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Transcriptional Analysis and Regulatory Signals of the *hom-thrB* Cluster of *Brevibacterium lactofermentum*

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Two genes, *hom* (encoding homoserine dehydrogenase) and *thrB* (encoding homoserine kinase), of the threonine biosynthetic pathway are clustered in the chromosome of *Brevibacterium lactofermentum* in the order 5′ *hom-thrB* 3′, separated by only 10 bp. The *Brevibacterium thrB* gene is expressed in *Escherichia coli*, in *Brevibacterium lactofermentum*, and in *Corynebacterium glutamicum* and complements auxotrophs of all three organisms deficient in homoserine kinase, whereas the *Brevibacterium hom* gene did not complement two different *E. coli* auxotrophs lacking homoserine dehydrogenase. However, complementation was obtained when the homoserine dehydrogenase was expressed as a fusion protein in *E. coli*. Northern (RNA) analysis showed that the *hom-thrB* cluster is transcribed, giving two different transcripts of 2.5 and 1.1 kb. The 2.5-kb transcript corresponds to the entire cluster *hom-thrB* (i.e., they form a bicistronic operon), and the short transcript (1.1 kb) originates from the *thrB* gene. The promoter in front of *hom* and the *hom*-internal promoter in front of *thrB* were subcloned in promoter-probe vectors of *E. coli* and corynebacteria. The *thrB* promoter is efficiently recognized both in *E. coli* and corynebacteria, whereas the *hom* promoter is functional in corynebacteria but not in *E. coli*. The transcription start points of both promoters have been identified by primer extension and S1 mapping analysis. The *thrB* promoter was located in an 87-bp fragment that overlaps with the end of the *hom* gene. A functional transcriptional terminator located downstream from the cluster was subcloned in terminator-probe vectors.

Threonine is synthesized from aspartic acid in five enzymatic reactions. The initial two reactions which convert aspartic acid to aspartate-β-semialdehyde are common to the lysine pathway. Conversion of aspartate-β-semialdehyde into homoserine (catalyzed by homoserine dehydrogenase [HD]) is common for threonine and methionine biosynthesis. Homoserine is converted into threonine by the action of two other enzymes, homoserine kinase (HK) and threonine synthase (TS).

In *Escherichia coli*, the genes encoding four of the five enzyme activities involved in threonine biosynthesis are clustered together in the *thrABC* operon. The *thrA* gene encodes the bifunctional enzyme aspartokinase-HD (AKI-HDI), whereas *thrB* and *thrC* encode HK and TS, respectively (7, 20). In corynebacteria there is one monofunctional HD (17), whereas in *E. coli* there are two isoenzymes (HDI and HDII, respectively) that form part of bifunctional polypeptides AKI-HDI and AKII-HDII.

We previously cloned and sequenced the *Brevibacterium lactofermentum hom* gene encoding the monofunctional HD (26) and the *thrB* gene encoding HK (24, 25) and showed that both are clustered. The *thrC* gene encoding TS was found to be at a separate position in the chromosome (21). An arrangement of the three genes identical to that in *B. lactofermentum* was found in *Corynebacterium glutamicum* (12, 13, 32). These two microorganisms are closely related nonpathogenic corynebacteria (7a). The nucleotide sequence of the *hom-thrB* cluster in both organisms is highly similar, since only 27 nucleotides in the *thrB* gene and three nucleotides in the *hom* gene are different, and therefore, the respective polypeptides are also very similar at the amino acid sequence level. For the *C. glutamicum hom-thrB* cluster, it was shown that both genes form a bicistronic operon transcribed from a promoter in front of *hom* (32). However, the fact that *E. coli* mutants defective in HK could be complemented by the *B. lactofermentum thrB* gene alone and that *thrB* alone could be expressed in *E. coli* (24) suggested that in *B. lactofermentum*, *thrB* is preceded by its own promoter and thus that the transcriptional organization of the *hom-thrB* cluster is different from that in *C. glutamicum*. In this report, we study the *hom-thrB* cluster in *B. lactofermentum* by heterologous and homologous complementation and expression studies and present a detailed analysis of the promoters, transcripts, and a functional terminator of the *hom-thrB* cluster.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli*, *Brevibacterium*, and *Corynebacterium* strains used in this work are listed in Table 1. *B. lactofermentum* ATCC 13869 (wild-type strain) was used as a source of DNA. *B. lactofermentum* R31, a strain that gives a high efficiency of transformation (38), was used as a host in protoplast transformation experiments. Corynebacteria were grown in trypticase soy broth (TSB; Difco) or TSA (TSB with 2% agar) complex medium at 30°C. Minimal medium for corynebacteria (MMC) was as described by Kaneko and Sakaguchi (16). *E. coli* strains were grown in Luria broth (LB) or Luria agar (LA) (35) or in VB minimal medium (40) at 37°C, except when other conditions are specified.

Complementation of *E. coli* auxotrophs was tested in solid and liquid VB minimal media. Complementation of *B. lactofermentum* auxotrophs in MMC was studied. The following supplements were added, when required, to the minimal media: 0.1 mM *meso*-diaminopimelic acid, 0.2 mM L-homo-

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype or characteristic[a]	Source[b] or reference
Strains		
E. coli C600-1	F⁻ *trpB thr-1 leuB6 thi-1 supE44 lacY1 tonA31 tonY1* r_k^- m_k^-	30
E. coli GT14[c]	*thrA1101 metLM1005 lysC1004 pro1001 serB22*	42
E. coli Gif106M1[c]	*thrA1101 metLM1000 lysC1001 arg1000 ilv296 thi-1 malA1 xyl-7 ara-13 mtl-2 supE44* λ⁻ λ^r	CGSC
E. coli GT15[d]	*thrA1015 metLM1005 lysC1004 pro1001 serB22*	42
E. coli Gif102[d]	*lysC1004 thrA1015 metLM1005 thi-1 relA1 spoT1* λ⁻	CGSC
E. coli GT201[c,d]	*thrA1A2 metLM1005 lysC1004 lacZU239*	42
E. coli Gif99[c,d]	*thr1100 metLM1000 arg1000 ilvA296 thi-1 malA1 xyl-7 ara-13 mtl-2 strA9 supE44* λ⁻ λ^r	CGSC
E. coli GT20	*thrB* Str⁺ HfrH	42
E. coli YA73	*thrB100 thi-1 relA1 spoT1* λ⁻	CGSC
E. coli GT121	*thrC metLM1005 lysC1004 lacZU239*	42
E. coli Gif41	*thrC1001 thi-1 relA1 spoT1* λ⁻	CGSC
E. coli JM101	*lac pro thi supE/F tra36 proAB laciZ*	28
E. coli DH5	F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17* r_k^- m_k^+ *sup44 relA1 deoR* λ⁻	19
E. coli DH5α	F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17* r_k^- m_k^+ *sup44 relA1* λ⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169	14
E. coli HB101	F⁻ *hsdS20* r_B^- m_B^- *recA13 ara14 proA2 lacY1 galK2 rpsL20* Sm^r *xyl-5 mtl-1 supE44* λ⁻	4
E. coli LE392	F⁻ *hsdR574* r_k^- m_k^+ *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*	Promega
B. lactofermentum ATCC 13869	Wild type	ATCC
B. lactofermentum R31	Aec^r Mly^r white	38
B. lactofermentum BL1035	*thrC* Aec^r	38
B. flavum ATCC 21474	*hom*	ATCC
B. ammoniagenes UL1	*thrB*	ULECC
C. glutamicum ATCC 13032	Wild type	ATCC
C. glutamicum CM1210	*leu hom*	ULECC
C. glutamicum UL9	*thrB*	ULECC
Plasmids		
pUC series	Ap *lacZ*	Laboratory stock
pULTH series	Plasmids containing the *thrB* gene	24
pKK232-8	Ap, promoterless *cat*	5
pKO-2	Ap, promoterless *galK*	2
pKM-2	Same as pKO-2 but containing a terminator before the promoterless *galK*	2
pKG1800	Ap *galK*	33
pRK238*c*Its	Tc *cI*	29
pEVvrf3	Ap	8
pULMM1	pEVvrf3 + *Hin*dIII (*hom* gene)	This work
pULP1	pKK232-8 + *Sma*I-*Hae*III (*hom* promoter)	This work
pULP2	pKK232-8 + *Sma*I-*Hin*dIII (*hom* promoter)	This work
pULPM1	pKM-2 + *Eco*RI-*Eco*RV (*hom* promoter)	This work
pULPM2	pKM-2 + *Eco*RI-*Hin*dIII (*hom* promoter)	This work
pULPO1	pKO-2 + *Eco*RI-*Eco*RV (*hom* promoter)	This work
pULPO2	pKO-2 + *Eco*RI-*Hin*dIII (*hom* promoter)	This work
pULMJ31	pBR322 derivative containing Ap, Hyg, and the promoterless Km resistance gene	6
pULP8	pULMJ31 containing the 87-bp *Sau*3AI-*Bam*HI *thrB* promoter	This work
pULP8T	Same as pULP8 but containing tandem copies of the *thrB* promoter	This work
pULP-BB1	pKK232-8 containing an 87-bp *Bam*HI fragment from pULP8T	This work
pULM880	*E. coli*-corynebacterium promoter-probe vector containing Ap and the promoterless Km resistance gene	21
pULM900	pULM880 containing the 87-bp fragment (*thrB* promoter) before the Km resistance gene	This work
pULT1	pKG1800-derivative containing a 185-bp *Nru*I-*Pvu*II terminator fragment from the *hom-thrB* cluster	This work
pULT2	pKG1800-derivative containing a 127-bp *Hin*cII-*Pvu*II terminator fragment from the *hom-thrB* cluster	This work
pGEM-3Z	Ap *lacZ*	Promega
pGEM-4Z	Ap *lacZ*	Promega
pGEMAP1	pGEM-4Z containing a 0.96-kb *Eco*RV-*Nhe*I fragment from pULR9	This work
pGEMAP2	pGEM-3Z containing a 1.7-kb *Hpa*I fragment from pULR9	This work

[a] Aec, *S*-aminoethylcysteine; Mly, methyllysine; Ap, ampicillin; Km, kanamycin; Hyg, hygromycin; Sm, streptomycin; Tc, tetracycline.
[b] ULECC, University of León Culture Collection; CGSC, *E. coli*: Genetic Stock Center (Yale University, New Haven, Conn.); ATCC, American Type Culture Collection.
[c] Aspartokinase ($thrA_1$) deficient.
[d] HD ($thrA_2$) deficient.

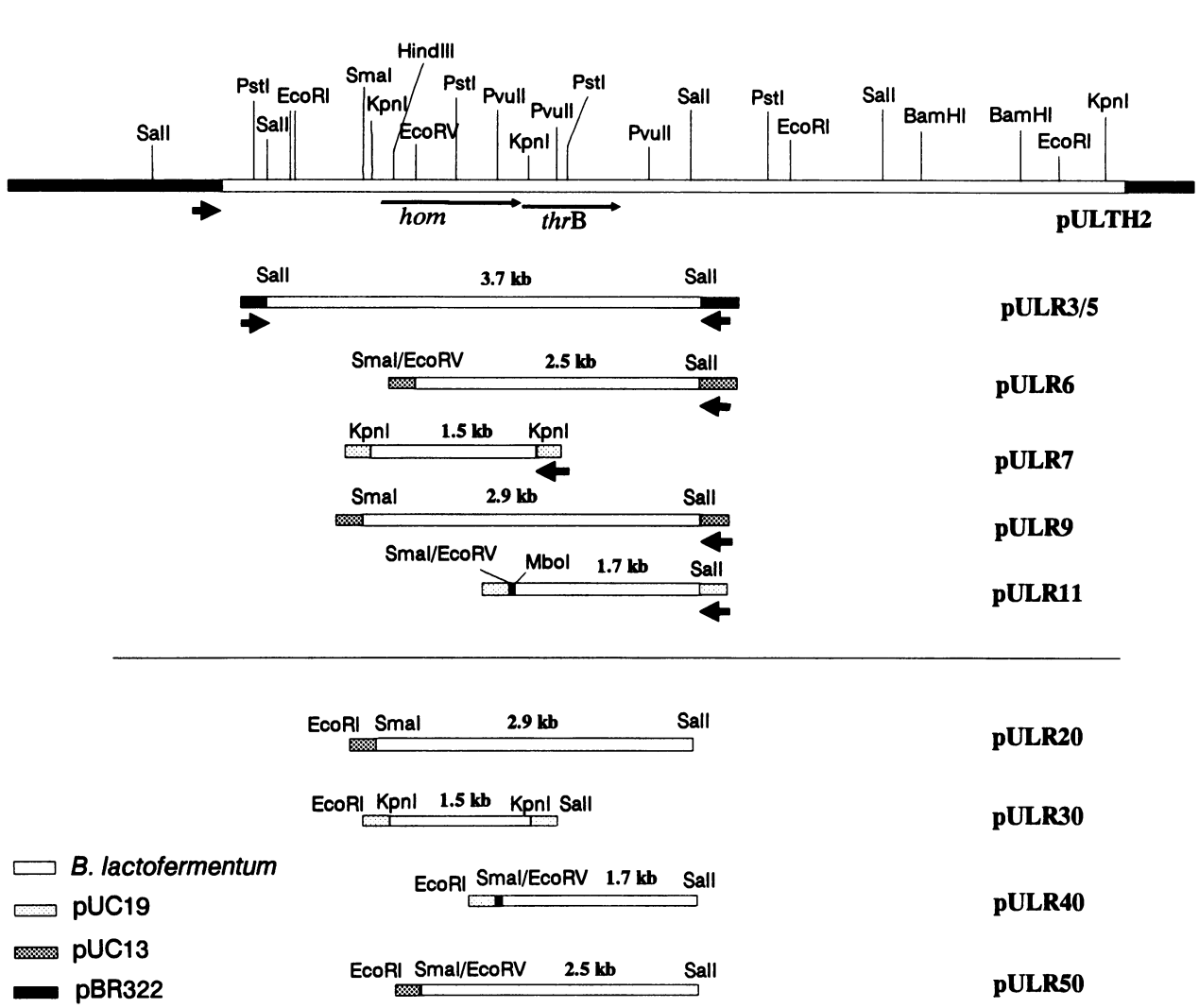


FIG. 1. Constructions used for homologous and heterologous expression of the *B. lactofermentum hom* and *thrB* genes. (A) DNA fragments were subcloned from pULTH2 into pBR322, pUC13, or pUC19 as indicated and used for complementation and expression studies in *E. coli* auxotrophs. The short arrows indicate the orientations of the *tet* or *lac* promoters in the vectors, and the long, thin arrows show the positions of the *hom* and *thrB* genes. (B) DNA fragments containing the *hom* and/or *thrB* genes subcloned into the corynebacterium vector pULRS6 by replacement of the *Eco*RI-*Sal*I fragment of the *hyg* gene of this vector.

serine, and 0.15 mM (final concentration) L-threonine. Other amino acids were used at 0.3 mM.

DNA techniques and transformation of *E. coli* and corynebacteria. Total DNA from *B. lactofermentum* was obtained as described previously (24). Plasmid DNA from *B. lactofermentum* was purified as described by Kieser (18), except that treatment with lysozyme was extended for 3 h. Polyethylene glycol-assisted transformation of protoplasts and electroporation of corynebacteria were done as described before (9, 38).

Plasmid DNA from *E. coli* was isolated by the alkaline lysis procedure, and transformation was carried out by the RbCl method (35). Restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were used as recommended by the manufacturers. Electrophoresis and isolation of DNA fragments were performed as described by Sambrook et al. (35).

DNA sequencing. DNA of *B. lactofermentum* subcloned in pBR322, pUC13, or pUC19 was digested with several restriction enzymes, and the resulting fragments were subcloned in phage M13-derived vectors mp10 and mp11 (27). Single-stranded DNA was isolated after transformation of *E. coli* JM101. Sequencing was done by the dideoxy method (36), using the Amersham sequencing kit. The DNA sequencing of cloned fragments in promoter probe vectors was done by the supercoiled DNA sequencing technique (2).

Expression of the *hom* gene from the p_L promoter. The expression system consists of two compatible plasmids, pRK 238c*I*(Ts), which contains the Tc^r gene and the gene for the heat-sensitive repressor of lambda phage c*I*(Ts) (29), and pEVvrf3 (8), and the host strain *E. coli* DH5. For expression, the *hom* gene was inserted into the plasmid pEVvrf3, which contains the Ap^r gene, the lambda p_L promoter, and the consensus ribosome-binding sequence (SD) of *E. coli*. *E. coli* DH5RK cells transformed with pEVvrf3 or pULMM1 (the latter containing the *hom* gene) were grown at 30°C in LB medium supplemented with ampicillin (50 μg/ml) and tetracycline (25 μg/ml) to an optical density at 600 nm of 0.3 (about 6×10^7 cells per ml); the cultures were divided into two flasks,

and one of them was kept at 30°C and the other was given a heat shock (1 min at 60°C) and further incubated for 30, 60, or 90 min at 42°C. Cells were harvested by centrifugation and lysed. Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Mateos et al. (24).

Subcloning in promoter-probe and terminator-probe vectors. Four vectors, pKK232-8 (which contains a polylinker region located upstream from the promoterless chloramphenicol acetyltransferase [*cat*] reporter gene), pKO-2 and pKM-2 (with the promoterless galactokinase gene), and pULMJ31 carrying the promoterless kanamycin resistance gene, were used for promoter studies with *E. coli* (Table 1). Different fragments located upstream from the *hom* and *thrB* genes from *B. lactofermentum* were subcloned into these vectors to study whether they are functional in *E. coli*. pKK232-8 derivatives containing functional promoter sequences were selected by chloramphenicol and ampicillin resistance (final concentration, 100 and 50 μg/ml, respectively). The *galK* gene present in pKM and pKO vectors offers the advantages of a readily assayable enzyme activity. A Gal$^-$ host cell cannot metabolize galactose and cannot grow on minimal plates containing galactose as the sole carbon source. On MacConkey galactose indicator plates (Mac-*gal*), they grow as white colonies. Complementation with a promoter-containing plasmid (*galK*$^+$) allows growth on minimal galactose plates (positive selection) and produces red colonies on Mac-*gal* plates (positive screen). pULMJ31 was used to clone the *thrB* promoter; clones carrying inserted promoters were selected as kanamycin (50 μg/ml)- and ampicillin-resistant transformants. The *E. coli*-corynebacterium bifunctional plasmid pULM880 containing the ampicillin resistance gene and the promoterless kanamycin resistance gene preceded by the *B. lactofermentum trp* terminator (21) was used to test the promoter activity of the *thrB* promoter in *B. lactofermentum* R31. The transcriptional terminator activity was tested by using the vector pKG1800, which contains the complete *galK* gene and a polylinker between the promoter and the structural gene.

RNA isolation, Northern (RNA) hybridization, primer extension experiments, and S1 endonuclease mapping. Total RNA from *B. lactofermentum* and from *C. glutamicum* was extracted essentially as described by Börmann et al. (3), except that the phenol extraction temperature was 65°C.

For Northern hybridization, about 10 μg of total RNA from *B. lactofermentum* or from *C. glutamicum* was treated with 1 U of DNase (RNase free) for 15 min at 37°C, mixed with 10 μl of loading dye, heated for 10 min at 95°C, cooled on ice, loaded, and separated by agarose-formaldehyde gel electrophoresis (35).

After electrophoresis, the RNA was transferred onto a nylon membrane (Nytran 13; Schleicher & Schuell), using a VacuGene apparatus from Pharmacia. For hybridization, three digoxigenin-dUTP-labeled mRNA probes (see Fig. 5A) were prepared as follows: a 0.96-kb *Eco*RV-*Nhe*I fragment from plasmid pULR9 was cloned into the *Sma*I-*Xba*I sites of the transcription vector pGEM-4Z (Promega), resulting in pGEM AP1, and a 1.7-kb *Hpa*I fragment from pULR9 was cloned into the *Sma*I site of the transcriptional vector pGEM-3Z (Promega), resulting in pGEMAP2. After linearization of pGEMAP1 with *Eco*RI and of pGEMAP2 with *Acc*I and *Hin*dIII, respectively, three digoxigenin-labeled antisense transcripts covering *hom*, *thrB*, and *hom-thrB*, respectively, were generated by using T7 RNA polymerase and the SP6/T7 transcription kit from Boehringer. Hybridization of the antisense probes was carried out at 46°C. After a washing, detection was performed by using the DIG Nucleic Acid Detection Kit from Boehringer. The 0.24- to 9.5-kb RNA ladder from Gibco/BRL was used as a size marker.

TABLE 2. Complementation of threonine auxotrophs of *Brevibacterium* and *Corynebacterium* spp. by the subcloned DNA fragments of *B. lactofermentum*

Strain	Complementation of transformants[a] with:			
	pULR20	pULR30	pULR40	pULR50
B. lactofermentum BL1035 (*thrC*)	−	−	−	NT
B. flavum ATCC 21474 (*hom*)	+	+	−	NT
B. ammoniagenes UL1 (*thrB*)	+	−	+	+
C. glutamicum CM1210 (*leu hom*)	+ (Leu$^-$)	NT	−	NT
C. glutamicum UL9 (*thrB*)	+	NT	+	+

[a] In all cases, transformants were selected by resistance to kanamycin (50 μg/ml). NT, not tested.

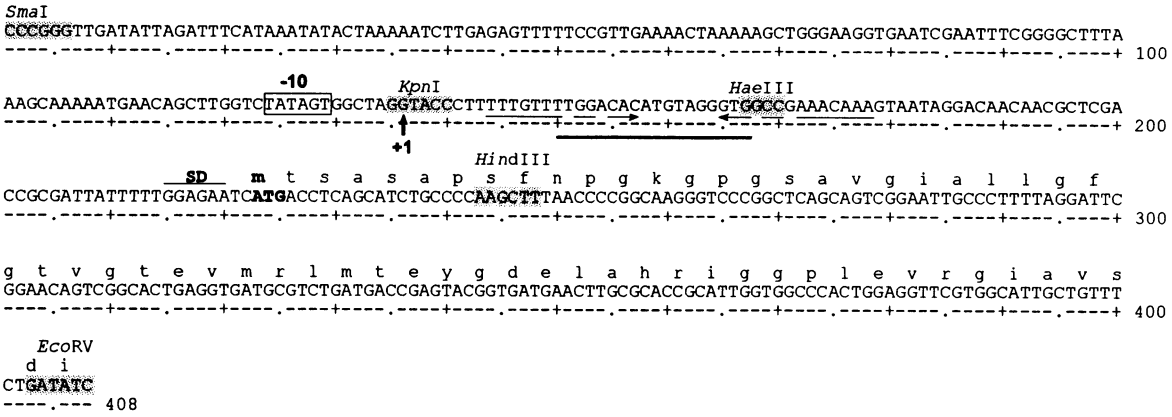
For primer extension analysis, about 70 μg of total RNA from *B. lactofermentum* or from *C. glutamicum* was dried, dissolved in 0.1 ml of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA (pH 8), 0.4 M NaCl in 80% formamide], denatured together with primer DNA for 10 min at 95°C, and annealed for 12 h at 42°C. The nucleic acids were precipitated with ethanol and dissolved in 20 μl of avian myeloblastosis virus (AMV) reverse transcriptase (RT) mixture containing 4 μl of 5× AMV-RT buffer (Promega); a 250 μM concentration (each) of dCTP, dGTP, and dTTP; 2.5 μM dATP; and 40 U of RNasin (Promega). This mixture was supplemented with 1 μl of actinomycin (5 mg/ml) and 2.6 μl of [α-^{35}S]dATP. The reaction mixture was heated at 42°C for 2 min, and the primer extension was carried out by adding 3 U of AMV RT and incubating for 2 h at 42°C. The reaction was stopped with 1 μl of 0.5 M EDTA (pH 8). Free RNA was removed then by treatment with RNase for 30 min at 37°C and the DNA was precipitated with ethanol (−20°C) and redissolved in 3 μl of TE buffer plus 3 μl stop buffer (U.S. Biochemicals sequencing kit). A total of 3 μl of the sample was loaded and separated on a 6% denaturating polyacrylamide gel. The sequencing ladder was generated by using the same primer used for the primer extension reaction. All primers were synthesized with the Gene Assembler Plus and the appropriate chemicals from Pharmacia.

For S1 endonuclease mapping, a 417-bp *Eco*RI-*Eco*RV DNA fragment from pULR9 was labeled with [γ-^{32}P]ATP at the 5′ ends and used for hybridization with total RNA of *B. lactofermentum*. Increasing amounts of RNA were mixed with the labeled probe in 20 μl of hybridization buffer. After being heated for 10 min at 95°C, the samples were allowed to hybridize at 67°C for 10 h (with very slow decreases in the temperature). A total of 300 μl of ice-cold S1 nuclease buffer containing 200 U of S1 nuclease was added to the hybridization reaction mixture and incubated for 1 h at 37°C (10). After ethanol precipitation, samples were loaded on a 6% (wt/vol) acrylamide–7 M urea DNA-sequencing gel. RNA from *E. coli* was used as a negative control in the hybridization experiments. A sequencing ladder of M13 phage (44) was used as a control of band size.

RESULTS

The *hom-thrB* cluster of *B. lactofermentum* does not complement *E. coli thrA*$_2$ mutants. Several fragments of different size

A



B

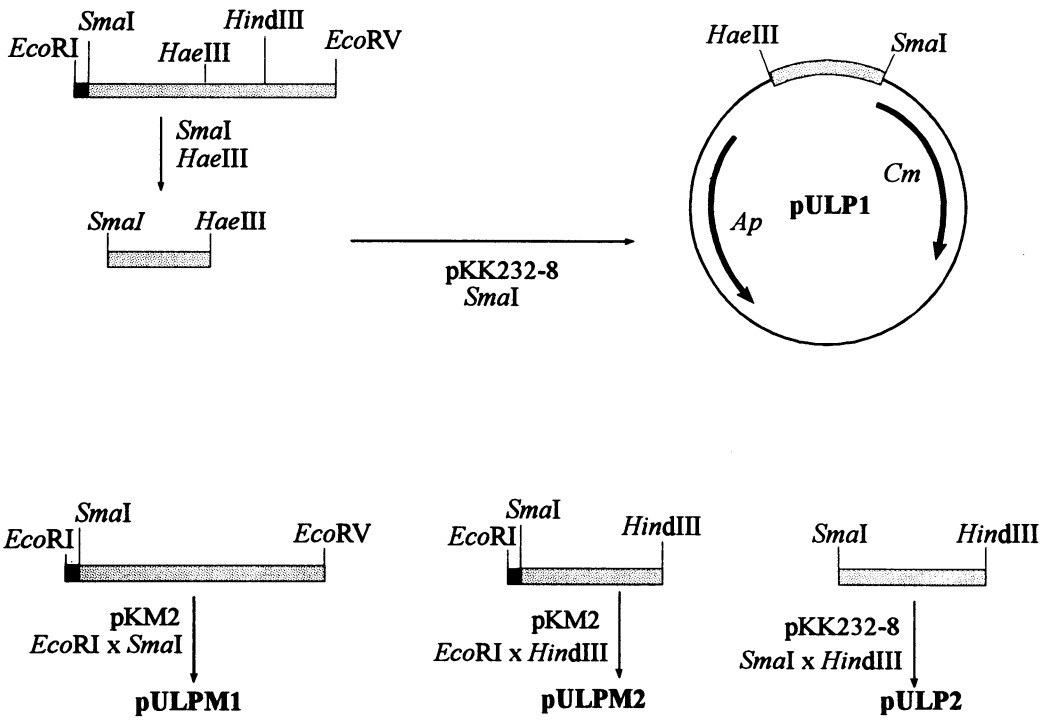


FIG. 2. (A) Nucleotide sequence of the *B. lactofermentum hom* gene upstream region (*Sma*I-*Eco*RV) subcloned in the *E. coli* promoter-probe vectors pKK232-8 and pKM2 for expression studies. The transcription start point (+1) is indicated by a vertical arrow. The putative −10 region is boxed, and the first translated codon of the *hom* gene is shown in boldface type. Convergent arrows indicate an inverted repeat in the leader transcript region. The target sequences of the different restriction endonucleases are shadowed. SD, hypothetical ribosome-binding sequence. The nucleotides corresponding to the primer used for primer extension analysis are underlined (black bar). The *hom* nucleotide sequences have been deposited in the EMBL gene library under accession numbers X77191 and Y00476. (B) Strategies used to subclone the upstream region of the *hom* gene in the promoter-probe vectors. The black fragment originates from the pUC polylinker.

were subcloned from the original plasmid pULTH2 (24) in pBR322, pUC13, or pUC19 vectors, giving rise to plasmids pULR3 to pULR11 (Fig. 1A), which were used to transform the *E. coli thr* auxotrophs shown in Table 1. *E. coli thrB* auxotrophs (C600-1, YA73, and GT20) but not $thrA_2$ (= *hom*) strains were complemented, although the *hom* gene is complete in pULR3, pULR5, and pULR9 (see sequence in reference 26). There are two possible explanations for the lack of complementation of the *E. coli* $thrA_2$ mutations: either the promoter and regulatory sequences of the *B. lactofermentum hom* gene are not recognized in *E. coli* or the *B. lactofermentum* HD is not functional in *E. coli*.

Four *Eco*RI-*Sal*I DNA fragments from plasmids pULR6, pULR7, pULR9, and pULR11 were subcloned into the corynebacterium vector pULRS6 (39), resulting in plasmids pULR50, pULR30, pULR20, and pULR40, respectively (Fig. 1B). pULR20 complemented the *hom* and *thrB* mutations in the corynebacteria listed in Table 2. pULR30 was able to complement the *hom* mutation of *Brevibacterium flavum* ATCC 21474, and pULR40 and pULR50 complemented the *thrB* auxotrophies of *Brevibacterium ammoniagenes* and *C. glutamicum*. The lack of complementation of *B. lactofermentum* BL1035, an auxotroph that has been identified as a *thrC* mutant deficient in TS (21), by pULR20 indicates that the *thrC* gene is not located in the 2.9-kb *Sma*I-*Sal*I fragment of native DNA.

Lack of expression of the *hom* promoter region in *E. coli*. Since the *hom-thrB* cluster did not complement *E. coli* $thrA_2$ mutants, expression of the *hom* gene was studied in detail. Blunt-ended *Sma*I-*Hae*III (165-bp) and *Sma*I-*Hin*dIII (247-bp) DNA fragments from the upstream region of the *hom* gene were subcloned into pKK232-8 carrying the promoterless *cat* gene as a reporter (Fig. 2) and transformed into *E. coli* DH5. Chloramphenicol-resistant colonies could only be isolated with plasmids carrying the *Sma*I-*Hae*III insert. However, all the clones carried the plasmid pULP1 with the fragment in an orientation opposite to that of the *hom* promoter (Fig. 2B). Ampicillin-resistant transformants containing the *Sma*I-*Hae*III fragment or the *Sma*I-*Hin*dIII fragment in the expected orientation in pKK232-8 were also obtained; however, they were not resistant to chloramphenicol.

Two other fragments, *Eco*RI-*Eco*RV (420 bp) and *Eco*RI-*Hin*dIII (262 bp), carrying the *hom* promoter region were subcloned into the promoter probe vectors pKO-2 (resulting in plasmids pULPO1 and pULPO2) and pKM-2 (giving rise to plasmids pULPM1 and pULPM2, respectively). In these constructions, the *hom* promoter was in the proper orientation with respect to the reporter *galK* genes. All the plasmids were used to transform *E. coli* HB101 *galK*. The recombinant strains containing pULPM1 and pULPM2 plasmids were unable to grow on minimal medium with galactose as a carbon source, and when plated on MacConkey medium, the colonies were white, confirming that galactose was not utilized.

An active HD is formed in *E. coli* from the *hom* gene. Strong expression of the *hom* gene in *E. coli* was observed by coupling the *B. lactofermentum hom* gene to the expression vector pEVvrf3 (8) (Fig. 3A). In the resulting plasmid (pULMM1), the fused open reading frame encodes a protein lacking 6 amino acids from the N-terminal end of the HD but contains 9 amino acids corresponding to the linker of the pEVvrf3 vector. As shown in Fig. 3B, strong expression of a protein of the expected size, 47 kDa, was observed at 30, 60, and 90 min after heat induction but not in uninduced cells or in transformants with the control vector pEVvrf3 without an insert.

The *E. coli* auxotrophs GT15 and Gif102 deficient in HD activity were complemented after transformation with pUL MM1 but not with pEVvrf3 when they were grown at 40°C.

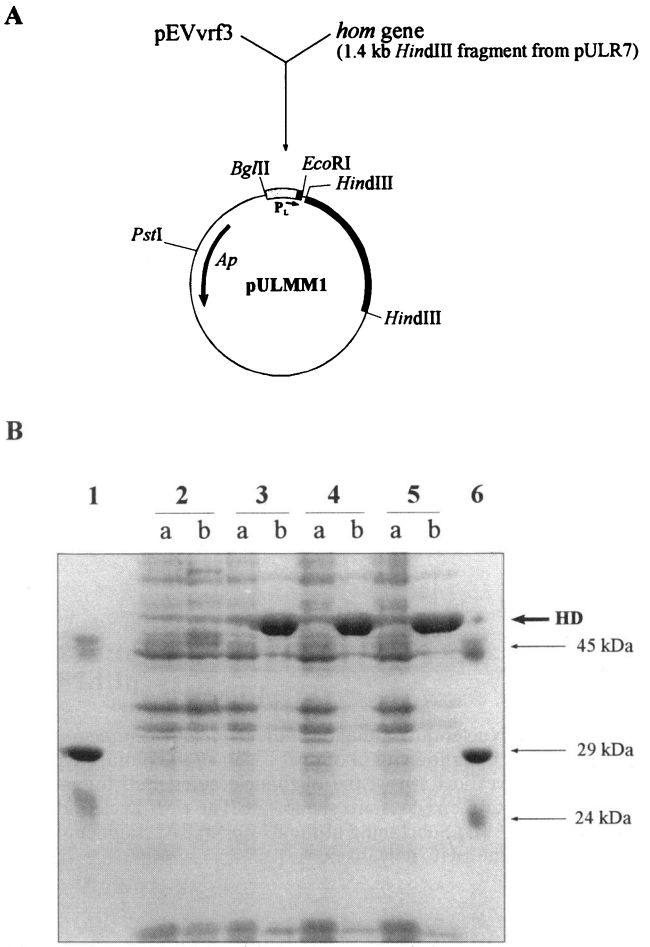


FIG. 3. Expression of the HD encoded by the *hom* gene of *B. lactofermentum* as a fusion protein in *E. coli*. (A) Details of the construction in the pEVvrf3 expression vector. (B) SDS-PAGE of the proteins formed before and after heat induction. Lanes: 1 and 6, size markers (ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; and trypsinogen, 24 kDa); 2, *E. coli* transformed with pEVvrf3 without insert; 3, 4, and 5, *E. coli* transformed with pULMM1 induced for 30, 60, and 90 min, respectively (lanes b), or without induction (lanes a). The gel was stained with Coomassie blue.

These results prove that the *B. lactofermentum* HD is functional in *E. coli* and confirm that the lack of complementation observed initially was due to inability of *E. coli* to express sufficient amounts of the *B. lactofermentum hom* gene product. Since *thrB* did complement *E. coli thrB* mutants, these data also suggest that this gene, in contrast to *hom*, is expressed at high levels in *E. coli*, presumably from a promoter other than *hom*.

The *thrB* promoter is located in an 87-bp DNA fragment overlapping the *hom* gene. According to the nucleotide sequence of the *hom-thrB* cluster region (25, 26), the ATG start codon of the *thrB* is separated by 10 bp from the TAA termination codon of the *hom* gene (Fig. 4A). To determine if there is a separate promoter for the *thrB* gene (internal to the *hom* gene), a 467-bp *Pvu*II-*Bam*HI fragment was isolated from pULR7 (the *Bam*HI site belongs to the pUC19 polylinker) (Fig. 4B). This fragment was partially digested with *Sau*3AI and the fragments ligated to the *E. coli* promoter probe vector pULMJ31 which contains the promoterless kanamycin resistance gene as a reporter. All *E. coli* clones resistant to

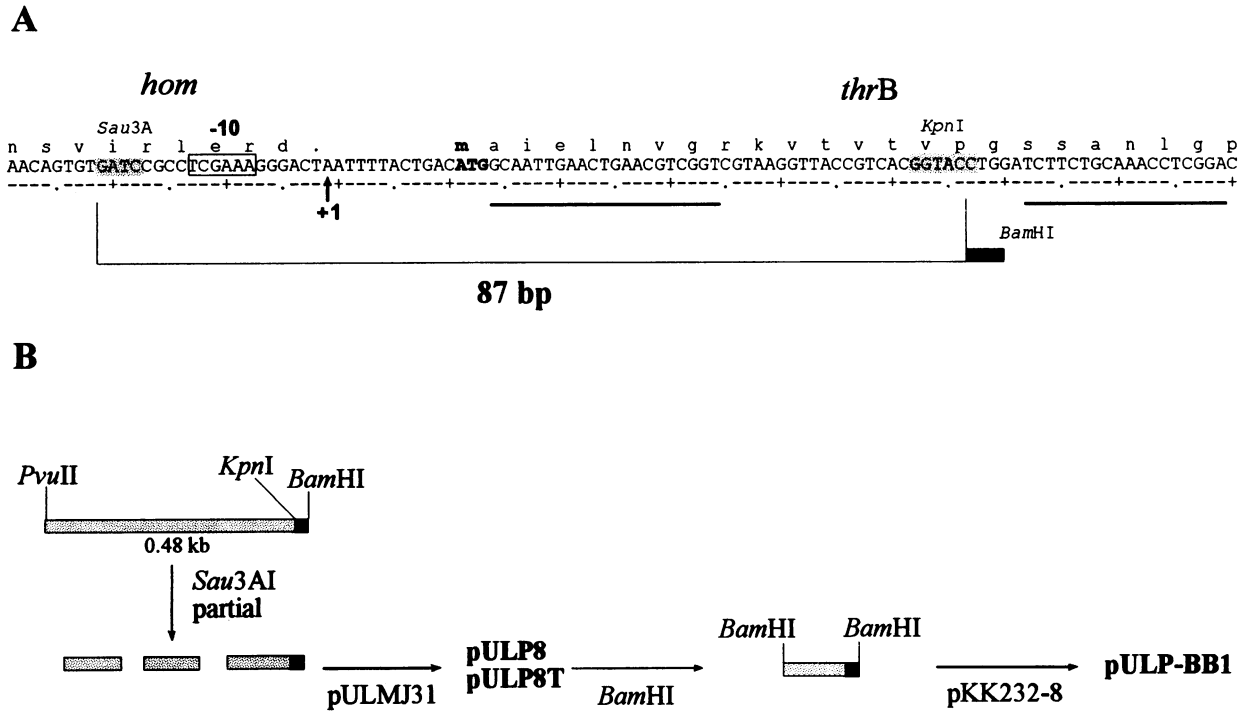


FIG. 4. (A) Nucleotide sequence of the *B. lactofermentum hom-thrB* intergenic region showing the 87-bp DNA fragment with promoter activity. The translation initiation codon of *thrB* is shown in boldface type. The putative −10 region is boxed, and the first transcribed nucleotide (+1) is indicated by a vertical arrow; the oligonucleotides used for primer extension studies are underlined (black bar). The target sequences of the different restriction enzymes are shadowed. The *thrB* nucleotide sequence have been deposited in the EMBL gene library under the accession number Y00140. (B) Subcloning of the 87-bp *Sau*3AI fragment in the promoter probe vectors pULMJ31 and pKK232-8. The black bar fragments originate from the pUC polylinker.

kanamycin carried a plasmid (pULP8) containing the 87-bp fragment shown in Fig. 4A. In some clones, two tandemly repeated 87-bp fragments (plasmid pULP8T) from which the 87-bp fragment was isolated as a *Bam*HI casette were found.

The 87-bp fragment also showed promoter activity in *E. coli* when subcloned into the *E. coli* promoter probe vector pKK232-8 (resulting in plasmid pULP-BB1) and into corynebacteria, using the bifunctional promoter probe vector pULM880 (resulting in plasmid pULM900). The kanamycin resistance marker in pULM900 is efficiently expressed in *E. coli* and corynebacterium conferring resistance to 50 μg of kanamycin per ml, which confirms our initial findings which showed that *B. lactofermentum thrB* is expressed in *E. coli*.

Northern hybridization analysis. The results obtained by complementation analysis and by cloning of *hom* and *thrB* upstream regions into promoter-probe vectors suggested the presence of functional promoters in front of both the *hom* and the *thrB* genes of *B. lactofermentum* and thus that both genes are transcribed independently. In order to study the transcriptional organization of these genes, we performed Northern blot hybridization with total RNA from *B. lactofermentum* and antisense RNA probes covering *hom-thrB*, *thrB*, or *hom* (Fig. 5A). As shown in Fig. 5B, two transcripts of 2.5 and 1.1 kb were observed on hybridization to the *hom-thrB*-specific probe (lane 1) and to the *thrB*-specific probe (lane 3). On hybridization to the *hom*-specific probe (lane 5) only the 2.5-kb signal was obtained. Hybridization with total RNA from *C. glutamicum* revealed an identical hybridization pattern (lanes 2, 4, and 6, respectively). These results indicate (i) that a bicistronic mRNA covering *hom* and *thrB* is formed, (ii) that additionally, a monocistronic mRNA covering only *thrB* is synthesized, (iii) that upstream from both *hom* and *thrB* there are promoters, and (iv) that the transcriptional organization of the *hom-thrB* cluster is identical in *B. lactofermentum* and in *C. glutamicum*.

Transcriptional start point of the *hom* and *thrB* genes. S1 mapping studies with a 417-bp *Eco*RV-*Eco*RI probe to protect the *hom* transcript revealed a protected fragment of 270 nucleotides (Fig. 6A). This protected fragment upstream from *Eco*RV indicated that the transcription start point is located at or around a G 87 bp upstream from the ATG initiation codon of the *hom* gene (Fig. 2A). This G as the first transcribed nucleotide of the *hom* gene was confirmed by primer extension studies (Fig. 6B) with a primer covering a region 56 to 73 nucleotides upstream of the translational start codon. The selection of the primer used for primer extension studies of the *hom* promoter was made to avoid premature and unspecific stopping of the primer extension due to the secondary structure. The signals at the bottom of Fig. 6B, lane 2, are also probably due to the secondary structure.

On the basis of the S1 mapping and primer extension identification of the transcriptional start point, we have defined the −10 region, TATAGT, which conforms quite well to the consensus Pribnow box of *E. coli* and corynebacteria. If we assume a standard 16- to 18-bp spacing region, a putative −35 region, AAAGCA, can be defined.

The transcriptional start site in front of *thrB* was also identified by primer extension with a primer covering the codons 2 to 8 of the *thrB* gene. The main signal obtained (Fig. 7) corresponds to a T residue and to an A residue located 13 and 12 bp, respectively, upstream of the translational start. Primer extension experiments using a different primer (codons 18 to 23) gave a signal corresponding to the same position

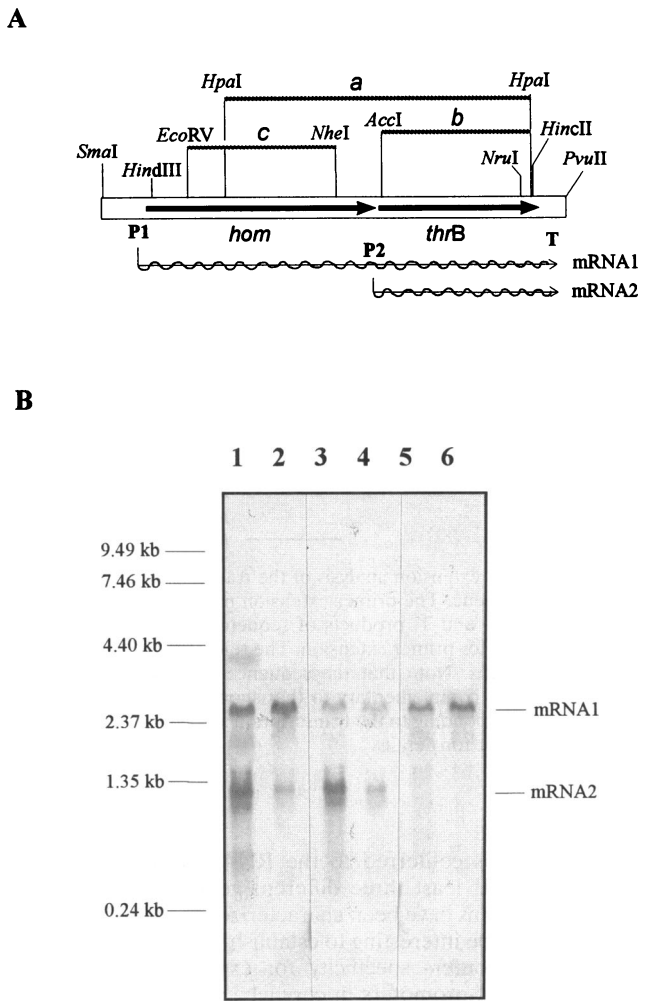


FIG. 5. Transcriptional analysis of the *hom-thrB* cluster. (A) Organization of the cluster and probes used for hybridization. P1 and P2, promoter regions; T, terminator. The *Eco*RV-*Nhe*I and *Hpa*I-*Hpa*I fragments used to prepare the antisense RNA probes are indicated by bars at the top. The two transcripts (mRNA1 and mRNA2) are shown by wavy lines with arrows. (B) Hybridization of RNA of *B. lactofermentum* (lanes 1, 3, and 5) and *C. glutamicum* (lanes 2, 4, and 6) with probe *a* (lanes 1 and 2), probe *b* (lanes 3 and 4), and probe *c* (lanes 5 and 6).

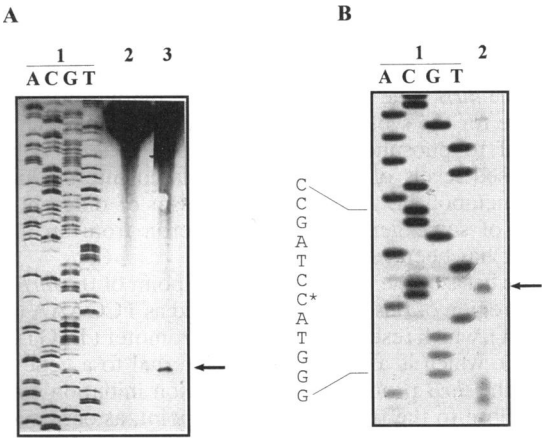


FIG. 6. (A) S1 endonuclease protection analysis of the 5′ end of the transcript initiating at the *hom* promoter. Lanes: 1, sequence of phage M13mp18 used as a control; 2, labelled probe used in the protection studies; 3, protected fragment (arrow). (B) Primer extension analysis of the transcriptional start site in front of the *hom* gene. The primer extension product is shown in lane 2. Lanes A, C, G, and T, products of sequencing reactions with the same primer used for primer extension. The relevant DNA sequence is shown on the left. Note that the sequence represents the coding strand and thus is complementary to that shown in Fig. 2A. The +1 nucleotide is indicated (asterisk and arrow). See text for details.

(−12) and thus confirmed the result obtained with the first primer. Bands of minor intensity observed in Fig. 7, lane 1, appear to be due to nonspecific termination of primer extension due to the secondary structure of the transcript.

A functional transcriptional terminator is located downstream from the *hom-thrB* cluster. Two different DNA fragments (185-bp *Nru*I-*Pvu*II and 127-bp *Hinc*II-*Pvu*II) from the downstream region of the *thrB* gene (Fig. 5A) were subcloned in the terminator probe vector pKG1800 (plasmids pULT1 and pULT2, respectively) and used to transform *E. coli* HB101 and LE392.

E. coli HB101 transformed with the recombinant plasmids did not express the reporter galactokinase gene as shown by the white colonies in MacConkey agar plates compared with the control red colonies transformed with pKG1800 without an insert. A further proof of the presence of a transcriptional terminator in these DNA fragments was provided by the lack of toxicity of pULT1 and pULT2 when introduced in the *galK galT* mutant *E. coli* LE392. Clones transformed with pULT1 and pULT2 grew perfectly on MacConkey agar (white colonies) compared with transformants of pKG1800 which do not grow on Mac-*gal* because of the accumulation of toxic phosphorylated sugar derivatives. The transcriptional terminator is, therefore, active in *E. coli* and prevents expression of the galactokinase gene.

Analysis of the nucleotide sequence of the DNA fragment with terminator activity revealed a perfect 12-bp inverted repeat, AAGGCCCCTTC (25), that may form in the RNA a stem and loop structure typical of terminators with a ΔG of −33.2 kcal/mol (43). The inverted sequence is followed by a T-rich sequence which may be transcribed into a run of U's typical of *rho*-independent terminators.

DISCUSSION

The *hom* and *thrB* genes of corynebacteria form a bicistronic operon under the control of the upstream promoter and regulatory signals. A transcript covering both genes was reported for *C. glutamicum* (12, 32) and has also been observed in *B. lactofermentum* in this work. Coordinate expression of both genes most likely confers an ecological advantage to corynebacteria by favoring synthesis and interaction of HD and HK.

We have observed, however, that *thrB* is additionally expressed from its own promoter to give a monocistronic transcript both in *B. lactofermentum* and *C. glutamicum*. This is in contrast to the results described for *C. glutamicum* by Follettie et al. (12). It remains unclear why those authors failed to observe the additional *thrB* transcript, although a faint 1.5-kb band appears to be present in their Northern analysis. It is interesting that in *E. coli*, the *thrB* gene is also expressed from the *thrABC* operon promoter as well as from its own promoter (34), which is located in the coding region of the $thrA_2$ gene. The *thrB*-specific promoter in corynebacteria is also located in the *hom* structural gene with the transcriptional start site

located within the translational stop codon of *hom* (Fig. 4A). Since the *hom-thrB* operon promoter is known to be repressed by methionine in *C. glutamicum* (32), the separate transcription of the *thrB* gene from the internal promoter may provide an escape for the synthesis of basal levels of HK, which might be useful to phosphorylate the homoserine formed by the unrepressed levels of HD or other related intermediates in central metabolism. A similar mechanism of differential expression of some genes of the *trp* operon from an internal promoter has been reported (15, 37).

On the basis of the transcription start point of the *thrB* gene, the −10 region of this gene was identified as TCGAAA (boxed in Fig. 4A), which resembles the *lysC*β promoter (TCGTCT) of *C. flavum*, which is also a promoter internal to a gene (*lysC*) (11). In the *thrB* promoter, the translation initiation codon is located close to the transcription start point, as occurs also in the *thrC* gene (21). These leaderless promoters belong to a group of transcription initiation sequences that are well known in species of *Streptomyces* (41). The −35 region of the *thrB* promoter shows the sequence GCCAAT, which is similar to that of other corynebacterial promoters (22). However the relevance of the −35 region in corynebacterial promoters is unclear. In this case, the −35 region is not contained in the 87-bp promoter that shows activity both in *E. coli* and corynebacteria. Although it might be tempting to conclude that the −35 region is not essential for transcription initiation at the *thrB* promoter, it is important to note that a functional −35 region may be provided by upstream sequences in the vector.

The lack of complementation of two different *E. coli* $thrA_2$ strains by the *hom* gene of *B. lactofermentum* indicated that the HD was not functional or that the *hom* gene was not expressed in *E. coli*. The first possibility was intriguing, since the *thrA* of *E. coli* encodes a bifunctional polypeptide AK-HD, whereas the *hom* gene from corynebacteria encodes a monofunctional HD activity. Comparison of the amino acid sequences of the HD of *B. lactofermentum* (26) with those of the AKI-HDI and AKII-HDII of *E. coli* (45) revealed that the similarity starts at amino acid 440, which is known to be the junction between the two domains of the bifunctional enzyme; the HD from corynebacteria is, therefore, entirely analogous to the HD domain of *E. coli*. The protein encoded by the *B. lactofermentum hom* gene was shown to be functional (by complementation of *E. coli* $thrA_2$ [HD] mutants) when expressed as a fusion protein from the lambda p_L promoter and the consensus ribosome-binding sequence of *E. coli*.

Further support for the lack of expression of the *hom* gene of *B. lactofermentum* in *E. coli* was provided by the inability to synthesize HD in *E. coli* minicells under conditions in which the HK is efficiently formed (24). In addition, the *hom* promoter gene was not expressed in *E. coli* when the *hom* promoter region was cloned in three different promoter-probe vectors by using the reporter chloramphenicol acetyltransferase or galactokinase genes. Assuming that complementation of Hom^- mutants of *B. lactofermentum* with DNA fragments containing the same 5′ ends as used in *E. coli* expression studies is a valid indicator of promoter activity, these results suggest that the *hom* gene is not expressed in *E. coli*. Although complementation of the $thrA_2$ mutation of *E. coli* by *C. glutamicum* DNA was reported, this appeared to occur by expression from *E. coli* promoters endogenous to the vector (1).

The lack of expression of the *hom* gene in *E. coli* supports our previous observation that there are at least two types of promoters in corynebacteria: those which are recognized both in *E. coli* and corynebacteria (i.e., *E. coli*-like promoters) and those that are used only in the gram-positive corynebacteria (corynebacterium-specific promoters) (23). Promoter recognition specificity is conferred to the RNA polymerase by the sigma factors. At least three different genes encoding sigma factor-like proteins have been characterized for *B. lactofermentum* (31). It will be interesting to establish whether any of those sigma factors confers specificity for expression of the two different types of promoters in corynebacteria represented by the *hom* and *thrB* promoters. Alternatively the lack of expression in *E. coli* may be due to a requirement for positive regulatory proteins that interact with the promoter region.

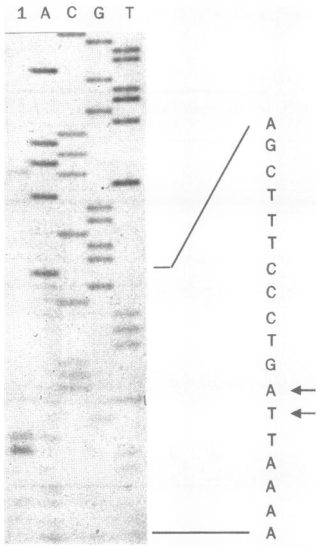


FIG. 7. Primer extension analysis of the transcriptional start site in front of the *thrB* gene. The primer extension product is shown in lane 1. Lanes A, C, G, and T, products of sequencing reactions with the same primer used for primer extension. The relevant DNA sequence is shown on the right. Note that the sequence represents the coding strand and thus is complementary to that shown in Fig. 4A. The two nucleotides corresponding to the transcription start point are indicated by arrows. See text for details.

The *hom* transcript contains a long inverted repeat in its 5′ end that may form a stem and loop structure (ΔG −16.2). It is tempting to believe that such a structure may be involved in an attenuation-like mechanism which requires additional proteins for expression, as proposed for *Streptomyces* spp. and other gram-positive bacteria. Such a requirement might explain why the *hom* gene of corynebacteria is not expressed in *E. coli*.

The presence of a functional transcriptional terminator in the *B. lactofermentum hom-thrB* operon confirms that both genes are controlled by common promoter and terminator signal sequences. A similar inverted repeat sequence that may also act as a functional terminator was reported downstream of the *thrB* gene of *C. glutamicum* (32). The inverted repeat from *C. glutamicum* lacks one of the G-C pairs of the *B. lactofermentum* terminator, and its estimated ΔG would be −28.2 kcal/mol compared with −33.2 kcal/mol for the *B. lactofermentum* terminator.

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