Lactococcus lactis takes up di- and tripeptides via a proton motive force-dependent carrier protein. The gene (dtpT) encoding the di-tripeptide transport protein of L. lactis was cloned by complementation of a dipeptide transport-deficient and proline auxotropic Escherichia coli strain. Functional expression of the dipeptide transport gene was demonstrated by uptake studies of alanin-[14C]glutamate and other peptides in E. coli cells. The di-tripeptide transport catalyzes proton motive force-driven peptide uptake and dipeptide exchange activity. The nucleotide sequence of dtpT was determined and the translated sequence corresponds with a protein of 463 amino acid residues. Hydrophathy profiling indicates that the protein could form 12 membrane-spanning segments with the amino and carboxyl termini at the outer surface of the membrane. A secondary structure model is presented which is substantiated by analysis of DtpT-PhoA fusion constructs. Amino acid sequence comparisons showed no significant homology with other bacterial peptide transport systems nor with any other known protein. Flanking regions of the di-tripeptide transport gene were used to delete dtpT from the chromosome of L. lactis. Genetic and biochemical characterization of this mutant indicates that DtpT is the only transport protein in L. lactis for hydrophilic di- and tripeptides.

Peptides can serve as sole carbon and/or nitrogen sources for most species of bacteria, fungi, plants, and animals (Payne, 1980). The best understood bacterial peptide transport systems are those from Salmonella typhimurium and Escherichia coli. These Gram-negative bacteria possess three distinct peptide transport systems with overlapping substrate specificities (Higgins and Gibson, 1986). The oligopeptide transport system (Opp) transports almost any peptide containing 2–5 amino acid residues (Hiles et al., 1987). Apart from transporting nutrient peptides, Opp also serves an important function in the recycling of cell wall peptides which are released from peptidoglycan during growth (Goodell and Higgins, 1987). Furthermore, it plays a role in the sensitivity of enterobacteriaceae to aminoglycoside antibiotics (Kaishwagi et al., 1992). The second peptide transport system (Tpp) has a more restricted substrate specificity. It only transports hydrophobic tripeptides and some dipeptides (Higgins and Gibson, 1986). This system is expressed only under anaerobic growth conditions. It is positively regulated by the gene products of ompR and envZ (Gibson et al., 1987). The third peptide transport system is the dipeptide permease (Dpp). This transport system is rather specific for dipeptides but has also been shown to transport some tripeptides (Manson et al., 1986). Dpp also serves as a chemoreceptor for peptide chemotaxis (Manson et al., 1986).

For Gram-positive bacteria different peptide transport systems have also been described, i.e. the ami locus of Streptococcus pneumoniae (Alloing et al., 1990), the Opp system of Lactococcus lactis (Kunji et al., 1993) and the dici and spoOk loci of Bacillus subtilis (Mathiopoulos et al., 1991; Perego et al., 1991; Rudner et al., 1991). All bacterial peptide transport systems described so far are members of a larger family, the ABC transporter or traffic ATPase superfamily. A typical ABC transporter consists of four membrane-associated domains (Higgins, 1992). Two of these domains are highly hydrophobic and span the membrane (normally) six times. These domains form the pathway through which the substrate crosses the membrane. The two other domains contain the ATP-binding cassette and are located at the cytoplasmic face of the membrane. All bacterial ABC transporters that mediate solute uptake require a substrate-binding protein. Binding proteins of Gram-negative bacteria are located in the periplasm. The substrate-binding proteins of the Gram-positive bacteria have a signal peptide with a sequence typical for lipomodification (Perego et al., 1991).

The di-tripeptide carrier of L. lactis (DtpT) is a secondary transport system in contrast to the binding protein-dependent ATP-driven peptide transport systems. Accumulation of the dipeptide alaninylglutamate in peptidase-free membrane vesicles of L. lactis has been shown to be driven by the electrical potential (∆Ψ) and the chemical gradient of protons (∆pH) across the membrane (Smid et al., 1989a). The DtpT system has a broad substrate specificity, but size recognition is restricted to di- and tripeptides only.

In this study, the gene encoding the lactococcal di-tripeptide transport system was cloned and characterized genetically and biochemically. The high specificity for proline-containing dipeptides was used to complement the proline auxotrophic, dipeptide transport negative E. coli E1772. The cloned gene encodes an integral membrane protein with a unique primary sequence and some unusual secondary structure features.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were grown at 37 °C, with vigorous aeration, in Luria Broth or in M9 minimal medium (Sambrook et al., 1989) supplemented with carbenicillin (50 μg/ml), tetracycline (10 μg/ml), or kanamycin sulfat (50 μg/ml) when appropriate and essential nutrients as indicated by the auxotropic markers. L. lactis strains were grown at 28 °C in M17 (Difco) or in a chemically defined medium (Poolman and Konings, 1988), both at pH 6.4, and supplemented with 0.5% (w/v) glucose.

Cloning of the Transport Gene—Chromosomal DNA was isolated from L. lactis ML3 according to Leenhouts et al. (1990) and partially digested withendonuclease Sau3A. The cleaved DNA was fractionated by polyacrylamide gel (5% w/v) electrophoresis, after which fragments...
Hybridization, washing, and staining steps were performed in three subsequent sucrose gradient centrifugations (Meager et al., 1977). De-energized cells were washed twice and resuspended in 100 mM potassium phosphate, pH 6.5, to a final OD<sub>600</sub> of approximately 25. The cells were de-energized with 10 mM 2-deoxy-glucose for 20 min at 28°C. This procedure results in the depletion of intracellular amino acid pools and facilitates cloning of this fragment, and fragments derived thereof, E. coli BX2, which reduces the copy number of Col E1 ori plasmids, was used as host. Subclones of pSKF3 (3.5-kb HindIII-SalI insert of pDT5 in plasmid II SK') were also obtained by exonuclease digestion using the Erase-a-base method (Promega). A T7 DNA sequencing kit (Pharmacia) was used for sequencing of double-stranded DNA by the dideoxy chain termination method (Sanger et al., 1977). PGENE (release 6.26, Genofit) was used for computer-assisted sequence analysis. Protein homology searches were performed in the EMBL SWISSPROT (release 23) database, using the FASTA algorithm from Pearson and Lipman (1988).

**TABLE I**

<table>
<thead>
<tr>
<th>Bacterium/plasmid</th>
<th>Relevant characteristics</th>
<th>Source/refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>lacY1 min A1 min B2</td>
<td>Olson et al. (1991)</td>
</tr>
<tr>
<td>P678-54</td>
<td>LE8392, pCNSnc:Tek</td>
<td>Adler et al. (1967)</td>
</tr>
<tr>
<td>BX2</td>
<td>3aara,leu7687 lacZXY 74 phoA 120 galE galK thi rpsE rpsB argE (am) resA</td>
<td>François et al. (1987)</td>
</tr>
<tr>
<td>CC118</td>
<td>M9, purified in three subsequent sucrose gradient centrifugations (Meager et al., 1977). De-energized cells were washed twice and resuspended in 100 mM potassium phosphate, pH 6.5, to a final OD&lt;sub&gt;600&lt;/sub&gt; of approximately 25. The cells were de-energized with 10 mM 2-deoxy-glucose for 20 min at 28°C. This procedure results in the depletion of intracellular amino acid pools and facilitates cloning of this fragment, and fragments derived thereof, E. coli BX2, which reduces the copy number of Col E1 ori plasmids, was used as host. Subclones of pSKF3 (3.5-kb HindIII-SalI insert of pDT5 in plasmid II SK') were also obtained by exonuclease digestion using the Erase-a-base method (Promega). A T7 DNA sequencing kit (Pharmacia) was used for sequencing of double-stranded DNA by the dideoxy chain termination method (Sanger et al., 1977). PGENE (release 6.26, Genofit) was used for computer-assisted sequence analysis. Protein homology searches were performed in the EMBL SWISSPROT (release 23) database, using the FASTA algorithm from Pearson and Lipman (1988).</td>
<td>This work</td>
</tr>
<tr>
<td>JM101</td>
<td>M9, purified in three subsequent sucrose gradient centrifugations (Meager et al., 1977). De-energized cells were washed twice and resuspended in 100 mM potassium phosphate, pH 6.5, to a final OD&lt;sub&gt;600&lt;/sub&gt; of approximately 25. The cells were de-energized with 10 mM 2-deoxy-glucose for 20 min at 28°C. This procedure results in the depletion of intracellular amino acid pools and facilitates cloning of this fragment, and fragments derived thereof, E. coli BX2, which reduces the copy number of Col E1 ori plasmids, was used as host. Subclones of pSKF3 (3.5-kb HindIII-SalI insert of pDT5 in plasmid II SK') were also obtained by exonuclease digestion using the Erase-a-base method (Promega). A T7 DNA sequencing kit (Pharmacia) was used for sequencing of double-stranded DNA by the dideoxy chain termination method (Sanger et al., 1977). PGENE (release 6.26, Genofit) was used for computer-assisted sequence analysis. Protein homology searches were performed in the EMBL SWISSPROT (release 23) database, using the FASTA algorithm from Pearson and Lipman (1988).</td>
<td>This work</td>
</tr>
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</table>

**Molecular Cloning and DNA Sequencing**—Molecular cloning techniques were performed essentially as described by Sambrook et al. (1989). Plasmids from *L. lactis* were isolated by the method of Birnboim and Doly with modifications described by Leenhouts et al. (1980). *L. lactis* was transformed by electroporation as described by Holo and Nes (1989). For DNA sequencing, the 3.5-kb HindIII-SalI insert of pDT5 (see Fig. 2) was transferred to plasmid II SK' (Stratagene). To facilitate cloning of this fragment, and fragments derived thereof, E. coli BX2, which reduces the copy number of Col E1 ori plasmids, was used as host. Subclones of pSKF3 (3.5-kb HindIII-SalI insert of pDT5 in plasmid II SK') were also obtained by exonuclease digestion using the Erase-a-base method (Promega). A T7 DNA sequencing kit (Pharmacia) was used for sequencing of double-stranded DNA by the dideoxy chain termination method (Sanger et al., 1977). PGENE (release 6.26, Genofit) was used for computer-assisted sequence analysis. Protein homology searches were performed in the EMBL SWISSPROT (release 23) database, using the FASTA algorithm from Pearson and Lipman (1988).

**Construction of the Integration Plasmid**—The integration plasmid pINT300, which contains the 5'- and 3'-flanking sequences of *dtpT* was constructed as follows. *Sal*I-digested pSKF3 was treated with appropriate restriction enzyme(s) and fractionated by agarose gel electrophoresis. DNA was labeled with digoxigenin-dUTP by using Nonradioactive DNA Labeling and Detection Kit (Boehringer). Hybridization, washing, and staining steps were performed according to instructions of the manufacturer.

**Transport Assays**—Cells grown to an OD<sub>600</sub> of 0.6 were harvested by centrifugation, washed twice and resuspended to 100 mM potassium phosphate, pH 6.5, to a final OD<sub>600</sub> of approximately 25. The cells were de-energized with 10 mM 2-deoxy-glucose for 20 min at 28°C. This procedure results in the depletion of intracellular amino acid pools and facilitates cloning of this fragment, and fragments derived thereof, E. coli BX2, which reduces the copy number of Col E1 ori plasmids, was used as host. Subclones of pSKF3 (3.5-kb HindIII-SalI insert of pDT5 in plasmid II SK') were also obtained by exonuclease digestion using the Erase-a-base method (Promega). A T7 DNA sequencing kit (Pharmacia) was used for sequencing of double-stranded DNA by the dideoxy chain termination method (Sanger et al., 1977). PGENE (release 6.26, Genofit) was used for computer-assisted sequence analysis. Protein homology searches were performed in the EMBL SWISSPROT (release 23) database, using the FASTA algorithm from Pearson and Lipman (1988).
Di-tripeptide Transport in *L. lactis*

**RESULTS**

Cloning of the Di-tripeptide Transport Gene—The di-tripeptide transport gene of *L. lactis* ML3 was cloned by complementation of *E. coli* E1772, which is proline auxotroph and dipeptide transport negative. Although *E. coli* E1772 is Dpp, it still transports various proline-containing dipeptides (e.g. prolylglycine) and this property was used in our complementation assay. To ascertain that Pro-Gly is a substrate of the di-tripeptide carrier of *L. lactis*, uptake of Pro-Gly was assayed in washed cell suspensions and accumulation was quantitated from the increase in intracellular proline as measured by reversed-phase HPLC. The results presented in Fig. 1 show that Pro-Gly is taken up by the wild-type ML3 strain, while uptake is not observed with the di-tripeptide transport negative mutant *L. lactis* MGD71 (Kunji et al., 1993).

For shotgun cloning, fractionated *L. lactis* chromosomal DNA was ligated in the expression vector pTAQI. pTAQI was used as the cloning vector since DNA fragments could be inserted downstream of the tac promoter. This allows expression of proteins in case the cloned genes do not possess a promoter or in case the *L. lactis* promoter is not functional in *E. coli*. The ligation mixtures were used to transform *E. coli* E1772, after which the cells were spread on M9 plates containing carbencillin and Pro-Gly as sole source of proline. Colonies can only grow on these selective plates when *E. coli* E1772 is functionally complemented by the di-tripeptide transport gene of *L. lactis*. Five colonies were found on the selective plates after transformation. These colonies did not grow on plates without a proline source, which excludes the possibility that genes coding for proline biosynthesis functions were cloned. Analysis of plasmid DNA of one of the colonies showed that the pTAQI vector contained a 4.2-kb insert. This chimeric plasmid was designated pDT5.

To confirm that the gene for di-tripeptide transport originated from the *L. lactis* chromosomal, chromosomal DNA of *L. lactis* was digested with HindIII and KpnI and fractionated by agarose electrophoresis. A digoxigenin-11-dUTP-labeled 2.1-kb HindIII-KpnI fragment of the 4.2-kb insert of pDT5 (see Fig. 2) hybridized with a 2.1 chromosomal fragment. This probe did not hybridize to chromosomal DNA of *E. coli* E1772 (data not shown).

Nucleotide Sequence and Coding Regions of the Di- and Tripeptide Transport Gene—A restriction map of the 4.2-kb insert was constructed by double and triple digestions. The map is shown together with the flanking regions of the pTAQI vector in Fig. 2. Restriction sites for the enzymes *SalI*, *SacI*, EcoRI, *SphI*, and *Smal* were not present in the 4.2-kb fragment. The restriction map was used to locate the di-tripeptide transport gene on pDT5 by subcloning fragments. Maps of the plasmid derivatives and the growth characteristics of *E. coli* E1772 transformed with these plasmids on selective plates with prolylglycine are shown in Fig. 2B. Only *E. coli* E1772 bearing plasmid pDT3 was able to grow on the selective plates. A 3.5-kb HindIII-SalI fragment was transferred to pBluescript II SK', yielding pSKF3. Subclones of pSKF3 were obtained by exonuclease digestion, and the nucleotide sequence was determined.

The nucleotide sequence of the di-tripeptide transport gene and the 5'- and 3'-flanking regions is shown in Fig. 3. Between positions 283 and 1672 an open reading frame (ORF) of 1,389 bp is found. This ORF could encode a protein (DtpT) of 463 amino acids, corresponding with a molecular mass of 50,630 daltons. The ORF is preceded by a putative ribosome-binding site, GGAG at position 261 (Fig. 3). This ribosome-binding site is complementary to the 3' end of the lactococcal 16 S rRNA (Ludwig et al., 1985) and has a ΔG of -9.4 kcal (Tinoco et al., 1973). If translation occurs at position 283, the spacing between the ribosome-binding site and the ATG is 18 bases which is rather long for efficient translation of the mRNA. Another potential translation initiation site is the ATG at position 364, with a putative ribosome-binding site AAAG (ΔG = -4.6 kcal) at bp 352. To establish whether the first ribosome-binding site and the ATG at position 283 could serve as start signal in *E. coli*, the 5' region of the putative ORF and part of the coding region (bp 78-300) were fused to lacZ lacking translation initiation signals. The corresponding construct (pDTZ1) yielded high β-galactosidase activity (425 Miller units) when present in *E. coli* JM101 (control cells without pDTZ1 had a β-galactosidase activity of 5 Miller units). These results suggest that translation initiation of the gene in *E. coli* does occur at the position...
Di-tripeptide Transport in L. lactis

Fig. 2. Restriction endonuclease map of pDT5 (A) and characterization of the deletion derivatives of pDT5 (B). E. coli E1772 cells transformed with these plasmids were analyzed for growth on selective media with prolylglycine as sole source of proline. P, and MCS refer to the tac promoter and the multiple cloning site of pTAQI, respectively. Symbols: open bars, deletions in the cloned fragment; dotted bars and striped bars, Pm and vector DNA, respectively. The position and the direction of transcription of the di-tripeptide transport gene are indicated by the arrow. H, HindIII; P, PstI; K, KpnI; B, BamHI; S, Sall.

The discrepancy between the molecular mass (50,630 Da) deduced from the nucleotide sequence and the apparent molecular mass determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is probably due to the abnormally high binding of sodium dodecyl sulfate to the di- and tripeptide transport system, similar to that observed for other hydrophobic proteins (Poolman et al., 1989; Tolner et al., 1992). The concentration of β-lactamase and lac repressor protein (LacI) in minicells harboring pDT5 is much lower than in minicells containing pTAQI, suggesting that the insert reduces the copy number of the plasmid.

Functional Properties of the Di-tripeptide Transport Protein—Alanylglutamate (Ala-Glu) has previously been used to characterize the L. lactis di-tripeptide transport protein in membrane vesicles (Smid et al., 1989a). For this purpose L-alanyl-[^14]C]glutamate was synthesized. Ala-Glu transport activity was assayed in the dipeptide transport deficient E. coli E1772 to demonstrate that the di-tripeptide transport gene of L. lactis is functionally expressed. Uptake of Ala[^14]C]Glu by E1772 harboring pDT5, which carries the cloned di-tripeptide transport gene, is severalfold higher than in cells harboring pTAQI (Fig. 5A). To establish that Pro-Gly (used in the complementation assay) and Ala-Glu are taken up via the same transport system, competition experiments were performed. The initial rate of uptake and steady-state level of accumulation of the radioactive Ala-Glu decreased significantly upon addition of a 10-fold excess of unlabelled Pro-Gly to the reaction mixture (Fig. 5B). Complete inhibition of Ala[^14]C]Glu uptake was observed with a 500-fold excess of Pro-Gly, indicating that Pro-Gly and Ala-Glu indeed compete for the same transport system. To assure that Ala[^14]C]Glu was the transported species rather than[^14]C]glutamate, which could have been formed from extracellular or periplasmic peptidase activity, unlabeled glutamate was added to the reaction mixture. Addition of glutamate had no effect on the uptake of labeled Ala-Glu (data not shown), indicating that hydrolysis of the dipeptide had not occurred prior to transport. These results confirm the specificity of the system for dipeptides. The specificity for peptides is also shown by the
exit of accumulated Ala-[14C]Glu in E. coli strain E1772 harboring pDT5 upon addition of a 10-fold excess of unlabeled Ala-Glu. Exit of accumulated Ala-[14C]Glu was not observed upon addition of unlabeled glutamate (Fig. 6). Reversed-phase HPLC analysis of tator sequences gene and the flanking regions. The 12 transmembrane α-helical segments as predicted by the hydropathy pro-

Fig. 4. Expression of DtpT in minicell-producing E. coli P678-54. Proteins were labeled in the presence of [35S]methionine and resolved by 12.5% SDS-polyacrylamide gel electrophoresis. Lane 1, P678-54 containing pDT5; lane 2, P678-54 containing pTAQI (vector control). Dipeptide transport protein (DtpT), lac repressor protein (LacI), β-lactamase (β-lac), and molecular size markers (kDa) are indicated.

Fig. 5. Alanyl-[14C]glutamate uptake in E. coli E1772 cells harboring pTAQI (A) or pDT5 (B). Concentrated cell suspensions were diluted to a final protein concentration of ~0.33 mg/ml into 50 mM potassium phosphate, 5 mM MgSO4, pH 6.5. After 1 min of pre-energization with 10 mM phosphate in the presence of oxygen, Ala-[14C]Glu was added to a final concentration of 478 μM. Closed squares show the uptake of Ala-[14C]Glu in the presence of 55 μM CCCP. Transport was stopped at different time intervals as described under “Materials and Methods.” Open symbols indicate the uptake of Ala-[14C]Glu in the presence of a 10-fold (○) and 100-fold (V) excess of unlabeled Pro-Gly.

tentatively that the cloned dtpT gene encodes the H+-linked peptide transport system that was previously identified in L. lactis (Smid et al., 1989).

Construction and Analysis of a Dipeptide Transport-negative Mutant of L. lactis—To prove that DtpT is the lactococcal dipeptide transport protein, and to obtain a stable dipeptide transport-negative mutant suitable for expression of various dtpT alleles in L. lactis, the dtpT gene was deleted from the chromosome via homologous recombination (Fig. 7A). For this purpose plasmid pINT300 was constructed, which is a derivative of pORI280 (Leenhouts and Venema, 1993). Plasmid pORI280 contains a functional P-galactosidase gene (p-lac), and molecular size markers (kDa) are indicated.
Di-tripeptide Transport in L. lactis

pORI280 yielding pINT300. L. lactis MG1363 was transformed with pINT300 and transformants were selected on M17, supplemented with X-gal and erythromycin. Blue colonies arise from cells in which recombination between the chromosome and one of the flanking regions has occurred. Southern hybridization analysis of chromosomal DNA isolated from several transformants demonstrated that the plasmid had integrated into the genome at each of the homologous loci (data not shown). Fig. 7B (lane 2) shows a 2.6- and a 5.2-kb fragment, as expected upon integration in region A (strain MG1363-3). Subsequently, L. lactis MG1363-3 was grown for 30 generations in nonselective media, i.e. without erythromycin. This allows the plasmid to resolve at the other homologous region (B), which leads to deletion of the transport gene from the chromosome. L. lactis lacking dtpT was selected on M17 X-gal plates. A number of white colonies were selected and further analyzed by Southern hybridization. The results of the analysis of one of these colonies is presented in Fig. 7B (lane 3). The detection of a 4.2-kb chromosomal fragment indicates that strain MG1363-3 has lost all vector sequences, but also most (1088 bp) of the dtpT gene. This mutant was designated L. lactis AG300.

To characterize the dtpT deletion strain, growth experiments were performed first. L. lactis AG300 grew normally on chemically defined medium containing 0.25 mM of the dipeptide analog alanyl-/3-chloro-alanine (diACA), which is toxic for the wild-type L. lactis. DiACA has previously been shown to be a substrate of the di-tripeptide transport system of L. lactis (Smid et al., 1989b). On the other hand, the mutant was not able to grow on chemically defined medium containing 1 mM alanylglutamate as sole source of glutamate. Glutamate is an essential amino acid for L. lactis (Smid, 1991). These growth experiments indicate that the di- and tripeptide transport system is not functional in the deletion mutant AG300. Second, uptake of prolylalanine (Pro-Ala) was monitored in MG1363 (wild-type) and AG300 (AdtpT). Transport of Pro-Ala is completely abolished in the mutant, while initial uptake rates of 15 nmol/min·mg of protein could be estimated in the parent (wild-type) strain (Fig. 8). Finally, L. lactis AG300 was transformed with plasmid pGKF5, which contains the di-tripeptide transport gene. L. lactis AG300/pGKF5 had the same growth characteristics (sensitivity to diACA, growth on Ala-Glu) as the wild-type. These results demonstrate that the dtpT gene has been deleted from the chromosome of L. lactis MG1363, result-
ing in the deletion mutant *L. lactis* AG300, and that complementation occurs with the *dtpT* gene in trans.

**Sequence Comparison and Membrane Topology**—The amino acid sequence of DtpT has been compared with those of proteins in the EMBL SWISSPROT sequence data base. No striking similarity with any other protein in the data base was found, indicating that *dtpT* encodes a new type of bacterial peptide transport protein. Hydropathy analysis of DtpT reveals 12 hydrophobic stretches of at least 20 residues (Fig. 3, underlined) and these regions most likely span the membrane in α-helical configuration (Poolman and Konings, 1993). The transmembrane segments III-XII are indicated by different hydropathy analysis methods but differences are predicted for the amino-terminal portion of the protein (see Fig. 9, two alternative models). For both models, the "positive-inside-rule" (Von Heyne and Gavel, 1988) predicts that the amino- and carboxyl-terminal ends of the DtpT protein are located at the outer surface of the membrane. It should be noted, however, that the proposed interhelix loop XI-XII (about 44 amino acids) is long enough to span the membrane in α-helical configuration which would then bring the carboxyl terminus, with a surplus of two positive charges, at the inner surface of the cytoplasmic membrane. To discriminate between the two topology models (Fig. 9, A and B) and to obtain preliminary information about the location of the amino terminus, a number of in frame fusions of the 5′ region of *dtpT* and *phaA* were made. The positions of the fusion points and the corresponding alkaline phosphatase activities are shown in Fig. 9. The results support the model depicted in Fig. 9B, i.e. high alkaline phosphatase activity (43 Miller units) is observed with the fusion at residue Asp-50 of DtpT, which locates the corresponding loop region to the outside of the cytoplasmic membrane. The fusion at residue Val-104 yields low alkaline phosphatase activity (1.5 Miller units), indicating that interhelix loop III-IV is on the cytoplasmic side of the membrane.

**DISCUSSION**

Lactococci are multiple amino acid auxotrophs that require an exogenous nitrogen source for growth. They can use amino acids, peptides, and/or caseins to satisfy this requirement (Law, 1978). Di-tripeptide transport was shown to be essential for growth on the milk protein θ-casein, indicating that one or more essential or growth-stimulating amino acids are released as di- or tripeptides during casein hydrolysis (Smid et al., 1989b).

In this paper, the cloning of the di-tripeptide transport gene (*dtpT*) of *L. lactis* is described. The gene encodes a protein which shares no homology to components of any other bacterial peptide transport system nor with any other protein in the EMBL SWISSPROT sequence data base to date. The catalytic activity of DtpT also differs from other known bacterial peptide transport systems since it transports a wide variety of di-tripeptides in symport with proton(s).

The following observations indicate that the di-tripeptide transport gene of *L. lactis* was cloned. (i) Alanylglutamate uptake by dipeptide transport deficient *E. coli* cells harboring pDT5 is significantly higher than in cells which contain the vector pFAQ1 only (Fig. 5A). (ii) Substrate specificity studies indicate that DtpT specifically transports di- (and tri) peptides. (iii) A dipeptide transport-negative mutant, designated *L. lactis* AG300, has been constructed by homologous recombination using flanking regions of the cloned *dtpT* gene. (iv) Transformation of *L. lactis* AG300 with pDT5 resulted in growth characteristics similar to those of wild-type MG1363.

A few observations regarding peptide transport activities in
whole cells require further explanation. In our analysis of transport activities using intact cells the peptides are in most cases hydrolyzed rapidly in the cytoplasm. Since the amino acids formed can be metabolized and/or excreted at different rates, the estimated rates of peptide uptake will always be an underestimate and the observed accumulation of amino acids will be different for the various residues present in the peptide (see Fig. 8). A surprise has been the observation that E. coli E1772 is unable to hydrolyze Ala-Glu, which has allowed us to detect peptide exchange in whole cells. Finally, in our transport assays using washed cells of L. lactis or E. coli and the peptides indicated in this study, we have never detected any dipeptide hydrolysis outside the cell membrane.

Known bacterial peptide transport systems other than DtpT are encoded by an operon of (in many cases) five genes, and these systems all belong to the superfamily of ABC transporters. The oligopeptide transport system of L. lactis which transports peptides of 4 up to at least 46 amino acid residues (Konings et al., 1993) also belongs to this family (Tynkkynen et al., 1993). In contrast to the oligopeptide transport system, the di- and tripeptide transport system of L. lactis is encoded by a single gene (dtpT). On basis of the longest ORF found, DtpT consists of 651 amino acid residues. The loop that separates the helices I-VI and VII-XII of (most) sec-}

**REFERENCES**


Di-tripeptide Transport in L. lactis