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Published in:
Biochemistry

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

Publication date:
1997

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Cloning and Functional Expression in *Escherichia coli* of the Gene Encoding the Di- and Tripeptide Transport Protein of *Lactobacillus helveticus*

HAJIME NAKAJIMA, ANJA HAGTING, EDMUND R. S. KUNJI, BERT POOLMAN, AND WIL N. KONINGS

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Received 3 February 1997/Accepted 26 March 1997

The gene encoding the di- and tripeptide transport protein (*dpt*) of *Lactobacillus helveticus* (*dpt*<sub>LH</sub>) was cloned with the aid of the inverse PCR technique and used to complement the dipeptide transport-deficient and proline-auxotrophic *Escherichia coli* E1772. Functional expression of the peptide transporter was shown by the uptake of propyl-[<sup>14</sup>C]alanine in whole cells and membrane vesicles. Peptide transport via *Dpt*<sub>LH</sub> in membrane vesicles is driven by the proton motive force. The system has specificity for di- and tripeptides but not for amino acids or tetrapeptides. The *dpt*<sub>LH</sub> gene consists of 1,491 bp, which translates into a 497-amino-acid polypeptide. *Dpt*<sub>LH</sub> shows 34% identity to the di- and tripeptide transport protein of *Lactococcus lactis* and is also homologous to various peptide transporters of eukaryotic origin, but the similarity between these proteins is confined mainly to the N-terminal halves.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used are listed in Table 1. *L. helveticus* was grown at 37°C in MRX broth plus 0.5% glucose (19). *E. coli* strains were grown aerobically at 37°C in Luria broth with KCl (19). 5% glucose was used as the carbon source. The plasmids used are listed in Table 1.

**Chromosomal DNA preparation.** Chromosomal DNA was isolated from *E. coli* strains by the alkaline lysis method (1). Other methods were performed according to standard procedures (17). In vitro amplification of DNA was performed with PWO polymerase by using the recommended buffer plus 2 mM MgSO<sub>4</sub> (Boehringer Mannheim GmbH). Amplified DNA was purified with the QIAquick PCR purification kit (QIAGEN) and analyzed by agarose gel electrophoresis.

**Cloning of *dpt*<sub>LH</sub> by inverse PCR.** The 5' region of an open reading frame downstream of *pepN* in *L. helveticus* (2) was found to be homologous to *dpt* of *Lactococcus lactis* (*dpt*<sub>LL</sub>). The PCR technique was used to clone a 2.4-kb DNA fragment that encompassed the 3' end of *pepN* and part of the *L. helveticus* *dpt* gene (*dpt*<sub>LH</sub>). The oligonucleotides PF (5'-GGTCATCGAAAGCAAGGT) and PR (5'-GTGTTITTTAAGGCCCAT) were used to synthesize this fragment, which was subsequently used to identify a 2.4-kb *Hpa*I fragment by Southern hybridization; the *Hpa*I fragment contained the entire *dpt*<sub>LH</sub> gene. *Hpa*I fragments were extracted from an agarose gel and ligated with T4 DNA ligase (Boehringer Mannheim GmbH). By using oligonucleotides IF (5'-CCCGAGTCTTTTGGACAGCGGAGAGGC, EcoRV site introduced) and IR (5'-CCCGAGTGTCCAATTGCTTAGCACTG, BarnHI site introduced), the DNA was amplified by inverse PCR, following digestion with EcoRV and BarnHI, and ligated into pTAQI (digested with *Eco*<sub>Nd</sub>I; removal of *Eco*<sub>RV</sub> and *Bam*<sub>HI</sub> and *Pst*I fragments were added to the indicated concentrations).

**Southern hybridization.** Chromosomal DNA, digested with the appropriate restriction enzymes, was separated by agarose gel electrophoresis and transferred to a Zeta-plus blotting membrane (Bio-Rad). DNA was labelled with digoxigenin by the Klenow reaction, hybridized at stringent conditions (65°C), and visualized with the CSPD luminescent detection kit (Boehringer Mannheim GmbH).

**DNA sequence.** DNA was sequenced by the dideoxy method using 35S-ATP plus the T7 DNA polymerase kit (Pharmacia) or an automatic sequencing apparatus (Waters) using fluorescent oligonucleotides and cycle sequencing. Prior to the sequencing, fragments were cloned with the aid of the inverse PCR technique and used to complement the dipeptide transport-deficient and proline-auxotrophic *E. coli* E1772. The transformed bacteria were spread on M9 agar, supplemented with ampicillin (100 μg/ml) plus 100 μM Pro-Gly as sole source of proline.

**Membrane vesicles.** *E. coli* E1772 harboring the appropriate plasmid vector was grown in M9 medium supplemented with 1.0 g of yeast extract and 10 g of sodium succinate per liter and 100 μg of ampicillin per ml. The cells were grown at 37°C in a jar fermentor with an airflow of 15 liters/min until an *A<sub>600</sub>* of 0.6. Membrane vesicles were prepared by the method of Kaback (9) and suspended in 1 ml of 10 mM Tris·HCl (pH 7.8) containing 1 mM EDTA and treated with protease K (250 μg/ml) and proteinase E (500 μg/ml) for 30 min at 37°C. The cells were washed with TE (10 mM Tris·HCl plus 1 mM EDTA [pH 7.5]) and resuspended in 1 ml of TE. After addition of mutanolysin (160 U), the mixture was incubated at 37°C for 30 min. Subsequently, sodium dodecyl sulfate, EDTA, and protease K were added to final concentrations of 0.1%, 75 mM, and 200 μg/ml, respectively, and the incubation continued for 2 hr at 65°C. After a wash with phenol-chloroform (50:50 vol/vol; three times), chromosomal DNA was precipitated and dissolved in TE with DNase-free RNase (20 μg/ml) overnight at 4°C.

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used are listed in Table 1. *L. helveticus* was grown at 37°C in MRX broth plus 0.5% glucose (19). *E. coli* strains were grown aerobically at 37°C in Luria broth (17a). When needed, ampicillin (100 μg/ml) and kanamycin (40 μg/ml) were added to the indicated concentrations.

**Chromosomal DNA preparation.** Chromosomal DNAs of *L. helveticus* NCD02712 and SBT2171 were prepared essentially as described by Delley et al. (3). Exponentially growing cells (10 ml) were harvested by centrifugation and washed twice with 100 mM potassium phosphate (pH 7.0). The cells were re-suspended in 1 ml of 10 mM Tris·HCl (pH 7.8) containing 1 mM EDTA and treated with protease K (250 μg/ml) and proteinase E (500 μg/ml) for 30 min at 37°C. The cells were washed with TE (10 mM Tris·HCl plus 1 mM EDTA [pH 7.5]) and resuspended in 1 ml of TE. After addition of mutanolysin (160 U), the mixture was incubated at 37°C for 30 min. Subsequently, sodium dodecyl sulfate, EDTA, and protease K were added to final concentrations of 0.1%, 75 mM, and 200 μg/ml, respectively, and the incubation continued for 2 hr at 65°C. After a wash with phenol-chloroform (50:50 vol/vol; three times), chromosomal DNA was precipitated and dissolved in TE with DNase-free RNase (20 μg/ml) overnight at 4°C.

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**Membrane vesicles.** *E. coli* E1772 harboring the appropriate plasmid vector was grown in M9 medium supplemented with 1.0 g of yeast extract and 10 g of sodium succinate per liter and 100 μg of ampicillin per ml. The cells were grown at 37°C in a jar fermentor with an airflow of 15 liters/min until an *A<sub>600</sub>* of 0.6. Membrane vesicles were prepared by the method of Kaback (9) and re-suspended...
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Bacterium or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td><em>E. coli</em> E1772</td>
<td>W3110 <em>dpkC::kan proC::Tn10</em>Δ</td>
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<td></td>
<td>lacI169 RecAΔ HsdR Δ</td>
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<td>GyrAΔ DeorΔ lacZAM15</td>
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<tr>
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<td>DH5 Δ</td>
<td></td>
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<td><em>L. helveticus</em></td>
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<td>Institute of Food</td>
</tr>
<tr>
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<td>Research</td>
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<tr>
<td></td>
<td>SBT2171 Industrial strain</td>
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<td><strong>Plasmids</strong></td>
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<td>Gencor</td>
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<td>pTDH</td>
<td>pTAQI carrying <em>dtpT</em>Δ</td>
<td>This study</td>
</tr>
<tr>
<td>pTD5</td>
<td>pTAQI carrying <em>dtpT</em>Δ</td>
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<tr>
<td>pBluescript II SK*</td>
<td>Amp' cloning vector</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

* Reading, United Kingdom.
* South San Francisco, Calif.
* Paisley, United Kingdom.
* La Jolla, Calif.

in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH with 2 mM MgSO₄ (pH 6.5) and stored in liquid nitrogen until use.

**Transport assay using nonradio-labelled peptides.** Overnight cultures of *L. helveticus* NCDO2712 were diluted 20-fold into glucose-MRX medium and incubated for 5 h at 37°C. The cells were washed twice and resuspended in 50 mM potassium phosphate (KP) (pH 6.5). Subsequently, the cells were de-energized with 10 mM 2-deoxyglucose for 20 min at 37°C. The de-energized cells were washed twice and resuspended in MES-KOH supplemented with 2 mM MgSO₄ (pH 6.5). The transport assays with alanine, di- or tripeptide transported as a substrate were carried out as described by Kunji et al. (12), with glucose (0.5%, wt/vol) as energy source. The amino acid and peptides accumulated were separated by C18 reverse-phase high-pressure liquid chromatography (HPLC) after labeling with dansyl chloride (12).

**Transport assay using *l*-prolyl-[¹⁴C]alanine.** *E. coli* strains were incubated with 10 mM L-L-threotolactate in the presence of oxygen to generate a proton motive force, and transport of L-Pro-[¹⁴C]Ala was assayed as described by Hagting et al. (6). For transport in membrane vesicles, the electron donor system potassium ascorbate-phenazine methosulfate (PMS) plus oxygen was used to generate a proton motive force. Membrane vesicles were resuspended to a final concentration of 60 μg/ml in 50 mM MES-KOH–2 mM MgSO₄–50 μM PMS. One minute after the addition of 10 mM K-ascorbate, transport was initiated by the addition of Pro-[¹⁴C]Ala.

**Miscellaneous.** Protein was measured by the method of Lowry et al. (13) using bovine serum albumin as a standard. Aminopeptidase activity was measured as described by Sasaki et al. (18) with 2 μM alanyl-[¹⁴C]alanine as substrate. L-Pro-[¹⁴C]Ala (5.7 GBq/mmol) was synthesized by Hagting et al. (7). All peptides and di- or tripeptide transported as a substrate were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland); other chemicals were of reagent grade. All peptides and amino acids used were reconstituted in HPLC-grade water. Aminopeptidase activity was measured as described by Sasaki et al. (18) with 2 mM alanyl-[¹⁴C]Ala (5.7 GBq/mmol) as substrate.

**Cloning and functional expression of *dtpT*Δ in *E. coli*.** Sequence comparisons showed that a gene encoding a putative di- or tripeptide transport protein was present downstream of *pepN* in *L. helveticus* (2). The N-terminal 55 amino acids of DtpTΔ are 55% identical with DtpTΔ. The entire gene encoding the peptide transport protein of *L. helveticus* was isolated by inverse PCR and ligated into pTAQI, yielding pTDH. Plasmid pTDH was used to transform the peptide transport-deficient and proline-auxotrophic *E. coli* E1772 (16). The recombinant strain was able to grow on a medium containing Pro-Gly as source of proline, suggesting that the peptide transport gene was expressed functionally.

Subsequent transport experiments confirmed this suggestion. *E. coli* E1772/pTDH takes up Pro-[¹⁴C]Ala at a higher rate than the parent strain (E1772/pTAQI) (Fig. 2). Figure 2 also shows the uptake of Pro-[¹⁴C]Ala by E1772/pDT5 (carrying *dtpT*Δ) for comparison. Since the tac promoter region of pTAQI is missing in pTDH, the cloned gene (*dtpT*Δ) is most likely expressed from its own promoter.

Southern hybridization experiments with the *dtpT*Δ gene as probe confirmed that *dtpT*Δ is present in both *L. helveticus* NCDO2712 and SBT2171.

The DNA sequence of *dtpT*Δ and the translated amino acid sequence are shown in Fig. 3. An open reading frame of 1,491 nucleotides, encoding a 497-amino-acid polypeptide, was found. The translation initiation site was selected on the basis of the alignment of the protein sequence to DtpTΔ (see below). A putative ribosome binding site (5'-GGGGAG) is found 19 nucleotides upstream of the start codon. Putative −35 (5'-TGGACA) and −10 (5'-TTTATT) regions, separated by 18 nucleotides, are found in the noncoding region between *pepN* and *dtpT*Δ. Terminator-like structures are present downstream of *pepN* and *dtpT*Δ, suggesting that the two genes are

**RESULTS**

**Characterization of peptide transport in *L. helveticus*.** *L. helveticus* NCDO2712 takes up in the presence of an energy source the l-alanine and the peptides di- or tripeptide, transported as a substrate was monitored in glucose-metabolizing cells.

Two deoxynucleotide-treated cells. In order to eliminate the possibility that the peptides were degraded outside the cells, i.e., prior to uptake, the extracellular aminopeptidase activity was measured. Aminopeptidase activity (0.178 at A₅₆₂) was detected in the cell extract (following sonication), but no such activity was found in the extracellular medium, not even after prolonged incubation of the cells in the buffer used for the transport assays. The same observations were made for the industrial strain SBT2171, which possesses very high proteolytic activity (18).

**Cloning and functional expression of *dtpT*Δ in *E. coli*.** Sequence comparisons showed that a gene encoding a putative di- or tripeptide transport protein was present downstream of *pepN* in *L. helveticus* (2). The N-terminal 55 amino acids of DtpTΔ are 55% identical with DtpTΔ. The entire gene encoding the peptide transport protein of *L. helveticus* was isolated by inverse PCR and ligated into pTAQI, yielding pTDH. Plasmid pTDH was used to transform the peptide transport-deficient and proline-auxotrophic *E. coli* E1772 (16). The recombinant strain was able to grow on a medium containing Pro-Gly as source of proline, suggesting that the peptide transport gene was expressed functionally.

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transcribed separately. The terminator downstream of \textit{dtpT}_{LH} consists of a stem of 12 nucleotides and has a $\Delta G$ of $-71$ kJ/mol at 25°C.

The overall G+C content of \textit{dtpT}_{LH} is 38.6%, which matches well with that of the genome of \textit{L. helveticus} (from 38 to 40%). From the \textit{dtpT}_{LH} gene a primary amino acid sequence of the DtpT LH protein was deduced; the protein has 68% nonpolar amino acids, a value typical of a membrane protein. Hydropathy profiling suggested 12 hydrophobic stretches that are long enough to span the membrane in a zigzag fashion; the transmembrane segments are indicated in Fig. 3.

\textbf{Properties of DtpT}_{LH}. Membrane vesicles prepared from \textit{E. coli} E1772 do not transport Pro-[^14C]Ala in the presence of the electron donor system ascorbate-PMS (data not shown). However, membrane vesicles of strain E1772/pTDH transport this peptide well under those conditions (Fig. 4). This shows that DtpT_{LH} indeed codes for the di- and tripeptide transporter and that transport of Pro-[^14C]Ala is driven by the proton motive force. The role of the proton motive force was further demonstrated by using ionophores. Valinomycin, which dissipates the membrane potential (K$^+$ ionophore), and nigericin, which dissipates the pH gradient (K$^+$/H$^+$ ionophore), inhibited transport of Pro-[^14C]Ala significantly (Fig. 4). The presence of both ionophores completely collapsed the proton motive force and reduced the uptake of Pro-[^14C]Ala to equilibration.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Functional expression of \textit{dtpT}_{LH} in \textit{E. coli} E1772. Prolyl[^14C]alanine uptake was measured in \textit{E. coli} E1772 harboring pTDH (●) or pTAQI (vector control) (○) and \textit{E. coli} E1772 harboring pDT5 with \textit{dtpT}_{LH} (▲). Transport experiments were performed aerobically at 30°C in the presence of the electron donor Li-\alpha-lactate (10 mM); the concentration of Pro[^14C]Ala was 2.9 μM.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Nucleotide sequence of \textit{dtpT}_{LH} and deduced amino acid sequence. The putative promoter regions (−35 and −10), the ribosome binding site (RBS), and putative transmembrane-spanning segments (TMS) are underlined; putative terminator sequences are indicated by dotted lines.}
\end{figure}
levels. These observations indicate that transport of Pro-$^{[14]C}$Ala is most likely electrogenetic and occurs in symport with a proton(s).

Information about the substrate specificity of DtpT LH was obtained by following the transport of Pro-$^{[14]C}$Ala in the presence of an excess (50-fold) of amino acids or peptides (Table 2). Alanine, tetra-alanine, and Leu-enkephalin (pentapeptide) did not affect the transport of Pro-$^{[14]C}$Ala, while di- and trialanine, Pro-Gly, and Phe-Ala strongly inhibited the uptake. Also the branched-chain amino acid-containing peptides di-Leu and tri-Leu inhibited Pro-$^{[14]C}$Ala uptake to a great extent, whereas Phe-Val and Leu-Val had much smaller inhibitory effects. These results show that DtpT LH is a di- and tripeptide transporter just as DtpT LL.

Sequence alignments. The sequence alignment of DtpT LH and DtpT LL is shown in Fig. 5. Overall, DtpT LH has 34.0% amino acid identity to DtpT LL. Interestingly, the identity is confined largely to the N-terminal halves of the proteins (47.0% identity for the N-terminal 243 amino acids). Not only the sequences but also the hydropathy profiles of DtpT LH and DtpT LL were found to be significantly different at the extreme C-terminal ends of the proteins. Database searches showed that DtpT LH is a member of the peptide transport (PTR) family (19), which includes various peptide transporters of eukaryotic origin. The PTR consensus motif, F-(Y/V/F)-(W/F/h)-X-I-N-(h/M)-G-(S/L)-(L/F)-(h/S) (h indicates a hydrophobic residue) (19), is present in the fifth putative membrane-spanning region of DtpT LH as well as in eukaryotic members of the PTR family. Also in this family the similarity is confined mainly to the N-terminal halves of the proteins.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative uptake of Pro-$^{[14]C}$Ala (%)</th>
</tr>
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<tbody>
<tr>
<td>None (500 μM)</td>
<td>100</td>
</tr>
<tr>
<td>Ala</td>
<td>115</td>
</tr>
<tr>
<td>Di-Ala</td>
<td>30</td>
</tr>
<tr>
<td>Tri-Ala</td>
<td>20</td>
</tr>
<tr>
<td>Tetra-Ala</td>
<td>98</td>
</tr>
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<tr>
<td>Tri-Leu</td>
<td>44</td>
</tr>
<tr>
<td>Leu-enkephalin$^a$</td>
<td>96</td>
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<td>Pro-Gly</td>
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<td>Phe-Val</td>
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<tr>
<td>Leu-Val</td>
<td>89</td>
</tr>
<tr>
<td>Phe-Val</td>
<td>38</td>
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</tbody>
</table>

$^a$ Membrane vesicles were prepared from E. coli E1772 expressing DtpT LH. Transport of Pro-$^{[14]C}$Ala (10 μM) was measured for 30 s at pH 6.5 in the absence or presence of 500 μM compound. The electron donor system K-ascorbate-PMS was used to generate a proton motive force.

$^b$ Tyr-Gly-Gly-Phe-Leu.

FIG. 4. Pro-$^{[14]C}$Ala transport in membrane vesicles of E. coli E1772/pTDH. Uptake of Pro-$^{[14]C}$Ala (final concentration, 29 μM) was measured at 37°C by using K-ascorbate (10 mM)-PMS (50 μM) as electron donor system in the presence of O2. Transport experiments were performed in the presence of 1 μM valinomycin (●), 0.5 μM nigericin (△), 1 μM valinomycin plus 0.5 μM nigericin (■), or no ionophore (○). The equilibration level of uptake is indicated by the dotted line.

![Graph showing Pro-Ala uptake](image)

**TABLE 2. Substrate specificity of DtpT LH**

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DISCUSSION

In this article, the cloning and characterization of the di- and tripeptide transport system of \textit{L. helveticus} (\textit{dtpT}_{LH}) are described. The \textit{dtpT}_{LH} gene is located downstream of the gene encoding the general aminopeptidase N (\textit{pepN}). The \textit{dtpT}_{LH} gene can be functionally expressed in \textit{E. coli}, presumably from its own promoter.

Studies of peptide transport in \textit{L. helveticus} suggested the presence of a di-, tri-, and also oligopeptide transport system(s). The cloning of the \textit{dtpT}_{LH} gene and the subsequent characterization of \textit{DtpT}\textsubscript{LH} established that this system is specific for transport of di- and tripeptides. Substrate specificity studies suggest that \textit{DtpT}\textsubscript{LH} has a preference for more hydrophilic peptides just like \textit{DtpT}\textsubscript{L}. (4). Di- and tripeptides containing hydrophobic amino acids, such as Phe-Val and Leu-Val, inhibit Pro-[\textsuperscript{14}C]Ala uptake only slightly, although the hydrophobic peptides Leu-Leu and Leu-Leu-Leu are quite effective inhibitors of Pro-Ala transport. Foucaud et al. (4) reported that the substrate specificities of \textit{DtpT} and an ATP-dependent di- and tripeptide uptake system (\textit{DtpP}) in \textit{Lactococcus lactis} overlap but that hydrophobic branched-chain amino acid-containing peptides are transported preferentially by \textit{DtpP}. Similar to what is suggested by this study, peptides like Leu-Leu and Leu-Leu-Leu are transported efficiently by \textit{DtpT} of \textit{Lactococcus lactis}, whereas other branched-chain-amino-acid-containing peptides are poor substrates.

Recently, Klein et al. (10) reported the cloning of a gene, \textit{dppE}, encoding a protein involved in dipeptide transport in \textit{Lactobacillus delbrueckii} subsp. \textit{lactis}. Although the primary sequence has not been published, this transporter appears to be different from \textit{DtpT}\textsubscript{LH} because \textit{DppE} has been reported to be 30\% identical to \textit{DciAE} (14), which encodes the binding protein of the dipeptide transport system of \textit{Bacillus subtilis}. \textit{DciAE} is a member of the ATP-binding cassette (ABC) transporter superfamily (8). Possibly, \textit{DppE} encodes a \textit{DtpP}-like di- and tripeptide transporter.

The amino acid transport systems of \textit{L. helveticus} were found to have properties similar to those of \textit{Lactococcus lactis} (15). This study shows also that the di- and tripeptide transport system \textit{DtpT}\textsubscript{LH} is very similar to that of \textit{Lactococcus lactis} (6). On the other hand, the protease gene of \textit{B. delbrueckii} subsp. \textit{bulgaricus} (\textit{prtB}) is quite different from the one of \textit{Lactococcus lactis} (5). The proteins of \textit{Lactococcus lactis} and \textit{B. delbrueckii} subsp. \textit{bulgaricus} belong to different serine protease subfamilies, i.e., subtilisin and cysteine-subtilisin, respectively. However, the peptides released from \textit{\beta}-casein by the two proteases appear to be very similar (11). To what extent the proteolytic pathways of \textit{Lactococcus lactis} and \textit{L. helveticus} (or other lactobacilli) are similar or different remains to be determined.

ACKNOWLEDGMENTS

We thank André Boorsma for assistance with automatic DNA sequencing.

This work was supported by a grant from Snow Brand Milk Products Co. Ltd.

REFERENCES