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ORIGINAL PAPER

Factors enhancing L-valine production by the growth-limited L-isoleucine auxotrophic strain *Corynebacterium glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*)

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Abstract Cell growth limitation is known to be an important condition that enhances L-valine synthesis in *Corynebacterium glutamicum* recombinant strains with L-isoleucine auxotrophy. To identify whether it is the limited availability of L-isoleucine itself or the L-isoleucine limitation-induced *rel*-dependent ppGpp-mediated stringent response that is essential for the enhancement of L-valine synthesis in growth-limited *C. glutamicum* cells, we deleted the *rel* gene, thereby constructing a relaxed (*rel*$^-$) *C. glutamicum* Δ*ilvA* Δ*panB* Δ*rel ilvN*M13 (pECKA*ilvBNC*) strain. Variations in enzyme activity and L-valine synthesis in *rel*$^+$ and *rel*$^-$ strains under conditions of L-isoleucine excess and limitation were investigated. A sharp increase in acetohydroxy acid synthase (AHAS) activity, a slight increase in acetohydroxyacid isomeroreductase (AHAIR) activity, and a dramatic increase in L-valine synthesis were observed in both *rel*$^+$ and *rel*$^-$ cells exposed to L-isoleucine limitation. Although the positive effect of induction of the stringent response on AHAS and AHAIR upregulation in cells was not confirmed, we found the stringent response to be beneficial for maintaining increased AHAS, dihydroxyacid dehydratase, and transaminase B activity and L-valine synthesis in cells during the stationary growth phase.

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Introduction

L-valine is an essential amino acid for vertebrates. It is used as a feed additive and is a component of infusion solutions and cosmetics. There is also a great demand for L-valine as a precursor in the chemical synthesis of herbicides [8, 27].

Currently, the most economic route to amino acid production is via microbial synthesis, predominantly using *Corynebacterium glutamicum*. However, the industrial production of L-valine by microbial synthesis is not yet well developed [9], primarily because bacterial cells possess complicated control mechanisms for the synthesis of branched-chain amino acids (BCAA) (L-valine, L-leucine and L-isoleucine). The synthesis of L-valine competes with other BCAAs for common precursors and the same enzymes (Fig. 1). Moreover, the activity of the enzymes involved in L-valine synthesis and the expression of the respective genes is feedback-regulated by all of these amino acids [10, 32].

The pathway of L-valine formation involves four reaction steps (Fig. 1): 2 mol of pyruvate are condensed to acetolactate by acetohydroxyacid synthase (AHAS, encoded by the *ilvB* and *ilvN* genes) [20]; NADPH + H^+ is used as a cofactor for the subsequent reaction catalysed by acetohydroxyacid isomeroreductase (AHAIR, encoded by *ilvC*) [7, 20]; in the third step, dihydroxyacid dehydratase (DHAD, encoded by *ilvD*) [36] forms α-ketoisovalerate from α, β- dihydroxyisovalerate which, in the fourth and final step, is transaminated to L-valine by transaminase B (TmB, encoded by *ilvE*) using L-glutamate as an amino group donor [36]. Although the L-alanine-dependent

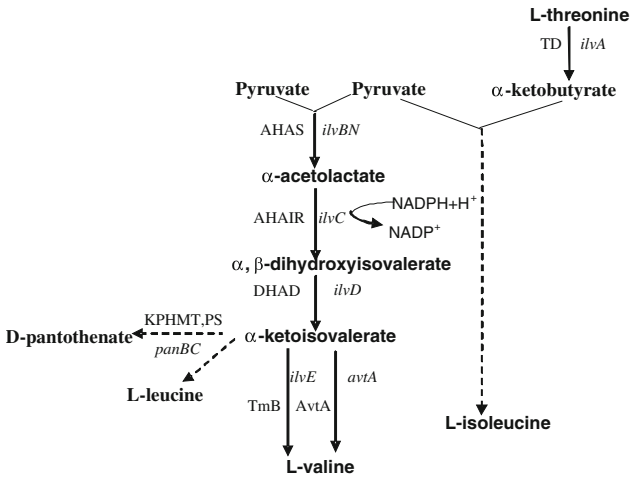

Fig. 1 Simplified scheme of biosynthetic pathways of L-valine, L-isoleucine, L-leucine, and D-pantothenate in *Corynebacterium glutamicum*. The enzymes and their respective genes (*in parenthesis*) are: *TD* L-Threonine dehydratase (*ilvA*), *AHAS* acetohydroxyacid synthase (*ilvBN*), *AHAIR* acetohydroxyacid isomeroreductase (*ilvC*), *DHAD* dihydroxyacid dehydratase (*ilvD*), *TmB* L-glutamate-dependent transaminase B (*ilvE*), *KPHMT* ketopantoate hydroxymethyltransferase, *PS* D-pantothenate synthase (*panBC*). L-Alanine-dependent transaminase AvtA is encoded by *avtA*

transaminase (AvtA, encoded by *avtA*) can transaminate α-ketoisovalerate to L-valine [30, 31], AvtA may play a minor role in L-valine synthesis in *C. glutamicum* [28, 30].

L-valine overproduction in the wild-type strain *C. glutamicum* (ATCC 13032) have been achieved by deletion of the *ilvA* and the *panBC* genes, resulting in the L-isoleucine and D-pantothenate auxotrophy of strains and the plasmid-bound overexpression of the *ilvBNC* or *ilvBNCD* genes [12, 36, 42]. Further improvement in L-valine production by recombinant strains has been achieved by site-directed mutagenesis of the chromosomal *ilvN* gene, thus removing AHAS inhibition by all three BCAAs [12].

The second generation of *C. glutamicum* L-valine producers was constructed by gene modifications, which resulted in a substantial increase in intracellular pyruvate and $NADPH + H^+$ concentrations. Such modifications include the overexpression of *pyc,* encoding pyruvate carboxylase and the deletion of the *aceE*, *pqo* and *pgi* genes, encoding the pyruvate dehydrogenase complex, pyruvate:quinone oxidoreductase and phosphoglucose isomerase, respectively [2, 3]. Although these strains are not auxotrophs, when cultured, they produce very high levels of L-valine [3].

The rate of amino acid synthesis by recombinant strains varies significantly depending on variations in the physiology of the cell culture. A transition in the cellular growth rate to below maximum during batch- and fed-batch cultivations has been determined to be a precondition for increasing AHAS activity and L-valine synthesis by *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*), which is auxotrophic for L-isoleucine and D-pantothenate [41]. L-Valine oversynthesis due to the high AHAS activity in various recombinant *C. glutamicum* strains auxotrophic for L-isoleucine has also been observed under conditions of L-isoleucine limitation [17, 25, 36]. The production of L-valine by isoleucine-auxotrophic or isoleucine-bradytrophic strains was observed to drop sharply when an excess of isoleucine was added to the minimal medium [17]. However, the mechanism of L-valine production under an L-isoleucine-limited growth condition remains unclear.

The increased transcription of genes and increased translation rate of L-valine synthesis enzymes in bacterial cells may result from the *rel*-dependent ppGpp-mediated stringent response induced by L-isoleucine starvation. The stringent response is required for the transcription of the *ilv–leu* operon as well as for the increased translation rate of enzymes involved in BCAA synthesis in *Bacillus subtilis, Escherichia coli* and *Salmonella typhimurium* [13, 46].

There is as yet no evidence that the upregulation of enzymes involved in L-valine synthesis in *C. glutamicum* are also under the positive control of the stringent response. Induction of this response by DL-serine hydroxamate only causes a stronger expression of genes involved in the synthesis of L-histidine and L-serine [4]. Also, *rel* gene deletion was found only to result in auxotrophy for L-histidine and L-serine but not for L-valine [45]. Consequently, bearing in mind that L-isoleucine limitation enhances L-valine synthesis in *C. glutamicum* [17, 25] but that the expression of genes involved in this synthesis are not affected by the stringent response, we would expect that L-valine production in stringent positive and negative strains *(rel*$^+$ and *rel*$^-$, respectively) should be similar. However, the elevated L-valine concentration that we achieved with the *rel*$^+$ strain compared to the *rel*$^-$ strain in preliminary experiments indicates that the stringent response has a positive effect on L-valine production in *C. glutamicum*. Thus, the overall influence of the stringent response on L-valine synthesis in *C. glutamicum* is still unclear. A significant difference in global gene expression, including some sigma factor genes, between the *rel*$^+$ and *rel*$^-$ *C. glutamicum* strains due to the stringent response induced by DL-serine hydroxamate has been reported [4]. Thus, the effect of the stringent response on L-valine synthesis in this bacterium may also result from its effect on global gene expression and respective changes in cellular metabolism.

The aim of our study was to investigate the extra- and intracellular factors enhancing L-valine production by a growth-limited *C. glutamicum* recombinant strain, auxotrophic for L-isoleucine, and to elucidate the role of the stringent response in L-valine overproduction.

Materials and methods

Microorganisms and culture conditions

The strain used in this study was a derivative of the *C. glutamicum* wild type (WT; ATCC 13032), *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*), which is auxotrophic for L-isoleucine and D-pantothenate, with feedback-resistant AHAS and the *ilvBNC* operon present on the multicopy plasmid pECKA [12]. To elucidate the effect of the *rel*-dependent ppGpp-mediated stringent response on cellular growth and L-valine synthesis, we deleted the *rel* gene. The resulting relaxed strain *C. glutamicum* Δ*ilvA* Δ*panB* Δ*rel ilvN*M13 (pECKA*ilvBNC*) was unable to accumulate ppGpp.

The strains were cultured at 30°C. Brain heart infusion complex medium (BHI) was used for pre-culture growth in baffled shake flasks on a rotary shaker (220 rpm). Slightly modified CGXII medium [20] supplemented with deferoxamine (3 mg l^{-1}) instead of protocatechuic acid was used to grow the main culture in baffled shake flasks or in a bioreactor. The medium was supplemented with L-isoleucine (1.52 mM), D-pantothenate (0.75 μM), biotin (2 mg l^{-1}) and kanamycin (30 mg l^{-1}).

Batch- and fed-batch cultivations of cells were carried out in a Mini-type bioreactor (MBR, Zurich, Switzerland) with a working volume of 1.8 l, as described previously [41], in modified CGXII medium with initial glucose concentrations of 40 or 20 g l^{-1}, respectively. When the glucose concentration in the cell culture fell to 6.0 ± 0.5 g l^{-1}, glucose supplementation was initiated to maintain the concentration of glucose in the range of 6.0–8.0 g l^{-1}. The pH (7.0) of the cell culture medium was maintained by the automatic addition of 12 M NH_4OH to the bioreactor. In flask cultivations, the pH of the medium was buffered with 0.2 M MOPS. Short-term cultivations were carried out in baffled shake flasks under conditions of L-isoleucine excess or limitation, respectively, as follows. The cells were precultured in a modified CGXII minimal medium with an initial glucose concentration of 20 g l^{-1} and L-isoleucine concentration of 0.57 mM. During the exponential growth phase, the cell culture was harvested by centrifugation, then washed with CGXII minimal medium lacking L-isoleucine and resuspended in a medium either with or without 1.52 mM L-isoleucine and recultured for 4 h. L-Isoleucine-limited cultures were additionally supplemented with 10 mM L-valine to block the uptake of residual L-isoleucine by the cells [25].

Construction of the rel$^-$ mutant

Inactivation of the *rel* gene within the *C. glutamicum* chromosome was achieved by deleting a 200-bp internal fragment using the crossover PCR method [18] as follows. The PCR fragments (A and B) of the upstream and downstream sequences of the intended gene region to be deleted extended by mutually complementary 20-bp sequences were amplified with the primers RELAF1 + DELRELAR1 (591 bp) and DELRELAF2 + RELAR2 (577 bp), respectively. Fragments A and B were purified and mixed, and a single large fragment C (1148 bp) was amplified by PCR using the outer primers RELAF1 + RELAR2. Primer-generated *Eco*RI sites were used to clone the resulting product with the 200-bp deletion in the vector pKSAC45. The construct was transferred to *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 by electrotransformation, and the clones in which the double recombination event occurred were detected by positive selection based on the conditional lethal effect of the *sacB* gene in *C. glutamicum* [19]. The clones, free of the vector sequences, were analysed to confirm that the correct deletion within the chromosomal *rel* gene had been generated. Electrophoretic analysis of the PCR products amplified with the primers RELAF3 + RELAR3 (designed outside the original fragment C used for the construction of the deletion) showed the expected fragment sizes: 1.5 kb with the *C. glutamicum* WT genome DNA as a template and 1.3 kb with the Δ*rel* strain genome as a template. The final evidence of a 200-bp deletion was provided by sequencing the fragment from the Δ*rel* strain (data not shown). The nucleotide sequences of the primers used and the positions of their 5′ ends within the complete *C. glutamicum* ATCC13032 genome sequence (NCBI Reference Sequence NC_006958) are:

RELAF1: AGGAATTCGTAGTGAGCGAACAAG (1 756 962);
DELRELAR1: AAGACGTTCCCTTTCGATCCTTCT ACTGAG (1 756 172);
DELRELAF2: GGATCGAAAGGGAACGTCTT CATGATGGTG (1 756 391);
RELAR2: GTGAATTCATCAATAATTTCTTTGA GG (1 755 615);
RELAF3: AGATCGCACAGTCGGTAA (1 757 042);
RELAR3: TGCGGATGCCAACAAGAT (1 755 484).

Physiological parameters

The growth of the cultures was followed by measuring the optical density of the cell culture at 600 nm (OD_{600}) using a Helios UV-Visible spectrophotometer (Thermo Spectronic, Cambridge, UK). The biomass concentration was calculated from OD_{600} values using an experimentally determined correlation factor of 0.3 g cell dry mass (CDM) l^{-1} for $OD_{600} = 1$.

The cellular oxygen consumption rate (Q_{O2}) was measured with a Clark-type Radiometer oxygen electrode.

Samples of the cell culture from the bioreactor or flasks were diluted with the same growth medium up to 1 mg CDM ml^{-1} and transferred to the electrode chamber. To measure the cyanide-resistant respiration ($Q_{O2\ CN\text{-}resistant}$), which characterizes oxygen consumption uncoupled from ATP synthesis, we supplemented 1 mM KCN to the diluted cell culture sample. The cyanide-sensitive respiration ($Q_{O2\ CN\text{-}sensitive}$), which characterizes oxygen consumption coupled with ATP synthesis, was calculated as the difference between $Q_{O2\ summary}$ and $Q_{O2\ CN\text{-}resistant}$. The effect of hydrogen peroxide on cellular respiration was measured after a 10-min exposure of the cell culture to 10 mM H_2O_2. The $Q_{O2\ summary}$ and $Q_{O2\ CN\text{-}sensitive}$ was expressed in nanomoles per milligram CDM per minute taking into account that 1 unit corresponds to 0.75 nmol O_2 ml^{-1}. $Q_{O2\ CN\text{-}resistant}$ was expressed as the percentage of total respiration.

The viability of the cells after hydrogen peroxide stress was determined by plating the appropriate dilutions of growing cultures on BHI agar. The survival of cells was expressed as the percentage of the viability of parallel stressed and unstressed cultures.

The experiments were conducted in triplicate. Values are averages ± standard deviation (SD) of data from two independent samples of each time point, with three replicates of each analytical measurement of one representative fermentation or three independent short-term recultivations.

Determination of amino acids

Processing of the cell culture samples to separate the intracellular and extracellular fluids was performed by silicon oil centrifugation [23]. The concentration of amino acids in the resulting intra- and extracellular fractions was quantified by reversed-phase high-performance liquid chromatography (HPLC; (Agilent 1100; Hewlett-Packard, Palo Alto, CA) with fluorimetric detection (excitation at 340 nm and emission at 450 nm) after automatic precolumn derivatization with *ortho*-phthaldialdehyde. Separation was carried out at 40°C on a Hypersil AA-ODS column (particle size 5 µm, 200 × 2.1 mm; Hewlett-Packard). The elution buffer consisted of a polar phase (20 mM sodium acetate (pH 7.2) supplemented with 0.018% TEA and 0.3% THF) and a nonpolar phase [100 mM sodium acetate (pH 7.2):acetonitrile:methanol; 1:2:2]. The intracellular concentration of amino acids was calculated taking into account the previously reported cell volume for *C. glutamicum* of 1.8 µl mg CDM^{-1} [16].

Using the experimental data, we were able to calculate the specific growth rate (μ) as well as the L-valine ($q_{L\text{-}valine}$), L-leucine ($q_{L\text{-}leucine}$) and L-alanine synthesis rates ($q_{L\text{-}alanine}$) [39].

Biochemical parameters

Enzyme assays

Preparation of cell-free extract The cells were harvested by centrifugation (4000 *g*, 4°C, 15 min). The cell pellet was washed twice with 0.2% KCl at 4°C, and the cells were resuspended in 100 mM potassium phosphate buffer (pH 7.3) supplemented with 0.5 mM dithiothreitol and 20% (v v^{-1}) glycerol.

Cell-free extracts were obtained by sonication (UPs200; Hielscher Ultrasonics, Stuttgart, Germany) of the cell suspension (50 kHz, 8 min of 0.5 s and 0.5 s of space-out). Cellular debris was removed by centrifugation (10,000 *g*, 4°C, 30 min). Protein concentrations in the cell-free extracts were determined spectrophotometrically (Helios Gamma spectrophotometer; Thermo Spectronic) according to the method of Lowry [29].

AHAS activity assay The activity of AHAS in the cell-free extracts was determined by a method based on the enzymatic conversion of pyruvate to acetoin through α-acetolactate, as modified by Leyval et al. [28]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 50 mM sodium pyruvate, 10 mM $MgCl_2$, 100 µM thiamine pyrophosphate and 100 µM flavin adenine dinucleotide. The reaction was initiated by the addition of 0.1 ml of cell-free extract to 0.9 ml of the enzyme assay mixture. The reaction mixture was then incubated at 37°C for 20 min, and the reaction was terminated by acidification (addition of 0.1 ml of 50% H_2SO_4). The reaction mixture was then incubated for an additional 30 min at 37°C to allow the α-acetolactate to be converted into acetoin. The acetoin formed was quantified using the Voges–Proskauer method [48]. The absorbance of the reddish reaction mixture was measured spectrophotometrically at 535 nm. The specific AHAS activity was expressed in nanomoles of α-acetolactate formed per milligram protein per minute.

AHAIR activity assay The activity of AHAIR was determined by the method developed by Arfin and Umbarger [1] and modified by Leyval et al. [28], which is based on determining the $NADPH + H^+$ oxidation rate during the enzymatic reaction. The reaction mixture (0.99 ml) consisted of 100 mM potassium phosphate buffer (pH 7.3), 10 mM α-acetolactate, 3 mM $MgCl_2$ and 0.05 ml cell-free extract. The reaction was initiated by adding 0.01 ml of 10 mM $NADPH + H^+$ to the reaction mixture and was performed at 30°C. $NADPH + H^+$ consumption was monitored spectrophotometrically at 340 nm**.** The specific AHAIR activity was expressed in nanomoles of $NADPH + H^+$ oxidized per milligram protein per minute.

DHAD activity assay The activity of DHAD was assayed using the method of Flint et al. [14]. The method is based on the formation of 2,4-dinitrophenylhydrazone from the keto acid product of the enzymatic reaction. The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$ and 10 mM DL-α,β-dihydroxyisovalerate. The reaction was initiated by the addition of 0.1 ml of the cell-free extract to 0.9 ml of the reaction mixture. The reaction run at 37°C for 10 min and terminated by the addition of 0.1 ml of 2,4-dinitrophenylhydrazine saturated in 2 N HCl. This hydrazine reacted with α-ketoisovalerate, the product of the DHAD reaction, to give α-ketoisovalerate-dinitrophenylhydrazone. The concentration of this product was then determined at 540 nm. The specific DHAD activity was expressed in nanomoles of α-ketoisovalerate formed per milligram protein per minute.

TmB activity assay The TmB assay is based on the determination of α-ketoglutarate produced by the amination of α-ketoisovalerate (TmB substrate) using L-glutamate as an amino group donor. The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 40 mM L-glutamate, 4 mM α-ketoisovalerate and 0.25 mM pyridoxal 5′-phosphate monohydrate. The reaction was initiated by the addition of 0.1 ml cell-free extract to 0.9 ml of reaction mixture. It was performed at 30°C for 10 min, and then terminated by the addition of 0.1 ml of 20% H_3PO_4. The sample was centrifuged (13,000 *g*, 4°C, 5 min) and the supernatant neutralized by the addition of 5 M KOH solution in 1 M triethanolamine. In the next step, α-ketoglutarate, produced in the TmB reaction, was quantified enzymatically with the help of $NADP^+$-specific L-glutamate dehydrogenase. The specific TmB activity was expressed in nanomoles of α-ketoglutarate formed per minute per milligram of protein.

Assays of intracellular compound concentrations

ppGpp was extracted from the cells with 0.2 M KOH as described by Zhang et al. [49]. The nucleotides in the cell extracts were separated by isocratic ion exchange HPLC (Waters 501; Waters, Milford, MA) on a 4.6 × 250-mm Hypersil Sax 5-μm column, using a 0.03 M potassium phosphate buffer (pH 3.4) supplemented with 14% acetonitrile and 0.01 M tetrabutylammonium phosphate as the mobile phase. ppGpp was quantified by measuring the absorbance at 254 nm using a Waters Tunable Absorbance Detector.

Pyruvate and NADPH + H^+ were extracted from the cells and determined using the previously described enzymatic assay [40].

Chemicals

The synthesis of α-acetolactate, which was used for the AHAIR assay and the synthesis of α,β-dihydroxyisovalerate, used for the DHAD assay, were carried out according to reported methods [6, 33]. All other chemicals used were of analytical grade and commercially available.

Results

Searching for possible extra- and intracellular factors enhancing L-valine synthesis in growth-limited *C. glutamicum*

The L-valine-producing strain *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*), auxotrophic for L-isoleucine and D-pantothenate, was selected as a model strain to investigate factors enhancing L-valine synthesis in this bacterium. The increased resistance of AHAS to inhibition by any of the BCAAs (due to the *ilvN*M13 mutation) is an advantage of this strain in terms of reducing the regulatory effects of varying concentrations of BCAAs on AHAS activity and their physiological consequences. Batch cultivations were carried out to follow the relationship between variations in cellular growth rate, intracellular concentrations of L-valine precursors and amino acid synthesis.

The kinetics of biomass and L-valine accumulation (Fig. 2a), the growth rate and L-valine synthesis rate during the batch cultivations (Fig. 2b) showed that growth rate downregulation triggers L-valine overproduction by *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*). However, an enhanced synthesis of L-leucine and L-alanine was also observed (Fig. 2b).

The increase in L-valine synthesis by this bacterium under conditions of cellular growth rate downregulation correlated with a drastic increase in AHAS activity in these cells (Fig. 2c). On the other hand, increased L-valine synthesis correlated with a significant decrease in intracellular pyruvate concentration, whereas no significant changes in NADPH + H^+ concentration were observed (Fig. 2c). Based on these results, the upregulation of AHAS activity can be considered to be a possible factor enhancing L-valine production in *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*).

The increase in AHAS activity and intracellular L-valine concentration in the growth-limited cell culture occurred in parallel with a significant decrease in L-isoleucine concentration in the medium and cells and with a sharp increase in the intracellular concentration of ppGpp, a signal molecule of the stringent response (Fig. 2d). Thus, the increased AHAS activity and L-valine synthesis in the growth limited

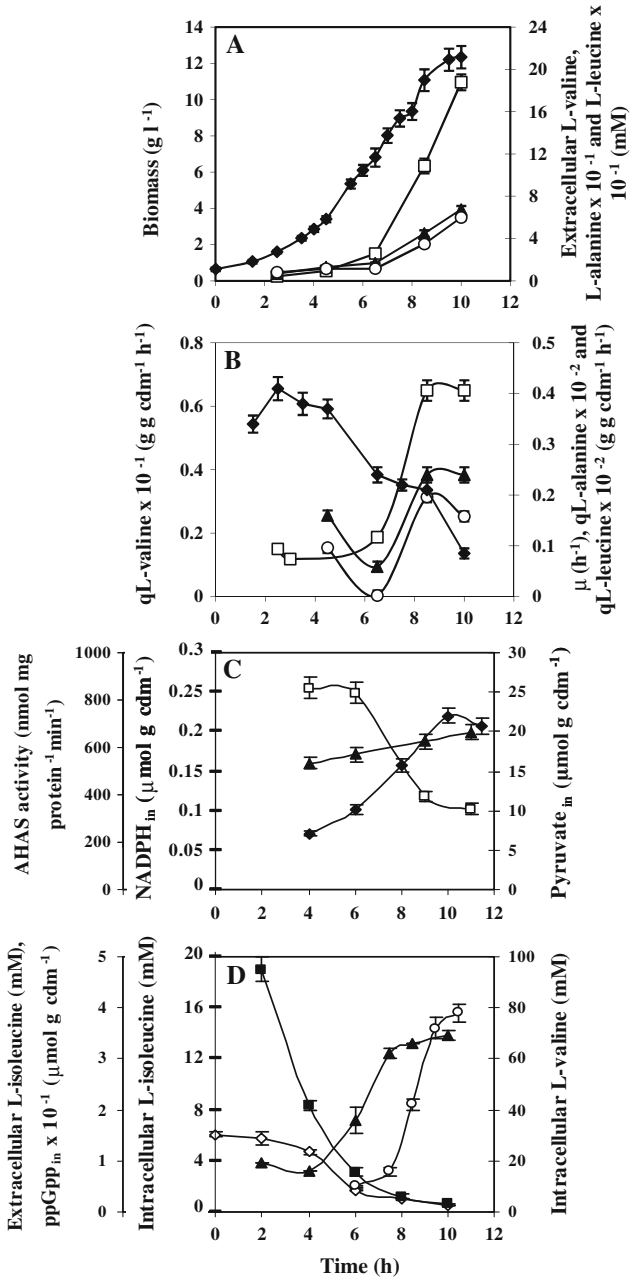

Fig. 2 Batch cultivation of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*). Kinetics of: **a** biomass (*filled diamond*), extracellular concentration of L-valine (*open square*), L-alanine (*filled triangle*) and L-leucine (*open circle*); **b** cellular growth rate (*filled diamond*), specific rate of L-valine (*open square*), L-alanine- (*filled triangle*) and L-leucine synthesis (*open circle*); **c** activity of AHAS (*filled diamond*) intracellular concentration of pyruvate (*open square*) and NADPH + H^+ (*filled triangle*); **d** extracellular- (*open diamond*) and intracellular concentration of L-isoleucine (*filled square*), intracellular concentration of ppGpp (*open circle*) and L-valine (*filled triangle*). Values are averages of data from two independent samples of each time point of one cell culture with three replicates of each analytical measurement ± standard deviation (SD)

C. glutamicum Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) may also indirectly result from the stringent response induced by L-isoleucine limitation.

Effects of L-isoleucine limitation and induction of the stringent response on the activity of L-valine forming enzymes and amino acid production

To investigate whether the L-isoleucine limitation itself or the L-isoleucine limitation-induced stringent response initiated the increase in AHAS activity (and probably also that of other enzymes involved in the L-valine synthesis pathway) and in L-valine production in *C. glutamicum*, we used fed-batch cultivations of *rel*$^+$ and *rel*$^-$ recombinant strains in medium with an initial glucose concentration of 20 g l^{-1}. The glucose concentration was kept in the range 6.0–8.0 g l^{-1} during the stationary growth phase to minimize the possible effects of varying glucose level on the physiology of the culture.

The exponential growth under an excess of L-isoleucine as well as the reduction in cellular growth rate that occurred as a consequence of L-isoleucine depletion in both the medium and inside the cells (after 6 h of cultivation; Fig. 3a, b) were comparable in the *rel*$^+$ and *rel*$^-$ strains. It should be noted that a similar—though temporary—drop in the growth rate of both strains after 4 h of cultivation also occurred. This temporary drop in growth rate was quite unexpected, as it was not related to L-isoleucine depletion. It coincided with a rather sharp increase in the level of intracellular L-valine in *rel*$^+$ and *rel*$^-$ cells, even though the extracellular accumulation of L-valine was low. The reason for the atypical growth kinetics of *rel*$^+$ and *rel*$^-$ recombinants of *C. glutamicum* during the fed-batch cultivations is not yet clear.

The growing cells of *rel*$^+$ and *rel*$^-$ strains also showed similar trends in L-valine synthesis: the intracellular level of L-valine and the rate of its extracellular accumulation were low during the exponential phase and significantly increased in the post-exponential growth phase (Fig. 3c, d). The increase in L-valine synthesis was related to increases in the activity of AHAS and AHAIR in the cells (Fig. 3e, f).

It was only the *rel*$^+$ strain, in which L-isoleucine starvation triggered fast ppGpp accumulation, as expected. ppGpp formation and cellular growth rate downregulation during the cultivations correlated with increased AHAS and AHAIR activity and increased L-valine synthesis in this strain (Fig. 3c, e). A similar increase in enzyme activity and L-valine synthesis under these conditions was also observed in the *rel*$^-$ strain (Fig. 3d, f). However, whereas the rate of L-valine synthesis remained high in the *rel*$^+$ strain, it significantly decreased in the *rel*$^-$ strain during the stationary growth phase (Fig. 3c, d). This decrease in L-valine synthesis by the *rel*$^-$ strain was related to the more significant decrease in AHAS activity and low DHAD and TmB activity as compared to the activity of these enzymes in the *rel*$^+$ strain (Fig. 3e, f).

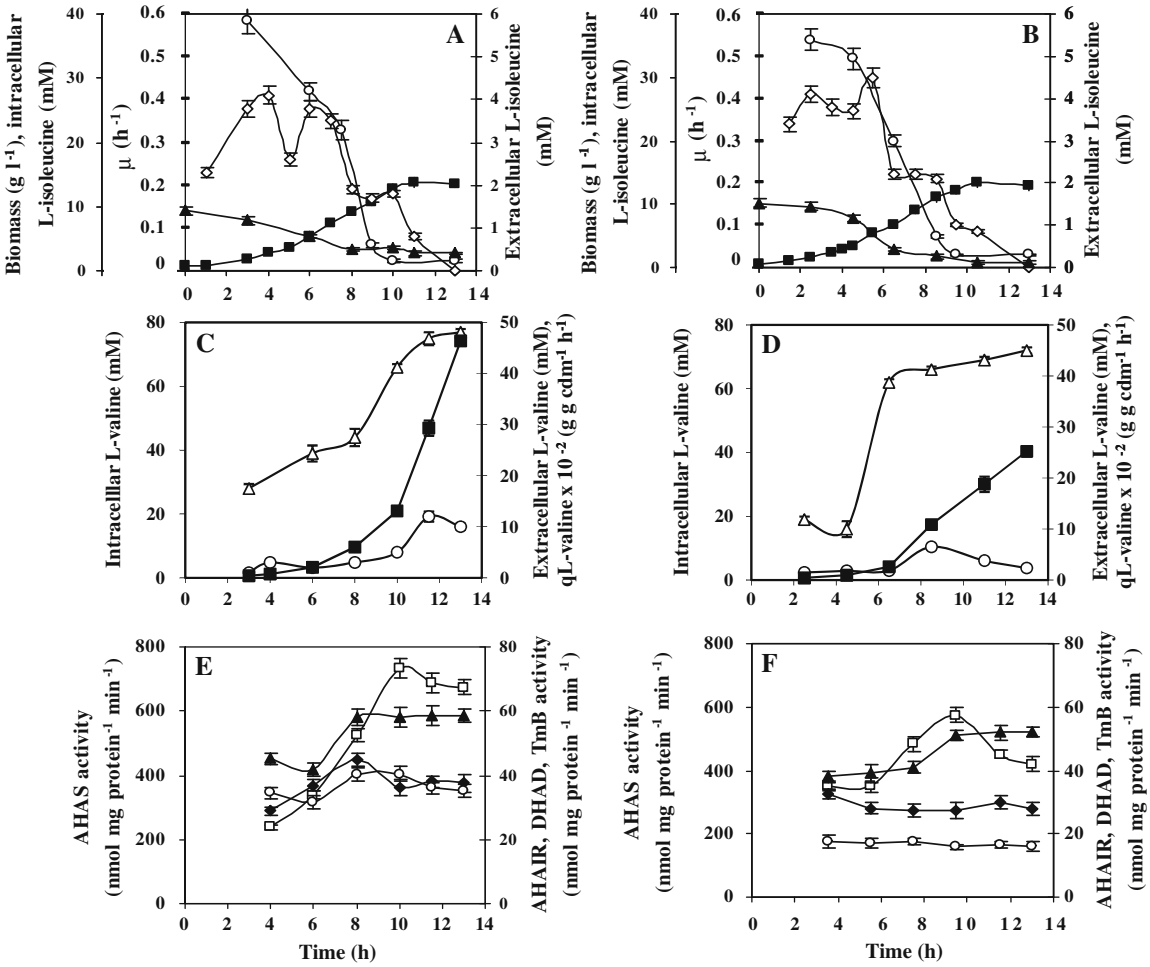

Fig. 3 Fed-batch cultivations of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) (**a**, **c**, **e**) and its *rel*⁻ derivative (**b**, **d**, **f**). **a**, **b** Biomass (*filled square*), cellular growth rate (*open diamond*), extracellular (*filled triangle*) and intracellular (*open circle*) L-isoleucine; **c**, **d** intracellular (*open triangle*) and extracellular (*filled square*) L-valine and rate of L-valine synthesis (*open circle*); **e**, **f** activity of AHAS (*open square*), AHAIR (*filled triangle*), DHAD (*filled diamond*) and TmB (*open circle*). Values are averages of data from two independent samples of each time point of one cell culture with three replicates of each analytical measurement ± SD

To document the observed effects of L-isoleucine limitation and the induction of the stringent response on the activity of the enzymes involved in the L-valine synthesis pathway in the tested *C. glutamicum* strains, we performed short-term recultivations of the exponentially growing *rel*⁺ and *rel*⁻ cells under conditions of L-isoleucine excess (medium supplemented with 1.52 mM L-isoleucine) and deficiency (L-isoleucine-free medium supplemented with 10 mM L-valine) (Table 1). Complete depletion of intracellular L-isoleucine was achieved after a 1-h recultivation of the cells in the L-isoleucine-free medium**.** The L-isoleucine limitation resulted in a lower biomass formation and a significant increase in AHAS and AHAIR activity in both the *rel*⁺ and *rel*⁻cells. In *rel*⁺ cells, it also resulted in a slight increase in DHAD activity, but in the *rel*⁻ cells a significant decrease in this enzyme activity was apparent (Table 1).

Variations in cellular respiration during cultivations of *rel*⁺ and *rel*⁻ strains were further investigated in order to determine possible reasons for low L-valine synthesis in slow-growing stringent negative cells. We found that increased enzyme activity and L-valine synthesis in *rel*⁺ cells was related to increased ATP synthesis-coupled and decreased ATP synthesis-uncoupled respiration. The $Q_{O2\ CN\text{-}sensitive}$ of *rel*⁺ cells subjected to L-isoleucine limitation was approximately twofold higher than that of *rel*⁻ cells, but the $Q_{O2\ CN\text{-}resistant}$ was approximately twofold lower (Table 1).

Increased cyanide-resistant respiration in *C. glutamicum* has been reported to result from reactions in which molecular oxygen chemically oxidizes redox enzymes, forming a superoxide [43]. Therefore, the aforementioned differences in the respiration of *rel*⁻ as compared to *rel*⁺ cells could be an indication of the increased oxidative stress

Table 1 Effect of L-isoleucine availability on biomass formation, cellular respiration and enzyme activity in *Corynebacterium glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) and its *rel*⁻ derivative

Parameters	Strain			
	rel⁺		*rel*⁻	
	L-isoleucine availability			
	Excess	Limitation	Excess	Limitation
X (g l^{-1})	6.87 ± 0.55	4.80 ± 0.32	6.45 ± 0.45	4.63 ± 0.40
$Q_{O2\ summary}$ (nmol mg CDM^{-1} min^{-1})	96.6 ± 5.8	89.5 ± 7.2	126.4 ± 8.8	64 ± 5.7
$Q_{O2\ CN\text{-}resistant}$ (% of total)	12.0 ± 0.7	29.0 ± 2.3	47.0 ± 3.2	50.0 ± 4.0
$Q_{O2\ CN\text{-}sensitive}$ (nmol mg CDM^{-1} min^{-1})	85.0 ± 5.0	63.6 ± 5.1	67.0 ± 5.0	32.0 ± 2.8
AHAS activity (nmol mg protein min^{-1})	451 ± 24	556 ± 32	364 ± 16	437 ± 18
AHAIR activity (nmol mg protein min^{-1})	68 ± 5	88 ± 7	43 ± 4	56 ± 3
DHAD activity (nmol mg protein min^{-1})	36 ± 4	44 ± 4	13 ± 2	7 ± 1
TmB activity (nmol mg protein min^{-1})	32 ± 2	16 ± 3	17 ± 2	16 ± 2

Q_{O2} cellular oxygen consumption rate, $Q_{O2\ CN\text{-}resistant\ (\text{-}sensitive)}$ cyanide-resistant (-sensitive) respiration, *CDM* cell dry mass, *AHAS* acetohydroxyacid synthase, *AHAIR* acetohydroxyacid isomeroreductase, *DHAD* dihydroxyacid dehydratase, *TmB* L-glutamate-dependent transaminase B

Data represent average values ± standard deviation for three independent cultures

Results of 4-h recultivation under conditions of L-isoleucine excess or limitation (L-isoleucine-free medium supplemented with 10 mM L-valine)

in stringent-negative cells exposed to L-isoleucine limitation. Moreover, the short-term treatment of cells with 10 mM hydrogen peroxide resulted in a complete elimination of cyanide-sensitive respiration and a drastic increase in cyanide-resistant respiration in *rel*⁻ cells, whereas only a 2.2-fold decrease and a 1.3-fold increase in the respective respirations in *rel*⁺ cells was observed. We also estimated a 22% lower viability of *rel*⁻ cells under hydrogen peroxide stress.

The comparatively low activity of AHAS, DHAD and TmB in *rel*⁻ cells subjected to L-isoleucine limitation or hydrogen peroxide stress (latter data not shown) could be an indication that the decrease in both enzyme activity and L-valine synthesis resulted from the increased oxidative stress in *rel*⁻ cells. Furthermore, a 33% lower L-valine production was achieved by the *rel*⁻ strain relative to the *rel*⁺ *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) strain during the fed-batch cultivations illustrated in Fig. 3 (L-valine concentration after a 24-h cultivation was 50 and 75 mM, respectively). It should be noted that the estimated biomass of *rel*⁻ was only approximately 8% less than that of the *rel*⁺ strain after the 24-h cultivation (11.5 and 12.5 g l^{-1}, respectively).

Discussion

Ruklisha et al. [41] found that a transition in the bacterial growth rate to below maximum during batch- and fed-batch cultivations is related to an increase in L-valine synthesis in *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*). Restricted cellular growth resulting from a limited supply of medium constituents [22, 35, 37, 40, 44] as well as intracellularly introduced growth limitations [11] have been demonstrated to be conditions that also enhance L-lysine synthesis in *C. glutamicum*.

In our study, we focused on investigating factors which could be responsible for the increase in L-valine synthesis in growth-limited cultures of a *C. glutamicum* strain auxotrophic for L-isoleucine. The increase in L-valine synthesis in growth-limited *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) in this study was, as in *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) [41], related to a sharp increase in AHAS activity in cells.

AHAS in *C. glutamicum* exhibits a weak affinity to pyruvate (Km pyruvate = 8.3 mM) [28]. Thus, intracellular pyruvate availability could be essential to maintaining AHAS activity and L-valine synthesis in cells. Increased intracellular pyruvate levels have been shown to have a positive effect on L-valine synthesis in other *C. glutamicum* recombinant strains [2]. However, increased AHAS activity and the overproduction of L-valine (and other pyruvate-derived amino acids) in growth-limited cells of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) was related to a decrease in the intracellular pyruvate level, suggesting that pyruvate was utilized efficiently for amino acid synthesis. Thus, we suggest that the upregulation of pyruvate-derived amino acid synthesis in the growth-limited cells of this strain was not due to an increased intracellular pyruvate concentration but to an increased rate of precursor utilization for amino acid synthesis.

The increase in AHAS activity in *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) was related to the

decrease in L-isoleucine concentration in the medium and cells. Since the expression of *ilvBN* is known to be controlled by transcriptional attenuation involving anti-termination in response to a deficiency in any of the three BCAAs [32], the increase in AHAS activity in cells could result from L-isoleucine limitation. On the other hand, it was found out that growth limitation by amino acid results in the induction of the stringent response in *C. glutamicum* cells [38, 45, 47]. Therefore, we speculated that the positive effect of L-isoleucine limitation on AHAS upregulation in *C. glutamicum* could also be due to ppGpp-mediated stringent response induction, as has been reported for *Escherichia coli* and *Bacillus subtilis* [13, 46]. We found that a rapid increase in the intracellular concentration of ppGpp under conditions of L-isoleucine limitation correlated with an increase in AHAS activity and an increased L-valine synthesis by *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*).

In order to distinguish whether it was the L-isoleucine limitation itself or the stringent response induced by this limitation that was crucial for upregulation of AHAS (and probably other L-valine synthesis enzymes as well), we constructed the *C. glutamicum* Δ*ilvA* Δ*panB* Δ*rel ilvN*M13 (pECKA*ilvBNC*) strain. The results of short-term recultivations, similarly to those of fed-batch cultivations, showed that AHAS and AHAIR activity and L-valine production under the conditions of L-isoleucine limitation were increased in both *rel*$^+$ and *rel*$^-$ strains. Consequently, we conclude that the stringent response was not involved in enzyme and L-valine synthesis upregulation in growth-limited *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*). Moreover, we observed a distinct increase in AHAS and AHAIR activity in plasmid-less *rel*$^+$ and *rel*$^-$ *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 strains subjected to L-isoleucine limitation (data not shown). The strong upregulation of the transcription of genes encoding L-lysine synthesis under conditions of amino acid limitation in an L-lysine-producing *C. glutamicum* strain has also been found to be independent of the stringent response induction [15]. However, the results of our study show that the presence of the *rel* gene is beneficial for L-valine synthesis by the late stationary phase of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) cell culture. AHAS, TmB and particularly DHAD activity as well as L-valine synthesis during the stationary growth phase was clearly higher in the *rel*$^+$ strain**.**

We show that the presence of the *rel* gene had a positive effect in maintaining ATP-synthesis-coupled respiration and in minimizing ATP-synthesis-uncoupled respiration in cells subjected to L-isoleucine starvation. This gene was beneficial in maintaining ATP-synthesis-coupled respiration as well as the viability of cells subjected to hydrogen peroxide stress. Since an increase in cyanide-resistant respiration in *C. glutamicum* has previously been reported to result from superoxide generation in cells [43], the comparatively low cyanide-resistant respiration could be an indication of a low oxidative stress in *rel*$^+$ cells.

Based on increased L-valine synthesis in the *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) *rel*$^+$ strain, we surmise that the positive effect of the stringent response on L-valine synthesis may result from its positive effect in reducing oxidative stress. The decrease in L-valine synthesis in bacterial cells under oxidative stress conditions could result from superoxide-caused damage in the DHAD enzyme [24] as well as from excess NADPH + H^+ utilization in oxidative stress-defence reactions [5].

The expression of genes involved in oxidative stress-defence reactions in *C. glutamicum* has been reported to be controlled by the sigma factors SigB, SigM and SigH [21, 26, 34]. Brockmann-Gretza et al. [4] found that the increased expression of *sigB* is dependent on the induction of the stringent response in this bacterium. Thus, the positive effect of the stringent response on oxidative stress-defence capability in *C. glutamicum* could result from the *rel*-dependent induction of *sigB*. However, there are as yet no data indicating that genes encoding SigM and SigH could also be under the positive control of the stringent response.

Taken together, our results demonstrate that AHAS upregulation, resulting from L-isoleucine limitation, is a major factor enhancing L-valine production in growth-limited *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*). The stringent response was not shown to be directly involved in the upregulaton of enzyme and L-valine synthesis in this strain. However, the stringent response was found to be essential to maintaining an increased enzyme activity and L-valine synthesis in slowly growing cells.

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