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Cloning and Nucleotide Sequences of the Homoserine Dehydrogenase Genes (*hom*) and the Threonine Synthase Genes (*thrC*) of the Gram-Negative Obligate Methylotroph *Methylobacillus glycogenes*

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We have cloned the homoserine dehydrogenase genes (*hom*) from the gram-negative obligate methylotrophs *Methylobacillus glycogenes* ATCC 21276 and ATCC 21371 by complementation of an *Escherichia coli* homoserine dehydrogenase-deficient mutant. The 4.15-kb DNA fragment cloned from *M. glycogenes* ATCC 21371 also complemented an *E. coli* threonine synthase-deficient mutant, suggesting the DNA fragment contained the *thrC* gene in addition to the *hom* gene. The homoserine dehydrogenases expressed in the *E. coli* recombinants were hardly inhibited by L-threonine, L-phenylalanine, or L-methionine. However, they became sensitive to the amino acids after storage at 4°C for 4 days as in *M. glycogenes*. The structures of the homoserine dehydrogenases overexpressed in *E. coli* were thought to be different from those in *M. glycogenes*, probably in subunit numbers of the enzyme, and were thought to have converted to the correct structures during the storage. The nucleotide sequences of the *hom* and *thrC* genes were determined. The *hom* genes of *M. glycogenes* ATCC 21276 and ATCC 21371 encode peptides with M_rs of 48,225 and 44,815, respectively. The *thrC* genes were located 50 bp downstream of the *hom* genes. The *thrC* gene of ATCC 21371 encodes a peptide with an M_r of 52,111, and the gene product of ATCC 21276 was truncated. Northern (RNA) blot analysis suggests that the *hom* and *thrC* genes are organized in an operon. Significant homology between the predicted amino acid sequences of the *hom* and *thrC* genes and those from other microorganisms was found.

Methanol is a good carbon source to use in amino acid fermentation because of its high purity and low price. With the use of methanol, raw material costs could be reduced greatly, and the purification of the product and the treatment of wastewater could be simplified. Despite the importance of methanol as a carbon source, reports on the breeding of amino acid producers from methylotrophs have been limited because isolation of mutants from methylotrophs is quite difficult for unknown reasons.

Recently we succeeded in the isolation of L-glutamic acid-hyperproducing mutants from the gram-negative obligate methylotrophs *Methylobacillus glycogenes* ATCC 21276 and ATCC 21371 and the derivation of L-threonine- and L-lysine-producing mutants from them (15). The L-threonine and L-lysine biosynthetic enzymes of the L-threonine- and L-lysine-producing mutants were characterized, and several regulatory enzymes were found to be desensitized to feedback inhibition by L-threonine and/or L-lysine (16).

Interestingly, some of the L-threonine and L-lysine biosynthetic enzymes of *M. glycogenes* were also sensitive to aromatic amino acids synthesized by a pathway different from that of the L-aspartate family amino acids. Homoserine dehydrogenase (HD), a major regulatory enzyme of L-threonine biosynthesis, is as sensitive to L-phenylalanine as it is to L-threonine. An interrelationship between the biosynthetic pathway of the L-aspartate family amino acids and that of the aromatic amino acids has been suggested (16).

In many microorganisms, HD is regulated by feedback inhibition or feedback repression, and its gene forms an operon with other L-threonine biosynthetic genes. In *Escherichia coli*, HD I is fused with aspartate kinase I (AK I) and is inhibited by L-threonine. The *thrA* gene encoding the fused protein AK I-HD I is organized in an operon, *thrABC*, with the *thrB* gene encoding homoserine kinase and the *thrC* gene encoding threonine synthase. The *thrABC* operon is regulated by an attenuation mechanism sensitive to L-threonine and L-isoleucine (6). In *Bacillus subtilis*, HD is a separate protein from AK and is inhibited by L-threonine (18) and the *hom* gene is organized in an operon, *hom-thrC-thrB* (17). The *hom* gene of *Corynebacterium glutamicum* forms an operon with *thrB* in the order *hom-thrB* (20) and is repressed by L-methionine (7). In *Pseudomonas aeruginosa* (5) and *Methylobacillus flagellatum* (13), HD is inhibited by L-threonine and the *hom* gene forms an operon, *hom-thrC*.

It is worthwhile to clone and investigate the *M. glycogenes hom* genes, whose products have unique properties including strong inhibition by L-phenylalanine. In this paper, we describe cloning of the *hom* genes and *thrC* genes found downstream of the *hom* genes from *M. glycogenes* ATCC 21276 and ATCC 21371 and some enzymatic properties of the HDs expressed in *E. coli* recombinants. The nucleotide sequences of the *hom* and *thrC* genes were determined, and the deduced amino acid sequences were compared with those of counterparts from other microorganisms.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)	Phenotype[a]	Reference
Strains			
M. glycogenes			
ATCC 21276		Wild type	
ATCC 21371		Wild type	
E. coli			
GT 5	*thrA1017 metLM1005 lysC1004 pro-1001 thi-1 relA1 spoT1* (λ^-)	AK^- HD^- HK^- TS^-	21
Gif 102	*thrA1015 metLM1005 lysC1004 thi-1 relA1 spoT1* (λ^-)	HD^-	21
Hfr 3000 YA 73	*thrB1000 thi-1 relA1 spoT1* (λ^-)	HK^-	21
Gif 41	*thrC thi-1 relA1 spoT1* (λ^-)	TS^-	21
Plasmids			
pBR322	Vector	Tc^r Amp^r	19
pUC19	Vector	Amp^r	24
pIHD-1	Derivative of pBR322 carrying 3.1-kb *Pst*I fragment cloned from *M. glycogenes* ATCC 21276	Tc^r HD^+	This study
pIK-1	Derivative of pUC19 carrying 2.1-kb *Sma*I fragment of pIHD-1	Amp^r HD^+	This study
pIK-2	Derivative of pUC19 carrying 2.1-kb *Sma*I fragment of pIHD-1 (the insert was ligated to pUC19 in the opposite direction from pIK-1)	Amp^r HD^+	This study
pIKD-21	Deletion derivative of pIK-2 overexpressing HD	Amp^r HD^+	This study
pIHD-5	Derivative of pUC19 carrying 1.2-kb *Sma*I-*Eco*RV fragment of pIHD-1	Amp^r	This study
pIHD-6	Derivative of pUC19 carrying 0.7-kb *Sma*I-*Eco*RV fragment of pIHD-1	Amp^r	This study
pTHD-1	Derivative of pBR322 carrying 4.15-kb *Pst*I fragment cloned from *M. glycogenes* ATCC 21371	Tc^r HD^+ TS^+	This study
pTHD-2	Derivative of pBR322 carrying 3.2-kb *Eco*RI-*Pst*I fragment of pTHD-1	Tc^r HD^+	This study
pTK-1	Derivative of pUC19 carrying 2.1-kb *Sma*I fragment of pTHD-1	Amp^r HD^+	This study
pTK-2	Derivative of pUC19 carrying 2.1-kb *Sma*I fragment of pTHD-1 (the insert was ligated to pUC19 in the opposite direction from pTK-2)	Amp^r HD^+	This study
pTHD-5	Derivative of pUC19 carrying 1.2-kb *Sma*I-*Eco*RV fragment of pTHD-1	Amp^r HD^+	This study
pTTS-1	Derivative of pUC19 carrying 2.3-kb *Eco*RV-*Pst*I fragment of pTHD-1	Amp^r TS^+	This study
pTTS-2	Derivative of pUC19 carrying 2.3-kb *Eco*RV-*Pst*I fragment of pTHD-1 (the insert was ligated to pUC19 in the opposite direction from pTTS-1)	Amp^r TS^+	This study

[a] Abbreviations: Amp^r, ampicillin resistance; HK, homoserine kinase; Tc^r, tetracycline resistance; TS, threonine synthase.

MATERIALS AND METHODS

Bacteria and plasmids. The bacteria and plasmids used in this study are listed in Table 1.

Media and culture methods. *M. glycogenes* ATCC 21276 and ATCC 21371 were cultivated in shake flasks as described in our previous study (16). *E. coli* cells were cultivated as described previously (12). One hundred milligrams of ampicillin per liter or 10 mg of tetracycline per liter was used to supplement liquid or agar media to cultivate *E. coli* strains containing plasmids. M9S1 medium (M9 medium [12] supplemented with 50 mg [each] of 18 amino acids [except L-threonine and L-methionine] and with 50 mg of 2,6-diaminopimelic acid per liter) was used for the complementation tests of the *E. coli* mutants Gif 102, Hfr 3000 YA 73, and Gif 41. M9S5 medium (M9 medium supplemented with 50 mg [each] of 20 amino acids per liter) was used for the complementation test of *E. coli* GT 5.

DNA manipulations. DNA restriction enzyme digestion, separation of DNA fragments by gel electrophoresis, DNA ligation, and transformation of *E. coli* strains were performed by standard methods as described previously (12). Restriction enzymes and DNA ligase were supplied by Takara Shuzo Co. Ltd. (Kyoto, Japan). Southern hybridization was done with a DNA labeling and detection kit (Boehringer Mannheim) according to the method recommended by the supplier.

Construction of gene libraries. Chromosomal DNAs of *M. glycogenes* were prepared by the method described by Marmur (14). The chromosomal DNAs of *M. glycogenes* ATCC 21276 and ATCC 21371 were partially digested with *Pst*I and separated by agarose gel electrophoresis. The 3- to 6-kb DNA fragments from each strain were purified from the gel, ligated into the *Pst*I site of pBR322 to construct gene libraries, and used to transform *E. coli* Gif 102.

Subcloning of plasmids. The 2.1-kb *Sma*I fragment of pIHD-1 was inserted into the *Sma*I site of pUC19 to form pIK-1 and pIK-2 in both orientations. pIKD-21, a plasmid with overexpression of HD, is a deletion derivative of pIK-2. pIHD-5 and pIHD-6 were constructed by ligating the 1.2-kb *Sma*I-*Eco*RV and 0.7-kb *Sma*I-*Eco*RV fragments of pIHD-1 into the *Sma*I site of pUC19, respectively. pTHD-2 was constructed by the self-ligation of the 6.8-kb *Eco*RI fragment of pTHD-1. The 2.1-kb *Sma*I fragment of pTHD-1 was inserted into the *Sma*I site of pUC19 to form pTK-1 and pTK-2 in both orientations. pTHD-5 was constructed by inserting the 1.2-kb *Sma*I-*Eco*RV fragment of pTHD-1 into the *Sma*I site of pUC19. The 2.3-kb *Eco*RV-*Pst*I fragment of pTHD-1 was treated with a blunting kit (Takara Shuzo Co. Ltd.) to create blunt ends and was inserted into the *Sma*I site of pUC19 to construct pTTS-1 and pTTS-2 in both orientations.

DNA sequence analysis. Both strands of DNA were sequenced by the dideoxy method with double-stranded DNA (4). The deletion derivatives of the inserts were obtained by treating pIK-1, pIK-2, pTK-1, pTK-2, pTTS-1, and pTTS-2 with a deletion kit (Takara Shuzo Co. Ltd.). DNA sequences were analyzed with GENIUS software (Mitsui Knowledge Industry Co. Ltd., Tokyo). Amino acid sequences were analyzed with PRINAS software (Mitsui Knowledge Indus-

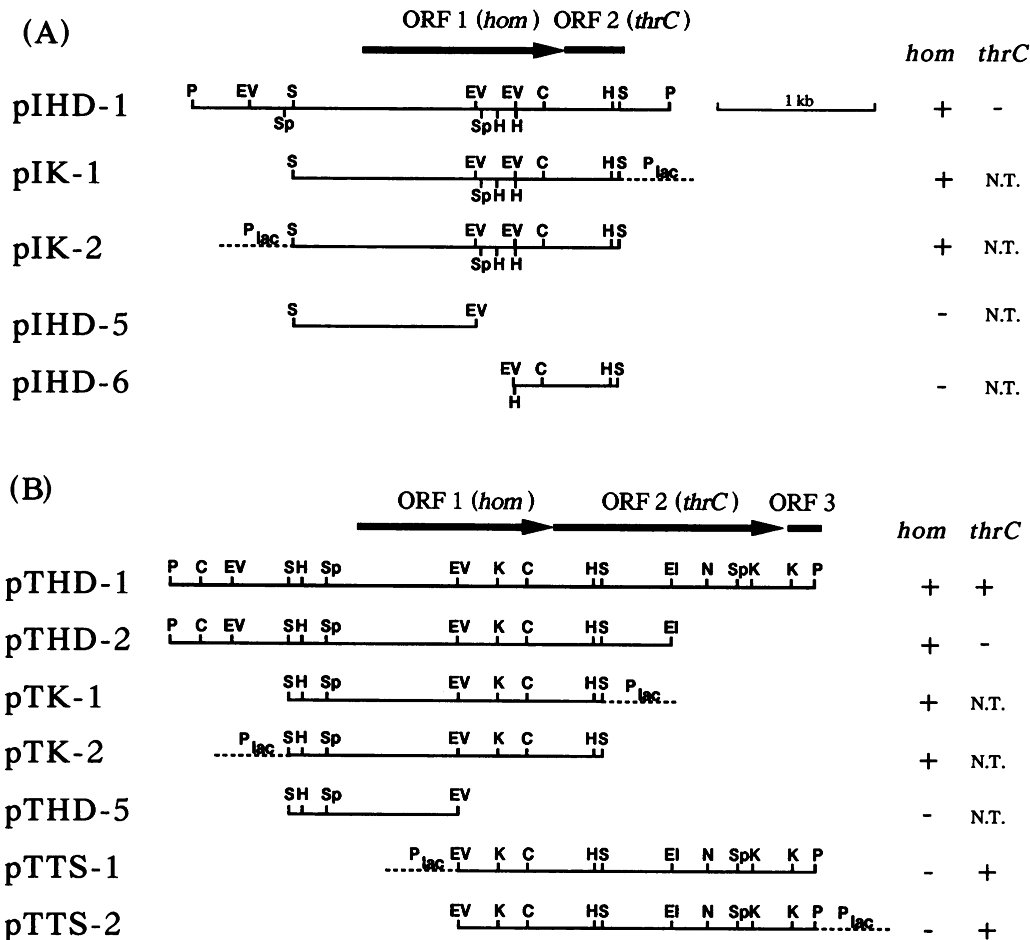

FIG. 1. Physical maps and subcloning of the plasmids harboring the *hom* and *thrC* genes of *M. glycogenes* ATCC 21276 (A) and ATCC 21371 (B). The positions of the ORFs determined by sequencing are shown by arrows. P_{lac} denotes the position of the *lac* promoter of pUC19. The complementation activities conferred by each plasmid in *E. coli* Gif 102 (*hom*) or Gif 41 (*thrC*) are indicated by + or −. Abbreviations: EI, *Eco*RI; EV, *Eco*RV; C, *Cla*I; H, *Hin*cII; K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sma*I; Sp, *Sph*I; N.T., not tested.

try Co. Ltd.). Homology analyses of amino acid sequences were done by a method described previously (11).

Northern blot analysis. Northern (RNA) blot analysis was performed as described previously (1). DNA probes were prepared by PCR with the Gene Amp kit (Perkin-Elmer Cetus) as follows. A 555-bp DNA fragment internal to the *hom* gene and a 541-bp DNA fragment internal to the *thrC* gene of *M. glycogenes* ATCC 21371 were amplified by PCR with the oligonucleotides 5′-AACCCATCAATGTTG GCCTG-3′ (5′ primer) and 5′-CCAGGCGTTGAGCTTC CTTA-3′ (3′ primer) for *hom* and 5′-ATCGCCTGCACTGTC TTTCT-3′ (5′ primer) and 5′-TTGCAGGCTGAACATCTG TG-3′ (3′ primer) for *thrC*, respectively, and pTHD-1 was used as a template. PCR was done with a thermal program: 30 cycles at 94°C for 1.5 min, 45°C for 2 min, and 72°C for 3 min and 1 cycle at 72°C for 4 min. The DNA fragments obtained were labeled with [α-^{32}P]dCTP (Amersham) with the *Bca* BEST labeling kit (Takara Shuzo Co. Ltd.) and used as probes.

Enzymatic methods. Preparation of cell extracts and measurements of HD activities were performed as described previously (16). One unit of HD was defined as the amount of enzyme which oxidized 1 μmol of NADH in 1 min. A 4.5% polyacrylamide gel was used to separate proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (9). Protein concentrations were determined with a Bio-Rad protein assay kit with bovine gamma globulin as the standard protein.

Purification of HD from *E. coli* recombinants. The cell extracts from *E. coli* Gif 102/pIKD-21 or Gif 102/pTK-2 were applied to a DEAE-Sepharose CL-6B column (2.5 by 40 cm) (Pharmacia) preequilibrated with MET buffer (20 mM Tris-HCl [pH 7.0], 2 mM $MgCl_2$, 2 mM EDTA, 20 mM 2-mercaptoethanol, 0.02 mM phenylmethylsulfonyl fluoride), washed extensively with MET buffer, and eluted with a linear 0.1 to 0.4 M KCl gradient. The active fractions collected were applied to a hydroxyapatite column (1.5 by 20 cm) (Seikagaku Kogyo, Tokyo, Japan) preequilibrated with MEP buffer (10 mM potassium phosphate buffer [pH 7.0], 2 mM $MgCl_2$, 2 mM EDTA, 20 mM 2-mercaptoethanol, 0.02 mM phenylmethylsulfonyl fluoride), washed extensively with MEP buffer, and eluted with a linear gradient of 10 to 70 mM potassium phosphate.

Immunochemical methods. Preparation of the antiserum against the HD purified from *E. coli* Gif 102/pIKD-21 and Western blotting (immunoblotting) were done according to the method described previously (8).

TABLE 2. Inhibitory effects of amino acids on HD activities in *E. coli* Gif 102 containing *M. glycogenes hom* genes

Strain	Amino acid addition	Relative activity (%)[a]	
		Before storage	After storage
Gif 102/pIHD-1	None	100	100
	10 mM Thr	88	29
	10 mM Phe	93	6
	10 mM Met	108	72
M. glycogenes ATCC 21276	None	100	ND
	1 mM Thr	4	ND
	1 mM Phe	4	ND
	1 mM Met	102	ND
	10 mM Met	33	ND
Gif 102/pTHD-1	None	100	100
	30 mM Thr	58	33
	10 mM Phe	62	25
	10 mM Met	97	65
M. glycogenes ATCC 21371	None	100	ND
	1 mM Thr	6	ND
	1 mM Phe	3	ND
	1 mM Met	106	ND
	10 mM Met	19	ND

[a] Inhibitory effects of amino acids on the HD activities in the cell extracts of the *E. coli* recombinants were examined soon after the extracts were prepared (before storage) and after storage at 4°C for 4 days (after storage). ND, not determined.

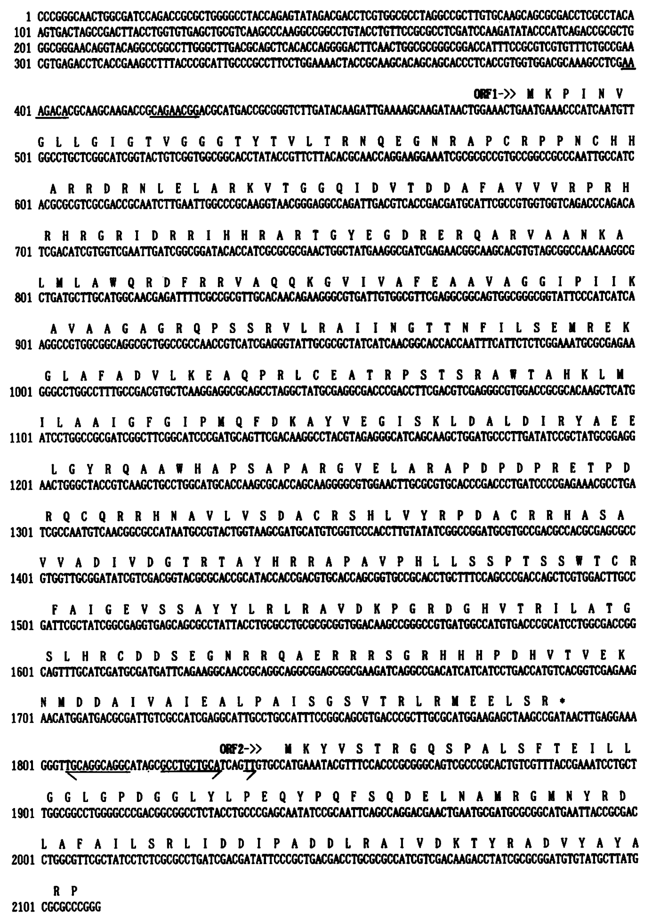

FIG. 2. Nucleotide sequence of the 2.1-kb *Sma*I fragment of *M. glycogenes* ATCC 21276. A putative promoter sequence is underlined. A stem-loop structure is indicated by arrows.

Nucleotide sequence accession number. The nucleotide sequences of the 2.1-kb *Sma*I fragment encoding the HD and the N-terminal sequence of the threonine synthase of *M. glycogenes* ATCC 21276 and the 3.4-kb *Sma*I-*Pst*I fragment encoding the HD, threonine synthase, and N-terminal sequence of thymidylate synthase of *M. glycogenes* ATCC 21371 will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under the accession no. D14070 and D14071, respectively.

RESULTS AND DISCUSSION

Cloning of the *hom* and *thrC* genes from *M. glycogenes* ATCC 21276 and ATCC 21371. Several genes of gram-negative methylotrophs have been cloned by heterologous complementation of *E. coli* mutants (10), so we tried to clone the *hom* genes of *M. glycogenes* ATCC 21276 and ATCC 21371 by complementation of an *E. coli* HD-deficient mutant. Gene libraries of *M. glycogenes* ATCC 21276 and ATCC 21371 were constructed as described in Materials and Methods, and an *E. coli* HD-deficient mutant, Gif 102, was transformed. The transformed cells were plated onto M9S1 medium with L-threonine and L-methionine omitted, and plasmids from the colonies that appeared on the medium were analyzed.

A plasmid containing a 3.1-kb *Pst*I fragment, designated pIHD-1, and a plasmid containing a 4.15-kb *Pst*I fragment, designated pTHD-1, were obtained from *M. glycogenes* ATCC 21276 and ATCC 21371, respectively (Fig. 1). The specific activities of the HD in *E. coli* Gif 102 carrying pIHD-1 and pTHD-1 were 0.078 and 0.06 U/mg of protein, respectively, whereas no activity was detected in *E. coli* Gif 102 containing pBR322. The chromosomal DNAs of the methylotrophs were digested completely with *Pst*I, separated by agarose gel electrophoresis, and hybridized with probes prepared from the cloned *Pst*I fragments. Only the expected 3.1- and 4.15-kb bands were detected in the *Pst*I-digested cDNAs of ATCC 21276 and ATCC 21371, respectively, suggesting the absence of any other homologous DNA in the chromosomal DNAs (data not shown). The 3.1-kb fragment of pIHD-1 and the 4.15-kb fragment of pTHD-1 were hybridized with one another, indicating that the DNA sequences of the fragments were homologous.

To examine whether pIHD-1 and pTHD-1 contain other L-threonine biosynthetic genes, pIHD-1 and pTHD-1 were used to transform other *E. coli* L-threonine auxotrophic mutants, GT 5 (*thrA metLM lysC*), Hfr 3000 YA 73 (*thrB*), and Gif 41 (*thrC*), and the complementation of the L-threonine auxotrophy by the plasmids was tested. Although pIHD-1 could not complement any other L-threonine-auxotrophs, pTHD-1 complemented the L-threonine auxotrophy of Gif 41, suggesting that pTHD-1 contains the *thrC* gene in addition to the *hom* gene.

Subcloned fragments from pIHD-1 and pTHD-1 were tested for the complementation of Gif 102 and Gif 41 to localize the *hom* and *thrC* genes (Fig. 1). Both of the *hom* genes of pIHD-1 and pTHD-1 were localized in the 2.1-kb *Sma*I fragments, and the *thrC* gene of pIHD-1 was localized in the 2.3-kb *Eco*RV-*Pst*I fragment, respectively.

Properties of the HD activities produced in *E. coli*. The molecular masses of the HDs of *E. coli* Gif 102 containing pIKD-21 and pTK-2, purified with DEAE-Sepharose CL-6B and hydroxyapatite columns, were estimated by SDS-PAGE

to be 50 and 48 kDa (data not shown), close to the values deduced from the nucleotide sequences described below.

The HDs of *M. glycogenes* ATCC 21276 and ATCC 21371 are inhibited strongly by L-threonine or L-phenylalanine and are inhibited weakly by L-methionine (Table 2). The HDs in the cell extracts of Gif 102 harboring pIHD-1 or pTHD-1 were hardly inhibited by these amino acids (Table 2). However, they became sensitive to the amino acids after storage at 4°C for 4 days.

The HDs in the cell extracts of the *E. coli* recombinants before and after storage at 4°C for 4 days were separated by SDS-PAGE, and the molecular masses were compared by Western blotting with the antiserum against the purified HD prepared from Gif 102/pIKD-21. The molecular masses of the enzymes after storage were the same as those before storage (data not shown), indicating that the HDs in *E. coli* recombinants were neither digested nor greatly modified during the storage, although the possibility of covalent modification with a small molecule such as phosphate cannot be ruled out.

Turano et al. (23) reported that the carrot HD was interconvertible between an L-threonine-nonsensitive homodimer and an L-threonine-sensitive homotrimer. We speculate that the L-threonine-nonsensitive HDs of the *E. coli* recombinants are different from the L-threonine-sensitive ones of *M. glycogenes* in the subunit numbers of the enzyme, as observed in the carrot. Since the HDs were overproduced 10- to 20-fold in *E. coli* compared with in *M. glycogenes*, it might take a long time to form the correct structures susceptible to inhibition by the amino acids in *E. coli*.

Nucleotide sequences of the 2.1-kb *Sma*I fragment of pIHD-1 and the 3.4-kb *Sma*I-*Pst*I fragment of pTHD-1. The 2.1-kb *Sma*I fragment of pIHD-1 was sequenced on both strands, and two open reading frames (ORFs) were found (Fig. 2). ORF1 encodes a predicted peptide with an M_r of 48,225 initiated at the ATG codon (nucleotides 483 to 485) and terminated at the TAA (nucleotides 1788 to 1790). ORF2 encodes a truncated peptide of 89 amino acid residues initiated at the ATG codon (nucleotides 1842 to 1844). The amino acid sequence deduced from ORF2 is quite homologous to the N-terminal region of the predicted peptide of the threonine synthase of *M. glycogenes* ATCC 21371, thought to be the N-terminal domain of the *thrC* gene of *M. glycogenes* ATCC 21276. A putative promoter sequence, 5'-AAAGACA-3' (−35) 5'-CAGAAC-3' (−10), homologous to a proposed consensus promoter sequence of gram-negative methylotrophs, 5'-AAAGACA-3' (−35) 5'-TAGAAA-3' (−10) (10), was found 80 to 50 bp upstream of the initiation codon of the *hom* gene (underlined in Fig. 2). A promoterlike sequence was not found upstream of the *thrC* gene.

Similarly, the nucleotide sequence of the 3.4-kb *Sma*I-*Pst*I fragment of pTHD-1 was determined, and three ORFs were found (Fig. 3). ORF1 encodes a deduced peptide with an M_r of 44,815 initiated at the ATG codon (nucleotides 491 to 493) and terminated at the TAA (nucleotides 1727 to 1729). ORF2 encodes a predicted peptide with an M_r of 52,111 initiated at the ATG codon (nucleotides 1779 to 1781) and terminated at the TGA (nucleotides 3204 to 3206). ORF3 encodes a truncated peptide of 64 amino acid residues initiated at the ATG codon (nucleotides 3219 to 3221).

ORF1 and ORF2 are suggested to be the coding sequences of the *hom* and *thrC* genes of *M. glycogenes* ATCC 21371, respectively, by the complementation analysis results shown in Fig. 1. The deduced amino acid sequence of ORF3 is homologous to those of thymidylate synthase genes (*thyA*) from other microorganisms, assumed to be the N-terminal

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   1 CCCGGGCAATTGGCTATCCAGACCGCGCTTGGTGGTTACCAGAGCATCGATGACCTGGTGGCACCGACTGGGCGCTTGTGCAAGCAGCGCGACCTTGCCT
 101 ACAAGATGTTGACAGATATTCCAGGGGTTAGCTGTGTCAAGCCCAAGGCTGGCCTGTACTTGTTCCCGCGCCTTGACCCCAAGATCTACCCGATTGAGGA
 201 CGACCAGCAGTTCATCCTGGATTTGTTGCTGGAAGAAAAGTATTGCTGGTGCAGGCTCAGGTTTAATTGGCATGCTCCGACCACTTCCGCGTGGTGTTCC
 301 TGCCGAATGTGGATGATCTCACCGAGGCATTCACCCGGATTGCCCGCTTCCTGGAAAATTATCGCAAGCAGCACAGCACGCTTACGGTGGTGGATGGCAA

                                                                                   ORF1->> M  K  P  I
 401 AGCGGCAAAAGAAATACAAACAAAGCCACACAATGCAACTGCATGATATGGCTGGTCTATAAGATTGAAAAGCAAGAAACTGGAAACTGAATGAAACCCA

      N  V  G  L  L  G  I  G  T  V  G  G  G  T  Y  T  V  L  T  R  N  Q  E  E  I  A  R  R  A  G  R  P  I
 501 TCAATGTTGGCCTGCTCGGCATCGGTACTGTCGGTGGCGGCACCTATACTGTTTTAACCCGTAACCAGGAAGAAATCGCACGCCGTGCTGGACGCCCAAT

      A  I  T  R  V  A  D  R  N  L  E  L  A  R  Q  V  T  G  G  K  I  D  V  T  D  D  A  F  A  I  V  S  D
 601 CGCCATTACCCGTGTTGCCGATCGTAATCTGGAGCTGGCTCGCCAGGTGACTGGTGGAAAAATTGATGTCACCGATGATGCTTTTGCCATCGTGTCTGAT

      P  A  I  D  I  V  V  E  L  I  G  G  Y  T  V  A  R  E  L  V  L  K  A  I  E  N  G  K  H  V  V  T  A  N
 701 CCGGCAATTGATATTGTTGTTGAACTGATCGGTGGCTACACCGTGGCGCGTGAACTGGTGCTGAAGGCCATTGAGAATGGCAAGCACGTGGTCACGGCCA

      K  A  L  I  A  C  M  A  M  K  F  L  P  L  R  R  K  K  A  S  S  S  L  L  K  L  P  L  L  V  V  S  P
 801 ATAAGGCCTTGATTGCCTGCATGGCAATGAAATTTTTGCCGCTGCGCAGAAAAAAGGCGTCATCGTCGCTTTTGAAGCTGCCGTTGCTGGTGGTATCCCC

      L  F  K  A  V  R  E  G  L  A  A  N  R  I  E  W  I  A  G  I  I  N  G  T  T  N  F  I  L  S  E  M  R
 901 ATTATTCAAGGCCGTACGTGAAGGCCTGGCGGCCAATCGTATTGAGTGGATTGCTGGCATCATCAATGGCACGACCAATTTCATTCTCTCGGAAATGCGT

      E  K  G  L  A  F  A  D  V  L  K  E  A  Q  R  L  G  Y  A  E  A  D  P  T  F  D  V  E  G  I  D  A  A  H
1001 GAAAAGGGTCTGGCGTTTGCTGATGTGCTTAAGGAAGCTCAACGCCTGGGTTATGCCGAGGCAGACCCGACTTTCGATGTCGAAGGCATTGATGCTGCGC

      K  L  M  I  L  A  A  M  L  W  L  F  V  H  S  L  C  R  G  I  T  K  L  D  A  V  D  I  T  K  R  T  D
1101 ACAAGCTCATGATCCTTGCTGCGATGCTTTGGCTATTCGTGCACAGCTTATGTCGAGGCATCACGAAGCTGGATGCCGTGGATATCACCAAGCGTACCGA

      K  G  V  E  L  R  V  H  P  T  L  I  P  E  K  R  L  I  C  Q  C  E  W  R  N  E  C  C  A  G  Q  G  R
1201 TAAGGGCGTGGAGTTGCGTGTGCACCCAACCTTGATCCCGGAAAAGCGCTTGATTTGCCAATGTGAATGGCGCAATGAATGCTGTGCTGGTCAAGGGCGA

      C  C  W  P  T  L  Y  Y  G  A  G  A  G  A  E  P  T  A  S  A  V  A  D  L  V  D  G  T  D  R  G  I  S  C
1301 TGCTGTTGGCCTACCTTGTATTATGGTGCCGGTGCTGGTGCAGAACCTACCGCTAGTGCGGTTGCCGACTTGGTCGATGGTACCGACCGTGGCATCAGCT

      P  H  L  A  F  Q  P  D  R  L  V  D  L  P  I  L  P  I  G  E  I  S  S  A  Y  Y  L  R  L  R  A  V  D
1401 GTCCACACCTGGCTTTCCAGCCAGACCGCTTGGTAGACTTGCCCATCCTGCCTATCGGCGAGATTAGCAGTGCCTATTACCTGCGCCTGCGTGCAGTGGA

      K  P  G  V  L  A  D  V  T  R  I  L  G  D  R  Q  I  S  I  D  A  M  I  Q  K  E  P  Q  E  G  E  D  Q
1501 TAAGCCAGGCGTGCTCGCTGACGTGACCCGTATCCTCGGTGACCGTCAGATTTCGATCGATGCGATGATCCAGAAAGAGCCACAAGAGGGCGAAGACCAG

      A  D  I  I  I  L  T  H  V  T  V  E  K  N  M  D  D  A  I  A  A  I  E  A  L  P  A  I  S  G  K  V  T  R
1601 GCCGACATCATCATTCTCACCCACGTCACAGTTGAGAAAAACATGGATGATGCCATCGCCGCCATTGAGGCACTACCTGCCATTTCCGGCAAGGTCACGC

      L  R  M  E  E  L  S  R  *                                            ORF2->> M  K  Y  I  S  T  R  G
1701 GTTTGCGCATGGAAGAACTAAGCCGATAACTTGAGAAAAGGGTTGCAGGCAGGCCTATTGCCTCCTGCATCAGTTGCAATGAAATACATTTCCACCCGCG

      Q  S  P  A  L  S  F  S  E  I  L  L  G  G  L  A  P  D  G  G  L  Y  L  P  E  Q  Y  P  Q  F  S  A  D
1801 GGCAATCGCCTGCACTGTCTTTCTCTGAAATTCTGCTTGGCGGCTTGGCGCCTGATGGCGGCTTGTATTTGCCCGAGCAATACCCGCAGTTTAGCGCTGA

      A  L  S  A  M  R  G  M  N  Y  R  D  L  A  F  T  I  L  S  R  L  I  D  D  I  P  A  D  D  L  R  I  I
1901 CGCACTGAGCGCGATGCGCGGCATGAATTACCGCGACCTGGCGTTCACCATCCTCTCGCGTCTGATCGACGATATTCCCGCTGACGACCTGCGCATCATC

      V  D  K  T  Y  R  A  D  V  Y  A  Y  A  R  P  G  Q  D  A  E  D  I  T  P  T  Y  K  L  E  D  D  L  Y  L
2001 GTCGACAAGACCTATCGCGCGGATGTATATGCCTATGCTCGCCCGGGCCAGGATGCCGAAGACATTACGCCGACCTATAAGCTGGAGGACGACCTCTACC

      L  S  L  S  N  G  P  T  L  A  F  K  D  M  A  M  Q  L  L  G  N  L  F  E  Y  V  L  A  Q  K  G  E  T
2101 TGCTTTCCCTGTCCAATGGCCCAACCCTGGCGTTCAAGGATATGGCGATGCAGTTATTGGGCAACCTGTTCGAATACGTGTTGGCGCAAAAGGGCGAGAC

      T  N  I  L  G  A  T  S  G  D  T  G  S  A  A  E  Y  A  M  R  G  K  Q  G  V  K  V  F  M  L  S  P  H
2201 GACTAATATTCTCGGCGCGACCTCCGGCGATACCGGTTCTGCGGCGGAATACGCCATGCGCGGCAAGCAGGGCGTCAAGGTGTTCATGCTCTCGCCGCAC

      Q  K  M  S  R  F  Q  T  A  Q  M  F  S  L  Q  D  D  N  I  F  N  I  A  V  K  G  V  F  D  D  C  Q  D  I
2301 CAGAAGATGAGCCGTTTCCAGACCGCACAGATGTTCAGCCTGCAAGACGACAATATCTTCAATATCGCGGTCAAGGGCGTGTTTGACGACTGCCAGGACA

      V  K  A  V  S  N  D  H  A  F  K  A  K  N  K  I  G  A  V  N  S  I  N  W  A  R  V  A  A  Q  V  V  Y
2401 TCGTCAAGGCCGTGTCGAACGACCATGCTTTCAAGGCCAAGAATAAGATCGGTGCCGTGAATTCCATCAACTGGGCACGCGTGGCTGCTCAGGTAGTGTA

      Y  F  K  G  Y  F  A  V  T  A  D  N  A  Q  Q  V  S  F  A  V  P  S  G  N  F  G  N  V  C  A  G  H  I
2501 TTACTTCAAGGGTTATTTCGCCGTCACTGCGGATAATGCGCAGCAGGTCAGCTTTGCCGTGCCTTCCGGCAACTTCGGCAATGTCTGTGCCGGGCATATC

      A  R  M  M  G  L  P  I  A  K  L  V  V  A  T  N  E  N  D  V  L  D  E  F  F  K  T  G  V  Y  R  P  R  G
2601 GCCCGCATGATGGGCTTGCCGATTGCCAAGCTGGTGGTAGCGACCAACGAAAACGACGTGCTGGATGAGTTCTTCAAAACTGGCGTCTACCGTCCGCGCG

      S  A  N  T  Y  H  T  S  S  P  S  M  D  I  S  K  A  S  N  F  E  R  F  V  F  D  L  V  G  R  D  A  A
2701 GCTCCGCCAATACCTACCATACTTCCAGCCCCTCCATGGATATTTCCAAGGCCTCCAATTTCGAGCGTTTCGTCTTCGACCTAGTGGGCCGTGACGCCGC

      K  V  R  E  L  W  G  K  V  D  A  G  G  S  F  D  L  N  D  G  G  W  F  A  K  V  A  D  Y  G  F  V  S
2801 CAAGGTGCGCGAGCTATGGGGCAAGGTGGATGCGGGCGGCAGTTTCGACCTTAACGACGGTGGCTGGTTTGCCAAGGTAGCGGATTACGGCTTCGTTTCC

      G  S  S  N  H  A  N  R  M  Q  T  I  K  A  T  H  E  R  Y  G  V  T  I  D  T  H  T  A  D  G  L  K  V  A
2901 GGCAGCAGCAACCATGCCAACCGCATGCAGACCATCAAGGCGACGCATGAGCGCTACGGTGTCACCATTGATACCCACACCGCGGACGGTCTCAAGGTGG

      L  E  H  R  E  A  G  T  P  M  L  V  L  E  T  A  L  P  A  K  F  E  D  A  I  V  E  A  L  G  H  K  P
3001 CGCTGGAACACCGCGAGGCAGGTACCCCGATGCTGGTGCTGGAAACCGCATTGCCGGCCAAATTCGAGGACGCGATTGTCGAGGCGTTAGGGCATAAACC

      E  R  P  H  S  L  E  G  L  E  S  L  P  Q  R  F  E  V  M  E  A  D  A  A  V  I  K  Q  F  I  V  E  H
3101 CGAGCGTCCGCACAGCCTGGAAGGCCTGGAATCCCTGCCTCAGCGCTTTGAGGTGATGGAGGCCGATGCAGCCGTCATCAAACAGTTCATTGTTGAGCAT

      I  *     ORF3->> M  K  V  Y  H  D  L  M  R  H  V  L  E  H  G  H  K  K  E  D  R  T  G  T  G  T  L  S
3201 ATTTGATCGGGAGATGTCATGAAGGTCTATCACGACTTGATGCGCCACGTACTGGAGCACGGCCACAAGAAGGAAGACCGTACCGGTACCGGCACCTTAT

      V  F  G  Y  Q  M  R  F  D  L  A  E  G  F  P  L  L  T  T  K  K  V  H  L  K  S  I  I  H  E  L  L  W
3301 CAGTGTTTGGCTACCAGATGCGTTTTGACTTGGCCGAGGGCTTCCCGCTACTTACCACCAAGAAGGTGCACCTGAAATCCATCATCCATGAGTTGCTTTG

      F  L  Q
3401 GTTCCTGCAG
```

FIG. 3. Nucleotide sequence of the 3.4-kb *Sma*I-*Pst*I fragment of *M. glycogenes* ATCC 21371. A stem-loop structure is indicated by arrows.

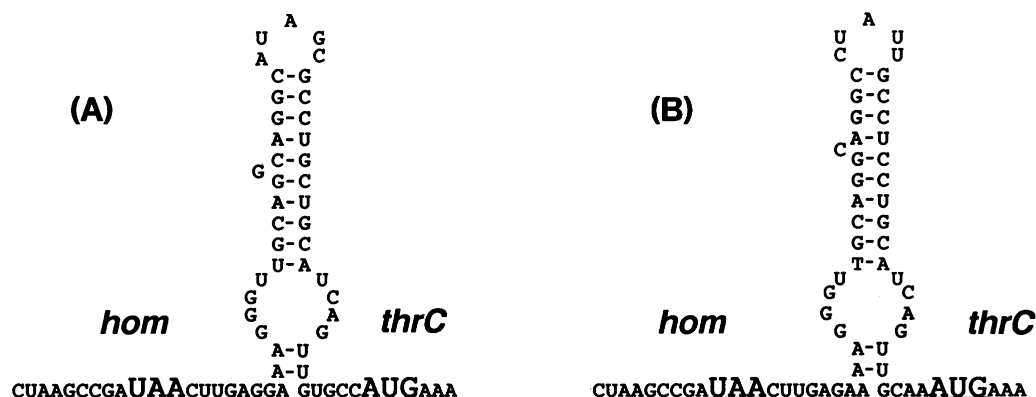

FIG. 4. Putative secondary structures of mRNA in the intergenic regions of the *hom* and *thrC* genes from *M. glycogenes* ATCC 21276 (A) and ATCC 21371 (B). The stabilities of the stem-loop structures of ATCC 21276 and ATCC 21371 are calculated as ΔGs of −17.8 and −19.6 kcal (1 cal = 4.184 J), respectively, according to the method of Tinoco et al. (22).

sequence of the *thyA* gene of the strain (data not shown). A promoterlike sequence was not found in the upstream regions of the *hom*, *thrC*, and *thyA* genes.

The formation of stem-loop structures is possible in the mRNAs between the *hom* and *thrC* genes of ATCC 21276 and ATCC 21371 (ΔG = −17.8 and −19.6 kcal [1 cal = 4.184 J], respectively) (Fig. 4). They may play roles in regulating the expression of the *hom* and *thrC* genes or stabilizing the transcripts. Similar structures of mRNA were reported in the intergenic regions between the *hom* and *thrC* genes from *M. flagellatum* (13), between the same genes from *P. aeruginosa* (5), and between the *arcA*/*arcB* and *arcB*/*arcC* genes of *P. aeruginosa* (3). The structure between the *arcA*/*arcB* and *arcB*/*arcC* genes was assumed to have a role in protection of mRNA against 3′-exonuclease degradation.

Northern blot analysis. Northern blots of mRNAs were made to examine whether the *hom* and *thrC* genes of *M. glycogenes* are organized in an operon (Fig. 5). RNAs extracted from *M. glycogenes* ATCC 21276 and ATCC 21371 were separated by 1.2% agarose gel electrophoresis and hybridized with probes prepared from a 555-bp fragment internal to the *hom* gene (bp 495 to 1049) or a 541-bp fragment internal to the *thrC* gene (bp 1805 to 2345) of *M. glycogenes* ATCC 21371. The probes could hybridize not

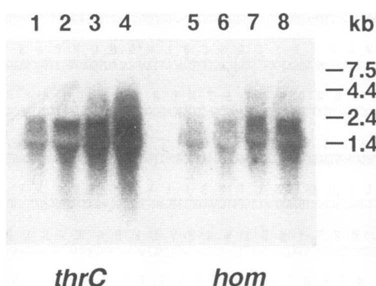

FIG. 5. Northern blots of mRNA transcribed from the *hom-thrC* region. The RNAs extracted from *M. glycogenes* ATCC 21276 (lanes 1, 2, 5, and 6) and ATCC 21371 (lanes 3, 4, 7, and 8) were hybridized with probes prepared from a 555-bp DNA fragment internal to the *thrC* gene in lanes 1 to 4 and a 540-bp DNA fragment internal to the *hom* gene in lanes 5 to 8, respectively. The amounts of the RNAs used were 5 (lanes 3, 5, and 7), 10 (lanes 1, 4, 6, and 8), and 22.5 μg (lane 2). The positions of size markers are indicated on the right side.

only the RNA from ATCC 21371 but also the RNA from ATCC 21276, although the intensities of the bands in the latter samples were weaker than those in the former ones, and the sizes of the bands detected by each probe were similar in the two samples.

The approximately 2.4-kb mRNAs were hybridized with both probes, suggesting that the *hom* and *thrC* genes are organized in an operon, as in *M. flagellatum* (13) and *P. aeruginosa* (5). Shorter bands (ca. 1.4 kb) detected by each probe were thought to be digested or processed products of the 2.4-kb mRNAs, but another explanation cannot be excluded. That is, the transcription of the *hom* genes could terminate at the intergenic regions of the *hom* and *thrC* genes, and the transcription of the *thrC* genes could initiate in the intergenic region, although promoterlike sequences were not found upstream of the *thrC* genes. Further studies are necessary to give a sufficient explanation of the results.

Comparison of the predicted amino acid sequences of the *hom* genes. The predicted amino sequences of the *hom* genes of *M. glycogenes* were compared with those of other microorganisms (Fig. 6). Those of the two methylotrophs were highly homologous; that is, 47.4% (206 of 435 amino acid residues) of the amino acid sequence of *M. glycogenes* ATCC 21276 is identical to that from ATCC 21371. Comparison of the sequence of ATCC 21371 with those of *P. aeruginosa*, *B. subtilis*, *C. glutamicum*, *E. coli* HD I, and *E. coli* HD II revealed identities of 49.0% (215 of 439 amino acid residues), 31.9% (138 of 433 amino acid residues), 31.5% (140 of 445 amino acid residues), 18.4% (66 of 358 amino acid residues), and 17.3% (62 of 358 amino acid residues), respectively.

E. coli HD I and HD II are less homologous to the *M. glycogenes hom* gene products than other HDs are. *E. coli* HD I and HD II are bifunctional proteins of AK-HD, and others are monofunctional HDs. This bifunctional HD, only found in members of the family *Enterobacteriaceae*, is thought to have evolved in a way different from those of other monofunctional HDs (18). The structural and evolutionary differences explain the differences in homology. The high degree of homology between the *M. glycogenes hom* gene product and other monofunctional *hom* gene products suggests that they have a common ancestor.

Several well-conserved domains were found in the amino acid sequences that were compared. The amino acid sequence motif at positions 10 to 15, G-X-G-X-X-G, sur-

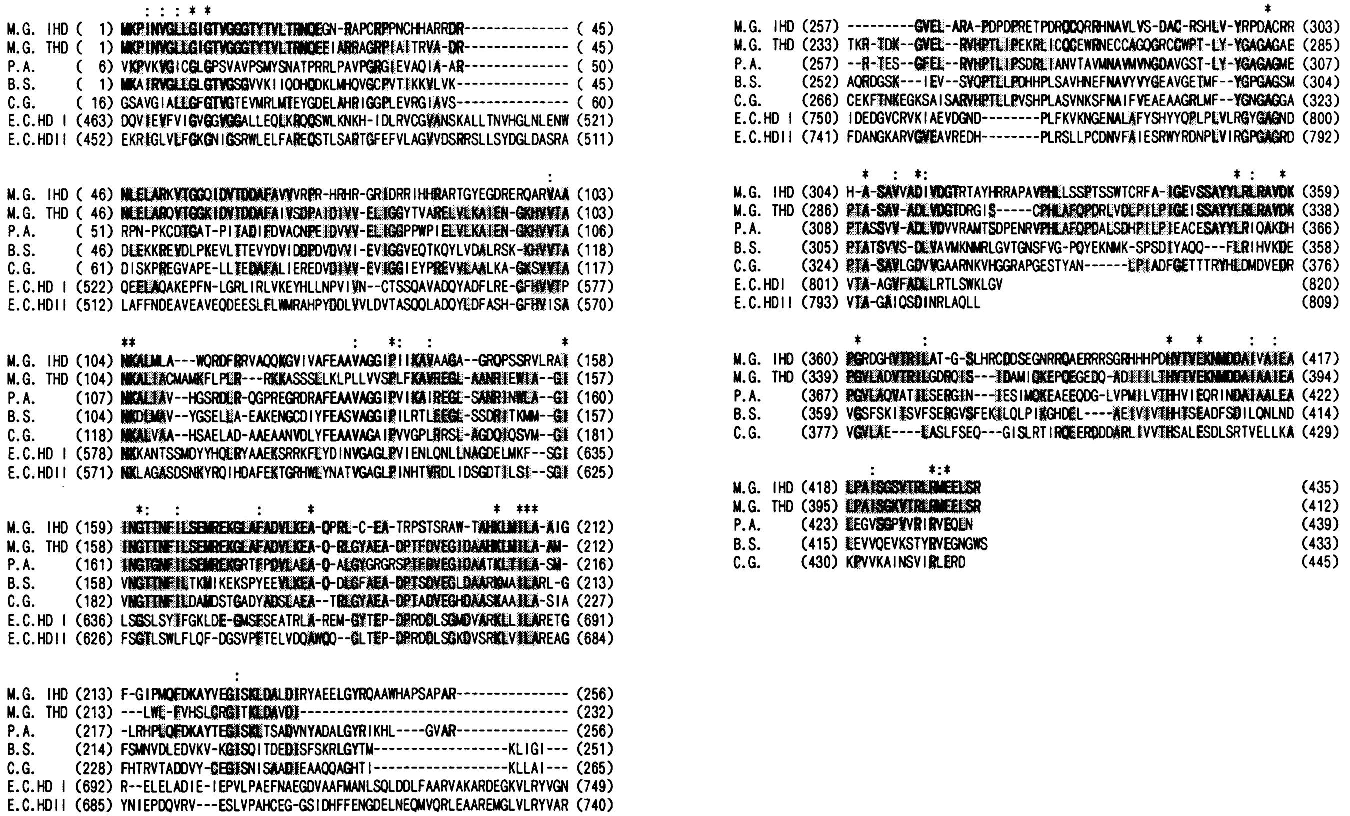

FIG. 6. Homology between the amino acid sequence of the HDs of *M. glycogenes* ATCC 21371 and ATCC 21276 and those of other microorganisms. Shaded letters and boldface letters show amino acid residues identical to those of *M. glycogenes* ATCC 21371 and *M. glycogenes* ATCC 21276, respectively. Conserved residues in all of the amino acid sequences are noted with asterisks, and homologous residues (I-L-V-M, D-E, R-K, S-T, F-Y) are noted with colons. Abbreviations: M.G., *M. glycogenes*; P.A., *P. aeruginosa*; B.S., *B. subtilis*; C.G., *C. glutamicum*; E.C., *E. coli.*

rounded by small hydrophobic amino acid residues, which is characteristic of the NAD(P) binding site of HDs (18, 20), is found in the N-terminal region. Like other HDs, NAD, the cofactor of the enzyme, should bind this region in *M. glycogenes.*

Parsot and Cohen (18) showed that the amino acid sequence between positions 200 and 221 of *B. subtilis* was well conserved in *E. coli* AK I-HD I and AK II-HD II counterparts and suggested that this region could be involved in substrate binding or could be a catalytic region. The amino acid sequence motif K-X-X-I-L-A in this region is conserved in all of the sequences compared, suggesting the importance of this region in substrate binding or catalytic activity.

The regulatory domains involved in feedback inhibition by L-threonine were postulated to be present in the C-terminal regions in *B. subtilis* (18), *C. glutamicum* (2), and *M. flagellatum* (13). The C-terminal regions of the *M. glycogenes* HDs show some homology with those of other HDs. Alteration in this region of the *M. glycogenes hom* genes by site-directed mutagenesis will provide useful information with which to investigate the regulatory domains concerned with inhibition by L-threonine, L-phenylalanine, and L-methionine.

Comparison of the predicted amino acid sequences of *thrC* genes. The predicted amino acid sequences of the *thrC* genes of the *M. glycogenes* strains were compared with those of other microorganisms (Fig. 7). The N-terminal peptides of two *M. glycogenes* strains are quite homologous; that is, 88 of 89 amino acid residues are identical. Comparison of the sequence of ATCC 21371 with those of *P. aeruginosa*, *B. subtilis*, *C. glutamicum*, *Serratia marcescens*, *E. coli*, and *Saccharomyces cerevisiae* showed identities of 40.3% (197 of 489 amino acid residues), 39.2% (138 of 352 amino acid residues), 29.1% (140 of 481 amino acid residues), 32.4% (139 of 429 amino acid residues), 34.3% (147 of 428 amino acid residues), and 32.5% (167 of 514 amino acid residues), respectively.

The homology observed throughout the amino acid sequences suggests that the structure of the catalytic mechanism of the threonine synthase of *M. glycogenes* is similar to those of the threonine synthases of other microorganisms (17). Threonine synthase is a pyridoxal phosphate enzyme. Parsot (17) found significant homology between the amino acid sequences of threonine synthase from *E. coli* and *B. subtilis* and those of other pyridoxal phosphate enzymes, such as threonine dehydratase in *S. cerevisiae* and serine dehydratase in *E. coli*. He suggested that, prior to the separation of those organisms, an ancestral microorganism had a pyridoxal phosphate enzyme with a wide substrate specificity, and subsequent mutation led it to acquire its current substrate specificity. The high degree of homology between the threonine synthase of *M. glycogenes* and those of other microorganisms supports his idea. The motif at positions 115 to 121, P-T-X-X-F-K-D, is well conserved in all of the sequences. Pyridoxal phosphate, the coenzyme for synthase reaction, may bind the lysine residue

FIG. 7. Homology between the amino acid sequence of the threonine synthase of *M. glycogenes* ATCC 21371 and those of other microorganisms. Shaded letters show amino acid residues identical to those of *M. glycogenes* ATCC 21371. Conserved residues in all of the amino acid sequences are noted with asterisks, and homologous residues (I-L-V-M, D-E, R-K, S-T, F-Y) are noted with colons. S.M., *S. marcescens*. All other abbreviations are as defined in the legend to Fig. 6.

at position 120 by analogy with other threonine synthases (17).

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