# Molecular Characterization of Genes Coding for Wild-Type and Sulfonylurea-Resistant Acetolactate Synthase in the Cyanobacterium *Synechococcus* PCC 7942

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Cyanobacterial ALS Gene, Sequence, Herbicide Resistance, Point Mutation, Expression in *E. coli*

We present here the isolation and molecular characterization of acetolactate synthase (ALS) genes from the cyanobacterium *Synechococcus* PCC 7942 which specify a sulfonylurea-sensitive enzyme and from the sulfonylurea-resistant mutant SM 3/20, which specify resistance to sulfonylurea herbicides. The ALS gene was cloned and mapped by complementation of an *Escherichia coli ilv* auxotroph that requires branched-chain amino acids for growth and lacks ALS activity. The cyanobacterial gene is efficiently expressed in this heterologous host. The ALS gene codes for 612 amino acids and shows high sequence homology (46%) at the amino acid level with ALS III of *E. coli* and with the tobacco ALS. The resistant phenotype is a consequence of proline to serine substitution in residue 115 of the deduced amino acid sequence. Functional expression of the mutant gene in wild-type *Synechococcus* and in *E. coli* confirmed that this amino-acid substitution is responsible for the resistance. Yet the deduced amino-acid sequence as compared with other ALS proteins supports the notion that the amino-acid context of the substitution is important for the resistance.

## Introduction

The first step in the biosynthesis of branched-chain amino acids is catalyzed by acetolactate synthase. Three ALS isoenzymes I, II, and III coded by separate operons *ilv*NB, *ilv*MG and *ilv*HI were characterized in enteric bacteria. Each enzyme is a tetramer composed of two large subunits carrying the catalytic activity, and two small subunits that confer sensitivity to inhibition by the end products and are required for efficient catalysis (for review see [1, 2]). This enzyme is a target for three classes of herbicides, the sufonylureas, imidazolinones and sulphonanilides. ALS II and III are sensitive whereas ALS I is resistant to these herbicides. Resistant forms of ALS have been identified in sulfonylurea-resistant mutants of enteric bacteria, yeast, and more recently, in plants (for review see [3]). Sequence analysis of the resistant forms, indicates that in each case a single amino-acid substitution confers the resistance [3, 4].

Cyanobacteria are considered to be a living chloroplast and offer an excellent system for studying at the molecular genetics level chloroplast genes and nuclear encoded chloroplast proteins. Since the plant ALS is localized in the chloroplast [5], we have sought to use the cyanobacteria for generation and analysis of sulfonylurea-resistant genes.

Recently, we have isolated sulfonylurea resistant mutants from *Synechococcus* PCC 7942, whose ALS activity was 300- to 3000-fold more resistant to sulfometuron (SM) as compared to that of the wild-type (WT). The cyanobacterial SM 3/20 mutation did not affect the enzyme activity nor its sensitivity to feed back inhibition by valine [6]. Here we present the isolation and molecular characterization of ALS genes from the wild-type cyanobacterium *Syncechococcus* PCC 7942 and from the sulfonylurea-resistant mutant SM 3/20.

## Materials and Methods

Synechococcus PCC 7942 was cultivated as previously described [6]. *E. coli* CU 888 kindly given by G. W. Hatfield is a *ilv*BN, *ilv*MG and *ilv*HI mutant, devoid of detectable ALS activity and requires branched-chain amino acids for growth. This strain was used for complementation studies and was grown in mineral medium containing glucose (0.5%), valine, leucine and isoleucine to final concentrations of 50 μg/ml each. *E. coli* cultures

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complemented by plasmids carrying *Synechococcus* ALS clones were grown in the above medium without valine, in the presence of ampicilline (100 μg/ml). Transformations and DNA methods were performed as previous described [7]. ALS activity was measured in sonicated cell-free extracts as previously described [6] or in toluenized cells [3].

## Results and Discussion

*Isolation of a cyanobacterial ALS gene and functional expression in E. coli*

Southern blot analysis of *Synechococcus* PCC 7942 WT DNA digested by various restriction enzyme and hybridized with *Anabaena* ALS gene (kindly given by B. Mazur) as a probe, revealed major hybridization bands of the size of 3.8, 9.0 and 6.0 kb of *Hinc* II/*Eco* RI, *Hinc* II and *Eco* RI digests respectively of either WT (lanes a, b, c) or the mutant (lanes d, e, f) DNA respectively (Fig. 1). Two approaches were undertaken for the cloning of the *Synechococcus* wild-type (WT) ALS gene: a) complementation of *E. coli* CU 888 which lacks any detectable ALS activity and required branched-chain amino acids for growth, with our gene-bank of *Synechococcus* PCC 7942 [7], and b) identification of ALS genes in the gene bank by hybridization with the *Anabaena* probe. In both cases positive clones were obtained. A plasmid containing 12 kb insert (designated pSM 18) was isolated by complementation, and further analyzed.

The restriction analysis of pSM 18 and subcloning of the cyanobacterial ALS gene is presented in Fig. 2. Mapping the WT ALS gene was based on the ability of pSM 18 subclones to complement the requirement of *E. coli* CU 888 for valine, and on the expression of ALS activity in this strain. Transformation of *E. coli* CU 888 by pSM 18 and plasmids carrying the 6 kb *Eco* RI, 3.8 kb *Eco* RI/*Hinc* II and the 2 kb *Xba* I fragments enabled the cells to grow in the absence of valine. These transformants expressed significant levels of ALS activity (150 to 200 nmol·hr$^{-1}$·mg prot$^{-1}$) which are 2–3-folds higher than the level in *Synechococcus* extracts. Similar results were obtained with inserts subcloned in opposite orientations (not shown), suggesting that the ALS gene is within the 2 kb *Xba* I fragment.

Although in *E. coli* the cyanobacterial ALS gene is on a multicopy plasmid it is evident that the cyanobacterial gene is efficiently expressed in the heterologous host. A recombinant ALS plant gene has recently been shown to function in *E. coli* [8].

*Isolation, mapping and functional expression of the herbicide resistant SM 3/20 ALS gene*

Based on the restriction analysis of the putative ALS WT gene, a gene bank of SM 3/20 DNA was constructed as 3.8 kb *Eco* RI/*Hinc* II fragments cloned in pUC 118. Using the WT 3.8 kb *Hinc* II/*Eco* RI DNA fragment as a probe, a positive clone was identified, from which a plasmid designated pSM 320 was isolated.

As shown in Fig. 2, pSM 320 contains a 3.8 kb *Hinc* II/*Eco* RI fragment identical by its restriction map to the equivalent fragment in pSM 18. Transformation of *E. coli* CU 888 by pSM 320 not only complemented the valine auxotrophy but also re-

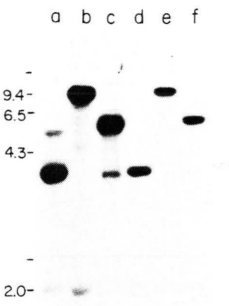


Fig. 1. Southern-blot analysis of *Synechococcus* PCC 7942 and SM 3/20 mutant DNA. The DNA was cleaved by *Hinc* II/*Eco* RI, *Hinc* II and *Eco* RI (lanes a–c, and d–f respectively) and hybridized with $^{32}$P-labelled nick-translated 1.2 kb *Hinc* II fragment of Anabaena ALS gene (kindly given by B. Mazur). The hybridization was performed at a low stringency (37% formamide, 37 °C, 5 × SSC (0.75 M NaCl, 0.75 M Na-Citrate) followed by washings with 2 × SSC at a maximum temperature of 48 °C).

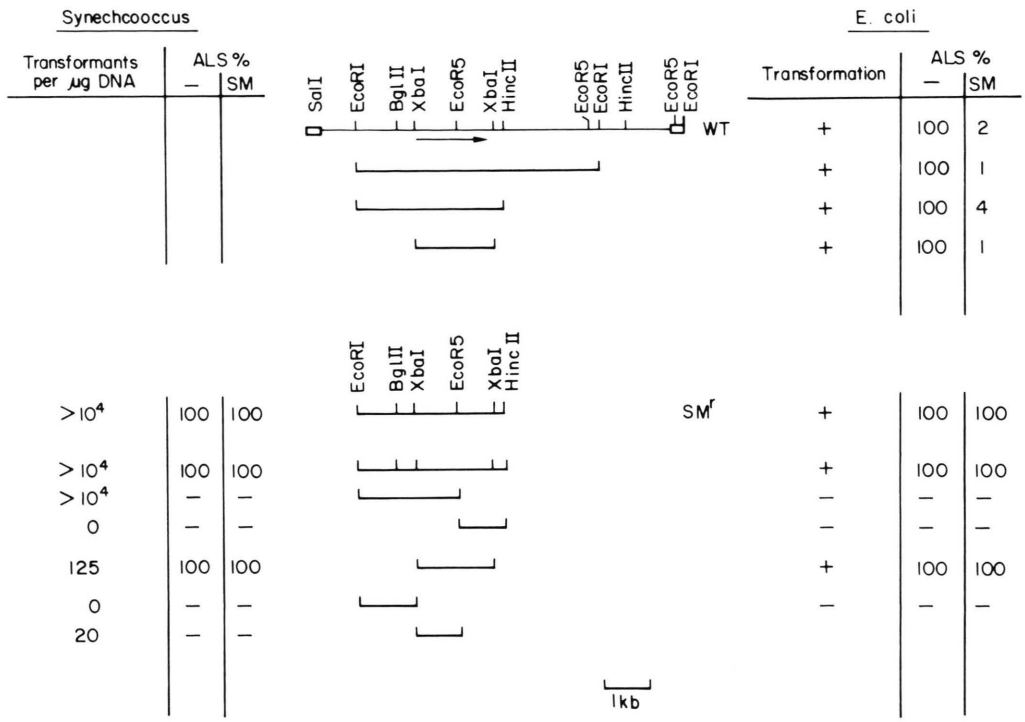


Fig. 2. Subcloning of the WT and SM 3/20 ALS genes, mapping the SM 3/20 mutation and expression of *Synechococcus* ALS in *E. coli* CU 888. The subclones in pUC 118 or pUC 119 [16] or the linear fragments were used to transform WT *Synechococcus* or *E. coli* CU 888. Typical 100% activity in *Synechococcus* is: 70 nmol acetoin · $h^{-1}$ · mg protein$^{-1}$ and in *E. coli* is 200 nmol · $hr^{-1}$ · mg protein$^{-1}$.

sulted in ALS activity which was resistant to sulfometuron ($I_{50}$ = 400 µM), whereas the ALS activity of pSM 18, or its derivative transformants was sensitive ($I_{50}$ = $2 \times 10^{-1}$ µM). As in the case of the wild-type clones, the smallest fragment of pSM 320 which complemented *E. coli* CU 888 was the 2.0 kb *Xba*I fragment.

*Synechococcus* PCC 7942 is a recombinant proficient host and upon transformation with homologous DNA and under appropriate selection the transformable DNA replaces the homologous region in the genome *via* a double crossing-over event [9]. This mechanism was used to map the SM 3/20 mutation. Fig. 2 further shows that transformation of wild-type *Synechococcus* cells by pSM 320 subclones and selection for the ability to grow in the presence of SM, resulted in resistant transformants which exhibited resistant ALS activity. Based on the transformation studies, the SM 3/20 mutation was mapped within the 1.1 kb *Eco*R 5/*Xba*I DNA fragment.

Table I is a dose response of the ALS activity of a typical *Synechococcus* WT clone transformed by pSM 320, as compared with the activity of the SM 3/20 mutant and the WT. As expected, the transformant exhibited a level of resistance similar to that of the mutant, thereby confirming the mapping of the SM 3/20 mutation in an isogenous strain. The enhancement of the apparent $I_{50}$ in the case of the mutant or the transformant as compared to the WT value was: 5000, 800 and 1500-folds for three variants of sulfonylureas: SM, metsulfuron (MS) und chlorosulfuron (CS) respectively. It demonstrates that the SM 3/20 mutation which was isolated by selection for SM resistance [6], confers resistance also to MS and CS. Among the three herbicides the CS was the weakest inhibitor.

Table I further demonstrates that the cyanobacterial ALS activity was less sensitive to SM in the *E. coli* host, as compared to the activity in the natural host, a phenomenon which has recently been

Table I. Inhibition of cyanobacterial ALS activity by sulfonylureas.

| Strain | $I_{50}$ [μM][a] | | |
|---|---|---|---|
| | SM | MS | CS |
| WT | $1.4 \times 10^{-2}$ | $2.6 \times 10^{-2}$ | $2.0 \times 10^{-1}$ |
| SM 3/20 | $7.0 \times 10^{1}$ | $5.0 \times 10^{1}$ | $3.0 \times 10^{2}$ |
| WT/pSM 320[b] | $6.0 \times 10^{1}$ | $7.0 \times 10^{1}$ | $3.5 \times 10^{2}$ |
| CU 888/pSM 18 HR[c] | $2.0 \times 10^{-1}$ | – | – |
| CU 888/pSM 320 | $4.0 \times 10^{2}$ | – | – |

[a] $I_{50}$ is the herbicide concentration which inhibits ALS activity by 50%.
[b] WT transformed by pSM 320 (see Fig. 2).
[c] *E. coli* CU 888 transformed by pSM 18 HR which is a derivative of pSM 18 carrying a *Hinc*II/*Eco*RI 3.8 kb fragment (see Fig. 2).

observed also in *E. coli* transformed by a recombinant ALS plant gene, and is inexplicable as yet [8].

*Sequence analysis of the ALS gene and the molecular basis of the herbicide resistance*

Sequence analysis of the WT ALS gene (Fig. 3) revealed an open reading frame of 612 amino acids with GTG as the initiation start codon. The role of GTG as a translation start codon is known in other organisms [10]. Potential ribosomal binding site and promoter sequence are underlined. Sequences which can form a stem and loop structure and might serve as transcription terminator are underlined with arrows.

The deduced amino-acid sequence is 46% homologous with the tobacco ALS gene [11] and with the ALS III gene of *E. coli* [12]; the homology with other ALS genes is lower (not shown).

The tobacco gene is longer and codes for 670 amino acids, however the mature chloroplast protein is expected to be shorter and it was suggested that 81 amino acids at the N′ terminal comprise a leader peptide which is processed off upon entry to the chloroplast [11]. Alignment of the cyanobacterial gene with the tobacco gene suggests that the putative leader sequence of the tobacco ALS gene is not present in the cyanobacterial gene (not shown).

Sequence analysis of the SM 3/20 ALS gene reveal a point mutation, resulting from a C to T

Table II. Comparison of the deduced amino-acid sequence around the cyanobacterial SM resistance mutation with the sequences of ALS genes from other organisms.

| Strain | Sequence | SM resistance |
|---|---|---|
| *Synechococcus* | TGQVPRTAIGTDAFQET | – |
| *Synechococcus* mutant | S | + |
| Tobacco | TGQVPRrmIGTDAFQET | – |
| Tobacco mutant | Q | + |
| Yeast | TGQVPtsAIGTDAFQEa | – |
| Yeast mutant | S | + |
| *E. coli* I | TGQVPasmIGTDAFQEV | + |
| *E. coli* II | TGQVsapfIGTeAFQEV | – |
| *E. coli* ALS III | SGQVatslIGyDAFQEC | – |

The sequence of tobacco [11, 13], yeast [4, 14] and *E. coli* [4, 12, 14, 15] genes were described previously. Small letters indicate nonconserved amino acids.

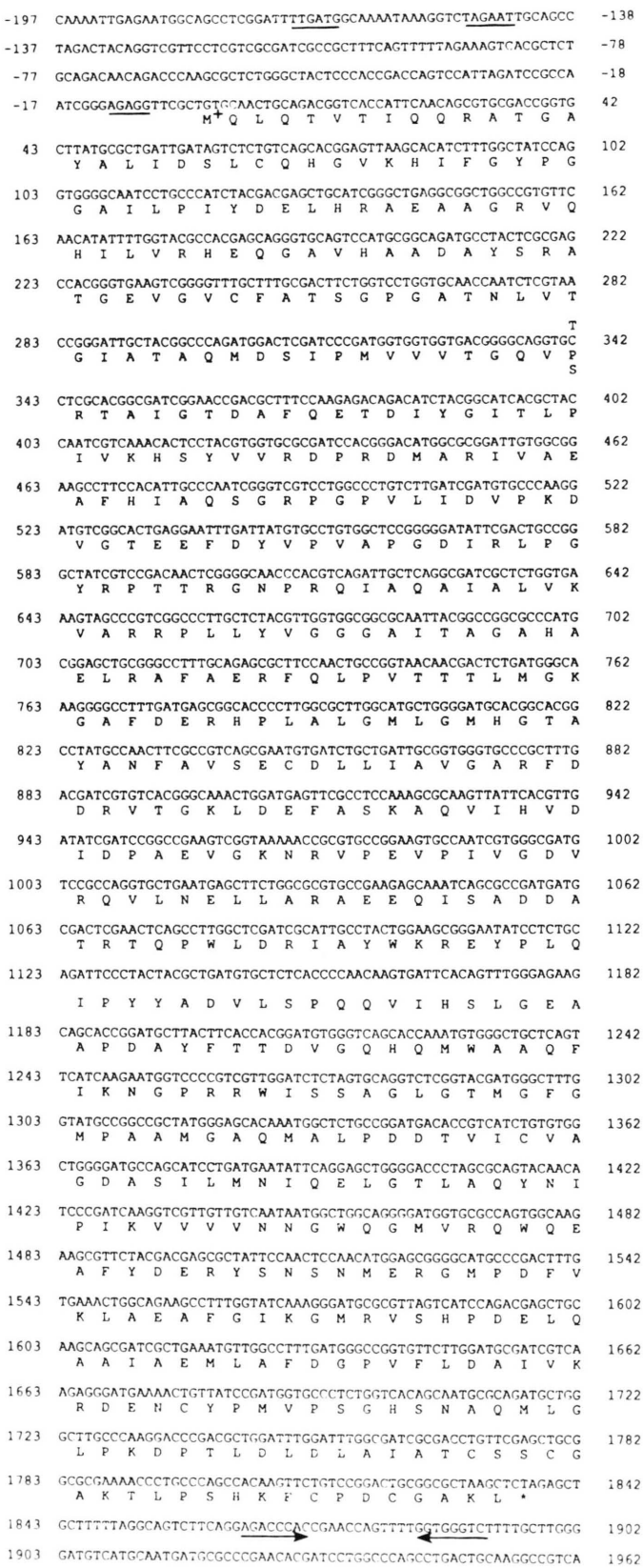


Fig. 3. Sequencing the ALS genes of *Synechococcus* WT and the SM 3/20 mutant. Putative promoter and Shine-Dalgarno sequences are underlined. Sequences which can form stem and loop and might serve as a transcription terminator are underlined with arrows.

change of nucleotide 342 which caused a proline to serine substitution in position 115 of the deduced amino-acid sequence (Fig. 3).

The SM 3/20 mutation is in a conserved region of the ALS gene (Table II). Proline to serine substitution in this conserved region resulted in herbicide resistance also in yeast [4]; substitution of the equivalent proline to glutamine resulted in resistant tobacco [13]. Analysis of the deduced amino-acid sequence around the SM 3/20 mutation supports the notion that the amino-acid context of the substitutions is important for the resistance as implicated from the data showing that serine is present in the indicated position in resistant *Synechococcus* mutant as presented here as well as in resistant yeast [4] and in the moderate sensitive ALS II of *E. coli* [14], whereas proline is present in the sensitive WT *Synechococcus* and in tobacco [11], as well as in the resistant ALS I of *E. coli* [15].

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