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Carbohydrate Utilization in *Streptococcus thermophilus*: Characterization of the Genes for Aldose 1-Epimerase (Mutarotase) and UDPglucose 4-Epimerase

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The complete nucleotide sequences of the genes encoding aldose 1-epimerase (mutarotase) (*galM*) and UDPglucose 4-epimerase (*galE*) and flanking regions of *Streptococcus thermophilus* have been determined. Both genes are located immediately upstream of the *S. thermophilus lac* operon. To facilitate the isolation of *galE*, a special polymerase chain reaction-based technique was used to amplify the region upstream of *galM* prior to cloning. The *galM* protein was homologous to the mutarotase of *Acinetobacter calcoaceticus*, whereas the *galE* protein was homologous to UDPglucose 4-epimerase of *Escherichia coli* and *Streptomyces lividans*. The amino acid sequences of *galM* and *galE* proteins also showed significant similarity with the carboxy-terminal and amino-terminal domains, respectively, of UDPglucose 4-epimerase from *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, suggesting that the yeast enzymes contain an additional, yet unidentified (mutarotase) activity. In accordance with the open reading frames of the structural genes, *galM* and *galE* were expressed as polypeptides with apparent molecular masses of 39 and 37 kilodaltons, respectively. Significant activities of mutarotase and UDPglucose 4-epimerase were detected in lysates of *E. coli* cells containing plasmids encoding *galM* and *galE*. Expression of *galE* in *E. coli* was increased 300-fold when the gene was placed downstream of the *tac* promoter. The gene order for the *gal-lac* gene cluster of *S. thermophilus* is *galE-galM-lacS-lacZ*. The flanking regions of these genes were searched for consensus promoter sequences and further characterized by primer extension analysis. Analysis of mRNA levels for the *gal* and *lac* genes in *S. thermophilus* showed a strong reduction upon growth in medium containing glucose instead of lactose. The activities of the *lac* (lactose transport and β -galactosidase) and *gal* (UDPglucose 4-epimerase) proteins of lactose- and glucose-grown *S. thermophilus* cells matched the mRNA levels.

Streptococcus thermophilus transports lactose by means of a proton motive force-linked mechanism (33). Lactose enters the cell as a free sugar, and the disaccharide is hydrolyzed into glucose and galactose by β -galactosidase (20, 33). Glucose enters the glycolytic pathway, whereas in the presence of excess lactose, the galactose moiety of lactose is excreted into the medium (39).

The *lac* genes of *S. thermophilus* have recently been cloned, sequenced, and partially characterized (20, 33; C. J. Schroeder, C. Robert, G. Lenzen, L. L. McKay, and A. Mercenier, submitted for publication). The lactose transport gene (*lacS*) encodes a 69,454-dalton (Da) protein consisting of an amino-terminal domain with homology to the melibiose carrier of *Escherichia coli* and a carboxy-terminal domain with homology to enzyme III or enzyme III domains of various phosphoenolpyruvate-dependent phosphotransferase systems from gram-positive and gram-negative organisms. A similar transport protein has been found in *Lactobacillus bulgaricus* (33, 38), and the function(s) of the different domains of the transport proteins is currently under investigation (unpublished data). Contrary to the *E. coli lac* operon, the β -galactosidase gene (*lacZ*) is found downstream of the lactose transport gene in *S. thermophilus* and *L. bulgaricus*. In these last two organisms, the genes for lactose transport and β -galactosidase are separated by a 3-base-pair (bp) intercistronic region (33).

The *gal* genes of *S. thermophilus* have not yet been studied at the molecular level. Enzyme activity measurements have established that the Leloir pathway enzymes UDPglucose 4-epimerase and UDPglucose-hexose-1-phosphate uridylyltransferase are present in *S. thermophilus* but that the organism cannot express high levels of galactokinase (23, 39). The excretion into the medium of the galactose moiety of lactose and the apparent Gal⁻ phenotype of *S. thermophilus* have been attributed to a defect in the induction mechanism for galactokinase. Under appropriate selective pressure, Gal⁻ cultures can become Gal⁺. However, the Gal⁺ phenotype of *S. thermophilus* is rapidly lost upon subculturing in milk medium (39). Galactose transport in *S. thermophilus* has been studied to some extent (22). Most likely, galactose enters the cell via the lactose transport protein (33). The lactose transport protein not only catalyzes sugar-cation symport but also homologous and heterologous exchange of β -galactosides (33). This raises the interesting possibility that during lactose metabolism, the transport reaction proceeds as lactose-galactose exchange independent of the proton motive force.

This study was undertaken in an attempt to find the 5' end, and possible regulatory sequences or genes, of the *S. thermophilus lac* operon. However, DNA sequencing data and gene expression studies showed the presence of additional genes coding for enzymes involved in carbohydrate metabolism, i.e., a mutarotase and a UDPglucose 4-epimerase. Thus far, *S. thermophilus* genes other than those involved in lactose and galactose metabolism have not been cloned and sequenced or characterized genetically.

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TABLE 1. *E. coli* strains used

Strain	Relevant characteristics	Source or reference
HB101	<i>lacYI</i> Sm ^r <i>galK2</i>	7
P678-54	<i>lacYI minA1 minB2</i>	3
JM101	$\Delta(lac-proAB)(F' lacI^q\Delta M15)$	46
SA500	<i>gal</i> ⁺	S. Adhya
SA599	<i>galT</i> _{amb79}	S. Adhya
SA1287	<i>gal(ET)</i> $\Delta_{\Delta 7}$	S. Adhya
E274	<i>galE</i>	S. Adhya
MC1061	$\Delta lacIPOZYA galU galK$	33

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study are listed in Table 1. *S. thermophilus* A147 was obtained from the Centre International de Recherche Daniel Carasso, BSN group. The organisms were grown as described previously (33).

Nomenclature. We have chosen to denote *S. thermophilus* genes by using three lowercase letters followed by a capital letter. For the *lac* and *gal* genes of *S. thermophilus*, the *E. coli* nomenclature was used when genes encoded homologous proteins with similar functions, e.g., *lacZ* for the β -galactosidase gene and *galE* for the UDPglucose 4-epimerase gene. For nonhomologous genes encoding proteins with similar functions, a different capital letter was used, e.g., *lacS* rather than *lacY* for the lactose transport gene of *S. thermophilus*. The mutarotase gene of *S. thermophilus* was arbitrarily named *galM*, since it has no known counterpart in *E. coli*.

DNA manipulations. Chromosomal DNA was isolated essentially as described previously (38), except that after lysis of the cells an equal volume of isopropanol was added for spooling of the DNA. Upon removal from the isopropanol phase, the DNA was dried and dissolved in 1 to 5 ml of TE (10 mM Tris hydrochloride [pH 7.5], 1 mM disodium EDTA) containing 0.5% sodium dodecyl sulfate and 100 μ g of proteinase K per ml. After incubation for 30 to 60 min at 50°C, the suspension was extracted several times with phenol-chloroform. Plasmid DNA was isolated from *E. coli* strains by the alkaline lysis method (6). DNA sequencing was performed as described previously (33). Synthetic oligonucleotides were synthesized by the Genentech Organic Synthesis Group (Genentech Inc., South San Francisco, Calif.). Other methods, including enzymatic reactions, electrophoresis, and Southern hybridizations, were performed according to standard procedures (27).

Transformation. Routinely, *E. coli* strains were transformed by the calcium chloride-rubidium chloride method, as described previously (27). For transformation with complex ligation mixes (three- and four-way ligations), cells were transformed by electroporation (14) (electrotransformation [9]). Electrotransformation was also used for *E. coli* E274, SA500, SA599, and SA1287, which are only poorly transformable by other procedures. For electrotransformation, cells were grown to mid-exponential phase, harvested by centrifugation, washed twice with distilled H₂O, and suspended to an A₆₀₀ of 100 to 400 in 10% (vol/vol) glycerol. Samples (50 to 100 μ l) were quickly frozen in a dry ice-ethanol bath and stored at -70°C. The cells were transformed with the Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) by using cuvettes with a 0.2-cm electrode gap. The voltage and capacitance settings were 2,500 V and 25 μ F, respectively. The resistance of the pulse controller was set at 200 Ω .

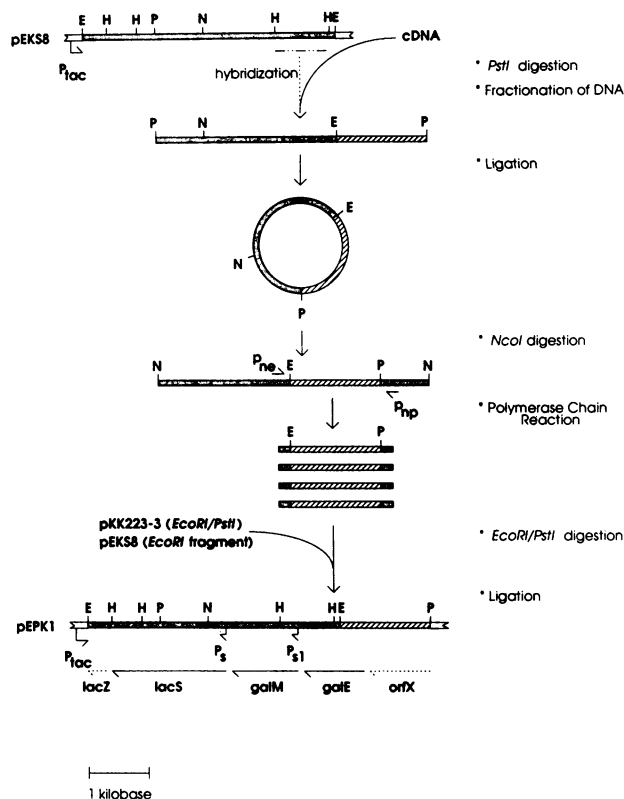


FIG. 1. Cloning of *galE*. The procedure for the cloning of *galE* is described in Materials and Methods. To identify the DNA fragment containing an intact *galE* gene, *PstI*-digested chromosomal DNA was fractionated by agarose gel electrophoresis and transferred to Nytran filters for Southern hybridization. A 0.9-kb *HindIII* fragment from pEKs8 (—) was used to identify the *PstI* fragment of about 4.5 kb. The fragment to be amplified, containing the 5' end of *galE*, is indicated (▨). The gene order and direction of transcription of the genes are indicated at the bottom of the figure. The ORFs for *lacZ* and *orfX* are incomplete (.....). *P_{tac}*, *tac* promoter; *P_s*, *lacS* promoter; *P_{si}*, putative *galM* promoter; *E*, *EcoRI*; *H*, *HindIII*; *N*, *NcoI*; *P*, *PstI*.

Cloning of *galE*. The procedure for cloning a DNA fragment upstream of *galM* is schematically shown in Fig. 1 (41). Briefly, chromosomal DNA was digested to completion with *PstI*. The cleaved DNA was fractionated by agarose gel electrophoresis, and, on the basis of Southern hybridization experiments, fragments of about 4.5 kilobases (kb) were electroeluted. The DNA fragments were ligated at a concentration of <0.1 ng/ μ l. With these dilute DNA suspensions, the formation of DNA circles is favored over the formation of DNA concatemers (11). Subsequently, the DNA ligase was inactivated and the DNA was digested with *NcoI*. Following phenol-chloroform extraction and ethanol precipitation in the presence of 100 ng of carrier tRNA per μ l, DNA (1 to 5 ng) was used for the polymerase chain reaction. The approximate positions of the forward (*p_{ne}*) and reverse (*p_{np}*) primers are indicated in Fig. 1. The sequences of *p_{ne}* and *p_{np}* are 5'ACCATCAGGTTTGCACCCGCCACATTA AA and 5'GGTATCGGAATATTTAGTATTGCAGGTAC, respectively. A portion (10 to 20 μ l) of the polymerase chain reaction-amplified DNA was extracted with chloroform prior to digestion with *EcoRI* and *PstI*. The *EcoRI*-*PstI* (1.5 kb) fragment together with the 4.2-kb *EcoRI* fragment of pEKs8 was ligated into pKK223-3 that had been linearized with *EcoRI* and *PstI*. The ligation mix was used to transform

E. coli HB101. The transformed organisms were spread on MacConkey agar (Difco Laboratories, Detroit, Mich.) plates containing 1% (wt/vol) lactose, 50 µg of carbenicillin per ml, and 25 µg of streptomycin per ml.

Polymerase chain reaction. Amplification of DNA (29, 35) was carried out for 30 cycles in the DNA Thermal Cycler (Perkin Elmer-Cetus Instruments, Norwalk, Conn.) after an initial denaturation cycle at 94°C for 10 min. Subsequent cycles were carried out for 90 s at 94°C (denaturation of DNA), 60 s at 50°C (annealing of primers), and 4 min at 70°C (primer extension). The reaction mixture (total volume, 100 µl) consisted of 10 mM Tris hydrochloride, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.1% (wt/vol) gelatin, 1 to 5 ng of DNA, 500 µM deoxynucleoside triphosphates, ~1 µM forward primer, and ~1 µM reverse primer. To prevent evaporation and refluxing of the solution, 20 to 50 µl of mineral oil was layered on top of the reaction mixture. *Thermus aquaticus* DNA polymerase (5 U; Perkin Elmer-Cetus) was added immediately after the initial denaturation step. To analyze the amplification products, 2 µl of the reaction mixture was electrophoresed on a 0.8% agarose gel.

Isolation of RNA and Northern (RNA) hybridizations. *S. thermophilus* A147 was grown to an A₆₀₀ of 0.2 to 0.8 in MRS medium (12) containing 1% (wt/vol) lactose or glucose as the sole carbohydrate source. Cultures (100 to 200 ml) were quickly harvested by centrifugation, suspended in 5 ml of ice-cold TE, and frozen in liquid nitrogen. The frozen cells were ground in a coffee grinder, and the powder was added immediately to 5 ml of 2× extraction buffer (prepared by mixing predissolved solutions of *para*-aminosalicylic acid sodium salt [2.4 g per 7.5 ml] and triisopropyl-naphthalene sulfonic acid [0.4 g/7.5 ml] to which 10 ml of 1 M Tris hydrochloride, pH 8.5, 1.25 M NaCl, and 0.25 M disodium EGTA were added). The suspension was vortexed and extracted with phenol-chloroform until protein was no longer detectable at the interface. The RNA was ethanol precipitated, and the pellet was dissolved in 50 µl of diethylpyrocarbonate-treated H₂O containing 1 U of RNase inhibitor (RNasin) per µl.

For Northern analysis, 2 to 5 µl of RNA was dissolved in a mixture of 50% formamide, 6% formaldehyde, 50 mM 3-(*N*-morpholino)propanesulfonic acid-NaOH (pH 7.0), 3 mM sodium acetate, and 0.1 mM disodium EDTA (total volume, 20 µl). The RNA was denatured for 4 min at 65°C, chilled on ice, and electrophoresed on 0.8% (wt/vol) agarose containing 2.2 M formaldehyde (24). RNA transfer to Nytran (Schleicher & Schuell, Inc., Keene, N.H.) filters and nick translation of DNA fragments were carried out as described previously (27).

Primer extension assay. For mapping of the *lacS* and *galM* promoters, oligonucleotides pext (5'GACGTCGTTACCAA AAGCACCAGCTGCGTA) and pexm (5'GTGAGTGTGGA AATGACAACACCATTTA), respectively, were used. Oligonucleotide pexg (5'AACAATCTTTGGATCGTTTAA ATAAGTTT) was used for mapping the region immediately upstream of *lacZ*. The primers (0.2 to 0.4 pmol) were end labeled by standard procedures (27), extracted with phenol-chloroform, ethanol precipitated, and dissolved in 5 µl of 100 mM Tris hydrochloride (pH 8.0) buffer containing 2 mM disodium EDTA, 800 mM NaCl, and 5 U of RNasin. RNA was added (up to 5 µl) and the mixture was incubated for 2 min at 90°C and allowed to cool to room temperature. Subsequently, 10 µl of 200 mM Tris hydrochloride (pH 8.3) buffer containing 20 mM MgCl₂, 100 mM KCl, 20 mM dithiothreitol, 200 µg of deoxynucleoside triphosphates per ml, 200 µg of actinomycin D per ml, 2 U of RNasin per µl,

and 10 U of avian myeloblastosis reverse transcriptase (Boehringer GmbH, Mannheim, Federal Republic of Germany) was added. The mixture was incubated for 60 min at 42°C, after which the reaction was stopped by phenol-chloroform extraction. Following ethanol precipitation, the pellet was dissolved in 5 µl of sequencing loading buffer. The cDNAs were separated on a 5% (wt/vol) polyacrylamide-8 M urea sequencing gel parallel to sequencing runs of the appropriate DNA fragments in M13mp18/mp19. The primers used for primer extension, i.e., pext and pexm, were also used for sequencing the promoter regions.

Transport assay. *S. thermophilus* A147 cells were grown to an A₆₀₀ of about 0.7 in lactose- and glucose-MRS (see above). The cells were harvested by centrifugation, washed twice with 100 mM K-PIPES [K-piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.0, buffer containing 5 mM MgSO₄, and suspended to an A₆₀₀ of about 20 in the same buffer with or without 1 mM thiomethyl-β-D-galactopyranoside (TMG). The cells were incubated at room temperature for about 2 h and concentrated by centrifugation. For [¹⁴C] TMG/TMG exchange experiments, 5 µl of concentrated cells (A₆₀₀ of about 200) were diluted 40-fold into buffer containing [¹⁴C]TMG (final concentration, 45 µM). Further manipulations were the same as those described previously (34).

Enzyme assays. Enzyme assays were performed on lysates of *S. thermophilus* A147 and different *E. coli* strains transformed with various plasmid constructions. Cell-free lysates were prepared as described previously (32). Mutarotase activity was determined polarographically at 30°C in a coupled enzyme system by using α-D-glucose (Sigma Chemical Co., St. Louis, Mo.) as the substrate and β-D-glucose: oxygen oxidoreductase (Boehringer GmbH) as the coupling enzyme (28). The assay mixture consisted of 100 mM Tris hydrochloride, 10 mM EDTA, 0.002% NaN₃, 200 µM α-D-glucose, 0.5 mg of β-D-glucose oxidase per ml, and 0.2 to 0.4 mg (total protein) of cell extract per ml. UDPglucose 4-epimerase, UDPglucose-hexose-1-phosphate uridylyl-transferase, and β-galactosidase activities were determined by standard procedures (see references 45, 39, and 38, respectively; see also footnote to Table 2).

Miscellaneous. Protein was measured by the method of Lowry et al. (26), with bovine serum albumin as the standard. Minicells were isolated and purified by sucrose gradient centrifugation, and proteins were labeled in vivo with [³⁵S]methionine, as described previously (33).

RESULTS

Nucleotide sequence of the mutarotase gene. In a previous paper, the cloning, sequencing, and characterization of the lactose transport gene (*lacS*) of *S. thermophilus* were described (33). The chimeric plasmid pEKS8 encoding the lactose transport protein consists of the expression vector pKK223-3 and a 4.2-kb *EcoRI* insert of *S. thermophilus* DNA. Expression of pEKS8-encoded proteins in a minicell-producing *E. coli* strain indicated the presence of an open reading frame (ORF), corresponding with a 38- to 39-kDa protein, in addition of the *lacS* ORF. Construction of a deletion derivative (pEKS8-7) of pEKS8 showed that the 38- to 39-kDa protein was not essential for lactose transport (33). The nucleotide sequence and the deduced amino acid sequence of the ORF are described below.

Translation of the ORF predicts a polypeptide of 348 amino acids corresponding to a calculated molecular mass of 39,095 Da (Fig. 2). The translation initiation site at position 1204 (Fig. 2) is proposed on the basis of the start of the ORF

A		B		
<i>galE_S</i>	1	MAILVGGAGYIGSHMVDRLVEKQEKVVVVDSLVTHGRAHV----	mutA	< signal sequence > 1 MKKLAIIGVTVYSFAQLANAATLNKVSYGTGQNGQKVDLYTMSNNNGVSVSFISFGGVIT
<i>galE_E</i>	1	MRVLVPTGGSGVIGSHTCVQLQNG-HDVIILDNLNSKRSVLPVIERLG	<i>galM</i>	1 MKISCEIIGKVDGSG-DVSKISMENNGVIVISTLTGTATGAT
<i>galE_S</i>	1	MSGYLVTTGGAGVYVQAHLVEAGNE-VVVLHNLSYGFREVCRRVFP--	<i>gal10S^C</i>	x+56GYQLRGEVAFRSADMRYDARFVTIGAGTRFQATFANGL
<i>gal10Kⁿ</i>	1	MSEDKYCVLVGGAGYIGSHTVVELCEAG-YKCVIVDNLSNYSVESVARMELLT	<i>gal10K^C</i>	346KGVDSKFFGDKGDFSSVRVSLGKGTFSFEVKLANLGATV
<i>gal10Sⁿ</i>	1	MTAQIQSESTSKIVLVTTGGAGYIGSHTVVELIENG-YDCVVDNLNSNS		
<i>galE_S</i>	44	-PDAIFYQGDLSQDFMRKFVKENPDVDAVIFAAYSILVGESMEKPLKFDNNTAGMVK	<i>mutA</i>	61 QILTPDAQKQNNVILGFDDLKGYEVDTT-KEGIFHFGGLIGRYANRIGNAKFSLDGKTYN
<i>galE_E</i>	49	GKHPTFVEGDIRNEALMTELLHDH-AIDTVIHFAGLKAVGESVQKPLEYVDNNGVTGLRL	<i>galM</i>	40 EFLVPMETGALKNIVLGFSDFDY-----YKNNLCACQSIGRVAIGKASYTHNMVLYS
<i>galE_S</i>	48	-----RSSRRPHGRQVRKLSFGVLIHFAPFSQVGESVVKPEKIVDNNVGGTAL	<i>gal10S^C</i>	x+92 ASIVDLKLVNG-QSVVLGYENEEGY-----LNPDAYSIGATIGRYANRISKGKFSLCKNDYQ
<i>gal10Kⁿ</i>	53	GQEIKFAKLDLCELEPLNKLFDVY-KIDSVLHFAGLKAVGESYQPLTYVYFNIVGTINL	<i>gal10K^C</i>	385 DIVV-----NGCSVVASLONETEKYD-----QSNPFPGATVGYANRIANGTFEINGKRQ
<i>galE_S</i>	103	LEVMECEGVKIVFSSSTAATYGIPEE----IPILETTPQN-PINPYGESKMMETIMK-W	<i>mutA</i>	120 LE-KNNGPNSLHSGNPGFDKRVVQVKPLVSKGETV-KASLKLTSPNGDQGFPGKLDVEVI
<i>galE_E</i>	108	ISAMRAANVKNFIFSSSATVYGDQPK----IPYVESFPPTGTPQSPYKSKLMVEQIILTDL	<i>galM</i>	95 LP-KNEGDNCLHGGPKGMQVQW-NVVTNLDVYV-ETKPIRRLYSSVDGFPDGVTVSIS
<i>galE_S</i>	99	LEAMRGAVRRLVFSSTAATYGEPEQ----VPIVESAPTR-PTNYPYGASKLAVDHMIT-G	<i>gal10S^C</i>	x+150 LTV-NGVNAVHSSIGSLHRRKFLGPIIQNPSNVVFTAEYMLIDNEKOTEFQDGLLVTVQ
<i>gal10Kⁿ</i>	112	LEMCKSHDVKLVFSSSATVYGDATRPFENMIPETPCPTCG-PTNYPYGKTLIEDMMRD	<i>gal10K^C</i>	436 LTVSKEDGNNVHSGINSYHSSKFLGPIVKNPEAHIWTDAP-KYVDETFEPARISLTVR
<i>galE_S</i>	157	SDQAYGIVYVPLRYFNAGANLMLVRLVTRTS--ETHLLPIILQVAAQVREKIMIFGGDYN	<i>mutA</i>	178 YSLSDQNEFKIEYKAKTD--QPTVNLITNHSYFNLSGAGNYPG--VLDHVQVLNAGRI
<i>galE_E</i>	164	QKAQPDWSIALRLRYFNPGAHSPGDMGEDPQGINNLMPIYIAQVAGRRDSLAIIFGNDYP	<i>galM</i>	152 YRLN-NNRRLTILFEA-FDVTSTVFNTNHSYFNLS--DK--QDLSHSELQIYSYDR
<i>galE_S</i>	153	EAAAHGLGAVSVPYFNVAAGVGEYGERHDPE--SHLIPLVLQVAGQRRREISVYGGDDYP	<i>gal10S^C</i>	x+209 YTVNVAQKSLIYVYKGLTAGEATPINLTMHSYFNLS--KPYGDTIEGTEIMVRSKKS
<i>gal10Kⁿ</i>	171	HFSDKSFSFALLRYFNPIGAHSPGVIGE-AFGIPNNLLPFMAQVAIGRRPKLVYFGDDYD	<i>gal10K^C</i>	494 YKVDEKKTLTVEYESKAVAGQGSTGANITNHTYFNLS--KFNATIKGSVKQLIDMTG
<i>galE_S</i>	215	TPDGTNVRDVIHVPFDLADAHLLAVEYLKRGNES-TAFNIGSSGTCFSLNILEAARKVTGK	<i>mutA</i>	234 LVTQDQNSLPTGEIASVAGTFFDFMFKPAIKVDIRANNQQLAYGYDQTVHWNKQSQGL
<i>galE_E</i>	224	TEDCTGVRDVIHVMDLADGHVVMKLANPKGV-HYINLGAQVGNVSLVDVNFVNSKAGCK	<i>galM</i>	204 LEIDSELIPTGQKINVDNETNYDFRKTLDLPLRIEANN-----GFDDAFVGGGTCDHV
<i>galE_S</i>	210	TPDRFVCAATSTSPVTPRPCTPW---CAAAPGEHLICNLGNMGFSVREVEVTVRRVVTGH	<i>gal10S^C</i>	x+265 VDVKNNMIPGTNVDREIATFNSTKPTVLGPKNP-----QDFCCYVDVANA--KP
<i>gal10Kⁿ</i>	230	SDVGTPIRDVIHVDLAKGLHALLKYLEYAGTCREWNLGTGHTTVLQMYRAFCAIDGF	<i>gal10K^C</i>	550 LEVNNKLPTLGNLKYQVATFNSPTEITKEFVPL-----DFCVSLGPAKLD
<i>galE_S</i>	274	EIPAEKADRRPGPDILIASSEKARTVLGKQKQDFNIEKI IASAWAHSHPKGYDDRG	<i>mutA</i>	294 NLAIVVDPKSKRTMQLVTEPESVQMYTADHLLGNIVGAN---GVLYRQADALALETQH
<i>galE_E</i>	283	PVNHFAFPRECEGAFRPTGRTPAKFTVNLNWRVTRT-LDEMAQDTHWQSRHPCGYPD	<i>galM</i>	257 KEVAILHDKESGDGIEIFSNRNGLIVTMDIDENIYFARD--KGMKAKRREIAMEAQT
<i>galE_S</i>	267	PIPELHAPRRGRDPALVASAGRAREKLGNPSRADL-AIVSDAMELQRRAQG	<i>gal10S^C</i>	x+313 SQINTLNNLTLIVKAFHPSNSITLVLVSTPTEYQFYTGDFL--SAGYEARQGFALIEPCR
<i>gal10Kⁿ</i>	290	NFEVYVTRRRQDVLNLTAKDRAETNELEWKTELD-VNKACVDLAKWTKQDNPFYQYI..	<i>gal10K^C</i>	600 RSPPLTPVFKLSNEDARLEVEVAT-----TEPTFQVYTGVDVVDKDYENRAGICEPGR
<i>gal10Sⁿ</i>	x	WSSVVRVTRRRAGDVLNLTAKPDRAKREKMQTELQ-VEDSKDLNWKWTENPFYQYI..	<i>mutA</i>	350 FPDSPNQPTFPS-TRLNPHQTVNSVTVFKFVQK
			<i>galM</i>	315 LPDAVNHKGFQD-IILDKHGSVNVYELGFQYFNSSR
			<i>gal10S^C</i>	x+371 YIDAINQENKDCVTLNKGETYSGKTLVRF
			<i>gal10K^C</i>	655 YIDAVNNPEWSSVLPAGETVYGHKLSYVTRTL

FIG. 3. Computer alignment of the amino acid sequences of *galE* protein (A) and *galM* protein (B) with those of various proteins. Identical (*) and conserved (:) amino acids in the sequences are indicated. (A) *galE_S*, *galE_E*, *galE_S*, *gal10K*, and *gal10S* denote the UDPglucose 4-epimerases from *S. thermophilus*, *E. coli* (25), *S. lividans* (1), *K. lactis* (43), and *S. cerevisiae* (10), respectively. The superscript n refers to the amino-terminal domain of the UDPglucose 4-epimerases of *K. lactis* and *S. cerevisiae*. (B) *mutA*, Mutarotase from *A. calcoaceticus* (18); *galM*, putative mutarotase from *S. thermophilus*; *gal10K^C* and *gal10S^C*, carboxy-terminal domains of UDPglucose 4-epimerase from *K. lactis* (43) and *S. cerevisiae* (10), respectively. The signal sequence of *mutA* is indicated above the protein sequence. In the numbering of the *gal10S* sequence, an x is present since the gene has not entirely been sequenced (10).

Since the size of the protein is about half the size of the UDPglucose 4-epimerases (*gal10*) of *K. lactis* and *S. cerevisiae*, it was hypothesized that the gene encoding a protein homologous to the amino terminus of UDPglucose 4-epimerase of *K. lactis* and *S. cerevisiae* might be located upstream of *galM*. To test the hypothesis, an additional fragment of *S. thermophilus* chromosomal DNA was cloned.

Cloning of *galE*. To isolate a DNA fragment upstream of *galM*, a new method was employed in which the polymerase chain reaction was used to enrich a chromosomal DNA library for the appropriate DNA fragment prior to cloning (see Materials and Methods; Fig. 1) (30, 36, 41). For the construction of pEPK1, a 1.5-kb *EcoRI-PstI* polymerase chain reaction-derived fragment and the 4.2-kb *EcoRI* fragment of pEKS8 were ligated into pKK223-3 (*EcoRI-PstI*) (Fig. 1). The ligation mixture was used to transform *E. coli* HB101 (*lacYI*), and *lac⁺* clones were selected on lactose-MacConkey agar plates. Plasmid DNA was isolated from a few of these clones, and the construction of pEPK1 was confirmed by restriction analysis. Subsequently, pEPK1 was used to transform *E. coli* E274 (*galE*) and the transformed organisms were spread on galactose-MacConkey agar plates. None of the transformants had a *gal⁺* phenotype, indicating either that the UDPglucose 4-epimerase gene of *S. thermophilus* was not present on this DNA fragment or that the gene was not (functionally) expressed in *E. coli*. Since in the construction of pEPK1 the *tac* promoter runs in the opposite direction of *lacS* and *galM* (and possibly the

UDPglucose 4-epimerase gene [*galE*]), plasmids in which the *tac* promoter was placed upstream of these genes were constructed (Fig. 4). Plasmid constructions pPK1/*tac* and pHP1 (but not pPK1/anti-*tac*) yielded *gal⁺* phenotypes in the *E. coli* E274 background, indicating that *galE* was present in these constructions and that the *tac* promoter was required for sufficient expression of the gene in *E. coli* E274 (Table 2).

Nucleotide sequence of the UDPglucose 4-epimerase gene. The nucleotide sequence and the deduced amino acid sequence of *galE* are shown in Fig. 2. Errors in the sequence could occur, since *galE* was constructed from a 1.5-kb *EcoRI-PstI* fragment obtained after amplification by the polymerase chain reaction (Fig. 1) and the fidelity of DNA synthesis in vitro by *Taq* DNA polymerase is suspect (15, 40). Therefore, the 500-bp *EcoRV-EcoRI* fragment (Fig. 2) from two independent *galE⁺* clones, comprising the amino-terminal part of *galE*, was subcloned into M13mp18/19. The DNA sequences from both templates were identical, indicating that no errors were introduced by the *Taq* DNA polymerase (data not shown).

The ORF for *galE* most likely consists of 996 bp which correspond to a protein monomer of 332 amino acids with a calculated molecular mass of 37,023 Da. The translation initiation site at position 136 (Fig. 2) is proposed on the basis of the start of the ORF (at bp 133), the position of the putative ribosome-binding site (AGGAG at bp 124), and the amino acid sequence homology between the *galE* protein, the corresponding enzymes of *E. coli* and *Streptomyces*

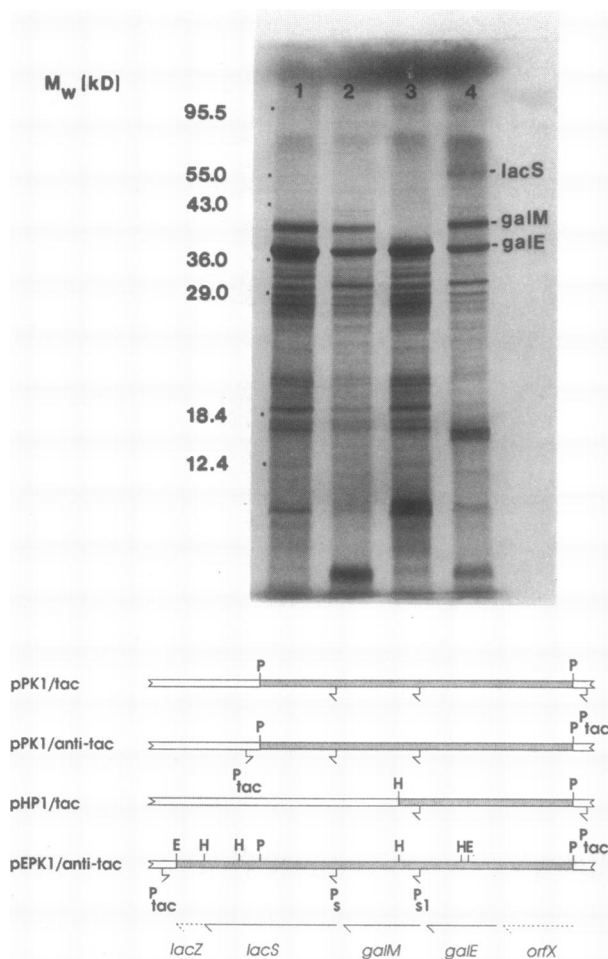
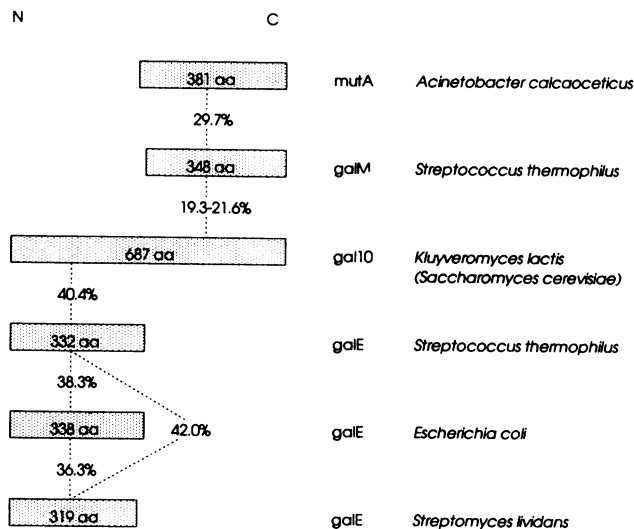


FIG. 4. Expression of *galM* and *galE* in minicell-producing *E. coli* P678-54. Upper half: ^{35}S -labeled proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis, after which the proteins were identified by autoradiography. Lane 1, pPK1/tac; lane 2, pPK1/anti-tac; lane 3, pHP1/tac; lane 4, pEPK1/anti-tac. Bottom half: Plasmid constructions containing *galM* or *galE* or both. Symbols are the same as those defined in the legend to Fig. 1.

lividans, and the amino-terminal domain of UDPglucose 4-epimerase of *K. lactis* (Fig. 3A and Fig. 5). Upstream of *galE*, another ORF (*orfX*) was found, consisting of at least 1,008 bp (data not shown). The intergenic region between *orfX* and *galE* was only 32 bp long. A search for -10 and -35 promoter regions by using consensus sequences reported for gram-positive and gram-negative bacteria (19) did not reveal strong promoter sequences upstream of *galE*, consistent with the observation that without an exogenous promoter *galE* is weakly expressed in *E. coli*. The sequence data together with the Northern analysis of *S. thermophilus* mRNA (see below) suggest that *galE* and *orfX* might be transcribed as a single message with the promoter being located upstream of *orfX*. To check whether *orfX* possibly coded for UDPglucose-hexose-1-phosphate uridylyltransferase (*galT*), the other *gal* enzyme known to be present in *S. thermophilus* (39), pPK1/tac, was used to transform *E. coli* SA1287 (*gal(ET)*_{ΔA7}) and SA599 (*galT*_{amb79}). Complementation was not observed and UDPglucose-hexose-1-phosphate uridylyltransferase activity could not be detected in lysates prepared from these organisms, indicating that *orfX* does not



The percent amino acid identity between pairs of proteins. The length of the proteins is indicated in the boxes.

mutA: mutarotase (aldose-1-epimerase)
gal10: UDPglucose-4-epimerase (yeast)
galE: UDPglucose-4-epimerase

FIG. 5. Comparison of *galM* and *galE* proteins with other homologous proteins. The position and size of *galM* (and *mutA*) and *galE* relative to *gal10* are indicated by the boxes. aa, Amino acids.

contain a complete ORF and/or proper ribosome-binding site or that *orfX* does not encode the uridylyltransferase.

Amino acid sequence homology. UDPglucose 4-epimerase of *S. thermophilus* was homologous to the corresponding enzymes of *E. coli* (25) (38% amino acid identity), *S. lividans* (1) (42% amino acid identity), and the amino-terminal domain of *K. lactis* UDPglucose 4-epimerase (43) (40% amino acid identity) (Fig. 3A and 5). UDPglucose 4-epimerase from *S. cerevisiae*, which has been only partially sequenced (10), is also similar to *S. thermophilus galE* at the amino terminus. No similarity was found between *orfX* and *galT* from *E. coli* or between *orfX* and any other protein in the Dayhoff protein bank.

Expression of *galE* and UDPglucose 4-epimerase activity. Different plasmids containing *galM* or *galE* or both were introduced into the minicell-producing *E. coli* P678-54. *galM* was expressed as a 39-kDa protein with the constructions

TABLE 2. UDPglucose 4-epimerase activity measurements

Strain	Orientation of <i>P</i> _{tac} - <i>galE</i>	IPTG ^a	Activity ^b (μmol/min per mg of protein)
E274		-	0.0
E274(pPK1)	tac	+	14.9
E274(pPK1)	Anti-tac	+	0.05
E274(pPK1)	tac	-	17.9
E274(pPK1)	Anti-tac	-	0.06
E274(pHP1)	tac	-	15.1

^a IPTG, Isopropyl-β-D-thiogalactopyranoside.

^b Activity was measured as UDPgalactose 4-epimerase activity. The assay mixture consisted of 100 mM glycine-NaOH (pH 8.6), 10 mM NAD⁺, 1 mM UDPgalactose, 0.3 U of UDPglucose dehydrogenase per ml, and, depending on the final UDPgalactose 4-epimerase activity, 2 to 100 μg of cell-free lysate per ml. Measurements were done at room temperature.

pPK1 and pEPK1, and expression was more or less independent of the orientation of *galM* towards the *tac* promoter (Fig. 4, lanes 1, 2, and 4). *galM* was absent in the deletion derivative pHP1/*tac* (Fig. 4, lane 3). A 37-kDa protein, corresponding with the size of *galE* (see above), was observed in all plasmid constructions. Expression of *galE* was greatly increased when the gene was placed downstream of the *tac* promoter (Fig. 4, lanes 1 and 3). Finally, a faint band corresponding to expression of *lacS* protein, a protein with an apparent molecular mass of 55 kDa (33), was observed in pEPK1 only (Fig. 4, lane 4).

For UDPglucose 4-epimerase activity measurements, lysates were prepared from *E. coli* E274 alone or transformed with different plasmid constructions (Table 2). UDPglucose 4-epimerase activity could not be detected in *E. coli* E274. Low but significant activity was present in lysates prepared from *E. coli* E274 (pPK1/anti-*tac*). This activity, i.e., 50 to 60 nmol of UDPglucose formed per min per mg of protein, was insufficient to complement *E. coli* E274 (see above). UDPglucose 4-epimerase activity was increased about 300-fold when *galE* was placed downstream of the *tac* promoter, and this activity was irrespective of expression of *galM* (compare E274/pPK1-*tac* with E274/pHP1-*tac*). Activities were not affected by the presence of isopropyl- β -D-thiogalactopyranoside during growth of the organisms, indicating that the *tac* promoter is hardly regulated or not regulated in *E. coli* E274. Although it is difficult to quantitate the protein expression levels precisely, there seems to be a discrepancy between the difference in protein levels (Fig. 4) and enzyme activities (Table 2) in *tac* and anti-*tac* constructions of *galE*. It is not clear whether the discrepancy is due to differences in the strains used.

mRNA levels and 5'-end mapping of mRNA. Total RNA isolated from lactose- and glucose-MRS-grown *S. thermophilus* cells was analyzed by Northern hybridizations. To identify mRNAs, appropriate gene fragments, i.e., 1,450-bp *PvuII*, 646-bp *BstXI-EcoRV*, and 540-bp *PvuII* fragments for *lacS*, *galM*, and *galE*, respectively, were nick translated and used as probes. For lactose-MRS-grown cells, major mRNAs of about 5.5 and 3.5 kb were identified for *lacS* and *galE*, respectively (data not shown). Very little mRNA was detected in glucose-MRS-grown cells. *galM* mRNA could not be detected (reliably) by Northern analysis, irrespective of whether cells were grown in lactose- or glucose-MRS.

The size of the *lacS* mRNA suggests that *lacS* is transcribed together with the β -galactosidase gene (*lacZ*) as a single message. To locate the transcriptional start point of the *lac* genes, the primer extension method was used. Two major transcripts were observed (Fig. 6A, vertical bars); one (Fig. 6A, m1) starts 10 bp downstream of the inferred -10 sequence (indicated as +1), whereas the other (Fig. 6A, m2) is located 34 bp downstream of the -10 region. For the latter transcript, no sequences similar to promoters from gram-positive and gram-negative bacteria could be found. In accordance with the Northern analysis, the amount of DNAs synthesized with the mRNA template from glucose-MRS-grown cells was reduced at least 10-fold compared with lactose-MRS-grown cells (Fig. 6A). The two major transcripts as well as the effect of glucose on the transcript levels were observed with four different preparations of RNA, i.e., RNA isolated from cells harvested at an A_{600} of 0.2 to 0.8 (data not shown). Primer extension analysis in the 300-bp region immediately upstream of *lacZ* did not reveal a major transcriptional start point, supporting the contention that *lacZ* and *lacS* are transcribed from a single promoter(s) located upstream of *lacS*.

TABLE 3. Effects of lactose and glucose on expression of *lac* and *gal* genes in *S. thermophilus*

Protein	Gene	Activity (nmol/min per mg of protein)	
		Lactose grown	Glucose grown
β -Galactosidase	<i>lacZ</i>	10,800	600
Lactose transport	<i>lacS</i>	18 ^a	1 ^a
UDPglucose 4-epimerase	<i>galE</i>	2,070	20
UDPglucose-hexose-1-phosphate uridylyltransferase	<i>galT</i>	492	19

^a Lactose transport was measured as [¹⁴C]TMG/TMG exchange activity (see Materials and Methods).

For *galM*, a minor transcript, starting 19 bp downstream of a putative -10 sequence, was detected (Fig. 6B, vertical bar). The *galM* transcript was observed in lactose- but not glucose-MRS-grown cells (Fig. 6B). The size of the *galE* transcript combined with the observation that a DNA probe derived from *galM* did not hybridize with the transcript suggests that the true promoter is located upstream of *orfX*.

Enzyme and lactose transport activities. Since the mRNA levels for the *lac* and *gal* genes of *S. thermophilus* were highly reduced in glucose-MRS-grown cells, 10-fold or more compared with lactose-MRS-grown cells, the corresponding enzyme and transport activities were measured. Table 3 shows that the activities for β -galactosidase, lactose transport, UDPglucose 4-epimerase, and UDPglucose-hexose-1-phosphate uridylyltransferase in lactose-MRS-grown cells are 10- to 20-fold higher than in glucose-MRS-grown cells. These data together with the effect of glucose on the mRNA levels indicate that expression of the *lac* and *gal* genes is regulated at the level of transcription. Mutarotase activities in cell lysates prepared from lactose- and glucose-MRS-grown cells are too low to be measured accurately, i.e., on top of the rate of spontaneous mutarotation.

DISCUSSION

The cloning of *galE* was achieved by using a special polymerase chain reaction for which a region of interest can be amplified when only 5' or 3' flanking sequences are known (30, 41). The advantage of the method over classical cloning procedures is that transformants do not have to be screened by laborious hybridization protocols in order to identify the desired recombinant clone. A potential disadvantage of the method relates to the relatively low fidelity of *Taq* DNA polymerase (lack of detectable exonucleolytic proofreading activity) (40), which can result, among other errors, from base substitution errors in the amplified DNA fragment. However, such errors have not been observed in the fragment (500-bp *EcoRV-EcoRI*) needed for the construction of *galE* (see Results).

In aqueous solution, many saccharides exist in two different isomeric forms, i.e., α and β , differing in specific rotation. The anomeric interconversion of D-glucose and other aldoses which can occur spontaneously is called mutarotation. Spontaneous mutarotation is acid-base catalyzed and consequently strongly affected by pH and chemical composition of the aqueous medium. On the basis of the kinetics of spontaneous mutarotation, pH dependence, and catalysis by amino acids (in particular histidine [and other

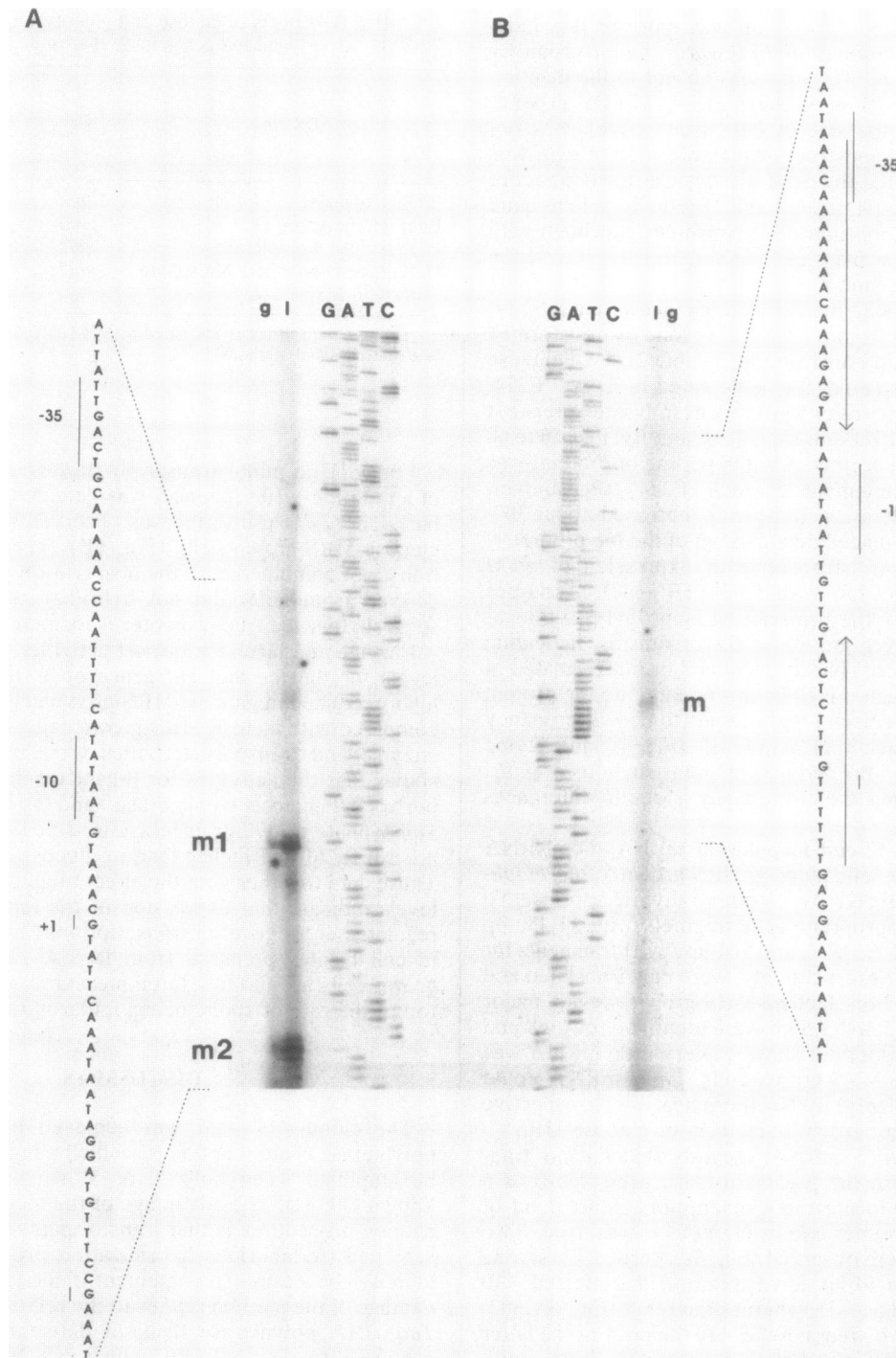


FIG. 6. Determination of the 5' ends of *lacS* (A) and *galM* (B) transcripts. Primer extension on mRNA isolated from glucose-MRS- and lactose-MRS-grown *S. thermophilus* A147 is indicated by g and l, respectively. Equal amounts of total RNA were loaded in all lanes. To calibrate the transcriptional start points, M13 clones of the corresponding fragments were sequenced in parallel. Relevant nucleotide sequences of the promoter regions are given on either side of the figure. Putative promoter regions and transcriptional start points are indicated by vertical bars. For the *galM* promoter region, the inverted repeat is indicated by arrows.

solutes containing an imidazole group)), it has been proposed that one component of the active site of enzyme-catalyzed mutarotation involves a histidine residue (4, 5). In the alignment of *galM* with *mutA* and *gal10ⁿ* proteins (Fig. 3B), two histidine residues are conserved, i.e., His-105 and His-180 (indicated by arrow), of which the latter is present in

a region highly conserved between the four proteins. This suggests that His-105 or (more likely) His-180 furnishes the imidazole group needed for catalysis.

Mutarotase of *A. calcoaceticus* has been shown to contain a leader peptide sequence which directs the secretion of the enzyme into the periplasmic space (17, 18). Interestingly,

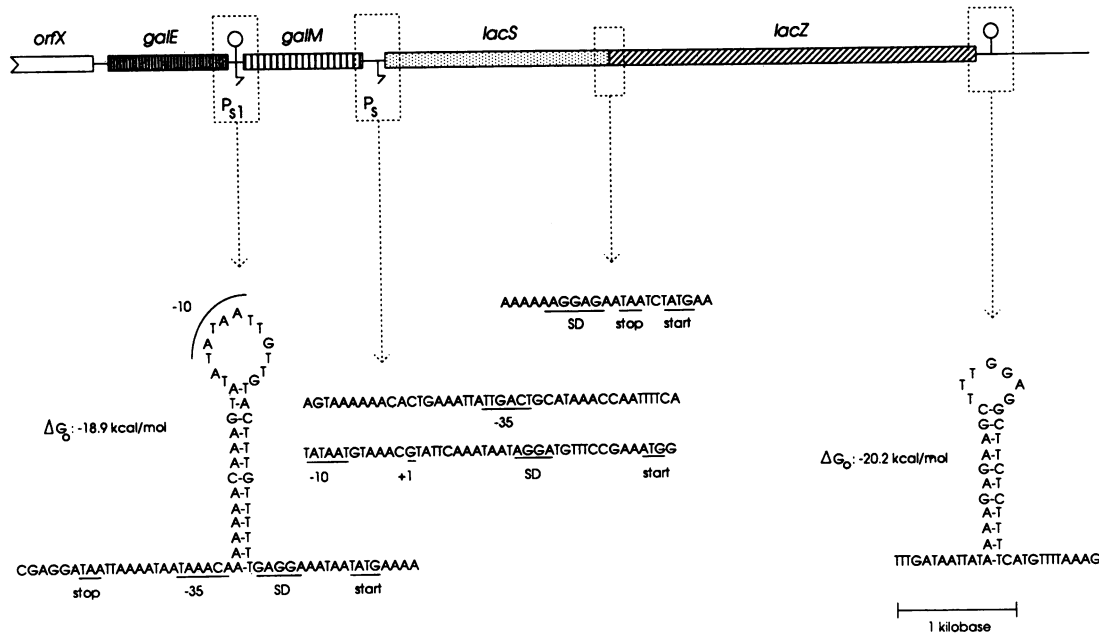


FIG. 7. *gal-lac* gene cluster of *S. thermophilus*. The gene order and nucleotide sequences of intercistronic regions of the *gal* and *lac* genes of *S. thermophilus* A147 are shown. The nucleotide sequences for the region between *lacS* and *lacZ* were taken from reference 33, whereas the sequence for the region 3' of *lacZ* was taken from Schroeder et al. (submitted). Sequences are given as DNA rather than RNA.

the first amino acid of the *galM* protein aligns with the first amino acid of the mature mutarotase protein (Fig. 3B).

An interesting outcome of this study is the sequence homology between *galM* (and *mutA*) and the carboxy-terminal domain of UDPglucose 4-epimerase (*gal10*) of yeasts and that between *galE* and the amino-terminal domain of the same enzymes (Fig. 5). On the basis of these similarities, we speculate that the *gal10* enzymes possess mutarotase activity in addition to UDPglucose 4-epimerase activity. The presence of an apparent hybrid epimerase in *K. lactis* and *S. cerevisiae* could suggest that *galM* and *galE* are expressed functionally only as a hetero-multimeric enzyme complex. On the other hand, the data indicate that *galM* and *galE* can be expressed as functionally active proteins independent of each other.

In many organisms, the genes for galactokinase (*galK*), UDPglucose 4-epimerase (*galE*), and UDPglucose-hexose-1-phosphate uridylyltransferase (*galT*) are present in a single operon, constituting the Leloir pathway for galactose metabolism (1, 2, 44). Alternatively, it has been shown for *Erwinia stewartii* that *galE* is not linked to *galK* and *galT*; rather, *galE* is linked to genes encoding enzymes involved in the biosynthesis of extracellular polysaccharides (13). *galE* is expressed constitutively, whereas *galK* and *galT* are inducible in *E. stewartii*. The fact that *orfX* is not homologous to *galT* or *galK* from *E. coli* could argue against the presence of the complete *gal* operon upstream of the *lac* operon. On the other hand, in accordance with an operonlike structure, *galE* and *galT* appear to be regulated similarly in *S. thermophilus* (Table 3; Fig. 6). Future experiments will be directed towards characterizing the function of *orfX* and cloning genes involved in the regulation of carbohydrate metabolism.

UDPglucose 4-epimerase is required during galactose metabolism to convert UDPgalactose to UDPglucose, which enters the glycolytic pathway. Since most strains of *S. thermophilus* are *gal* mutants (23, 39), the role of UDPglu-

cose 4-epimerase in this organism could be to convert UDPglucose into UDPgalactose for the biosynthesis of extracellular polysaccharides (8, 13) or galactosyl lipids (31).

The gene order and possible regulatory elements of the *gal* and *lac* genes are depicted in Fig. 7. Although the inverted repeat, indicated as a stem-loop structure, downstream of *lacZ* does not fully resemble a typical rho-independent terminator, the region could well be involved in transcription termination, considering the size of the *lac* transcript and the fact that an 860-bp region downstream of *lacZ* is noncoding (Schroeder et al., submitted). Putative *lac* and *galM* promoter regions are indicated by *p_s* and *p_{s1}*, respectively. On the basis of the primer extension assay, the *p_s* -35/-10 promoter region seems to be functional in *S. thermophilus*, although a second transcript (Fig. 6A, m2), for which no consensus promoter sequence can be identified, appears to be present too. A possible start of the *galM* transcript is found 19 bp downstream of a putative -35/-10 promoter (Fig. 6B, m). Whether this promoter region is indeed used or whether an as yet unrecognizable promoter region is used by RNA polymerase from *S. thermophilus* remains unclear. Also, it cannot be excluded that *galM* is transcribed from a promoter located upstream of *galE* and *orfX*. In that case, the role of the inverted repeat could be to decrease the expression of the distally located *galM* (21). Finally, the level of the putative *galM* transcript in *S. thermophilus* is too low to direct the synthesis of sufficient mutarotase activity to be detected under the conditions employed.

The codon usage in the mutarotase and UDPglucose 4-epimerase genes was compared with the codon usage in the lactose transport gene (33) and the β-galactosidase gene (Schroeder et al., submitted) of *S. thermophilus* (Table 4). In general, the codon usage is very similar in the four genes. A strong preference for A or U in the third position can be observed, resembling the average codon usage in *E. coli* genes (16, 42). Major exceptions are the codons for Leu and Pro.

TABLE 4. Condon usage in the β -galactosidase (*lacZ*), lactose transport (*lacS*), mutarotase (*galM*), and UDPglucose 4-epimerase (*galE*) genes of *S. thermophilus*

Codon	Amino acid	No. of times codon used in:			
		<i>lacZ</i>	<i>lacS</i>	<i>galM</i>	<i>galE</i>
UUU	Phe	28	20	13	8
UUC	Phe	24	25	5	4
UUA	Leu	8	4	9	4
UUG	Leu	22	21	4	7
CUU	Leu	31	32	9	11
CUC	Leu	4	6	2	2
CUA	Leu	10	2	1	1
CUG	Leu	1	0	1	1
AUU	Ile	28	34	18	13
AUC	Ile	21	23	4	8
AUA	Ile	0	2	3	1
AUG	Met	15	17	8	11
GUU	Val	58	27	11	13
GUC	Val	6	8	6	3
GUA	Val	12	17	6	9
GUG	Val	3	2	2	3
UCU	Ser	20	15	2	6
UCC	Ser	1	1	2	0
UCA	Ser	19	19	9	9
UCG	Ser	0	1	1	0
AGU	Ser	16	10	7	3
AGC	Ser	11	3	3	1
CCU	Pro	17	7	3	5
CCC	Pro	0	0	0	0
CCA	Pro	23	10	4	11
CCG	Pro	0	0	1	1
ACU	Thr	26	16	4	4
ACC	Thr	4	7	1	2
ACA	Thr	24	20	13	9
ACG	Thr	0	3	2	0
GCU	Ala	36	16	8	14
GCC	Ala	3	9	4	3
GCA	Ala	18	14	3	8
GCG	Ala	2	1	0	3
UAU	Tyr	39	8	11	8
UAC	Tyr	23	10	4	6
UAA	Ochre	0	1	1	1
UAG	Amber	1	0	0	0
CAU	His	11	4	7	4
CAC	His	13	7	1	5
CAA	Gln	37	12	5	8
CAG	Gln	2	2	4	1
AAU	Asn	33	16	22	4
AAC	Asn	23	10	8	10
AAA	Lys	47	28	16	15
AAG	Lys	18	6	3	5
GAU	Asp	53	19	24	16
GAC	Asp	21	4	5	6
GAA	Glu	75	27	13	19
GAG	Glu	7	3	8	2
UGU	Cys	8	1	2	1
UGC	Cys	1	0	3	0
UGA	Opal	0	0	0	0
UGG	Trp	19	9	1	4
CGU	Arg	19	14	2	9
CGC	Arg	2	2	1	1
CGA	Arg	2	0	1	2
CGG	Arg	0	0	0	0
AGA	Arg	10	0	8	1
AGG	Arg	1	0	2	2
GGU	Gly	50	42	12	15
GGC	Gly	5	3	5	2
GGA	Gly	13	14	9	5
GGG	Gly	3	1	2	3

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