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Leucine Synthesis in *Corynebacterium glutamicum*: Enzyme Activities, Structure of *leuA*, and Effect of *leuA* Inactivation on Lysine Synthesis

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Enzymes and genes of the isopropylmalate pathway leading to leucine in *Corynebacterium glutamicum* were studied, and assays were performed to unravel their connection to lysine oversynthesis. The first enzyme of the pathway is inhibited by leucine (K_i = 0.4 mM), and all three enzyme activities of the isopropylmalate pathway are reduced upon addition of this amino acid to the growth medium. Three different DNA fragments were cloned, each resulting in an oversynthesis of one of the three enzymes. The *leuA* complementing fragment encoding the isopropylmalate synthase was sequenced. The *leuA* gene is 1,848 bp in size, encoding a polypeptide with an M_r of 68,187. Upstream of *leuA* there is extensive hyphenated dyad symmetry and a putative leader peptide, which are features characteristic of attenuation control. In addition to *leuA*, the sequenced fragment contains an open reading frame with high coding probability whose disruption did not result in a detectable phenotype. Furthermore, the sequence revealed that this open reading frame separates *leuA* from *lysC*, which encodes the asparate kinase initiating the synthesis of all amino acids of the aspartate family. The *leuA* gene was inactivated in three lysine-secreting strains by insertional mutagenesis. Fermentations were performed, and a roughly 50% higher lysine yield was obtained when appropriate leucine concentrations limiting for growth of the constructed strains were used.

Some *Corynebacterium glutamicum* strains classically bred for lysine production are leucine auxotrophs (21, 27), and it is suggested that in these strains, mutations in leucine biosynthetic genes are linked to high lysine productivity. The few mutants studied so far, however, were all obtained by nonspecific *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Moreover, it is not clear which Leu^- mutation is present in the lysine producers. Only in a single case in a recent analysis of a hyperproducer was it possible to attribute the Leu^- mutation to the isopropylmalate (IPM) dehydratase (27). Leucine is synthesized by the IPM pathway (Fig. 1). The first enzyme of the sequence is the IPM synthase encoded by *leuA*. This enzyme is feedback controlled in its activity by leucine in *Salmonella typhimurium* (31) and *Saccharomyces cerevisiae* (1). In addition, expression of *leuA* is regulated in the members of the family *Enterobacteriacae* by transcription attenuation in response to the level of charged $tRNA^{Leu}$. The two further enzymes unique to leucine synthesis are IPM dehydratase, encoded by *leuC* and *leuD* in *Escherichia coli*, and IPM dehydrogenase, encoded by *leuB*.

Because of the apparent lack of information on the possible connection of Leu^- mutations and lysine formation, it is necessary to apply molecular techniques to permit a defined analysis. Such studies, together with biochemical studies, have shown unexpected characteristics of lysine synthesis in *C. glutamicum*, namely, its control by *lysC* (14, 27), the importance of *dapA* (8), a split pathway (28), and the existence of a specific export system for lysine (2). Here we present an analysis of the regulation of the leucine biosynthetic enzymes in this industrially important bacterium. We investigated *leuA* in detail to permit its directed mutagenesis and therefore to prevent any possible accumulation of intermediates of the IPM pathway which can occur in the case of mutation of other leucine biosynthetic genes. We finally used the constructed *leuA* strains to study the consequences of leucine limitation on lysine accumulation.

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MATERIALS AND METHODS

Strains, plasmids, and growth. All bacterial strains and plasmids used are listed in Table 1. The minimal medium used for *C. glutamicum* was CGXII (15). The medium used for *E. coli* consisted of (per liter) 7 g of KH_2PO_4, 3 g of K_2HPO_4, 1 g of $(NH_4)_2SO_4$, 10 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 5 mg of $MnSO_4 \cdot 4H_2O$, 5 mg of $ZnSO_4 \cdot H_2O$, 0.1 mg of $CuSO_4 \cdot 5H_2O$, 15 g of agar-agar (pH 6.5), 10 mM $MgSO_4$, 0.05 mg of thiamine, and 0.55 g of glucose · H_2O. When appropriate, kanamycin (25 μg/ml) or ampicillin (50 μg/ml) was added.

Genetic engineering. DNA was isolated from *C. glutamicum* by a modified alkaline extraction procedure with lysozyme (28, 29). In vitro procedures, as well as the analysis of plasmid and chromosomal DNA, were carried out by standard procedures (24). Plasmids were introduced into *C. glutamicum* via electroporation (17) or by conjugation with plasmid-carrying *E. coli* cells (26). This latter method was used when nonreplicative plasmids with homologous sequences were integrated in the chromosome. Transconjugants were selected on LB containing 2% glucose, 25 μg of kanamycin per ml, and 50 μg of nalidixic acid per milliliter.

DNA sequencing and analysis. The *Xba*I-*Xho*I fragment of pKK7 (see Fig. 2) was cloned into the *Bam*HI site of pUC18 in both orientations, and two sets of deletion clones were prepared by exonuclease III treatment (Promega, Madison,

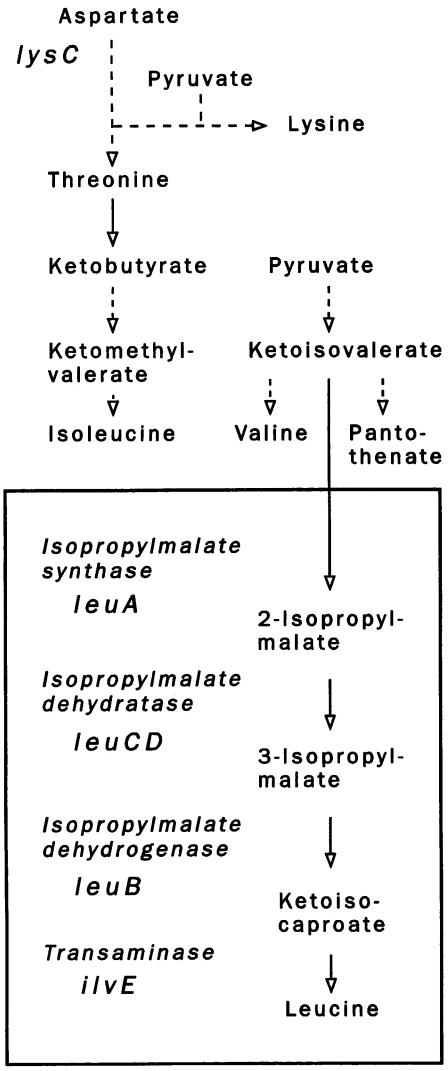

FIG. 1. Simplified flow scheme of the aspartate family of amino acids with selected genes and detailed flow scheme (boxed) of the IPM pathway of leucine synthesis. The gene symbols are in accord with the *E. coli* nomenclature.

Wis.). DNA sequence determination was performed by the dideoxy-chain termination method (25) with Sequenase, version 2.0, from U.S. Biochemicals, Cleveland, Ohio. Sequence data were compiled and analyzed by using the HUSAR program package, release 2.0 (EMBL, Heidelberg, Germany). Multiple alignments were carried out by using the algorithm of Myers and Miller (20).

Biochemical methods. Preparation of cell extracts and determination of enzyme activities were done as described previously (27). The molecular weight of the IPM synthase was determined as described previously (19) by gel filtration of extracts of *C. glutamicum* pKK73 grown on minimal medium CGXII.

The lysine concentration was determined fluorometrically after precolumn derivatization with *o*-phthaldialdehyde and separation on a reversed-phase column by high-pressure liquid chromatography (HP 1090A; Hewlett-Packard, Avondale, Pa.).

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence of *C. glutamicum leuA* (see Fig. 3) is X70959.

RESULTS

Regulation of enzyme synthesis and activity. The activities of IPM synthase, IPM dehydratase, and IPM dehydrogenase in crude extracts of *C. glutamicum* ATCC 13032 (wild type) were determined. The effect of addition of leucine to minimal medium was to reduce the specific activity of the enzymes to about half of the value determined in cells grown without leucine (Table 2). This effect is comparable to that found for the enzymes of *S. typhimurium* (3, 4). An even more reduced activity of IPM synthase and IPM dehydrogenase was found in cells grown in complex medium. The leucine-auxotrophic (Leu^-) strain *C. glutamicum* ATCC 14310, which was also characterized, was devoid of IPM synthase activity (Table 2).

For the IPM synthase of *C. glutamicum* we determined a molecular mass of 160,000 ± 20,000 Da by gel filtration (data not shown). From this value, together with the sequence information on the respective gene (data see below), we concluded that the native IPM synthase is a dimer. This was also shown for the enzyme of *S. cerevisiae* (1). The enzyme is strongly inhibited by leucine (apparent K_i = 0.4 mM; data not shown), providing *C. glutamicum* with a strict feedback control of metabolite flux through the IPM pathway.

Cloning of *leu* fragments. Recombinant plasmids carrying *C. glutamicum* DNA (6) were screened to make *E. coli leu* mutants Leu^+. From a *leuB*-complementing cosmid we derived pBS152 (Fig. 2). In *C. glutamicum* this yielded a specific IPM dehydrogenase activity of 0.28 μmol/min/mg of protein (26 times more than the wild-type level), thus confirming that the *leuB* genes of *E. coli* and *C. glutamicum* are functionally equivalent.

From a *leuD*-complementing cosmid, we obtained pBS10 and pBS13 (Fig. 2). Plasmid pBS10 complemented both *leuD* and *leuC*, whereas the subclone pBS13 complemented only the *leuC* mutation of *E. coli*. Both plasmids complemented the IPM dehydratase mutation of *C. glutamicum* MH20-22B (27) as well and consistently yielded two- to threefold higher IPM dehydratase activities in *C. glutamicum* ATCC 13032 than the plasmid-free strain.

Plasmid pKK7, containing a *Sau*3A-generated fragment of chromosomal DNA in pBR322, complemented the *leuA* mutation of *E. coli* (Fig. 2). No further *leu* mutation could be complemented. A 7.4-kb fragment of pKK7 was subcloned in shuttle vector pJC1 (6), and the resulting plasmid, pKK71, was introduced into *C. glutamicum*. In the recombinant strain a high specific IPM synthase activity was determined (about eightfold higher than wild-type levels), whereas no increase in the activity of IPM dehydratase or IPM dehydrogenase was detected. Complementation mapping indicated that *leuA* is located on the 3.5-kb internal *Xho*I-*Xba*I fragment cloned in pKK76 (Fig. 2). The integrity of this fragment was confirmed by Southern hybridization (results not shown).

Sequence analysis. The entire nucleotide sequence of both strands of the *Xho*I-*Xba*I fragment (3,492 bp) containing *leuA* was determined. Using a computer-assisted analysis we found a large open reading frame (ORF) extending from nucleotide (nt) 1524 to nt 3371 (Fig. 3). Since this ORF spans the DNA region functionally proven to result in IPM synthase synthesis, it was designated *leuA*. The translational start was assigned to nt 1524 (instead of nt 1630), since a deletion analysis (data not shown) revealed that the clones

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5	F⁻ *supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	12
CV512	F⁺ *leuA371*	30
CV514	F⁺ *leuB501*	30
CV522	F⁺ *leuC222*	30
CV526	F⁺ *leuD101*	30
C. glutamicum		
ATCC 13032	Type strain	Culture collection
DG 52-5	ATCC 13032 *lysC3*(Fbr)	18
MH20	ATCC 13032 *lysC2*(Fbr)	27
ATCC 14310	ATCC 13032 Leu⁻	Culture collection
MH20-22B	MH20 Leu⁻	27
ATCC 21127	*C. glutamicum* subsp. *flavum*, Hom⁻	Culture collection
Plasmids		
pUC18	Cloning vector; Ap^r	33
pEM1	Mobilizable cloning vector; Km^r, oriT$_{RP4}$	28
pZ1	*E. coli-C. glutamicum* shuttle vector; Km^r	18
pJC1	*E. coli-C. glutamicum* shuttle vector; Km^r	6
pEKEx2	*E. coli-C. glutamicum* expression vector; Km^r *tacP lacI*^{q}	10
pBS10	pJC1::[*leuCD*, 16-kb insert of chromosomal DNA of *C. glutamicum*]	This work
pBS13	pJC1::[*leuC*, 4.6-kb *Sal*I subclone of pBS10]	This work
pBS152	pEKEx2::[*leuB*, 3.2-kb *Sma*I-*Sal*I fragment of chromosomal DNA of *C. glutamicum* blunted with Klenow enzyme]	This work
pKK7	pBR322::[*leuA*, 9.4-kb insert of chromosomal DNA of *C. glutamicum*]	This work
pKK71	pJC1::[*leuA*, 7.5-kb *Sau*3A subclone of pKK7]	This work
pKK75	pJC1::[2.8-kb *Bam*HI subclone of pKK7]	This work
pKK76	pZ1::[3.4-kb *Xho*I-*Xba*I subclone of pKK7]	This work
pKK73	pJC1::[3.0-kb *Sal*I subclone of pKK71]	This work
pKK73d	pJC1::[*Pvu*I deletion derivative of pKK73]	This work

with successive removal of the 5′-flanking region of *leuA* as far as nt 1450 still carried the functional *leuA* gene, whereas the clone in which the deletion extended to nt 1556 did not. In addition, a Shine-Dalgarno structure is apparent (5′-TAAGAAGGT-3′), appropriately spaced 8 nt upstream of the start codon. Twenty-nine nucleotides downstream of the stop codon of the gene is an inverted repeat (Fig. 3), whose transcript would form a stem-loop structure ($\Delta G = -30$ kcal/mol [−125.5 kJ/mol]) likely to represent a rho-independent transcription terminator. The structural features in the 5′-flanking region of *leuA* point to its transcriptional control by an attenuation mechanism (see Discussion).

Further upstream of *leuA* an ORF (ORFX) extending from nt 440 to 1270 was detected. Its deduced polypeptide has characteristics typical of a membrane protein with six transmembrane α-helices, flanked on either side by charged or helix-breaking amino acids. However, upon inactivation of this putative gene by insertional mutagenesis, no altered phenotype was detected (data not shown). Therefore, no functional assignment can be given.

Most interestingly, on the opposite strand in front of ORFX we identified the start of an incomplete ORF identical to the 5′ end of the *lysC* gene of *C. glutamicum*, which encodes the aspartate kinase (14). This implies that *lysC*, which is of the upmost importance for the flux control of lysine synthesis (7), is located in close proximity to *leuA*. Both genes are separated by ORFX and are transcribed in opposite directions.

Disruption of *leuA*. The described molecular characterization of *leuA* was used for inactivation of this gene in the three lysine-secreting strains of *C. glutamicum*, namely, strain MH20 (27), strain DG52-5 (18), and strain ATCC 21127. For this purpose gene disruption via integration of a vector carrying an internal *leuA* fragment was used (29). The basis

TABLE 2. Activities of leucine biosynthetic enzymes in *C. glutamicum*

Strain	Growth medium	Sp act[a] (μmol/min/mg of protein) of:		
		IPM synthase	IPM dehydratase	IPM dehydrogenase
ATCC 13032	Minimal	0.140 ± 0.02	0.013 ± 0.001	0.011 ± 0.001
ATCC 13032	Minimal plus Leu[b]	0.063 ± 0.015	0.006 ± 0.002	0.006 ± 0.002
ATCC 13032	Complex	0.014 ± 0.01	0.005 ± 0.002	≤0.001
ATCC 14310	Minimal[c]	≤0.001	0.008	0.012
MH20-22B[d]	Minimal[c]	0.11	≤0.001	0.015

[a] Values for strain 13032 are the means of at least three measurements of different extracts.
[b] Leucine concentration was 20 mM.
[c] Leucine concentration was 4 mM.
[d] The data for this strain are from reference 27 and are included for comparison.

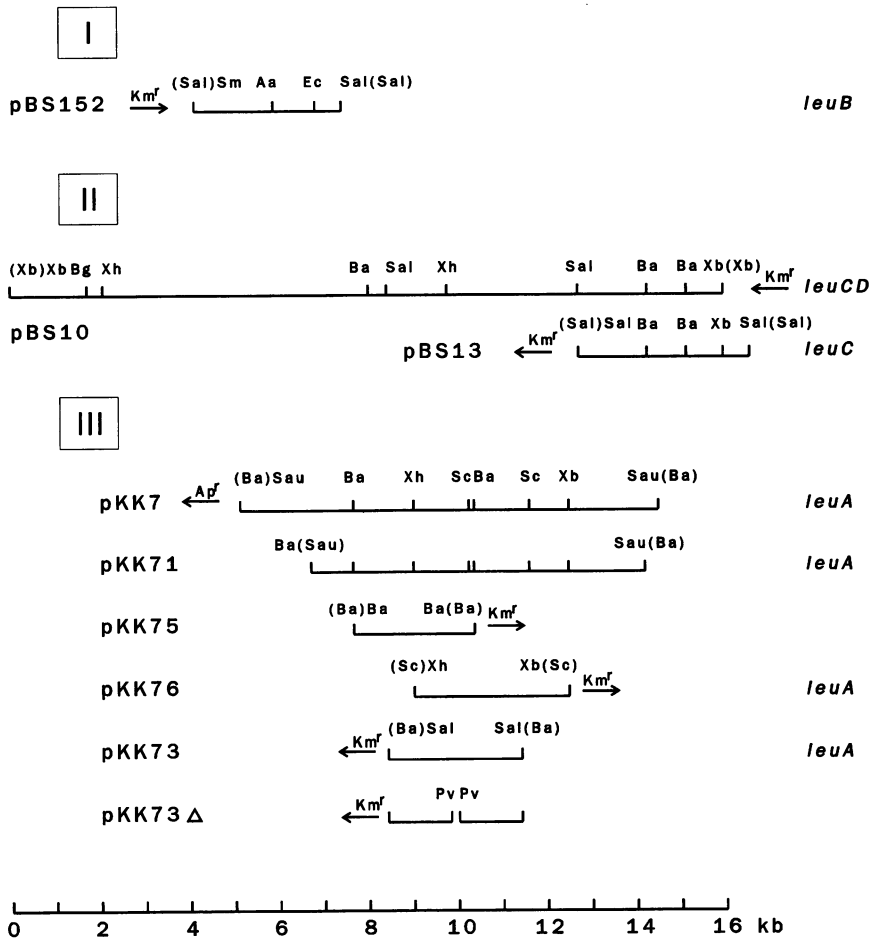

FIG. 2. Restriction maps of *leu*-complementing fragments, and deletion mapping of *leu* genes of *C. glutamicum*. (I) Cosmid-derived fragment complementing *leuB*. (II) Cosmid-derived fragments complementing *leuC* or *leuD*. (III) Fragment complementing *leuA* and its deletion analysis. *leuA*, *leuB*, *leuC*, or *leuD* to the right indicates that the subclone complemented the corresponding mutation in *E. coli*. Restriction sites given in parentheses are those of the vector used for cloning of the corresponding fragment. The arrow gives the orientation of the insert in the vector. Abbreviations of restriction enzyme sites: Aa, *Aat*I; Ba, *Bam*HI; Bg, *Bgl*I; Ec, *Eco*RI; Pv, *Pvu*II; Sal, *Sal*I; Sau, *Sau*3A; Sc, *Sca*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I.

vector was pEM1 (Km^r), which is nonreplicative in *C. glutamicum* but can be transferred by conjugation from *E. coli* to *C. glutamicum* (26). This vector was digested with *Bam*HI and *Pst*I and ligated with the 647-bp *Bam*HI-*Pst*I fragment (Fig. 3) of the *leuA* gene. The resulting plasmid, pKK74I2, was introduced into the donor strain, *E. coli* S17-1 (26), and mating with the *C. glutamicum* strains was performed. Km^r transconjugants were obtained, and several randomly chosen clones proved to be plasmid free and auxotrophic for leucine, confirming that pKK74I2 was integrated in the *leuA* locus. Strains MH20::pKK74I2, DG52-5::pKK74I2, and ATCC 21127::pKK74I2 were selected. They proved to lack IPM synthase activity and were chosen for further studies.

In addition, a second integrative plasmid (pKK74I1) carrying a different internal *leu* fragment (*Pst*I-*Bam*HI 190-bp fragment [Fig. 3]) was constructed. Again, transconjugants were obtained, characterized as IPM synthase negative, and included in the physiological studies.

Effect of *leuA* inactivation on lysine formation. In initial experiments the constructed strains were grown with various leucine concentrations, and the amount of accumulated lysine was determined. It was readily apparent that the large amounts of leucine supplied were detrimental for lysine accumulation. Therefore, strain DG52-5::pKK74I2 was grown on minimal medium CGXII and both growth and lysine formation were observed over time. As can be seen from Fig. 4, lysine accumulation decreased with increasing leucine concentrations. The largest amount of lysine was obtained in the presence of 1.15 mM leucine. At this concentration, however, growth is severely limited. Therefore, the growth limitation exerted by the availability of leucine is of major importance for lysine accumulation.

All six *leuA* strains constructed, together with their ancestor strains, were assayed for finally accumulated lysine at one distinct leucine concentration (Table 3). They all displayed the positive effect of the *leuA* mutation on lysine formation, showing the general suitability of this mutation. Strain ATCC 21127 required a higher leucine concentration to reach high lysine accumulation, probably because this strain is already homoserine auxotroph. This particular feature of strain ATCC 21127 also enabled us to assay whether varying the homoserine concentrations influences lysine formation as well (Table 3). As can be seen, lysine

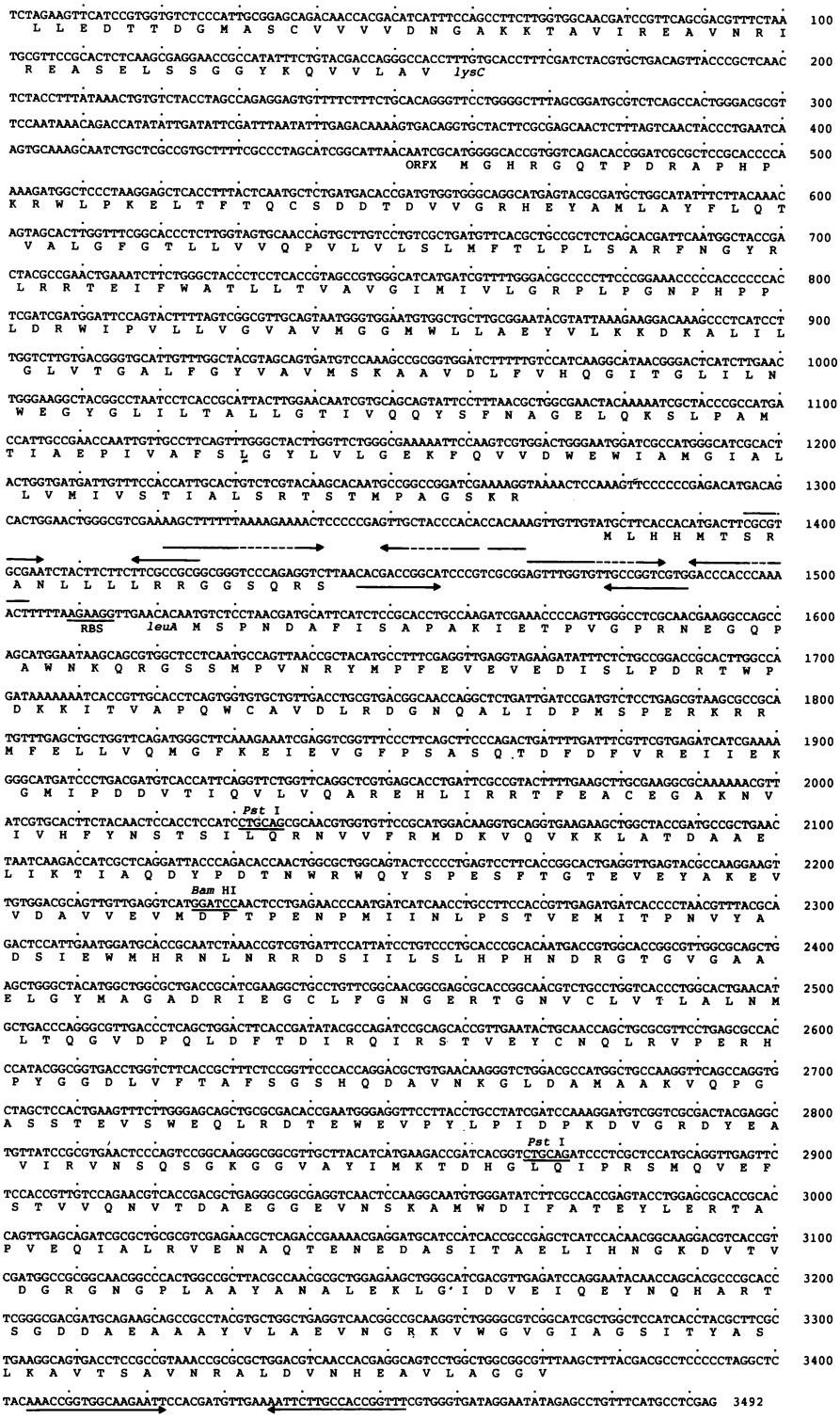

FIG. 3. Nucleotide sequence and selected restriction sites of *C. glutamicum leuA* and flanking regions. Numbers at the right refer to nucleotides. The deduced amino acid sequence of *leuA*, the putative leader peptide, part of *lysC*, and the putative membrane protein are given above the sequence. Four imperfect inverted repeats potentially involved in secondary structures of attenuator are indicated by arrows. Putative transcription terminator and ribosome-binding site (RBS) are also shown (underlined). The GenBank accession number of the nucleotide sequence shown is X70959.

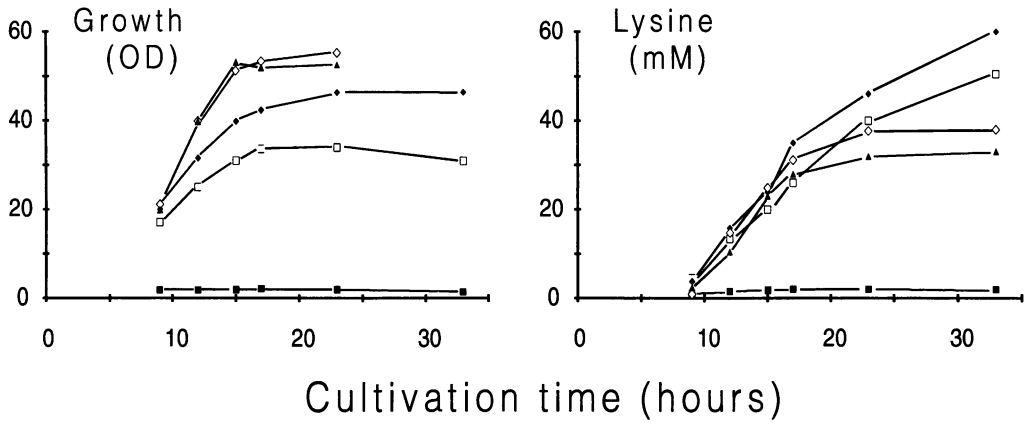

FIG. 4. Growth (OD, optical density) and lysine formation with strain DG52-5::pKK74I2 *leuA* supplied with no L-leucine (■) or 1.15 mM (□), 1.5 mM (◆), 3.0 mM (◇), or 4.6 mM (▲) L-leucine.

formation was also increased by limiting homoserine concentrations. It is thus evident that in addition to leucine limitation, a homoserine limitation is effective for increasing lysine accumulation.

DISCUSSION

As shown, the IPM synthase of *C. glutamicum* is highly similar to that of *S. cerevisiae* (44% identical amino acids), but it has only 22% identical amino acids to the *S. typhimurium* (22) or *Lactococcus lactis* (11) protein. The proteins of the last two organisms have high homology to each other (39%). Despite these differences in homology and size, conserved regions of all four polypeptides now known are distributed over their entire length (Fig. 5). A remarkable feature within all four sequences is a block of about 40 amino acids (amino acids 278 to 319 of the *C. glutamicum* sequence). This block contains three of the four conserved histidine residues, which could be important for the mechanism of the enzyme reaction. It also contains six conserved glycines, which are important for structural reasons because of the extreme dihedral angle allowed by the glycine residues. Therefore, this highly conserved region might well be the reaction center of IPM synthase.

TABLE 3. Lysine accumulation by *C. glutamicum* strains with inactivated IPM synthase, *leuA*

Strain[a]	Phenotype	Concn of amino acid supplied (mM)	Amt of lysine accumulated (mM)
MH20		Leu, 1.5	40
MH20::pKK74I1	Leu$^-$	Leu, 1.5	57
MH20::pKK74I2	Leu$^-$	Leu, 1.5	59
DG52-5		Leu, 1.5	36
DG52-5::pKK74I1	Leu$^-$	Leu, 1.5	55
DG52-5::pKK74I2	Leu$^-$	Leu, 1.5	61
ATCC 21127	Hom$^-$	Hom, 6.7; Leu, 3.0	47
ATCC 21127::pKK74I1	Hom$^-$ Leu$^-$	Hom, 6.7; Leu, 3.0	59
ATCC 21127::pKK74I2	Hom$^-$ Leu$^-$	Hom, 6.7; Leu, 3.0	57
ATCC 21127	Hom$^-$	Hom, 3.4; Leu, 3.0	61
ATCC 21127	Hom$^-$	Hom, 5.0; Leu, 3.0	56
ATCC 21127	Hom$^-$	Hom, 6.7; Leu, 3.0	47

[a] Strain ATCC 21127 was additionally supplied with 0.2 g of thiamine per liter.

Knowledge of the sequence of the *leuA* gene and flanking regions enables us to propose possible mechanisms of expression control. Thus upstream of *leuA* the sequence suggests the presence of a leader peptide containing four consecutive leucine residues, which is indicative of transcription attenuation, as known for several amino acid biosynthetic operons of *E. coli* (16). Further indications of such kind of control of *leuA* expression are the regions of dyad symmetry in front of *leuA*, which partially overlap the putative leader peptide with the potential to form alternative stem-and-loop structures of the transcript (Fig. 3). However, a distinct rho-independent terminator structure, similar to *E. coli* terminators (23), is not apparent. Two further features of *leuA* are apparently optimally suited for sensitive and rapid response to regulate expression of IPM synthase in *C. glutamicum*: the low leucine content of only 6% in the protein (the lowest of all *C. glutamicum* polypeptides known) and the strong bias of the *leu* codons toward the most frequently used one, CTG (9). This would ensure that the less frequent $tRNA^{Leu}$ species are not a rate-limiting factor in the translation of *leuA*.

The inactivation of *leuA* definitely shows that influencing the leucine synthesis results in increased lysine formation when the appropriate leucine concentration is supplied. This knowledge, together with the molecular structure of *leuA*, could therefore be of advantage to further increase lysine formation with classically bred *C. glutamicum* strains. As a connection between the *leuA* mutation and increased lysine formation, two general possibilities are conceivable. Thus a direct molecular link between *leuA* and *lys* genes could exist, or the Leu$^-$ mutation (together with the special growth conditions) could result in an additional cellular supply of metabolites for lysine synthesis. Regarding a transcriptional connection, the sequence has in fact revealed a surprisingly close proximity of *leuA* to *lysC*, which is of the upmost importance for flux control toward lysine (8, 14, 27). However, since *lysC* (together with the clustered *asd* gene) is transcribed divergently from *leuA*, and *leuA* and *lysC* are separated by ORFX, we have no indication of this. This also holds for unknown genes downstream of *leuA* since disruption of *leuA* resulted in a leucine requirement only. Also, the fact that the one hyperproducer strain MH 20-22B characterized by us (27) is defective in the IPM dehydratase (*leuCD*) and not in the IPM synthase (*leuA*) is clear evidence against a connection on the transcriptional level with *leuA*

FIG. 5. Aligned polypeptide sequences of *leuA* of *C. glutamicum*, *S. typhimurium* (22), and *L. lactis* (11) and *LEU4* of *S. cerevisiae* (1). Gaps are introduced to achieve maximal homology; identical amino acids are boxed.

downstream genes, since both strains exhibit leucine-dependent lysine accumulation. Another possibility would be that the Leu$^-$ mutation results in a global effect on gene expression, like the stringent response as known for enterobacteria, where amino acid starvation results in the stimulation of the transcription of a large number of genes, including several amino acid biosynthetic operons (16). Also, a situation similar to that in Lrp-controlled systems is possible (13). However, on comparison of 20 enzymes of the hyperproducer MH 20-22B with the wild-type *C. glutamicum* (27), no significant difference in enzyme synthesis (except for IPM dehydratase) was detected. This makes a direct molecular connection between *leuA* and *lys* genes unlikely but points to a general physiological effect. The simplest idea would be that the two pyruvate molecules saved for leucine synthesis are additionally available for lysine synthesis (Fig. 1). However, the experiment with increasing leucine concentrations clearly shows that it is the growth limitation which is important, otherwise a high final lysine concentration would have accumulated at high leucine concentrations. Also, the fact that the homoserine limitation with *C. glutamicum* subsp. *flavum* influences lysine accumulation argues in favor of a positive effect of limiting growth conditions. Therefore, the probably higher availability of metabolites and also of energy might be related to higher lysine accumulation. In fact, even such nonspecific constraints on growth as a limited supply of phosphate in the medium increases lysine yield in *C. glutamicum* (5). Moreover, metabolite secretion is known for *Klebsiella pneumoniae* as a result of limitation for any nutrient component (32). Taken together, the data currently available can be best explained phenomenologically by the idea that the major effect of leucine limitation on lysine formation is a general effect due to constrained growth.

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