Genetic and Biochemical Characterization of the Oligopeptide Transport System of *Lactococcus lactis*

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The nucleotide sequence of a chromosomal DNA fragment of *Lactococcus lactis* subsp. *lactis* SSL135, previously implicated in peptide utilization, has been determined. The genes *oppDFBCA*, encoding the oligopeptide transport system (Opp), and that encoding the endopeptidase PepO were located on this 8.9-kb DNA fragment. The *oppDFBCA* and *pepO* genes are probably organized in an operon. Analysis of the deduced amino acid sequences of the genes indicated that the oligopeptide transport system consists of two ATP-binding proteins OppD and OppF, two integral membrane proteins OppB and OppC, and a substrate-binding protein OppA. On the basis of the homology of OppF and OppD of *L. lactis* with other ABC (ATP-binding cassette) transporter proteins, the *L. lactis* Opp system can be classified as a member of this group. Two integration mutants, one defective in OppA and the other defective in PepO, were constructed. Growth of these mutants in a chemically defined medium with oligopeptides showed that the transport system, but not the endopeptidase, is essential for the utilization of peptides longer than three residues. Uptake of the pentapeptide Leu-enkephalin in glycolyzing lactococcal cells was followed by rapid hydrolysis of the peptide intracellularly. Importantly, extracellular hydrolysis of Leu-enkephalin is not observed. The OppA-deficient mutant was unable to transport Leu-enkephalin. Growth experiments with pasteurized milk revealed that transport of oligopeptides forms an essential part of the proteolytic system in lactococci.

For growth in milk, lactococci depend on the presence of a proteolytic system composed of a cell envelope-associated proteinase (PrtP), several peptidases, and amino acid and peptide transport systems (17, 19, 24, 42). The first step in the utilization of milk proteins (caseins) is their degradation by a proteinase to (oligo-) peptides of various sizes (32, 33, 56). All lactococcal proteinases are capable of degrading β-casein, while some strains produce a proteinase that also degrades α- and κ-casein. The size limit for peptide uptake in lactococci was suggested to be 5 or 6 amino acid residues (23, 40); longer peptides produced by PrtP are believed to be subject to further degradation by extracellular peptidases before transport into the cell can take place. Several peptidases from lactococci have been described, and most of these have been purified to homogeneity and characterized biochemically (19). The genes encoding aminopeptidase C (pepC) (5), aminopeptidase N (pepN) (45, 47, 53), X-prolyl-dipeptidylaminopeptidase (pepXP) (30, 34), endopeptidase PepO (pepO) (31), and tripeptidase PepT (pepT) (30a) have been cloned and characterized. The nucleotide sequence analyses, together with immunological studies (46) strongly suggest that PepN, PepC, PepXP, PepO, and PepT are located intracellularly.

Biochemical evidence shows that separate transport systems for amino acids, a single di- and tripeptide transporter and an oligopeptide transport system, are operative in lactococci (40, 42). The di- and tripeptide transport system is driven by the proton motive force and is essential for growth of lactococci on casein-containing media (43, 44). The gene (*dipT*) for the di- and tripeptide transport system encodes a protein typical of secondary polytopic membrane proteins (11). The transport of oligopeptides is, most likely, coupled to ATP hydrolysis (21).

A spontaneous mutant of *Lactococcus lactis* subsp. *lactis* MG1614, MG1614V, is not capable of growth in milk, despite the presence of a functional proteinase gene. We have previously described the cloning of a chromosomal DNA fragment of *L. lactis* subsp. *lactis* SSL135 in plasmid pVS8 which is needed for rapid growth and acid production of strain MG1614V in milk (58). The chromosomal fragment in pVS8 enabled strain MG1614V to also grow on tryptic peptides of casein. Since the presence of pVS8 in MG1614V did not result in the enhanced release of amino acids from casein, it was proposed that pVS8 specified a combined peptidase-peptide transport system (51).

In this article, we present the nucleotide sequence of part of the chromosomal insert in pVS8 and demonstrate that it, indeed, encodes an endopeptidase and a functional oligopeptide transport system, as was demonstrated in peptide uptake experiments. Moreover, the genes encoding the oligopeptide-binding protein and the endopeptidase were disrupted in the chromosome of *L. lactis* to assess their roles in the utilization of casein and oligopeptides.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture media.** The bacterial strains and plasmids used in this study are listed in Table 1. Lactococcal strains were grown at 30°C in M17 broth (49) supplemented with 0.5% glucose or lactose. Growth in milk was tested in pasteurized low-fat milk as described previously (58). *Escherichia coli* was grown in Luria-Bertani broth (27) at 37°C. When needed, erythromycin (5 μg/ml for *L. lactis* and 10 μg/ml for *E. coli*), chloramphenicol and rifampin (5 and 50 μg/ml, respectively, for *L. lactis*), and ampicillin (100 μg/ml for *E. coli*) were added.
**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant description*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis subsp. lactis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML3</td>
<td>Lac+ Prt+ Opp+ PepO+</td>
<td>21, 44</td>
</tr>
<tr>
<td>MLDT1</td>
<td>Di- and tripeptide transport-deficient mutant of ML3</td>
<td>21, 44</td>
</tr>
<tr>
<td>MG1363</td>
<td>Lac− Prt− Opp− PepO−; plasmid-free derivative of NCDO 712</td>
<td>8</td>
</tr>
<tr>
<td>MG1614V</td>
<td>Lac− Prt− PepO−; plasmid-free derivative of NCDO 712; a spontaneous oligopeptide transport deficient-mutant of MG1614 (8)</td>
<td>51, 52, 58</td>
</tr>
<tr>
<td>VS230</td>
<td>Cm′; MG1614V transformed with pVS8</td>
<td>58</td>
</tr>
<tr>
<td>VS772</td>
<td>MG1363opp::pVS19A Opp− PepO−</td>
<td>This study</td>
</tr>
<tr>
<td>VS798</td>
<td>MG1363pepO::pLS19B Opp+ PepO−</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>endA1 recA1 gyrA96 thi hsdR17 (rK− mK+) relA1 supE44 Δ(lac-proAB) [F− traD36 proAB lacIqZΔM15]</td>
<td>61</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pVS8</td>
<td>Cm′; contains oppDFBCEpapO</td>
<td>58</td>
</tr>
<tr>
<td>pLS19</td>
<td>Amp’ Em’; pUC19 carrying the erm gene of pE194 in the NdeI site</td>
<td>K. Leenhouts, University of Groningen</td>
</tr>
<tr>
<td>pVS19A</td>
<td>1,130-bp XbaI-EcoRV fragment of opp4 ligated to pLS19</td>
<td>This study</td>
</tr>
<tr>
<td>pLS19B</td>
<td>800-bp PstI-EcoRI fragment of pepO ligated to pLS19</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Lac+, lactose-fermenting phenotype; Prt+, ability to produce proteinase; Opp+, functional oligopeptide transport system; OppA−, deficiency in the substrate-binding protein of the oligopeptide transport system; PepO+ and PepO−, production and lack of production of the endopeptidase PepO, respectively; Cm′, Amp′, and Em′, resistance to chloramphenicol, ampicillin, and erythromycin, respectively.

**General DNA techniques and DNA sequencing.** Plasmid DNA and chromosomal DNA from *L. lactis* were isolated by the methods of Anderson and McKay (3) and Leenhouts et al. (25), respectively. Plasmid DNA from *E. coli* was isolated as described by Maniatis et al. (27). *L. lactis* was electrophoresed as described previously (15). Conjugation of lactococci was done according to the method of Gasson and Davies (9). DNA modification enzymes were obtained from Boehringer GmbH (Mannheim, Germany) and New England Biolabs Inc. (Beverly, Mass.). Fragments of the chromosomal DNA insert in pVS8 were cloned into pGEM4Z (Promega Corp., Madison, Wis.) for plasmid sequencing. Both strands were sequenced by the dideoxy chain termination method using SP6 or T7 promoter sequencing primers and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Part of the sequencing was also done with synthetic oligonucleotide primers (Applied Biosystems, Inc., Foster City, Calif.) designed on the basis of known sequences. Homology searches in the EMBL Swiss-Prot protein sequence data base (release 23) were done by using the FASTA algorithm of Pearson and Lipman (35). Free energies of ribosome binding sites were calculated by the method of Tinoco et al. (50) using the lactococcal 16S-rRNA sequence (26).

Plasmid pLS19, carrying the origin of replication of pBR322, was used as an integration vector in *L. lactis* (25). The 1,130-bp XbaI-EcoRV fragment of pVS8 was ligated into pLS19 digested with XbaI and SmaI, resulting in plasmid pLS19A. Plasmid pLS19B contains the 800-bp PstI-EcoRI fragment of pVS8 ligated to the PstI-EcoRI fragment of pLS19. *E. coli* was used as a host for the plasmid constructions. Southern hybridizations were performed by using the Dig DNA labelling and detection kit (Boehringer GmbH) according to the instructions of the manufacturer.

**Growth on leucine-containing peptides.** Growth of *L. lactis* was tested in the chemically defined medium described by Poolman and Konings (38), except that cysteine and tyrosine were added at final concentrations of 250 and 287 mg/liter, respectively. Growth on leucine-containing peptides was tested in chemically defined medium lacking leucine. Leucine-containing peptides were added to final concentrations of 100 μM.

Leu-Gly, Leu-Gly-Gly, Gly-Leu-Gly-Leu, and Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) were purchased from Bachem (Bubendorf, Switzerland). Tyr-Gly-Gly-Phe-Leu-Lys (Leu-enkephalin-Lys), Ser-Ile-Gly-Leu-Ala-Lys, and Val-His-Leu-Thr-Pro-Val-Gly-Lys were purchased from Sigma Chemical Co. (St. Louis, Mo.). Growth was checked visually after an 18-h incubation at 30°C.

**Transport studies.** Transport of Leu-enkephalin was monitored as described by Kunji et al. (21). Prior to transport, cells were washed twice with 100 mM potassium phosphate (pH 6.5) and resuspended to a final concentration of 10 mM and the mixture was incubated for 20 min at 30°C. Cells were subsequently washed twice and resuspended in 100 mM potassium phosphate, pH 6.5. For transport assays, cells (A660~4) were preincubated for 5 min in the presence of 0.5% (wt/vol) glucose, after which Leu-enkephalin was added to a final concentration of 1 mM. At various time intervals, cells (1 ml) were collected on a 0.45-μm pore size cellulose acetate filter (Schleicher & Schuell GmbH, Dassel, Germany) by using a manifold filtration apparatus (Hoefer, San Francisco, Calif.) with vacuum applied by a Divac 2.4 L pump (Leybold AG, Köln, Germany). The cells were washed three times with ice-cold potassium phosphate (100 mM; pH 6.5). The filter was subsequently transferred to a vial (20 ml) (Packard, Cranbury Industries, Meriden, Conn.) which contained 300 μl of 5% (vol/vol) perchloric acid and 10 mM Na-EDTA. After 30 min of incubation, 110 μl of acidic cell extract was pipetted into an Eppendorf tube containing 100 μl of 1 M KOH–1 M KHCO3 to adjust the pH to 9.5. Samples were stored at −20°C. Amino acids and peptides were analysed after derivatization with dansyl chloride, essentially by methods described by Tapui et al. (48) and Wiedmeier et al. (60). The dansylated neutralized cell extracts were separated by high-performance liquid chromatography as described previously (39).

**Nucleotide sequence accession number.** The oppDFB CApepO sequence has been assigned the GenBank accession number L18760.
LACTOCOCCAL OLIGOPEPTIDE TRANSPORT SYSTEM

RESULTS

Nucleotide sequence analysis of the chromosomal insert in pVS8. The nucleotide sequence of an 8,918-bp region of chromosomal DNA of *L. lactis* in pVS8 revealed the presence of six open reading frames (ORFs), which are schematically drawn in Fig. 1. The entire sequence is presented in Fig. 2. With the exception of ORF2 (GTG codon), all ORFs start with an ATG codon. All ORFs are preceded by possible ribosome binding sites with ΔG° ranging from −9.4 to −20.4 kcal/mol (ca. −39,000 to −85,400 J/mol). ORF1 contains 338 codons, has the potential to encode a 37.5-kDa protein, and is preceded by potential lactococcal −35 and −10 promoter sequences (54, 55). The start codon of ORF2 and the stop codon of ORF1 overlap. ORF2 contains 319 codons and could encode a 35.9-kDa protein. ORF2 and ORF3 are overlapping for seven codons. ORF3, containing 319 codons (putative protein, 35.9 kDa), and ORF4, with 294 codons (32.8-kDa protein), are separated by a 9-bp intercistronic region. ORF4 and ORF5 are separated by 109 nucleotides. A second putative promoter is found directly upstream of the fifth ORF, ORF5, which comprises 600 codons. The 65.8-kDa protein putatively encoded by ORF5 contains a signal sequence typical for prokaryotic oligopeptides (Fig. 2) (57). ORF5 and ORF6 are separated by a 124-bp intercistronic region. ORF6 contains 627 codons (71.5-kDa putative protein). In the 370 bp downstream of ORF6, two weak stem-loop structures that could act as rho-independent terminators (−2.3 and −9.4 kcal/mol [ca. −9,600 and −39,000 J/mol, respectively]) were found (Fig. 2).

Plasmid pVS8 encodes the lactococcal oligopeptide transport system and the endopeptidase PepO. The EMBL Swiss Prot protein sequence data base (release 23) was searched for proteins homologous to those deduced from ORF1 to ORF6. The proteins specified by ORF1 and ORF2 have significant homology with several ATP-binding proteins. The highest similarity scores were found with proteins that are part of oligopeptide transport systems. Identical amino acids were found throughout the protein sequences. The sequences conserved include the Walker A motif, the P-loop or glycine-rich loop involved in phosphoryl transfer, and the Walker motif B that is associated with many nucleotide-binding proteins (Fig. 3) (12, 59). On the basis of the similarity to the ATP-binding proteins of the oligopeptide transport system of *Salmonella typhimurium*, ORF1 and ORF2 were named OppA and OppF, respectively. Part of the alignment of OppD and OppF of *Lactis* with the ATP-binding proteins of oligopeptide transport systems of *S. typhimurium* (14), *Bacillus subtilis* (36, 41), the dipeptide transport system of *B. subtilis* (28), and the ami locus of *Streptococcus pneumoniae* (1) is shown in Fig. 3. When OppD is aligned with these four proteins, 25% identical and 48% similar residues are found. The corresponding values in the alignment for OppF are 25% and 57%, respectively. The lactococcal OppD and OppF proteins share 34.5% identical residues.

Hydropathy analyses by the method of Kyte and Doolittle (22) revealed that ORF3 and ORF4 each have six potential membrane-spanning segments (not shown). The polypeptides are homologous to OppB and OppC from *S. typhimurium* (14), AmiC and AmiD from *S. pneumoniae* (1), OppB (SpoOKB) and OppC (SpoOKC) from *B. subtilis* (36, 41), and DciAB and DciAC from the *B. subtilis* dipeptide transport system (28). On the basis of its similarity to the amino acid level with OppB, SpoOKB, AmiC, and DciAB (ranging from 27 to 32%), ORF3 was named OppB. Also, on the basis of its similarity with OppC, SpoOKC, AmiD, and DciAC (ranging from 29 to 39%), ORF4 was named OppC. Lactococcal OppB and OppC share only 21% identical residues.

Upon screening of the data base for proteins homologous to OppC, it was found that a protein identical to OppC from *L. lactis* had been cloned and sequenced by Petzell and McKay (37). These authors characterized a 1.65-kb XbaI-XhoI fragment from the *L. lactis* subsp. cremoris lactose plasmid pSK11L, which facilitated the Campbell-like integration of the thermosensitive replicating plasmid pSK11L into the lactococcal chromosome (7, 16). It appears that this entire XbaI-XhoI fragment is present in the lactococcal piece of DNA cloned in pVS8 (Fig. 1).

ORF5 is homologous to the substrate-binding proteins of oligopeptide transport systems. The deduced amino acid sequence of ORF5 shares 24% and 26% identical residues with the OppA proteins of *S. typhimurium* (14) and *B. subtilis* (36, 41), respectively, and 23% identical residues with AmiA from *S. pneumoniae* (1). On the basis of this homology, ORF5 was designated OppA.

The deduced amino acid sequence of ORF6 is identical to that of the lactococcal neutral endopeptidase PepO (31). Mierau et al. (31) cloned the gene encoding PepO from *L. lactis* subsp. cremoris P8-2-47 using antibodies raised against the purified enzyme. Although the pepO genes of both strains are virtually identical, the nucleotide sequences immediately downstream of the genes diverge. It was demonstrated that the expression of PepO in *E. coli* depends on the presence of a thermosensitive plasmid pSK11L, which facilitated the Campbell-like integration of the thermosensitive replicating plasmid pSK11L into the lactococcal chromosome (57). A consensus lactococcal −35 and −10 region immediately downstream of the genes diverge. It was demonstrated that the expression of PepO in *E. coli* depends on the presence of a 2.5-kb region upstream of pepO, and Mierau et al. (31) suggested that pepO is part of an operon. As indicated above, a consensus lactococcal −35 and −10 region immediately upstream of oppA could be identified (Fig. 2). Apparently, this promoter is operative in *E. coli*, but its functionality in *L. lactis* remains to be established.

Construction of OppA- and PepO-deficient mutants of *L. lactis*. Plasmid pVS8, which contains all the genes for oligopeptide transport (opp) and for the endopeptidase PepO, is able to complement the deficiency of the ability of the spontaneous mutant *L. lactis* MG1614V to grow in milk. To establish whether opp, pepO, or both are critical for casein utilization, two integration mutants were constructed. Plasmid pLS19A, carrying an internal fragment of oppA, and plasmid pLS19B, containing an internal fragment of pepO, were inserted in the chromosome of the plasmid-free wild-type strain *L. lactis* MG1363. One of the erythromycin-resistant transformants obtained with each integration plasmid was analyzed by Southern hybridization. Chromosomal DNAs of strains VS772 (oppA disruption) and VS798 (pepO disruption) and that of the recipient strain MG1363 were digested with EcoRI or HindIII.
The 1,130-bp \( XbaI-EcoRV \) fragment of \( pVS8 \) (Fig. 1) was used as a probe in the analysis of the \( oppA \) mutant \( VS772 \) (Fig. 4), whereas the 800-bp \( PstI-EcoRI \) fragment (Fig. 1) was used to analyze the \( pepO \) mutant \( VS798 \) (Fig. 5). The hybridizing restriction fragments of the chromosomal DNAs of both mutants have sizes expected when the \( oppA \) and \( pepO \) genes are disrupted by integration. These mutants were used to assess the role of \( OppA \) and \( PepO \) in peptide transport, peptide degradation, and growth in milk (see below).

**opp genes encode a functional oligopeptide transport system.** The uptakes of Leu-enkephalin in different strains of \( L. \) *lactis* were compared to demonstrate that \( opp \) is involved in oligopeptide uptake. Addition of Leu-enkephalin (Tyr-Gly-Phe-Leu) to glucose-metabolizing cells of the wild-type strain \( L. \) *lactis* ML3 resulted in the accumulation of the corresponding amino acid residues inside the cell (Fig. 6A). Apparently, Leu-enkephalin is rapidly hydrolyzed by high intracellular aminopeptidases, and/or other peptidase activities. Changes in internal concentrations of other amino acids were not observed (data not shown). Accumulation of amino acid residues from Leu-enkephalin was observed neither in \( L. \) *lactis* MG1614 (Fig. 6B), which lacks

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**Fig. 2.** Nucleotide and deduced amino acid sequences of the \( L. \) *lactis* subsp. *lactis* SSI135 oligopeptide transport system and the endopeptidase \( PepO \). The putative ribosome binding sites (RBS) and promoter regions are boldfaced and underlined, respectively. The consensus lipoprotein signal peptidase cleavage site in \( OppA \) is also underlined. Possible stem-loop structures are indicated (dashed arrows).
The uptake rates based on tyrosine and glycine accumulation (the latter value was divided by two, because Leu-enkephalin contains two glycine moieties) were approximately the same, suggesting that these residues are transported across the cytoplasmic membrane in the form of the pentapeptide Leu-enkephalin (Fig. 6). The apparent uptake rates and final accumulation levels of leucine and phenylalanine are somewhat lower than those of tyrosine and glycine. Based on the rates of tyrosine and glycine accumulation, the rates of Leu-enkephalin uptake were 56 and 20 nmol min⁻¹ mg⁻¹ of protein⁻¹ for the strain carrying opp on a plasmid (VS230) and the wild-type strain ML3, respectively. From these results, we conclude that pVS8, indeed, encodes an oligopeptide transport system. Apparently, the expression of the opp genes is not tightly controlled, since an increase of the number of copies of the opp genes led to an enhanced uptake rate.

**Opp is essential for uptake of peptides larger than 3 amino acids and for growth in milk.** Leucine is essential for growth of *L. lactis* ML3, MG1363, and MG1614V (42, 52). To establish the role of the oligopeptide transport system and the endopeptidase in the utilization of peptides, growth of wild-type and mutant lactococcal strains was tested in a chemically defined medium in which leucine was replaced by leucine-containing peptides. As is illustrated in Table 2, all strains tested were able to grow on di- and tripeptides, except for the ML3-derived di- and tripeptide transport mutant (21, 44). The fact that the di- and tripeptide transport mutant of ML3 (strain MLDT1) can grow on oligopeptides supports the view that the growth observed on these peptides does not result from extracellular breakdown and subsequent uptake of smaller peptides. The oligopeptide transport mutant *L. lactis* MG1614V was unable to use peptides longer than 3 residues, whereas the parent strain MG1363 grew on all peptides provided. The phenotype of the mutant MG1614V can be restored to wild type by the introduction of the *opp* genes on plasmid pVS8: strain VS230 grows on all Leu-containing peptides. When the chromosomal gene for the substrate-binding protein OppA is interrupted, as is the case in strain VS772, only growth on di- and tripeptides was observed. Growth of the pepO integration mutant VS798 was similar to that of MG1363. These results show that the oligopeptide transport system is essential for growth on peptides larger than 3 residues, whereas the endopeptidase PepO does not appear to be essential for oligopeptide utilization. As the strains used so far lacked both the proteinase and the lactose genes, the possible requirement of the *opp* and *pepO* genes for growth in milk could not be examined. Therefore, the lactose-proteinase plasmid pLP712 was introduced in the

FIG. 3. Part of the aligned amino acid sequences of ATP-binding proteins from various oligopeptide transport systems: OppF and OppD from *L. lactis*, OppF and OppD from *S. typhimurium* (SALTY), SpoKE and SpoKD from *B. subtilis* (BACSU), AmiF and AmiE from *S. pneumoniae* (STRPN), and DciAD, the dipeptide transport system of *B. subtilis*, identified (asterisks) and similar (dots) residues are indicated. The two Walker motifs, A (WA) and B (WB), characteristics of many nucleotide-binding proteins, are also indicated.

the entire opp operon, nor in the oppA disruption mutant VS772 (Fig. 6D). Interestingly, *L. lactis* carrying the opp operon on plasmid pVS8 (strain VS230) accumulates the amino acid residues with a much higher rate than the wild-type strain, in which the opp operon is located on the chromosome (Fig. 6C).

FIG. 4. Schematic representation of Campbell-like integration of plasmid pLS19A into the chromosomal (chr.) oppA gene in *L. lactis* MG1363 (left) and Southern hybridization of chromosomal DNA from the parental strain MG1363 and the integrant VS772 digested with EcoRI and HindIII (right). The 1,130-bp *XbaI-EcoRV* fragment of pVS8 was used as a probe. The internal fragment of oppA present in pLS19A is represented by a shaded bar. The sizes of the expected hybridizing restriction fragments of the integrant, VS772, are indicated. The parental strain MG1363 is expected to give a hybridizing band of 3.8 kb with EcoRI and 4.1 kb with HindIII. Lanes: 1, MG1363 digested with HindIII; 2, VS772 digested with HindIII; 3, MG1363 digested with EcoRI; 4, VS772 digested with EcoRI. Phage lambda DNA digested with HindIII was used as a marker (lane 5); sizes in kilobases are indicated on the right. E, EcoRI; H, HindIII.
various strains by conjugation, by using *L. lactis* subsp. *lactis* NCDO 712 as a donor (8). Growth in milk of the transconjugants was monitored by a plating assay and by measuring the acid production. The results (Fig. 7) compare well to those obtained in the chemically defined medium. The wild-type strain MG1363, MG1614V carrying multiple copies of *opp* and *pepO* on pVS8 (VS230), and the endopeptidase-deficient mutant VS798 grew well in milk. The *opp*-deficient strain MG1614V did not grow in milk. As is illustrated in Fig. 7, strain VS772 (OppA−) did grow to some extent, albeit extremely slowly. Apparently, the *oppA* mutation results in a leaky phenotype. These results indicate that oligopeptide transport is essential for rapid growth of lactococci in milk.

**FIG. 5.** Schematic representation of the integration of plasmid pLS19B into the chromosome (chr.) of *L. lactis* MG1363 (left) and Southern hybridization of chromosomal DNA from the parental strain MG1363 and the integrant VS798 digested with EcoRI and HindIII (right). The internal fragment of *pepO* present in pLS19B is represented by a shaded bar. The same 800-bp Psrl-EcoRI fragment of pVS8 was used as a probe. The sizes of the expected hybridizing restriction fragments of the integrant VS798 are shown. The parental strain MG1363 is expected to give hybridizing bands of 3.8 kb with EcoRI and 1 and 0.7 kb with HindIII. Lanes: 1, MG1363 digested with HindIII; 2, VS798 digested with HindIII; 3, MG1363 digested with EcoRI; 4, VS798 digested with EcoRI. Phage lambda DNA digested with HindIII was used as a marker (lane 5); sizes in kilobases are indicated on the right. E, EcoRI; H, HindIII.

**FIG. 6.** Time course of internal amino acid pools in glycolyzing cells of *L. lactis* ML3 (Opp+ PepO+) (A), MG1614V (Opp− PepO+) (B), VS230 (Opp+ PepO+) (C), and VS772 (OppA− PepO−) (D) upon the addition of 1 mM Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu). Accumulation of tyrosine (△), glycine (●), phenylalanine (○), leucine (△), and Leu-enkephalin (○) is shown.

**DISCUSSION**

We have sequenced over 8.9 kb of chromosomal DNA of *L. lactis* subsp. *lactis* SSL135 and have proven that it encodes a functional oligopeptide transport system (Opp) and an endopeptidase (PepO). Because of their tight genetic organization, the genes *oppDFBCapepO* seem to be located in an operon-like structure. Putative promoters are present in the nucleotide sequences immediately upstream of *oppD* and *oppA*. Using in vitro transcription-translation studies, at least four proteins were shown to be encoded by this chromosomal DNA fragment as present in pVS8 (52).

The proteins of the oligopeptide transport system of *L. lactis*...
are similar to the corresponding proteins of *S. typhimurium* (14), *B. subtilis* (36, 41), and *S. pneumoniae* (1), although the gene organization in these operons is different. In *B. subtilis*, *S. pneumoniae*, and *S. typhimurium* oppA is the first gene of the operon. In *L. lactis*, however, oppA is the last of the opp genes. A gene encoding an endopeptidase not involved in oligopeptide transport, pepO, is transcribed from the same promoter as oppA, since the OppA-deficient mutant (strain VS772) also lacks PepO (30a, 31). In the case of the *B. subtilis* dipeptide transport system, the gene for the substrate-binding protein DciAE is the last gene in the operon, which contains an additional gene of unknown function (28). Similarly, the AmiB protein of the *S. pneumoniae* oligopeptide transport system has no counterpart in other known systems (1).

Oligopeptide transport systems are members of a large family of so-called ABC (ATP-binding cassette) transporters or traffic ATPases, which includes uptake and excretion systems of both prokaryotic and eukaryotic origins (2, 13, 18). On the basis of the homology between OppF and OppD of *L. lactis* and other ABC transporter proteins, the *L. lactis* Opp system can be classified as a member of this group. The basic organization of ABC transporters consists of four distinct domains. Two highly hydrophobic integral membrane proteins that span the membrane five to six times mediate substrate translocation across the cytoplasmic membrane. Two highly conserved proteins on the cytoplasmic side of the membrane most likely couple ATP hydrolysis to transport. The bacterial ABC uptake systems also encompass a substrate-binding protein which is located in the periplasm in gram-negative bacteria. The substrate-binding proteins of the gram-positive species (i.e., OppA and DciAE of the *B. subtilis* oligopeptide and dipeptide transport systems, respectively, AmiA of *S. pneumoniae*, and OppA of *L. lactis*) all have a signal peptide typical for prokaryotic preproteins. Perego et al. (36) have shown that OppA of *B. subtilis* is bound to the cell, and it is expected that all of these proteins are anchored to the cell membrane by an amino-terminal lipoyl group. In addition to serving as a transporter for the uptake of nutrients (oligopeptides), oligopeptide transport systems have been shown to be involved in the recycling of cell wall peptides in gram-negative bacteria (10) and in sporulation in *B. subtilis* (36, 41).

The present study convincingly shows that the pentapeptide Leu-enkephalin is transported into the cell by the oligopeptide transport system encoded by oppDFBCA. Subsequent hydrolysis leads to accumulation of the amino acid residues constituting Leu-enkephalin. No detectable uptake of Leu-enkephalin in the oligopeptide transport-deficient strains MG1614V and VS772 was observed. Therefore, the accumulation of tyrosine, glycine, phenylalanine, and leucine in strain VS230 is the result of Leu-enkephalin uptake and is not due to extracellular breakdown of the oligopeptide and subsequent uptake of the hydrolysis products. Uptake of Leu-enkephalin is increased in a strain carrying multiple copies of opp (strain VS230 with pVS8) relative to uptake by the wild-type strain ML3, indicating that the proteins are overproduced. The differences in the apparent uptake rates and the accumulation levels of glycine and tyrosine versus leucine and phenylalanine could be due to the more lipophilic nature of the latter two amino acids. The higher hydrophobicity of leucine and phenylalanine could lead to passive leakage of these amino acids from the cells (6, 38). In addition, if the concentration gradients of the amino acids exceed the driving force imposed by the amino acid carrier mechanism, the residues may also leave the cell by facilitated diffusion (20). Therefore, the estimated uptake rates of Leu-enkephalin based on the concentration of intracellular amino acids will always be underestimates of the actual oligopeptide uptake rate. Nevertheless, the transport assays provide convincing evidence that the transport system encoded by the oppDFBCA genes indeed facilitates the uptake of the pentapeptide Leu-enkephalin. All present evidence suggests that the opp genes described in this article encode the oligopeptide transport system whose energetics has been described recently (21).

The growth experiments using chemically defined medium supplemented with leucine-containing peptides clearly demonstrate that the oligopeptide transport system is essential for growth of lactococci on peptides longer than 3 residues. It has been suggested that the size restriction for peptide uptake by lactococci is 5 or 6 amino acids (23, 40). In the present study, however, normal growth of *L. lactis* utilizing a leucine-contain-

### TABLE 2. Growth of *L. lactis* strains in chemically defined medium lacking leucine supplemented with leucine-containing peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MG1363 (Opp+ PepO+)</th>
<th>MG1614V (Opp+ PepO+)</th>
<th>VS230 (Opp+ PepO+)</th>
<th>VS772 (OppA PepO-)</th>
<th>VS798 (Opp+ PepO-)</th>
<th>MLDT1 (Opp+ Dpp-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Gly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Leu-Gly-Gly-Leu</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Gly-Leu-Gly-Leu</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyr-Gly-Gly-Phe-Leu</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tyr-Gly-Gly-Phe-Leu-Lys</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ser-Ile-Gly-Ser-Leu-Ala-Lys</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Val-His-Leu-Thr-Pro-Val-Gly-Lys</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Growth of strain</strong>:</td>
<td><strong>OP+</strong></td>
<td><strong>PepO+</strong></td>
<td><strong>PepO-</strong></td>
<td><strong>PepO-</strong></td>
<td><strong>PepO-</strong></td>
<td><strong>PepO-</strong></td>
</tr>
</tbody>
</table>

*a* Opp+, functional oligopeptide transport system; Opp−, deficiency in oligopeptide transport; OppA+, deficiency in the substrate-binding protein of the oligopeptide transport system; PepO+ and PepO−, production and lack of production of the endopeptidase PepO, respectively; Dpp+, deficiency in di- and tripeptide transport. +, good growth; −, no growth; ND, not done.

FIG. 7. Growth (a) and acid production (b) of *L. lactis* strains in pasteurized milk. Plasmid pLP712, carrying the genes for lactose utilization and proteinase production, was introduced via conjugation in all strains used in the experiment. ■, MG1614V (Opp+ PepO+); ▲, VS772 (OppA PepO-); ▼, MG1363 (Opp+ PepO+); ●, VS230 (Opp+ PepO+); ◆, VS798 (Opp+ PepO−).
ing octapeptide was observed. Current studies are directed towards a further biochemical analysis of the substrate specificity and size restriction of the oligopeptide-binding protein. Growth and acid production of the various strains in milk clearly show that the transport of oligopeptides is an essential part of the proteolytic system of lactococci.

The close genetic linkage of the opp genes and pepO suggests that the gene products are also physiologically linked: it would make sense if oligopeptides taken up by the Opp system are subsequently hydrolyzed by the endopeptidase PepO. However, whereas the opp genes are essential, disruption of the endopeptidase gene pepO had no apparent effect on the utilization of milk protein or peptides by \( L. \text{ lactis} \). It is possible, of course, that the activity of PepO can be taken over by other peptidases with overlapping specificities (29).

Because of their presumed role in casein degradation, an extracellular location has been envisaged for a number of lactococcal proteolytic enzymes. However, PrtP, the lactococcal protease, remains the only proteolytic enzyme whose extracellular location is certain. Recent genetic, biochemical, and immunological data strongly suggest an intracellular location for all of the lactococcal peptides (4, 46). The present results indicate that the presence of an oligopeptide transport system allows lactococci to utilize oligopeptides containing up to 8 amino acid residues. Therefore, the emerging picture of the lactococcal proteolytic system assumes that extracellular hydrolysis of casein is accomplished by the protease PrtP alone and that this hydrolysis produces sufficient ingestible di-, tri-, and oligopeptides for the sustenance of growth of lactococci in milk.

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