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Analysis and Expression of the *thrC* Gene of *Brevibacterium lactofermentum* and Characterization of the Encoded Threonine Synthase

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The *thrC* gene of *Brevibacterium lactofermentum* was cloned by complementation of *Escherichia coli thrC* auxotrophs. The gene was located by deletion mapping and complementation analysis in a 2.9-kb *Sau*3AI-*Hin*dIII fragment of the genome. This fragment also complemented a *B. lactofermentum* UL1035 threonine auxotroph that was deficient in threonine synthase. A 1,892-bp DNA fragment of this region was sequenced; this fragment contained a 1,446-bp open reading frame that encoded a 481-amino-acid protein having a deduced M_r of 52,807. The gene was expressed in *E. coli*, by using the phage T7 system, as a 53-kDa protein. The promoter region subcloned in promoter-probe plasmids was functional in *E. coli*. A Northern analysis revealed that the gene was expressed as a monocistronic 1,400-nucleotide transcript. The transcription start point of the *thrC* gene was located by S1 mapping 6 bp upstream from the translation initiation codon, which indicated that this promoter was one of the leaderless transcription-initiating sequences. The threonine synthase overexpressed in *B. lactofermentum* UL1035 was purified almost to homogeneity. The active form corresponded to a monomeric 52.8-kDa protein, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme required pyridoxal phosphate as its only cofactor to convert homoserine phosphate into threonine.

Coryneform bacteria are widely used for industrial production of amino acids, and their biosynthetic pathways are being studied to improve amino acid yields. Threonine is synthesized from aspartic acid in five enzymatic steps in all of the microorganisms that have been studied to date (8). The two first steps, which are catalyzed by aspartokinase and aspartate semialdehyde dehydrogenase, are common to diaminopimelate, lysine, and methionine biosynthesis and result in the conversion of aspartic acid to aspartate semialdehyde. Further reduction of the semialdehyde by homoserine dehydrogenase (*thrA* or *hom* gene) produces homoserine, which is also an intermediate in the biosynthesis of methionine. The homoserine hydroxyl group is then rearranged to produce threonine in two steps: (i) phosphorylation by homoserine kinase (*thrB* gene), resulting in homoserine phosphate, and (ii) rearrangement of the hydroxyl group (from C-4 to C-3) and dephosphorylation by threonine synthase (*thrC* gene), yielding threonine. The molecular mechanism of threonine synthase, a pyridoxal phosphate-requiring enzyme, is complex and requires further study.

In *Escherichia coli* the threonine operon is composed of three genes, *thrA*, *thrB*, and *thrC*, which code for aspartokinase I-homoserine dehydrogenase I (E.C. 2.7.2.4; E.C. 1.1.1.3), homoserine kinase (E.C. 2.7.1.39), and threonine synthase (E.C.4.2.99.2), respectively (7, 24). The threonine operon of *E. coli* maps at 0 min on the chromosome (47). A similar organization of the *thr* cluster occurs in *Serratia marcescens* (22). In *Bacillus subtilis* the three threonine biosynthetic genes are clustered in a different order (*hom-thrC-thrB*), and the *hom* gene differs from the *thrA* gene in encoding only homoserine dehydrogenase activity (33, 35). Recently, a new organization of the threonine biosynthetic genes has been found in *Pseudomonas aeruginosa*. In this microorganism, the *hom* and *thrC* genes are clustered in an operon at 31 min on the chromosome, whereas the *thrB* gene is located at 10 min. This *thrB* gene exhibits no homology with other *thrB* genes, and it has been proposed that there are at least two isoenzymes of homoserine kinase (*thrB*) in *P. aeruginosa* (6).

The threonine biosynthetic genes of *Corynebacterium glutamicum* have been also cloned and sequenced (16, 21, 37). In *Brevibacterium lactofermentum*, the *hom* and *thrB* genes are linked, but the *thrC* gene is not located in the same cluster (28, 29). Therefore, we decided to clone and characterize the *thrC* gene of *Brevibacterium lactofermentum* in order to determine the organization and expression of the *thr* genes of this organism.

In this paper we describe the molecular characteristics of the *Brevibacterium lactofermentum thrC* gene and the transcription and translation of this gene in *E. coli* and corynebacteria. In this study we examined the threonine synthase, and the protein was partially purified from corynebacterial cultures. The deduced amino acid sequence revealed that threonine synthase has a consensus pyridoxal phosphate-binding motif and supported the proposal that there is a molecular mechanism similar to the mechanism involving threonine dehydratase and tryptophan synthase.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The corynebacteria and *E. coli* strains used in this study are listed in Table 1. *Brevibacterium lactofermentum* ATCC 13869 was used as the source of DNA and *Brevibacterium lactofermentum* R31 (a strain exhibiting a high efficiency of transformation) was used as the recipient strain in transformation experiments. Coryne-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or description[a]	Reference or source[b]
Corynebacteria		
Brevibacterium lactofermentum ATCC 13869	Wild type, yellow	ATCC
Brevibacterium lactofermentum R31	MeLys^r Aec^r, white	40
Brevibacterium lactofermentum UL1035	Aec^r *thrC*, white	40, this study
C. glutamicum AS019	Rifampin-resistant mutant of ATCC 13059	K. Dunican
E. coli strains		
DH5α	F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17* (r_k^- m_k^+) *sup44 relA1* λ$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	Laboratory stock
WK6*mutS*	Δ(*lac-proAB*) *galE strA mutS215*::Tn*10*/F′ *lacI*^{qZ} ΔM15 *proA*$^+$*B*$^+$	R. Zell
Gif41	*thrC1001 thi-1 relA spoT1*	B. Bachmann
GT121	*thrC metLM1005 lysC1004 lacZU239*	I. Saint-Girons
GT15	*thrA1015 metLM1005 lysC1004 pro1001 serB22*	I. Saint-Girons
GT20	*thrB str*$^+$ HfrH	I. Saint-Girons
Gif102	*lysC1004 thrA1015 metLM1005 thi-1 relA1 spoT1* λ$^-$	B. Bachmann
YA73	*thrB1000 thi-1 relA1 spoT1* λ$^-$	B. Bachmann
C-600-1	*leuB6 thr1 thy1 supE44 lacY1 tonY1 tonA31 trpB* r_k^- m_k^-	S. Aiba
Hfr G6MD2	*hisA323* Δ(*bioH-asd*)*29* λ$^-$	B. Bachmann
Hfr3000 U482	*relA1 asd-1 spoT1 thi-1* λ$^-$	B. Bachmann
K38	HfrC *relA1 pit-10 spoT1 tonA22 ompF627* (T^r_2) *phoA4* λ$^-$	S. Tabor
Plasmids		
pUC13	ColE1 Ap^r	Laboratory stock
pUC118/pUC119	ColE1 Ap^r, with M13 origin of replication	Laboratory stock
pT7-5/pT7-6	ColE1 Ap^r, with the T7-RNA polymerase promoter in a different configuration with respect to the β-lactamase	S. Tabor
pGP1-2	Km^r, with the T7 RNA polymerase gene under the control of the λ P_L promoter and the c*I*857 gene	46
pKK232-8	*E. coli* promoter-probe vector	4
pUL609M	*Brevibacterium lactofermentum-E. coli* shuttle vector, Ap^r Km^r Hyg^r	27
pUL880M	*Brevibacterium lactofermentum-E. coli* promoter-probe vector, Ap^r, with the promoterless Km^r gene	24a
pULTH series	pBR322 derivatives containing the *hom-thrB* cluster of *Brevibacterium lactofermentum*	28
pULM101	pUC13/*Bam*HI containing a 10-kb *Sau*3AI fragment (*thrC*$^+$) from *Brevibacterium lactofermentum*	This study
pULM102	pUC13/*Hin*dIII containing a 6-kb *Hin*dIII fragment (*thrC*$^+$) from *Brevibacterium lactofermentum*	This study
pULM121/122	pUC13/*Hin*dIII containing a 2.9-kb *Hin*dIII fragment (*thrC*$^+$) from pULM101	This study
pULM131/132	pUC119/118 containing a 2.9-kb *Hin*dIII fragment (*thrC*$^+$)	This study
pULM123/124	pUC119/118 containing a 4.5-kb *Sph*I fragment from pULM101	This study
pULM125/126	pUC119/118 containing a 4.0-kb *Hin*dIII-*Sma*I fragment from pULM101	This study
pULM127/128	pUC119/118 containing a 4.0-kb *Sph*I fragment from pULM102	This study
pULM15/16	pT7-5 containing a 2.9-kb *Sal*I fragment (*thrC*$^+$) from pULM122 in the correct (pULM15) and opposite orientations (pULM16)	This study
pULM20	pUL609 containing a *Sal*I fragment (*thrC*$^+$) of pULM122	This study

[a] MeLys, methyllysine; Aec, *S*-aminoethylcysteine; Ap, ampicillin; Km, kanamycin; Hyg, hygromycin.
[b] ATCC, American Type Culture Collection.

bacterium cells were grown in tryptic soy broth (Difco) and tryptic soy agar (tryptic soy broth supplemented with 2% agar) complex media at 30°C. Minimal medium for corynebacteria has been described previously by Kaneko and Sakaguchi (20).

The *E. coli* strains were grown in Luria broth or on Luria agar (26) or in VB minimal medium (49). The following supplements were added to the minimal media when necessary: 3 mM (final concentration) thiamine and 0.1 mM (final concentration) *m*-diaminopimelic acid. Ampicillin, kanamycin, and hygromycin were added, when required, at a final concentration of 50 μg/ml (for ampicillin) or 100 μg/ml (for kanamycin and hygromycin).

DNA isolation and manipulation. Total DNA from *Brevibacterium lactofermentum* was obtained as described previously (9). Plasmid DNAs from *Brevibacterium lactofermentum* and *E. coli* were purified by the alkaline lysis method essentially as described by Maniatis et al. (26), except that the corynebacteria were treated with lysozyme for 3 h. Plasmid DNA was screened rapidly by using the boiling method (19).

DNA restriction and modification enzymes were purchased from Boehringer, Promega, and Amersham and were used according to the manufacturers' instructions. DNAs were separated and visualized in 0.8 to 1.0% agarose gels by using standards methods. DNA fragments were isolated from low-melting-point agarose (Sigma). Plasmids and restriction fragments were transferred from 0.8% agarose gels to nitrocellulose (Schleicher & Schuell) or nylon (Amersham) filters and hybridization was performed as described by Southern (44). Plasmid DNA or DNA fragments were nick translated with [α-^{32}P]dCTP (3,000 Ci mmol^{-1}; Amersham) by using a nick

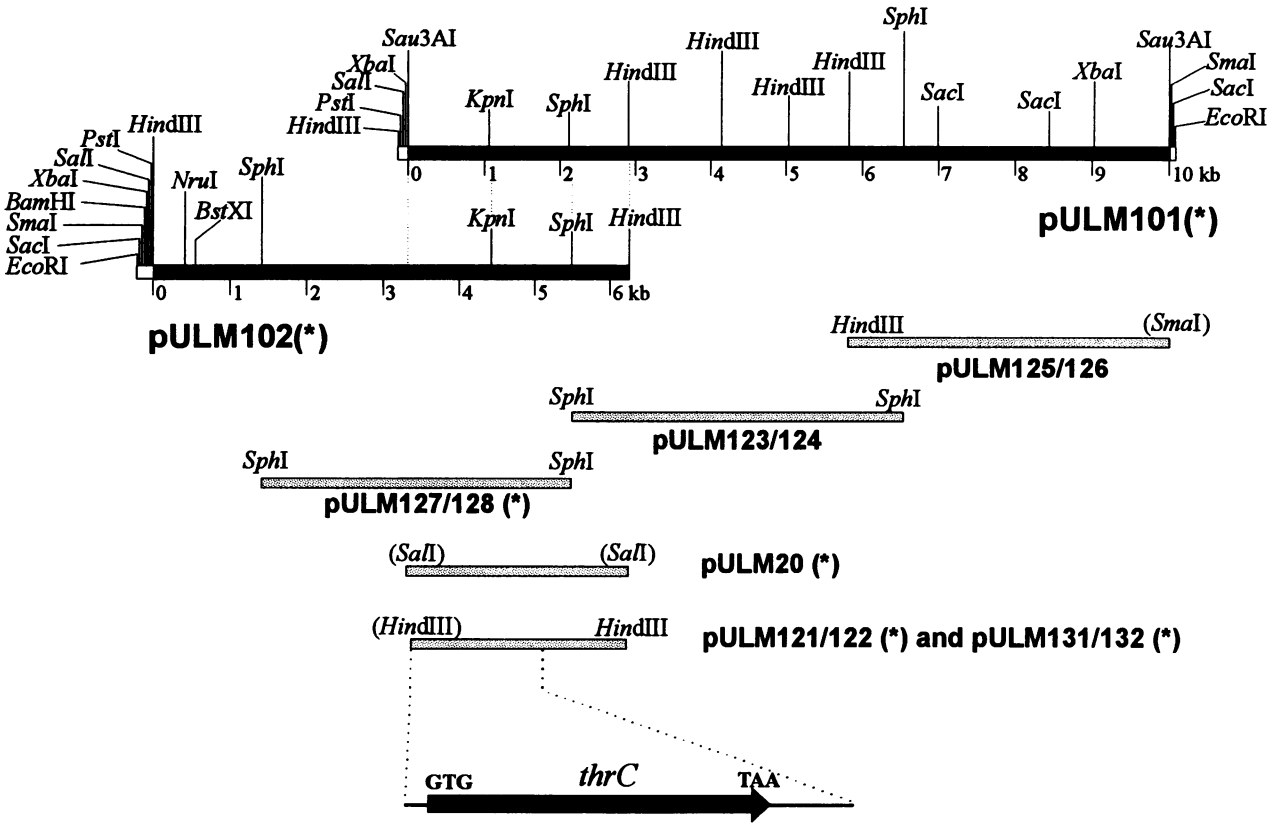


FIG. 1. *Brevibacterium lactofermentum* DNA fragments that complement *E. coli thrC* auxotrophs (solid bars) isolated from the *Sau*3AI (pULM101) and *Hin*dIII (pULM102) libraries and fragments subcloned for complementation mapping (shaded bars). The *Sal*I restriction sites of pULM20 and the *Hin*dIII site of pULM131/132 and pULM121/122 shown in parentheses belong to the polylinker. Asterisks indicate plasmids that complement *thrC* mutants of *E. coli*.

translation kit obtained from Boehringer. Labeled DNA was purified and concentrated by using a Minicolumn-D column (Sigma).

Construction of libraries and cloning of the *thrC* gene. *Brevibacterium lactofermentum* chromosomal DNA was partially digested with *Sau*3AI or totally digested with *Hin*dIII. *Sau*3AI fragments ranging in size from 4 to 10 kb were isolated by sucrose gradient ultracentrifugation. Equal amounts of *Sau*3AI-digested or *Hin*dIII-digested chromosomal DNA were ligated with *Bam*HI- or *Hin*dIII-digested pUC13, respectively, at 14°C for 16 h by using T4 DNA ligase. Each ligation mixture was originally transformed into *E. coli* DH5α for library amplification. The *Sau*3AI and *Hin*dIII libraries were used to transform the *thrC* mutants *E. coli* GT121 and *E. coli* Gif41 (Table 1).

Transformation procedures. Transformation of *E. coli* by plasmid DNA was performed by using the RbCl method (17) or the transformation and storage solution method of Chung et al. (5), with slight modifications. *E. coli* cells were inoculated in Luria broth and were grown to an optical density at 600 nm of 0.4. After centrifugation, each cell pellet was directly resuspended in 3 ml of transformation and storage solution, and small aliquots were frozen at −70°C until they were used for transformation.

Polyethylene glycol-mediated transformation of *Brevibacterium lactofermentum* protoplasts was carried out as described previously (40), and electroporation of corynebacteria was performed as described by Dunican and Shivnan (10).

DNA sequencing. DNA inserts were digested with several restriction enzymes, and the resulting fragments were subcloned in pUC119 and pUC118 phagemids (Table 1). Single-stranded plasmid DNA was isolated after transformation of *E. coli* WK6*mutS* (Table 1) with the recombinant phagemids and helper virus M13*K07* (31). Sequencing was performed by the dideoxy method (39), using the Sequenase system (United States Biochemical Co.). α-^{35}S-labeled dATP (>600 Ci mmol^{-1}) was purchased from Amersham. The DNASTAR computer program (DnaStar, Inc., Madison, Wis.) was used to analyze the DNA and protein sequences. Multiple alignment was performed by using the CLUSTALV (18) and the PHYLIP (11) software programs.

Gene expression in *E. coli*. The *Brevibacterium lactofermentum thrC* gene was expressed in *E. coli* by using the bacteriophage T7 RNA polymerase-promoter system (46). A 2.9-kb *Sal*I DNA fragment from plasmid pULM122 was cloned downstream of the strong T7 ϕ10 promoter in pT7-5 and pT7-6 (Table 1), resulting in plasmids pULM15 and pULM16, respectively. The hybrid plasmids were introduced by transformation into *E. coli* K38, which contained compatible plasmid pGP1-2 encoding the T7 RNA polymerase gene under the control of the inducible lambda P_L promoter and the gene encoding heat-sensitive lambda repressor c*I*857. Thermal induction of *E. coli* K38(pGP1-2) transformed with the constructions described above was performed for 30 min at 43°C. Rifampin was added to a final concentration of 300 μg/ml, and the cells were incubated for an additional 60 min at 43°C and

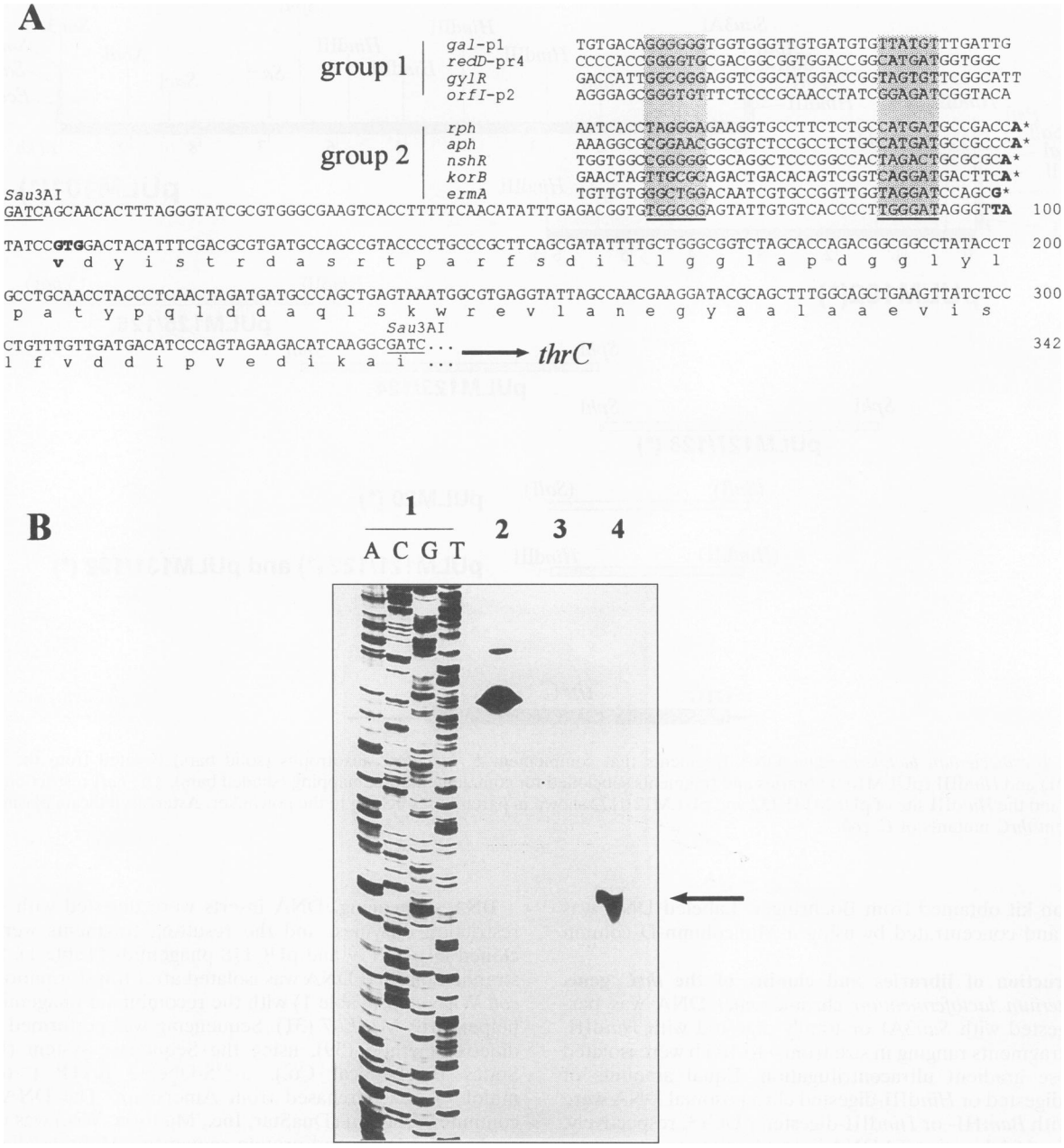


FIG. 2. (A) Nucleotide sequence of the 340-bp *Sau*3AI fragment upstream from the *thrC* gene showing the GTG initiation codon at position 106 (boldface type). The putative −10 and −35 regions, as established by S1 mapping, are shaded and are compared with the regions of the promoters having a G-rich −35 box (group 1) and the leaderless promoters (group 2) of several *Streptomyces* species (see Discussion). The first transcribed nucleotides are in boldface type and are indicated by asterisks in the *Streptomyces* sequences. (B) Determination of the transcription initiation site by S1 mapping. Lanes 1, sequence (ACGT) of M13mp18; lane 2, labeled probe (340 bp) used in the protection experiments; lane 3, lack of protection with the *E. coli* C600-1 total RNA; lane 4, protected DNA fragment (arrow).

then for 8 h at 30°C. Cell samples were centrifuged, disrupted, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12% (wt/vol) acrylamide.

RNA isolation and analysis. Corynebacterial cells were grown to an optical density at 600 nm of 1.0 and were collected by centrifugation. The cells were disrupted once with a French press at 60 lb/in^2. The lysate was placed in a CsCl solution and ultracentrifuged as described by Glisin et al. (13). A Northern analysis was performed by using nylon filters essentially as described by the Hybond-N suppliers (Amersham protocols). A 0.45-kb *Eco*RI-*Pst*I DNA fragment was radiolabeled with [α-^{32}P]dCTP and used as a probe.

To determine the site of initiation of transcription by S1 mapping, a 340-bp *Sau*3AI DNA fragment upstream of the *thrC* sequence was labeled at the 5′ end by using polynucleotide kinase and [γ-^{32}P]dATP (3,000 μCi/mmol). The radioactive DNA fragment was hybridized with 100 μg of RNA by using the method described by Berk (3). A total of 40 U of S1

nuclease (Amersham) was used to digest the nonhybridized DNA or RNA, and the products of the reaction were loaded onto a 6% polyacrylamide sequencing gel. The sequence of M13mp18 (51) was used as a control in the S1 mapping experiment.

Purification and activity of the *Brevibacterium lactofermentum* threonine synthase. Threonine synthase activity was determined essentially by the method of Shames et al. (42). The homoserine phosphate used as the substrate was obtained from crude extracts of the *thrC* mutant *E. coli* Gif41 (Table 1). The release of phosphate was measured by the method of Fiske and Subbarow (23). Protein concentrations were measured by using a Bradford assay kit (Bio-Rad, Inc.).

Threonine synthase was partially purified from *Brevibacterium lactofermentum* R31(pULM20) (Table 1) cultures. Corynebacterial cells were disrupted with the French press, and the cell lysate was treated as described by Han et al. (16), with some modifications. The supernatant was treated with 0.1 volume of 1% protamine sulfate, and the supernatant was fractionated by precipitation with ammonium sulfate (45 to 65%). The protein precipitate was resuspended in 50 mM Tris-HCl (pH 7.5) and subjected to chromatography in DEAE-Sepharose (Column K 15/30; Pharmacia) in 50 mM Tris-HCl buffer (pH 7.5). Threonine synthase was eluted with a linear 0.3 to 0.7 M KCl salt gradient. The active fractions were concentrated by ammonium sulfate precipitation and applied to a Superose 12 HR 10/30 fast protein liquid chromatography (FPLC) column (Pharmacia) that had been stabilized previously with 50 mM Tris-HCl (pH 7.5).

RESULTS

Complementation of *E. coli thrC* mutants with *Brevibacterium lactofermentum* DNA. DNA from *Brevibacterium lactofermentum* ATCC 13869 was partially digested with *Sau*3AI or fully digested with *Hin*dIII and ligated with pUC13. The ligation mixtures were used to transform *E. coli thrC* auxotrophs Gif41 and GT121 (Table 1). Transformants were selected in VB medium containing ampicillin (and supplemented with diaminopimelic acid, lysine, and methionine for strain GT121). Plasmids pULM101 and pULM102 (Fig. 1), which complemented the *thrC* auxotrophy of both *E. coli* strains, were isolated from the *Sau*3AI and *Hin*dIII libraries, respectively; these plasmids contained 10- and 6-kb inserts of *Brevibacterium lactofermentum* DNA, respectively. The two inserts shared a 2.9-kb region, which should have contained the *thrC* gene.

Total DNA from *Brevibacterium lactofermentum* was digested with *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I, blotted, and hybridized with labeled pULM102. All of the fragments present in the cloned inserts were consistent with the hybridized bands (data not shown), confirming that the inserts in pULM101 and pULM102 were not rearranged during cloning. Hybridizations were also performed with total DNA from *C. glutamicum* digested with the same enzymes. Most of the hybridization bands were identical in *Brevibacterium lactofermentum* and *C. glutamicum* AS019; the exceptions were the *Bcl*I bands, which were different in the two corynebacteria.

Deletion mapping and complementation of *thrC*. DNA fragments from plasmids pULM101 and pULM102 were subcloned in pUC118 or pUC119 as shown in Fig. 1 and were used to transform *E. coli thrC* auxotrophs. The 2.9-kb DNA fragment common to both pULM101 and pULM102 was subcloned as a *Hin*dIII fragment in pUC13 in both orientations (resulting in pULM121 and pULM122) and also in pUC119 (resulting in pULM131 and pULM132). Other fragments of pULM101 were subcloned either as a 4.5-kb *Sph*I fragment (plasmids pULM123 and pULM124) or as a 4.0-kb *Hin*dIII-*Sma*I fragment (plasmids pULM125 and pULM126). The *thrC* auxotrophy of *E. coli* Gif41 and GT121 was complemented only by pULM121, pULM122, pULM131, and pULM132. A 4.0-kb *Sph*I fragment subcloned from pULM102 (resulting in plasmids pULM127 and pULM128) also complemented *thrC* auxotrophy. These results suggest that the *thrC* gene of *Brevibacterium lactofermentum* is located in the 2.0-kb *Hin*dIII-*Sph*I region of pULM101 (Fig. 1).

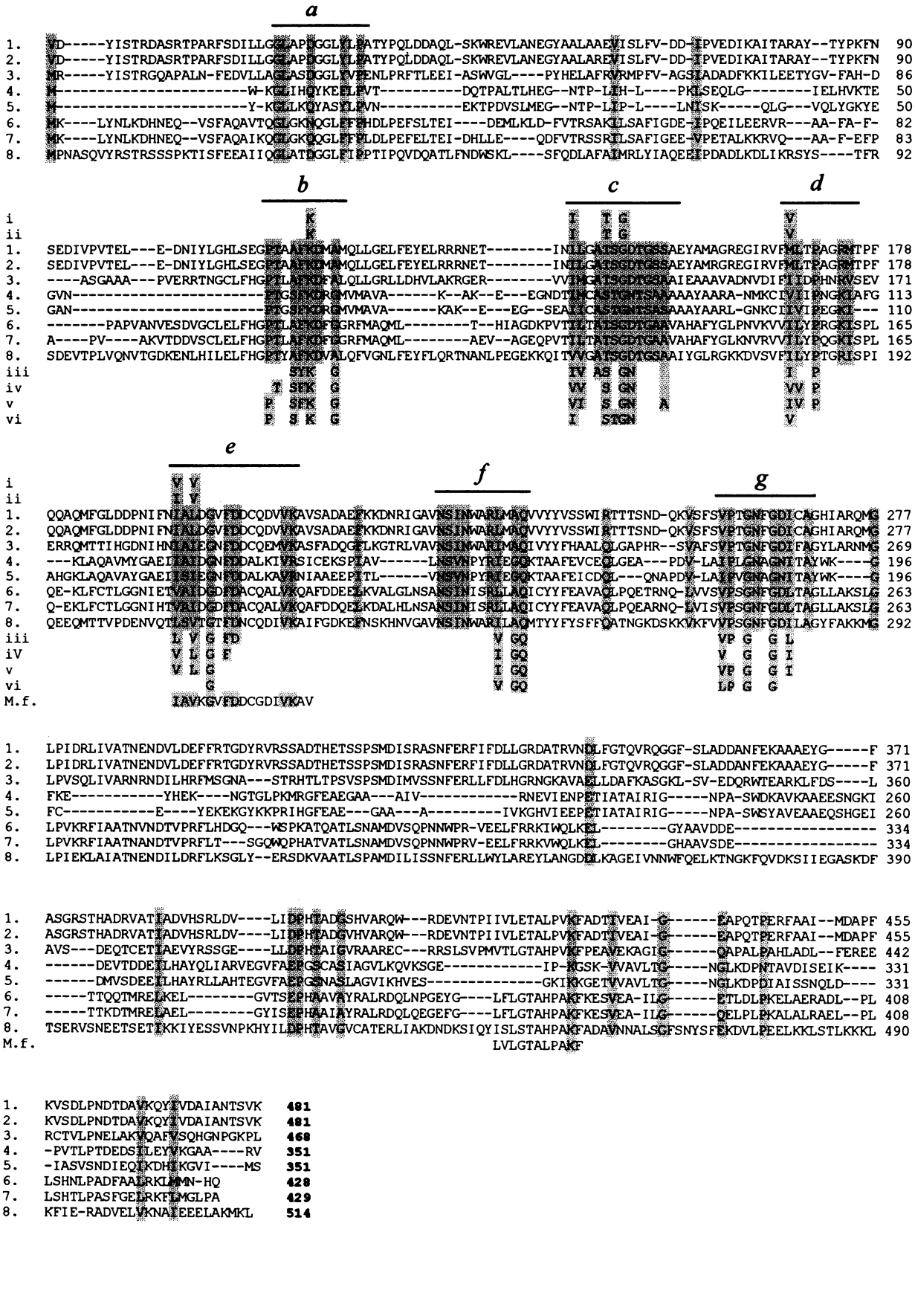
The 2.9-kb *Hin*dIII fragment subcloned as a *Sal*I fragment from the pUC13 polylinker was subcloned in corynebacterial plasmid pUL609M (Table 1), resulting in plasmid pULM20, which complemented the *thr* auxotroph *Brevibacterium lactofermentum* UL1035.

Since the *Brevibacterium lactofermentum* DNA fragments cloned in original plasmids pULM101 and pULM102 were relatively large and may have included other genes, they were used to complement *E. coli thrA*, *thrB*, *metL*, *lysC*, and *asd* auxotrophs. None of the strains tested (Table 1) was complemented, suggesting that the *thrC* gene is not linked to the threonine, methionine, and lysine biosynthetic genes or that if these genes are linked, the encoded proteins are not functional in *E. coli*.

***thrC* gene is not linked to *hom* or *thrB*.** In *E. coli*, *Serratia marcescens*, and *Bacillus subtilis* the three threonine biosynthesis genes (*thrA* [*hom*], *thrB*, and *thrC*) are linked together in a single cluster. The *Brevibacterium lactofermentum hom* and *thrB* genes are known to be linked (28). To establish whether the three *thr* genes are also linked in corynebacteria, plasmids pULTH2, pULTH4, pULTH8, pULTH11, and pULTH18 containing the *hom-thrB* cluster were digested with *Pst*I, and the resulting preparations were resolved by electrophoresis, blotted onto nylon filters, and hybridized with labeled pULM 101 or pULM102. None of the inserts of the pULTH plasmids hybridized with pULM101 or pULM102, which indicated that the *thrC* gene is separated by at least 10 kb from the *hom-thrB* cluster.

Nucleotide sequence and deduced amino acid sequence of the *thrC* gene. Both strands of a 1,892-bp *Sau*3AI (left end of pULM101 in Fig. 1)-*Nci*I DNA fragment were sequenced. The nucleotide sequence obtained has been deposited in the EMBL, GenBank, and DDJB data bases under accession number Z29563. An analysis of the coding region revealed that there was 1,446-bp open reading frame (ORF), designated ORF1, located between positions 106 (G) and 1551 (A). The coding potentials of the different ORFs were studied by using the algorithm of Gribskov et al. (14), taking into account the codon usage of *Brevibacterium lactofermentum* and *C. glutamicum* (25). ORF1, in contrast to other, smaller ORFs, exhibited a very high average Gribskov algorithm value.

ORF1 contained two in-frame GTG codons at nucleotide positions 64 to 66 and 106 to 108 (Fig. 2). If we assumed that the first GTG was the initiation codon, the ORF encoded a 495-amino-acid protein with an M_r of 54,413. If we assumed that the second GTG was the initiation codon, the encoded protein contained 481 amino acids and had a deduced M_r of 52,807. The results of an analysis of the Gribskov algorithm data strongly supported the hypothesis that the second GTG triplet was the real translation initiation codon since the Gribskov index value was quite low for the first 100 triplets and increased sharply around nucleotide 106; i.e., the Gribskov algorithm data clearly indicated that the triplets in the region from position 64 to position 106 had very low coding potential (they were not consistent with the codon usage of corynebac-


a
b
c
d
e
f
g
1.
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3.
4.
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i
ii
iii
iv
v
vi
M.f.

FIG. 3. Alignment of the deduced amino acid sequences of the threonine synthases of *Brevibacterium lactofermentum* (sequence 1), *C. glutamicum* (sequence 2), *P. aeruginosa* (sequence 3), *Bacillus subtilis* (sequence 4), *Bacillus* sp. (sequence 5), *E. coli* (sequence 6), *Serratia marcescens* (sequence 7), and *Saccharomyces cerevisiae* (sequence 8), the tryptophan synthase of *Bacillus subtilis* (sequence i), the tryptophan synthase of *Brevibacterium lactofermentum* (sequence ii), the threonine dehydratase of *E. coli* (sequence iii), the threonine dehydratase of *Saccharomyces cerevisiae* (sequence iv), the serine dehydratase of *E. coli* (sequence v), and the previously described fragment of the threonine synthase of *Methylophilus flagellatum* (sequence M.f.). All of the sequences except the *M. flagellatum* sequence (48) were obtained from the PIR data base. Note the seven conserved regions (regions *a* to *g*) that are overlined. Motif *b* (PTXAFKDXG) (pyridoxal phosphate binding sequence) surrounds Lys-118 of the *Brevibacterium lactofermentum* enzyme.

terial ORFs). The amino acids in positions 2 to 6 obtained when the second GTG triplet was used as the initiation codon, Asp-Tyr-Ile-Ser-Thr, are the amino acids at the amino-terminal end of the threonine synthase of *C. glutamicum* (16).

The G+C content of ORF1 was 55.7 mol%, a value similar to the average G+C content of the *Brevibacterium lactofermentum* chromosome (see Discussion).

The nucleotide sequence of *thrC* was compared with the sequences in the GenBank and EMBL data bases, and the deduced amino acid sequence was compared with the sequences in the PIR protein data bank. Six other *thrC* genes are known, and a seventh one, in a *Bacillus* sp., was described during the course of this work (see below) (Fig. 3). All of the encoded proteins have seven conserved domains, some of which are also found in the tryptophan synthase and threonine dehydratases (see Discussion). At the nucleotide level the level of homology between the *Brevibacterium lactofermentum thrC* gene and the *C. glutamicum thrC* gene was very high, and the levels of homology between the *Brevibacterium lactofermentum* gene and the genes of other microorganisms were lower. There are 14 differences in nucleotides between the *C. glutamicum* and *Brevibacterium lactofermentum thrC* genes; one of these 14 differences results in a change of a *Not*I restriction site to a *Nco*I site, which allowed us to distinguish clearly the DNAs of the two corynebacteria in this region.

Expression of *thrC* in *E. coli* by using the pT7 system: formation of a 53-kDa protein. The 2.9-kb *Hin*dIII fragment that overlapped in plasmids pULM101 and pULM102 was subcloned with *Sal*I ends into the polylinker of pT7-5 to give pULM15 (in the same orientation as the φ10 promoter) and pULM16 (in the opposite orientation). After heat induction of *E. coli* K38(pGP1-2) containing either pULM15 or pULM16 for 1 h at 43°C in the presence of rifampin (500 μg/ml), we observed a band at about 53 kDa (Fig. 4) in *E. coli*(pGP1-2)(pULM15) that was not observed in control *E. coli*(pGP1-2) or *E. coli*(pGP1-2)(pT7-5) preparations without the *thrC* insert or in *E. coli*(pGP1-2) transformed with pULM16. These results indicated that the molecular weight of the protein encoded by ORF1 was about 53×10^3, which was consistent with the hypothesis that the second in-frame GTG triplet was the translation initiation codon.

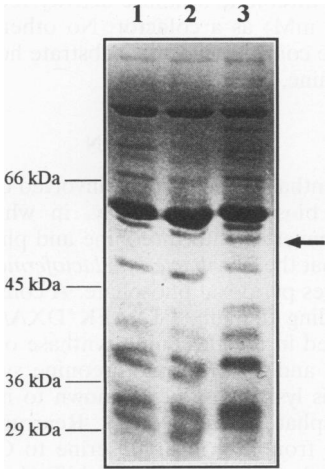

FIG. 4. Expression of ORF1 in *E. coli* by using the phage T7 promoter and the RNA polymerase system: SDS-PAGE of proteins from *E. coli* transformed with different constructions. Lane 1, *E. coli* K38(pGP1-2); lane 2, *E. coli* K38(pGP1-2)(pT7-5); lane 3, *E. coli* K38(pGP1-2)(pULM15). Note the 53-kDa band in *E. coli* transformed with plasmid pULM15 (arrow). The positions of size markers are indicated on the left.

Subcloning of the *thrC* promoter. The 2.9-kb *Hin*dIII fragment is expressed in both orientations in *E. coli*(pULM121 or pULM122); since there are only 100 bp upstream from the GTG in the DNA fragment cloned in pULM101, expression in *E. coli* suggests that the *thrC* promoter is located in this small upstream region. A 340-bp *Sau*3AI fragment was subcloned from the 2.9-kb *Hin*dIII fragment into the *Bam*HI site of the *E. coli* promoter-probe vector pKK232-8. The presence of the 340-bp fragment (Fig. 2A) was confirmed by *Pvu*II digestion. All clones carrying the insert in pKK232-8 were able to grow in the presence of chloramphenicol concentrations of more than 200 μg/ml.

The *thrC* promoter was also subcloned in bifunctional promoter-probe plasmid pUL880M (Table 1). This plasmid is able to replicate in *E. coli* (with an origin of replication from pUC19) and *Brevibacterium lactofermentum* (with an origin of replication from pBL1) and carries the promoterless Tn*5* kanamycin resistance gene with the upstream tryptophan terminator to prevent readthrough expression. The resulting construction conferred resistance to kanamycin (100 μg/ml) in both *E. coli* and corynebacteria; i.e., the *thrC* promoter was functional in both *E. coli* and corynebacteria.

Transcription start point analysis. S1 endonuclease protection studies were performed by using the 340-bp *Sau*3AI fragment labeled at its 5′ end with [γ-^{32}P]dATP. RNA was extracted from *Brevibacterium lactofermentum*(pULM20) carrying *thrC* in multicopy plasmid pULM20, since the chromosomal gene appeared to be expressed poorly and the *thrC* transcript could not be detected (see below). After digestion with S1 endonuclease, the protected fragment was 98 or 99 nucleotides shorter than the probe (Fig. 2B); i.e., the transcription start point was located at or around an adenine that was 6 bp upstream from the first GTG codon of ORF1.

A transcription start point close to the translation initiation site is well known in a subgroup of *Streptomyces* promoters and has also been described in the *pheA* gene of corynebacteria (12) (see Discussion).

Inverted repeat sequences downstream from the *thrC* ORF. Two different regions containing inverted repeats were found downstream from ORF1; one of these regions was located at nucleotides 1557 to 1647, and the other was located at positions 1735 to 1813 (Fig. 5). The free energy released if stem and loop structures were formed in the transcript was calcu-

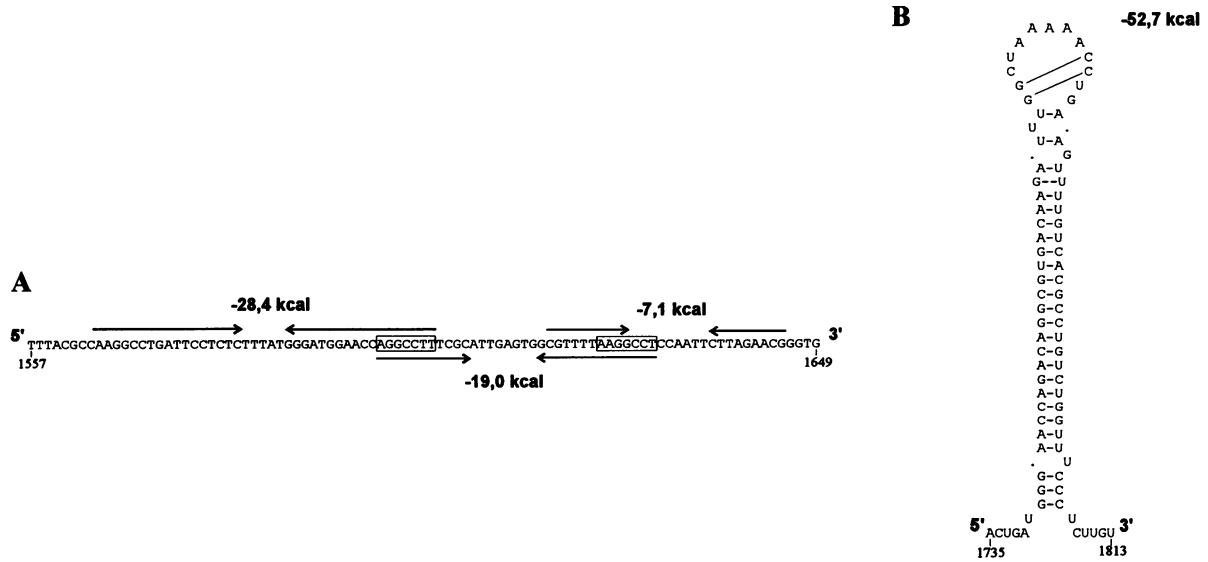

FIG. 5. Inverted repeats at positions 1557 to 1649 (A) and 1735 to 1813 (B) and a possible stem and loop structure in the mRNA (see text). The released free energy values as determined by the algorithm of Zuker and Stiegler (52) are indicated.

lated by using the algorithm of Zuker and Stiegler (52). The first putative termination region had three inverted repeats (ΔG, −28.4, −19.0, and −7.1 kcal/mol). The second putative terminator region (nucleotides 1735 to 1813) had a long 24-nucleotide inverted repeat with a mismatch and a calculated ΔG of −52.7 kcal/mol. The third loop of the first putative terminator region had four U residues in seven nucleotides, and the second hypothetical terminator was followed by four uridines in six residues (see Discussion).

***thrC* gene is transcribed as a monocistronic transcript.** A Northern analysis in which a 0.45-kb *Eco*RI-*Pst*I probe internal to *thrC* was used revealed a single transcript that was 1,400 nucleotides long (Fig. 6). The 1,400-nucleotide messenger was only poorly observed in RNA preparations extracted from *Brevibacterium lactofermentum* ATCC 13869, but was clearly observed when the RNA was extracted from *Brevibacterium lactofermentum*(pULM20) grown in either defined or complex medium. The size of the transcript correlated well with the size of the gene extending from the transcription start point at nucleotide 98 or 99 to the first putative terminator at positions 1550 to 1650. If transcription to the second hypothetical terminator had occurred, the expected size of the transcript would have been 1,700 nucleotides.

Threonine synthase activity is encoded by *thrC*. The levels of threonine synthase activity were determined in *E. coli* Gif41 with and without transformation with pULM121 and in *Brevibacterium lactofermentum* ATCC 13869 and UL1035 with and without transformation with pULM20.

As shown in Table 2, *E. coli* Gif41 exhibited no threonine synthase activity, whereas when this strain was transformed with pULM121, it exhibited 67 U of activity per mg of protein. Similarly, *Brevibacterium lactofermentum* UL1035 exhibited no threonine synthase activity and wild-type strain ATCC 13869 exhibited a very low level of activity (0.5 U/mg of protein), whereas *Brevibacterium lactofermentum* UL1035(pULM20) exhibited 45 U of activity per mg of protein. These results definitively established that *Brevibacterium lactofermentum* UL1035 is deficient in threonine synthase.

Purification of the threonine synthase encoded by *thrC*. Since *Brevibacterium lactofermentum* transformed with pULM20 exhibited a high level of threonine synthase activity, the enzyme was purified by removing nucleic acids with protamine sulfate and subjecting the preparation to ammonium sulfate (45 to 65%) precipitation, ion-exchange filtration through DEAE-Sepharose, and FPLC gel filtration through Superose 12. The enzyme was purified 80-fold (Table 3). The molecular weight of the native protein as determined by gel filtration (K_{av} value, 0.454) was 53×10^3. SDS-PAGE gels (Fig. 7) contained a 52-kDa protein that was progressively enriched during the purification procedure. The molecular weight estimated by gel filtration or SDS-PAGE corresponded exactly to the expected size (M_r, 52,807) deduced from the cloned *thrC* gene; i.e., the native threonine synthase appears to be a 52.8-kDa monomer.

The purified threonine synthase activity required pyridoxal phosphate (0.1 mM) as a cofactor. No other cofactors were required for the conversion of the substrate homoserine phosphate to threonine.

DISCUSSION

Threonine synthase is the enzyme involved in the last step of the threonine biosynthetic pathway, in which homoserine phosphate is converted into threonine and phosphate (8, 43). We observed that the *Brevibacterium lactofermentum* threonine synthase requires pyridoxal phosphate. A consensus pyridoxal phosphate binding domain, PTXAFK*DXA/G, around Lys-118, is conserved in the threonine synthase of *Brevibacterium lactofermentum* and in all other threonine synthases (Fig. 3, domain *b*). This lysine residue is known to interact with the pyridoxal phosphate cofactor (41). Rearrangement of the hydroxyl group from C-4 in homoserine to C-3 in threonine proceeds through formation of a Schiff's base between the pyridoxal phosphate and the reaction intermediate α-aminocrotonate. The same intermediate is involved in the threonine dehydratase reaction (which converts threonine into α-ketobutyrate and ammonium) and also in the condensation of indole and serine to form tryptophan catalyzed by tryptophan synthase (involving the α-aminocrotonate intermediate). All of

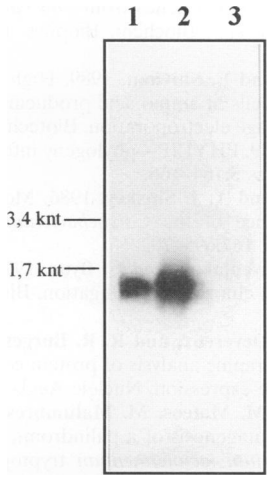

FIG. 6. Northern analysis of the transcripts from the *thrC* gene in which a 0.45-kb *Eco*RI-*Pst*I DNA fragment was used as the probe. Total RNA was obtained from *Brevibacterium lactofermentum* R31(pULM20) grown in minimal medium for corynebacteria (lane 1) or tryptic soy broth complex medium (lane 2). *E. coli* rRNAs were used as a control (lane 3). The sizes of the *E. coli* rRNAs (in kilonucleotides [knt]) are indicated on the left.

these enzymes have the pyridoxal phosphate consensus sequence, and it has been proposed that they all originated from a common ancestral gene (33, 34).

By comparing the conserved amino acids in the threonine synthase of *Brevibacterium lactofermentum* with the conserved amino acids in the threonine synthases of *P. aeruginosa* (6), *Bacillus subtilis* (33), *E. coli* (36), *Serratia marcescens* (32), *C. glutamicum* (16), and *Saccharomyces cerevisiae* (1), it is possible to establish a phylogenetic tree based on genetic distances by using the neighbour-joining method (38). The *Brevibacterium lactofermentum* and *C. glutamicum* threonine synthases are very similar to each other and are more closely related to the *P. aeruginosa* enzyme than to the enzymes of other bacteria. During this research a different *thrC* gene was cloned and sequenced from a contaminant *Bacillus* sp. that was initially thought to correspond to *Brevibacterium lactofermentum*. An analysis of the amino acid sequence revealed that this gene is closely related to the *Bacillus subtilis* gene (unpublished data).

The codon usage in the *thrC* gene of *Brevibacterium lactofermentum* is biased toward the utilization of codons having C or G in the third position (25). AUA, one of the less common codons in many bacteria, is not present in the *Brevibacterium lactofermentum thrC* gene but appears once in the *thrC* gene of *C. glutamicum*; which may be a limiting factor for high levels of expression of this gene if the AUA-interacting tRNAs are scarce in corynebacteria, as is true in enterobacteria.

TABLE 2. Threonine synthase activities in *E. coli* and *Brevibacterium lactofermentum* strains transformed with the *thrC* gene of *Brevibacterium lactofermentum*

Strain	Threonine synthase activity (U/mg of protein)[a]
E. coli Gif41	<0.1
E. coli Gif41(pULM121)	67.0
Brevibacterium lactofermentum ATCC 13869	0.5
Brevibacterium lactofermentum UL1035	<0.1
Brevibacterium lactofermentum UL1035(pULM20)	45.0

[a] One unit of activity was defined as 1 nmol of phosphate released from homoserine phosphate per min.

TABLE 3. Purification of the *Brevibacterium lactofermentum* threonine synthase

Step	Total activity (U)[a]	Amt of protein (mg)	Sp act (nmol/min/mg of protein)	Purification (fold)	% Recovery
Crude extract	26,530	585.0	45	1.0	100
Protamine sulfate	27,010	420.0	64	1.4	100
$(NH_4)_2SO_4$ (45-65%)	26,320	125.0	210	4.6	99
DEAE-Sepharose	18,740	19.1	980	21.7	70
Superose 12	6,510	1.8	3,610	80.2	24

[a] 1 U = 1 nmol of phosphate released from homoserine phosphate per min.

An S1 mapping analysis of the transcription start point revealed a very short leader transcript before the first translated codon. A similar situation has been described for the *pheA* gene of *C. glutamicum* (12). An entire group of 11 leaderless promoters has been described in actinomycetes (45). It has been proposed that in several of these promoters transcription and translation are initiated at the same nucleotide. Several of these leaderless promoters have a relatively highly conserved −10 hexamer similar to that recognized by the σ^{70} *E. coli* sigma factor of the RNA polymerase, but they have a consensus GGGGGG hexamer in the −35 region separated by a standard 17 nucleotides from the Pribnow box (−10 region). Alterations of the Gs in this −35 region result in promoter down mutations (45). The *thrC* promoter of *Brevibacterium lactofermentum* has the hexamer TGGGGG separated by exactly 17 nucleotides upstream from the −10 box defined by the transcription start point (Fig. 3). The similarity between the *thrC* promoter and the leaderless promoters of *Streptomyces* strains suggests that functionally related promoters recognized by special forms of the RNA polymerase may occur in the genus *Streptomyces* and in corynebacteria.

Two inverted repeats were found downstream from the *thrC* ORF; these inverted repeats may act as transcriptional terminators, as occurs in the tryptophan operon of *E. coli* (50). The first region (nucleotides 1557 to 1649) (Fig. 5) may form three

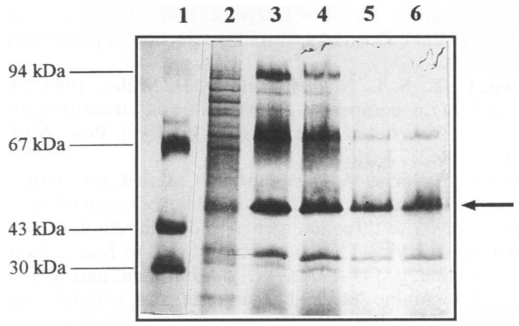

FIG. 7. SDS-PAGE of extracts of *Brevibacterium lactofermentum* UL1035(pULM20) obtained after different steps of the purification procedure (see Table 3). Lane 1, size markers; lane 2, fraction collected after precipitation with 45 to 65% ammonium sulfate; lanes 3 and 4, fractions collected after DEAE-Sepharose filtration; lanes 5 and 6, fractions recovered after gel filtration through Superose. The 53-kDa protein band (arrow) was enriched throughout the purification procedure. The positions of the size markers are indicated on the left.

alternative stem and loop structures that are reminiscent of the attenuators that are present in the tryptophan operon of *Brevibacterium lactofermentum* (15, 30) and other bacteria. The second inverted repeat (nucleotides 1735 to 1813) would form, if it were transcribed, a very stable stem and loop structure (ΔG, −52.7 kcal/mol). In the *E. coli trp* operon the first repeat acts as a rho-independent terminator, and the second repeat (separated by 250 bp) acts as a rho-dependent terminator. It seems that this tandem arrangement of terminators has evolved as a mechanism to secure transcriptional termination of those transcripts that fail to stop at the first terminator. An alternative explanation is that these tandem inverted repeat structures may protect the 3′ end of the mRNA molecule from RNase digestion (2). Similar mechanisms may occur in the two stem and loop structures downstream from the *Brevibacterium lactofermentum thrC* gene.

The *thrC* gene of *Brevibacterium lactofermentum* is not linked to the *hom-thrB* cluster and is transcribed as a monocistronic messenger. Hybridization of the previously cloned *hom-thrB* genes (28) with the fragments cloned in this study revealed that there is no homology between these fragments. This result was confirmed by the results of a detail analysis of a cosmid library of *Brevibacterium lactofermentum* DNA (8a). The *thrC* gene is not located in the cosmids that contain the *hom-thrB* cluster; i.e., they are separated by at least 40 kb on the *Brevibacterium lactofermentum* chromosome. The organization of the *thr* genes in corynebacteria is therefore different from the organization of these genes in other gram-negative and gram-positive (*Bacillus subtilis*) species. We are currently studying whether the type of organization of *thr* genes that occurs in corynebacteria also occurs in the related nocardioform bacteria and in actinomycetes.

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