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The *lac* Operon of *Lactobacillus casei* Contains *lacT*, a Gene Coding for a Protein of the BglG Family of Transcriptional Antiterminators

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The 5′ region of the *lac* operon of *Lactobacillus casei* has been investigated. An open reading frame of 293 codons, designated *lacT*, was identified upstream of *lacE*. The gene product encoded by *lacT* is related to the family of transcriptional antiterminator proteins, which includes BglG from *Escherichia coli*, ArbG from *Erwinia chrysanthemi*, SacT, SacY, and LicT from *Bacillus subtilis*, and BglR from *Lactococcus lactis*. Amino acid sequence identities range from 35 to 24%, while similarities range from 56 to 47%. The transcriptional start site of the *lac* operon was identified upstream of *lacT*. The corresponding mRNA would contain in the 5′ region a sequence with high similarity to the consensus RNA binding site of transcriptional antiterminators overlapping a sequence capable of folding into a structure that resembles a rho-independent terminator. LacT was shown to be active as an antiterminator in a *B. subtilis* test system using the *sacB* target sequence. *lacT* directly precedes *lacEGF*, the genes coding for enzyme IICB, phospho-β-galactosidase, and enzyme IIA, and these genes are followed by a sequence that appears to encode a second rho-independent transcription terminator-like structure. Northern hybridizations with probes against *lacT*, *lacE*, and *lacF* revealed transcripts of similar sizes for the *lac* mRNAs of several *L. casei* strains. Since the length of the *lac* mRNA is just sufficient to contain *lacTEGF*, we conclude that the *lac* operon of *L. casei* does not contain the genes of the accessory tagatose-6-phosphate pathway as occurs in the *lac* operons of *Lactococcus lactis*, *Streptococcus mutans*, or *Staphylococcus aureus*.

Uptake of lactose into bacterial cells and initiation of its metabolism can be mediated by several pathways: ABC protein-dependent systems, lactose-galactose antiporters, lactose-H$^+$ symport systems, or the lactose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) (20). While ABC protein-dependent lactose transport has been demonstrated in *Agrobacterium radiobacter* (59), lactose-galactose antiport has been described for *Streptococcus thermophilus* (41). The lactose permease-H$^+$ symport system, the genetics of which represent the paradigm of bacterial operon organization and regulation (31), is found in the enterobacteria, and its function has been studied in *Lactococcus lactis* (34). Lactose permease–β-galactosidase systems have also been identified in other gram-positive species, including lactobacilli (23, 32). The alternative pathway for lactose uptake, however, the lactose-specific PTS (Lac-PTS), has so far been confirmed only in gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus mutans*, dairy lactococci, and *Lactobacillus casei* strains (2, 3, 19, 20, 29, 37, 40, 42, 46). In some *Lactobacillus* species, two pathways, Lactose permease–β-galactosidase and Lac-PTS, have been found to coexist (23, 44).

During PTS-mediated transport, lactose is phosphorylated and then hydrolyzed by phospho-β-galactosidase (P-β-Gal) to glucose and galactose-6-P. While glucose is channeled into the Embden-Meyerhof glycolytic pathway by phosphorylation, galactose-6-P is converted to the triosephosphates via the tagatose-6-P pathway before it also enters the glycolytic pathway, as was first described for *S. aureus* (10).

The plasmid-encoded Lac-PTS operon of *Lactococcus lactis* (19, 55, 56), as well as the chromosomally encoded Lac-PTS operons of *S. aureus* (11, 12, 45) and *S. mutans* (29, 46), recently have been investigated at the molecular level, and their genetic organizations have been elucidated. The operons contain the genes coding for the enzymes of the tagatose-6-P pathway in the same order in addition to the genes of the lactose-specific transport proteins and P-β-Gal. Their transcription is regulated by repressors, with tagatose-6-P being the molecular inducer in *L. lactis* (56).

We have previously reported the isolation and sequencing of *lacE*, *lacG*, and *lacF*, the genes coding for the enzyme IIBC (EIIBC; formerly enzyme II), P-β-Gal, and EIIA (formerly factor III), respectively, of the plasmid-encoded Lac-PTS of *L. casei* 64H (2, 3, 42). We had found a sequence resembling a rho-independent transcription terminator downstream of *lacF*, indicating the 3′ end of the operon. Upstream of *lacE*, we found part of an unidentified open reading frame (ORF), suggestive of an extension of the operon in this direction. To continue the characterization of the Lac-PTS operon with respect to its size, the encoded genes, the gene order, and potential regulatory functions, we investigated the region upstream of the *lacE* gene.

We now present evidence that the *lac* operon in *L. casei* is composed of *lacT*, a gene coding for a protein with similarity to the BglG family of transcriptional antiterminators, and the genes *lacE*, *lacG*, and *lacF*. It does not contain the genes of the tagatose-6-P pathway as do the other known Lac-PTS operons. Mapping of the 5′ end of the *lac* mRNA allowed the identification of the transcriptional start site and the assignment of the putative −10 and −35 regions of the *lac* promoter.

MATERIALS AND METHODS

Bacterial strains and media. *L. casei* subsp. *casei* 64H (14, 24), ATCC 393, ATCC 4646, and ATCC 11578 were a kind gift of B. M. Chassy (University of Illinois at Urbana-Champaign). Stock cultures were maintained at 4°C in calcium carbonate-fortified litmus milk containing 1% glucose (15). Experimental cultures were grown in *Lactobacillus* carrying medium (LCM) supplemented with either 0.5% glucose or 0.5% lactose (21). *Escherichia coli* JM109 was grown in LB (47). Bacteria were plated on media solidified with 1.2% agar. Selection for *E. coli* cells transformed with the appropriate plasmids was by using 100 μg of ampicillin per ml, 1 mM IPTG, and 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. *Bacillus subtilis* GM1042 genotype (*sacXY*Δ*3*

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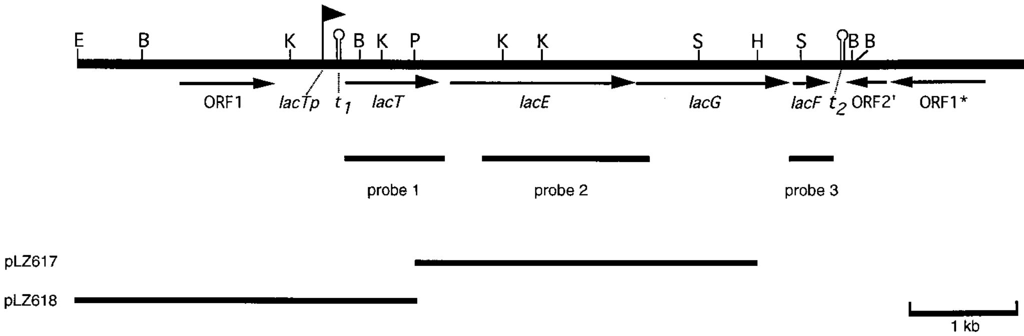

FIG. 1. The genetic organization of the *lac* region of pLZ64 is shown at the top. Marked restriction sites: *Bgl*II (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), and *Sph*I (S). Arrows below the heavy line indicate the extent and direction of identified reading frames. *lacTp* indicates the promoter and transcriptional start site of the operon. t_1 and t_2 represent rho-independent terminators. *lacT* encodes the transcriptional antiterminator, *lacE* encodes EIICB, *lacG* encodes P-β-Gal, and *lacF* encodes EIIA. Lines labeled probe 1, probe 2, and probe 3 indicate the extents of the DNA probes used in the Northern analysis of the mRNA of the operon. Below are indicated the fragments contained in pLZ617 and pLZ618.

*sacB*Δ*23 sacT*Δ*4 amyE::sacB'-lacZ* Phl^r) (36, 52), plasmid pDG148 carrying the *spac* promoter fused to *lacO* preceding a polylinker followed by P_{pen} and *lacI* (53), and a pDG148 derivative with an insert coding for the *E. coli bglG* gene, preliminarily named pDG148-bglG (7), were a kind gift from D. Le Coq, Institut National Agronomique Paris-Grignon, Thiverval-Grignon, France. Selection for *B. subtilis* transformed with pDG148 and derivatives was on LB containing 5 μg of kanamycin per ml. Cultures for β-galactosidase assays were grown in CgCH medium (17) supplemented with tryptophan (50 μg per ml) and kanamycin (5 μg per ml). The test system was induced by adding IPTG to a final concentration of 0.5 mM. β-Galactosidase assays were performed as described by Miller (39).

DNA manipulations. Plasmid DNA was isolated from *L. casei* 64H by the method of Chassy (13). Restriction enzyme digests were performed as recommended by the suppliers of the enzymes. Cloning of PCR products was done with a SureClone ligation kit from Pharmacia (Freiburg, Germany). Other DNA manipulations were performed according to standard procedures as described by Sambrook et al. (47).

DNA sequencing and analysis. The region 5′ to the previously characterized part of the *lac* operon of pLZ64 was cloned as an *Eco*RI-*Pst*I fragment into pUC18, resulting in pLZ618 (3). Using the restriction sites for *Bgl*II and *Kpn*I on the insert, fragments were subcloned into either pUC18 (60) or pTZ18U (38) and sequenced with a ^{T7}Sequencing kit from Pharmacia with universal and reverse primers from GIBCO-BRL (Gaithersburg, Md.). Where necessary, either *Rsa*I subclones were established in pUC18 in order to obtain overlapping sequences or specifically synthesized primers were used. The DNA sequence was established for both strands up to a *Bgl*II site 2,562 nucleotides (nt) upstream of the previously reported sequence (3) that contains a *Pst*I site at the 5′ end. Sequence continuity at the *Pst*I site was confirmed by cloning and sequencing the product of a thermocycling reaction that was performed with primers CA21 (TCTTGTAAGACTGCGG) and CA22 (AGACGATGGCGAGCAGG), using pLZ64 DNA as a template.

DNA sequence assembly and analyses were performed on a VAX computer using the Genetics Computer Group software package (25), while data bank searches were performed by using the BLAST service at the National Center for Biotechnology Information (4).

Cloning of *lacT* for expression and labeling in *E. coli*. The *lacT* gene was amplified in a thermocycling reaction with primers CA40 (GGGATATACATA TGCCAAAAATAGCTCAGA) and CA31 (CTGCAAGCTTGTTATAATGAA TCGT). Primer CA40 introduces an *Nde*I site at the position of the original translation start codon and allows the product to be cloned into expression vectors with an *Nde*I site at the position of the translation start site. Primer CA31 introduces a *Hin*dIII site behind the stop codon of *lacT*. The amplified DNA was cloned in two steps into the expression vector pT7-7. The first construct, pT7-7LacTΔ1, contains the *Nde*I-*Pst*I fragment. In a second step, pT7-7LacT was created by inserting the *Pst*I-*Hin*dIII fragment into pT7-7LacTΔ1. The sequence identity of the amplified inserted DNA was verified by sequencing with specific primers. Expression and labeling of the product with [^{35}S]methionine were performed as described by Tabor and Richardson (54). The bacterial strains and plasmids pGP1-2 and pT7-7 used in these experiments were a kind gift of Stanley Tabor, Harvard Medical School, Boston, Mass.

Cloning of *lacT* for affinity purification. The modified *lacT* gene from pT7-7LacT was cloned into the protein fusion vector pET16b as an *Nde*I-*Hin*dIII fragment. The fusion results in the addition of a decahistidine-containing peptide to the amino-terminal end of the protein, which allows the purification of the modified protein (His-tag-LacT) from crude cell extracts on a metal chelation column essentially in one step (28). Expression in *E. coli* BL21/pLysS and column purification of the protein were performed as recommended by the suppliers of the system (Novagen, Madison, Wis.). Purified protein was dialyzed against 50 mM NH_4HCO_3 and lyophilized.

Preparation of antisera against LacT. Purified His-tag-LacT was used to raise polyclonal antisera in rabbits against LacT. Immunization was performed by Eurogentec, Seraing, Belgium, using standard immunization protocols.

Western blot analysis of LacT expression. Cells were grown in either LCM–0.5% glucose or LCM–0.5% lactose to mid-logarithmic phase, washed with 50 mM Tris-HCl (pH 7.5), and broken by shaking with Zirkonia glass beads in a Retsch Mill MM2 (Retsch, Haan, Germany). Crude cell extracts were separated on 12% polyacrylamide gels, transferred to nylon membranes by semidry electroblotting, and detected with antiserum against LacT according to standard procedures (6).

Cloning of *lacT* for expression in the *B. subtilis* reporter system. The *lacT* gene was amplified in a thermocycling reaction with primers CA63 (CTTTAAGCTT AAGGAGGTGATCTAGATGCCAAAAATAGCTCAGATTTTTAACAACA ACGT) and CA62 (TTCTACGACTGCATGCGCGTTATAATGAATCGTT T). CA63 changes the original translational start codon to an ATG and adds a *B. subtilis* ribosome binding site (RBS) and a *Hin*dIII site to the 5′ end of *lacT*. CA62 introduces behind the stop codon of *lacT* an *Sph*I site. The newly introduced restriction sites allowed the cloning of the amplified fragment in the appropriate orientation into pDG148, resulting in pLACT2801.

RNA isolation and primer extension analysis. The procedures were performed essentially as described by van Rooijen and de Vos (55) except that cells were grown in LCM–20 mM DL-threonine containing 0.5% ribose, glucose, or lactose. For primer extension, the oligonucleotide PLac2 (GCGATTTGTGGTCTCGC CTAATT) was labeled with T4 polynucleotide kinase by using [γ-^{32}P]ATP (3,000 Ci/mmol) and extended with avian myeloblastosis reverse transcriptase (6). The product of the extension reaction was electrophoresed on a sequencing gel parallel to a standard sequencing reaction which was primed with the same oligonucleotide.

Hybridization. Probes used in the Northern blot experiments were labeled in a thermocycling reaction using the deoxynucleotide triphosphate-labeling mix of a DIG-DNA Labeling and Detection kit from Boehringer (Mannheim, Germany) and pLZ64 as a template. Primers were CA40 and CA31 for probe 1, which hybridizes with *lacT*; CA6 (CCATGGGCTTGCTGGCACTG and CA8 (GCCCCGCCCATTACAAA) for probe 2, which hybridizes with *lacE* and overlaps the 5′ part of the reading frame of *lacG* for 42 nt; and LacF1 (GAGTGA GACCCACAT) and P7 (GCGAATCATGTAGCGTGTTTCATCA) for probe 3, which hybridizes with *lacF* and overlaps the 3′ end of the reading frame of *lacG* for 24 nt. RNA was glyoxylated and size fractionated on 1% agarose gels that were also loaded with an RNA size standard (0.24- to 9.5-kb RNA ladder; GIBCO BRL). The lane containing the standard was cut off, stained with ethidium bromide, and used as a reference. The RNA in the remaining portion of the gel was transferred by capillary blotting to a nylon membrane (Quiagen GmbH, Düsseldorf, Germany). Hybridization of the probes and visualization of the bands were done according to the instructions for the Boehringer DIG Luminescent Detection kit.

Nucleotide sequence accession number. The reported nucleotide sequence has been assigned GenBank accession number U21391.

RESULTS

DNA sequence determination. The DNA sequence upstream of the previously reported *lacEGF* operon of *L. casei* was determined from the *Eco*RI-*Pst*I fragment of pLZ64 contained in pLZ618 (3) up to the *Bgl*II site which is located 2.6 kb upstream of the *Pst*I site (Fig. 1). Continuity of the sequence with the fragment of pLZ64 contained in pLZ617 (3) was confirmed by determining the sequence of an overlapping fragment obtained by using pLZ64 as a template in a thermocycling reaction and primers that hybridize on either side of the *Pst*I site. We report here the results for the sequence determination up to the *Kpn*I site 1.16 kbp upstream of the *Pst*I site which are relevant to the organization of the *lac* operon.

DNA sequence analysis. An extended intergenic region precedes putative −35 (TTTACA) and −10 (TACAAT) sequences of a potential transcriptional start site that can be identified at nt 360 to 365 and from nt 382 to 387, respectively, by their similarity to −35 and −10 sequences recognized by σ^{70} of *E. coli* and σ^A of *B. subtilis* (Fig. 2). The putative promoter region is followed by an imperfect inverted repeat at nt 441 to 481 and a hexanucleotide set of T's, indicating that this sequence could represent a potential rho-independent terminator. The free energy for the folding of this stem-loop structure was calculated to be −99.7 kJ.

A putative RBS sequence, GGAGG, is located at nt 497 to 501, leaving a distance of 10 nt to an ORF, which starts at nt 512 with the codon TTG and continues beyond the *Pst*I site to nt 1390, where it stops with the codon TAA. While the spacing of 10 nt between the RBS and the translation initiation site falls within the typical range of 6 to 10 nt, the start codon TTG appears to be used infrequently by lactobacilli. Of 70 *Lactobacillus* genes described at the sequence level, representing more than 10 species, 4 have been found to start with GTG and three have been found to start with TTG (43).

The ORF codes for a protein of 292 amino acids with a molecular mass of 33.9 kDa. The translated polypeptide has similarity to the family of proteins that have been identified as transcriptional antiterminators in the regulation of the transcription of several PTS operons. Pairwise comparisons of the amino acid sequences reveal 35, 32, 31, 29, 24, and 24% identities and 56, 53, 50, 52, 51, and 47% similarities to BglG (50), LicT (49), ArbG (22), SacT (18), SacY (61), and BglR (9), respectively (Fig. 3). The ORF was assigned the mnemonic *lacT*.

The identification of LacT as a member of the family of transcriptional antiterminators, as well as the location of a putative transcriptional start site upstream of a rho-independent terminator, is complemented by the identification of a region with high similarity to the ribonucleic antiterminator (RAT) sequences which have been previously demonstrated to be the target regions for the binding of, e.g., BglG, SacT, SacY, and LicT (8, 30, 36, 49) (Fig. 4). The RATs have been proposed to form alternative, less stable stem-loop structures than the following and partly overlapping terminators. The binding of the antiterminator proteins to these RATs was proposed to prevent the formation of the more stable terminator structure and to allow the transcription to proceed beyond the stop signal. As was observed with other RAT sequences, the putative *lacT* RAT partially overlaps the 5′ stem of the terminator. In the case of the *lacT* RAT, this stem of the folded RAT could be extended for another 3 bp (Fig. 2 and 4).

Determination of the size of the Lac-PTS operon. The arrangement of the putative promoter in front of a RAT and a potential rho-independent terminator which is followed by *lacT* indicated that this is probably the first gene of the Lac-PTS operon. To confirm this hypothesis, the sizes of the *lac* mRNAs of different *L. casei* strains were determined. Hybridization with probes against *lacT*, *lacE*, and *lacF* revealed bands migrating identically (Fig. 5). Since the probes are directed against sequences which are near the presumed 5′ and 3′ ends as well as against a region within the operon, it is unlikely that smaller alternative transcripts would not have been detected, except if the amount of an alternate *lac* mRNA is significantly lower than that of the complete operon. The size of 4.4 kb determined for the *lac* mRNA is in good agreement with the expected size of 4.8 kb for the transcript, if the operon extends from *lacTp* to the rho-independent terminator t_2 (Fig. 1).

```
        KpnI     .         .         .         .         .
    1   GGTACCATCAGTAGAGGCATCGTCGATGAGCCAAAGTTCAAAATCAATTGTTTGTTGCTG   60

                 .         .         .         .         .         .
   61   GATACTTTTTAATGTCTCAGGCAGATAATGACCTAAATTGTAAGTTGGCACAACGATGGT   120

                 .         .         .         .         .         .
  121   TAAATCTAAGTTCGACGTAATTGCCTCCTTGAAGCTCGTTTTGATCATTATCGTCAAATT   180

                 .         .         .         .         .         .
  181   AACGGAGGCGTCAAATAAAGTGATTCGTTTGTAGAGACATGACTAGCACTGATCATTAAA   240

                 .         .         .         .         .         .
  241   GAACAAAAGGCAACACAGATAATTGTCCTAAAAAACTGACGATGGCTTGCTGGATGGACA   300

                 .         .         .         .         .         T
  301   CTGTTGAAAAAGAAAAAAGAACAGCAAAACTAAAGATGCTACAAAAATAAAATAAAACGT   360
                                                           ATAAAGCGT
         -35                  -10
        TGACA    .         . TATAAT  .   ===>  .         . •••******
  361   TTACATTCTGTTCAGATATGGTACAATTTGTTTGTTGATAGGAATATTCAATGTGGATTG   420
        TTTCA
                            >>>>>>>>>>>> >>>>>> t1 <<<<<<<<<<<<<<<<<
        ***********************•••   .         .         .         .
  421   TGACTATTTAATTAGGCGAGACCACAAATCGCAGTGCTGATCGCAGCGCGTGATTTGTGG   480

        <
                 .      RBS          . lacT--->.         .         .
  481   TTTTTTCTTTGCACTGGGAGGGGATGACAACTTGCCAAAAATAGCTCAGATTTTTAACAA   540
    1                                   M  P  K  I  A  Q  I  F  N  N   10

                 .         .         .         .         .         .
  541   CAACGTGGCCTTGGTTGATCTAGACAACCGCGGCCAAGCCGTTGTAAGGGGACGTGGCAT   600
   11     N  V  A  L  V  D  L  D  N  R  G  Q  A  V  V  R  G  R  G  I   30
                 .         .         .         .         .BglII    .
  601   CGCTTTTCAGAAGAGGCGAGGAGATGTTATTCCGACAAAGCAGATAGAGAAGATCTTTTA   660
   31     A  F  Q  K  R  R  G  D  V  I  P  T  K  Q  I  E  K  I  F  Y   50

                 .         .         .         .         .         .
  661   TCTAGCGAACGAGACTTCCCGACAAAATTTGTACTTTCTCTTAAAAAATATTCCGATTGA   720
   51     L  A  N  E  T  S  R  Q  N  L  Y  F  L  L  K  N  I  P  I  D   70

                 .         .         .         .         .         .
  721   CGTGGTGACGACTACCTATGAAATTATTGATGTTGCCCAGAAACAATATCGACTGAAAGT   780
   71     V  V  T  T  T  Y  E  I  I  D  V  A  Q  K  Q  Y  R  L  K  V   90

                 .         .         .         .         .         .
  781   GCTTGATTATATCTACATTACCTTGAGTGATCATATTTACGAGGCATATAAACGCTATCA   840
   91     L  D  Y  I  Y  I  T  L  S  D  H  I  Y  E  A  Y  K  R  Y  Q   110
                 .         .    KpnI .         .         .         .
  841   GGCAGGGACTTATCAAGAAACAATGGTACCAGATTTTCATATTCAATATCCGGCCGAATA   900
  111     A  G  T  Y  Q  E  T  M  V  P  D  F  H  I  Q  Y  P  A  E  Y   130

                 .         .         .         .         .         .
  901   TGCGGTGGCTAAACAGGCACTGCAAATCATTGCCACGAACCTTGGCGTTCAGTTTCCACA   960
  131     A  V  A  K  Q  A  L  Q  I  I  A  T  N  L  G  V  Q  F  P  Q   150

                 .         .         .         .         .         .
  961   GTCGGAAATAAAAAATCTGGCGTTGCACTTTATCAACGCTTCCGGCGAAGACGATGGCGA   1020
  151     S  E  I  K  N  L  A  L  H  F  I  N  A  S  G  E  D  D  G  E   170

                 .         .         .         .         .         .
 1021   GCAGGTTTTTGGTAAAAGCAATGAAGCCTCACTTAGTCAACTTGTACAAGAAGTGTTGAA   1080
  171     Q  V  F  G  K  S  N  E  A  S  L  S  Q  L  V  Q  E  V  L  K   190

                 .         .         .         .         .         .
 1081   GCGTCATCACATTACTCGCTCTCATTCAAATGGCAACTACTATGACCGATTTATGATTCA   1140
  191     R  H  H  I  T  R  S  H  S  N  G  N  Y  Y  D  R  F  M  I  H   210
                 .         .   PstI  .         .         .         .
 1141   TCTCCAGTATCTCATCGACCGACTGCAGCGTGTTGATACATATGCCGTTACCATTGTCCC   1200
  211     L  Q  Y  L  I  D  R  L  Q  R  V  D  T  Y  A  V  T  I  V  P   230

                 .         .         .         .         .         .
 1201   TGAGGTTGCCACTGAACTTAAGCAAAACTATCCGCAGTCTTACAAGATTGCCTCAGAAAT   1260
  231     E  V  A  T  E  L  K  Q  N  Y  P  Q  S  Y  K  I  A  S  E  I   250

                 .         .         .         .         .         .
 1261   TTTCGATGAAATTAAGGATCAACTCTATCGCAGTATGAGTGAGGACGAACGACTTTACTT   1320
  251     F  D  E  I  K  D  Q  L  Y  R  S  M  S  E  D  E  R  L  Y  F   270

                 .         .         .         .         .         .
 1321   CATCATCCACATTCAGCGATTGATAAACGAAGCACCAGCCCAGAATCATTCACAAAACGA   1380
  271     I  I  H  I  Q  R  L  I  N  E  A  P  A  Q  N  H  S  Q  N  D   290

                 .         .         .         .         .         .
 1381   TTCATTATAACGCGCTCGCAGTCGTAGAAGCCTACACATAAGGGCTTTGAAGCAATCTAC   1440
  291     S  L  *
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FIG. 2. The DNA sequence found upstream of *lacE* and predicted primary amino acid sequence of LacT. The sequence presented overlaps at the *Pst*I site with that previously reported (3). The potential RBS is underlined. Putative −35 and −10 regions are also underlined and indicated. *Lactobacillus* consensus sequences are given above the sequence. Boldface letters mark highly conserved nucleotides with conservation of more than 75%; the other nucleotides are between 50 and 75% conserved. The sequence of the O1 element of the *acuABC* promoter region of *B. subtilis* is given below the −35 region. ===>, the transcriptional start site of the operon (the first nucleotide of the mRNA is underlined); *, the RAT region; ·, flanking nucleotides potentially extending the stem of the folded RAT structure; >>> and <<<, residues of the inverted repeats of the rho-independent terminator.

Identification of the transcriptional start site of the operon. The results of the Northern hybridizations and the presence of

```
      1                                                      50
LacT  .MPKIAQIFN NNVALVDLDN RGQAVVRGRG IAFQKRRGDV IPTKQIEKIF
BglR  ..MKIKKVLN NNVV.IAQND NEETILMSLG LGFGKKAGEV VEDKKIEKIF
BglG  MNMQITKILN NNVVVVIDDQ QREKVVMGRG IGFQKRAGER INSSGIEKEY
ArbG  ..MKIAKILN NNVVTVMDEQ NNEQVVMGRG LGFKKRPGDT VNAALIEKIF
SacY  ..MKIKRILN HNAI.VVKDQ NEEKILLGAG IAFNKKKNDI VDPSKIEKTF
SacT  ..MKIYKVLN NNAA.LIKED DQEKIVMGPG IAFQKKKNDL IPMNKVEKIF
LicT  ..MKIAKVIN NNVISVVNEQ GKELVVMGRG LAFQKKSGDD VDEARIEKVF

      51                                                    100
LacT  YLANETSRQN LYFLLKNIPI DVVTTTYEII DVAQKQYRLK VLDYIYITLS
BglR  ALKVTPEQQN FSELLSEIPS GIVELSILTL AKAKTKFDKT ISDTVLVAFA
BglG  ALSSHELNGR LSELLSHIPL EVMATCDRII SLAQERLG.K LQDSIYISLT
ArbG  SLRSSELTAR LSDVLERIPL EVVTTADRII ALAKEKLGGN LQNSLYISLT
SacY  IRKDTPDYKQ FEEILETLPE DHIQISEQII SHAEKELNIK INERIHVAFS
SacT  VVRD..ENEK FKQILQTLPE EHIEIAEDII SYAEGELAAP LSDHIHIALS
LicT  TLDNKDVSEK FKTLLYDIPI ECMEVSEEII SYAKLQLGKK LNDSIYVSLT

      101                                                   150
LacT  DHIYEAYKRY QAG.TYQETM VPDFHIQYPA EYAVAKQALQ IIATNLGVQF
BglR  DHLNAAIIRE KDNITIKNFL LWDIKRFFPE EFALCLETLQ KVQEKLNISL
BglG  DHCQFAIKRF QQNVLLPNPL LWDIQRLYPK EFQLGEEALT IIDKRLGVQL
ArbG  DHCHFAIERH RQGVDIRNGL QWEVKRLYQK EFAIGLDALD IIHRRLGVRL
SacY  DHLSFAIERL SNGMVIKNPL LNEIKVLYPK EFQIGLWARA LIKDKLGIHI
SacT  DHLSFAIERI QNGLLVQNKL LHEIKALYKK EYEIGLWAIG HVKETLGVSL
LicT  DHINFAIQRN QKGLDIKNAL LWETKRLYKD EFAIGKEALV MVKNKTGVSL

      151                                                   200
LacT  PQSEIKNLAL HFINASGEDD GEQVFGKSNE ASLSQLVQEV LKRHHITRSH
BglR  PEDEAGFLAM HIVNG..TLG .SGHEYATEL TKLMEEILTT LKYTLQVNFN
BglG  PKDEVGFIAM HLVSA..QMS .GNMEDVAGV TQLMREMLQL IKFQFSLNYQ
ArbG  PEDEAGFIAL HLVNA..QLD .SHMPEVMRI TRVMQEILNI VKYQLNLDYN
SacY  PDDEIGNIAM HIHTA..RNN AGDMTQTLDI TTMIRDIIEI IEIQLSINIV
SacT  PEDEAGYIAL HIHTA..KMD AESMYSALKH TTMIKEMIEK IKQYFNRKVD
LicT  PEDEAGFIAL HIVNA..ELN .EEMPNIINI TKVMQEILSI VKYHFKIEFN

      201                                                   250
LacT  SNGNYYDRFM IHLQYLIDRL QRVDTYAVTI VPEVATELKQ NYPQSYKIAS
BglR  EQDIYFQRFI THLKFFTERI LSNTKSDEST DEDLFLLITR KYPRAYIGTK
BglG  EESLSYQRLV THLKFLSWRI LEHA.SINDS DESLQQAVKQ NYPQAWQCAE
ArbG  EQAFSYHRFV THLKFFAQRL LGRT.PVFSE DESLHDVVKE KYTLAYHCAE
SacY  EDTISYERLV THLRFAIQHI .KAGESIYEL DAEMIDIIKE KFKDAFLCAL
SacT  ENSISYQRLV THLRYAVSRL .ESNEALHRM DEEMLYFIQK KYSFAYQCAL
LicT  EESLHYYRFV THLKFFAQRL FNGT.HMESQ DDFLLDTVKE KYHRAYECTK

      251                                         295
LacT  EIFDEIKDQL YRSMSEDERL YFIIHIQRLI NEAPAQNHSQ NDSL*
BglR  KSVSFLNRQV RTKFLKMDKY I*
BglG  RIAIFIGLQY QRKISPAEIM FLAINIERVR KEH*
ArbG  KIQDHIMLHY DYTLTKEELM FLAIHIERVR SELQEQTAE*
SacY  SIGTFVKKEY GFEFPEKELC YIAMHIQRFY QRSVAR*
SacT  ELAEFLKNEY QLHLPESEAG YITLHVQRLQ DLSE*
LicT  KIQTYIEREY EHKLTSDELL YLTIHIERVV KQA*
```

FIG. 3. Alignment of LacT with other identified antiterminator proteins. Amino acid residues identical in LacT and at least one other protein are indicated by a shaded background.

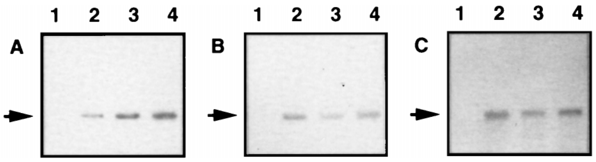

FIG. 5. Northern blot analysis of *lac* mRNA from different *L. casei* strains. (A) Signals obtained with probe 1; (B) results obtained with probe 2; (C) results obtained with probe 3. Lanes: 1, RNA from *L. casei* 64H grown on glucose; 2, RNA from *L. casei* 64H grown on lactose; 3, RNA from *L. casei* ATCC 393 grown on lactose; 4, RNA from *L. casei* ATCC 4646 grown on lactose. Arrows indicate positions of *lac* mRNA. For descriptions of the probes, see Fig. 1 and the text.

putative −35 and −10 regions upstream of *lacT* led to the hypothesis that the transcriptional start site must be located in front of the potential terminator. Therefore, a primer extension analysis was performed with primer PLac2, which anneals at nt 452 to 430. The size of the major product of the reaction indicates that the 5′ end of the mRNA corresponds to the G in position 394 of the DNA sequence (Fig. 2 and 6), leaving a distance of 6 nt between the last base of the −10 region and the transcriptional start site, which falls within the range of previously determined distances of mapped *Lactobacillus* transcription start sites (43). The amounts of products are comparable for the reactions from cells grown on the noninducing, nonrepressing substrate ribose and the inducing substrate lactose, while the result of the glucose reaction could suggest that a potential catabolite repressing effect acts at the level of transcription initiation.

```
                  1       10                        20       29
                  *        *                         *        *
L.ca. lacT     UGUGGAUUGUGACUAUUUAA                UUAGGCGAGACCACA
B.su. sacB        GGUUUGUUACUGAUAAA                GCAGGCAAGACC
B.su. sacPA       GGAUUGUGACUGGUAAA                GCAGGCAAGACC
B.su. bglF        GGAUUGUUACCGCACUA A              GCGGGCAAAACC
B.su. bglP        GGAUUGUUACUGCGAAA                GCAGGCAAAACC
B.su. licS        GGAUUGUUACUGAUAAA                GCAGGCAAAACC
L.la. bglR        GGAUAGUGAUUAUUAAG                UUAAGCUAGACC
E.co. bglG        GGAUUGUUACUGCAUUC                GCAGGCAAAACC
E.ch. stb1        GGGUUGCUACUGCCAUU G              GCAGGCAAAACC
E.ch. stb2A       GGAUUGCGACUGUAUAU CCCUCA         GCGGGAAAUACA
E.ch. stb2B       GGAUUGCGACUGUAUAU CCCUCAGCGGAAAU ACAGGCAAAACC
```

FIG. 4. Alignment of the RAT sequences of other antiterminators with the *lacT* RAT sequence. Nucleotides identical in *lacT* RAT and at least one other sequence within the RAT region are indicated by a shaded background. Numbers refer to positions within the RAT region. *L. ca.*, *L. casei*; *B. su.*, *B. subtilis*; *L. la.*, *L. lactis*; *E. co.*, *E. coli*; *E. ch.*, *E. chrysanthemi.*

Expression of *lacT* in *L. casei*. Although LacT is produced in *E. coli* when the gene is under control of the T7 expression system (results not shown), it remained to be shown that it is actually expressed in *L. casei*. Probing of extracts from *L. casei* cells in Western blots with polyclonal antiserum raised against LacT demonstrated that the level of *lacT* expression is low in *L. casei* 64H and too low to be detected in several other strains when growth is supported by glucose, while the protein is synthesized in the presence of lactose (Fig. 7).

Antitermination activity of LacT. The RAT sequences of various antitermination systems are similar (Fig. 4), and several antiterminators have been shown to recognize heterologous RAT sequences (9, 36, 49). Since there is so far no efficient system for the introduction of specific mutations in lactobacilli, the *B. subtilis* reporter system of Le Coq et al. (36) was used to demonstrate the antitermination activity of LacT. The chromosome of *B. subtilis* GM1042 carries a tripartite fusion: the constitutive promoter *trpEp* is fused to part of the *sacB* leader region which contains the *sacB* RAT(4M)-termi-

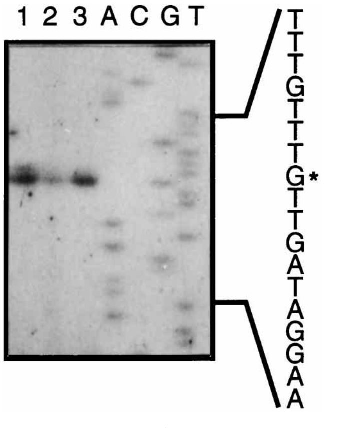

FIG. 6. Primer extension analysis of *lac* mRNA. Lanes 1, 2, and 3 contain the products of extension reactions obtained with total RNA isolated from ribose-, glucose-, and lactose-grown cells of *L. casei* 64H, respectively. Lanes A, C, G, and T contain the reaction products of a sequencing reaction performed with the same primer. The DNA sequence of the relevant region is shown at the right. The asterisk indicates the nucleotide corresponding to the main extension product in lanes 1 to 3.

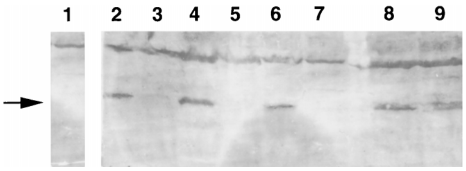

FIG. 7. Western blot analysis of cell extracts from different *L. casei* strains separated on a sodium dodecyl sulfate–12% polyacrylamide gel. Lanes: 1, extract of a plasmid-cured strain of *L. casei* 64H; lanes 2 and 3, ATCC 4646; 4 and 5, ATCC 393; 6 and 7, ATCC 11578; 8 and 9, 64H. Lanes 2, 4, 6, and 8 contain extracts of cells grown lactose; lanes 3, 5, 7, and 9 contain extracts of cells grown on glucose.

nator module and the very beginning of the *sacB* coding sequence fused in frame to *lacZ* (8, 36). Furthermore, the genes coding for the antiterminators SacY and SacT have been deleted from the chromosome. LacT is expressed from pLACT2801 carrying the *spac* promoter fused to *lacZo*, which is followed by *lacT*. It is controlled by the gene product of *lacI*, the transcription of which is driven by the constitutive P_{pen} promoter, which is also present on the plasmid. Expression of β-galactosidase activity, i.e., antitermination at the *sacB* RAT, was dependent on the expression of LacT or of BglG, the prototype antiterminator protein. While expression levels with the *bglG* clone were higher in the induced and uninduced samples than in the *lacT* samples, the ratios of induction were comparable with the two antiterminators (Table 1).

DISCUSSION

Several Lac-PTSs have recently been characterized by molecular cloning and sequencing, and their transcriptional regulation has been studied. The gene organizations of the *lac* operons of *S. aureus*, *S. mutans*, and *L. lactis* were found to be almost identical. The three operons include the genes coding for the proteins of the tagatose-6-P pathway (*lacABCD*) that are required after hydrolysis of the lactose-P by LacG for channeling the galactose-6-P moiety into the glycolysis, in addition to the genes coding for the transport proteins (Fig. 8). Regulation of transcription of these operons is mediated by repressors. Comparison of the previously identified genes of *L. casei* that are related to lactose metabolism, i.e., *lacE*, *lacG*, and *lacF*, revealed that although there is significant similarity to the proteins of the other systems, the *lac* genes and gene products of the lac-PTSs of *S. aureus*, *S. mutans*, and *L. lactis* are more closely related to one another than to those of the *L. casei* system, while the *L. casei* PTS is almost equidistantly related to the others. A different gene order was observed in *L. casei* as well. *lacF* is the last gene in *L. casei*, while in the other systems, *lacE* is situated at the 3′ terminus.

Since we have cloned and analyzed the genes of the lactose-specific transport proteins of the PTS of *L. casei*, we were interested to find out if this operon also contains the genes of the tagatose-6-P pathway and a repressor similar to those found in other bacteria. Information about the details of these regulatory elements would be of interest, as knowledge about regulation of gene expression in lactobacilli at the molecular level is still very limited. Since previous results indicated that the operon probably ends downstream of *lacF*, we investigated the region upstream of *lacE*. We report here the identification of the 5′ end of the operon and of *lacT* as its 5′ gene.

TABLE 1. Expression of *sacB′-lacZ* fusion in GM1042

Plasmid	β-Galactosidase activity[a]		Ratio of induction
	−IPTG	+IPTG[b]	
pDG148	2.1	2.2	1.0
pDG148-bglG	3.6	21.3	5.9
pLACT2801	2.5	15.4	6.2

[a] Expressed in Miller units (39).

[b] Induction was with 0.5 mM IPTG. Only extracts from induced cells showed a LacT signal in Western blots (results not shown).

The identification of *lacT* as the first gene of the *lac* operon in *L. casei* is based on the following findings. (i) Preceding the *lacT* gene there is an extended region of apparently noncoding sequence, which extends 142 nt beyond the *Kpn*I site for a total of more than 500 nt with no identifiable function before it is interrupted by an ORF (Fig. 1) (1). (ii) In the hybridization experiments, one species of *lac* mRNA, the size of which is in agreement with the expected value when the translation starts in front of *lacT* and ends at the terminator downstream of *lacF*, was detected with three probes which anneal at different positions within the operon. (iii) A transcriptional start site has been identified just upstream of the terminator which precedes *lacT*. As the hybridization experiments do not give any indication for another transcript of the operon, we propose that it is the only transcriptional start site of the operon of any significance.

The Northern blots show only signals with RNA isolated from induced cells, while the primer extension indicates that mRNA is present in induced and uninduced cells. This result would be expected if LacT is active as an antiterminator at the *lacT* RAT-t_1 element. The part of the message which served as the template in the primer extension experiments should always be synthesized, while the extension beyond t_1 would be dependent on the antitermination activity of LacT. Northern blotting and primer extension experiments cannot exclude the possibility that a large transcript can be synthesized and then processed and degraded before expression of the *lac* genes is effective in the uninduced state, leaving intact only the small transcript as a template for primer extension. LacT would then in the induced state prevent processing at t_1 by binding to the RAT, allowing in turn efficient translation of the structural genes. This scenario seems very unlikely, however, since Arnaud et al. showed recently in an elegant in vitro transcription-antitermination experiment with purified RNA polymerase holoenzymes from either *E. coli* or *B. subtilis* that SacT or SacY is required as an additional component in order to obtain transcription beyond the SacT-regulated terminator of the *sacPA* operon of *B. subtilis* (5). Without the antiterminator proteins, transcription would be efficient only up to the terminator. Since the structural elements of the various antitermination systems are all very similar, one might postulate that they all act similarly on their specific RATs and terminators and that no processing of the mRNA is involved in the regulation of these systems.

The activity of LacT as an antiterminator in the test system of Le Coq et al. (36) compares favorably with the activity of the prototype antiterminator BglG in this system. This finding is in agreement with the observations for several other antiterminators, which have been demonstrated to be able to antiterminate at RAT sequences other than their genuine target sequences (9, 36, 49).

The hybridization experiments indicate that the *lac* operons of the tested *L. casei* strains are similarly organized. They all contain *lacT*, *lacE*, and *lacF* and very probably also *lacG*, since this gene is contained at least in the *lac* operon of 64H, and they all demonstrated mRNA species of identical size with the different probes. Based on the 4.4-kb transcript observed in the strains studied, it appears that the genes of the tagatose-6-P

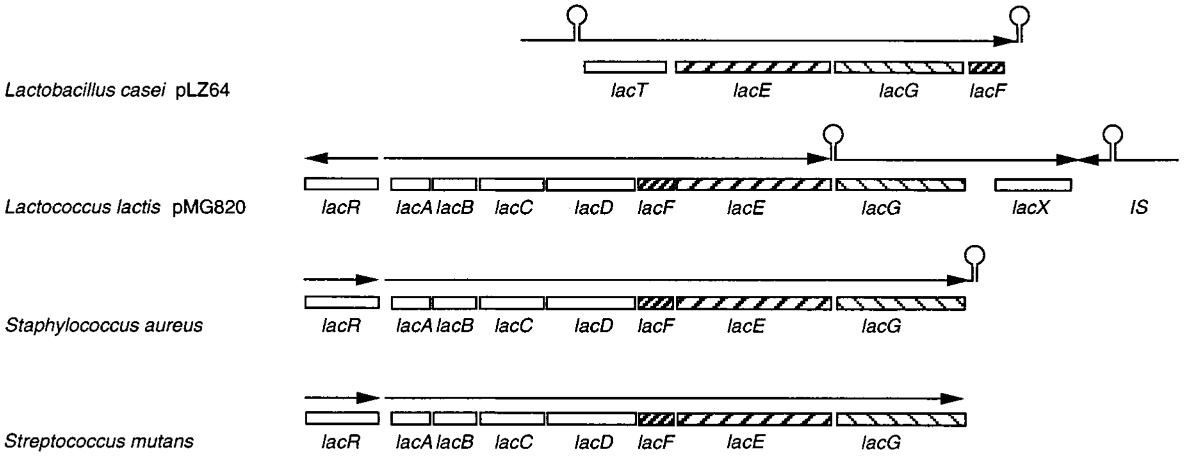

FIG. 8. Comparison of the organizations of the Lac-PTS operons. Arrows indicate directions of transcription and the lengths of transcriptional units. In *L. lactis*, two mRNA sizes for the *lac* operon, one comprising *lacA* to *lacE* and the other comprising *lacA* to *lacX*, were detected. Hairpins indicate rho-independent terminators.

pathway are not part of the operon. This finding is strengthened by the observation that directly downstream of t_2 there is a sequence of 366 nt with greater than 95% identity to IS*1165* from *Leuconostoc mesenteroides* (33) containing a partial ORF that runs opposite to the transcriptional direction of the *lac* operon. It is followed by a sequence of 870 nt which includes an ORF with similarity to the transposase of insertion sequence elements from *Acetobacter xylinum* and *Rhizobium meliloti* which also runs in opposite direction to the *lac* operon (1, 16, 51) (Fig. 1). PCR and hybridization experiments indicate that this arrangement is true for other strains as well (1). From this finding, it appears very unlikely that the genes of the tagatose-6-P pathway, if they were present on pLZ64, could be part of the *lac* operon. Since the ORF upstream of the *lac* operon is identical to the putative insertion sequence element on the 3′ side, it is tempting to speculate that this arrangement could constitute a transposable *lac* element.

lacT is located downstream of the conditional terminator t_1 (Fig. 1). Its expression would therefore be predicted to be dependent on the presence of lactose and should correlate with the expression of the Lac-PTS proteins. This is demonstrated by the Western blots, which essentially gave positive signals only with extracts from cells grown on lactose, not from cells grown on glucose. An exception is strain 64H, which exhibited some LacT expression and Lac-PTS activity during growth on glucose. Because the operons of the strains tested appear to be equally equipped with respect to their gene contents, the differences in Lac-PTS expression could depend on point mutations or small differences in sequence in the regions 5′ to the operon or even within the operon as well as in other components potentially interacting with the regulatory components.

The Lac-PTS has been found also to be subject to carbon catabolite repression, which could be mediated by a component of the glucose transport system of the mannose-specific PTS type or by intermediates of glucose metabolism (15, 57), since mutations in the mannose-specific PTS were found to relieve the repressive effect of glucose metabolism on Lac-PTS induction. It is interesting in this regard that the EIIA domains of the Nag and Bgl systems in *E. coli* can functionally replace EIIA^{Glc} (58) and that the *bgl* operon has been found to be subject to EIIA^{Glc}-dependent cross-regulation: EIIA^{Glc} was demonstrated to be able to complement a deleted EIIA^{Bgl} domain of EIIBCA^{Bgl} not only in its function during β-glucoside transport but also in the reaction leading to the phosphorylation of BglG in the absence of β-glucosides and/or glucose (48). It is tempting to speculate that the repressive effect of glucose metabolism in *L. casei* depends on a mechanism similar to that for the Bgl system in *E. coli*. An alternative or even additional effect of catabolite repression might be mediated by a mechanism similar to the *ccpA*/*amyE* system of *B. subtilis*, in which the gene product of *ccpA* binds to the *cis*-acting element *amyO* during glucose repression (27). Several elements of the *amyO* type have been identified in gram-positive operons such as the O1 element in the *acuABC* promoter region of *B. subtilis* (26). A sequence similar to this *cis*-acting element is located at nt 352 to 365 of the sequence of the *lacT* region (Fig. 2). It overlaps the complete −35 region of the *lac* promoter, an arrangement very similar to those of O1/*acuABC* and especially the *bglPH* operon in *B. subtilis*. The LicT-controlled *bglPH* operon has recently been demonstrated to be subject to carbon catabolite-dependent regulation at the level of initiation of transcription in addition to dependence on antitermination activity of LicT (35). The results of the primer extension experiment presented here suggest that this mechanism might be active in *L. casei* as well: the reaction products from cells grown on the noninducing, nonrepressing substrate ribose and the inducing substrate lactose show comparable signal levels, while the signal from glucose-grown cells is clearly fainter.

The construction of defined mutants of the components supposedly involved in the regulatory mechanisms will be necessary in order to study in detail their interaction and to elucidate the regulation of the Lac-PTS activity in *L. casei*. This will be subject of further investigations of this system.

Knowledge about the molecular mechanisms relating to the regulation of gene expression in lactobacilli is still rather limited. We now present evidence for the first time that an antiterminator could regulate a major carbohydrate uptake system in *L. casei*. Antiterminators have so far been demonstrated for PTSs involved in β-glucoside transport in *E. coli*, *Erwinia chrysanthemi*, and *L. lactis* and for operons involved in sucrose or β-glucan utilization in *B. subtilis*. The *lac* operon of *L. casei* is an example for a system with an alternate substrate specificity and is of special interest since a group of highly related *lac* operons are regulated by different mechanisms.

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