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Lactose Metabolism in *Lactobacillus bulgaricus*: Analysis of the Primary Structure and Expression of the Genes Involved

P. LEONG-MORGENTHALER,* M. C. ZWAHLEN, AND H. HOTTINGER

Nestec Ltd. Research Centre, Vers-Chez-Les-Blanc, P.O. Box 44, 1000 Lausanne 26, Switzerland

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The genes coding for the lactose permease and β-galactosidase, two proteins involved in the metabolism of lactose by *Lactobacillus bulgaricus*, have been cloned, expressed, and found functional in *Escherichia coli*. The nucleotide sequences of these genes and their flanking regions have been determined, showing the presence of two contiguous open reading frames (ORFs). One of these ORFs codes for the lactose permease gene, and the other codes for the β-galactosidase gene. The lactose permease gene is located in front of the β-galactosidase gene, with 3 bp in the intergenic region. The two genes are probably transcribed as one operon. Primer extension studies have mapped a promoter upstream from the lactose permease gene but not the β-galactosidase gene. This promoter is similar to those found in *E. coli* with general characteristics of GC-rich organisms. In addition, the sequences around the promoter contain a significantly higher number of AT base pairs (80%) than does the overall *L. bulgaricus* genome, which is rich in GC (GC content of 54%). The amino acid sequences obtained from translation of the ORFs are found to be highly homologous (similarity of 75%) to those from *Streptococcus thermophilus*. The first 460 amino acids of the lactose permease shows homology to the melibiose transport protein of *E. coli*. Little homology was found between the lactose permease of *L. bulgaricus* and *E. coli*, but the residues which are involved in the binding and the transport of lactose are conserved. The carboxy terminus is similar to that of the enzyme III of several phosphoenolpyruvate-dependent phosphotransferase systems.

Lactobacillus delbrueckii subsp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* are dairy lactic acid bacteria that are widely used in the fermentation of milk (25). During their growth in milk, lactose is used as the primary energy source. Two systems for transport and metabolism of lactose are known in lactic acid bacteria: (i) a phosphoenolpyruvate (PEP) lactose phosphotransferase system (PTS) with a phospho-β-galactosidase enzyme (21) and (ii) a lactose permease system with a β-galactosidase. The PEP-PTS system is found in many species, including lactococci (reviewed in references 30 and 31).

L. bulgaricus utilizes lactose via the second pathway. Lactose is brought into the cell as the free sugar and cleaved by β-galactosidase. The glucose moiety of the sugar is further metabolized while the galactose moiety is released (8, 23). The lactose operon of *Escherichia coli*, which also has a lactose permease (*lacY*) and β-galactosidase (*lacZ*), has been extensively studied, and the proteins involved are being characterized (1–4, 11, 13, 24). Not much is known, however, about the system in lactic acid bacteria. Recently, there has been a report on the cloning and sequencing of the β-galactosidase gene from *L. bulgaricus* (28). This group found that β-galactosidase from *L. bulgaricus* has an average similarity of 34% to that from *E. coli*, with stretches of high homology around the regions involved in activity.

In this report, we describe the cloning, expression, and nucleotide sequence determination of the lactose permease and β-galactosidase genes from *L. bulgaricus*. We identify a promoter sequence in front of the lactose permease gene, and we confirm the use of this promoter in *L. bulgaricus* by primer extension of RNA transcripts. The homology of the lactose permease to other sugar transport proteins and the similarity of the lactose operon to that from *S. thermophilus* are also discussed.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and media. The *L. bulgaricus* strain used was ATCC 11842, obtained from the American Type Culture Collection. It was grown in MRS medium (Difco) supplemented with 2% glucose at 42°C without aeration. *E. coli* strains used in cloning were JM105-8, a cured F^- derivative of JM105 (33), and MB406 (EMBL3 cloning kit; Promega). The strains were grown in Luria broth (LB) or minimal E media with glucose and proline, supplemented with 70 μg of ampicillin per ml when appropriate, at 37°C with vigorous aeration.

Construction of an *L. bulgaricus* genomic library. Genomic DNA from *L. bulgaricus* was prepared by conventional methods (9). The DNA was digested with *Sau*3A (0.1 U/μg of DNA at 37°C for 30 min) to maximize fragments of 6 to 20 kb. The partially digested DNA was size fractionated in a 10 to 40% sucrose gradient centrifuged at 70,000 × *g* for 16 h (6). Fractions containing fragments of 9 to 18 or 16 kb were pooled and precipitated in the presence of 0.3 M sodium acetate and 2 volumes of ethanol. Two banks, one with fragments from 9 to 18 kb and the other with fragments of 16 kb, were constructed by using the EMBL3 cloning system (Promega). The ligation mix was packaged in vitro with packaging mix from Promega. The packaged phages were then used to infect MB406 or JM105-8. A total of 1,000 individual plaques were picked, gridded onto 10 master plates, and used for subsequent screening for genes of interest.

Screening for β-galactosidase-positive plaques. The plaques from the master plates were picked and gridded onto LB plates supplemented with 50 μg of 5-bromo-4-chromo-3-indolyl-β-D-galactoside (X-Gal) (14) with an overlay of JM105-8 as the host strain for the phages. Blue plaques, considered β-galactosidase positive, were picked, purified, and used for further analysis.

Subcloning of the β-galactosidase gene. Different *Sal*I-

digested fragments of a β-galactosidase phage (B37) were subcloned into a pBR322 vector digested with *Sal*I and transformed into JM105-8. Transformants were selected on minimal-glucose plates containing X-Gal (50 μg/ml) and ampicillin (70 μg/ml). Blue colonies were picked and purified for further analysis. Further subcloning and deletion analysis using exonuclease III (12) were done to localize the active β-galactosidase gene.

Subcloning of the lactose permease gene. Different *Bam*HI-digested fragments of B37 were subcloned into a pBR322 vector digested with *Bam*HI and transformed into JM105-8. Transformants were selected on MacConkey-lactose (Difco) plates containing 0.5% lactose and 70 μg of ampicillin per ml. Transformants containing active lactose permease and β-galactosidase genes are recognized as red colonies (14).

Sequencing of the β-galactosidase and lactose permease genes. The fragments containing the active β-galactosidase and permease genes were sequenced by the dideoxy-chain termination method (27), using a kit from Pharmacia. Primers were made as 21-mers on an Applied Biosystems oligonucleotide synthesis machine. DNA sequences were analyzed with the University of Wisconsin Genetics Computer Group sequence analysis software package, version 6.1 (5).

Restriction enzyme analysis of phages and plasmids. DNA from plasmids and phages of interest were prepared by conventional methods (12). Restriction patterns of the plasmids and phages were determined by digestion of the DNA with restriction enzymes under conditions suggested by the supplier. Restriction enzymes were obtained from Boehringer Mannheim.

Mapping of the RNA transcripts. RNA was prepared from *L. bulgaricus* by the hot phenol protocol. *L. bulgaricus* was grown to mid-exponential phase, and growth was inhibited by the addition of a frozen and crushed solution containing 0.02 M Tris (pH 7), 5 mM $MgCl_2$, 0.02 M sodium azide, and 400 μg of chloramphenicol per ml (29). The cells were centrifuged and suspended in 4 ml of a solution containing 25% sucrose, 50 mM Tris (pH 8), 0.25 mM EDTA, and 0.5 mg of lyzozyme per ml. After 5 min on ice, the suspension was centrifuged and suspended in 0.6 ml of lysis buffer (20 mM Tris [pH 8], 3 mM EDTA, 0.2 M NaCl) and 1% sodium dodecyl sulfate. The suspension was heated to 95°C and extracted with phenol that had been heated to 65°C twice. The RNA was then precipitated in the presence of potassium acetate and 2 volumes of ethanol.

The 5′ end of the RNA was mapped by the primer extension method with slight modifications, using murine reverse transcriptase (10). An oligonucleotide (21-mer) 88 bp away from the putative start site of the lactose permease gene was used as a primer. A 10-μg sample of RNA was annealed to 40 pg of primer that had been labeled with [^{32}P]dATP by the kinase reaction (12) in 30 μl of hybridization buffer [80% formamide, 0.4 M NaCl, 1 mM EDTA, 0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.4] overnight at 47°C. The annealed mix was then precipitated in the presence of ethanol and dissolved in 30 μl of reverse transcriptase buffer (50 mM Tris-HCl [pH 8.3], 60 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). The reaction was carried out at 42°C in the presence of 1 mM each of the four deoxynucleotides and 50 U of murine reverse transcriptase for 1 h. The reaction mix was then precipitated in the presence of ethanol, resuspended in 2 μl of loading buffer, and run on a 4% denaturing polyacrylamide gel.

Amplification of the flanking DNA by inverted PCR. Genomic DNA was digested to completion with *Eco*RV and ligated at a concentration of 1 ng/μl under standard conditions (12). The ligated DNA was precipitated in the presence of 0.8 M lithium chloride and 2 volumes of ethanol and suspended to a concentration of 10 ng/μl. Fifty nanograms of the ligated DNA and 1 μg of each of the primers shown in Fig. 2 was used in the inverted polymerase chain reaction (PCR) under standard conditions (32).

Nucleotide sequence accession number. The sequence reported has been entered into GenBank under accession number M38754.

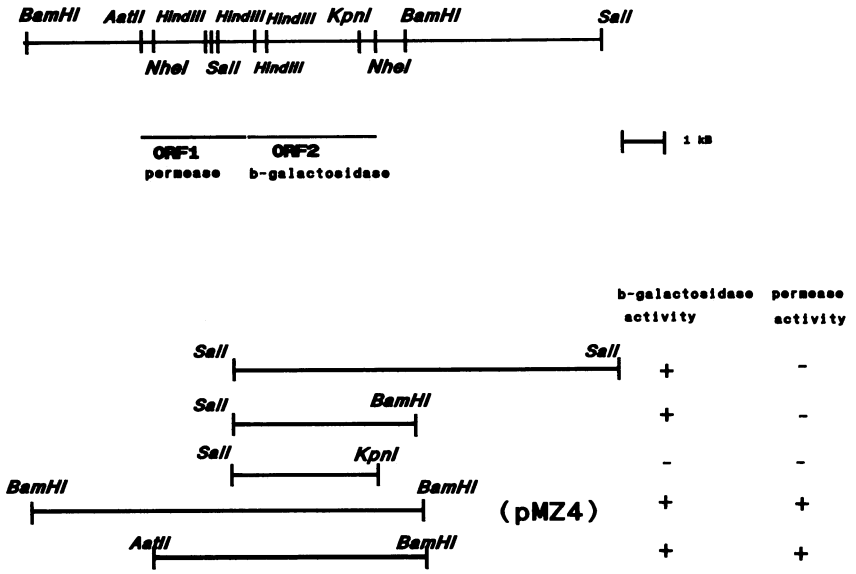

FIG. 1. Restriction map of the region containing the lactose permease and β-galactosidase genes. A fragment of B37 that contained the lactose permease and β-galactosidase activities was subcloned into pBR322. A detailed restriction map was generated, and deletion analysis was conducted to localize the coding regions of the lactose permease and β-galactosidase genes. The region of activity is indicated with dark lines below the map.

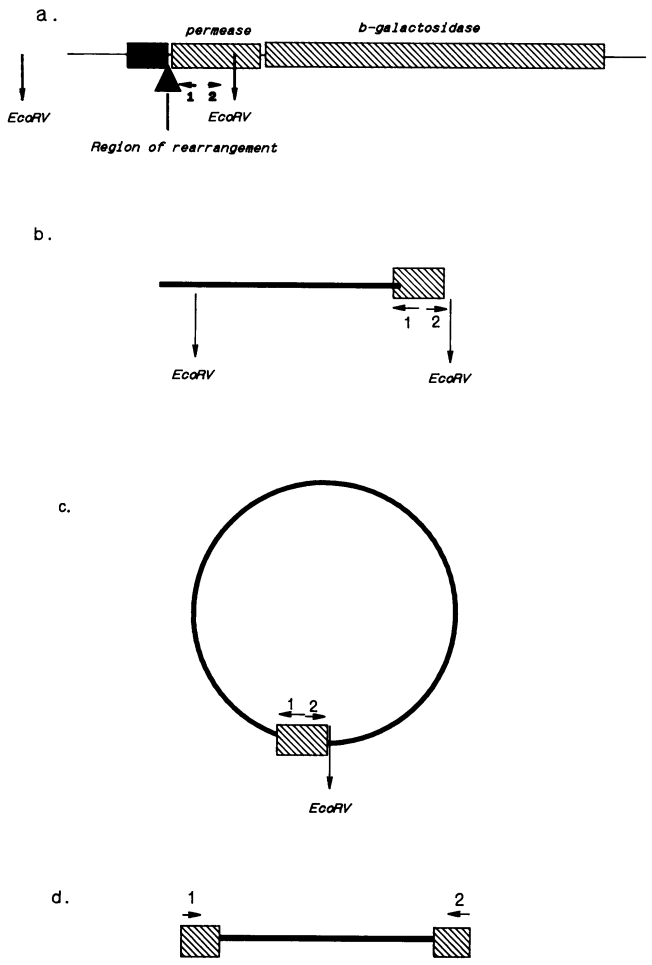

FIG. 2. Amplification of the flanking sequence of the lactose permease gene from *L. bulgaricus* genomic DNA. (a) The insert of pMZ4 containing part of the lactose permease and β-galactosidase genes. The shaded area represents the nucleotide sequences of the lactose permease and β-galactosidase genes that have been determined. The large arrow shows the site where a rearrangement has been found to be present, and the dark area represents the DNA introduced following the rearrangement. The primers to be used in the inverted PCR reaction are represented by 1 and 2, and the arrowheads show their 5′-to-3′ direction. (b) Digestion of genomic DNA with *Eco*RV. The shaded area and primers used are as shown in panel a. The dark line represents undetermined sequence of interest. (c) Ligation. Following digestion of genomic DNA with *Eco*RV, the DNA was ligated under conditions that maximize circularization. The shaded area, dark line, and small arrows are as explained for panels a and b. (d). Product following PCR amplification. The shaded area, dark line, and small arrows are as explained for panels a and b.

RESULTS

Cloning of β-galactosidase and lactose permease genes. From the EMBL3 lambda library, two β-galactosidase-positive plaques (B37 and C29) were found. A 7-kb *Sal*I fragment from B37, when subcloned into pBR322, was found to contain an active β-galactosidase gene. The lactose permease gene was cloned by complementation of an *E. coli* strain whose lactose operon was deleted (JM105-8). Clones that contained the 8-kb *Bam*HI fragment from B37 as the insert appeared as red colonies and thus contained active permease and β-galactosidase genes. From deletion and exonuclease III analysis, the lactose permease and β-galactosidase activities were found to be within an *Aat*II-*Bam*HI fragment (Fig. 1).

Nucleotide sequence analysis of the β-galactosidase gene. Analysis of the nucleotide sequence of the *Sal*I-*Bam*HI fragment showed the presence of an open reading frame (ORF) of 3,024 bp (data not shown). Deletion analysis localized the β-galactosidase activity to this fragment (Fig. 1). Therefore, this ORF (ORF2) is the coding region of the β-galactosidase gene. Comparison of our sequence with the one published by Schmidt et al. (28) showed 11 differences at the nucleotide level. All of the changes were the consequence of an AT-GC or GC-AT transition and did not result in any changes at the amino acid level. However, there was a difference of one amino acid, a lysine at position 905, which was absent in the published sequence.

Nucleotide sequence analysis of the lactose permease gene. Part of another ORF (ORF1) was found 5′ to the β-galactosidase gene. The *Bam*HI subclone (pMZ4), which contained a large part of ORF1 and the complete β-galactosidase gene, was found to complement an *E. coli* strain whose whole lactose operon had been deleted. Therefore, this clone contained an active lactose permease gene. Although it codes for an active permease gene, the restriction pattern of this clone was found not to be in complete agreement with that from detailed Southern analysis of *L. bulgaricus* genomic DNA (data not shown). To date, we have not been able to clone the amino terminus and the promoter region of this gene in its intact form in *E. coli* either from a clone bank or from an EMBL3 library (unpublished observations).

To obtain the nucleotide sequence of the complete lactose permease gene and its 5′-flanking region, an alternative method was used. The sequence of interest was amplified directly from the genomic DNA by using inverted PCR (32) and confirmed to be the correct fragment by Southern analysis. This amplified fragment was used as a template for sequencing (Fig. 2). With the nucleotide sequence information from pMZ4 and that obtained with inverted PCR, an ORF of 1,881 bp coding for a protein of 627 amino acids was found (Fig. 3). Comparison of the nucleotide sequence obtained from pMZ4 and that of the fragment obtained from inverted PCR showed that the lactose permease gene carried on pMZ4 did not include the first 135 bp of the coding sequence or the promoter of the gene.

Nucleotide sequence analysis of the flanking regions. By using the amplified fragment, 100 bp of the flanking sequences of the permease gene was obtained. Analysis of the flanking sequence showed the presence of a putative ribosome binding site 4 bp from the start (ATG) of the gene. Furthermore, two sets of 6 bp resembling −10 and −35 consensus sequences of promoters were found (Fig. 3). Primer extension analysis of RNA transcripts showed these consensus sequences to be the promoter used by *L. bulgaricus* (Fig. 4). The transcript starts 31 bp upstream from the first ATG codon of the lactose permease gene. The 30 bp immediately upstream from the −35 promoter sequence are highly AT rich compared with the whole genome, 87% versus 48%. A 9-bp direct repeat noted in Fig. 3 was also present.

In contrast to the lactose permease gene, the region immediately preceding the coding region of the β-galactosidase gene does not contain any sequences that resemble −10 or −35 consensus sequences. Primer extension analysis of RNA transcripts confirmed the lack of a promoter (results not shown). A putative Shine-Dalgarno sequence was found

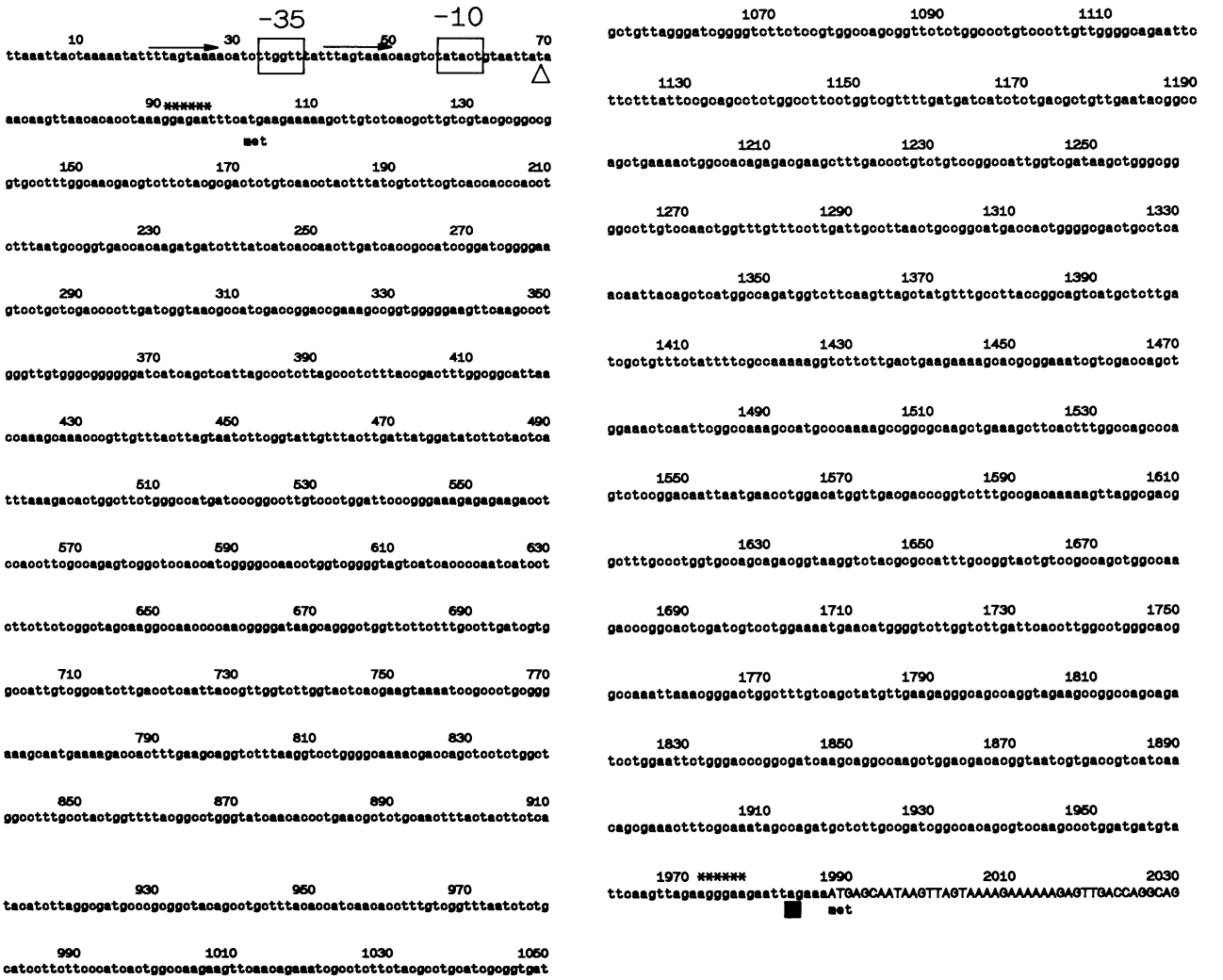

FIG. 3. Nucleotide sequence of the lactose permease gene, its 5′-flanking sequence, and its 3′-flanking sequence which contains the β-galactosidase gene. Only 44 bp of the coding sequence of the β-galactosidase gene are shown (uppercase letters). The complete coding sequence of this gene has been published in reference 27. The −10 and −35 consensus sequences of promoters are boxed. The promoter of the permease gene has been mapped to this region. The start of the transcript is 7 bp away from the −10 consensus sequence (△). The first methionines of the lactose permease and β-galactosidase genes are indicated, as is the stop codon of the lactose permease gene (■). Arrows represent the direct repeats which are present in the flanking sequence of the lactose permease gene. The putative ribosome binding sites of the genes is marked by asterisks.

9 bp from the initiation (ATG) codon. Only 3 bp are present in the intergenic region; therefore, the putative Shine-Dalgarno sequence is present within the coding frame of the permease gene (Fig. 3).

The regions following the stop codons of the permease and β-galactosidase genes were analyzed for possible terminator sequences. Since the β-galactosidase gene immediately follows the permease gene and no terminator was found, it is likely that these two genes are transcribed as one operon. The region following the β-galactosidase gene also did not contain any classical rho-independent terminator sequences. However, no ORF was found downstream as far as 800 bp away.

Amino acid composition and sequence homology of the lactose permease gene. The amino acid composition of the protein was obtained by translation of the nucleotide sequence. Eleven histidine residues were found in the protein. This amino acid residue has been found to play an important role in the transport of lactose (11, 24). The protein contains only one cysteine residue, at position 312.

A homology search with proteins in the NBRF and Swiss protein banks showed several proteins having significant homology to the sequence. Alignment of these proteins by using the GAP program showed that the protein has homology at its amino end, i.e., its first 460 amino acids, to the melibiose transport protein of *E. coli* (34). At the carboxy end it is similar to the PTS phosphoprotein III^{Glc} (16), the *N*-acetylglucosamine transport protein of the PTS system (19, 26), and the β-glucoside transport protein. One protein, the lactose transport protein of *S. thermophilus*, showed a very high homology (80% similarity) throughout the whole sequence. Sequence alignment of these two proteins together with the melibiose transport protein from *E. coli* is shown in Fig. 5. The protein from *S. thermophilus*, which contains 634 amino acids, is slightly larger than that from *L. bulgaricus*, which contains 627 amino acids. The protein

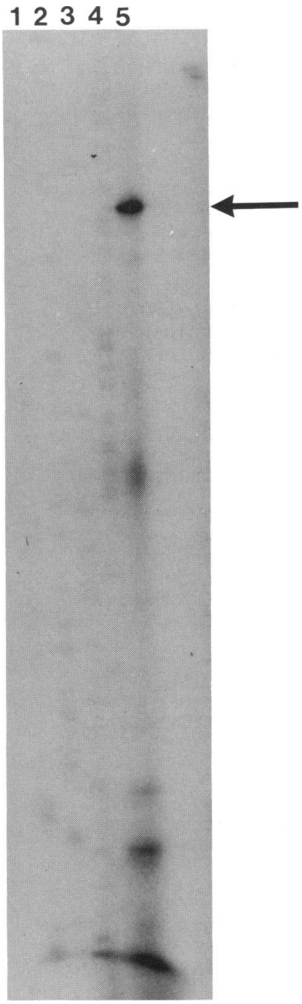

FIG. 4. Primer extension analysis of RNA transcripts from *L. bulgaricus*. The first four lanes are the dideoxy sequencing reactions ACGT, using a PCR-amplified fragment as a template and the kinase-reacted primer that was used for the primer extension reaction as a marker for the start of the transcript. Lane 5 contains the primer extension reaction. The start of the transcript is noted by an arrow.

from *S. thermophilus* was also found to be a hybrid protein with homology to the melibiose transport protein of *E. coli* at the amino terminus and similarity to the PTS transport system at the carboxy end (20). It is important to note that the histidine residues of the melibiose transport protein are not conserved. In particular, His-94, which was found to be the important histidine residue of the melibiose transport protein (22), is missing.

Although the lactose permease (*lacY*) of *E. coli* does not show significant homology, comparison of this protein with the permease from *L. bulgaricus* shows some conserved residues. Two important residues of the lactose permease, His-322 and Glu-325 (2, 3, 11, 24), are both conserved. The third residue, Arg-302, which is also involved in the putative charge relay system (13), is not conserved.

DISCUSSION

We have presented the cloning and sequencing of the lactose permease and β-galactosidase genes from *L. bulgaricus*. The amino acid sequence of the β-galactosidase obtained from translation of ORF2 from pMZ4 is identical to the published sequence from another strain of *L. bulgaricus* (B131) (28) with the exception of an additional lysine at position 905.

The nucleotide sequence of the lactose permease gene was obtained by a combination of sequence information obtained from a clone that contained the lactose permease activity and from an inverted PCR-amplified fragment (see Results). The amino acid sequence obtained from translation of the lactose permease gene had homologies to sequences of several proteins in the data bank. Sequence alignment of the lactose permease with other homologous proteins suggests that its lactose transport function resides at the amino terminus and that there is an as yet uncharacterized control function at the carboxy terminus. The observation that pMZ4 could complement the lactose permease lesion in *E. coli* implies that the first 45 amino acids of the lactose permease are not critical for the lactose transport activity of the protein.

The first 460 amino acids have a net positive charge of 8. This is similar to what is found for the melibiose transport protein, which has a net positive charge of 4, and the lactose permease of *E. coli*, which has a net positive charge of 7. Although the lactose permease bears homology to the melibiose transport protein, comparison of the two proteins shows that His-94, the residue involved in the binding and transport activities of the melibiose transport protein (22), is not conserved. The glutamate residue, Glu-361, which is involved in the Na^+ methyl-β-D-thiogalactopyranoside symport and H^+ melibiose symport of this protein, is conserved (11). No significant homology with the *lacY* protein was found. The comparison of these two proteins shows that the important residues are conserved, suggesting that transport of lactose by this permease is similar to that of the *lacY* protein from *E. coli*.

The carboxy terminus of the protein shows homology to the enzymes of the bacterial PEP-PTS. This second half, containing 210 amino acids, is found to be negatively charged and is less hydrophobic than the amino terminus. Also, the histidine residue of the PTS proteins which are phosphorylated is found to be conserved in this protein. Because the soluble PTS protein III^{Glc} has been found to be involved in the control of lactose transport (16) by binding to the lactose permease (18), this part of the protein may have a role in the control of lactose transport in *L. bulgaricus*.

Analysis of the 5′-flanking region of ORF2 showed several features highlighted in Fig. 3. The −10 and −35 sequences which have been identified upstream from the lactose permease gene have been found to be the promoter used in *L. bulgaricus* by primer extension of the RNA transcripts. The sequences of the promoter used by *L. bulgaricus* are similar to the consensus sequences of those used by *E. coli* and have characteristics of those found in GC-rich organisms (15, 17). Though *L. bulgaricus* is a GC-rich organism, the sequence around the promoter is found to contain a high number of AT base pairs (80%, versus 40% for the coding sequence).

The lactose permeases from *S. thermophilus* and *L. bulgaricus*, both encoded by the lactose operon, are highly homologous. They have homology to the melibiose transport protein and contain the important residues of the lactose permease of *E. coli*. The arrangements of these operons in the two organisms are similar; the lactose permease gene is located in front of the β-galactosidase gene. This arrangement differs from that of the lactose operons of other organisms which also metabolize lactose as a non-PTS

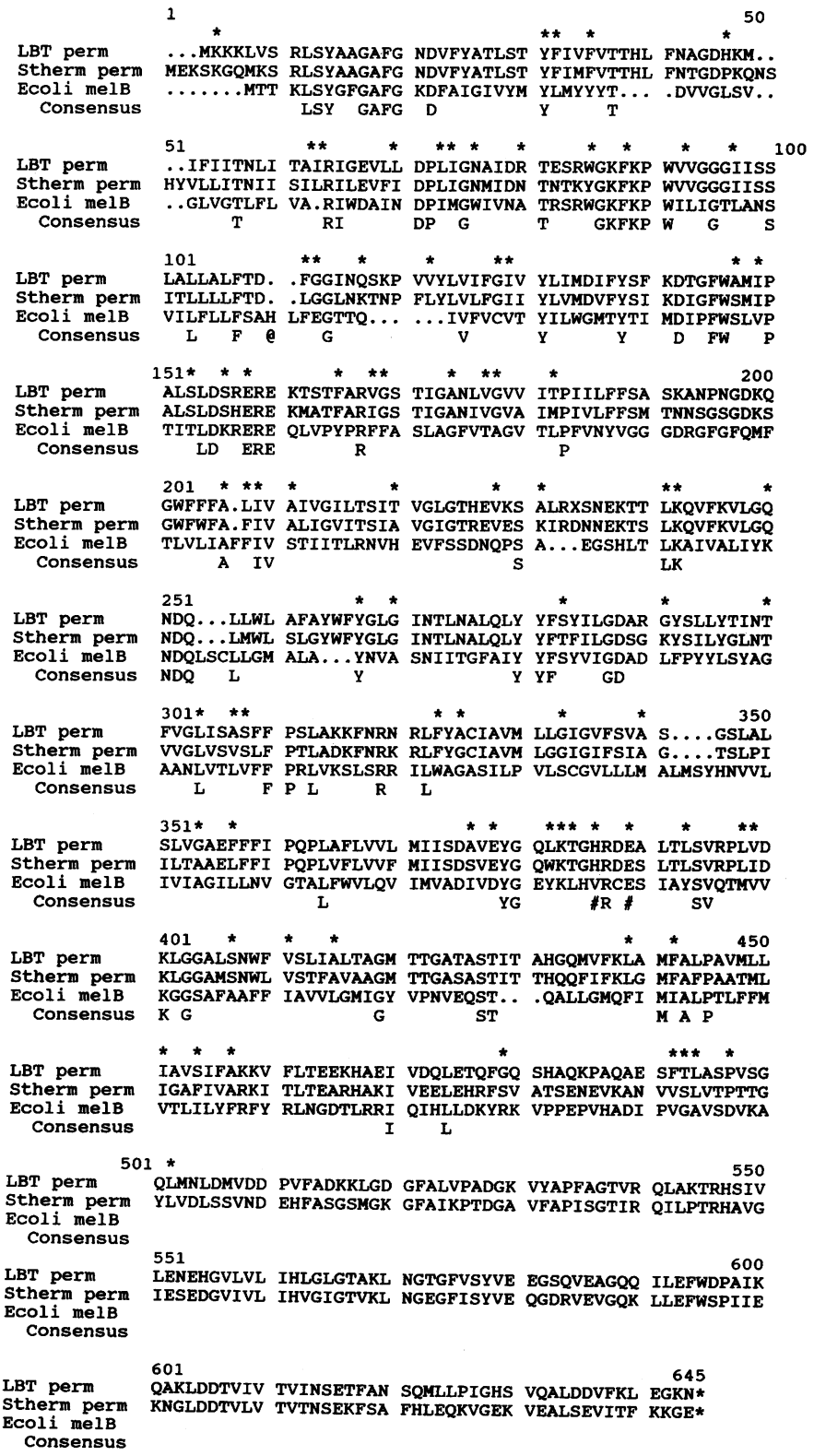

FIG. 5. Sequence alignment of the lactose permease of *L. bulgaricus* with the lactose permease of *S. thermophilus* and melibiose transport protein of *E. coli*. The consensus shows the conserved amino acids among the three proteins. Symbols: *, residue of the lactose permease of *E. coli* that is found to be conserved in the lactose permease of *L. bulgaricus*; @ His-94, the important histidine residue of the melibiose transport protein; #, His-322 and Glu-325, the important residues of the lactose permease of *E. coli*. Alignment of the lactose permease with the PTS enzymes at its carboxy terminus is not shown and is similar to that in reference 20.

sugar; the lactose permease genes in these operons are located after the β-galactosidase genes (1, 6). Because of the similarity of the proteins and unique arrangement of their genes, the lactose operons of *S. thermophilus* and *L. bulgaricus* could have a common origin. Analysis of the flanking sequences around these genes showed no homology to any transposons or insertion sequences. However, there is a repeat of 12 bp which is found before the promoter and after the β-galactosidase gene in *L. bulgaricus*. Interestingly, this repeat is also found in front of the promoter region of the lactose transport gene of *S. thermophilus*.

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