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

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The gene encoding the di- and tripeptide transport protein (DtpT) of *Lactobacillus helveticus* (DtpT_{LH}) 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 prolyl-[¹⁴C]alanine in whole cells and membrane vesicles. Peptide transport via DtpT 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 *dtpT_{LH}* gene consists of 1,491 bp, which translates into a 497-amino-acid polypeptide. DtpT_{LH} 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.

Lactobacillus helveticus is a gram-positive, facultatively anaerobic thermotolerant lactic acid bacterium that is highly adapted to growth in milk. Although milk contains free amino acids, the amounts are too limited for growth to high cell densities. In order to obtain the amino acids needed for growth, lactic acid bacteria, in general, use a proteolytic system that consists of an extracellular proteinase, peptide transport systems, and intracellular peptidases (11, 17).

The proteolytic system of *L. helveticus* has recently received a lot of attention (reviewed in reference 11), and the available data suggests that this system is very comparable to that of *Lactococcus lactis*. The proteinase and peptidases of *L. helveticus* are similar to those of *Lactococcus lactis*, but the overall proteolytic activity of *L. helveticus* is higher than that of *Lactococcus lactis* (18). *L. helveticus* also possesses several amino acid transport systems with properties similar to those of *Lactococcus lactis* (15). In our studies on peptide metabolism in *L. helveticus*, we found an open reading frame (ORF) downstream of the *pepN* gene whose deduced amino acid sequence is homologous to that of the di- and tripeptide transport protein of *Lactococcus lactis* (6). In this article, we report the cloning of this gene, the functional expression of the protein in *Escherichia coli*, and the characterization of this protein as a di- and tripeptide transporter.

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 (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* NCDO2712 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 proteinase 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 proteinase K were added to final concentrations of 0.1%, 75 mM, and 200 µg/ml, respectively, and the incubation continued for 2 h 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.

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).

Other DNA manipulations. Plasmid DNA was isolated from *E. coli* strains by the alkaline lysis method (1). Other methods were performed according to standard procedures (17a). In vitro amplification of DNA was performed with PWO polymerase by using the recommended buffer plus 2 mM MgSO₄ (Boehringer Mannheim GmbH). Amplified DNA was purified with the QIA quick PCR purification kit (QIAGEN) and analyzed by agarose gel electrophoresis.

Cloning of *dtpT_{LH}* by inverse PCR. The 5' region of an open reading frame downstream of *pepN* in *L. helveticus* (2) was found to be homologous to *dtpT* of *Lactococcus lactis* (*dtpT_L*). 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* *dtpT* gene (*dtpT_{LH}*). The oligonucleotides PF (5'-GGTCATCGAAAGCAAGT) and PR (5'-GTTTTTCTTAGAGCCCAT) were used to synthesize this fragment, which was subsequently used to identify a 2.4-kb *HpaI* fragment by Southern hybridization; the *HpaI* fragment contained the entire *dtpT_{LH}* gene. *HpaI* fragments were extracted from an agarose gel and ligated with T4 DNA ligase (Boehringer Mannheim GmbH). By using oligonucleotides IF (5'-CCGGATA TCTTTGGACAGCCAAGGGC; *EcoRV* site introduced) and IR (5'-CGCG GATCCAATTGCTTTAGCAACTGC; *BamHI* site introduced), the DNA was amplified by inverse PCR, following digestion with *EcoRV* and *BamHI*, and ligated into pTAQI (digested with *EcoRV* plus *BamHI*; removal of *tac* promoter region). The recombinant plasmids were used to transform 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.

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 subcloned or shortened by exonuclease III digestion using the Erase-Base system (Promega).

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 jar fermentors with an airflow of 15 liters/min until an *A*₆₆₀ of 0.6. Membrane vesicles were prepared by the method of Kaback (9) and resuspended

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TABLE 1. Bacterial strains and plasmids

| Bacterium or plasmid | Relevant characteristics | Source or reference |
|--------------------------------|---|---|
| Bacteria | | |
| <i>E. coli</i> | | |
| E1772 | W3110 <i>dppA::Kan proC::Tn10Δ lacU169 RecA⁻ HsdR⁻ GyrA⁻ DeoR⁻ lacZΔM15</i> | 16 |
| DH5α | | |
| <i>L. helveticus</i> | | |
| NCDO2712 | Standard strain | Institute of Food Research ^a |
| SBT2171 | Industrial strain | 18 |
| Plasmids | | |
| pTAQI | pBR322, <i>lacI</i> behind penicillinase promoter | Genecor ^b |
| pTDH | pTAQI carrying <i>dtpT_{LH}</i> | This study |
| pTD5 | pTAQI carrying <i>dtpT_{LL}</i> | 6 |
| pUC18 | Amp ^r cloning vector | Gibco BRL ^c |
| pBluescript II SK ⁺ | Amp ^r cloning vector | Stratagene ^d |

^a Reading, United Kingdom.^b South San Francisco, Calif.^c Paisley, United Kingdom.^d 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 nonradiolabelled 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_i) (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, dialanine, trialanine, and tetra-alanine as 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 labelling with dansyl chloride (12).

Transport assay using L-prolyl-[¹⁴C]alanine. *E. coli* strains were incubated with 10 mM Li-D-lactate 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 mM alanyl-*p*-nitroanilide as substrate. L-Pro-[¹⁴C]Ala (5.7 GBq/mmol) was synthesized by Hagting et al. (7). All peptides were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland); other chemicals were of reagent grade. All peptides and amino acids used were in the L configuration.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence reported in this paper is U77486.

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 dialanine, trialanine, and tetra-alanine. However, after dansylation and HPLC of the intracellular fraction only free alanine could be detected (Fig. 1). This suggests that the peptides are degraded immediately after uptake. A lag phase in the accumulation of alanine during uptake of alanine or peptides was found to be caused by the ATP synthesis and proton motive force generation during glucose metabolism, i.e., there is a lag in the energy generation of

2-deoxyglucose-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 A₄₁₀) 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_{LH}* in *E. coli*. Sequence comparisons showed that a gene encoding a putative di- and tripeptide transport protein was present downstream of *pepN* in *L. helveticus* (2). The N-terminal 55 amino acids of DtpT_{LH} are 55% identical with DtpT_{LL}. 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_{LL}*) for comparison. Since the *tac* promoter region of pTAQI is missing in pTDH, the cloned gene (*dtpT_{LH}*) is most likely expressed from its own promoter.

Southern hybridization experiments with the *dtpT_{LH}* gene as probe confirmed that *dtpT_{LH}* is present in both *L. helveticus* NCDO2712 and SBT2171.

The DNA sequence of *dtpT_{LH}* 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 DtpT_{LL} (see below). A putative ribosome binding site (5'-GGGAG) 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_{LH}*. Terminator-like structures are present downstream of *pepN* and *dtpT_{LH}*, suggesting that the two genes are

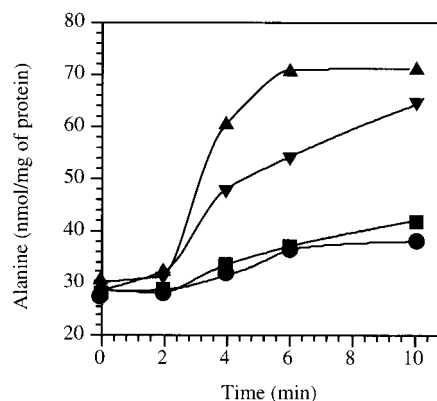


FIG. 1. Uptake of alanine and alanine-containing peptides by *L. helveticus* NCDO2712. Cells were de-energized with 2-deoxyglucose as described in Materials and Methods, and the uptake of alanine (▼), dialanine (●), trialanine (■), or tetra-alanine (▲) as a substrate was monitored in glucose-metabolizing cells; the final concentration of alanine and peptides was 0.5 mM. Glucose was added 2 min prior to the addition of alanine and alanine-containing peptides.

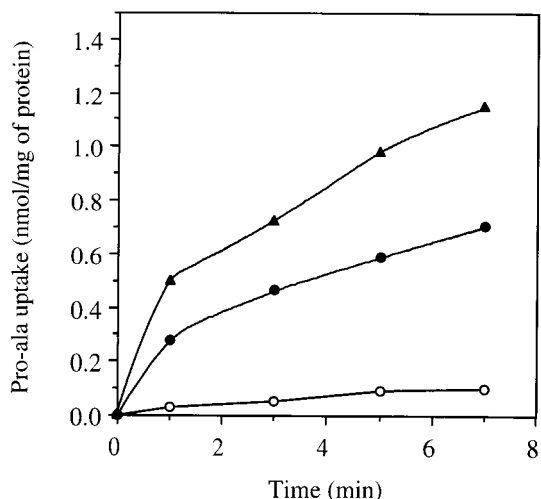


FIG. 2. Functional expression of *dtpT_{LH}* in *E. coli* E1772. Prolyl-[¹⁴C]alanine uptake was measured in *E. coli* E1772 harboring pTDH (●) or pTAQI (vector control) (○) and *E. coli* E1772 harboring pDT5 with *dtpT_{LL}* (▲). Transport experiments were performed aerobically at 30°C in the presence of the electron donor Li-d-lactate (10 mM); the concentration of Pro-[¹⁴C]Ala was 2.9 μM.

transcribed separately. The terminator downstream of *dtpT_{LH}* consists of a stem of 12 nucleotides and has a Δ*G* of -71 kJ/mol at 25°C.

The overall G+C content of *dtpT_{LH}* is 38.6%, which matches well with that of the genome of *L. helveticus* (from 38 to 40%). From the *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. Hydrophathy 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.

Properties of DtpT_{LH}. Membrane vesicles prepared from *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

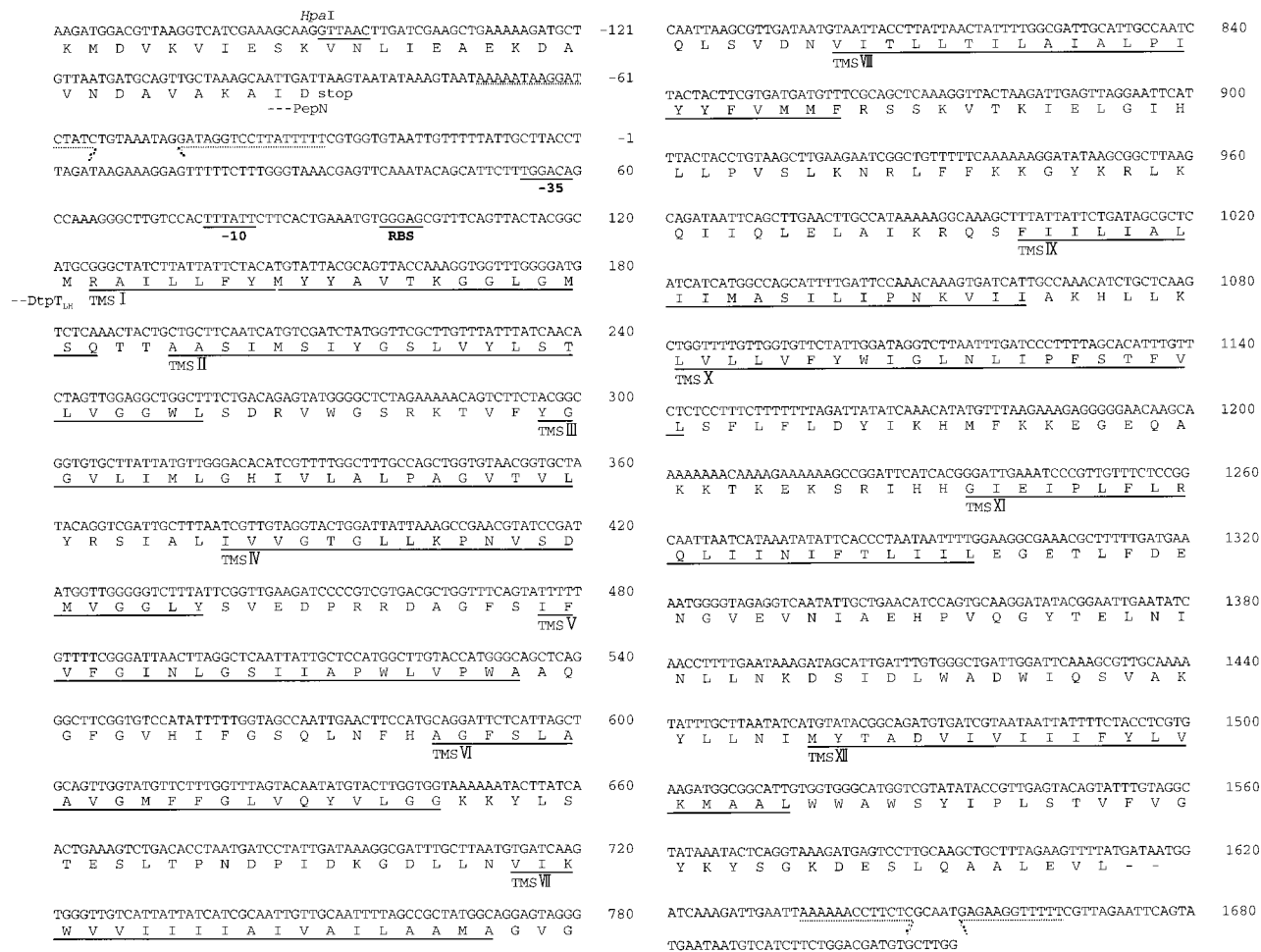


FIG. 3. Nucleotide sequence of *dtpT_{LH}* and deduced amino acid sequence. The putative promoter regions (-35 and -10), the ribosome binding site (RBS), and putative transmembrane-spanning segments (TMS) are underlined; putative terminator sequences are indicated by dotted lines.

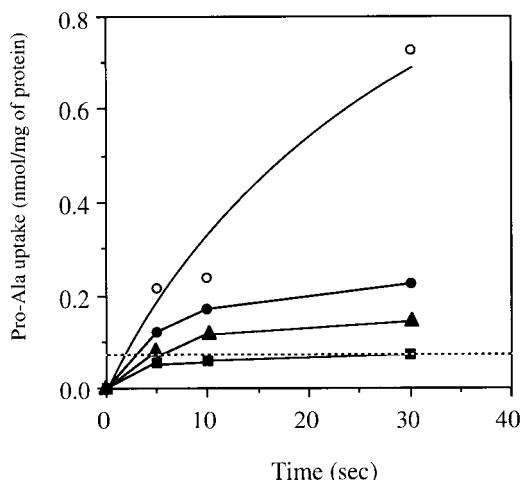


FIG. 4. Pro-¹⁴CAla transport in membrane vesicles of *E. coli* E1772/pTDH. Uptake of Pro-¹⁴CAla (final concentration, 29 μM) was measured at 37°C by using K-ascorbate (10 mM)-PMS (50 μM) as electron donor system in the presence of O₂. Transport experiments were performed in the presence of 1 μM valinomycin (●), 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.

levels. These observations indicate that transport of Pro-¹⁴CAla is most likely electrogenic and occurs in symport with a proton(s).

Information about the substrate specificity of DtpT_{LH} was obtained by following the transport of Pro-¹⁴CAla 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-¹⁴CAla, 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-¹⁴CAla 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

TABLE 2. Substrate specificity of DtpT_{LH}

| Addition (500 μM) | Relative uptake of Pro- ¹⁴ CAla (%) ^a |
|-----------------------------------|---|
| None..... | 100 |
| Ala..... | 115 |
| Di-Ala..... | 30 |
| Tri-Ala..... | 20 |
| Tetra-Ala..... | 98 |
| Di-Leu..... | 42 |
| Tri-Leu..... | 44 |
| Leu-enkephalin ^b | 96 |
| Pro-Gly..... | 34 |
| Phe-Val..... | 86 |
| Leu-Val..... | 89 |
| Phe-Ala..... | 38 |

^a Membrane vesicles were prepared from *E. coli* E1772 expressing DtpT_{LH}. Transport of Pro-¹⁴CAla (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.

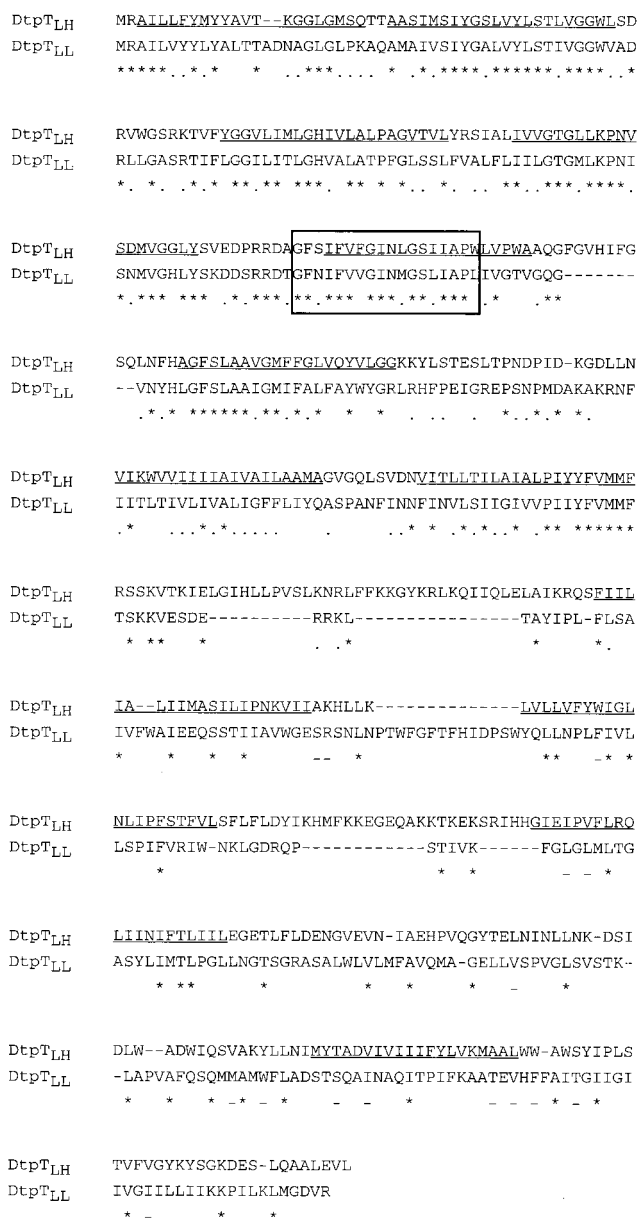


FIG. 5. Sequence alignment of DtpT_{LH} and DtpT_{LL}. Asterisks and dots, identical and similar amino acid residues, respectively. Putative membrane-spanning regions, as suggested by hydropathy profiling, are underlined. The PTR consensus sequences are boxed.

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.

DISCUSSION

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

Studies of peptide transport in *L. helveticus* suggested the presence of a di-, tri-, and also oligopeptide transport system(s). The cloning of the *dtpT_{LH}* gene and the subsequent characterization of DtpT_{LH} established that this system is specific for transport of di- and tripeptides. Substrate specificity studies suggest that DtpT_{LH} has a preference for more hydrophilic peptides just like DtpT_{LL} (4). Dipeptides containing hydrophobic amino acids, such as Phe-Val and Leu-Val, inhibit Pro-[¹⁴C]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 DtpT and an ATP-dependent di- and tripeptide uptake system (DtpP) in *Lactococcus lactis* overlap but that hydrophobic branched-chain amino acid-containing peptides are transported preferentially by DtpP. Similar to what is suggested by this study, peptides like Leu-Leu and Leu-Leu-Leu are transported efficiently by DtpT of *Lactococcus lactis*, whereas other branched-chain-amino-acid-containing peptides are poor substrates.

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

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

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