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Molecular Characterization of *AceB*, a Gene Encoding Malate Synthase in *Corynebacterium glutamicum*

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The *aceB* gene, encoding for malate synthase, one of the key enzymes of glyoxylate bypass, was isolated from a pMT1-based *Corynebacterium glutamicum* gene library *via* complementation of an *Escherichia coli aceB* mutant on an acetate minimal medium. The *aceB* gene was closely linked to *aceA*, separated by 598 base pairs, and transcribed in divergent direction. The *aceB* expressed a protein product of *Mr* 83,000 in *Corynebacterium glutamicum* which was unusually large compared with those of other malate synthases. A DNA-sequence analysis of the cloned DNA identified an open-reading frame of 2,217 base pairs which encodes a protein with the molecular weight of 82,311 comprising 739 amino acids. The putative protein product showed only limited amino acid-sequence homology to its counterparts in other organisms. The N-terminal region of the protein, which shows no apparent homology with the known sequences of other malate synthases, appeared to be responsible for the protein's unusually large size. A potential calcium-binding domain of EF-hand structure found among eukaryotes was detected in the N-terminal region of the deduced protein.

Corynebacterium glutamicum is a Gram positive non-sporulating organism and has been widely used for the industrial production of amino acids (see 15, 17, 20, and 35 for reviews). Increasing and optimizing the final yield of metabolites by strain manipulation has long been a major interest in the food and feed industry. The availability of genetic and molecular biological tools developed for *Corynebacterium* and related species has made possible designing and controlling novel pathways at molecular level (23, 30, 37). In addition, the availability of isolated genes facilitated precise control over the target pathway at gene and protein levels (12, 26).

The glyoxylate bypass of C. *glutamicum* comprises two enzymes (12, 24; Fig. 1). Isocitrate lyase which is encoded by *aceA* catalyzes the conversion of the Krebs cycle intermediate, isocitrate, to glyoxylate and succinate. Malate synthase, the product of *aceB*, catalyzes the condensation of glyoxylate with acetyl-CoA to produce malate. In *Escherichia coli*, the genes that encode metabolic and regulatory enzymes of the bypass are organized into the *aceB,A,K* operon (5, 6, 21). Expression of the glyoxylate bypass enzymes is essential for growth on acetate as the sole carbon source, since it prevents the net loss of the acetate carbon as CO_2 in the Krebs cycle.

As an initial step towards understanding the role and significance of the glyoxylate bypass in amino-acid production and the mechanisms which control the expression of the glyoxylate bypass genes, we isolated and characterized *aceB* gene from C. *glutamicum*. In this paper we describe the cloning and sequencing of *aceB* and show its close linkage with *aceA*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. C. *glutamicum* and *E. coli* strains were routinely grown in MB (9) and LB (22) broth, respectively. Unless otherwise specified, acetate was added to the final concentration of 2%. Antibiotics were added in the following amounts (micrograms per milliliter): ampicillin, 50; kanamycin, 25. When needed, M9 minimal medium was supplemented with ampicillin to a final concentration of 25 μg/ml. Amino acids, vitamins, and other supplements were added in the following amounts: methionine, 9.3 mM; thiamine, 0.05 mM; spermidine and putrecine, 0.05 mM. C. *glutamicum* and *E. coli* cells were routinely grown at 30°C and 37°C, respectively.

DNA Manipulations.

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Standard molecular cloning, transformation, and electrophoresis procedures were used (2, 22). *E. coli* cultures were screened for plasmid content by the alkaline lysis procedure (3). Mini plasmid preparation for *C. glutamicum* cells were performed as described (37). Chromosomal DNA from *C. glutamicum* ASO19 was prepared as described (12). *Corynebacterium* strains were transformed by electroporation as described (9). Restriction endonucleases and DNA modifying enzymes were purchased from New England BioLabs, Boehringer Mannheim Biochemicals, and Bethesda Research Laboratories (BRL) and used as recommended by the manufacturers.

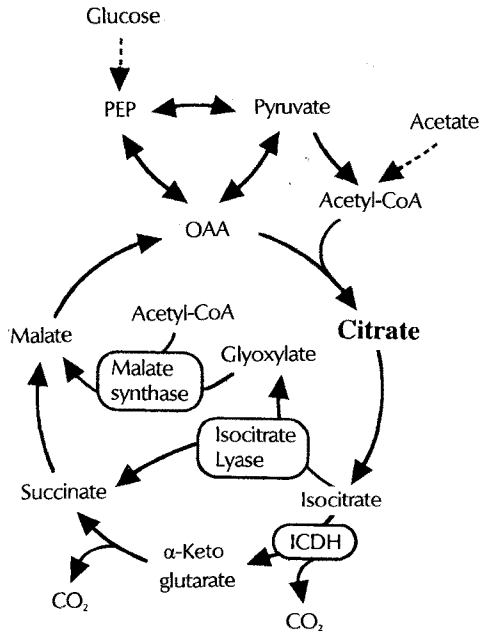

Fig. 1. The Krebs cycle, glyoxylate bypass, and associated pathways of *C. glutamicum*.
Abbreviations: OAA, oxaloacetate; PEP, phosphoenolpyruvate; ICDH, isocitrate dehydrogenase.

Cloning of *AceB*.

A *C. glutamicum* genomic library was made of 4- to 13-kb *Mbo*I fragments cloned into the *E. coli-Corynebacterium* shuttle vector pMT1. *C. glutamicum* ASO19 chromosomal DNA partially digested with restriction enzyme *Mbo*I was size fractionated by 10 to 40% sucrose gradient centrifugation, ligated with the *Bam*HI-digested vector, and transformed into *E. coli* DH5α. A total of approximately 5,000 recombinants were obtained. The recombinants were pooled and subjected to plasmid isolation. *E. coli* CGSC5236 cells were transformed with the plasmid DNA and plated onto the M9 minimal medium containing acetate (0.68%), ampicillin, and appropriate supplements (methionine, thiamine, spermidine, and putrecine). The plates were incubated at 37°C for 5 days. Colonies were isolated and screened for the plasmid content.

Subcloning.

Plasmid pSL14 was made by ligating the 1.5-kb *Eco*RI-*Kpn*I fragment of pSL08 (*Kpn*I site was provided by the vector) into pUC19. Plasmid pSL17 was made by ligating the 1.5-kb *Eco*RI-*Sal*I fragment of pSL08 into pUC19. Plasmid pSL22 was made by ligating the 1.4 kb *Pst*I-*Sal*I fragment of pSL08 into pUC19. Plasmid pSL28 was made by ligating the 2.8 kb *Eco*RI-*Kpn*I fragment of pSL05 into pUC19. Plasmid pSL12 was made by deleting a 2-kb *Kpn*I fragment from pSL08.

DNA Sequence Determination.

The complete nucleotide sequences of *aceA* and *aceB* were determined by the dideoxynucleotide chain termination method (30) using a commercially available

Table 1. Bacterial strains and plasmids

Strains or plasmids	Relevant genotypes or phenotypes[a]	Sources or references
E. coli		
CGSC5236	*aceB glc ppc*	CGSC[b]
DH5α	F⁻ ϕ *80dlacZDM15* △*(lacZYA-argF)U169 deoR endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* λ⁻	Bethesda Research Laboratories
C. glutamicum		
ASO19	Spontaneous rifampin resistant mutant of ATCC 13059	37
ASO19E12	Restriction-deficient varient of ASO19	10
Plasmids		
pMT1	Shuttle vector; Ap[r] (*E. coli*), Km[r] (*C. glutamicum*)	9
pUC19	Ap[r]	36
pSL05	pMT1 with 5.3-kb insert carrying *aceA* and *aceB*; Ap[r]Km[r]	This work
pSL08	pMT1 with 4.3-kb insert carrying *aceB*; Ap[r]Km[r]	This work
pSL12	pMT1 with 2.3-kb insert partially carrying *aceB*; Ap[r]Km[r]	This work
pSL14	pUC19 with 1.5-kb *Eco*RI-*Kpn*I fragment; Ap[r]	This work
pSL17	pUC19 with 1.5-kb *Eco*RI-*Sal*I fragment; Ap[r]	This work
pSL22	pUC19 with 1.4-kb *Pst*I-*Sal*I fragment; Ap[r]	This work
pSL28	pUC19 with 2.8-kb *Eco*RI-*Kpn*I fragment; Ap[r]	This work

[a]r superscripts indicate resistance. Ap, ampicillin; Km, kanamycin, [b] CGSC, E. coli Genetic Stock Center, Yale University, New Haven, Conn., USA

Sequenase II kit (United States Biochemical). A series of unidirectional deletions were generated from plasmids pSL14, pSL22, and pSL28 using Exonuclease III and S1 nuclease, and the deletion products were used as sequencing templates (2). The remaining *aceA* and *aceB* sequences were determined by using synthetic oligonucleotide primers. Sequences were analyzed using GCG (University of Wisconsin).

Partial Purification of Malate Synthase.

Malate synthase was partially purified from a *C. glutamicum* strain carrying plasmid pSL08 grown to the stationary phase in 1 liter of MB containing acetate. Cells were harvested at 6,000 *g* for 10 min. and the resulting pellet (approximately 6 g) was washed once with buffer A (50 mM Tris-HCl, 20 mM KCl, and 5% glycerol) and resuspended in 15 ml of the same buffer containing 33 mM $MgCl_2$. The cells were disrupted by sonication, and the lysate was centrifuged at 40,000 *g* for 30 min. at 4°C. The supematant was loaded onto a Q-Sepharose column (Biorad, 3 by 10 cm) which had been equilibrated with buffer A. Proteins were eluted by a linear gradient of KCl (0.02 to 0.5 M) at a flow rate of 2 ml/min. The fractions containing malate synthase activity were pooled and subjected to 60-90% ammonium sulfate fractionation. The pellet was resuspended in 2 ml buffer A and loaded onto a gel filtration column (Pharmacia; Sephacryl S200-HR, 2.5 by 85 cm). The flow rate was 0.25 ml/min. and 10 ml fractions were collected. Fractions containing malate synthase activity were pooled and concentrated by ultrafiltration. Throughout the purification, samples were monitored by SDS-PAGE. The protein was approximately 80% pure as estimated by SDS-PAGE (data not shown).

N-terminal Sequence Determination.

Approximately 5 μg of partially purified malate synthase was run on SDS-PAGE and transferred onto a PVDF membrane (Millipore Corp.). The protein band corresponding to malate synthase was subjected to protein sequencing by Edman degradation.

Enzyme Assays.

Crude extracts were prepared as described (16). Malate synthase (12), isocitrate lyase (12), and isocitrate dehydrogenase (11) were assayed as described.

Nucleotide Sequence Accession Number. The sequence reported has been assigned the GenBank accession number L27123.

RESULTS

Cloning of *AceB*.

A *C. glutamicum* ASO19 genomic library constructed in *Corynebacteriun-E. coli* shuttle vector pMT1 was screened for the complementation of an *E. coli aceB* mutant. Several positive clones which allowed the *E. coli aceB* mutant to grow on acetate as the sole carbon source were found and analyzed further. Approximately one positive clone was found per every 2,000 recombinants screened. Among the positive clones, plasmid pSL08 (Fig. 2) carried the smallest insert DNA which was 4.3 kb in size and the plasmid was analyzed further. Plasmid pSL05 (Fig. 2) carried a 5.3 kb-insert DNA.

Expression of Malate Synthase.

The ability of the plasmids pSL05 and pSL08 to express malate synthase was tested by enzymatic assays. Crude

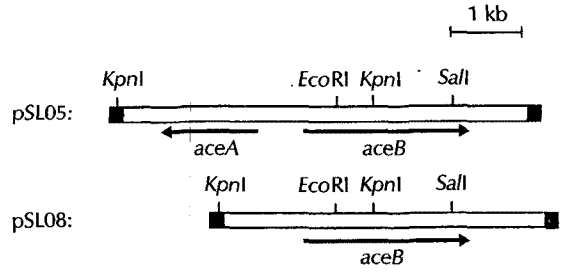

Fig. 2. Restriction map of the inserts in plasmid pSL05 and pSL08.
Malate synthase and isocitrate lyase coding regions which were identified by DNA-sequence analysis are indicated. Vector pMT1 (closed box) is not in scale.

Table 2. Expression of isocitrate lyase and malate synthase[a]

Strains	Genotypes or phenotypes	Plasmids	Specific activity[b], μmol min^{-1} mg^{-1}			Growth on acetate[c]
			MS	ICL	ICDH	
C. glutamicum ASO19E12	Wild type					
		pMT1	0.54	0.27	0.55	ND[d]
		pSL05	2.0	1.0	0.36	ND[d]
		pSL08	4.3	0.05	0.34	ND[d]
E. coli CGSC5236	*aceB*, *glc*					
		pMT1	0	0.32	0.80	−
		pSL05	0.55	0.14	0.63	+
		pSL08	0.35	0.26	0.65	+

[a] MS, malate synthase; ICL, isocitrate lyase; IDCH, isocitrate dehydrogenase, [b] The enzymes were induced by growth to the stationary phase on MB broth containing 2% sodium acetate, [c] Growth was tested on M9 minimal plate containing acetate as the carbon source, [d] ND, not determined.

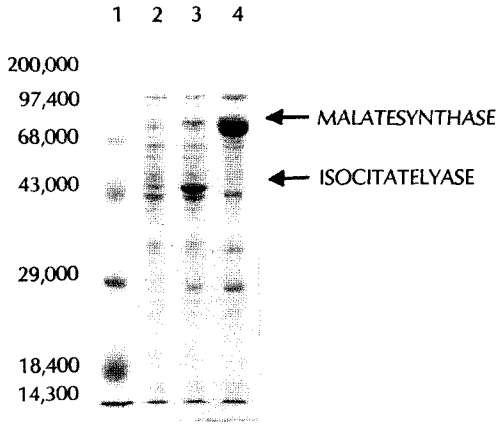

Fig. 3. Expression of malate synthase and isocitrate lyase from plasmids pSL05 and pSL08.
Crude extracts were prepared from cells grown on MB media containing acetate. Proteins were separated on 12% SDS-PAGE. Lanes: 1, Molecular weight markers; 2, C. *glutamicum* ASO19E12 (pMT1); 3, C. *glutamicum* ASO19E12 (pSL05); 4, C. *glutamicum* ASO19E12 (pSL08).

extracts were prepared from *E. coli aceB* mutant cells harboring plasmid pSL08 (or pSL05) and they were assayed. As shown in Table 2, plasmids pSL05 and pSL08 restored the malate synthase activity in *E. coli aceB* strain and allowed the strain to grow using acetate as the sole carbon source. The activities of isocitrate lyase and isocitrate dehydrogenase decreased upto 56%. Introduction of plasmid pSL05 into C. *glutamicum* ASO19E12 increased malate synthase and isocitrate lyase activity approximately 4-folds (Table 2). Introduction of plasmid pSL08 into C. *glutamicum* ASO19E12 increased the malate synthase activity approximately 8-folds (Table 2). However, presence of the plasmid appeared to suppress the isocitrate lyase activity in C. *glutamicum*. The isocitrate dehydrogenase activities decreased upto 38%.

SDS-PAGE analysis of the crude extract obtained from C. *glutamicum* ASO19E12 harboring plasmid pSL08 revealed a highly expressed protein band with approximate *Mr* of 83,000 (Fig. 3, lane 4). The protein showed the malate synthase activity (data not shown). Introduction of plasmid pSL05 into C. *glutamicum* ASO19E12 resulted in the expression of additional polypeptide with approximate *Mr* of 48,000 (Fig. 3, lane 3), which showed the isocitrate lyase activity (R. Williams, unpublished data). The intensity of the protein bands was almost proportional to the activities observed in Table 2. To locate the approximate region of *aceB* and verify the identity of the cloned DNA, plasmid pSL12, a deletion derivative of plasmid pSL08 (see Materials and Methods for the construction of pSL12), was constructed and tested for the expression of malate synthase in *E. coli* and C. *glutamicum* by enzymatic assays. No activity was observed in both organisms (data not shown).

Sequence of *AceB.* and Analysis of Encoded Protein product.

Sequence analysis of plasmid pSL05 revealed two major open reading frames which were separated by 598 bps and transcribed in divergent directions (Fig. 2). One of the open reading frames was identical to the recently published *aceA* sequences (28). The 2,217 nucleotide-long open reading frame which appeared as *aceB* (see below) was found in the central region of the insert in plasmid pSL08 (Fig. 2). The complete nucleotide sequences of *aceB* and flanking regions are shown in Fig. 4. The N-terminal 10 amino-acid sequences, MTEQEL-LSAQ, determined from the partially purified protein were used to identify the correct initiation site for the *aceB* gene. A potential ribosome binding site (31) of AGGAG was located 7 bp upstream from the translation initiation codon ATG. Like other known *Corynebacterium* genes, TAA was identified as the stop codon. The G+C content of *aceB* was 56%, which is similar to those of other genes from C. *glutamicum* species (7, 20, 25, 34). The codon preferences were very similar to the previously reported that of C. *glutamicum* genes (7, 25, 34).

The open reading frame encoded a polypeptide of the molecular weight 82,311 comprising 739 amino acids. This is in good agreement with the observed *Mr* value of 83,000 (see Fig. 3). The predicted isoelectric point of the mature peptide was 4.86. The encoded sequence contained an appreciable content of charged amino acids (33%) which were fairly evenly distributed throughout the sequence (data not shown).

Sequence Comparison with Other Proteins.

The translated amino-acid sequence of *aceB* was compared with the protein data base by using the BLAST software which uses the algorism developed by Altschul et al (1). Although the malate synthases of other organisms, such as *E. coli* (4) and *Saccharomyces cerevisiae* (8, 13), showed the highest similarity scores, the extent of the similarity was low. Fig. 5 shows the comparison between malate synthases of C. *glutamicum* and *E. coli*. The overall similarity between the compared region was 47% with 22% identity. Approximately the first 150 N-terminal amino-acid residues of C. *glutamicum* malate synthase appeared to be unique to the organism and showed no apparent similarity with the counterparts in other organisms (data not shown).

As shown in Fig. 6, a stretch of amino acids in the N-terminal region of C. *glutamicum* malate synthase showed the pattern of conserved residues anticipated for calcium-binding domains of EF-hand structure which is widely found among eukaryotes. The predicted secondary structure of the region suggested helix-loop-helix (EF-hand), which is typical of structures found in calcium-binding and calcium-modulated proteins (19).

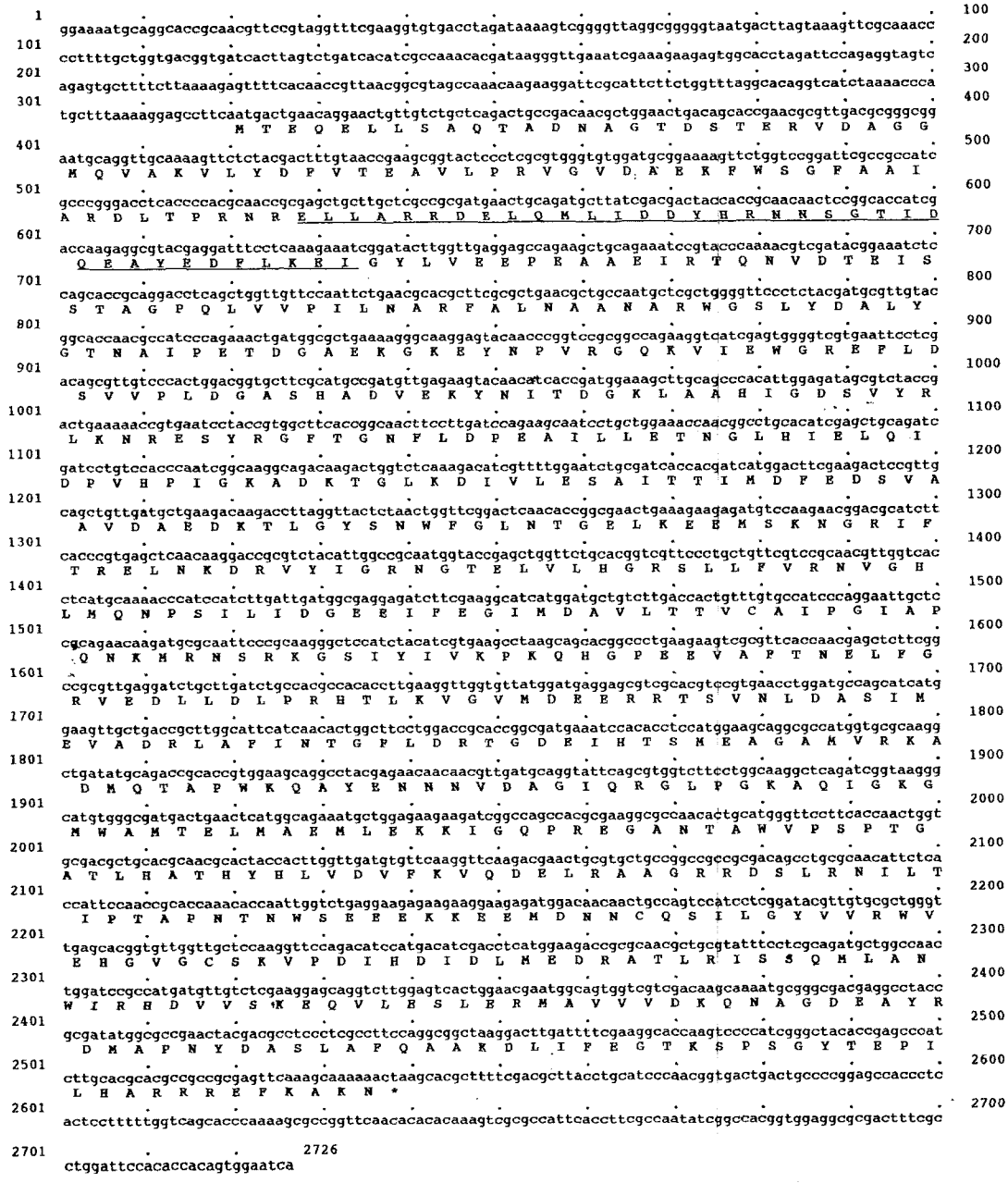

Fig. 4. Nucleotide sequence and deduced amino acid sequence of the *aceB* gene from *C. glutamicum*.
Potential calcium-binding domain of helix-loop-helix structure is underlined.

DISCUSSION

The glyoxylate pathway of *C. glutamicum* consists of malate synthase and isocitrate lyase. The experiments described here were designed to answer questions about the physiological role of glyoxylate bypass in amino-acid production and the mechanisms which control the expression of the glyoxylate bypass genes. As an initial step towards that goal, the *aceB* gene of *C. glutamicum*, which encodes the second enzyme of the bypass, was cloned and characterized.

The identity of the 4.3-kb insert in plasmid pSL08 as *aceB* clone was shown by 1) complementation of an *E. coli aceB* strain, 2) expression of malate synthase activity in *E. coli*, 3) expression of malate synthase activity in *C. glutamicum*, 4) expression of malate synthase in *C. glutamicum* as demonstrated by SDS-PAGE, and 5) studying sequence similarity with other malate synthases.

Interestingly, the expression of malate synthase from cloned *aceB* (plasmid pSL08) in *C. glutamicum* resulted

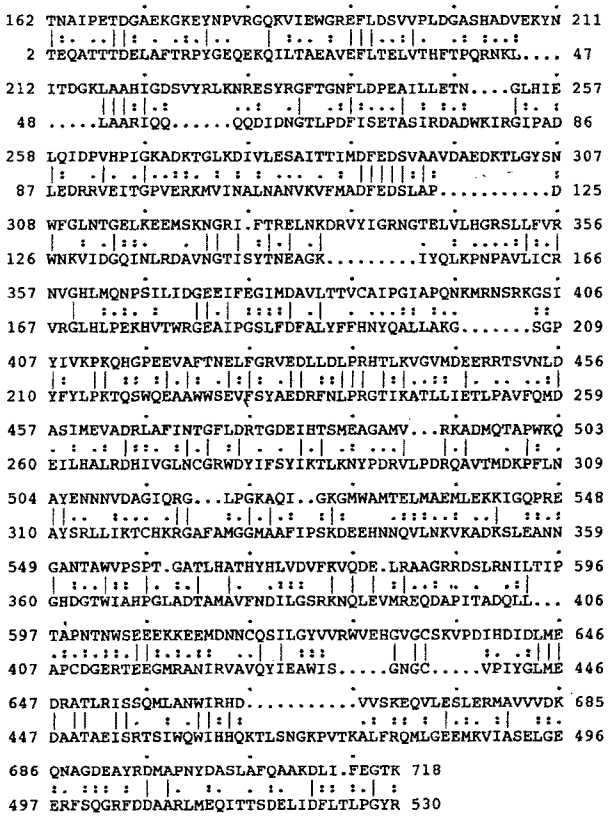

Fig. 5. Comparison of the deduced amino acid sequence of *aceB* to that of *aceB* of *E. coli.*

The sequences were aligned to yield maximum homology with respect to identical (vertical line) and functionally similar (double and single dots) amino acids. Sequences for *E. coli aceB* (4) are from published data.

in the suppression of isocitrate lyase activity (Table.2).

This may suggest the involvement of one or more common regulatory proteins which influence the expression of malate synthase and isocitrate lyase. Presence of multiple copies of the promoter region may titrate this regulatory factor(s), resulting in the overexpression of malate synthase, while decreasing the expression of isocitrate lyase. This is further supported by the results obtained with plasmid pSL05 (Table 2). Plasmis pSL05 which appeared to carry both *aceA* and *AceB* did not show any suppression of isocitrate lyase activity. Reduction in the expression of isocitrate lyase by plasmid pSL08 was not evident when the cloned gene was expressed in *E. coli.* Involvement of the regulatory elements in the expression of glyoxylate bypass enzymes in *E. coli* has been reported (18, 21, 33). Even though the cloned DNA expressed malate synthase in the *E. coli,* the level of expression was low (data not shown, as judged by SDS-PAGE) compared to that of *C. glutamicum,* indicating that the Corynebacterial *aceB* promoter may not be recognized efficiently in *E. coli.*

The molecular mass of the deduced protein product

	n nn n X Y ZG I-X -Z n nn n
Caltractin (C)	**EIKKMISEI DKDGSGTIDFEE FLTMMTAKM**
Caltractin (Sd)	**EIKKMIADI DKDGSGTIDFEE FLQMMTAKM**
TroponinC	**ELDAIIEEV DEDGSGTIDFEE FLVMMVRQM**
Calbindin-32	**DIEKVFSLY DRDNSGTIENEE LKGFLKDLL**
Malate synthase	**ELQMLIDDY HRNNSGTIDQEA YEDFLKEIG**

Fig. 6. Comparison of the putative calcium-binding domain of C. *glutamicum* malate synthase with calcium-binding domains of *Chlamydomonas* (C) caltractin (14), frog skeletal muscle troponin C (32), and *Drosophila melanogaster* calbindin (27), and *Scherffelia dubia* (Sd) caltractin (reference not available).

A typical structure of the EF-hand (19) consists of approximately 30 amino acids where two perpendicularly oriented α-helixes flank a 12-residue calcium-binding loop. Each α-helix consists of uniformly-spaced core hydrophobic residues (labeled n). The central calcium-binding loop consists of oxygen-containing side chains (labeled *X, Y, Z, -X,* and *-Z*) which acts as the ligands for bound Ca^{++}. The glycine (labeled G) which is believed to form a sharp bend in the loop and isoleucine (labeled *I*) are highly conserved among various calcium-binding domains.

obtained from the sequence data agreed very well with molecular mass of the partially purified malate synthase. The codon usage and relatively high G+C content at the degenerate codon position, which is typical for *Corynebacterium* spp., confirmed the correctness of the reading frame.

The malate synthase of C. *glutamicum* appears to be distinct from its counterparts in other organisms including the one from *E. coli.* Although the *aceB* and *aceA* of *E. coli* are organized into an operon, *aceBAK* (5, 6, 21), no such structure was found in the C. *glutamicum aceB* region. Apparently, in C. *glutamicum,* the *aceA* and *aceB* genes are transcribed divergently. The functional and evolutionary importance of the organization requires further investigation. The molecular weight of malate synthase of C. *glutamicum* (82,311) appears to be exceptionally large when compared with those of other malate synthases, which are close to 60,000. The deduced amino-acid sequence of C. *glutamicum* malate synthase showed only limited sequence similarity with those of *E. coli.* and *S. cerevisiae.* Approximately 150 amino acids located at the N-terminal region of the C. *glutamicum* malate synthase appeared to be unique to this organism, showing no apparent sequence similarity with other malate synthases. This region appears to be responsible for the protein's unusual size.

The significance of the calcium-binding domain like structure of EF-hand family found in the N-terminal region of the protein remains speculative. Even though regions of high homology were detected, the functional, topological, and evolutionary significance of the sequences need further investigation.

Acknowledgments

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