Regulation of branched-chain amino acid synthesis in actinomycetes

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Abstract

A set of *Streptomyces cinnamonensis* mutants with an increased metabolite flow through the branched chain amino acid biosynthetic pathway was analyzed by assessing the activities of enzymes catalyzing the pathway steps. Three mechanisms of the pathway deregulation were identified, all of them affecting acetohydroxy acid synthase (AHAS): (i) insensitivity to the feedback inhibition, (ii) elevated enzyme activity, or (iii) combination of the both.

In the parental strain and selected regulatory mutants, the genes *ilvB* and *ilvN* encoding the large (catalytic) and small (regulatory) AHAS subunits, respectively, and their regulatory region were sequenced. No changes were found in the regulatory region, i.e. promoter, ribosome binding site, leader peptide, and attenuator, providing no explanation for the increased AHAS activity. On the contrary, in the mutants with AHAS insensitive to feedback inhibition, mutations were found in either regulatory or catalytic subunits, and were proved to be responsible for the observed phenotype. The changes in the sequence of *ilvN* in mutant strains were of two different types. Certain point mutations were located in the conserved domain near the N terminus, resulting in amino acid residue substitutions G16D, V17D and L18F. Mutations in other strains conferring the same phenotypic change shortened the protein by inserting stop codone behind V104 or V107. Changes in the catalytic subunit were found in the mutants, where AHAS was apparently activated by valine *in vitro*. Deletion of Q217 affected a distant helix in β-domain, while substitution E139A occurred in a conservative loop near the active center. To reveal possible impact of individual mutations on self-association of the AHAS holoenzyme, the strength of the subunit-subunit interactions were assayed. The catalytic subunit mutations, E139A and ΔQ217, as well as regulatory subunit mutations G16D and V17D and shortening of the regulatory subunit to 107 residues affected the interaction indicating that the affected regions might be in contact with the matching subunit during allosteric regulation. In contrast, replacement of the adjacent L18 residue pointing inwards with phenylalanine did not influence the interaction implying that L18 may participate in valine binding or conformational change transfer.

Key words: acetohydroxy acid synthase; allosteric inhibition; *ilvN*; *ilvB*; *Streptomyces cinnamonensis*

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Abstrakt

U sady mutantů *Streptomyces cinnamonensis* se zvýšeným tokem metabolitů biosyntetickou drahou aminokyselin s rozvětveným řetězcem byly analyzovány aktivity enzymů katalyzujících jednotlivé stupně dráhy. Byly popsány tři mechanismy deregulace, vždy prostřednictvím změny vlastností synthasy acetohydroxykyselin (AHAS): (i) necitlivost ke zpětnovazebné inhibici, (ii) zvýšená aktivita enzymu, (iii) kombinace obou předchozích.

Z rodičovského kmene a vybraných mutantů byly sekvenovány geny *ilvB* a *ilvN* kódující velkou (katalytickou) a malou (regulační) podjednotku AHAS, a jejich regulační oblast. Žádné změny nebyly nalezeny v regulační oblasti, t.j. promotoru, ribosomálním vazebném místě, leader peptidu a atenuátoru, nebylo tedy možné vysvětlit zvýšení aktivity AHAS. Naproti tomu u mutantů, kde byla AHAS necitlivá ke zpětnovazebné inhibici, byly nalezeny mutace v regulační nebo katalytické podjednotce, a byla prokázána jejich odpovědnost za zjištěný fenotyp. Změny v sekvenci genu *ilvN* byly u mutantních kmenů dvojího typu. Část mutací byla lokalizována v konzervované oblasti u N-konce a vedla k substitucím G16D, V17D a L18F. Mutace v jiných kmenech způsobující stejnou změnu fenotypu zkracovaly protein insercí stop kodónu za V104 nebo V107. Změny v katalytické podjednotce byly nalezeny u mutantů, u nichž byla AHAS zdánlivě aktivována valinem v podmínkách *in vitro*. Delece Q217 ovlivnila odlehlou šroubovici v β-doméně, zatímco k substituci E139A došlo v konzervované smyčce poblíž aktivního centra.

Pro posouzení případného vlivu mutací na asociaci holoenzymu AHAS byla měřena síla interakce mezi podjednotkami. Mutace v katalytické podjednotce, E139A a ΔQ217, podobně jako mutace v regulační podjednotve G16D a V17D a zkrácení podjednotky na 107 aminokyselinových zbytků, interakci ovlivňovaly, a měly tedy pravděpodobně vliv na regiony zajišťující kontakt s odpovídající podjednotkou při allosterické regulaci. Naproti tomu nahrazení zbytku L18, jehož postranní řetězec směřuje dovnitř, nemělo vliv na sílu interakce. L18 se tedy může účastnit buď přímo vazby valinu, nebo přenosu konformační změny.

Klíčová slova: synthasa acetohydroxykyselin; allosterická inhibice; *ilvN*; *ilvB*; *Streptomyces cinnamonensis*

Contents

Preface

The present thesis is formed by selection of 6 papers unified by a common theme, the regulation of branched chain amino acid biosynthesis in *Streptomyces cinnamonensis* and interfering mutations in acetohydroxy acid synthase. The work was done during my stay in the laboratory of physiology and genetics of actinomycetes, Institute of Microbiology AS CR.

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1 Introduction

1.1 Actinomycetes in the environment

Actinomycetes are high GC content, Gram-positive bacteria and include often filamentous, but also coccoid and rod-shaped bacteria. They occupy multiple niches of all environments. They are, however, most important in soil, where they act as efficient degraders of plant, animal and fungal detritus because of their capability to utilize organic polymers such as lignin or cellulose (Goodfellow and Williams, 1983; Abdula *et al.*, 2007) and chitin (Manucharova *et al.*, 2007) as carbon sources. For example in forests soils they represented up to 63% of the bacterial communities (Hackl *et al.*, 2004), in grasslands enumerated to10^7 CFU per g of soil (Cho *et al.*, 2007), and in cropped fields to 10^4 CFU per g of soil (Shirokikh *et al.*, 2002).

Actinomycetes also colonize the rhizosphere, where they can parasite on plant roots or benefit from plant exudates, which represent easily available food source. Phytopathogenic actinomycetes, namely streptomycetes, produce phytotoxin thaxtomin which inhibits cell wall formation and enables entering the plants (Loria *et al.*, 1997). Actinomycetes also support the growths of plants for example by nitrogen fixation (Trujillo *et al.*, 2007) or antagonism to fungal pathogens (Coombs *et al.*, 2004). Actinomycetes seemed to be very adaptive and are believed to assist in development of the climax state of plant communities by supporting the growth of plants and associated bacteria (Kurtboke *et al.*, 2007), *Micromonosporineae* being the pioneering group while *Streptomycineae* the second group in the succession.

Finally, actinomycetes are major producers of secondary metabolites (Baltz, 2006), which inhibit or kill other microorganisms or serve as communication agents (Davies *et al.*, 2006). Many of them are pharmaceutically important because they represent antibacterial, antiviral, antiparazitic, antitumor or immunomodulating compounds. Secondary metabolites of actinomycetes are also used in agriculture as herbicides or insecticides (Thompson *et al.*, 2002). In the natural environment secondary metabolites have the potential to modify the structure and functioning of microbial communities by antibiosis or their assumed function as communication agents.

Actinomycetes are receiving considerable research attention in human and veterinary medicine. To a lesser extent, actinomycetes have also been used for

biodegradation of pollutants (Pasti-Grigsby *et al.*, 1996; Rehfuss and Urban, 2005), as biocontrol inoculants in commercial greenhouse (Greer and Diver, 1999; Coombs, 2004), and *via* their symbiotic association with actinorhizal plants in recovering programs for degraded soils (Dommergues, 1997). In addition, actinomycetes are also important human pathogens (Laurent *et al.*, 2007) and soil was reported as a reservoir (Polonelli *et al.*, 1981; De Groote, 2006). However, the major biotechnological importance of actinomycetes also means that they have mainly been studied from a limited number of scientific perspectives, and we have only a narrow understanding of their ecological roles.

1.2 Actinomycete diversity

In recent years, significant progress has been made in the description of novel species, in relation to the discovery of new secondary metabolite products (Donadio *et al.*, 2002, Fenical and Jensen, 2006, Gontang *et al.*, 2007) or human health (Laurent *et al.*, 2007). In parallel, it has become clear that actinomycete diversity is much wider than previously thought. Also, actinomycetes have been evidenced in other ecosystems than soils, e.g. in marine environment (Fenical and Jensen, 2006), in association with aquatic macrophytes (Wohl and McArthur, 1998), or in hypogean layers (Carlsohn *et al.*, 2007), as well as in less-studied soils *e.g.* under tropical rainforest (Wang *et al.*, 1999), acidic (Zakalyukina *et al.*, 2002; Kopecký *et al.*, 2011) and alkaline soils (Selyanin *et al.*, 2005), or sand dunes (Kurtboke *et al.*, 2007). However, these and other studies were often performed using only cultivation methods, so a major part of the information on actinomycete diversity and abundance in those environments is still missing. Also, usually only description of the actinomycete diversity in particular ecosystem was assessed rather than seeking for relationships between actinomycete abundance and diversity and environmental factors such as bedrock, particle size, organic matter content or climate.

More comprehensive actinomycete ecology studies have been performed by analyzing phospholipid ester-linked fatty acids (PLFA) and answering questions on actinomycete gradients in forest soils (Fritze *et al.*, 2000), the effect of roots on distribution of soil actinomycetes (Brant *et al.*, 2006), or their response to nitrogen and phosphorus fertilizers (Zhang *et al.*, 2007). In addition, the terminal RFLP (T-RFLP)

analysis of the 16S rRNA gene *rrs* was used to assess the endemicity of actinomycete communities (Wawrik *et al.*, 2007) or their distribution in soil particles (Sessitsch *et al.*, 2001). Finally, *rrs*-based denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) were used to describe actinomycete diversity in potato rhizosphere (Heuer *et al.*, 1997). These *rrs*-based molecular fingerprinting methods (*i.e.* T-RFLP, DGGE and TGGE) have proved useful to access a wider part of actinomycete diversity when comparing experimental treatments or ecological conditions. However, unless subsequent cloning/sequencing of bands is performed, their ability to provide direct information on the identity of the individual bacterial populations involved, at the genus or species level, is limited. In recent years, microarray technology has been proposed as a mean to combine the discriminating power of fingerprinting methods with direct taxonomic identification of a wide range of bacterial populations in the environment. In particular, microarrays constructed specifically to assess functional and/or taxonomic group(s) of bacteria became popular in studying various groups of bacteria (Loy *et al.*, 2002; Bodrossy *et al.*, 2003; Stralis-Pavese *et al.*, 2004; Kelly *et al.*, 2005; Kostic *et al.*, 2005; Brodie *et al.*, 2006; Sanguin *et al.*, 2006a, b) including actinomycetes (Kyselková *et al.*, 2008) in complex ecosystems.

1.3 Actinomycete life cycle

Actinomycetes possess many specific morphological traits and a complicated life cycle (Kieser *et al.*, 2000; Anderson and Wellington, 2001) although by their 16S rRNA sequences they belong close to gram positive rods and cocci *e.g. Micrococcus*, or *Arthrobacter* (Miyadoh *et al.*, 1997). Colonies of actinomycetes can be differentiated and capable of space and time control over gene expression, morphogenesis or metabolism resembling functioning of eukaryotic organisms (Kieser *et al.*, 2000). Life cycle of streptomycetes, the most studied actinomycetes, represented by *S. coelicolor*, starts on solid medium by spore germination. Spores are developed to long branched filaments, which are called vegetative mycelium (fast growth I period). Vegetative hyphae are multinucleate, only irregularly divided by incomplete septa (Wildermuth, 1970). After vegetative mycelium is developed, biomass accumulation is decreasing (transition period T) and some compartments of vegetative mycelium are lysed. In hyphae located on the colony surface, the supplies are accumulated, at the same time the production of some

extracellular proteins is increased and the synthesis of secondary metabolites and development of aerial mycelium begins. Aerial hyphae have a hydrophobic surface and show a typical spiral growth (Van Wenzel and Vijgenboom, 2004; Kieser *et al.*, 2000) (fast growth II period). Finally, aerial hyphae are converted to the chains of spores carrying one copy of the chromosome (sporulation period). In liquid media, actinomycetes grow along a typical growth curve, in which four growths phases can be distinguished similarly as on the solid medium (Puglia *et al.*, 1995; Vohradský *et al.*, 2000). Aerial hyphae are not differentiated in the liquid medium and some species *e.g. S. coelicolor* do not sporulate there. The T period is represented in both types of cultures and serves as a "control point" for physiological signaling leading to the onset of differentiation. The T period is also connected to activation of genes regulating antibiotic biosynthesis (Holt *et al.*, 1992) and mechanisms triggering expression of resistance genes induced by antibiotic production (Salah-Bey *et al.*, 1995). Antibiotic production on solid medium coincides with the development of the aerial hyphae and is generally connected to the stationary period in the liquid medium (Bibb, 2005). Regulation of secondary metabolism is to some extend coordinated with the regulation of their life cycle.

1.4 Actinomycete genome, actinomycetes as antibiotic producers

The genome of *S. coelicolor* represented the first complete actinomycete genome (Redenbach *et al.*, 1996; Bentley *et al.*, 2002) consisting of 8, 667 Mb. With its 7 825 putative genes belongs to the largest genomes known in bacteria. There are many regulation genes in comparison to other bacterial genomes. They are most likely responsible for the species good adaptability to stress and wide cooperation with the environment. Sequence analysis predicted many transporters and also drug efflux proteins most likely participating again in reaction to the environmental conditions. Capability to effectively utilize nutrients from the soil is demonstrated by many proteins, often hydrolases, with represent above described climax adaptation of this taxonomic group.

As it was mentioned, *S. coelicolor* genome emphasizes the function of regulations because there were identified 965 (12.3%) genes with putative regulatory function. This finding is in agreement with an observation that the larger the genome the higher the percentage of regulatory genes (Stover *et al.*, 2000). Another interesting trait of the *S.*

coelicolor genome is the presence of more than twenty potential gene clusters determining synthesis of known and predicted secondary metabolites. The number of secondary metabolite gene clusters was thirty in the second complete genome of actinomycetes *S. avermitilis* (Ikeda *et al.*, 2003). Demonstration of so many gene clusters in one strain supports the opinion that the diversity of secondary metabolites in actinomycetes is enormous and that its variability must be facilitated by horizontal transfer, which enables exchange of large amount of DNA (Donadio *et al.*, 2002) enabling different combinations of genes. Characteristic traits of secondary metabolite regulation in streptomycetes are diversity and complexity. Secondary metabolites provide adaptive advantages which are not completely understood because many of them may have different than antibiotic function in natural circumstances (Bibb, 2005).

2 Regulation of the branched-chain amino acid biosynthesis in actinomycetes

Valine metabolism serves as alternate source of *n*-butyrate, 2-methylmalonate and propionate building units in aglycons of macrolide antibiotics, *e.g.* tylosin (Omura *et al.*,1983a) and leucomycin (Omura *et al.*,1983b), or polyether-type antibiotics, *e.g.* monensin (Pospíšil *et al.*,1983) and lasalocid (Sherman *et al.*,1986).

Streptomyces cinnamonensis is a producer of oligoketide polyether antibiotics monensin A and B containing acetate, propionate and butyrate building units (Fig. 1). Branched-chain amino acids may serve as precursors of those units. Valine is degraded to butyrate, which is incorporated to monensin A only (Pospíšil *et al.*, 1983). Metabolism of isoleucine may provide a source of acetate and propionate building units (Pospíšil *et al.*, 1986). Acetohydroxy acid synthase (AHAS) is the first enzyme in the branched-chain amino acid biosynthesis. For valine biosynthesis, the enzyme catalyses a condensation of pyruvate with activated acetaldehyde arising from second pyruvate molecule. In the beginning of isoleucine biosynthesis, 2-oxobutyrate is condensed with activated acetaldehyde molecule giving rise to acetohydroxy butyrate. The ultimate step in both valine and isoleucine biosynthesis is amination of the respective 2-oxo acid. The reaction is catalysed by transaminase B. Valine dehydrogenase may also participate in the ultimate conversion, although it is rather considered as catabolic enzyme catalysing an oxidative deamination in streptomycetes. A higher concentrations of 2-oxobutyrate may

interfere with metabolic pathways, in which it can compete with their natural substrates, *e.g.* 2-oxoisovalerate (Powers and Snell, 1976). 2-Oxobutyrate is also considered as possible alarmone in *Escherichia coli* (Danchin *et al.*, 1984).

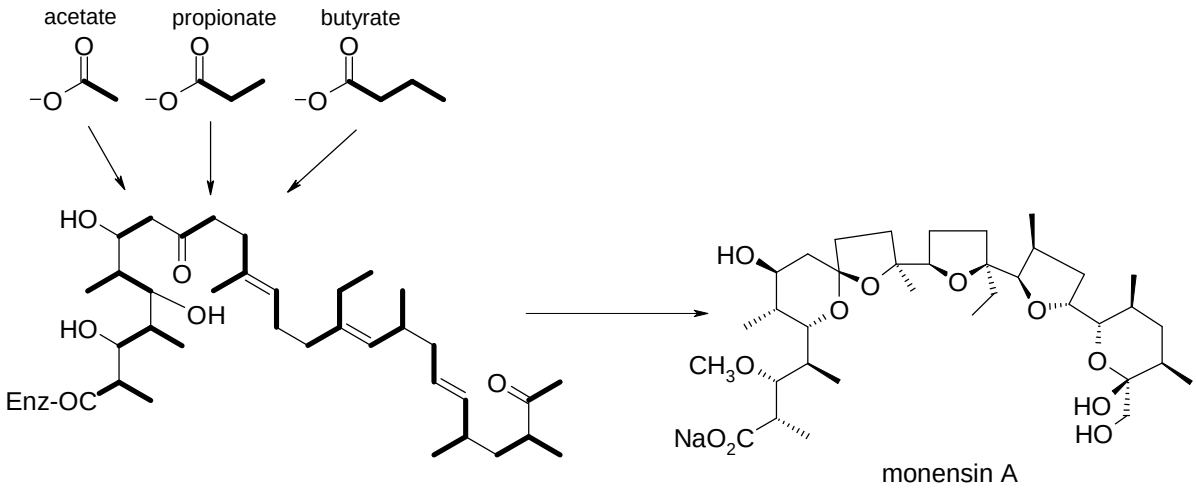

Fig. 1. Monensin A biosynthesis

Excretion of 2-ketoisovaleric acid (KIV) was demonstrated in *Streptomyces cinnamonensis* mutants resistant to valine analogues 2-amino-3-chlorobutyrate, 2-aminobutyrate and norleucine, respectively. The highest KIV concentrations of 170-230 μg ml^{-1} were found in cultivation liquids of norleucine resistant strains. Biochemical analyses of the acetohydroxy acid synthase (AHAS), valine dehydrogenase (VDH) and branched-chain amino acid aminotransferase activities revealed the deregulation of valine-synthesizing pathway, resulting in KIV excretion. In the 2-amino-3-chlorobutyrate resistant strain, the activity of AHAS increased 23- to 31-fold compared to the parental strain. The norleucine resistant mutants combined both 10- to 23-fold increase in AHAS activity and lack of efficient feedback regulation by valine. In double 2-amino-3-chlorobutyrate plus norleucine resistant mutant, the AHAS activity was only 4- to 8-fold higher, but release of feedback regulation was conserved. Similarly, feedback regulation was inefficient in 2-aminobutyrate resistant strains; however the AHAS activity was lower than in parental strain. A strong induction of VDH was observed in all regulatory mutants (Pospíšil *et al.*, 1998).

A set of *Streptomyces cinnamonensis* mutants resistant to 2-oxobutyrate in combination with valine or isoleucine was prepared. The selection process was strictly dependent on the presence of nitrate. Sixteen representatives of three groups of mutants

were biochemically analyzed as to the activities of enzymes of the branched-chain amino acid metabolism: acetohydroxy acid synthase, transaminase B and valine dehydrogenase. A majority of mutants resistant to 2-oxobutyrate and valine (BVR strains) exerted no feedback regulation of the acetohydroxy acid synthase by valine and proved to be potent producers of 2-ketoisovaleric acid. The BVR-13 strain produced up to 2.4 g l^{-1} of 2-ketoisovaleric acid during a 72-h cultivation in a nitrate-glucose medium. The excreted valine reached only 2.6% of 2-ketoisovaleric acid concentration. Bentonite present in the culture medium reduced NH_4^+ concentration and augmented the 2-ketoisovaleric acid production 1.3-fold. Half of the tested strains cultivated in a soya-bean medium exerted a substantially higher production of antibiotic monensins with elevated proportion of the monensin A (Pospíšil *et al.,* 1999).

The activity and structure of AHAS was elucidated in *S. cinnamonensis* mutants resistant to 2-oxobutyrate. AHAS is tetramer consisting of subunits of two types, large subunit responsible for catalytic activity, and a small (regulatory) one binding the AHAS allosteric inhibitor valine. With exception of enterobacteria, which posess several AHAS isozymes differing in substrate specificity and sensitivity to allosteric inhibition, most bacteria including streptomycetes express a single AHAS, sensitive to the end-product inhibition. Genes *ilvBN* encoding AHAS subunits are clustered with the gene *ilvC* coding for acetohydroxy acid isomeroreductase, the second enzyme of the metabolic pathway. Sequences of the actinobacterial *ilvBNC* cluster were first described from two streptomycetes, *Streptomyces avermitilis* (DeRossi *et al.*, 1995) and *Streptomyces coelicolor* (Redenbach *et al.*, 1996).

Acetohydroxy acid 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. The assays of reconstituted

acetohydroxy acid synthase confirmed that the described mutations conferred the altered biochemical properties of the native enzyme (Kopecký *et al.*, 1999).

In two *Streptomyces cinnamonensis* mutant strains ACB-NLR-2 and BVR-18, AHAS shoved an apparent activation by valine, which normally acted as an allosteric inhibitor. Sequencing the *ilvB* genes coding for the AHAS catalytic subunit revealed two distant changes in the mutants, ΔQ217 and E139A, respectively. Homology modeling was used to propose the structural changes caused by mutations. In mutant strain ACB-NLR-2 (resistant to 2-amino-3-clorobutyrate and norleucine), deletion of Q217 affected a helix in β-domain, distant from the active center. As no mutation was found in the regulatory subunit of this strain, ΔQ217 in IlvB was supposed to be responsible for the observed valine activation, probably *via* changed properties on the proposed regulatory-catalytic subunit interface. In mutant strain BVR-18 (resistant to 2-oxobutyrate), substitution E139A occurred in a conservative loop near the active center. *In vitro* AHAS activity assay with the enzyme reconstituted of the wild-type regulatory and BVR-18 catalytic subunits proved that the substitution in the catalytic subunit led to the apparent activation of AHAS by valine. We suggested that the conservative loop participated in a conformational change transfer to the active center during allosteric regulation (Kopecký *et al.*, 2008).

The impact of mutations found in AHAS on interactions between the catalytic and the regulatory subunits was examined using yeast two-hybrid system. Mutations in the catalytic and the regulatory subunits were projected into homology models of the respective proteins. Two changes in the catalytic subunit, E139A (α domain) and ΔQ217 (β domain), both located on the surface of the catalytic subunit dimer, lowered the interaction with the regulatory subunit. Three consecutive changes in the N-terminal part of the regulatory subunit were examined. Changes G16D and V17D in a loop and adjacent α-helix of ACT domain affected the interaction considerably indicating that this region might be in contact with the catalytic subunit during allosteric regulation. In contrast, the adjacent mutation L18F did not influence the interaction at all. Thus, L18 might participate in valine binding or conformational change transfer within the regulatory subunits. Shortening of the regulatory subunit to 107 residues reduced the interaction essentially, suggesting that the C-terminal part of the regulatory subunit is also important for the catalytic subunit binding (Kyselková *et al.*, 2010).

In *Streptomyces cinnamonensis* parental strain and two mutants with 10 to 20fold increased expression level of acetohydroxy acid synthase the *ilvBNC* operon upstream

DNA sequence was determined. An open reading frame that might code for a short leader peptide followed by a transcription terminator were identified within this region suggesting that the expression of *ilvBNC* operon may be controlled by attenuation. However, no mutation within this region was found in the mutants with increased expression level of AHAS implying that they must be subject to other mechanism of deregulation (Kyselková *et al.*, unpublished).

3 Conclusions

- A set of *Streptomyces cinnamonensis* regulatory mutants distinguished by an increased metabolite flow through the branched chain amino acid biosynthetic pathway was analyzed to reveal the mechanism of deregulation.

- The activities of selected enzymes participating in the pathway were assessed in crude extracts of the mutant strains and compared with the wild type. Three basic mechanisms of the pathway deregulation were identified, all of them affecting acetohydroxy acid synthase: (i) insensitivity to the feedback inhibition, (ii) elevated enzyme activity, or (iii) combination of the both.

- Changes in the sequence of *ilvN* conferring the insensitivity to valine in mutant strains were found in two distinct regions. Certain point mutations, namely G16D, V17D and L18F 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).

- In the mutant *Streptomyces cinnamonensis* strains where AHAS was apparently activated by valine *in vitro*, two changes were found in the catalytic subunit, ΔQ217 and E139A, respectively. Deletion of Q217 affected a helix in β-domain, distant from the active center. Substitution E139A occurred in a conservative loop near the active center. *In vitro* activity assay with the enzyme reconstituted of the wild-type regulatory and mutant catalytic subunits proved that the substitution E139A led to the observed apparent activation by valine.

- The upstream region of the AHAS encoding *ilvBN* genes was sequenced in the mutants with increased expression level of the enzyme. A relevant change was found

neither in the promoter region nor in attenuator suggesting that a different yet unknown process may be involved in AHAS expression control.

- The strength of the subunit-subunit interactions were studied in AHAS mutant forms to reveal possible impact of individual mutations on self-association of the AHAS holoenzyme. The catalytic subunit mutations, E139A and ΔQ217, as well as regulatory subunit mutations G16D and V17D and shortening of the regulatory subunit to 107 residues affected the interaction considerably indicating that the affected (or missing) regions might be in contact with the matching subunit during allosteric regulation. In contrast, replacement of the adjacent L18 residue pointing inwards with phenylalanine did not influence the interaction implying that L18 may participate in valine binding or conformational change transfer.

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5 Appendices

5.1 Derepression and altered feedback regulation of valine biosynthetic pathway in analogue-resistant mutants of *Streptomyces cinnamonensis* resulting in 2-ketoisovalerate excretion.

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Derepression and altered feedback regulation of valine biosynthetic pathway in analogue-resistant mutants of *Streptomyces cinnamonensis* resulting in 2-ketoisovalerate excretion

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S. POSPÍŠIL, J. KOPECKÝ AND V. PŘIKRYLOVÁ. 1998. Excretion of 2-ketoisovaleric acid (KIV) was demonstrated in *Streptomyces cinnamonensis* mutants resistant to valine analogues 2-amino-3-chlorobutyrate, 2-aminobutyrate and norleucine, respectively. The highest KIV concentrations of 170–230 mg l^{-1} were found in cultivation liquids of norleucine-resistant strains. Biochemical analyses of the acetohydroxyacid synthase (AHAS), valine dehydrogenase (VDH) and branched chain amino acid aminotransferase activities revealed the deregulation of the valine-synthesizing pathway, resulting in KIV excretion. In the 2-amino-3-chlorobutyrate-resistant strain, the activity of AHAS increased 23- to 31-fold compared with the parental strain. The norleucine-resistant mutants combined both a 10- to 23-fold increase in AHAS activity and lack of efficient feedback regulation by valine. In the double 2-amino-3-chlorobutyrate plus norleucine-resistant mutant, the AHAS activity was only four to eight-fold higher, but release of feedback regulation was conserved. Similarly, feedback regulation was inefficient in 2-aminobutyrate-resistant strains, however the AHAS activity was lower than in the parental strain. A strong induction of VDH was observed in all regulatory mutants.

INTRODUCTION

Streptomyces cinnamonensis produces a group of oligoketide polyether antibiotics monensins, the most important of which are monensin A and monensin B (Haney and Hoehn 1968). Incorporation experiments have established that the carbon backbone of monensins is constructed from precursors derived from acetate, propionate and butyrate. One butyrate unit is incorporated in monensin A whereas monensin B consists only of acetate and propionate building units (Day *et al.* 1973). Valine is a specific precursor for monensin A biosynthesis (Pospíšil *et al.* 1982) since the butyrate unit is formed from this amino acid via isobutyryl-CoA (Pospíšil *et al.* 1983; Birch and Robinson 1995). Knowledge of the butyrate unit origin was applied in the preparation of several groups of *S. cinnamonensis* analogue-resistant regulatory mutants overproducing valine. This was reflected in substantial changes in the quantitative ratios of monensin A and B produced by these mutants (Pospíšil *et al.* 1984) and in the cellular fatty acid profile (Pospíšil *et al.* 1985). During a study of aldehyde production by *S. cinnamonensis* (Pospíšil *et al.* 1994) we found that these regulatory mutants also excreted another major substance reacting with 2,4-dinitrophenylhydrazine, identified as described thereinafter.

The first enzyme of the branched chain amino acid biosynthesis is acetohydroxyacid synthase (AHAS), which in the valine pathway condenses two molecules of pyruvate to yield acetolactate. Acetohydroxyacid synthase undergoes feedback regulation by valine (Weinstock *et al.* 1992). Transaminase of branched chain amino acids (TAB) and valine dehydrogenase (VDH) catalyse the last step in this biosynthetic pathway as well as the first step of valine catabolism (Priestly and Robinson 1989; Leiser *et al.* 1996).

In this study we report the ability of the *S. cinnamonensis* regulatory mutants to produce branched chain 2-ketoacids (BCKA) and also provide biochemical analysis of this phenomenon by the study of AHAS, TAB and VDH activities in the cells grown under various conditions.

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MATERIALS AND METHODS

Strains

Streptomyces cinnamonensis C-100-5, considered as a standard control strain for this study, is the parental strain of the regulatory mutants (Pospíšil 1984) listed in Table 1.

Cultivation

Submerged cultivations were conducted in 500 ml flat-bottomed flasks (fitted with a 13 mm diameter stainless steel spring) containing 60 ml minimal liquid growth medium (MM) consisting of (g l^{-1}): $NaNO_3$, 10; glucose, 25; $MgSO_4.7\ H_2O$, 0·5; K_2HPO_4, 0·05. A medium of the same composition supplemented with 8 g l^{-1} $CaCO_3$ for buffering (MMC medium) was used in experiments requiring the presence of 50 mmol l^{-1} NH_4^+, which was added as NH_4NO_3 solution after sterilization. In a similar way, L-valine, L-leucine or L-isoleucine (when needed) was added separately to reach a concentration of 10 mmol l^{-1}. The cultures were incubated at 34 °C on a rotary shaker at 180 rev min^{-1}. The seed culture obtained by inoculation of MM medium with spores from a slant culture was grown for 3 d and then used for inoculation (8%, v/v). Each cultivation was performed at least in triplicate. For enzymatic analysis the mycelial cells were harvested by centrifugation at 24 h, washed three times with cold phosphate buffer (0·05 mol l^{-1}, pH 7·4) and frozen.

Analysis of branched chain 2-ketoacids

Actual BCKA concentrations in the fermentation liquid were measured after derivatization. To 4 ml filtered fermentation broth 1 ml 0·1% solution of 2,4-dinitrophenylhydrazine in 2 mol l^{-1} HCl was added and allowed to react for 2 h. The reaction mixture was repeatedly extracted with hexane : chloroform : propanol (48 : 12 : 40). The solvent was evaporated and the phenylhydrazones analysed. For orientation analysis and preparative purposes, separations were performed on silica gel plates developed in heptane : ethyl acetate : methanol (5 : 4 : 1). Derivatives of new metabolites were scraped off, eluted with diethylether : methanol (9 : 1) and, after repeated chromatography, analysed by nuclear magnetic resonance (NMR) and mass spectrometry. Precise quantitative analyses were carried out in a Spectra Physics (San Jose, CA, USA) modular HPLC system under the following conditions : column, 250 × 4 mm packed with Nucleosil 100-5C18 AB (Machery-Nagel, Düren, Germany) ; mobile phase, methanol : 0.05 mol l^{-1} CH_3COONa (60 : 40, v/v), flow rate 0·6 ml min^{-1} ; ambient temperature ; injected volume 20 μl ; detection at 380 nm. Phenylhydrazones prepared from corresponding purchased ketoacids (Serva, Heidelberg, Germany) were used as external standards.

Enzyme and protein assays

Frozen cells were resuspended in buffer (potassium phosphate 50 mmol l^{-1}, pH 7·4, 20% (v/v) glycerol, 0·25 mmol l^{-1} phenylmethylsulphonylfluoride) and disrupted at 0 °C by a Cole-Parmer (Chicago, IL, USA) 4710 Ultrasonic disintegrator for three 30 s pulses separated by 120 s intervals. For valine dehydrogenase and transaminase assays, the buffer contained also 5 mmol l^{-1} 2-mercaptoethanol. The mycelial extracts were clarified by centrifugation (20 000 *g*) and desalted on a Sephadex (Uppsala, Sweden) G-25 column equilibrated with the buffer used for disintegration.

The activity of AHAS (EC 4.1.3.18) was assayed according to Singh *et al.* (1988). The reaction mixture, containing 50 mmol l^{-1} potassium phosphate buffer (pH 7·8), 10 mmol l^{-1} sodium pyruvate, 10 mmol l^{-1} $MgSO_4$, 0·1 mmol l^{-1} thiamine pyrophosphate, 0·02 mmol l^{-1} flavine adenine dinucleotide Na_2, proteins (0·05–2·50 mg ml^{-1}) and valine (0, 0·1, 10 or 100 mmol l^{-1}), was incubated for 30 min (or 50 min for samples from aminobutyrate-resistant strains), stopped by the addition of 3 mol l^{-1} H_2SO_4 and the acetolactate synthesized was determined according to Westerfeld (1945) after conversion to acetoin (60 °C, 20 min).

The assay mixture for measurement of transaminase activity (EC 2.6.1.6) contained 50 mmol l^{-1} pyrophosphate buffer (pH 8·6), amino acid (L-valine or L-leucine, 13 mmol l^{-1}), 2-ketoglutarate (13 mmol l^{-1}), pyridoxal-5-phosphate

Table 1 Regulatory mutants of *Streptomyces cinnamonensis* (Pospíšil *et al.* 1984)

Strain	Parent strain	Resistance to	Concentration (mg ml^{-1})
ACBR-2	C-100-5	L-*threo*-2-amino-3-chlorobutyrate	0·05
ABR-21, ABR-38	C-100-5	L-2-aminobutyrate	1·0
NLR-3, NLR-12	C-100-5	L-norleucine	10
ACB-NLR-7	ACBR-2	L-*threo*-2-amino-3-chlorobutyrate and L-norleucine	0·05 and 10

(0.27 mmol l^{-1}) and proteins (0·28–1·52 mg ml^{-1}). After 10 min reaction, the mixture was treated as described by Duggan and Wechsler (1973).

Valine dehydrogenase (EC 1.4.1.8) activity in the direction of oxidative deamination was assayed by measuring the initial rates of NAD^+ reduction as recorded by the increase in absorbance at 340 nm. The assay mixture contained glycine buffer (70 mmol l^{-1}, pH 10), amino acid (L-valine or L-leucine, 10 mmol l^{-1}), NAD^+ (2 mmol l^{-1}) and proteins 0·25–1·0 mg ml^{-1}).

All enzyme assays were conducted at 30 °C. Protein concentrations were determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as a standard. The enzyme activity data represent the mean of three measurements. Standard deviations were all within 8% of the mean values.

RESULTS

Excretion of ketoacids

The 2,4-dinitrophenylhydrazone of a dominant metabolite excreted by young submerged cultures of *S. cinnamonensis* regulatory mutants (Pospíšil *et al.* 1984) was isolated and identified by NMR and mass spectroscopy as derivative of 2-ketoisovaleric acid (KIV) (data not shown). In a preliminary thin-layer chromatography test we found that this ketoacid reached a maximum concentration in approximately 1-d old cultures. Therefore in 24-h cultures we measured the concentration of all three BCKAs as their 2,4-dinitrophenylhydrazone derivatives by high performance liquid chromatography.

The production of organic acids by streptomycetes is substantially influenced by nitrogen sources (Madden *et al.* 1996). Cultivation of *S. cinnamonensis* in basic nitrate-glucose medium supplemented with ammonium salts led to acidification of the culture and consequently growth stopped. Therefore we have developed MMC medium containing $CaCO_3$ to neutralize the acids produced. It enabled us to observe the influence of NH_4^+ on enzyme levels and ketoacid excretion. Experiments were conducted with the parent C-100-5 strain and its regulatory mutants in MM, MMC and MMC enriched with NH_4^+ media. In all media the influence of exogenous branched chain amino acids was also explored.

The results obtained in MM medium are summarized in Fig. 1. In unsupplemented media the highest KIV concentrations were observed with NLR-3 and NLR-12 strains. Exogenous valine, with the exception of the NLR-12 strain, caused a further increase in KIV excretion compared with the control. The presence of leucine and isoleucine added to cultural media gave rise to production of corresponding ketoacids, i.e. 2-ketoisocaproic acid (KIC) and 2-keto-3-methylvaleric acid (KMV), respectively. In addition, leucine also mostly increased the excretion of KIV.

According to the data from cultivation in MMC medium (Fig. 2), NLR-3 and NLR-12 strains again produced the highest amounts of KIV, similar to those obtained on MM medium; 50 mmol l^{-1} NH_4^+ reduced the excretion of this acid, but relative changes were different, e.g. with NLR-3, the reduction was 87%, but only 11% in ACB-NLR-7. The 10 mmol l^{-1} valine added together with NH_4^+ substantially increased the KIV production in ABR-21 and ABR-38. Again, leucine in combination with NH_4^+ provoked the production of KIC and in some cases supported the production of KIV. This effect was unambiguously demonstrated by ACB-NLR-7 in both MM and MMC media.

Irrespective of the medium used, the standard C-100-5 control strain produced BCKAs from exogenous branched chain amino acids only. In regulatory mutants of *S. cinnamonensis* the KIV was accumulated by *de novo* synthesis due to its altered regulation. 2-ketoisovaleric acid and the other BCKAs reached their maximum concentration in the fermentation fluid at approximately 24 h and then they were reutilized, gradually disappeared and became mostly undetectable in 48-h culture (data not shown).

Enzyme analysis

To characterize the regulatory mutants, we studied the enzyme activities of AHAS, TAB and VDH from *S. cinnamonensis* cells growing under the conditions used for determination of BCKA production. Optimal conditions for enzyme assays were similar to those used in published studies with the exception of TAB, which required pyrophosphate buffer, pH 8·6, while Priestly and Robinson (1989) used phosphate buffer, pH 7·0, for *S. cinnamonensis* TAB.

In the standard C-100-5 strain, valine added to the cultural medium increased the levels of all enzymes studied. In cells grown in MM medium (Table 2) the activity of AHAS rose sevenfold and that of VDH and TAB twofold. Valine exerted a similar effect on cells from MMC medium (Table 3) even in the presence of NH_4^+; the activity of VDH was increased by a factor of 6.

In regulatory mutants, higher activities of enzymes participating in the metabolism of branched chain amino acids, with the exception of AHAS from ABR-21, ABR-38 and transaminase from ABR-38 strains, were generally observed (Tables 2 and 3). The activity of AHAS from ACBR-2 was increased over the control parent strain by factors of 23 (MM medium) and 31 (MMC medium). The feedback regulation by valine was operating in standard *S. cinnamonensis* C-100-5 and ACBR-2 strains at a similar level. In ABR, NLR and ACB-NLR-7 strains this feedback regulation was inefficient. Furthermore, slightly elevated AHAS activities were recorded in the presence of 10 mmol l^{-1} valine in the assay

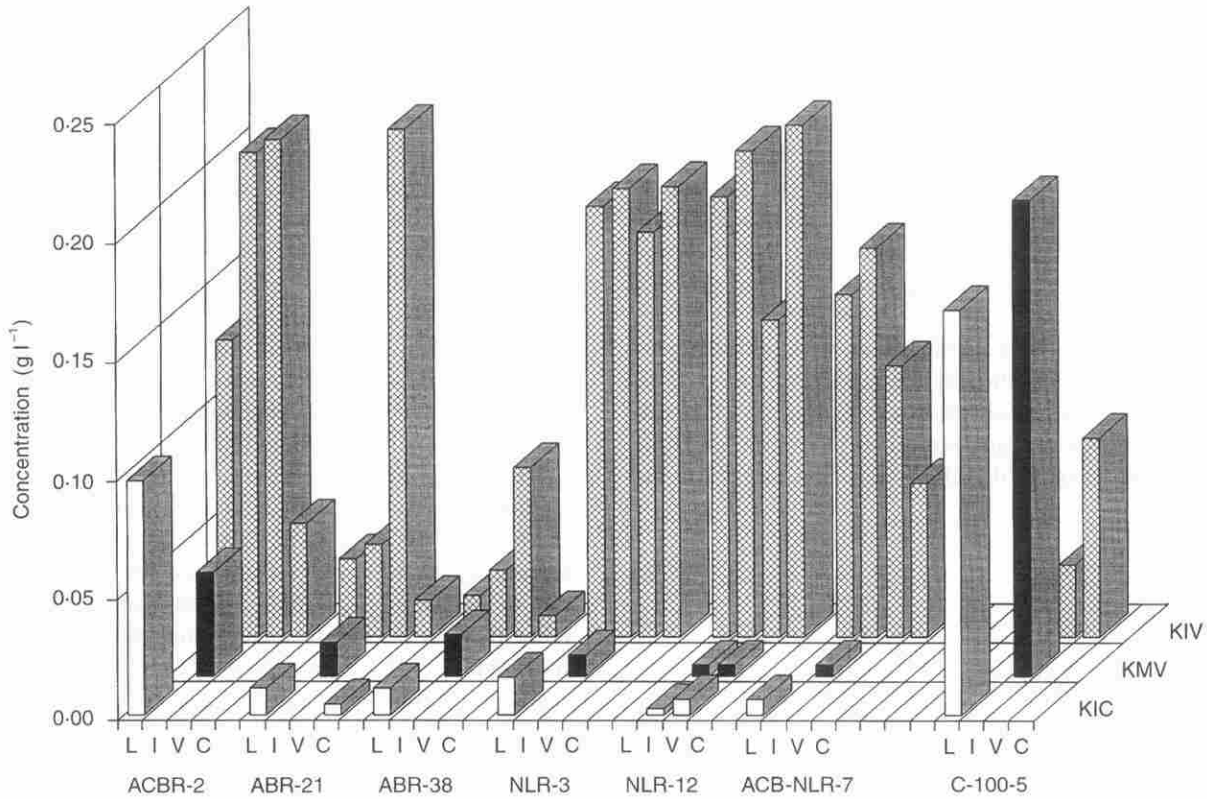

Fig. 1 Concentration of 2-ketoisovalerate (KIV), 2-keto-3-methylvalerate (KMV) and 2-ketoisocaproate (KIC) excreted by 24 h cultures of *Streptomyces cinnamonensis* strains in minimal liquid growth medium (MM) (C) and MM media supplemented with 10 mmol l^{-1} valine (V), isoleucine (I) and leucine (L), respectively

mixture in samples from NLR-3, NLR-12 and double resistant strain ACB-NLR-7, probably due to the influence of valine on the enzyme stability under the assay conditions. On the other hand, the same concentration of valine in C-100-5 and ACBR-2 AHAS caused approximately 50% inhibition.

Transaminase exhibited higher activities in extracts obtained from cells grown in media supplemented with valine. In the presence of NH_4^+ the TAB activity was slightly increased in ACBR-2, ACB-NLR-7 and both NLR strains.

Valine dehydrogenase is inducible by valine (Tang and Hutchinson 1995). In all regulatory mutants we could therefore expect VDH induction by an endogenous valine and this phenomenon occurred, e.g. with ABR-21 grown on MM medium, VDH activity was increased 22-fold compared with the C-100-5 strain. However, further valine added to MM medium of regulatory mutants had a negative effect on the VDH level. The influence of NH_4^+ on VDH expression was studied in MMC medium. In regulatory mutants a depression of synthesis of this enzyme was observed, in agreement with the observations of others (Navarrete *et al.* 1990; Tang and Hutchinson 1995), but in the standard C-100-5 strain an adverse effect of ammonia was not recorded and VDH activity even increased slightly for both valine and leucine used as substrates *in vitro*. This positive influence resembles observations in *Streptomyces aureofaciens* and *S. fradiae* (Nguyen *et al.* 1995).

DISCUSSION

The primary aim of the preparation of *S. cinnamonensis* regulatory mutants was to shift the ratio of monensins produced in favour of the desired monensin A through improved availability of butyrate derived from KIV. The standard C-100-5 strain produced equal amounts of both monensins, while in ABR and NLR strains the proportion of monensin A from total monensin production rose to 80%, in ACBR-2 to nearly 90% and in double-resistant ACB-NLR strains to 90–93% (Pospíšil *et al.* 1984). The overproduction of valine in regulatory mutants was also demonstrated by dramatic changes in cellular fatty acid composition (Pospíšil *et al.* 1985; Pospíšil and Řezanka 1994). The content of even *iso*-branched chain fatty acids, whose starting unit isobutyryl-CoA originates from valine, was significantly increased in regulatory mutants,

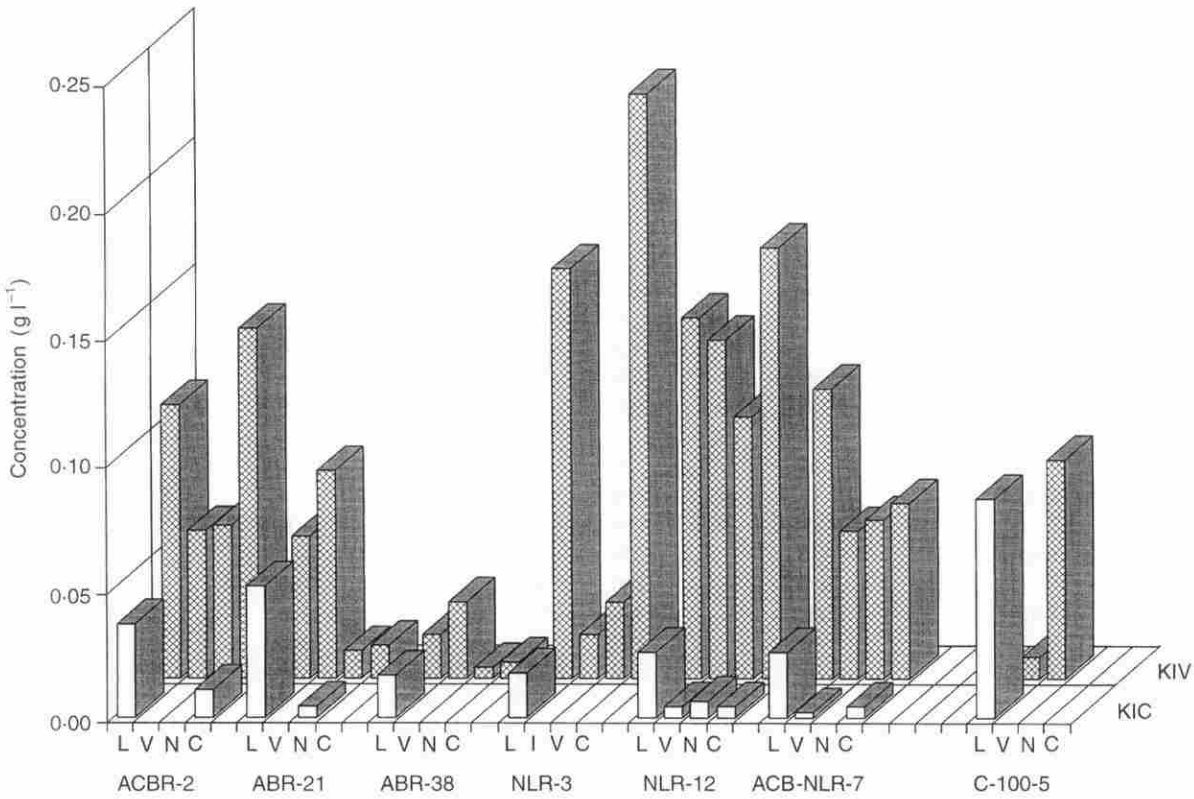

Fig. 2 Concentration of 2-ketoisovalerate (KIV) and 2-ketoisocaproate (KIC) excreted by 24 h cultures of *Streptomyces cinnamonensis* strains in minimal liquid growth medium buffered with $CaCO_3$ (MMC) (C), medium supplemented with 50 mmol l^{-1} NH_4^+ (N), 50 mmol l^{-1} NH_4^+ plus 10 mmol l^{-1} valine (V) and 50 mmol l^{-1} NH_4^+ plus 10 mmol l^{-1} leucine (L), respectively

e.g. in the double-resistant strain ACB-NLR-13 nearly threefold to 65·6% of total fatty acids (Pospíšil *et al.* 1985).

The KIV production reported here represents further evidence of altered regulation of the valine-isoleucine biosynthetic pathway. The excretion of KIV by *S. cinnamonensis* was the first example in streptomycetes, where a *de novo* synthesized BCKA was excreted due to changed metabolic regulation. Streptomycetes are known producers of pyruvate and 2-ketoglutarate (Doskočil *et al.* 1959; Gräfe *et al.* 1975; Ahmed *et al.* 1984; Surowitz and Pfister 1985; Madden *et al.* 1996). In *S. cinnamonensis* we have proved the pyruvate excretion in a similar way to the KIV production; it was not studied in detail. Occurrence of KMV in the cultivation media was reported in *S. avermitilis* after supplementation with isoleucine (Němeček *et al.* 1992). In *Salmonella typhimurium* excretion of KIV and KIC was observed and shown to be dependent on the iron level in the medium (Reissbrodt *et al.* 1997).

The production of KIV by *S. cinnamonensis* was stimulated by exogenous leucine. This phenomenon may be explained by feedback inhibition of isopropylmalate synthase (Pátek *et al.* 1994), the first enzyme of the leucine pathway, which condenses KIV with acetyl-CoA. Upon cultivation in the medium without strong buffering (MM) ammonium ions caused deleterious excretion of acids. In the MMC medium, the excretion of KIV was decreased in the presence of NH_4^+; however, an influence of Ca^{2+} ions released from $CaCO_3$ on metabolism cannot be ruled out.

Present biochemical analysis of *S. cinnamonensis* regulatory mutants revealed the way in which branched chain amino acid synthesis was released from regulation. In the ACBR-2 strain the substantially higher level of AHAS (23- and 31-fold) can be explained by derepression of the enzyme synthesis. 2-Amino-3-chlorobutyrate seems to be an efficient valine analogue for the isolation of desired mutants. In *S. coelicolor* 4-azaleucine-resistant mutants the AHAS activities were increased by factors of 3 to 7 over the wild type (Potter and Baumberg 1996). The proposed derepression of the valine–isoleucine pathway of the *S. cinnamonensis* ACBR-2 strain is in agreement with the ability of 2-amino-3-chlorobutyrate to bind to tRNA and repress the corresponding pathway (Williams and Freundlich 1969). Surprisingly, NLR mutants exerted both elevated AHAS activity and switched off feedback regulation by valine. The level of AHAS in NLR

Table 2 Activities (pmol s^{-1} mg^{-1}) of enzymes from *Streptomyces cinnamonensis* cells grown on minimal liquid growth medium

		AHAS				TAB		VDH	
		Valine concentration (mmol l^{-1})				Substrate		Substrate	
Strain	Cultivation	0	0·1	10	100	Valine	Leucine	Valine	Leucine
C-100-5	Control	130	100	76	—	420	700	39	9
	V*	900	770	530	—	1000	1400	88	23
	L†	77	56	41	—	640	1100	140	30
	I‡	130	110	78	—	750	1200	120	31
ACBR-2	Control	2900	2500	1600	—	2600	3500	530	91
	V	3500	2900	2000	—	3900	5200	220	29
ABR-21	Control	31	31	29	—	790	1200	870	150
	V	24	23	23	—	1500	2100	230	21
ABR-38	Control	47	47	44	22	560	1100	750	120
	V	108	108	99	61	1200	2100	230	16
NLR-3	Control	1400	—	1400	1100	2700	3800	270	45
	V	1100	—	1200	870	3500	4800	230	32
NLR-12	Control	1300	—	1300	970	3200	4300	300	46
	V	810	—	840	660	4000	5500	180	17
ACB-NLR-7	Control	520	—	530	380	3300	4700	470	100
	V	390	—	380	280	4600	6000	260	18

* Medium supplemented with 10 mmol l^{-1} valine.
† Medium supplemented with 10 mmol l^{-1} leucine.
‡ Medium supplemented with 10 mmol l^{-1} isoleucine.
Data represent the mean of at least three measurments, standard deviations were within 8%.
AHAS, acetohydroxyacid synthase; TAB, transaninase of branched chain amino acids; VDH, valine dehydrogenase; —, not determined.

mutants was lower than in ACBR-2, but was nevertheless 10- and 23-fold higher compared with the standard C-100-5 strain. Until now, norleucine has been used as the methionine analogue, e.g. for *Cephalosporium acremonium* (Matsumura *et al.* 1982) and *Escherichia coli* (Chattopadhyay *et al.* 1991). In the double-resistant ACB-NLR-7 strain the disturbed feedback regulation was conserved, but the total activity of AHAS was only four to eight times higher than in C-100-5. This observation was confirmed with two other ACB-NLR strains (data not shown).

In 2-aminobutyrate-resistant *S. cinnamonensis* strains a feedback control of AHAS also seems to be destroyed. The low AHAS activity in *S. cinnamonensis* ABR strains was surprising, but not without precedent; a substantial reduction in AHAS levels was observed in *S. fradiae* aminobutyrate-resistant strains (Vančura *et al.* 1989). Resistance to aminobutyrate was used for the selection of valine-producing *Serratia marcescens* strains; AHAS in these mutants was less sensitive to feedback inhibition by valine and furthermore a derepression of the corresponding biosynthetic pathway was observed (Kisumi *et al.* 1973).

It is generally accepted that AHAS consists of two kinds of subunits. The large ones (about 60 kDa polypeptides) are the catalytic subunits, while smaller polypeptides (9–17 kDa) are involved in the feedback control of the enzyme by valine (Weinstock *et al.* 1992). It is possible to deduce that resistance of *S. cinnamonensis* to 2-aminobutyrate and norleucine leads to selection of strains with mutation in the *ilvN* gene encoding the small subunits of AHAS. So far the detailed enzymological and genetic studies of acetohydroxy acid synthases have been conducted predominantly in enterobacteria. Among streptomycetes, only the sequence of AHAS coding *ilvBN* genes in *S. avermitilis* has been described and proven to be similar to that of other bacteria (DeRossi *et al.* 1995). The properties of AHAS in norleucine-resistant *S. cinnamonensis* mutants are provoking and worthy of further study.

In conclusion, the present paper highlighted the properties

Table 3 Activities (pmol s^{-1} mg^{-1}) of enzymes from *Streptomyces cinnamonensis* cells grown on minimal liquid growth medium buffered with $CaCO_3$

		AHAS				TAB		VDH	
		Valine concentration (mmol l^{-1})				Substrate		Substrate	
Strain	Cultivation	0	0·1	10	100	Valine	Leucine	Valine	Leucine
C-100-5	Control	54·0	39·0	31·0	—	790·0	1200·0	34·0	7·2
	N*	76·0	62·0	46·0	—	710·0	1000·0	53·0	13·0
	NV†	310·0	270·0	190·0	—	1400·0	1900·0	340·0	53·0
ACBR-2	Control	1700·0	1400·0	950·0	—	1300·0	1500·0	500·0	110·0
	N	1100·0	900·0	660·0	—	1600·0	1800·0	240·0	63·0
	NV	1200·0	1000·0	740·0	—	4300·0	4700·0	440·0	74·0
ABR-21	Control	2·8	2·7	2·8	1·9	1100·0	1900·0	310·0	78·0
	N	3·4	3·4	3·2	2·6	1100·0	1800·0	220·0	47·0
	NV	7·1	7·2	6·8	6·2	1200·0	1700·0	240·0	42·0
ABR-38	Control	3·7	3·6	3·2	3·2	780·0	1300·0	560·0	100·0
	N	3·5	3·4	3·1	3·2	680·0	1200·0	39·0	12·0
	NV	6·0	5·8	5·4	4·2	1000·0	1600·0	280·0	58·0
NLR-3	Control	1200·0	1300·0	1400·0	1100·0	4000·0	5400·0	300·0	58·0
	N	580·0	600·0	750·0	710·0	4800·0	6400·0	210·0	43·0
	NV	540·0	580·0	640·0	520·0	4300·0	5800·0	140·0	30·0
NLR-12	Control	1000·0	1100·0	1100·0	800·0	3000·0	3500·0	290·0	52·0
	N	560·0	590·0	640·0	520·0	3000·0	3600·0	170·0	39·0
	NV	610·0	640·0	700·0	550·0	4600·0	5700·0	240·0	42·0
ACB-NLR-7	Control	440·0	—	460·0	340·0	3900·0	4700·0	410·0	67·0
	N	230·0	—	260·0	200·0	4200·0	5200·0	270·0	47·0
	NV	200·0	—	230·0	190·0	4300·0	6000·0	140·0	31·0

* Medium supplemented with 50 mmol l^{-1} NH_4^+.
† Medium supplemented with 50 mmol l^{-1} NH_4^+ and 10 mmol l^{-1} valine.
Data represent the mean of at least three measurments, standard deviations were within 8%.
AHAS, acetohydroxyacid synthase; TAB, transaninase of branched chain amino acids; VDH, valine dehydrogenase; —, not determined.

of *S. cinnamonensis* regulatory mutants in which released control of branched chain amino acid synthesis was reflected in both primary and secondary metabolism.

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5.2 Overproduction of 2-ketoisovalerate and monensin production by regulatory mutants of *Streptomyces cinnamonensis* resistant to 2-ketobutyrate and amino acids.

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Overproduction of 2-ketoisovalerate and monensin production by regulatory mutants of *Streptomyces cinnamonensis* resistant to 2-ketobutyrate and amino acids

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Abstract

We have prepared *Streptomyces cinnamonensis* mutants resistant to 2-ketobutyrate in combination with valine or isoleucine. The selection process was strictly dependent on the presence of nitrate. Sixteen representatives of three groups of mutants were biochemically analyzed as to the activities of enzymes of the branched-chain amino acid metabolism: acetohydroxy acid synthase, transaminase B and valine dehydrogenase. A majority of mutants resistant to 2-ketobutyrate and valine (BVR strains) exerted no feedback regulation of the acetohydroxy acid synthase by valine and proved to be potent producers of 2-ketoisovaleric acid. The BVR-13 strain produced up to 2.4 g l^{-1} of 2-ketoisovaleric acid during a 72-h cultivation in a nitrate–glucose medium. The excreted valine reached only 2.6% of 2-ketoisovaleric acid concentration. Bentonite present in the culture medium reduced NH_4^+ concentration and augmented the 2-ketoisovaleric acid production 1.3-fold. Half of the tested strains cultivated in a soya-bean medium exerted a substantially higher production of antibiotic monensins with elevated proportion of the monensin A.

Keywords: *Streptomyces cinnamonensis*; 2-Ketoisovalerate; Acetohydroxy acid synthase; Valine; Metabolic regulation; Monensin

1. Introduction

Streptomyces cinnamonensis is a producer of oligoketide polyether antibiotics monensins A and B whose building units are acetate, propionate and butyrate [1]. Branched-chain amino acids (BCAA) can serve as precursors of these units. Valine is metabolized to butyrate, which is incorporated only into monensin A [2], isoleucine serves as a source of acetate and propionate building units [3]. Previously, *S. cinnamonensis* regulatory mutants resistant to valine analogs and producing the desirable monensin A were prepared [4] and a deregulation of valine-synthesizing pathway in these mutants was demonstrated [5].

The first enzyme in the pathway of BCAA is acetohydroxy acid synthase (AHAS). This enzyme in valine synthesis condenses pyruvate with active acetaldehyde, derived from the additional pyruvate, to yield acetolactate. In a parallel isoleucine pathway,

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2-ketobutyrate condenses with active acetaldehyde molecule leading to formation of acetohydroxybutyrate, the second AHAS metabolite. The biosynthesis of valine and isoleucine is completed by amination of the corresponding branched-chain 2-keto acids (BC2KA). This reaction is catalyzed by transaminase B (TAB). Valine dehydrogenase (VDH), an NAD^+-dependent enzyme, can also participate on the amination, although in *Streptomyces* VDH is rather considered as catabolic enzyme catalyzing the oxidative deamination.

AHAS is the target for various structurally diverse herbicides. The biochemical and genetic analyses suggested that the action of herbicide sulfometuron methyl on AHAS in *Salmonella typhimurium* results in toxic accumulation of 2-ketobutyrate [6,7]. 2-Ketobutyrate, in high concentrations, may interfere with metabolic pathways in which it competes with physiological substrates, such as 2-ketoisovalerate (KIV) in ketopantoate synthesis for CoA [8]. 2-Ketobutyrate is also considered as a putative alarmone of *Escherichia coli* [9].

Since germinating cells of *S. cinnamonensis* exert sensitivity to 2-ketobutyrate, we have used this substance in combination with amino acids for preparation of resistant strains. Among them, new types of regulatory mutants were found. They have been characterized as to changes in primary and secondary metabolism, i.e. by enzyme activities of BCAA pathway, BC2KA production and monensin A and B biosynthesis. Ketobutyrate-resistant strains presented here have been compared with regulatory mutants prepared and studied previously [4,5].

2. Materials and methods

2.1. Organism and isolation of mutants

S. cinnamonensis C-100-5 [4,5] has been used as the parental and control strain. Spore suspension of this strain, which was UV-irradiated to obtain a survival rate of 0.1–0.5%, was inoculated into minimal medium (MKB) consisting of (g l^{-1}): $NaNO_3$ 10, Na-salt of 2-ketobutyrate 10, $MgSO_4 \cdot 7H_2O$ 1.0, K_2HPO_4 0.5, $CaCl_2 \cdot 2H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01 and incubated at 34°C. A detailed protocol of isolation procedure and strain designation are presented in Fig. 1. Strains were maintained on a sporulation agar medium [4].

2.2. Culture conditions

Strains were cultivated in a soya-bean medium [3] for monensin production and minimal nitrate–glucose medium (MM; g l^{-1}: $NaNO_3$ 10, glucose 38, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 0.05) for BC2KA production [5]. The effect of 2-ketobutyrate (6.2 and 12.4 g l^{-1} concentration), bentonite (Bentonit BP 73, Lachema Brno, Czech Republic; 10 g l^{-1}) and valine (5 g l^{-1}) was studied in MM medium with substances added before inoculation.

2.3. Enzyme assays and other analyses

Enzyme activities of AHAS, TAB and VDH were

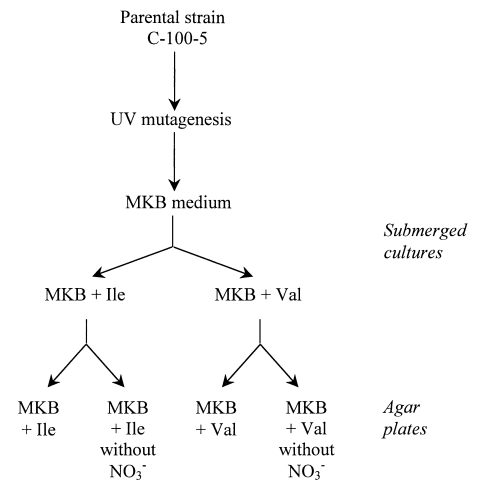

Fig. 1. Procedure for the isolation of *S. cinnamonensis* 2-ketobutyrate resistant mutants. UV-irradiated spore suspension of C-100-5 strain was incubated in MKB medium on a rotary shaker at 180 rpm. After 10 days of cultivation (without signs of visible growth), the culture was divided into two parts and the cells were transferred to a fresh MKB medium enriched with L-valine or L-isoleucine (3 g l^{-1}), and cultivated for another 3 weeks. The concentrated cell suspensions were then again divided into two parts, inoculated onto agarized MKB media with varied N-sources and incubated for 8 weeks.

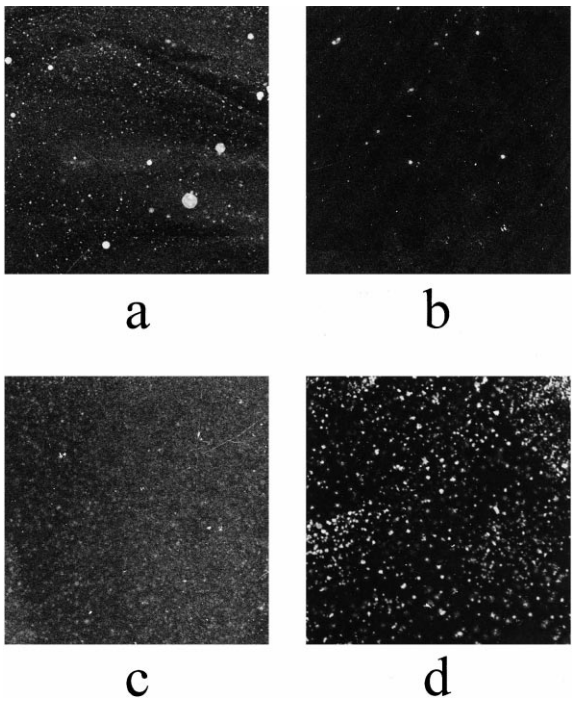

Fig. 2. Frequency and appearance of *S. cinnamonensis* colonies after an 8-week cultivation on the selection agar media: (a) MKB+Ile; (b) MKB without nitrate+Ile; (c) MKB+Val; (d) MKB without nitrate+Val.

measured as described before [5]. Assay of BC2KA [5] was modified owing to the higher production. The amount of derivatizing agent was increased proportionally (0.2 ml of the fermentation broth+4 ml of 0.1% solution of 2,4-dinitrophenylhydrazine in 2 M HCl). The concentration of nitrate, glucose and ammonium was measured using UV-Nitrate-Test (Boehringer Mannheim Cat. No. 905 658), Glucose GOD 250 (Lachema, Czech Republic), and spectrophotometrically according to Burck [10], respectively. The concentration of amino acid in fermentation broth was ascertained with a BIOCHROM-20 analyzer (Pharmacia, Uppsala, Sweden). Production of monensins A and B was assayed according to the previous work [4].

3. Results and discussion

Both morphology and number of *S. cinnamonensis* mutant colonies, appearing on agar plates at the end of selection process (Fig. 1), varied depending on the nitrogen source. Agar medium containing both valine and $NaNO_3$ suppressed growth, while valine alone permitted development of a number of colonies. On the both types of isoleucine media, the occurrence of resistant colonies was substantially lower (Fig. 2). Viable colonies were isolated by repeated cultivation on the corresponding selection agar media. The 83 isolates were inoculated on the sporulation agar and tested in submerged culture. According to our previous experience [4,5], the altered regulation of BCAA biosynthesis was reflected either by the increased proportion of monensin A produced, or by excretion of BC2KA. On the basis of these screening analyses, 16 strains from the three groups of resistant strains were investigated in more detail.

Enzyme activities and KIV production in 24-h cultures are presented in Table 1. Three of the BIR strains tested had about a 2-fold AHAS activity which might reflect a weak rise of the expression level, similar to that observed in TAB activities. VDH varied from undetectable activity in BIR-5 to 12-fold increase in BIR-13. Except for BIR-5, AHAS underwent a normal feedback valine regulation. The strain BIR-5 was the only KIV producer among BIR mutants. In BINR strains AHAS activities were generally lower compared to the standard parental strain C-100-5, on the contrary, VDH and TAB in these strains showed a slightly elevated activity. None of the BINR strains produced BC2KA.

All BVR strains exhibited a lower AHAS activity and the feedback regulation by valine was switched off, with the exception of the BVR-10 which resembled the parental strain in this respect. Valine at 100 mM inhibited the AHAS activity in four strains only by 8–14.3% and a negligible inhibition (2.1%) was recorded in BVR-18, 10 mM valine did not inhibit or even elevate AHAS activity under assay conditions. An extreme case of this elevation (by 38%) was observed again in BVR-18 strain. TAB activities were slightly (up to 2-fold) increased in the BVR group, while VDH activity varied over a wide range of 10–1600% of the level in the parental strain. The majority of the BVR strains excreted KIV.

The KIV production by BVR strains was explored during a 3-day cultivation (Fig. 3). With the exception of the BVR-18 strain, KIV accumulated during

Table 1
Activities of enzymes and production of KIV in 24-h cultures of *S. cinnamonensis* grown in MM medium

Strain	AHAS						VDH		TAB		Production of KIV (g l^{-1})
	Valine concentration (mM)						Activity (pkat mg^{-1})	Relative activity[a] (%)	Activity (pkat mg^{-1})	Relative activity[a] (%)	
	0		10		100						
	Activity (pkat mg^{-1})	Relative activity[a] (%)	Activity (pkat mg^{-1})	Inhibition[b] (%)	Activity (pkat mg^{-1})	Inhibition[b] (%)					
C-100-5	240.0	100.0	130.0	45.8	96.0	60.0	48	100.0	350	100.0	0
BIR-5	200.0	83.3	200.0	0.0	–[c]		n.d.[d]		620	177.1	0.62
BIR-10	500.0	208.3	330.0	34.0	–		380	791.7	800	228.6	0
BIR-11	440.0	183.3	270.0	38.6	–		110	229.2	620	177.1	0
BIR-12	280.0	116.7	170.0	39.3	–		58	120.8	620	177.1	0
BIR-13	550.0	229.2	340.0	38.2	–		590	1229.2	700	200.0	0
BIR-16	180.0	75.0	77.0	57.2	–		460	958.3	800	228.6	0
BINR-1	180.0	75.0	110.0	38.9	–		92	191.7	340	97.1	0
BINR-5	92.0	38.3	58.0	37.0	–		120	250.0	530	151.4	0
BINR-12	98.0	40.8	59.0	39.8	–		120	250.0	520	148.6	0
BINR-16	150.0	62.5	85.0	43.3	–		120	250.0	550	157.1	0
BVR-7	6.6	2.8	6.9	−4.5	6.0	9.1	4.7	9.8	520	148.6	0.13
BVR-10	140.0	58.3	84.0	40.0	–		56	116.7	360	102.9	0
BVR-13	25.0	10.4	25.0	0.0	23.0	8.0	8.9	18.5	620	177.1	1.21
BVR-14	21.0	8.8	21.0	0.0	18.0	14.3	8.3	17.3	670	191.4	1.10
BVR-15	32.0	13.3	32.0	0.0	28.0	12.5	4.1	8.5	500	142.9	0.77
BVR-18	94.0	39.2	130.0	−38.3	92.0	2.1	770	1604.2	560	160.0	0.12

Data represent the mean of at least three measurements, standard deviations were within 8%.
[a]Enzyme activity related to that in the control strain.
[b]Inhibition = 100−100×(activity with valine/activity without valine).
[c]Not determined.
[d]Not detected.

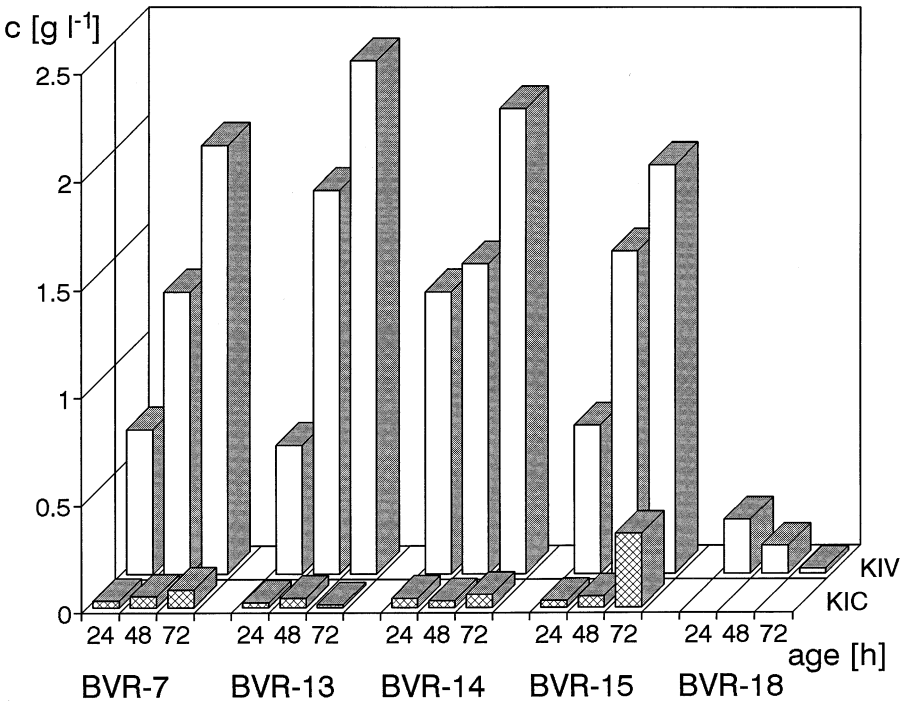

Fig. 3. The production of 2-ketoisovalerate (KIV) and 2-ketoisocaproate (KIC) by *S. cinnamonensis* BVR mutants in MM medium during a 3-day cultivation.

the cultivation and a maximum of 2.4 g l^{-1} was found at 72 h in BVR-13. At this point, the BVR strains differed from the previously described analog-resistant mutants [5], where the excreted KIV reached its maximum concentration in a 24-h culture and was rapidly reutilized. The excretion of 2-keto-isocaproate, a precursor of leucine derived from KIV, was also observed in BVR mutants, while 2-keto-3-methylvalerate, a precursor of isoleucine, has never been detected. BVR regulatory mutants explored in this study are very potent KIV producers. The concentration of the KIV excreted by the previously described norleucine-resistant strains [5] reached only about 10% of the current production.

In the BVR-13 strain, excreting the maximum amount of KIV, we tried to further increase the KIV production. Supplementation with the natural bentonite (10 g l^{-1}) reduced the extracellular NH_4^+ level and improved the KIV production to 133%, while nitrate (not shown) and glucose utilization rates were not affected (Fig. 4). Concentration of KIV reached its maximum in a 60-h culture and then decreased. In the culture without bentonite KIV concentration increased until 72 h. A 2-fold amount of bentonite (20 g l^{-1}) added had no further effect on the KIV production. The effect of bentonite on the control of ammonium level in the medium has already been described [11]; however, the positive effect on the KIV production is not clear. It probably influenced the overall metabolism, not only particular reactions in the KIV biosynthesis.

As KIV is the immediate precursor of valine, the amount of excreted amino acids was measured in 48- and 79-h fermentation broth in order to estimate the conversion of KIV to valine. In 48-h samples, the concentration of all amino acids was approximately 60 mg l^{-1}, irrespective of the bentonite presence, and valine proportion was about 33%. The samples taken at the end of the cultivation contained a higher amino acid concentration: 84 mg l^{-1} (control) and 130 mg l^{-1} (with bentonite) and valine proportion was also higher; viz. 42 and 63%, respectively. In all samples analyzed, valine was a dominating amino acid. Proportion of leucine was maximum 3.3% in the sample from 79 h with bentonite and concentration of isoleucine was lower by an order of magni-

tude. These data demonstrated that a minor amount (up to 2.6%) of overproduced KIV is transformed to the excreted valine.

In our previous work [5] we have shown that *S. cinnamonensis* is able to transform the exogenous BCAA to corresponding BC2KA. We tested this ability in the BVR mutants chosen on the basis of differences in enzyme activities and KIV production. In 24-h cultures of strains BVR-7, BVR-13 and BVR-18, supplementation with valine increased KIV production 2.2-, 1.3- and 9-fold, respectively. This experiment also foreshadows prevailing enzyme activities in the direction of valine deamination over the amination of KIV to valine. TAB requires glutamate and a corresponding system for its regeneration. In *Corynebacteriun glutamicum*, which was used for production of leucine from exogenous 2-ketoisocaproate, this requirement was fulfilled by glutamate dehydrogenase [12]. In *S. cinnamonensis* such or similar system might not operate at adequate level, leading to the KIV accumulation. It is difficult to compare the roles and possible predominance of VDH or TAB in the KIV conversion to valine. TAB activity in BVR-7 and BVR-13 was slightly elevated and VDH level in the both strains was very low (Table 1). However, the VDH activities in vivo are unknown and may differ from those assayed in cell-free extracts.

Table 2
Production of monensin A and B by *S. cinnamonensis* in soya-bean medium after a 6-day cultivation

Strain	Total production of monensins A and B (%)	Monensin A proportion (%)
C-100-5	100[a]	44
BIR-5	467	62
BIR-10	280	75
BIR-11	248	72
BIR-12	355	50
BIR-13	588	63
BIR-16	102	68
BINR-1	3	–[b]
BINR-5	139	44
BINR-12	135	40
BINR-16	397	41
BVR-7	95	59
BVR-10	76	69
BVR-13	544	64
BVR-14	726	63
BVR-15	264	59
BVR-18	44	72

Data represent the mean of three measurements.
[a]Control corresponds to 60 mg l^{-1}.
[b]Not determined.

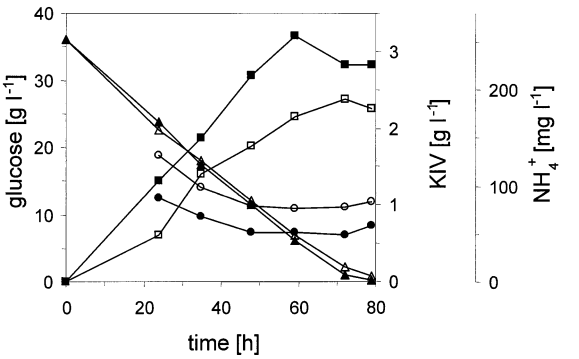

Fig. 4. Production of KIV (□, ■) by *S. cinnamonensis* BVR-13, utilization of glucose (Δ, ▲) and NH_4^+ level (○, ●) in the presence of bentonite (closed symbols) and in the control cultivation (open symbols). Samples (0.2 ml) were taken from each of even parallel flasks and pooled.

The sensitivity of germinating cells of *S. cinnamonensis* to ketobutyrate made the isolation of the resistant strains possible. On the contrary, 2-ketobutyrate added to the submerged culture of *S. cinnamonensis* in MM medium at 50 and 100 mM concentration caused no observable growth inhibition and in 24-h cells, the AHAS activity was elevated 2- and 10-fold, respectively. A similar effect was observed in *C. glutamicum* [13] and *S. typhimurium* [14] and was attributed to a derepression effect of 2-ketobutyrate.

Ketobutyrate-resistant strains represent a new type of regulatory mutants as for their isolation and properties. 2-Ketobutyrate possibly acted not only as a common metabolite during the selection process, and other functions, as described in *E. coli* [9], might be involved. The selection could consequently lead to the isolation of mutants changed not only in the regulation of BCAA synthesis, but also in whole metabolism supplying the corresponding intermediates and thus allowing the KIV overproduction. In our isolation procedure, we also cannot exclude that 2-ketobutyrate is aminated to 2-aminobutyrate which has been used in several cases as an amino acid ana-

log for the isolation of regulatory mutants [15]. *S. cinnamonensis* aminobutyrate-resistant mutants explored in our previous work [5] resembled the BVR strains in AHAS activity and insensitivity to valine inhibition, but differed by very low KIV excretion.

At least three different regulatory mechanisms appear to control the flow of carbon through the pathway: enzyme levels, availability of substrates, and feedback inhibition of AHAS. For the overproduction of KIV, impaired feedback regulation of AHAS seems to be more important than its expression level. According to the previous work, 2-amino-3-chlorobutyrate-resistant *S. cinnamonensis* ACBR-2 had a 31-fold level of AHAS with respect to the control strain; however, it was not an important KIV producer [5]. On the contrary, norleucine-resistant strains [5] and especially BVR mutants (Table 1) without the valine inhibitory effect on AHAS, exhibited a high ability to produce and accumulate KIV.

Production of KIV by *S. cinnamonensis* is a unique example among microorganisms. There are many producers of amino acids, but there is no information concerning the accumulation of their ultimate precursors. *S. cinnamonensis* regulatory mutants can thus serve for both KIV production and study of AHAS regulation. Bacterial AHAS consists of two types of subunits, the large one confers the proper catalytic activity, whereas the smaller participates in the feedback regulation by valine [16]. In our mutants with ineffective regulation of AHAS, it is reasonable to expect changes predominantly in the smaller subunit. Studies in this direction are now in progress.

Metabolism of BCAA in *S. cinnamonensis* provides precursors for the biosynthesis of the oligoketide polyether antibiotic monensins A and B [2–4]. Therefore, in all 16 strains, we assayed the production of both monensin derivatives. The results given in Table 2 show that the BIR and BVR strains produced an augmented proportion of the monensin A, in which valine is the key source of butyrate building unit. Additionally, the total monensins yield was increased in a majority of strains from both groups. All results obtained in this work indicate complex changes in the *S. cinnamonensis* ketobutyrate-resistant strains which can serve as a model for a detailed study of BCAA regulation and for production of primary and secondary metabolites.

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5.3 Mutations in two distinct regions of acetolactate synthase regulatory subunit from *Streptomyces cinnamonensis* result in the lack of sensitivity to end-product inhibition.

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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

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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. © 1999 Academic Press

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-*

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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.

```
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
```

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

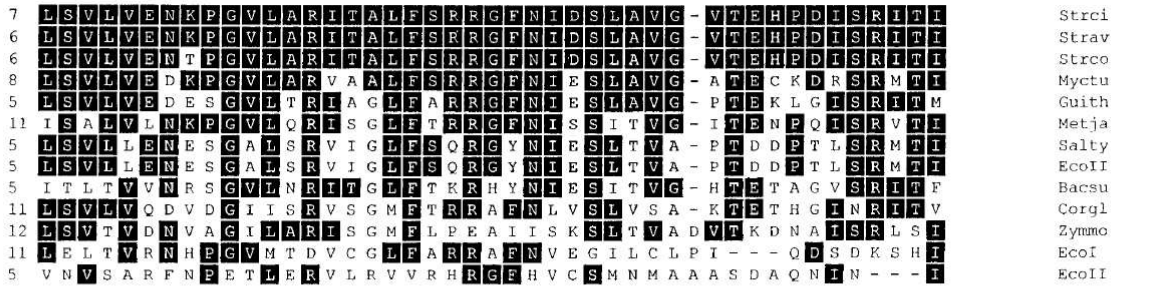

FIG. 2. Conserved domain near the N-terminus of acetolactate synthase small subunit. Number on the left indicates position of the first aa residue. Abbreviations used: Strci, *S. cinnamonensis;* Strav, *S. avermitilis;* Strco, *S. coelicolor;* Myctu, *Mycobacterium tuberculosis;* Guith, *Guillardia theta* (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

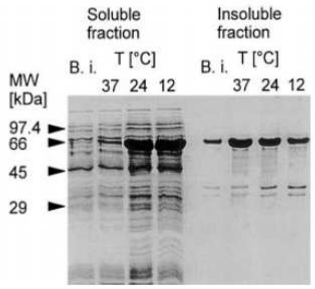

FIG. 3. Expression of the ALS catalytic subunit in *E. coli.* The yield of the soluble form in dependence on postinduction temperature.

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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5.4 Deregulation of acetohydroxy acid synthase: loss of allosteric inhibition conferred by mutations in the catalytic subunit.

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Deregulation of Acetohydroxy-Acid Synthase: Loss of Allosteric Inhibition Conferred by Mutations in the Catalytic Subunit

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ABSTRACT. Acetohydroxy-acid synthases (AHAS) of two mutant strains *Streptomyces cinnamonensis* ACB-NLR-2 and BVR-18 were chosen for this study for their apparent activation by valine, which regularly acts as an allosteric inhibitor. Sequencing the *ilvB* genes coding for the AHAS catalytic subunit revealed two distant changes in the mutants, ΔQ217 and E139A, respectively. Homology modeling was used to propose the structural changes caused by those mutations. In the mutant strain ACB-NLR-2 (resistant to 2-amino-3-chlorobutyrate and norleucine), deletion of Q217 affected a helix in β-domain, distant from the active center. As no mutation was found in the regulatory subunit of this strain, ΔQ217 in IlvB was supposed to be responsible for the observed valine activation, probably *via* changed properties on the proposed regulatory-catalytic subunit interface. In mutant strain BVR-18 (resistant to 2-oxobutyrate), substitution E139A occurred in a conservative loop near the active center. *In vitro* AHAS activity assay with the enzyme reconstituted from the wild-type regulatory and BVR-18 catalytic subunits proved that the substitution in the catalytic subunit led to the apparent activation of AHAS by valine. We suggest that the conservative loop participated in a conformational change transfer to the active center during the allosteric regulation.

The FAD-dependent acetohydroxy-acid synthase (AHAS; acetolactate synthase, EC 2.2.1.6) catalyzes formation of 2-acetolactate and 2-aceto-2-hydroxybutyrate, the intermediates of two parallel pathways of branched-chain amino-acid biosynthesis (Chipman *et al.* 1998). AHAS is a tetramer composed of two kinds of subunits, a catalytic one (IlvB) harboring the active center, and a regulatory one (IlvN) providing the feed-back inhibition of the enzyme by valine (Eoyang and Silverman 1986; Vyazmensky *et al.* 1996). The enzyme requires Mg^{2+} ion and cofactors flavin adenine dinucleotide (FAD) and thiamine diphosphate (TPP) (Chipman *et al.* 1998). Except for enterobacteria, where several isozymes with distinctive properties are present, most prokaryotes including streptomycetes seem to synthesize a single AHAS (Chipman *et al.* 1998), partially sensitive to the end-product inhibition.

Streptomyces cinnamonensis is a producer of oligoether ('polyether') antibiotics monensins A and B. Unlike its homologue, monensin A contains one butyrate unit (Day *et al.* 1973), derived from the valine precursor 2-oxoisovalerate in the course of the production on chemically defined media (Pospíšil *et al.* 1983). In some valine-analog resistant strains with higher metabolite flow through valine biosynthetic pathway, a proportion of monensin A was markedly elevated *via* increased availability of the butyrate unit (Pospíšil *et al.* 1984). Biochemical analysis of *S. cinnamonensis* mutants affected in the regulation of valine biosynthesis revealed the mechanisms of deregulation – insensitivity of AHAS to the end-product inhibition, increased level of AHAS activity, or combination of the two (Pospíšil *et al.* 1998, 1999). Mutations conferring the loss of sensitivity to valine were mostly identified in the AHAS regulatory subunit (Kopecký *et al.* 1999). The aim of the present study was: (*1*) to explain the loss of AHAS sensitivity to valine in two *S. cinnamonensis* strains, in which the regulatory subunit either did not harbor any mutation or the mutations did not prove to confer the observed change in the enzyme regulation; (*2*) to propose an explanation of the unexpected properties of the mutated AHAS using a homology model of the catalytic subunit.

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MATERIALS AND METHODS

Strains and plasmids. S. cinnamonensis parental strain C-100-5 and mutant strains ACB-NLR-2 double resistant to two valine analogs (Pospíšil *et al.* 1984) and BVR-18 growing on 2-oxobutyrate and valine as the sole C and N sources (Pospíšil *et al.* 1999) (Table I) were used. For cloning and heterologous expression *Escherichia coli* strains XL-1 blue, NM-522 and BL-21 (DE3) and vectors pBluescript II KS+ (*Stratagene*, USA) and pET-28b(+) (*Novagen*, USA) were employed.

Table I. *Streptomyces cinnamonensis* strains

Strain	Resistant to	Mutation in IlvN	Phenotype	Reference
C-100-5 (wt)	–	–	inhibition by valine	Pospíšil *et al.* 1984, 1998
ACB-NLR-2	2-amino-3-chlorobutyrate, norleucine	–	apparent valine activation	Pospíšil *et al.* 1984, 1998; Kopecký *et al.* 1999
BVR-18	2-oxobutyrate	I106V, A135P	apparent valine activation	Pospíšil *et al.* 1999; Kopecký *et al.* 1999

Cloning and expression of wild-type and mutant ilvB *and* ilvN *genes in* E. coli. Degenerated forward primer ilvBf with flanking *Nde*I site and reverse primer ilvBr with flanking *Hin*dIII site (Table II) were used for PCR amplification of a fragment harboring *ilvB* gene from genomic DNA of parental and mutant strains. The primers ilvBf and ilvBr were designed according to sequences of *ilvB* gene from *S. avermitilis* (De Rossi *et al.* 1995) and of the neighboring *ilvN* gene from *S. cinnamonensis* (Kopecký *et al.* 1999), respectively. The amplified fragment was treated with *Nde*I, *Hin*dIII and ligated to pET-28b(+) vector. The expression was carried out in *E. coli* BL-21 (DE3) at post-induction temperature 24 °C for 5 h (Kopecký *et al.* 1999). The IlvN protein from the parental *S. cinnamonensis* C-100-5 strain was prepared according to Kopecký *et al.* (1999).

Table II. Oligonucleotides

Primer	Sequence (5′ → 3′)	Position[a]
ilvBf	CCG GAA TCA TAT GAC SGA GCA GGC SAC	816–832
ilvBr	AAC CCA AGC TTG GTG GAC ATG ACG	42–26
ilvBleadf	CCG GAA TTC GAA AGC GCG TCG GCT GA	641–657
ilvBf1	CGA TGA TGG ACT CCA CCC	994–1011
ilvBf2	CTC GTG GAC ATC GCG AAG G	1359–1377
ilvBf3	CGA GAT CGG CAA GAA CCG	1805–1822
ilvBf4	CAT CCA GTA CGA GAA GCC	2099–2116
ilvBf5	GTC GTC GTC GAC TTC ATC G	2535–2553
ilvBr1	GAC CTT GTC GAG GTC TTC G	2501–2483
ilvBr2	CTC GTA CTG GAT GAA GTG C	2111–2093
ilvBr3a	TTG AGG TCG CTC CAC CAG G	1945–1927
ilvBr3b	GAT CAG GTC GGC CTT CTG C	1709–1691
ilvBr4	GTC GAG GCG ATG TGG AAG G	1339–1321
ilvBr5	CTC AAG AGA ACG AAT GAG G	932–914

[a]Numbered according to the sequences deposited in *GenBank* under accession nos. AF175526 (ilvBr) and AY785370 (all other primers).

Preparation of E. coli *crude extract and AHAS assay.* Frozen cells were resuspended in 20 mmol/L 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 used for AHAS reconstitution and activity assay as described by Pospíšil *et al.* (1998). For each assay, three repeats were performed.

Sequencing of the ilvB *genes.* The *ilvB* gene sequences were PCR amplified with primers ilvBleadf and ilvBr (Table II) using chromosomal DNA of *S. cinnamonensis* strains as template. The purified PCR fragments (MinElute PCR Purification Kit; *Qiagen*, Germany) were sequenced from primers ilvBf1–5 and

ilvBr1–5. The chain termination reaction (Sanger *et al.* 1977) was performed according to the cycle sequencing method (Murray 1989) with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 and analyzed on the ABI PRISM 3100 Genetic Analyzer (*Applied Biosystems*, USA).

Software. Sequence data were handled by Lasergene (*DNAStar*, USA) and Chromas (*Technelysium Pty. Ltd.*, Australia) software. Homology modeling was performed on SWISS-MODEL Protein Modeling server (Guex and Peitsch 1997; Schwede *et al.* 2003) using default parameters and crystal structures of *Arabidopsis thaliana* AHAS (*RCSB Protein Data Bank*, ID 1ybhA, 1yhyA, 1yhzA, 1yi0A, 1z8nA) as templates. Quaternary structure of the dimer was proposed based on crystal structure of the yeast AHAS (*RCSB Protein Data Bank*, ID 1jscA, 1jscB).

RESULTS

DNA sequence of the wild-type and mutant ilvB *genes.* Gene *ilvB* from the parental strain *S. cinnamonensis* C-100-5 and both mutant strains was PCR amplified and sequenced from a set of sequencing primers (Table II) covering both strands of the whole *ilvB* sequence. The *ilvB* nucleotide sequence from the parental strain (*GenBank* accession no. AY785370) includes 1848 bp corresponding to 615 amino acids in polypeptide sequence, which shares 92, 90 and 89 % identity with IlvB from *S. avermitilis*, *S. coelicolor* and *S. viridifaciens*, respectively. Nucleotide sequence of *ilvB* from the strain BVR-18 differed from the parental strain C-100-5 in a single substitution C574A resulting in an amino-acid change E139A. In *ilvB* nucleotide sequence from ACB-NLR-2, a triplet AGC at position 805–807 was deleted resulting in a deletion of glutamine 217 at the protein level (Table III).

Table III. Properties of native AHAS compared to heterologously prepared, *in vitro* reconstituted enzyme

Strain	Mutations in *ilvB* gene		Inhibition (%) of AHAS by 10 mmol/L valine	
	nucleotide	amino acid	*in vitro* reconstituted AHAS	crude extract of *S. cinnamonensis*
wt	–	–	45.9	45.8[a]
ACB-NLR-2	ΔAGC805–807	ΔQ217	nd[b]	–112.0
BVR-18	C574A	E139A	–42.0	–38.3[a]

[a]From Pospíšil *et al.* (1998, 1999). [b]Not determined.

Subunit expression and reconstitution of the holoenzyme. The wild-type and mutant *ilvB* ($ilvB_{wt}$, $ilvB_{BVR\text{-}18}$, $ilvB_{ACB\text{-}NLR\text{-}2}$) as well as wild-type *ilvN* ($ilvN_{wt}$) genes were heterologously expressed in *E. coli*. While IlvB proteins from parental strain and BVR-18 were expressed in a soluble form, the main portion of the protein from ACB-NLR2 remained insoluble (Fig. 1).

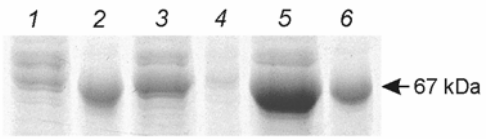

Fig. 1. Heterologous expression of IlvB (67 kDa) in *E. coli*; *1*, *2* – $IlvB_{ACB\text{-}NLR\text{-}2}$, *3*,*4* – $IlvB_{BVR\text{-}18}$, *5*,*6* – $IlvB_{wt}$; *1*,*3*,*5* – soluble fractions, *2*,*4*,*6* – insoluble fractions.

To reconstitute the holoenzymes, $IlvN_{wt}$ was combined with $IlvB_{wt}$, $IlvB_{BVR\text{-}18}$ or $IlvB_{ACB\text{-}NLR\text{-}2}$ and inhibition of the reconstituted enzymes by 10 mmol/L valine was measured. The AHAS containing $IlvB_{BVR\text{-}18}$ exhibited activity comparable to the wild type and activation by valine, confirming the phenotype of the mutation, while the reconstituted enzyme from $IlvB_{ACB\text{-}NLR\text{-}2}$ and $IlvN_{wt}$ exhibited significantly lower activity preventing further characterization of its sensitivity to valine inhibition.

Homology modeling. A homology model of the *S. cinnamonensis* AHAS (Fig. 2) was proposed based on the known three-dimensional structure of the dimer of yeast AHAS catalytic subunits (Pang *et al.* 2002; 40.5 % identity). In comparison with the yeast template, the main differences in the streptomycete IlvB protein sequence were observed in the domain β and its connection to domain α. Three gaps had a minor effect on the modeled 3D structure as they corresponded to the coiled regions (residues 394–402 and 439–440, numbered according to the yeast IlvB) and the flexible interdomain connection (residues 271–279).

In contrast, an insertion of 8 amino acids between the residues 446–447 was modeled as nonstructured loop on the subunit surface. The model revealed that the mutation E139A found in the strain BVR-18 was located in domain α within a loop at the IlvB–IlvB interface near the TPP harbored by the second catalytic subunit of the dimer, while the deletion of Q217 in ACB-NLR-2 occurred in a surface helix of β domain remote from the active center.

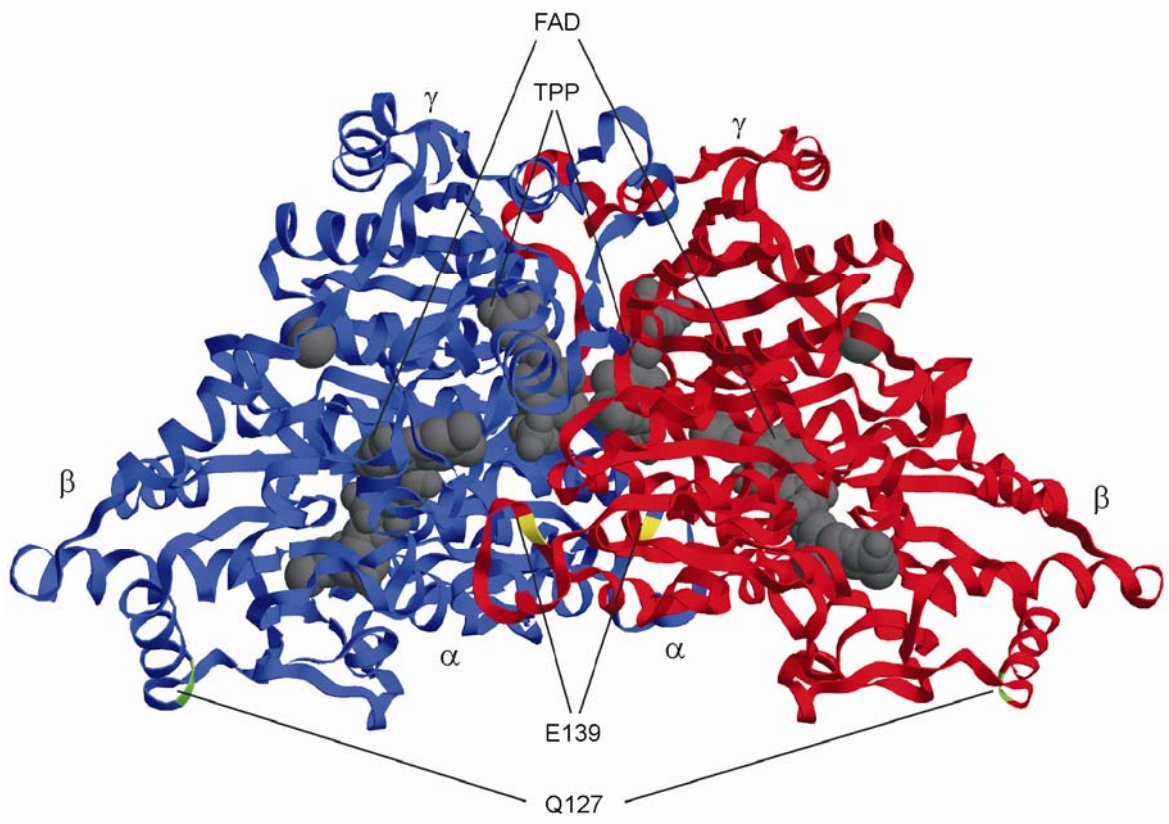

Fig. 2. Homology model of the wild-type AHAS catalytic subunit dimer from *S. cinnamonensis* with highlighted positions of the changes detected in mutant strains; cofactors are shown as spacefill models in *grey*; domains α, β, γ correspond to those in the yeast crystal structure.

DISCUSSION

We proposed an explaination of biochemical properties of AHAS in two regulatory mutants of *S. cinnamonensis*. In both strains, the valine biosynthesis was deregulated due to the lack of AHAS feedback inhibition resulting in 2-oxoisovalerate excretion. Differing from the other regulatory mutants, AHAS assayed *in vitro* in crude extracts of both ACB-NLR-2 and BVR-18 exhibited an apparent activation by its allosteric effector valine (Pospíšil *et al*. 1998, 1999; Kopecký *et al.* 1999).

In ACB-NLR-2, the sequence of *ilvN* gene coding for the AHAS regulatory subunit was identical with the wild type (*unpublished data*). The deletion of Q217 was found in the catalytic subunit; however, its impact on enzyme properties could not be directly verified *in vitro* as the reconstituted AHAS exhibited only low activity. In fact, the main proportion of the mutant IlvB remained in the insoluble fraction. The deletion probably caused an improper folding of the adjacent region or even the entire subunit in the heterologous expression system. However, as no mutation was found in the regulatory subunit, the deletion of Q217 found in the catalytic one was most probably responsible for the observed phenotype.

The deletion of Q217 occurred in β domain of the catalytic subunit, in a helix distant from the active centre. According to the homology model, it resulted in destabilization of the whole helix (*model not shown*). The region of the mutation represented one of major differences in the protein architecture between the allosterically regulated AHAS and the related pyruvate oxidase (POX; Muller and Schulz 1993), which lacked the allosteric regulation. The helix affected by Q217 deletion is not present in POX, where the corresponding region lacks any regular structure. Similarly, the main differences between IlvB in streptomyce-

tes and yeast were observed in this region. Considering the dissimilarity of eukaryotic and prokaryotic regulatory subunits of AHAS, the main difference in structure of the catalytic subunits would be expected within their surface regions touching the regulatory subunits. Thus, the region affected by Q217 deletion might be involved in an interaction with the regulatory subunit and in the transfer of the conformational change mediating the allosteric effect. This assumption was supported by recently published findings by Mitra and Sarma (2008) who identified the region surrounding the FAD binding site as that which is in contact with the regulatory subunits.

In BVR-18, sequencing of the *ilvN* gene revealed two mutations resulting in substitutions I106V and A135P in the protein sequence (Kopecký *et al.* 1999). However, the substitutions had no effect on valine inhibition of *in vitro* reconstituted AHAS containing the wild-type catalytic subunit (Kopecký *et al.* 1999). The enzyme reconstituted in an opposite way, *i.e.* from the mutant catalytic and wild-type regulatory subunits, exhibited biochemical properties fully comparable with the native AHAS assayed in crude extract. Thus, the substitution E139A found in the AHAS catalytic subunit was shown to be responsible for the observed insensitivity to the valine inhibitory effect.

The substitution E139A affected a conservative loop at the IlvB–IlvB interface near the TPP binding site of the second catalytic subunit. The only previously described mutation in IlvB conferring insensitivity to valine in *Nicotiana tabacum* (Hervieu and Vaucheret 1996) was located in the same region. The observed substitution S214L corresponded to the position of proline-148 in *Streptomyces*. Consequently, we assume a direct involvement of the loop in translocation of a conformation change from regulatory/catalytic subunit interface to the active site.

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5.5 Subunit-subunit interactions are weakened in mutant forms of acetohydroxy acid synthase insensitive to valine inhibition

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ORIGINAL PAPER

Subunit–subunit interactions are weakened in mutant forms of acetohydroxy acid synthase insensitive to valine inhibition

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Abstract In acetohydroxy acid synthase from *Streptomyces cinnamonensis* mutants affected in valine regulation, the impact of mutations on interactions between the catalytic and the regulatory subunits was examined using yeast two-hybrid system. Mutations in the catalytic and the regulatory subunits were projected into homology models of the respective proteins. Two changes in the catalytic subunit, E139A (α domain) and ΔQ217 (*β* domain), both located on the surface of the catalytic subunit dimer, lowered the interaction with the regulatory subunit. Three consecutive changes in the N-terminal part of the regulatory subunit were examined. Changes G16D and V17D in a loop and adjacent α-helix of ACT domain affected the interaction considerably, indicating that this region might be in contact with the catalytic subunit during allosteric regulation. In contrast, the adjacent mutation L18F did not influence the interaction at all. Thus, L18 might participate in valine binding or conformational change transfer within the regulatory subunits. Shortening of the regulatory subunit to 107 residues reduced the interaction essentially, suggesting that the C-terminal part of the regulatory subunit is also important for the catalytic subunit binding.

Keywords *Streptomyces cinnamonensis* · Acetohydroxy acid synthase · Subunit–subunit interaction · Yeast two-hybrid system · Allosteric regulation · ACT domain

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Introduction

Acetohydroxy acid synthase (AHAS; acetolactate synthase, EC 2.2.1.6) catalyses the first step of branched-chain amino acid biosynthesis (Chipman et al. 1998). The enzyme consists of two small regulatory and two large catalytic subunits, which both form dimers. The enzyme exhibits full activity only when regulatory subunits bind to the catalytic subunit dimer (Vyazmensky et al. 1996; Weinstock et al. 1992). Valine binds to the regulatory subunits and acts as an allosteric inhibitor of AHAS (Chipman et al. 1998). The crystal structure of the catalytic subunit dimer was described in yeast (Pang et al. 2002), and the crystal structure of the regulatory subunit dimer was determined in *Escherichia coli* AHAS III (Kaplun et al. 2006), *Thermotoga maritima* and *Nitrosomonas europea* (Petkowski et al. 2007). The crystal structure of the three latter orthologs revealed that they share the same fold: two *βαββαβ* ferredoxin domains at the N-terminal part of each monomer. The N-terminal domains were recognized to belong to ACT domain family, a group of structurally conserved domains involved in allosteric regulator binding (the family is

named after the first letters of three proteins harbouring the ACT domain, i.e. aspartate kinase, chorismate mutase and TyrA prephenate dehydrogenase; Aravind and Koonin 1999). A putative valine binding site was proposed on N-terminal domain interface of AHAS' regulatory subunits (Kaplun et al. 2006).

A set of *Streptomyces cinnamonensis* mutant strains was previously selected for their phenotypic expression of lost valine feed-back regulation and altered AHAS activity determined in cell crude extracts (Pospíšil et al. 1984, 1998, 1999). Comparison of wild type and mutant sequences of *ilvN* (coding for AHAS regulatory subunit) and *ilvB* (AHAS catalytic subunit) revealed that mutations in both subunits led to deregulation of AHAS. More precisely, the regulatory subunit mutations G16D, V17D and L18F in N-terminal domain and also a stop codon introduced to the positions 105 or 108 conferred AHAS insensitivity to valine inhibition (Kopecký et al. 1999). The substitution E139A and deletion of Q217 in the catalytic subunit were also responsible for AHAS deregulation (Kopecký et al. 2008).

In this study, we aimed to determine whether the mutations in *ilvN* and *ilvB* had some impact on interaction between the catalytic and regulatory subunits. Above that, an insight to the AHAS quaternary structure and allosteric inhibition mechanism was sought.

Materials and methods

Microorganisms and vectors

The *ilvN* and *ilvB* genes originated from *Streptomyces cinnamonensis* parental strain C-100-5 (wild type), and its spontaneous mutants resistant to valine analogues ABR-21, ABR-38, NLR-3, ACB-NLR-2 (Pospíšil et al. 1984) and BVR-7, BVR-13, BVR-18 (Pospíšil et al. 1999). *Escherichia coli* strain XL-1 Blue was employed for molecular cloning. *Saccharomyces cerevisiae* EGY 48 was used in the yeast two-hybrid system.

Vectors pRB1840, pLexA and pB42AD (Clontech, Palo Alto, CA) were employed in the yeast two-hybrid system. Genes *ilvN* and *ilvB* were introduced to vector pET28b(+) (Novagen, Madison, WI) in our previous work (Kopecký et al. 1999). Vector pJAKO was constructed in this work by inserting two complementary oligonucleotides (Supplementary Fig. S1) into pBluescript II SK(+) (Stratagene, La Jolla, CA) opened with *Hind*III and *Xba*I restriction endonucleases. Resulting vector pJAKO had a polylinker modified with an *Eco*RI site followed by *Nde*I and *Nco*I sites, allowing an insert carry-over between the expression vector pET28b(+) and the yeast two-hybrid system vectors pLexA and pB42AD.

Yeast two-hybrid system and statistical evaluation

To examine IlvB-IlvN interactions, Matchmaker LexA Two-Hybrid System (Clontech, Palo Alto, CA) was employed. *S. cerevisiae* strain EGY 48 carrying plasmid pRB1840 with reporter *lacZ* gene under the control of one LexA operator was transformed with plasmids pLexA carrying appropriate *ilvN* or *ilvB* and pB42AD carrying *ilvB* or *ilvN*, using a small-scale lithium acetate transformation protocol (Clontech Yeast Protocols Handbook; Clontech). Activity of β-galactosidase was determined according to the liquid culture assay protocol using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate (Clontech Yeast Protocols Handbook; Clontech). Presented results were obtained from parallel testing of four clones in assays with mutant IlvN and of eight clones in assays with mutant IlvB. One unit of β-galactosidase was determined as the amount that hydrolysed 1 μmol of ONPG to o-nitrophenol and D-galactose per minute per cell. The activity of β-galactosidase measured in all combinations was related to the value obtained for the $IlvN_{wt}$–$IlvB_{wt}$ interaction. Confidence intervals for β-galactosidase activities were determined at 95% confidence level. A two-sample *t*-test was used to compute the pairwise p-values. The Bonferroni correction was used to adjust for the problem of multiple comparisons.

Homology modelling

Homology modelling of IlvN structure was performed at SWISS-MODEL Protein Modelling Server, http://swiss-model.expasy.org/ (Schwede et al. 2003), using default parameters and *E. coli* IlvH (RCSB Protein Data Bank ID 2F1F) as a template. Homology modelling of IlvB structure was described in our previous work (Kopecký et al. 2008).

Results and discussion

Constructs in the yeast two-hybrid system

Both possible combinations of constructs, i.e. pB42AD-*ilvB,* pLexA-*ilvN* and pB42AD-*ilvN,* pLexA-*ilvB*, were examined for the *IlvB-IlvN* interactions in *S. cerevisiae* EGY 48. The transformants containing ilvN in pB42AD did not grow well on appropriate induction plates, so the combination of pB42AD-*ilvB* and pLexA-*ilvN* was used for β-galactosidase activity evaluation in liquid culture assay.

Interestingly, the adverse effect of IlvN fused to B42 was abolished in IlvN mutant forms from strains BVR-7 and BVR-13 having the same substitution V17D within the ACT domain, resulting in colonies of normal size. The ACT domain is considered to be structurally conserved, evolutionary mobile module, independently fused to a

variety of enzymes making them susceptible to the regulation by the respective ligands (Aravind and Koonin 1999). We therefore suppose that the ACT domain in fusion with B42 protein had an adverse effect on yeast growth and the substitution V17D abolished this effect possibly due to importance of V17 for general ACT domain functioning. However, this finding shows also possible limitations of in vivo interaction assays.

Mutations in IlvN

Mutations in two distinct regions of the regulatory subunit conferring AHAS insensitivity to valine were described: (1) substitutions in adjacent residues 16–18 and (2) a stop codon in the positions 105 or 108 (Kopecký et al. 1999). As the mutants were selected for the resistance to several valine analogues, it was supposed that their IlvN would not bind valine or the information of valine binding would not be transferred to the active centre of the enzyme. The transfer involves conformational changes within each subunit as well as a proper interaction between the regulatory and the catalytic subunit. The method of yeast two-hybrid system enabled to test whether the interaction between the subunits was affected (Table 1).

G16D and V17D

The substitution V17D occurred in two mutants: BVR-13 and, together with F30L, which was not separately tested, in BVR-7. In both strains, the interaction was strongly affected (reduction to 13.2 ± 1.4 and 14.1 ± 1.0%, respectively). The change G16D together with shortening of the polypeptide was detected in strain ABR-38 and lowered the interaction to 39.2 ± 8.9%. The effect of G16D was tested separately using an artificial construct (Kopecký et al. 1999) and resulted in lowering the interaction to 52.2 ± 12.4%. According to a homology model of *S. cinnamonensis* IlvN dimer, the two changes occurred at the boundary between a loop and an adjacent α-helix1 at the IlvN–IlvN dimer interface (Fig. 1a, b). The conserved G16 is the last residue of the loop (Fig. 1a). It is homologous to the amino acid residue G14 in *E. coli* AHAS III, which was previously described as important for valine binding (Mendel et al. 2001; Vyazmensky et al. 1996). According to our results, G16 might belong to the surface structures of the regulatory subunit that mediate the contact with the catalytic subunit. The residue points out of the ACT domain so its replacement by aspartate could affect the interaction with the catalytic subunit, spatially or due to its charge. Unlikely, the V17 residue points to the second alpha-helix (Fig. 1b) and is almost hidden by the residues of the second alpha-helix (Supplementary Fig. S2). It could not be excluded that the non-conservative G16D and V17D mutations affected the overall conformation of IlvN N-terminal domains and/or their dimerization (especially in the non-streptomycete host), and this could have an indirect effect on the interactions measured, providing that the regulatory subunit dimerization is necessary for binding of the catalytic subunit dimer.

L18F

The conservative change of adjacent L18F in strain ABR-21 did not influence the subunit–subunit interaction despite of its effect on AHAS inhibition level. It is in accordance with our homology model where L18 residue pointed

Table 1 Impact of mutations in IlvN and IlvB from *S. cinnamonensis* mutant strains on subunit–subunit interactions revealed by yeast two-hybrid assay and comparison with AHAS biochemical properties

Strain	Relative strength of IlvB–IlvN interaction (% of the wild type)[a]	Mutation		AHAS sensitivity to valine
		IlvB	IlvN	
wt	100.0 ± 10.0[b]	–	–	Sensitive
BVR-13	13.2 ± 1.4	–	V17D	Insensitive
BVR-7	14.1 ± 1.0	–	V17D, F30L	Insensitive
ABR-21	97.2 ± 8.5	–	L18F	Insensitive
ABR-38	39.2 ± 8.9	–	G16D, E105stop	Insensitive
	52.2 ± 12.4	–	G16D[c]	
NLR-3	25.6 ± 8.4	–	Q108stop	Insensitive
ACB-NLR-2	54.2 ± 4.1	ΔQ217	–	Apparent valine activation
BVR-18	77.8 ± 11.8	E139A	–	Apparent valine activation

Mutant IlvB was combined with wild-type IlvN and vice versa in the yeast two-hybrid assay

[a] Confidence intervals ($P < 0.05$) are indicated

[b] The absolute value for wt was 202.0 ± 20.2 β-galactosidase units

[c] Artificially prepared from IlvN ABR-38 (Kopecký et al. 1999)

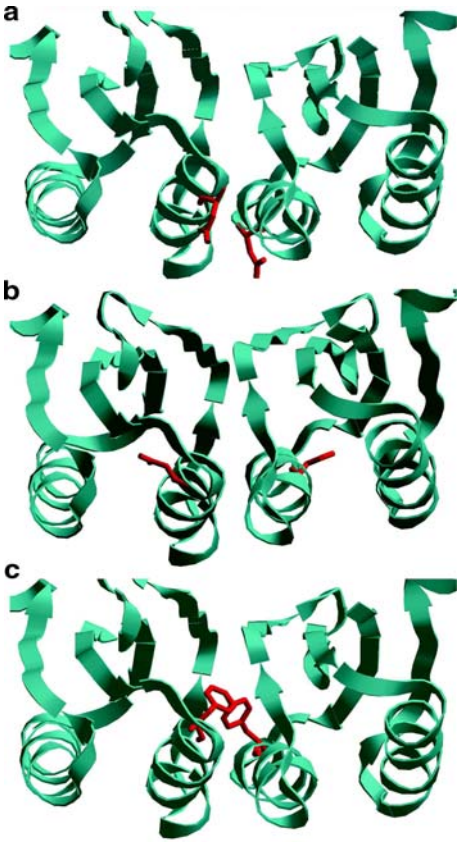

Fig. 1 Homology models of a dimer of the N-terminal part of IlvN from *Streptomyces cinnamonensis* based on *E. coli* IlvH crystal structure (RCSB Protein Data Bank ID 2F1F, Kaplun et al. 2006) as determined by SWISS-MODEL Protein Modelling Server (Schwede et al. 2003). Positions of side chains of aspartate 16 (replacing glycine, mutation G16D) (**a**), aspartate 17 (replacing valine, mutation V17D) (**b**) and phenylalanine 18 (replacing leucine, mutation L18F) (**c**) are indicated

inward and thus could not be employed in the catalytic subunit binding (Fig. 1c). L18 might therefore either directly participate in valine binding or participate in early conformation change propagation when valine was bound. Indeed, the residue (its homologue L16 in *E. coli* IlvH) was proposed to be a part of hydrophobic pocket binding valine side-chain (Kaplun et al. 2006). Its replacement with alanine (Kaplun et al. 2006) increased AHAS sensitivity to valine, while its replacement with phenylalanine (this study) abolished the AHAS sensitivity. Accepting the hypothesis that L18 binds the side-chain of valine, phenylalanine residue might disable valine binding sterically (while alanine might make the hydrophobic pocket more accessible).

E105stop and Q108stop

A stop codon at position 105 was detected in strain ABR-38 together with G16D. The effect of E105stop was not tested separately, as in another strain (NLR-3), shortening of IlvN at the position 108 reduced the interaction to 25.6 ± 8.4%. According to the homology model, the first beta sheet of C-domain (β5) was not affected in the absence of the rest of the polypeptide (not shown). Probably, the dimer of IlvN was less stable in this type of mutants as the interactions of β5 with β8 and β9, important for the dimer stabilization (Kaplun et al. 2006), were not possible. The role of C-domains in the catalytic subunit binding is puzzling. On one hand, Kaplun et al. (2006) suggested C-domains to provide interaction with the catalytic subunit based on regulatory subunit asymmetric charge. Our results are in concordance with this statement as IlvN shortening affected the interaction considerably. On the other hand, changes G16D and V17D lowered the interaction as well. Further, N-terminal part of the regulatory subunit was able to activate the catalytic one itself in the case of strain NLR-3 (Kopecký et al. 1999) as well as *E. coli* AHAS III (Mendel et al. 2003). Therefore, we suggest that both N- and C-domains may interact with the catalytic subunit. This is supported by following observations from homology models of IlvN and IlvB: First, parts of C-domains are accessible even from ACT domain direction (Supplementary Fig. S2a). Second, α and β domains of the catalytic subunit dimer, which were reported as interacting with the regulatory subunits Mitra and Sarma (2008), formed a 'bowl' in which the regulatory subunits might be plunged (Supplementary Fig. S2b). Thus, the bottom and the edge of the 'bowl' may be in contact with both N- and C-domains.

Mutations in IlvB

Changes in the catalytic subunit were found in two strains: substitution E139A in BVR-18 and deletion of Q217 in ACB-NLR-2. AHAS of both strains was activated with valine under in vitro assay conditions. In addition, the strain BVR-18 had two changes in the regulatory subunit, I106V and A135P (Table 1). However, valine activation in BVR-18 was found to be a result of the change in the catalytic subunit only (Kopecký et al. 1999; Pospíšil et al. 1999).

ΔQ217

The deletion of Q217 reduced the subunit–subunit interaction to 54.2 ± 4.1% (Table 1). The result was in agreement with our previous assumption that the helix harbouring Q217 (detail on model not shown) may interact with the regulatory subunit and/or be involved in transfer of the conformational change mediating the allosteric effect (Kopecký et al. 2008). The lowered binding of regulatory subunits might contribute to the low AHAS activity observed in ACB-NLR-2 (Kopecký et al. 2008) since regulatory subunit binding is required for AHAS activation

(Vyazmensky et al. 1996; Weinstock et al. 1992). However, the results could be also affected by improper folding of the mutated subunit in a non-streptomycete host. Mitra and Sarma (2008) also showed that this helix was in contact with the regulatory subunit of *E. coli* AHAS I, but the results are difficult to compare as AHAS I regulatory subunit lacks the C-terminal domain.

E139A

To assess the contribution of both mutant subunits to the BVR-18 phenotype, all possible combinations were tested. When $IlvB_{BVR-18}$ was combined with either $IlvN_{wt}$ or $IlvN_{BVR-18}$, the interaction was lowered to 77.8 ± 11.8 and $70.2 \pm 10.4\%$, respectively. In contrast, when $IlvN_{BVR-18}$ was combined with $IlvB_{wt}$, the interaction was $110.5 \pm 5.7\%$. Data analysis confirmed a significant effect of mutant in IlvB ($P = 0.016$) and no effect of mutant IlvN on IlvB–IlvN interactions in BVR-18. The change E139A in the catalytic subunit occurred in a loop located at the IlvB-IlvB dimer surface close to the ThDP binding site (not shown) that was supposed to participate in the transfer of the conformation change from the small subunit to the active site (Kopecký et al. 2008). Nevertheless, the results obtained from the yeast two-hybrid system revealed that this change could affect the subunit–subunit interaction itself.

The two mutations with an impact on regulatory subunit binding occurred in different domains (E139 in α and Q217 in β domain), but both were oriented to the inner surface of the 'bowl' (at the bottom and on the edge, respectively, Supplementary Fig. S2b) formed by the catalytic subunit dimer. They might thus be available for regulatory subunit binding, similarly to the previously reported residues close to the FAD-binding site (Mitra and Sarma 2008).

Conclusions and perspectives

We conclude that both N-terminal (particularly residues G16 and V17 being a part of the ACT domain) and C-terminal domains of AHAS regulatory subunit are involved in binding of the catalytic subunits. This may happen either indirectly, via regulatory subunit dimer stabilization providing that dimerization of regulatory subunits is required for catalytic subunit dimer binding, or directly, as proposed by homology modelling. According to the proposed model, the regulatory subunits might be plunged into the 'bowl' formed by α and in β domains of catalytic subunits, allowing a contact of both N- and C-terminal domains with catalytic subunit dimer. In addition, residue L18 in the ACT domain of the regulatory subunit seems to be involved in valine binding. The fact that four mutants with affected AHAS regulation had a mutation within ACT domain residues 16–18 points to the key role of this site for the enzyme regulation. Further biochemical tests are needed to confirm the effect of the mutations on regulatory subunit dimerization and interaction between regulatory and catalytic subunit. Also, more mutations within the susceptible regions should be tested by a site-directed mutagenesis to elucidate AHAS assembly dynamics and allosteric regulation.

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5.6 Mutants of *Streptomyces cinnamonensis* with acetohydroxy acid synthase expession level increased by one order of magnitude are not altered in the *ilvBNC* upstream region

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Mutants of *Streptomyces cinnamonensis* with acetohydroxy acid synthase expession level increased by one order of magnitude are not altered the *ilvBNC* regulatory region

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ABSTRACT. DNA sequence upstream the *ilvBNC* operon of *Streptomyces cinnamonensis* parental strain and two mutants with 10 to 20fold increased expression level of acetohydroxy acid synthase (AHAS) was determined. An open reading frame that might code for a short leader peptide followed by a transcription terminator were identified within this region suggesting that the expression of *ilvBNC* operon may be controlled by attenuation. Mutants with increased expression level of AHAS had no mutation within this region implying that they must be subject to other mechanism of deregulation.

The *ilvBNC* operon in streptomycetes and other bacteria codes for the catalytic (*ilvB*) and the regularory (*ilvN*) subunits of acetohydroxy acid synthase (AHAS) and for acetohydroxy acid isomeroreductase (*ilvC*). The enzymes catalyse the first two steps in branched-chain amino acid biosynthesis. Two catalytic subunits and two regulatory ones form a tetramer of AHAS The regulatory subunits bind the end product of the pathway,valine, which provides the inhibition of the enzyme (Eoyang and Silverman 1986; Vyazmensky *et al.* 1996). In addition to the allosteric regulation of AHAS by valine, the expression of the whole operon is supposed to be regulated by the mechanism of attenuation. A putative attenuator preceding *ilvB* was identified in *S. avermitilis* (De Rossi *et al.* 1995) and *S. coelicolor* (Bentley *et al.* 2002) and also in related *Mycobacterium avium* (Gusberti *et al.* 1996). A functional attenuator was demonstrated in *E. coli* (Friden *et al.* 1982) and *Corynebacterium glutamicum* (Morbach *et al.* 2000). An example of *ilvB* gene lacking the upstream attenuator was described in *Streptomyces viridifaciens* (Garg *et al.* 2002). However, an unusual operon containing *ilvBNCE* genes was adjacent to valanimycin biosynthetic gene cluster and, even if not essential for antibiotic production in heterologous host, could represent just additional gene copies increasing availability of valine as the antibiotic precursor in *S. viridifaciens*.

Several mutant strains of *Streptomyces cinnamonensis* were prepared in our laboratory in previous years. They are resistant to various valine analogues and have the valine biosynthetic pathway deregulated (Pospíšil *et al.* 1984). Biochemical analysis of the mutants revealed the

mechanisms of deregulation - insensitivity of AHAS to the end-product inhibition, increased level of AHAS activity, or combination of the both (Pospíšil *et al.* 1998; Pospíšil *et al.* 1999). Genetic analysis revealed mutations in *ilvN* (Kopecký *et al.* 1999) or in *ilvB* (Kopecký 2008) that were proved to be responsible for AHAS insensitivity to the inhibition by valine.

In this work, we focused on two strains, NLR-3 and ACBR-2, where increased level of AHAS activity was measured (Table I). No mutation was found in *ilvB* coding for the catalytic subunit in these strains (Kopecký 2008). AHAS of the strain NLR-3 is valine-insensitive (Pospíšil *et al.* 1998), which is a result of the stop codon introduction to the position 108 of the regulatory subunit, leading to the polypeptide shortening of about 70 amino acids (Kopecký *et al.* 1999). AHAS of the strain ACBR-2 is inhibited by valine in the same extent as in the wild type (Pospíšil *et al.* 1998). We suppose that the increased level of AHAS activity which was measured in these strains was rather due to increased expression level of the enzyme. We sequenced the *ilvBNC* upstream region of *S. cinnamonensis* parental and mutant strains and searched for some regulatory features that might be affencted by mutations in NLR-3 and ACBR-2.

MATERIALS AND METHODS

Strains, cultivation and DNA exctraction. Streptomyces cinnamonensis parental strain C-100-5 and mutant strains NLR-3, ACBR-2 were used in the study (Table I).

Isolation and sequencing of the ilvBNC upstream region. The upstream region of ilvBNC operon in the parental strain S. cinnamonensis C-100-5 was cloned using an adaptor PCR method (Willems 1998). The chromosomal DNA was digested with StuI, which cleaves the ilvB gene at the position 661, in combination with EcoRI, HindIII, SacI or SphI that do not cleave ilvB at all. The chromosomal DNA digested in these combinations were hybridized with a DIG-dUTP (Roche, Switzerland) labeled DNA probe, representing the 5´-terminal part of ilvB, prepared by PCR amplification from ilvBf1 and ilvBr4 primers (Table II). StuI/SacI restriction fragment of appropriate

length was isolated and ligated with an adaptor composed of the complementary PIlvBf and PIlvBfc oligonucleotides (Table II) creating the SacI complementary ends. The fragment was PCR amplified using PIlvBf and PIlvBr primers (Table II) and sequenced. In mutant strains NLR-3 and ACBR-2, ALSpromf and PIlvBr primers (Table II) were used for PCR amplification and sequencing. All the DNA manipulation were performed according to (Ausubel et al. 1995). The chain termination reaction (Sanger et al. 1977) was performed according to the cycle sequencing method (Murray 1989) with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 and analyzed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems-Hitachi).

Software. Sequence data were handled by Lasergene (DNAStar) and Chromas (Technelysium) software. RNA secondary structures were arranged using MBC Mfold 3.0 available at the internet site http://mfold.burnet.edu.au/rna_form. Protein-protein BLAST was performed at the internet site http://www.ncbi.nlm.nih.gov/BLAST/.

RESULTS AND DISCUSSION

Sequencing of the ilvBNC upstream region in the parental strain. Hybridization of *Stu*I/*Sac*I digested chromosomal DNA from the parental strain *S. cinnamonensis* C-100-5 with a labeled DNA probe representing the 5´-terminal part of *ilvB* gene revealed a fragment of approximately 1200 bp. The fragment, containing the 5´-terminal part of *ilvB* and the region upstream, was isolated, PCR amplified and sequenced.

In the parental strain, a sequence of 815 bp upstream the *ilvB* translation initiation codone was obtained (deposited in GenBank, accession no. **AY785370**). The first 582 bp of the sequence might code for a C-terminal part of a putative protein that shows similarity to the C-terminal part of a putative membrane associated phosphodiesterase of *S. coelicolor* (E-value 6e-68) and that of *S. avermitilis* (E-value 8e-65).

The intergenic region upstream *ilvB* in the parental strain *S. cinnamonensis* C-100-5 showed

79.7% and 75.1% identity with those of *S. avermitilis* and *S. coelicolor*, respectively. A putative ribosome binding site followed by a short open reading frame, which is absolutely conserved among streptomycetes, were identified 214 bp upstream the *ilvB* gene (Fig. 1). The ORF might code for a leader peptide containing a tandem of four branched-chain amino acid residues, three of them coded by rare codons. Also codons for arginine and glycine residues adjacent to this tandem are rare in streptomycetes. Another conserved sequence with dyad symmetry is located about 50 bp downstream of this ORF, directly followed by six T residues that can serve as transcription terminator. Presence of the leader peptide coding region and the terminator (similar to those in *S. avermitilis* and *S. coelicolor*) implies that *ilvBNC* transcription in streptomycetes may be controlled by attenuation as in *E. coli* (Friden *et al.* 1982) or *Corynebacterium glutamicum* (Morbach *et al.* 2000).

Modeling of RNA secondary structures upstream ilvB. Fig. 2 shows possible secondary structures of mRNA upstream *ilvB* gene, including the terminator hairpin. Similar secondary structures were proposed by (Craster *et al.* 1999) for mRNA sequence upstream *ilvB* of *S. coelicolor*. However, according to our model, stalling of the ribosome at any of four key codons would not prevent the terminator hairpin formation (not shown).

Sequencing of the ilvBNC upstream region in NLR-3 and ACBR-2. Sequences of approximately 700 bp upstream *ilvB* were obtained for NLR-3 and ACR-2. Neither of the mutant strains harbored any mutation within this region. The unaltered sequence was surprising especially in ACBR-2, which had about 20 fold increased AHAS level (Table I). It could be explained by the mode of action of a valine analogue 2-amino-3-clorobutyrate used during ACBR strains selection, which binds to the specific tRNAs rather than acting as a simple valine structural analogue (Williams and Freundlich 1969). $tRNA^{Val}$ recognizing GUA codon or particular aminoacyl tRNA synthetase could be affected in ACBR-2. Nevertheless, such explanation cannot be used for the NLR-3 strain, where norleucin was used for selection.

Other regulatory mechanism of *ilvB* expression in streptomycetes in place of classical

attenuation was considered by (Craster *et al.* 1999) as the replacement of Leu, Ile and Val codons in leader peptide sequence did not affect the regulation of the transcription level. Actually, another ways of AHAS expression control are possible. In *E. coli*, (p)ppGpp and cAMP regulation was described (Freundlich 1977; Friden *et al.* 1982). In *C. glutamicum*, direct repeats preceding the *ilvB* promoter are thought to play some role in AHAS expression control by 2-oxobutyrate. Also a regulatory protein binding mRNA may be involved in *ilvB* expression control similarly as in *B. subtilis* where RNA-binding attenuation protein (TRAP) plays a role in both attenuation and translation control of several operons (Babitzke and Gollnick 2001).

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Table I. *Streptomyces cinnamonensis* strains

Strain	Resistant to	Mutation in IlvN	AHAS activity (pmol s^{-1} mg^{-1}) measured in crude extract of *S. cinnamonensis* grown on minimal liquid growth medium	
			No valine added prior to enzyme assay	10 mM valine added prior to enzyme assay
C-100-5[abc] (wt)	–	–	130	76
ACBR-2[ab]	2-amino-3-clorobutyrate	Not sequenced	2900	1600
NLR-3[abc]	Norleucine	Q108stop	1400	1400

[a](Pospíšil *et al.* 1984)

[b](Pospíšil *et al.* 1998)

[c](Kopecký *et al.* 1999)

Table II. Oligonucleotides

Description	Sequence	Position [a]
PIlvBf	5'-CTG ATG GCA TAA CAG AGC T-3'	adapter
PIlvBfc	5'-CTG TTA TGC CAT CAG-3'	adapter
PIlvBr	5'-TCG CTG GGA TCC GGT GT-3'	695-679
ALSpromf	5'-ATC GAC GTG CTG AAG CTG-3'	97-114
ilvBr4	5'-GTC GAG GCG ATG TGG AAG G-3'	1339-1321

[a] Positions are numbered according to the sequences deposited in GenBank, accession no. **AY785370**

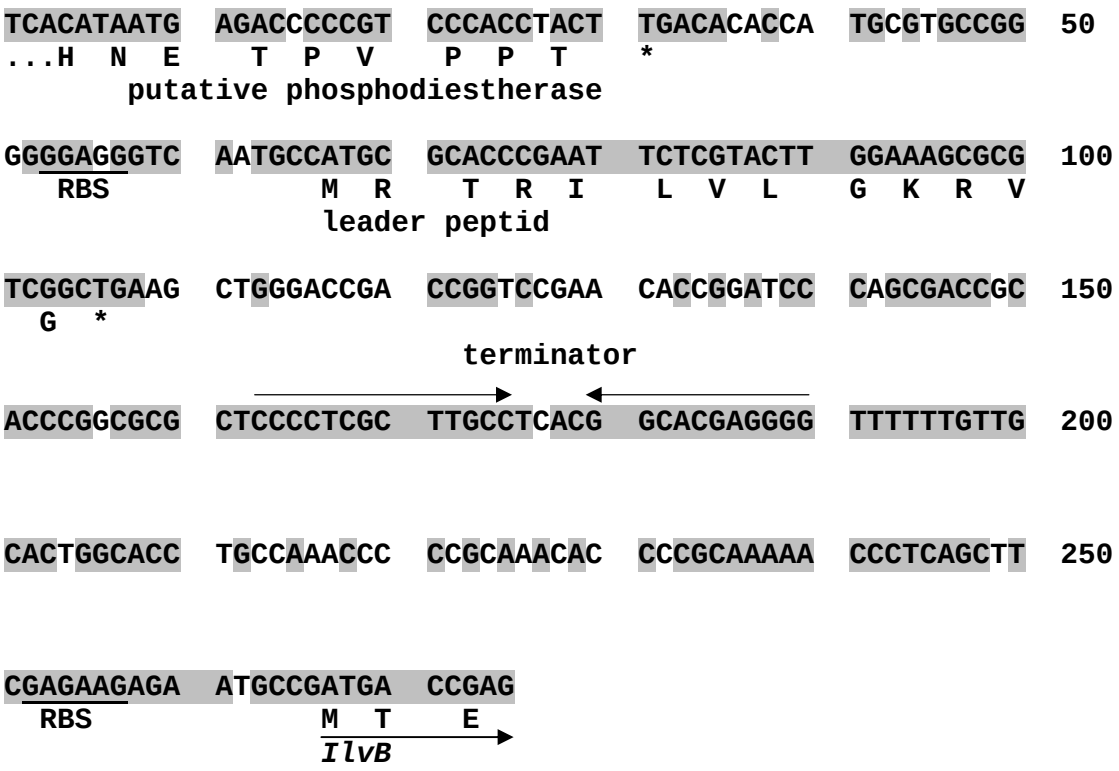

FIG. 1. The DNA sequence upstream *ilvB* of *S. cinnamonensis*. The nucleotides conserved in *S. coelicolor* and *S. avermitilis* are shadowed. RBS, ribosome binding site.

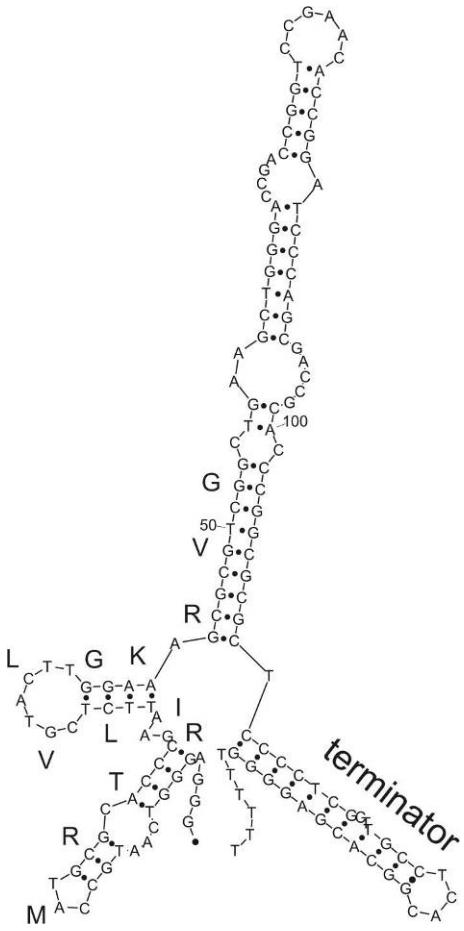

Fig. 2. Possible secondary structures upstream of *ilvB*.