FEBS Letters 348 (1994) 311–316

FEBS 14244

Molecular characterization of an *Escherichia coli* mutant with a temperature-sensitive malonyl coenzyme A-acyl carrier protein transacylase

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Received 19 May 1994; revised version received 9 June 1994

Abstract

The temperature-sensitive malonyl CoA-ACP transacylase found in the *Escherichia coli* strain LA2-89, carrying the *fabD89* allele, was shown to result from the presence of an amber mutation in the *fabD* gene, at codon position 257, in combination with the *supE44* genotype of this strain. The truncated form of the protein produced as the result of the amber mutation was demonstrated to be enzymatically inactive, whereas amber suppression rendered the resulting enzyme temperature labile. Site-directed mutagenesis of codon 257 revealed a requirement for an aromatic amino acid at this position in the polypeptide chain, to assure temperature stability of the enzyme.

Key words: Escherichia coli; Fatty acid biosynthesis; Temperature-sensitive; Malonyl CoA-ACP transacylase

1. Introduction

De novo synthesis of fatty acids in *Escherichia coli* is catalysed by a type II fatty acid synthetase (FAS II), a similar system to that found in plants. Each of the eight reactions involved in this system is carried out by separate, and structurally independent proteins. This is in contrast to the so-called type I FAS, as found in yeasts and vertebrates, in which the individual reactions are catalysed by domains of a multi-enzyme complex consisting of one or two polypeptide chains [1]. Elucidation of the lipid biosynthetic pathway of *E. coli* has made an important contribution to our understanding of the synthesis and function of lipids in general. The existence of *E. coli* fatty acid biosynthesis (*fab*) mutants has greatly facilitated this work. These mutants have not only allowed the detailed dissection of the individual enzymic steps of the pathway, but also provide a powerful tool to study the complex structure–function relationships between the fatty acid synthetase components that are essential for the function of this multi-enzyme complex. In this context, thermolabile mutant proteins are of particular interest since they often involve amino acid changes which affect the correct folding of the protein [2,3], and may also affect the interaction with other components of the multi-enzyme complex.

Recently, we have cloned the *E. coli fabD* gene [4], encoding malonyl CoA-ACP transacylase (MCAT), by complementation of a temperature-sensitive fatty acid biosynthesis mutant (LA2-89) [5]. MCAT catalyses the transacylation of malonate from coenzyme A to the acyl carrier protein (ACP) and thus provides the activated acetyl units required during the repeated condensation cycles of fatty acid synthesis. In this study we have analysed the complex nature of the mutations in the *E. coli* LA2-89 strain which are responsible for the temperature-labile MCAT activity and temperature-sensitive growth phenotype of this strain. In pursuit of the solution of MCAT's three-dimensional structure, the *E. coli* MCAT was recently crystallized [6]; this could further our study of structure–function relationships of FAS II components.

2. Materials and methods

2.1. Protein extract preparation

Protein extracts were prepared as described by Verwoert et al. [4], with minor modifications. After DNase treatment, enzyme assays or gel electrophoresis were performed on the total or the soluble fraction of the lysate. The latter was obtained by fractionation of the lysate into pellet and supernatant by centrifugation in a microfuge. The pellet was resuspended by vigorous pipetting, again followed by centrifugation. The first and second supernatant were pooled and constituted the soluble fraction.

2.2. Enzyme and protein assays

Protein and MCAT were routinely assayed as described in Verwoert et al. [4].

2.3. Cloning and nucleotide sequence analysis of the fabD89 gene

In order to clone the mutant MCAT coding region, as a *NcoI–HindIII* fragment, into vector pUC19 [7], a PCR was performed on chromosomal DNA of *E. coli* strain LA2-89 [5]. Primers used previously to clone the wild-type *E. coli* MCAT gene [4] served to introduce the appropriate restriction sites. Using the restriction sites present in

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SSDI 0014-5793(94)00630-E

the *fabD89* gene (the same as can be found in the wild-type *fabD* gene, Fig. 1), DNA fragments of the *fabD89* coding region were subcloned in M13mp18 and M13mp19, and dideoxy sequencing employing an Applied Biosystems DNA Sequencer model 370A was performed as described by Verwoert et al. [4].

2.4. Construction of pIV6 mutant plasmids by site-directed mutagenesis

The collection of mutant plasmids was obtained by oligonucleotide-directed mutagenesis on pIV6 [4] plasmid DNA. The modification involved two different PCR rounds, as shown in Fig. 1. In the first PCR (Fig. 1A) the M13 Forward primer (FW) was used in combination with either primer I (5′ CGGTTCAGCAGACGAAGTCTGTTG 3′), which changes the tryptophan (W) at codon position 257 of the *fabD* coding region into a glutamine (Q), or the mixed primer II (5′ CGGTTCAG-C/T/G-C/A/G-C/T/G-ACGAAGTCTGTTG 3′), which can introduce 12 different amino acids at codon position 257. The following thermal cycle was used: denaturation at 94°C for 30 s; annealing at 55°C for 60 s; and extension at 72°C for 90 s, repeated 30 times. The PCR product was a 400 bp fragment with the change of W-to-Q at the marked position (codon position 257 of the *fabD* coding region, Fig. 1) or, when mixed primer II was used, a collection of 400 bp double-stranded (ds) DNA molecules encoding different amino acids at the marked position (Fig. 1) in both strands. In order to exchange the mutated pIV6 fragments with the wild-type pIV6 fragment, the sequence of the pMIC1960 insert (a single stranded M13mp19 derivative, containing part of the *fabD* gene, Fig. 1) was modified by a second PCR (Fig. 1B). The M13 Reverse primer (Rev) was used in combination with the complementary strand of the 400 bp PCR products. The template:primer ratio was optimal at 1:10, and the thermal cycle used was the same as in the first PCR. This PCR resulted in ds 750 bp fragments with a *Pst*I site located 5′ upstream of the mutated codon (Fig. 1). The 400 bp PCR products used as a primer in the second PCR were purified over a Qiagen column (Qiagen Inc., USA) to remove primer residues from the first PCR. The 750 bp PCR products were digested with *Pst*I and *Sac*II and ligated to pIV6 digested with the same restriction enzymes, resulting in recombinant plasmids pIV6 ($W^{257}Q$) and pIV6 ($W^{257}X$). Since the clones obtained after the first round amplification may contain a mixture of two distinct mutants, a second round of transformation was necessary. The nucleotide sequence of the region between the *Pst*I and *Sac*II sites of the resulting mutants was determined for both strands by dideoxy-nucleotide sequence analysis as described above.

2.5. Expression studies

For over-expression in *E. coli* two type of vectors were used, pET11-d [8] and pIV6 [4]. In order to clone the wild-type and the mutant MCAT coding region into expression vector pET11-d [8] as a *Nco*I–*Bam*HI fragment, a PCR was performed on chromosomal DNA of *E. coli* strain XL1Blue ($recA^-$) (Stratagene) and LA2-89 [5]. The appropriate restriction sites were introduced, as described by Kater et al. [9], using the following primers: (1) 5′ CGTCTAGAATAAGGATTAAACC-ATGGCGC 3′, which introduces an *Xba*I and an *Nco*I site at the starting ATG of the coding region [4], and (2) 5′ GGGGATCC-CGAAGCTTGCACCGG 3′, which changes the *Eco*RI site at the end of the coding region [4] into a *Bam*HI site. Constructs derived from pET11-d were introduced into *E. coli* BL21 (DE3) [8], and pIV6 derivatives were introduced into *E. coli* XL1Blue ($recA^-$) by transformation. Protein extracts were made as described in section 2.1. and used for MCAT assays, SDS-PAGE and Western immunoblotting as described previously [4].

2.6. Data analysis

Kinetic parameters were determined by using Eadie-Hofstee plots, and all data were analysed by computer-assisted non-linear regression by using Enzfitter software. Standard errors were estimated by Enzfitter.

3. Results and discussion

3.1. Steady state kinetics

As shown by Harder et al. [5], the *E. coli* strain LA2-89 carrying the *fabD89*^{ts} allele is unable to grow at temperatures higher than 35°C unless supplemented with long-chain fatty acids. The MCAT activity in this strain was found to be almost completely inactivated by pre-incubation of the extract at 42°C, under conditions which do not alter the activity of wild-type *E. coli* cells [4,5]. In addition to this temperature sensitivity, the MCAT activity measured at the permissive temperature of 30°C was only 2.5% of that found in *fabD*$^+$ *E. coli* strains [4,5]. Using malonyl-CoA as substrate, we determined the Michaelis constant (K_m) of the wild-type and mutant MCAT protein in crude protein extracts of *E. coli* strain XL1Blue and LA2-89. Fitting the data in Eadie-Hofstee plots (not shown) gave identical values for the K_m of wild-type and mutant MCAT (11.0 ± 0.8 and 11.1 ± 0.8 μM, respectively). Consequently, the defect in the protein does not affect its ability to bind to the substrate. The V_{max} value for the mutant protein in crude extracts (6.8 ± 0.2 nmol/min/mg total soluble protein) represents only 2–3% of that of wild-type MCAT in crude extracts (320.0 ± 10.3 nmol/min/mg total soluble protein). Since the same relationship was found for the specific activity of mutant and wild-type extracts under standard assay conditions [4,5], and the kinetic properties of both enzymes are the same, the reduction in the V_{max} value of the mutant protein must be due to a decrease in the amount of functionally active protein within the extract.

3.2. The fabD89 allele contains an amber mutation at codon position 257

An earlier study [4] demonstrated the presence of two MCAT-related polypeptides in *E. coli* strain LA2-89 protein extracts (mol. wt. 32,000 and 27,000 Da), compared to a single one in wild-type strain extracts (32,000 Da). To establish whether the two forms were the result of a mutation in the *fabD89* gene, both strands were sequenced as described in section 2. A single G-to-A transition was found in the *fabD89* gene as compared to the wild-type sequence at position 1301 [4]. This mutation alters the tryptophan codon (TGG, W) at codon position 257 into an amber codon (TAG, termination) (Fig. 1 and [4]). Premature termination of translation caused by this amber mutation explains the truncated form (27,000 Da) of the enzyme found in LA2-89. The additional synthesis of a protein with an apparently normal size (32,000 Da) is most likely explained by the amber suppressor genotype (*SupE44*) of this strain. As demonstrated by Inokuchi et al. [10], the duplicated and mutated $tRNA^{gln}$ present in *supE* strains is capable of suppressing amber mutations by occasional insertion of a glutamine. Based upon these data, the full-length protein found in this strain is expected to contain a glutamine (Q) instead of a tryptophan residue (W) at position 257 of the amino acid sequence. To establish whether the truncated or the suppressed form of the protein is responsible for the temperature-labile MCAT activity

found in LA2-89, we studied both forms separately by site directed mutagenesis and over-expression studies.

3.3. *The 27 kDa truncated polypeptide is enzymatically inactive*

To study the truncated MCAT protein in more detail, the *fabD89* gene was over-expressed by the use of the *E. coli* expression vector pET11-d, as described in section 2. The wild-type *fabD* gene was used as a control. Fig. 2B shows a 12% SDS-PAGE gel of protein extracts of *E. coli* strain BL21 grown at 28°C, harboring these recombinant pET11-d plasmids. Due to the non-suppressor genotype of BL21, over-expression of the *fabD89* allele only results in the synthesis of the truncated polypeptide. Fractionation of the protein lysates into pellet and supernatant showed that, in contrast to the wild-type protein (Fig. 2B, lane 1), the truncated protein was undetectable in the soluble fraction and all the protein was found in the low-speed pellet (Fig. 2B, lanes 4 and 5). The over-production of a protein up to a concentration level rare in nature can often be the reason for the formation of aggregates of 'denatured', insoluble polypeptide chains (also termed inclusion bodies) in *E. coli* [3]. In this case, however, the reason for aggregation must lie within the mutation itself, as inclusion bodies of the truncated form were also observed in the single copy situation. Immunoblot analysis of protein extracts prepared from LA2-89 carrying the *fabD89* allele (Fig. 2A) demonstrated that the soluble fraction contained only the suppressed full-length protein (Fig. 2A, lane 2). Enzyme studies showed that MCAT levels of the total and soluble fraction of the LA2-89 protein extract, as well as that of the cells over-producing the truncated form, were identical (data not shown). The MCAT enzymatic activity can therefore be totally attributed to the suppressed form of the protein, whereas the truncated form is catalytically inactive. The activity of the suppressed form explains why LA2-89 is still able to grow at 28°C, albeit slower as compared to a wild-type strain [5]. The inactivity of the truncated polypeptide explains the anomalous results found in earlier co-transduction experiments [11] carried out to map the *fabD* locus, as introduction of the *fabD89* allele in a non-suppressor background is expected to be lethal.

3.4. *Site-directed mutagenesis of the tryptophan-257 of the wild-type* fabD *gene*

To investigate whether the incorporation of the glutamine (Q) by the *SupE* mutation of the LA2-89 strain rendered the suppressed protein heat labile, the nucleotide sequence of plasmid pIV6, carrying the wild-type *fabD* gene, was changed by site-directed mutagenesis, as described in section 2 (Fig. 1). A mutant protein was produced, pIV6(W^{257}→Q), in which Q replaced W-257. Since there is a clear relationship between temperature-sensitive mutations and protein structure and maturation [2,3], we have also used a degenerate oligonucleotide to introduce other amino acid codons at this position.

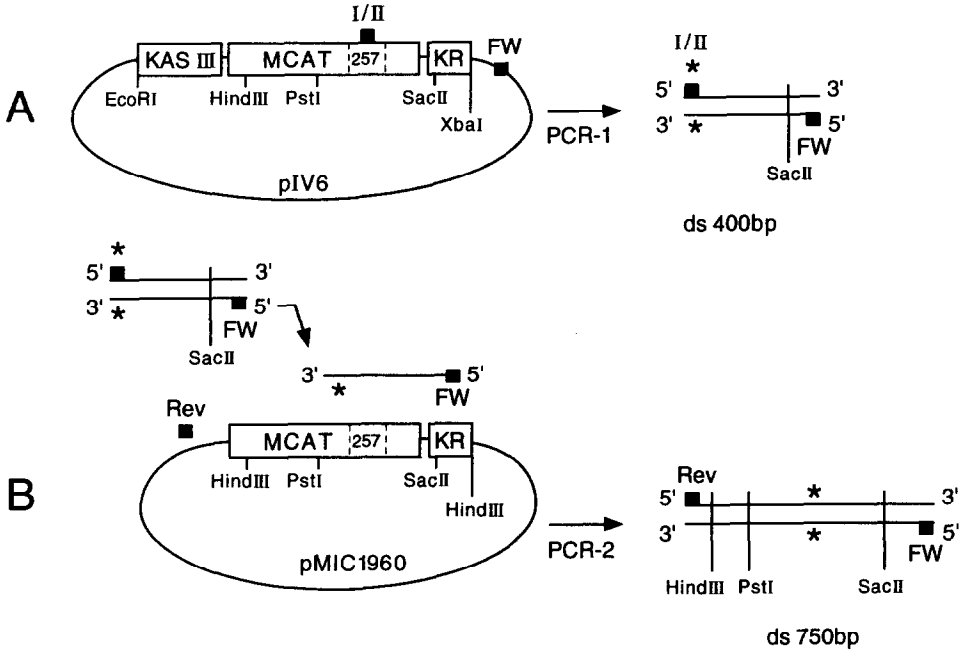

Fig. 1. Construction of pIV6 mutant plasmids by site-directed mutagenesis. (A) First PCR on pIV6 plasmid DNA, with the M13 Forward primer (FW) and either primer I or II, resulting in PCR products of 400 bp with a mutation at the marked position (*). (B) Second PCR on pMIC1960 ss DNA, with the M13 Reverse primer (Rev) and the complementary strand of the 400 bp PCR product from the first PCR, resulting in PCR products of 750 bp with a mutation at the marked position (*). Subsequently, the *PstI–SacII* fragment carrying the mutation (*) was exchanged with the *PstI–SacII* fragment of pIV6, resulting in a collection of pIV6 derivatives.

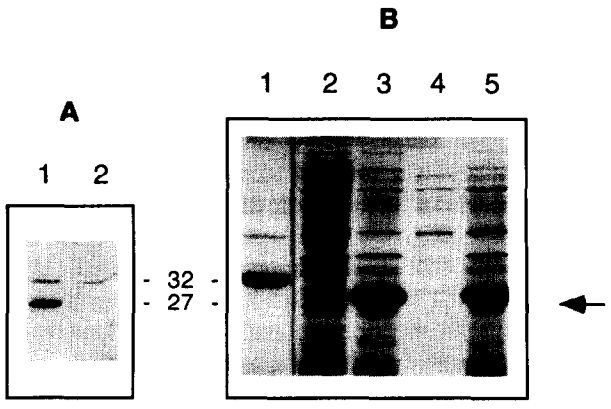

Fig. 2. (A) Western blot analysis of crude protein extracts prepared from *E. coli* LA2-89, grown at 28°C. Immunodetection of MCAT-related proteins was performed using anti-*E. coli* MCAT antibodies [4]. Lane 1, total protein fraction (approximately 10 μg protein); lane 2, soluble protein fraction. (B) 12% SDS-PAGE of approximately 10 μg of crude protein extracts of *E. coli* strain BL21, grown at 28°C, harboring different recombinant plasmids. Lane 1, pET50 (wild-type MCAT) with IPTG induction, soluble protein fraction; lane 2, pET60 (mutant MCAT) without IPTG, total protein fraction; lane 3, pET60 with IPTG, total protein fraction; lane 4, pET60 with IPTG, soluble protein fraction; lane 5, pET60 with IPTG, pellet fraction. Sizes are shown in kDa. The position of the truncated mutant MCAT protein is indicated by the arrow.

The sequence of the oligonucleotide was chosen such that a collection of mutants would be obtained that would enable us to assess whether this site of the polypeptide chain is crucial for correct folding or stability of the enzyme. The amino acid substitution mutants that were obtained (underlined) fall within 7 of the 8 amino acid groups with side chains of similar chemical properties: GAILV, ST, FYW, QN, ED, HKR, CM and P (W represents the wild-type situation).

3.5. *The suppressed full-length protein is heat labile*

E. coli strain XL1BLUE was transformed with pIV6 and the 8 amino acid substitution mutants of pIV6 indicated above. Cells were grown at 28°C and 39°C, and protein extracts were made and fractionated into low-speed supernatants and pellets. The proteins were separarated by SDS-PAGE. Fig. 3A displays the polypeptide distribution between the total and soluble fraction observed in extracts of cells over-expressing the wild-type (pIV6) and three of the mutant gene constructs (W-257→Q; W-257→R and W-257→Y) at the restrictive temperature of 39°C. The results show that, although the wild-type and mutant proteins accumulate to similarly high total quantities in these extracts (compare lanes 2, 4, 6 and 8), there is a marked difference in the solubility of these proteins at this restrictive temperature. The wild-type and the W-257→Y proteins were found in the soluble fraction of the protein extracts (lanes 3 and 9), whereas most of the W-257→Q and W-257→R polypeptide chains were recovered in the pellet as aggregated species (lanes 5 and 7). All other mutant proteins (see above) behaved very similarly to the W-257→Q and W-257→R mutants, and were predominantly found in the pellet fraction (data not shown). In cells over-expressing either the wild-type, or the mutant proteins, the level of MCAT proteins that accumulated was lower at the permissive temperature of 28°C than at 39°C. If the cells were grown at 28°C, however, all 8 mutant proteins were found almost exclusively in the soluble fraction. This was revealed by both enzyme studies (not shown) and Western blot analyses. Fig. 3B shows a comparison of the soluble and total fraction of extracts of cells over-expressing the wild-type or the W-257→Q mutant MCAT gene either at the restrictive (39°C) or the permissive temperature (28°C). This immunoblot not only demonstrates that the mutant protein W-257→Q is soluble when produced at 28°C (compare lanes 7 and 8), but also reveals that the mutant protein is almost exclusively found in the low speed pellet fraction when synthesized at 39°C (lanes 9 and 10). Recent work [12] has linked inclusion body formation to temperature-sensitive denaturation. Schein and Noteborn [12] have shown that formation of soluble recombinant proteins in *E. coli* is

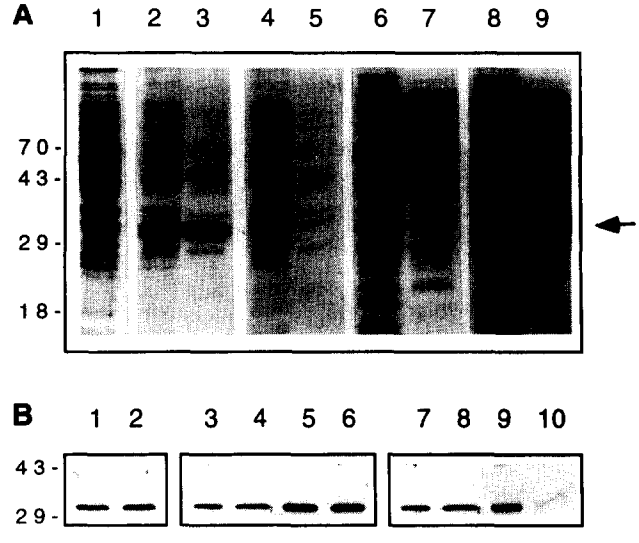

Fig. 3. (A) 12% SDS-PAGE of approximately 10 μg (lanes 1–5) and 15 μg (lanes 6–9) of crude protein extracts of *E. coli* XL1Blue, grown at 39°C, harboring various pIV6 recombinant plasmids. Lane 1, XL1Blue cells, total protein fraction; lanes 2 and 3, pIV6 total and soluble protein fraction; lanes 4 and 5, pIV6($W^{257}Q$) total and soluble protein fraction; lanes 6 and 7, pIV6($W^{257}R$) total and soluble protein fraction; lanes 8 and 9, pIV6($W^{257}Y$) total and soluble protein fraction. (B) Western blot analysis of proteins synthesized in *E. coli* XL1Blue, grown at 28°C and 39°C, expressing pIV6 and pIV6($W^{257}Q$). Immunodetection of MCAT-related proteins was performed, using anti-*E. coli* MCAT antibodies [4]. Lanes 1 and 2, XL1Blue grown at 39°C, 1 μg of total and soluble fraction; lanes 3–6, pIV6, respectively, 0.1 μg of 28°C total protein fraction, 28°C soluble protein fraction, 39°C total protein fraction, 39°C soluble protein fraction; lanes 7–10, pIV6($W^{257}Q$), respectively, 0.1 μg of 28°C total protein fraction, 28°C soluble protein fraction, 39°C total protein fraction, 39°C soluble protein fraction. Sizes are shown in kilodaltons. The position of the MCAT protein is indicated by the arrow.

favoured by lower growth temperature. A very similar situation is found with the mutant MCAT proteins. With the exception of the W257→Y mutant, in which the tryptophan residue is replaced by another aromatic amino acid (tyrosine), all other mutants are insoluble at 39°C whereas they are clearly soluble when synthesized at low temperature.

A direct correlation between solubility of the protein and enzyme activity was also obtained by enzyme studies. Table 1 shows the results obtained by analyzing MCAT activity of extracts of cells grown at 28°C or 39°C. The data presented in Table 1 are restricted to those obtained for the W257→Y, W257→Q and W257→R mutants. All other mutants gave similar results as the W257→Q and W257→R mutants and the data have therefore been omitted. The results show that, when grown at 28°C, expression of the mutant genes results in the synthesis of an active enzyme, at a level which is very similar to that of the wild-type gene construct (column 1). All except the wild-type and the W257→Y mutant enzyme, however, are inactivated by more than 90% during incubation at 39°C (column 2). When grown at 39°C and assayed at 28°C, the enzyme activity is a mere 1–2% of that found with the wild-type or the W257→Y construct (column 3). Although this result demonstrates that the inactive aggregated state of the mutant polypeptides is virtually irreversible, it also shows that the mutant polypeptides occasionally fold into an active protein when grown at 39°C. Moreover, it also implies that the structure of this catalytically active polypeptide is different from that of the bulk of the mutant enzyme produced at 28°C, since this residual activity found at 39°C is clearly temperature-resistant. This finding also explains why introduction of these mutant genes on high copy number plasmids still renders the mutant *E. coli* strain LA2-89 temperature-resistant (results not shown).

In summary, the results demonstrate that substitution of tryptophan at codon position 257 of the MCAT polypeptide chain with a non-aromatic amino acid affects its correct folding into an active form at 39°C. Moreover, when produced at 28°C, all mutant polypeptides fold into an active enzyme, but these amino acid substitutions render the enzyme heat labile. This heat inactivation results in aggregation and desolubilization of the soluble active enzyme as revealed by the formation of inclusion bodies by the temperature shift (not shown).

Studies of mutant proteins have revealed that amino acid replacements which substantially destabilize the protein structure are generally confined to the buried or rigid parts of the protein [13]. Tryptophan 257 is, as are most tryptophans, probably largely buried. Substitution of the tryptophan by a smaller amino acid will introduce a cavity, with a corresponding change in the free energy, which constitutes one of the reasons for the destabilization of the protein [14]. Also the introduction of any polar amino acid into a buried location is energetically unfavorable. The disruption of the hydrophobic core of the protein by the W257→Q mutation leads to spontaneous thermal unfolding of the protein above a certain temperature, i.e. the melting point of the protein has been lowered. Once the protein has unfolded, inclusion bodies are formed. It is suggested that the temperature-sensitive mutations only alter amino acids that have side chains of with low crystallographic thermal factors and low solvent accessibility in the X-ray crystal structure of the wild-type protein [13]. Elucidation of the three-dimensional structure of MCAT will explain the requirement for an aromatic amino acid at this position in the polypeptide chain, which assures the temperature stability of the enzyme.

Our results demonstrate that the defect in the *fabD89* gene, in combination with the *supE44* genotype, is responsible for the temperature-sensitive character of strain LA2-89. The amber suppressor tRNA, encoded by the *supE44* allele, must compete with the release factor that recognizes its target termination codon. This results in only 2–3% of full-length active MCAT protein, compared to a *fabD*$^+$ strain, when grown at the permissive temperature of 28°C (Table 1). Further heat inactivation of the low amount of suppressed protein results in insufficient MCAT activity to sustain de novo fatty acid biosynthesis and is responsible for the temperature-sensitive growth phenotype of LA2-89.

Finally, on the bases of our results it can be argued that temperature-sensitive mutants may be more effi-

Table 1
Malonyl CoA-ACP transacylase relative activities[a] in *E. coli* XL1blue transformants and LA2-89

Extract	Cells grown at 28°C		Cells grown at 39°C	
	28°C assay	39°C assay	28°C assay	39°C assay
LA2-89	$3.5 \cdot 10^{-2} \pm 7 \cdot 10^{-3}$	$7 \cdot 10^{-4} \pm 3 \cdot 10^{-4}$	–	–
XL1blue	1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.35 ± 0.1
pIV6	433 ± 29	547 ± 45.6	841 ± 43	1013 ± 160
pIV6($W^{257}Q$)	420 ± 33	18.3 ± 5.1	8.1 ± 0.8	10.1 ± 1.5
pIV6($W^{257}Y$)	385 ± 15	581 ± 30.5	895 ± 65	956 ± 185
pIV6($W^{257}R$)	413 ± 25.2	30.2 ± 3.3	11.3 ± 1.9	12.5 ± 2.0

[a] MCAT activity was measured in the soluble fraction of the protein extracts. MCAT activity of XL1Blue, grown at 28°C and incubated for 30 min at 28°C, is set at 1 ($1 = 1.1 \times 10^3$ nmol/min/mg). All other MCAT activities, ± S.D. ($n = 3$), are quantified to this value.

ciently isolated in nonsense-suppressor strains than in wild-type strains of *E. coli*.

Acknowledgements: We thank Drs. Z.S. Derewenda and M.C. Walsh for their helpful comments on the manuscript. This research was supported by grant VBI 80.1412 of the Dutch Stichting voor de Technische Wetenschappen.

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