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Mutations in Two Distinct Regions of Acetolactate Synthase Regulatory Subunit from *Streptomyces cinnamonensis* Result in the Lack of Sensitivity to End-Product Inhibition

Jan Kopecký,[1] Jiří Janata, Stanislav Pospíšil, Jürgen Felsberg, and Jaroslav Spížek
Institute of Microbiology, Prague, Czech Republic

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Acetolactate synthase small subunit encoding *ilvN* genes from the parental *Streptomyces cinnamonensis* strain and mutants resistant either to valine analogues or to 2-ketobutyrate were cloned and sequenced. The wild-type IlvN from *S. cinnamonensis* is composed of 175 amino acid residues and shows a high degree of similarity with the small subunits of other valine-sensitive bacterial acetolactate synthases. Changes in the sequence of *ilvN* conferring the insensitivity to valine in mutant strains were found in two distinct regions. Certain point mutations were located in the conserved domain near the N terminus, while others resulting in the same phenotype shortened the protein at V(104) or V(107). To confirm whether the described mutations were responsible for the changed biochemical properties of the native enzyme, the wild-type large subunit and the wild-type and mutant forms of the small one were expressed separately in *E. coli* and combined *in vitro* to reconstitute the active enzyme.

Key Words: **acetohydroxy acid synthase; *ilvN;* valine-insensitivity.**

The prokaryotic FAD-dependent acetolactate synthase (ALS, EC 4.1.3.18) catalyzes formation of 2-acetolactate and 2-aceto-2-hydroxybutyrate, the intermediates of two parallel pathways of branched-chain amino acid biosynthesis leading to valine and isoleucine, respectively (reviewed in 1). ALS is a tetramer composed of two kinds of subunits, a large one, responsible for the catalytic activity, and a small or regulatory one, containing the valine-binding site (2, 3). The presence and functionality of the ALS small subunit has recently been demonstrated also in *Saccharomyces cerevisiae* (4, 5).

Except for enterobacteria, where several isozymes with distinctive properties are present, most of prokaryotes including streptomycetes seem to synthesize a single ALS (1), partially sensitive to the end-product inhibition. ALS encoding *ilvBN* genes are clustered with *ilvC* coding for acetohydroxy acid isomeroreductase, the second enzyme of the pathway. Sequence of the cluster is known in two streptomycetes—*S. avermitilis* (6) and *S. coelicolor* (7).

Streptomyces cinnamonensis is a producer of polyether antibiotics monensins A and B. While monensin B is synthesised from acetate and propionate building units only, monensin A contains also one butyrate unit (8), derived from valine precursor 2-oxoisovalerate (9). A proportion of monensin A was markedly elevated through increased availability of the butyrate unit in mutant strains resistant to valine analogues (10). Biochemical analysis of two groups of *S. cinnamonensis* mutants affected in the regulation of valine synthesis has previously revealed basic mechanisms of deregulation—insensitivity of ALS to the end-product inhibition by valine, increased level of ALS activity, or combination of the both (11, 12).

The present paper describes mutations in the *ilvN* gene coding for ALS regulatory subunit in *S. cinnamonensis* and provides evidence that some of them confer the insensitivity of ALS to inhibition by valine.

MATERIALS AND METHODS

Strains and plasmids. Streptomyces cinnamonensis strains C-100-5, ABR-21, ABR-38, NLR-3 (11), BVR-7, BVR-13 and BVR-18 (12) were used in the study. For cloning and heterologous expression *Escherichia coli* strains XL-1 blue, NM-522 and BL-21 (DE3) and vectors pGEM-7Zf(+) (New England Biolabs), pBluescript II KS+ (Stratagene) and pET-28b(+) (Novagen) were employed.

Cloning of the fragment containing ilvN gene. The fragment was PCR amplified from genomic DNA isolated from *Streptomyces cin-*

[1] To whom correpondence should be addressed at Institute of Microbiology, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic. Fax: +420 2 4752347. E-mail: jkopecky@biomed.cas.cz.

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namonensis parental and mutant strains by salting out procedure (13). Degenerated primers ilvNf and ilvNr (Table 1) were synthesised according to the coding sequence of neighbouring genes in *ilvBNC* operon from *Streptomyces avermitilis* (6) with flanking *Eco*RI (5′) and *Hind*III (3′) sites, respectively. Under optimal conditions (glycerol added to a final concentration 10%; DNA denaturation at 94°C for 1 min, primer annealing at 58°C for 30 s and DNA extension at 72°C for 1 min; 40 cycles followed by 5 min incubation at 72°C) PCR amplification using Expand High Fidelity PCR System (Boehringer) yielded a single product. The fragment was treated with *Eco*RI and *Hind*III and ligated into a pGEM-7Zf(+) vector. The plasmids were transformed into *E. coli* NM-522 cells (14).

Sequencing. Isolates of ds plasmid DNA for sequencing were prepared from 5 ml of LB medium using the NucleoSpin DNA purification kit (Macherey-Nagel). The chain termination reaction (15) was performed with the ThermoSequenase sequencing system from Amersham International plc. in cycle sequencing technique (16) using TexasRed nonradioactive labelled, universal M13 forward and reverse sequencing primers. The sequencing gels were run on the Vistra DNA sequencer 725 (Molecular Dynamics & Amersham Life Science).

Cloning and expression of wild-type and mutant ilvN in E. coli. In order to prepare an expression construct and to confirm the sequences from an independent clone, the *ilvN* gene (528 bp) was PCR amplified (conditions were as described above, except for annealing temperature of 52°C) from genomic DNA of wild-type and mutant strains using a pair of primers ilvNef (Table 1) and ilvNer designed according to the previously determined sequence of N-terminal part of *S. cinnamonensis ilvN* gene and downstream-located noncoding region with flanking *Eco*RI and *Nde*I (5′) and *Hind*III (3′) sites, respectively. The fragments were treated with EcoRI and HindIII, ligated into pBluescript II KS+ vector and sequenced. From the clones with confirmed sequence, *Nde*I-*Hind*III fragments were cut off and introduced to pET-28b(+) vector. *E. coli* BL21(DE3) transformants bearing the respective pET-28b-ilvN constructs were inoculated in LB medium with kanamycin (30 μg/ml) and grown at 37°C until the culture reached OD_{550} of 0.6–0.7. The expression was induced by IPTG at a final concentration of 0.4 mM and the cells were cultivated for 1 h at 37°C. Cells with the overexpressed fusion protein were harvested, centrifuged, and washed with 20 mM Tris-HCl (pH 8.0).

Cloning and expression of wild-type IlvB in E. coli. Degenerated forward primer ilvBf with flanking *Nde*I site designed according to N-terminal part of *ilvB* gene from *S. avermitilis* (11) and reverse primer ilvBr with flanking *Hind*III site designed according to the known sequence of the neighbouring *ilvN* gene from *S. cinnamonensis* (Table 1) were used for PCR amplification (conditions as above, however, extension time was prolonged to 3 min) of *ilvB* gene from genomic DNA of parental *S. cinnamonensis* strain. The single PCR product was treated with *Nde*I, *Hind*III and introduced to pET-28b(+) vector. The initial conditions of expression were as described for *ilvN*.

Preparation of E. coli crude extract and ALS assay. Frozen cells were resuspended in 20 mM Tris-HCl buffer pH 8.0 and disrupted in a Cole-Parmer 4710 Ultrasonic disintegrator at 0°C for three 10 s pulses separated by 30 s intervals. The extracts were clarified by a 15 min centrifugation at 20 000 g. Cell free extracts containing the overexpressed subunits were combined just prior to assay in a ratio ensuring saturation by the regulatory subunit. The activity of ALS was assayed as described elsewhere (11).

RESULTS AND DISCUSSION

Sequence of the wild-type ilvN. The *ilvN* gene coding for the ALS regulatory subunit in *S. cinnamonensis* C-100-5, the parental strain, was sequenced from the both strands and the sequence was confirmed from an independent clone (Fig. 1). The deduced amino acid sequence of the wild-type IlvN in *S. cinnamonensis* showed 86.3% and 92% identity with the known sequences from *S. avermitilis* and *S. coelicolor,* respectively. Among other known sequences of ALS regulatory subunits the degree of identity varied from 17.4% (ALS II in *E. coli*) to 62.5% (*Mycobacterium tuberculosis*). The most conservative region over the group is located near the N-terminus (Fig. 2). In *E. coli* ALS III (38% identity with *S. cinnamonensis* IlvN), a mutation G16D was described (3), which prevented binding of valine by the isolated regulatory subunit. Based on these facts, the region seems to be important for binding of the allosteric inhibitor.

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ATGTCCACCAAGCACACGCTCTCCGTCCTGGTCGAGAACAAGCCCGGCGT      50
M  S  T  K  H  T  L  S  V  L  V  E  N  K  P  G  V      17

CCTCGCCCGGATCACCGCCCTGTTCTCGCGCCGCGGCTTCAACATCGACT     100
L  A  R  I  T  A  L  F  S  R  R  G  F  N  I  D  S      34

CGCTCGCCGTGGGCGTCACCGAGCACCCCGACATCTCCCGCATCACCATC     150
   L  A  V  G  V  T  E  H  P  D  I  S  R  I  T  I      50

GTGGTGAACGTCGAGGACCTGCCGCTCGAACAGGTGACCAAGCAGCTCAA     200
V  V  N  V  E  D  L  P  L  E  Q  V  T  K  Q  L  N      67

CAAGCTCGTCAACGTCCTCAAGATCGTCGAACTGGAGCCGGGCGCCGCGG     250
 K  L  V  N  V  L  K  I  V  E  L  E  P  G  A  A  V      84

TGGCCCGCGAGCTCGTCCTGGCGAAGGTCCGCGCCGACAACGAGACCCGC     300
   A  R  E  L  V  L  A  K  V  R  A  D  N  E  T  R      100

TCCCAGATCGTCGAGATCGTCCAGCTGTTCCGCGCCAAGACCGTCGACGT     350
S  Q  I  V  E  I  V  Q  L  F  R  A  K  T  V  D  V      117

CTCCCCGGAGGCCGTCACCATCGAGGCGACCGGCAGCAGCGACAAGCTGG     400
 S  P  E  A  V  T  I  E  A  T  G  S  S  D  K  L  E      134

AGGCCATGCTCAAGATGCTGGAGCCCTTCGGCATCAAGGAGCTCGTGCAG     450
   A  M  L  K  M  L  E  P  F  G  I  K  E  L  V  Q      150

TCCGGCACCATCGCGATCGGCCGCGGCTCCCGCTCCATCACGGACCGCAG     500
S  G  T  I  A  I  G  R  G  S  R  S  I  T  D  R  S      167

CCTGCGGGCGCTCGACCGCAGCGCCTGA                           528
 L  R  A  L  D  R  S  A  *                             175
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FIG. 1. DNA sequence of *ilvN* gene in the wild-type strain *S. cinnamonensis* C-100-5 and deduced amino acid sequence of its product, the regulatory subunit of ALS. This sequence has been deposited in GenBank under Accession No. AF175526. Underlined residues indicate the positions of mutations found in *S. cinnamonensis* regulatory mutants.

Sequences of ilvN mutant forms. The mutant strains *S. cinnamonensis* ABR-21, ABR-38 and NLR-3 were resistant to valine analogues 2-aminobutyrate and norleucine, respectively (10). Strains BVR-7, -13 and -18 were isolated as mutants overcoming the growth inhibition by 2-oxobutyrate in the presence of valine (12). ALS activity in crude extracts from all of them exerted a markedly decreased sensitivity to valine inhibition (11, 12). The *ilvN* genes from six mutant strains were sequenced from both the strands in two independent clones. The point mutations detected, resulting in the changes of amino acid sequence, are summarised in Table 1. The positions of mutations are

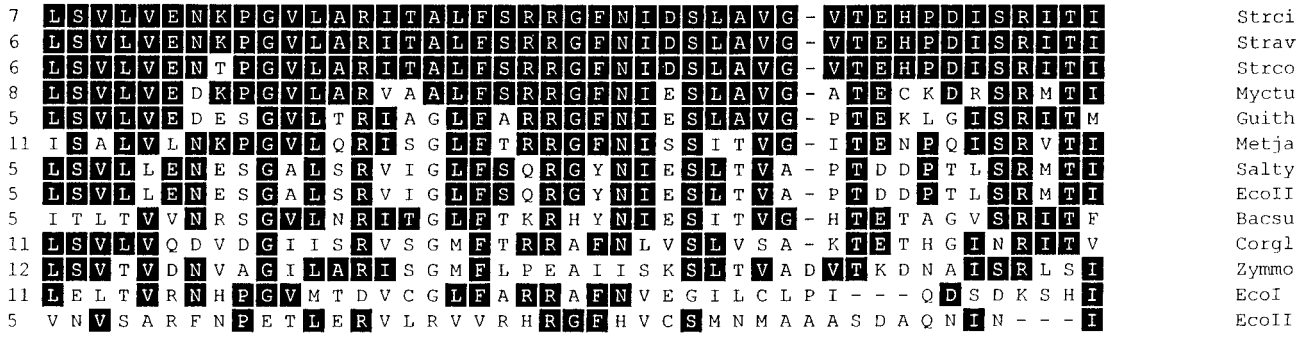

FIG. 2. Conserved domain near the N-terminus of acetolactate synthase small subunit. Number on the left indicates position of the first aa residue. Abbreviations used: Strci, *S. cinnamonensis;* Strav, *S. avermitilis;* Strco, *S. coelicolor;* Myctu, *Mycobacterium tuberculosis;* Guith, *Guillardia theta* (plastid); Metja, *Methanococcus jannaschii;* Salty, *Salmonella typhimurium;* EcoIII, *E. coli* ALS III; Bacsu, *Bacillus subtilis;* Corgl, *Corynebacterium glutamicum;* Zymmo, *Zymomonas mobilis;* EcoI, *E. coli* ALS I; EcoII, *E. coli* ALS II.

underlined in Fig. 1. Mutations detected in four of six strains (ABR-21, ABR-38, BVR-7, BVR-13) accumulated in the conserved N-terminal region (Fig. 2). According to GOR IV prediction (17), the residues at positions 16–18 are located at the interface between coiled and α-helical regions. The mutation which shortened the polypeptide by introducing the stop codon instead of residue 105 or 108 was found twice (ABR-38, NLR-3). Only in the strain BVR-18, substitutions of a different type were observed.

Heterologous expression of ALS subunits. The occurrence of mutations in the gene coding for the regulatory subunit may not be the only reason for the observed biochemical properties of the mutant enzymes. Another mutation might also be present in the large, catalytic subunit. In order to test the significance of the mutations identified in the regulatory subunit, wild-type and mutant ALS regulatory subunits and wild-type catalytic subunit were separately expressed in *E. coli* and used to reconstitute the ALS activity *in vitro.* The regulatory subunits were overexpressed in a soluble form at a post-induction temperature of 37°C, whereas the catalytic subunit was misfolded under the same conditions and appeared almost exclusively in inclusion bodies. The solubility of IlvB was achieved by decreasing the post-induction cultivation temperature as described for expression of human methylmalonyl-CoA mutase (18). After a 5-h post-induction cultivation at 24°C the prevailing part of IlvB was in a soluble form, a further decrease down to 12°C (24 h cultivation) minimized the proportion of IlvB in inclusion bodies, whereas the yield of the soluble protein remained almost the same (Fig. 3).

TABLE 1

Oligonucleotides Used for the PCR Amplification

Description	Sequence
IlvNf	5′-CCG GAA TTC ATG GT(GC) TGG CC(GC) ATG GT-3′
IlvNr	5′-AAC CCA AGC TTG CGT C(GC) G CGT CGT AG(AT) A-3′
IlvNef	5′-CCG GAA TTC ATA TGT CCA CCA AGC ACA C-3′
IlvNer	5′-AAC CCA AGC TTG TCT CGG TAT CCG AGA-3′
IlvBf	5′-CCG GAA TCA TAT GAC (GC)GA GCA GGC (GC)AC-3′
IlvBr	5′-AAC CCA AGC TTG GTG GAC ATG ACG-3′

Reconstitution of ALS. Crude extracts of *E. coli* containing overexpressed ALS subunits were combined as follows: $IlvB_{wt}$ + $IlvN_{wt}$ and $IlvB_{wt}$ + $IlvN_{mut}$, directly in the reaction mixture just before incubation. The artificial ALSs composed of wild-type catalytic and the respective regulatory subunit were assayed for sensitivity to valine inhibition. Crude extracts could be used as the *E. coli* ALS activity, which might interfere with the assay, did not exceed the basal level under assay conditions (always checked by controls). The data obtained with the regulatory subunits from five of the mutants tested (Table 2) proved that the described mutations were responsible for the loss of sensitivity to feedback inhibition observed in the native enzyme. On the contrary, the enzyme containing the regulatory subunit from *S. cinnamonensis* BVR-18 diverged absolutely from the properties of ALS detected in cell free extract of this strain. Sensitivity of the reconstituted enzyme to the inhibition was comparable with the wild-type ALS suggesting that the mutations I106V

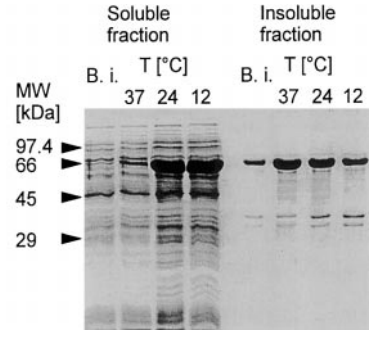

FIG. 3. Expression of the ALS catalytic subunit in *E. coli.* The yield of the soluble form in dependence on postinduction temperature.

TABLE 2

Properties of Native and *in Vitro* Reconstituted Wild-Type and Mutant Forms of ALS

Strain	Mutations detected in *ilvN* gene		Inhibition of ALS by 10 mM valine [%]	
	Nucleotide	Amino acid	*In vitro* reconstituted ALS	Crude extract of *S. cinnamonensis*[a]
WT	—	—	45.9	45.8
BVR-13	T50A	V17D	0	0
BVR-7	T50A, T88C	V17D, F30L	0	−4.5
ABR-21	C52T	L18F	0	0
ABR-38	G47A, G313T	G16D, E105stop	0	0
NLR-3	C322T	Q108stop	0	0
BVR-18	A316G, G403C	I106V, A135P	46.2	−38.3

[a] Data from (11, 12).

and A135P did not affect the ability to mediate the valine inhibition. This observation was similar to that in another analogue-resistant mutant *S. cinnamonensis* ACB-NLR-2 (data not shown). In BVR-18, another mutation has presumably occurred in the catalytic subunit, which might either change the region in contact with the regulatory subunit or otherwise disable the transmission of a conformational change induced by interaction of the regulatory subunit with valine. It remains unclear, whether the two mutations detected, although they alone did not abolish the inhibition by valine, had any significance in the native ALS. This should be clarified by cloning and expression of the (presumably) mutated catalytic subunit from this strain and its *in vitro* combination with the wild-type or mutant IlvN.

In the regulatory subunit from the strain ABR-38, the mutations were located in both the regions critical for the valine inhibition. Interestingly, the substitution of D for G16 in ABR-38 strain exactly matched the mutation described in *E. coli* ALS III and proved to prevent the binding of valine (3). Moreover, in ABR-38, this mutation occurred together with another one shortening the polypeptide, which alone might have disabled the regulation by valine, as observed in NLR-3 strain. To compare their effects, the mutation introducing the stop codon instead of E105 found in ABR-38 was removed using the unique *Bst*EII site located at position 183. The resulting artificially prepared gene coded for a polypeptide IlvN-ABR38L with a restored full length and a single mutation G16D. This form of IlvN was produced in *E. coli* and used to reconstitute the enzyme activity (Table 3). The regulatory subunit IlvN-ABR38L still differed from the wild type. However, inhibition by 11% was observed in the presence of 10 mM valine and at 100 mM, the inhibition by 72% was already comparable with the wild type, suggesting that the reconstituted enzyme at least partially recovered its ability to bind valine.

TABLE 3

Effect of Mutations Found in *ilvN* from the Strain ABR-38

Small subunit	Length	Mutation(s)	Inhibition of reconstituted ALS [%]	
			10 mM Val	100 mM Val
IlvN-ABR38	104 aa	G16D, E105stop	0	35.4
IlvN-ABR38L	175 aa	G16D	11.0	72.0

Our results suggested that two types of mutations were critical with respect to ALS end-product inhibition. Substitutions of residues in positions 16–18 with aspartic acid and phenylalanine, respectively, were most frequent. Interestingly, truncation of the polypeptide behind the residues 104–107, i.e., the loss of the whole, approximately 70 aa long C-terminus, resulted in the same phenotype.

Further experiments aimed at discrimination between possible effects of the described mutations on the association of subunits, affinity to valine or induction of conformational change mediating allosteric inhibition are currently in progress.

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