Acta Universitatis Latviensis, 2005, Vol. 691, Biology, pp. 83–91

The effect of stringent control on valine biosynthesis by *Corynebacterium glutamicum*

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Abstract

The present study focused on methods of stringent response induction and the investigations of its effect on valine synthesis by the isoleucine auxotroph *Corynebacterium glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*. The intracellular concentration of guanosine tetraphosphate increased and bacterial growth rate decreased, i.e. intracellular stringent response, was induced under conditions of (i) cell culture growth in medium lacking isoleucine and (ii) reduced isoleucine uptake by cells. The induction of the cellular stringent response was followed by an increase in the activity of acetoxydroxy acid synthase, e.g. *ilvBN*-encoded key enzyme of valine synthesis, and by a drastic increase in the cell specific rate of amino acid synthesis. It was demonstrated that induction of the stringent response can be used as method to increase valine overproduction by *C. glutamicum* cells.

Key words: *Corynebacterium glutamicum*, ppGpp, stringent control, valine biosynthesis.

Introduction

The stringent response is a pleiotropic physiological response of bacterial cells to amino acid or energy source limitation (Cashel et al. 1996). Many features of the stringent response are mediated by the accumulation of hyperphosphorylated guanosine nucleotide guanosine 5'-diphosphate 3'-diphosphate (ppGpp). Usually two related proteins RelA and SpoT are involved in the metabolism of ppGpp in bacterial cells. The RelA protein encoded by *relA* gene has synthase activity and is responsible for ppGpp biosynthesis during amino acid limitation. *RelA* is activated when uncharged tRNA binds to the acceptor site of a translating ribosome. The SpoT protein encoded by the *spoT*| gene is primarily a ppGpp hydrolase, but it also catalyzes ppGpp synthesis in response to glucose starvation (Cashel et al. 1996).

The *relA*- and *spoT*-regulated stringent mechanism has been described in gram negative bacteria *Escherichia coli* and *Salmonella typhimurium* (Cashel et al. 1996). In contrast, a *relA*/*spoT*| homologous gene (*rel*) that encodes a bifunctional enzyme with ppGpp synthase and ppGpp-degrading activities has been described in the gram positive bacterium *Corynebacterium glutamicum* (Wehmeier et al. 1998), also in *Streptococcus equisimilis* (Mechold, Malke 1997), *Bacillus subtilis* (Wendrich, Marahiel 1997) and *Mycobacterium tuberculosis* (Tauch et al. 2001).

Intracellular ppGpp in bacterial cells functions as an organiser of the adaptive cellular response to starvation. ppGpp co-ordinates the global transcriptional pattern with the

current growth conditions: it mediates feedback repression (negative control) or even induction of the expression (positive control) of many genes in bacterial cells. Since ppGpp serves as a specific alarmone of the stringent response, it may be used as an indicator of starvation for amino acids and an energy source in bacterial cells (Cashel et al. 1996).

The negative stringent control usually concerns reactions typical of growth and reproduction: an immediate reduction of the ribosomal RNA (*rrn*) operon transcription and a massive reduction of the synthesis of stable RNA (rRNA and tRNA; Cashel et al. 1996; Zhang et al. 2002), also a reduction of the synthesis of a certain mRNAs and an increase in the rate of protein degradation in bacterial cells (Lewin 2000). The accumulation of ppGpp in bacterial cells can also cause negative effects on the biosynthesis of lipids, polyamines and peptidoglycan (Cashel et al. 1996). All of these negative effects lead to changes in bacterial physiology, predominantly to the reduction of bacterial growth rate.

In contrast, in some cases ppGpp appears to have a positive regulatory effect on the expression of genes and on the translation level of enzymes involved in the biosynthesis of some amino acid. Induction of the ppGpp-mediated *relA*-dependent stringent control was found to be required to increase the expression of the genes encoding branched-chain amino acid synthesis in *E. coli*, *S. enterica* serovar typhimurium (Tedin, Norel 2001) and *B. subtilis* (Eymann et al. 2002). It has also been shown that induction of this control is required to elevate the translation level of branched chain amino acid synthesis enzymes in *B. subtilis* (Eymann et al. 2002). However, further research is required to establish whether the synthesis of branched-chain amino acids in other bacteria is under the strong control by stringent response mechanism as well.

The gram-positive soil micro-organism *Corynebacterium glutamicum* is of a special interest for the industrial production of amino acids. During the recent 40 years various mutants of *C. glutamicum* have been isolated with the capacity to produce significant amounts of different L-amino acids (glutamic acid, lysine, threonine and others). In spite of the great demand on the world-wide market, industrial production of L-valine with this or another bacteria is still not developed. The main reason is a complicated control of valine synthesis in bacterial cells. For example, acetohydroxy acid synthase (AHAS), the key enzyme of valine biosynthesis in *C. glutamicum* is common for the synthesis of all branched chain amino acids and is feedback inhibited by all of them (Morbach et al. 2000). Hence, it is very important to construct strains that will aid to increase our knowledge about the cellular metabolism control and to find methods to increase valine overproduction in bacterial cells.

It has been reported that lysine synthesis by *C. glutamicum* RC 115 can be significantly enhanced by the induction of the ppGpp-mediated stringent response (Ruklisha et al. 1995; Ruklisha et al. 2001). This effect was explained as an increase in lysine synthesis activity in cells as a consequence of an increased intracellular precursor availability.

The goal of this study was to identify methods of the stringent response induction in *C. glutamicum* cells and to investigate the effect of this response on valine synthesis by its producing bacteria.

Materials and methods

Bacterial strains and culture conditions

The strain used in this study was *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*. This strain,

auxotrophic for isoleucine, was shown to be able to excrete L-valine and D-pantothenic acid (Sahm, Eggeling 1999).

Brain heart infusion (BHI) medium was used for pre-culture growth. CG XII minimal medium (Keilhauer et al. 1993) with some modifications (isoleucine concentration 0.57 mM or 1.14 mM, 3 mg l^{-1} deferoxamine instead of 30 mg l^{-1} protocatechuic acid, 2 mg l^{-1} biotine instead of 0.2 mg l^{-1} and 50 mg ml^{-1} kanamycin) was used for main culture growth. The pH was maintained at 7.0 due to the addition of 42 g l^{-1} MOPS in the culture medium. The cultivations were performed at 30 °C in buffled Erlenmeyer flasks on a rotary shaker (220 rpm). At least three runs of experiments were performed in order to estimate kinetic parameters.

Physiological parameters

Bacterial growth was followed by measuring the optical density of the cell culture at 600 nm in a Helios UV-Visible spectrophotometer (Thermo Spectronic, UK). Concentrations of amino acids in the cell culture were quantified by HPLC method as described previously (Ruklisha, Paegle 2001). Using the kinetic experimental data for measured biomass and valine concentrations, the specific rates of bacterial growth (μ) and valine synthesis (q_p) were calculated (Ruklisha et al. 1995).

Determination of acetoxydroxy acid synthase activity

The cells were harvested by centrifugation for 15 min at 10 000 g_n at 4 °C, washed twice with 2 % KCl and resuspended in 100 mM potassium phosphate buffer (pH 7.3) containing 0.5 mM ditiothreitol and 20 % (v/v) glycerol. The cells were disrupted by pulsed sonication ('Dr. Hielscher' ultrasonic processor, Germany) of 8 min total duration, with 0.5-s pulses and 0.5-s intervals between them. Cell debris and intact cells were separated from the cell extract by centrifugation (30 min, 4 °C, 12 000 g_n). The activity of acetoxydroxy acid synthase (AHAS, EC 4.1.3.18) was assayed in the cell-free extracts as described by Leyval et al. (2003). The method is based on the conversion of pyruvate to α-acetolactate, which was subsequently decarboxylated to acetoin and detected by the colorimetric method of Westerfeld (1945) using a Helios UV-Visible spectrophotometer (Thermo Spectronic, UK). The AHAS specific activity was expressed in nmol of α-acetolactate formed per mg protein per min. The protein concentration in cell free extracts was determined by Lowry's method (Lowry et al. 1951).

Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) assays

ppGpp was extracted from the cells with 0.2 M KOH as described by Zhang et al. (2002). Nucleotides in cell extracts were separated by isocratic ion exchange HPLC (Waters 501, USA) with a 4.6 × 250 mm Hypersil Sax 5 μm column (Alltech, Belgium), 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 absorbance at 254 nm using a Tunable Absorbance Detector (Waters 486, USA).

Chemicals

ppGpp was obtained from TrilinkBiotech (USA). BHI was purchased from Liofilchem (Italy). All other biochemicals were of analytical grade and purchased from Sigma-Aldrich.

Results

In order to induce the stringent response in bacterial cells and to estimate the consequences of this response on valine synthesis, short-term experiments of *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* growth under isoleucine-unlimited or limited conditions (control and experimental version, respectively) were carried out.

Cells were precultured in a modified CGXII minimal medium with isoleucine concentration 0.57 mM. Cell cultures in the exponential growth phase ($\mu = 0.20 \pm 0.01$ h^{-1}) were harvested by centrifugation, then washed with CGXII minimal medium lacking isoleucine and resuspended in a medium either with or without 1.14 mM isoleucine. Cells were re-cultivated for 7 h.

The experimental results showed that bacterial growth was significantly influenced by isoleucine limitation (Fig. 1): the biomass concentration in the cell culture subjected to isoleucine starvation for 3.5 h was 1.9 times lower in comparison with the concentration achieved in a standard medium. Also, the calculated value of the specific growth rate of cells cultured under isoleucine limited conditions was low ($\mu = 0.06 \pm 0.00$ h^{-1}), compared to the value achieved by cells cultured in a standard medium ($\mu = 0.28 \pm 0.01$ h^{-1}). The intracellular concentration of ppGpp showed a slight increase after 1 to 2 h and a drastic increase after 3.5 h of cell culture growth under isoleucine limited conditions. The final intracellular concentration of ppGpp under the latter conditions reached 0.27 nmol mg^{-1} DM (Fig. 1). In contrast, intracellular ppGpp was not detected in cells grown under isoleucine-unlimited conditions.

To verify the effect of isoleucine limitation on ppGpp synthesis and the stringent response induction in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells, additional experiments were carried out: isoleucine uptake by cells was reduced by supplementation of the cell culture with valine. As competition occurs between isoleucine and valine for uptake by BrnQ, a carrier that transports all branched-chain amino acids into *C. glutamicum* cells (Lange et al. 2003), the inhibition of isoleucine uptake can be achieved by increased concentrations of valine in the medium. High concentrations of valine in the medium can eventually evoke the same isoleucine starvation effect in bacterial cells.

The scheme of experiments for investigating the effect of isoleucine uptake reduction on bacterial growth and ppGpp accumulation in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*

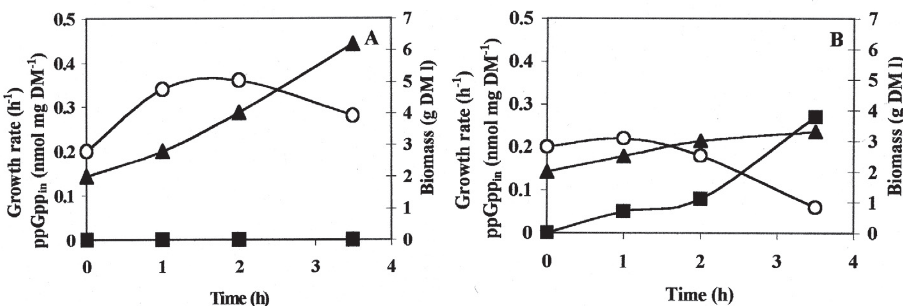

Fig. 1. Changes in biomass concentration (▲), bacterial growth rate (❍) and ppGpp concentration (■) in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under isoleucine unlimited (A) or isoleucine limited (B) conditions achieved by its extracellular limitation.

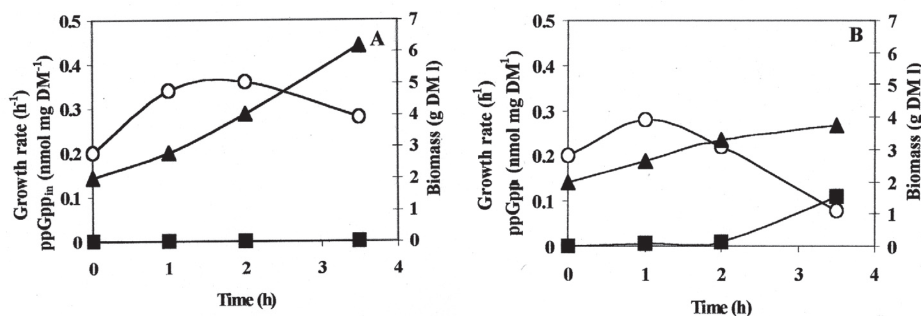

Fig. 2. Changes in biomass concentration (▲), bacterial growth rate (❍) and ppGpp (■) concentration in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under isoleucine unlimited (A) or isoleucine uptake limited (B) conditions achieved by cell culture supplementation with 175 mM valine.

cells was similar to that used to estimate the effect of isoleucine limitation in the medium. Cells were precultured in CGXII minimal medium with an isoleucine concentration of 1.14 mM for 4 h. Then the cell culture was collected and re-cultivated in CGXII medium without or with 175 mM valine for 7 h.

The re-cultivations showed a significant decrease in biomass formation and bacterial growth rate, as well as an increase in the intracellular concentration of ppGpp under conditions when cell culture was exposed with 175 mM valine (Fig. 2).

There was an inverse relation between biomass formation and intracellular ppGpp accumulation in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under conditions with extracellularly limited isoleucine or those with restricted isoleucine uptake, and ppGpp accumulation was absent in cells grown under isoleucine unlimited conditions. This indicated that *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* metabolism under both of these conditions might be regulated in a stringent response manner using ppGpp as an alarmone.

Further, the effect of the stringent response on valine biosynthesis by *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* was investigated. It was observed that an increase in ppGpp accumulation was followed by a significant increase in AHAS activity and an increase in the specific rate of valine synthesis of cells subjected to isoleucine starvation (Table 1). This suggested that the stringent response might lead to an increase in valine overproduction.

It should be noted that a more significant increase in the cell specific rate of valine synthesis observed under stringent response conditions induced in *C. glutamicum* cells by the restriction of isoleucine uptake. Thus, besides AHAS activity, other intracellular conditions (intracellular precursor concentrations or others) might also be important enhancing the valine synthesis activity of bacterial cells under the stringent response conditions.

Discussion

The intracellular concentration of ppGpp increased and the specific growth rate of *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells decreased under conditions of isoleucine limitation e.g. bacterial growth in a medium lacking isoleucine or those with reduced

Table 1. The effect of isoleucine limitation on 5'-diphosphate 3'-diphosphate ($ppGpp_{in}$) concentration, acetohydroxy acid synthase (AHAS) activity and cell-specific rate of valine synthesis of *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*. Parameters were estimated after 3.5 h ([a]) or 7.0 h ([b]) of cell culture growth under the specified conditions. The data shown are means and standard deviations from three runs of experiments with five replicates for each estimation

Culture conditions	Medium modification	ppGppin (nmol mg^{-1} DM)[a]	AHAS activity (nmol min^{-1} mg^{-1} protein)[a]	Valine synthesis (g h^{-1} g^{-1} DM)[b]
Isoleucine unlimited	–	0	362 ± 10	0.043 ± 0.002
Isoleucine limited	Isoleucine omittion	0.270 ± 0.008	590 ± 22	0.073 ± 0.003
Isoleucine limited	Supplementation with 175 mM valine	0.110 ± 0.005	692 ± 24	0.113 ± 0.004

isoleucine uptake by valine. In contrast, intracellular ppGpp was not detected in cells grown under isoleucine-unlimited conditions. Consequently, the $ppGpp_{in}$ concentration was increased and the stringent response was induced in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under conditions of isoleucine limitation.

Further, the effect of the stringent response on valine synthesis by *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* was investigated. An increase in the activity of AHAS e.g. a key enzyme of valine synthesis, and the cell specific rate of valine synthesis was enhanced under conditions with extracellular or intracellular limitation of cell culture growth by isoleucine. Therefore, an increase in AHAS activity in cells was directly related with an increase in ppGppin concentration and inverse related with the specific bacterial growth rate and biomass concentration achieved in the cell culture. Consequently an increase in valine synthesis might be a result of the stringent response induced in bacterial cells by isoleucine limitation.

It has been reported that transcription of *ilvBNC* operon encoding acetoxydroxy acid synthase and isomeroreductase, e.g. key enzymes of L-isoleucine, L-valine and L-leucine synthesis in *C. glutamicum*, is repressed by an excess of branched chain amino acids (5 mM each) and that this transcription may be increased under limiting conditions for any of these amino acids (0.5 mM each) (Morbach et al. 2000). It has also been reported that exposure of *C. glutamicum* cells with 40 mM valine, demonstrating the effect of isoleucine uptake restriction, causes an increase in *ilvBN* mRNA levels, and also an increase in the intracellular level of the *ilvB* protein product i.e. the large subunit of AHAS (Lange et al. 2003). Therefore, isoleucine limitation may be a pre-condition for the derepression of transcription of genes encoding enzymes of branched chain amino acid synthesis, also a pre-condition for the increase of enzyme translational level in *C. glutamicum*. However, transcriptome analysis of *B. subtilis* wild type strain (*relA*$^+$) and *relA*$^-$ mutant proved that functioning of the *relA* gene was absolutely required for the derepression of transcription of *ilvBNC* operon under branched-chain amino acid limited conditions (Eymann et al. 2002). Moreover, a *relA*$^-$ mutation in *B. subtilis* resulted in a strain auxotrophy for valine and a weaker one for isoleucine, leucine and methionine (Wendrich, Marahiel 1997). It

was also shown that *relA*- mutants of *E. coli* K-12 strains failed in derepression of *ilvBN* (Freundlich 1977). These investigations clearly demonstrated that transcription of operons encoding synthesis of branched chain amino acids in *B. subtilis* and *E. coli* K-12 was strongly dependent on *relA* and the stringent response induction. However it is not clear if synthesis of branched chain amino acids in other bacteria is under strong dependence on *relA* induction as well.

Our results suggest that an increase in AHAS activity in *C. glutamicum* under isoleucine limited conditions might be a consequence of the derepression of *ilvBN* operon. Correlation between the intracellular concentration of ppGpp, the activity of AHAS and the cell specific rate of valine synthesis, may indicate that the stringent response mechanism might directly control valine synthesis by this bacterium. However, further research is required to estimate whether *rel*-dependent, ppGpp mediated stringent response is required for induction of the transcription of operons encoding branched chain amino acid synthesis in *C. glutamicum*.

The effect of the stringent response on synthesis of branched chain amino acids and other metabolic processes in bacterial cells might also be indirect. Moreover, transcriptome and proteome analysis of *B. subtilis* wild type and *relA*- mutant strain showed that some genes may be induced or downregulated independently of *relA* even under the stringent response conditions.

The positive effect of isoleucine limited conditions on valine synthesis by *C. glutamicum*, shown in this study, might be a result of the derepression of *ilvBN* transcription in response to isoleucine limitation, also a result of *rel*-dependent induction of *ilvBN* transcription. This means that valine synthesis in this bacterium might be indirectly or directly controlled by the stringent control mechanism.

It can not be excluded that valine synthesis in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* might also be an indirect effect of the intracellular stringent response situation e.g. effect of an increased availability of precursors leading to increased activity of valine synthesis enzymes. AHAS in *C. glutamicum* exhibits a weak affinity for pyruvate (K_M = 8.3 mM) (Leyval et al. 2003). Consequently, the increase in intracellular pyruvate concentration might have a significant impact on the activity of this enzyme in bacterial cells.

Therefore, stringent response induction in *C. glutamicum* cells might be used as a method to enhance valine synthesis by this bacterium. However further research should be done to estimate whether an increase in valine overproduction by this bacterium is a direct consequence of the *rel*-dependent induction of valine synthesis encoding genes.

Acknowledgements

The present work was supported by a grant from EU (VALPAN, QLK3-2000-00497) and a grant from the Science Council of Latvia (grant No. 04.1112). I. Denina was supported by a grant from The European Social Fund (ESF). The authors wish to thank Dr. L. Eggeling from the Institut für Biotechnologie 1, Forschungzentrum-Jülich (Germany) for providing *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* and the company Degussa AG (Germany) for providing ppGpp as a generous gift.

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Stringent **mehānisma loma valīna biosintēzes regulācijā *Corynebacterium glutamicum* šūnās**

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Kopsavilkums

Pētījumu mērķis bija noskaidrot *stringent* kontroles nozīmi valīna biosintēzes regulācijā *Corynebacterium glutamicum* šūnās. *Stringent* kontroli izoleicīna auksotrofa *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* celma šūnās inducēja ārpusšūnas izoleicīna ierobežojuma apstākļos, kā arī samazinot šīs aminoskābes transportu šūnās: abos gadījumos palielinājās iekššūnas guanozīna tetrafosfāta koncentrācija un samazinājās šūnu augšanas ātrums. Vienlaicīgi šīs kontroles inducēšana izraisīja krasu valīna sintēzes regulatorā fermenta acetohidroksiskābes sintēzes aktivitātes, kā arī šūnu valīna sintēzes specifiskā ātruma palielināšanos. Pētījumi liecina, ka *stringent* kontroles inducēšanu var pielietot, lai palielinātu valīna virssintēzi *C. glutamicum* šūnās.