of these peptides was reduced in the ΔdtpT mutant AG300 but was unaffected by the opp mutation (VS772). The net accumulation rate of Leu was 4 to 18 times higher with Leu-Leu as substrate compared with the rate obtained with Met-Leu, Val-Leu, or Leu-Val as substrates (Table 3). The accumulation rates for Leu and Met were similar at initial substrate concentrations of the dipeptides Leu-Val and Met-Met of 0.5 or 1 mM (5 and 7 nmol/min/mg of protein for Leu-Val; 32 and 25 nmol/min/mg of protein for Met-Met) (Fig. 1 and Table 3; also results not shown), suggesting that the transport reaction occurs at or near saturation conditions. *L. lactis* MG1363 transports the tripeptide Leu-Leu-Leu at a higher rate than it does His-Gly-Gly. The di-, tri-, and oligopeptide-deficient transport mutant CV4 displayed reduced uptake of both peptides, but the effect was most pronounced for Leu-Leu-Leu. The net accumulation rate of Leu was lower with Leu-Leu-Leu as substrate than it was with the dipeptide Leu-Leu as substrate (Table 3).

In summary, the rates of di- and tripeptide transport in the di- and tripeptide transport-deficient mutant AG300 and the double mutant CV4 varied between <1 to 48% of the rate observed for the wild-type strain MG1363 (and the oligopeptide transport mutant VS772). These results strongly suggest that the uptake of di- and tripeptides is mediated by at least two distinct transport systems with overlapping specificities. Since the transport of the di- and tripeptides was completely abolished in the ΔdtpT FCA R mutant CF310 (Table 3), we propose that FCA resistance is due to a mutation in a second di- and tripeptide transport locus, designated dtpP.

**TABLE 3. Uptake of di-, tri-, and oligopeptides by glycolyzing *L. lactis* cells**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid accumulation rate (nmol/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MG1363 (wild type)</td>
</tr>
<tr>
<td>Glu-Val*</td>
<td>84</td>
</tr>
<tr>
<td>Gly*-Asp</td>
<td>24</td>
</tr>
<tr>
<td>Met*-Asp</td>
<td>50</td>
</tr>
<tr>
<td>Lys-Leu*</td>
<td>424</td>
</tr>
<tr>
<td>Arg-Gly*</td>
<td>89</td>
</tr>
<tr>
<td>His-Gly*</td>
<td>160</td>
</tr>
<tr>
<td>Pro-Gly*</td>
<td>24</td>
</tr>
<tr>
<td>Ala-Gly*</td>
<td>67</td>
</tr>
<tr>
<td>Phc-Ala*</td>
<td>15</td>
</tr>
<tr>
<td>Phc-Val*</td>
<td>15</td>
</tr>
<tr>
<td>Leu*-Val</td>
<td>148</td>
</tr>
<tr>
<td>Val-Leu*</td>
<td>284</td>
</tr>
<tr>
<td>Leu*-Leu*</td>
<td>491</td>
</tr>
<tr>
<td>Met-Leu*</td>
<td>50</td>
</tr>
<tr>
<td>Met*-Met*</td>
<td>190</td>
</tr>
<tr>
<td>His-Gly*-Gly*</td>
<td>89</td>
</tr>
<tr>
<td>Leu*-Leu*-Leu*</td>
<td>216</td>
</tr>
<tr>
<td>Tyr-(Gly)*,-Phc-Leu</td>
<td>94</td>
</tr>
</tbody>
</table>

*Peptide uptake rates were determined from the time-dependent increase of the intracellular amino acid pools as described in Materials and Methods. The amino acids marked with asterisks were used to estimate the accumulation rates. Each peptide was present at a concentration of 0.5 mM. Blanks indicate data that were not determined.
Energetics of the second di- and tripeptide transport system. To characterize the transport of di- and tripeptides by the putative DtpP system further, Leu-Leu-Leu was chosen as substrate for uptake assays with *L. lactis* AG300. In the absence of metabolic energy, no significant uptake of the peptide was detected. Addition of a fermentable sugar (glucose) resulted in a high rate of tri-leucine uptake. At pH 7.5, when the external and internal pH values are similar and the proton motive force is composed of $\Delta \psi$ only (16), Leu-Leu-Leu uptake was not significantly affected by the addition of either valinomycin, a potassium ionophore which dissipates the membrane potential in the presence of $K^+$ (data not shown), or nigericin, a potassium proton ionophore. However, the addition of valinomycin plus nigericin inhibited Leu-Leu-Leu uptake, whereas the uncoupler carbonyl cyanide–$m$-chlorophenylhydrazone (1 mM) did not. Monensin, which converts the $\Delta \psi$ into a chemical sodium gradient, did not significantly lower tri-leucine transport at pH 6.5 and in the presence of 5 mM sodium ions (data not shown). These results are at variance with the involvement of a proton or sodium motive force as the driving force for the DtpP system.

To investigate further the nature of the driving force of di- and tripeptide transport by DtpP, the effect of the ATPase inhibitor o-vanadate was studied. Since ATP production in *L. lactis* by the glycolytic pathway is affected by $o$-vanadate (11), the arginine deiminase pathway was used for the generation of metabolic energy. The addition of arginine led to a rapid uptake of tetra-alanine, tri-leucine, and leucine (Fig. 2A to C, respectively) in *L. lactis* AG300 cells. The addition of $o$-vanadate did not influence leucine uptake (Fig. 2C), which was expected since leucine transport is driven by the proton motive force (2). In contrast, arginine-energized tri-leucine uptake (Fig. 2B) and tetra-alanine uptake (Fig. 2A) were partly inhibited by $o$-vanadate. This finding suggests that tri-leucine uptake via DtpP, as tetra-alanine uptake via Opp (11), is most likely driven by ATP or a related energy-rich phosphorylated intermediate.

Expression of DtpP in *L. lactis* CV4. The activity of the second di- and tripeptide transport system (DtpP) varied with the growth conditions. The rate of Leu-Leu-Leu uptake increased two- to threefold when CDM was used instead of the complex medium M17 to culture the cells (Fig. 3). A further two- to threefold increase in the transport rate was observed when Leu-Leu or Leu-Leu-Leu was present in CDM, suggesting that these peptides serve as inducers of the DtpP system. Similar results were obtained with Leu-Leu as the substrate of the transport system (data not shown).

**pH dependence of DtpP-mediated transport.** The external pH dependence of Met-Met uptake was measured in glycolyzing *L. lactis* CV4 cells (Fig. 4). Met-Met uptake showed a broad optimum range from pH 5.5 to 7.5. Below pH 4, no significant Met-Met uptake was detected. The highest rates of Met-Met uptake were observed between pHs 6 and 7. The broad optimum pH range for DtpP indicates that this system is operative in the physiological pH range as observed for the DtpT and Opp transport systems (24).
The presence of a second di- and tripeptide transport system in *L. lactis* is indicated by the residual di- and tripeptide uptake in strains lacking DtpT (with or without functional Opp) and the complete loss of di- and tripeptide transport activity in the ΔdtpT FCA escorts. The accumulation of the amino acids present in the peptides was clearly not due to external breakdown of the peptides followed by transport of the amino acids since (i) no free amino acids were detected in the external medium, (ii) the accumulation of amino acids was observed only with transportable peptides, the nontransported peptides were hydrolyzed upon permeabilization of the cells, and (iii) the di- and tripeptide transport activity was completely lost in the ΔdtpT FCA escort mutant, the peptidase activities in the wild-type strain and in the ΔdtpT FCA escort mutant were similar. DtpT and the newly described DtpP system have overlapping specificities for di- and tripeptides but do not transport amino acids or oligopeptides (Table 2 and results not shown) (11, 25, 26). The DtpT carrier transports relatively hydrophilic di- and tripeptides such as Glu-Val, Gly-Asp, Met-Asp, Ala-Gly, and Pro-Gly (6, 26; also this study), whereas DtpP transports a system(s) (26). Although small peptides have not been detected in the degradation of β-casein by PrtP (9), it has been suggested that at least one essential amino acid is taken up in the form of a di- or a tripeptide which could be released from casein species other than the β form (e.g., κ-casein) (20, 23).

In *L. lactis*, peptide uptake is accompanied by rapid intracellular hydrolysis and the selective exodus of amino acids when the pools become excessively high. The high level of hydrophobicity of some amino acids can lead to passive leakage from the cell (2, 19). In addition, if the concentration of an amino acid exceeds the driving force imposed by the carrier mechanism, the amino acid may also leave the cell by facilitated diffusion. Therefore, the estimated uptake rates based on the concentrations of intracellular amino acids will always be underestimated of the actual peptide uptake rate (29). It is unlikely that amino acid loss as a result of protein synthesis affects the estimation of the peptide uptake rates, since amino acid incorporation into trichloroacetic acid-precipitable material is hardly or not at all observed under these conditions (15a). Furthermore the rates of peptide uptake are similar in the presence and absence of chloramphenicol.
In conclusion, the presence of a third lactococcal peptide transport system was demonstrated with the use of single and double transport mutants of \textit{L. lactis}. The newly described transporter DtpP is specific for di- and tripeptides that are composed of amino acids with relatively hydrophobic side chains and requires ATP or a related energy-rich, phosphorylated intermediate to drive the peptide uptake.

ACKNOWLEDGMENTS

We thank V. Juillard and S. D. Erhlich for helpful discussions.

REFERENCES