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ORIGINAL PAPER

S. Guillouet · A. A. Rodal · G-H. An · N. Gorret
P. A. Lessard · A. J. Sinskey

Metabolic redirection of carbon flow toward isoleucine by expressing a catabolic threonine dehydratase in a threonine-overproducing *Corynebacterium glutamicum*

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Abstract Carbon destined for lysine synthesis in *Corynebacterium glutamicum* ATCC 21799 can be diverted toward threonine by overexpression of genes encoding a feedback-insensitive homoserine dehydrogenase (*hom*^{dr}) and homoserine kinase (*thrB*). We studied the effects of introducing two different threonine dehydratase genes into this threonine-producing system to gauge their effects on isoleucine production. Co-expression of *hom*^{dr}, *thrB*, and *ilvA*, which encodes a native threonine dehydratase, caused isoleucine to accumulate to a final concentration of 2.2±0.2 g l^{-1}, five-fold more than accumulates in the wild-type strain, and approximately twice as much as accumulates in the strain expressing only *hom*^{dr} and *thrB*. Comparing these data with previous results, we found that overexpression of the three genes, *hom*^{dr}, *thrB*, and *ilvA*, in *C. glutamicum* ATCC 21799 is no better in terms of isoleucine production than the expression of a single gene, *tdcB*, encoding a catabolic threonine dehydratase from *Escherichia coli*. Co-expression of *hom*^{dr}, *thrB*, and *tdcB*, however, caused the concentration of isoleucine to increase 20-fold compared to the wild-type strain, about four times more than the corresponding *ilvA*-expressing strain. In this system, the apparent yield of isoleucine production was multiplied by a factor of two [2.1 mmol (g dry cell weight)$^{-1}$]. While the balance of excreted metabolites showed that the carbon flow in this strain was completely redirected from the lysine pathway into the isoleucine pathway, it also showed that more pyruvate was diverted into amino acid synthesis.

S. Guillouet · A.A. Rodal · G-H. An · N. Gorret · P.A. Lessard
A.J. Sinskey (✉)
Department of Biology 68-370,
Massachusetts Institute of Technology, 77 Massachusetts Avenue,
Cambridge MA 02139, USA
e-mail: Asinskey@mit.edu
Tel.: +1-617-2536721, Fax: +1-617-2538550

Present addresses:
S. Guillouet, INSA de Toulouse,
Departement de Genie Biochimique Alimentaire,
135 Avenue de Rangueil, 31077 Toulouse Cedex 4, France

A. A. Rodal, Department of Molecular and Cell Biology,
University of California at Berkeley, Berkeley CA 94720, USA

G-H. An, Division of Applied Biological Sciences,
Sunmoon University, 100 Kalsan-Ri, Tangjeong-Myon, Asan,
Chungnam 336-840, Korea

Introduction

Corynebacterium glutamicum is used in the commercial production of amino acids, primarily glutamic acid and lysine (Lessard et al. 1999). Metabolic engineering of this bacterium has sought not only to improve glutamic acid and lysine production, but also to divert carbon from these pathways into other products. The primary challenge in this research centers on developing strategies to overcome the mechanisms that regulate flux through these biosynthetic pathways. For example, *C. glutamicum* regulates lysine biosynthesis at a few key nodes. The first of these nodes is controlled by aspartokinase, a lysine- and threonine-sensitive enzyme that modulates flux toward all of the aspartate-derived amino acids, which include lysine, methionine, threonine and isoleucine (Fig. 1). Strains of *C. glutamicum*, such as ATCC strain 21799, that express feedback-insensitive forms of aspartokinase overproduce aspartate semialdehyde (ASA), which is then converted into lysine. Colon et al. (1995a) have shown that some of the ASA produced in this strain can be diverted from the lysine pathway toward threonine by overexpressing a feedback-insensitive form of homoserine dehydrogenase (encoded by *hom*^{dr}) in conjunction with homoserine kinase (encoded by *thrB*).

More recently, we have been able to convert a lysine-producing strain into an isoleucine-producing strain by introducing a catabolic threonine dehydratase that converts threonine into α-ketobutyrate, the first step toward isoleucine biosynthesis (Guillouet et al. 1999). Surprisingly, although Colón et al. (1995b) steered flux away from lysine and toward isoleucine by overexpressing the deregulated homoserine dehydrogenase, homoserine ki-

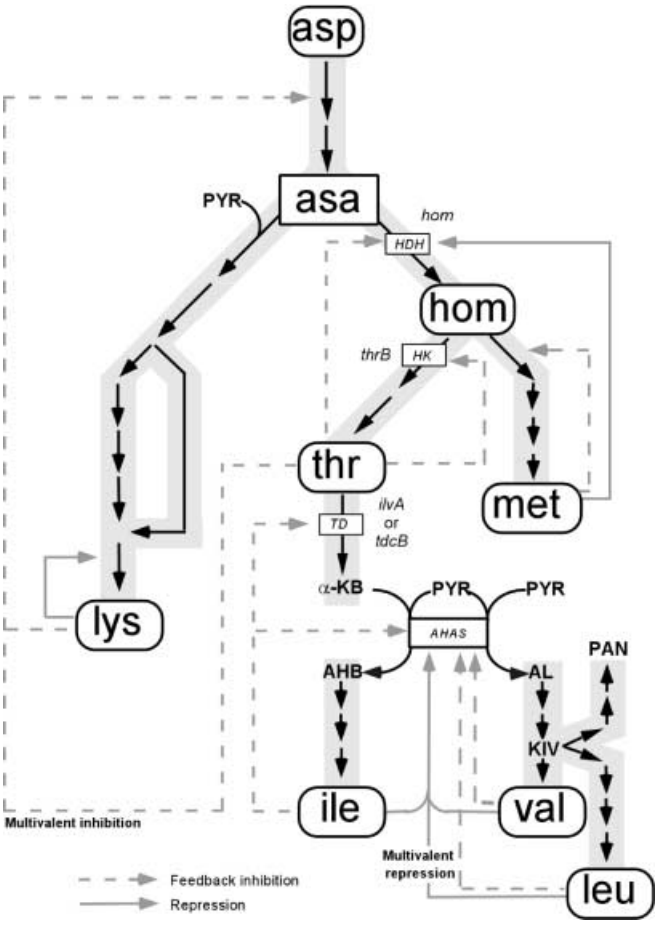

Fig. 1 Biosynthesis and regulation of the aspartate-derived amino acids. *asp* Aspartic acid, *asa* aspartate semialdehyde, *hom* homoserine, *thr* threonine, *met* methionine, *lys* lysine, *ile* isoleucine, *val* valine, *leu* leucine, *α-KB* α-ketobutyrate, *AHB* acetohydroxybutyric acid, *PYR* pyruvate, *AL* acetolactate, *KIV* keto-isovalerate, *PAN* pantothenate, *HDH* homoserine dehydrogenase, *HK* homoserine kinase, *AHAS* acetohydroxyacid synthase, *TD* threonine dehydratase; the genes *hom*, *thrB* and *ilvA* or *tdcB* (which encode *HDH*, *HK* and *TD*, respectively) are indicated alongside each enzyme

nase and threonine dehydratase (encoded by *ilvA*), we found that a catabolic threonine dehydratase alone could consume enough threonine (and therefore enough ASA) to support isoleucine biosynthesis (Guillouet et al. 1999). From this result, we surmise that activity of the catabolic threonine dehydratase (encoded by the *tdcB* gene from *Escherichia coli*) is sufficient to draw intermediates into the threonine-isoleucine pathway such that none of these intermediates rise to concentrations that engage the normal feedback regulatory mechanisms of the resident homoserine dehydrogenase or other enzymes.

Although the earlier work with the *tdcB*-expressing strain was encouraging in terms of isoleucine production, we noted that the strain involved still produced a substantial amount of lysine (more than 25% the amount produced by the strain lacking the *tdcB* gene) (Guillouet et al. 1999). It is possible that, despite its obvious strengths, the threonine-isoleucine biosynthetic pathway that is driven by the catabolic threonine dehydratase cannot fully compete for ASA. In the present study, we determined whether we could further improve isoleucine production by introducing the *hom*^{dr} and *thrB* genes used by Colón et al. (1995b) to draw ASA away from the lysine pathway. This strategy led to an increase in isoleucine levels, drawing enough carbon away from other pathways to further reduce lysine biosynthesis and to introduce a growth limitation to this strain.

Materials and methods

Strains, plasmids and media

Plasmids (Fig. 2) were maintained in *E. coli* XL1-Blue (Stratagene, La Jolla, CA) and subsequently electroporated into *C. glutamicum* ATCC 21799 as described elsewhere (Follettie et al. 1993).

Defined medium for *C. glutamicum* was prepared as described previously (Guillouet et al. 1999). When appropriate, kanamycin (150 mg/l for plasmid maintenance) and isopropyl-β-D-thiogalactopyranoside (IPTG; 150 mg/l to induce expression from the *tac* and *trc* promoters; see below) were used.

For the growth study with amino acid supplements, the defined medium was complemented with Bacto casamino acids (Difco, Detroit, Mich.) at a concentration of 2 g l^{-1} or with amino acid (alanine, glycine, methionine or valine) at a concentration of 0.5 g l^{-1} each.

Plasmid construction

hom^{dr} was amplified by PCR from pGC42 (Colon et al. 1995a) using primers hom1 (5′-CCAAAATGTCCTCACCGGGT-3′) and hom2 (5′-ACTTGTCAACAGCCATGTCAGTAAAATTAGTCCCT-3′). *tac:thrB* was also amplified from pGC42 using primers thrB1 (5′-TGACATGGCTGTTGACAAGTAATCATCGGCT-3′) and thrB2 (5′-CCACGCGTGTTCAGCGAATAATTGCT-3′). Splicing by overlap extension (Horton et al. 1989) was used to fuse the two resulting PCR products, taking advantage of the complementarity built into primers hom2 and thrB1. The joined *hom*^{dr}-*tac:thrB* PCR product was then ligated into pCR-Script (Stratagene). Because the insert in this plasmid was not in the desired orientation, the PCR product was excised from this plasmid via digestion with *Sfu*I and *Bam*HI. This fragment was ligated into the *Bam*HI and *Cla*I sites of pBluescript SK$^-$ (Stratagene), creating pAPE22. The *Xba*I-*Sal*I fragment of pAPE22, containing the *hom*^{dr}-*tac:thrB* PCR product, was then ligated into the *Xba*I and *Sal*I sites of pAPE7, pAPE12 and pAPE13 (Guillouet et al. 1999) to create pAPE18, pAPE19 and pAPE20, respectively (see also Fig. 2). In these constructs, *thrB* was under the control of the *tac* promoter, while *ilvA* and *tdcB* were under the control of the *trc* promoter, permitting induction of these genes by IPTG. Expression of *hom*^{dr} was driven by the native *hom* promoter.

Enzyme assays

Cell-free crude extracts were prepared as described previously (Guillouet et al. 1999) for use in enzyme assays. Protein concentrations were determined by the method of Bradford (1976) with the Bio-Rad (Hercules, Calif.) protein assay kit using bovine serum albumin as a standard.

Threonine dehydratase activity was measured with an assay that monitors the formation of α-ketobutyrate from threonine, as described in (Guillouet et al. 1999). This assay was reproducible within 15% in five-fold replicate measurements. The homoserine kinase assay was based on the coupling of enzyme activity to the oxidation of NADH monitored at 340 nm (ε=6.22 10^3 M^{-1} cm^{-1})

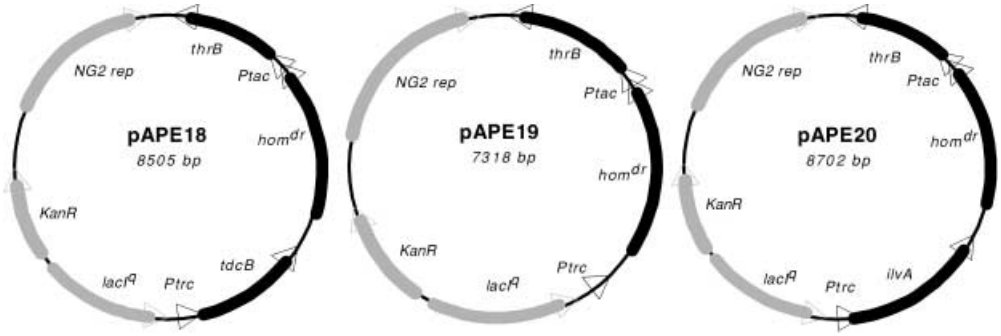

Fig. 2 Plasmid maps. *NG2 rep*, ORF from the NG2 replicon permitting plasmid replication in both *Escherichia coli* and *Corynebacterium glutamicum* (Zhang et al. 1994). P*trc*, *trc* promoter from pTrc99a; P*tac*, *tac* promoter from pGC42; *lacI*^{q}, ORF encoding *lac* repressor from pTrc99a; *KanR*, kanamycin resistance gene

using the protocol described by (Colon et al. 1995a). The reproducibility of the homoserine kinase assay in a given five-fold measurement was typically within 15%. Homoserine dehydrogenase activity was based on the coupling of enzyme activity to the oxidation of NADPH monitored at 340 nm (ε=6.22.10^3 M^{-1}cm^{-1}) using the protocol described by (Colon et al. 1995a). Five-fold repetition of the homoserine dehydrogenase assays showed that the assay was reproducible, typically within 10%.

Fermentations

Fermentations were carried out in duplicate in 2 l of defined medium in a 4-l Chemap CMF100 reactor (Alfa-Laval Chemap, Switzerland) as described previously (Guillouet et al. 1999).

Determination of biomass, sugars, organic acids, amino acids and carbon dioxide

During the fermentation, samples were collected and centrifuged at 4 °C and 10,000 *g* for 10 min. Cells from replicate fermentations were each processed separately. For biomass determination, cell dry weight was determined gravimetrically. Glucose, organic acids and amino acids were all quantitated by HPLC as described elsewhere (Guillouet et al. 1999). Carbon dioxide was measured in the outlet gases using a Compact Infrared Gas Analyzer RI-550A (CEA Instruments, Emerson, N.J., USA).

Results

Fermentation results

In order to determine the advantage of using the catabolic threonine dehydratase for the production of isoleucine in a threonine overproducer, fermentations were performed with *C. glutamicum* ATCC 21799 carrying pAPE19, the plasmid containing the *hom^{dr}-thrB* fusion (as a control), and two derivatives of the ATCC 21799 strain carrying either pAPE20, which contains the *hom^{dr}-thrB-ilvA* fusion, or pAPE18, which contains the *hom^{dr}-thrB-tdcB* fusion.

Throughout the fermentations the carbon balances were good, i.e. more than 95% of glucose, citrate and leucine consumed were accounted for in the major products (biomass, CO_2, amino acids, pyruvate and lactate). The remainder was in part associated with a number of minor products present at trace amounts (α-ketobutyrate, α-keto-methylvalerate, acetate).

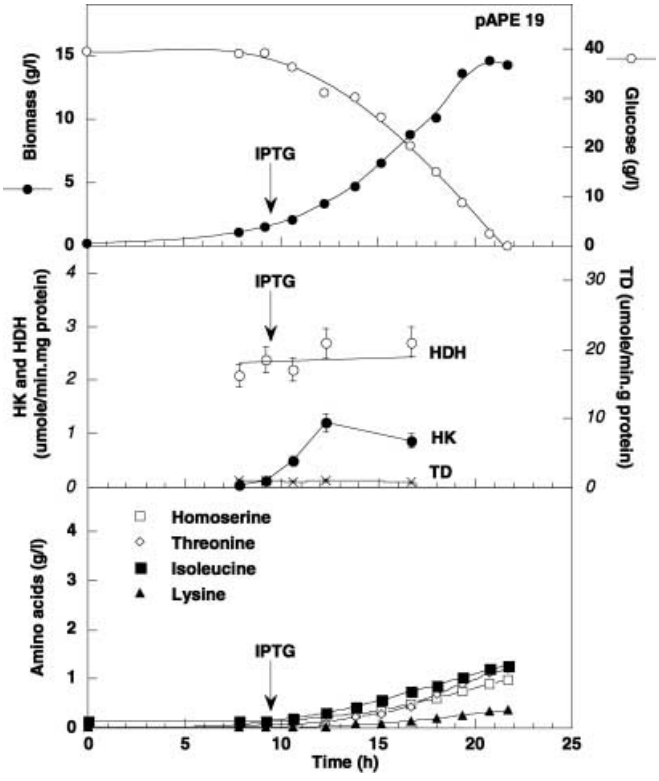

Fig. 3 Culture of *C. glutamicum* ATCC 21799 (pAPE19) in batch reactor on defined medium. Kinetics of growth, substrate consumption, amino acid production and specific activities of homoserine kinase (*HK*), homoserine dehydrogenase (*HDH*) and threonine dehydratase (*TD*)

Fermentation with the threonine-overproducing strain

Corynebacterium glutamicum ATCC 21799 carrying the pAPE19 plasmid was grown twice in defined medium in a 4-l reactor. IPTG (150 mg/l) was added when the biomass reached 1.5 g cell dry weight l^{-1}. Kinetics of growth, substrate consumption, threonine dehydratase, homoserine kinase and homoserine dehydrogenase specific activities and amino acid production are shown in Fig. 3. This strain grew exponentially with a specific growth rate of 0.26 h^{-1} and a glucose to biomass conversion yield of 0.4 g cell dry weight (g glucose)$^{-1}$. The basal levels of homoserine dehydrogenase and threonine dehydratase remained constant around 2.5 µmol NADH

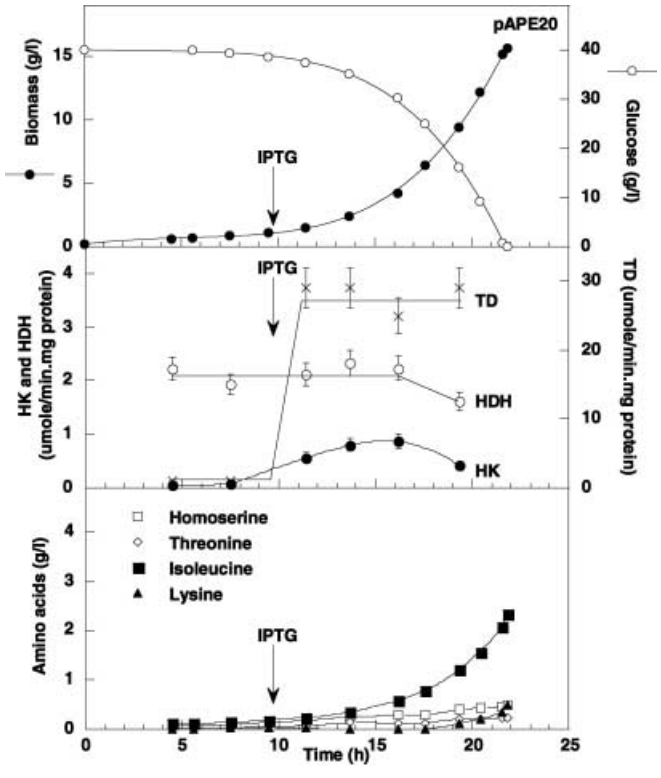

Fig. 4 Culture of *C. glutamicum* ATCC 21799 (pAPE20) in batch reactor on defined medium. Kinetics of growth, substrate consumption, amino acid production and specific activities of homoserine kinase (*HK*), homoserine dehydrogenase (*HDH*) and threonine dehydratase (*TD*)

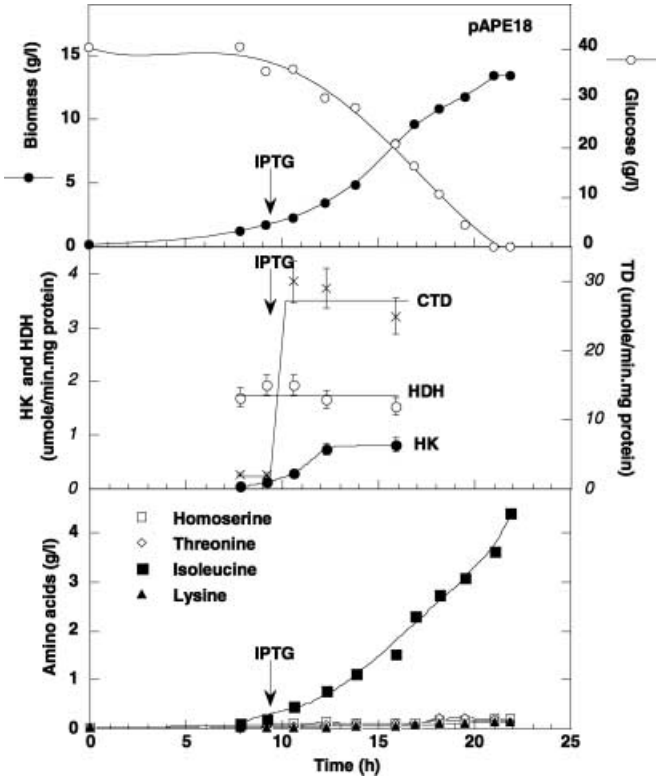

Fig. 5 Culture of *C. glutamicum* ATCC 21799 (pAPE18) in batch reactor on defined medium. Kinetics of growth, substrate consumption, amino acid production and specific activities homoserine kinase (*HK*), homoserine dehydrogenase (*HDH*) and catabolic threonine dehydratase (*CTD*)

min^{-1} mg protein^{-1} and 1 µmol α-ketobutyrate min^{-1} mg protein^{-1}, respectively, during the fermentation. This corresponds to a 20-fold increase in the level of homoserine dehydrogenase than that in the wild-type strain (ATCC 21799 carrying the control vector pAPE12), in which homoserine dehydrogenase activity was in the range of 0.12 µmol NADH min^{-1} mg protein^{-1}. In ATCC 21799 carrying pAPE19, IPTG induced more than 15-fold increase in homoserine kinase activity (rising from 0.06 to 1.21 µmol NADH min^{-1} mg protein^{-1}). The strain produced mainly threonine, isoleucine and homoserine at final concentrations of 1.1±0.1, 0.95±0.2 and 0.75±0.1 g l^{-1} in the two fermentations. The final concentration of lysine was 0.2±0.1 g l^{-1}.

Fermentation with the ilvA*-overexpressing strain*

Corynebacterium glutamicum ATCC 21799 harboring pAPE20 was grown twice in defined medium in a 4-l reactor. As before, IPTG (150 mg/l) was added when biomass reached 1.5 g cell dry weight l^{-1} (Fig. 4). The specific growth rate and the glucose to biomass conversion yield of this strain were identical to those of the strain carrying pAPE19. The level of homoserine dehydrogenase remained constant around 2.2 µmol NADH min^{-1} mg protein^{-1}. The addition of IPTG resulted in a 25-fold increase in the level of threonine dehydratase and a 15-fold increase in the level of homoserine kinase. ATCC 21799 (pAPE20) produced mainly isoleucine at final concentrations of 2.2±0.2 g l^{-1} in the two fermentations. The final concentrations of homoserine, threonine and lysine were low, at 0.4±0.1 g l^{-1}, 0.1±0.1 g l^{-1} and 0.45±0.1 g l^{-1}, respectively.

Fermentation with the *tdcB*-expressing strain

Corynebacterium glutamicum ATCC 21799 harboring pAPE18 was grown twice in defined medium in a 4-l reactor under the same conditions of agitation, aeration and IPTG addition as for the two strains described above. The maximum specific growth rate (0.19 h^{-1}) and the glucose to biomass conversion yield (0.31 g g^{-1}) of this strain were lower than those obtained with the other strains (Fig. 5). A linear growth phase was observed after 16 h of culture, indicating a growth limitation. Exponential growth could be restored by adding to the medium supplemental methionine and/or valine [as we observed previously (Guillouet et al. 1999)] or pantothenate (to a final concentration of 0.45 mg l^{-1}) (data not shown). The level of homoserine dehydrogenase remained constant around 2 µmol of NADH min^{-1} mg protein^{-1}. The addition of IPTG resulted in a 15-fold in-

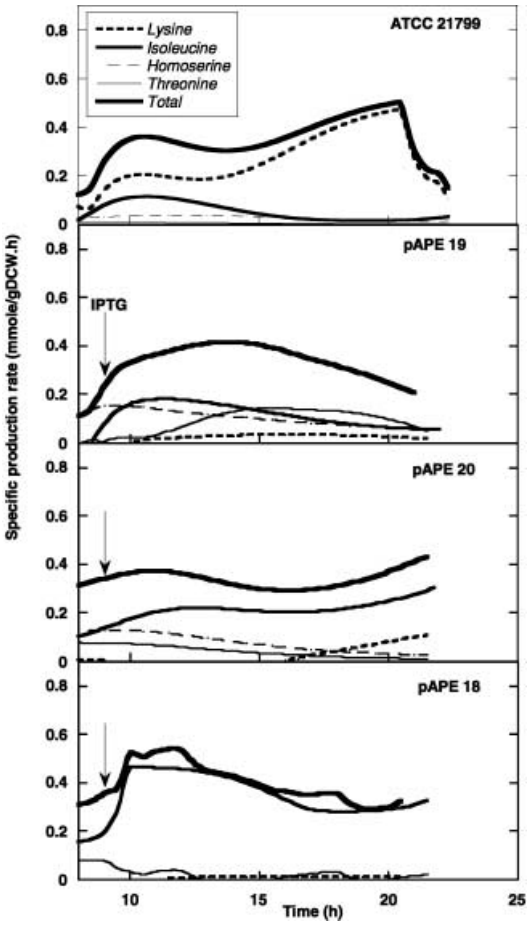

Fig. 6 Evolution of the specific productivities for amino acids during the cultivations of the wild-type strain ATCC 21799 and its derivatives carrying pAPE18, 19, or 20. Productivities were calculated from the same data that were used to construct the graphs in Figs.3, 4 and 5

crease in the level of homoserine kinase and 25-fold increase in catabolic threonine dehydratase activity. As a result, ATCC 21799 (pAPE18) produced 4.0±0.3 g l^{-1} isoleucine in the two fermentations. The concentrations of threonine, homoserine and lysine were very low, around 0.1 g l^{-1}.

Metabolic analysis

Dynamic analysis of amino acid production

The evolution of the specific amino acid production rates over the fermentation showed that for each strain the highest homoserine production occurred before IPTG addition due to the constitutive expression of *hom^{dr}* (Fig. 6). In the strain carrying the *hom^{dr}-thrB* plasmid (pAPE19), the addition of IPTG led to the production of isoleucine (with a maximum rate around 0.2 mmol g^{-1} h^{-1}) at the expense of homoserine. The production of threonine occurred 2–3 h after addition of IPTG, when the productivity of isoleucine declined presumably due to isoleucine feedback inhibition on threonine dehydratase. Co-expression of *ilvA* in such a strain (by introducing pAPE20) kept the isoleucine productivity high (at 0.2–0.3 mmol g^{-1} h^{-1}) during the whole fermentation. Some lysine production appeared at the end of the fermentation, showing a saturation of the threonine-isoleucine pathway possibly due to isoleucine-mediated feedback inhibition of the threonine dehydratase. The strain harboring pAPE18 (*hom^{dr}-thrB-tdcB*) showed a very high isoleucine productivity all along the fermentation with a maximum of 0.45 mmol g^{-1} h^{-1}.

Table 1 Final concentrations of byproducts during fermentation

Final concentrations (g/l)	Wild-type	pAPE19	pAPE20 (*ilvA*)	pAPE18 (*tdcB*)
Lactate	0.5	1.2	0.8	0.9
Alanine	0.2	1.0	0.5	0.3
α-Ketomethyl valerate	0.0	0.2	0.1	0.6

Byproduct excretion during fermentations

During the fermentations, the main byproducts excreted in the medium were alanine, lactate and α-keto-methylvalerate (Table 1). Whereas the wild-type excreted only lactate and alanine, the strains carrying pAPE20, pAPE19 and pAPE18 plasmids excreted higher amounts of lactate and alanine and they also excreted α-ketomethylvalerate. The presence of these byproducts indicates metabolic leakage at two levels, in glycolysis at the level of pyruvate, and in the isoleucine biosynthesis pathway. The overflow around the pyruvate node was not due to oxygen limitation since dissolved oxygen was maintained above 20% saturation.

Discussion

We previously reported the expression of an *E. coli* catabolic threonine dehydratase in a lysine producing *C. glutamicum* (Guillouet et al. 1999). The insensitivity to isoleucine feedback regulation of the *E. coli* catabolic threonine dehydratase enabled the redirection of 75% of the carbon flow from lysine to isoleucine. In order to improve this redirection of carbon flow toward isoleucine, we expressed the catabolic threonine dehydratase in a threonine-producing strain of *C. glutamicum*. Whereas homoserine dehydrogenase and homoserine kinase normally limit threonine production due to their sensitivity to feedback inhibition by threonine, we achieved threonine overproduction by overexpressing the *thrB* (encoding homoserine kinase) and *hom^{dr}* (encoding a deregulated homoserine dehydrogenase) in a manner similar to that used by Colon et al. (1995b). Because of differences in gene organization in the plasmids used, it would have

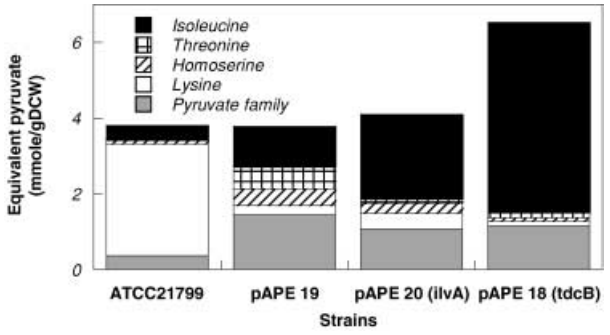

Fig. 7 Excreted metabolite balance for the wild-type strain ATCC 21799 and its derivatives carrying pAPE18, 19, or 20. Results were calculated from final fermentation titers and expressed in equivalent pyruvate per dry cell weight. One mol of threonine, homoserine, alanine, or lactate are each synthesized from 1 mol of pyruvate, whereas lysine and isoleucine are each synthesized from 2 mol of pyruvate. Pyruvate, lactate and alanine were grouped under the term "pyruvate family"

been difficult to compare the effectiveness of the isoleucine production strategies involving pAPE18 and pGC77 (Colon et al. 1995b). Placing the genes used by Colon et al. (1995b) into the same context as was used for *tdcB* expression makes it possible to directly compare the current strategy with previous work. Taken together with our earlier comparison of the *ilvA* and *tdcB* genes alone (Guillouet et al. 1999), it is possible to determine what component of the three-gene strategy is most responsible for the observed isoleucine benefit. From these studies, it is clear that expressing *tdcB* alone provides as much benefit as does the coexpression of the three gene system first described by Colon et al. (1995b).

Overexpression of homoserine kinase, deregulated homoserine dehydrogenase and the original *ilvA*-encoded threonine dehydratase in *C. glutamicum* grown on glucose led to a redirection of the carbon flow from the lysine pathway to the threonine and isoleucine pathways, as previously reported (Colon et al. 1995b). ATCC 21799 carrying pAPE20 produced five-fold more isoleucine than did the untransformed ATCC 21799 strain alone (Guillouet et al. 999). We monitored the appearance of several metabolites: the aspartate-derived amino acids, as well as byproducts alanine and lactate. The common precursor to each of these compounds is pyruvate. Therefore metabolite balances are expressed in equivalents of pyruvate per g dry cell weight. These data show that the expression of the *hom^{dr}-thrB-ilvA* genes in *C. glutamicum* redirected nearly 80% of the pyruvate from lysine toward threonine and isoleucine (Fig. 7, wherein 100% is defined as the amount of pyruvate that ends up as lysine in the wild-type). The remaining pyruvate was converted primarily into lysine, with only a small fraction being converted to alanine and lactate. The higher amounts of lactate and alanine produced in these two strains can be explained by the fact that an increase in the synthesis of homoserine and threonine at the expense of lysine (whose synthesis normally consumes 2 mol of pyruvate compared to 1 mol for threonine and homoserine synthesis) leads to a higher availability of pyruvate that can be converted into alanine and lactate. The apparent yield of isoleucine production per unit biomass in the strain harboring the *hom^{dr}-thrB-ilvA* plasmid was 1.05 mmol (g dry cell weight)$^{-1}$. In comparison, a strain expressing the catabolic threonine dehydratase alone had an apparent yield of 1.08 mmol (g dry cell weight)$^{-1}$ (Guillouet et al. 1999). This demonstrates that overexpression of *tdcB* alone in a lysine-producing strain is sufficient to drive isoleucine overproduction to a level comparable to that demonstrated with a three-gene system (*hom^{dr}*, *thrB*, *ilvA*).

The expression of *tdcB* gene product in combination with *hom^{dr}* and *thrB* led to a much higher isoleucine production. The isoleucine concentration increased 20-fold compared to the wild-type strain, and the apparent yield of isoleucine production was multiplied by a factor of two [2.1 mmol (g dry cell weight)$^{-1}$]. While the balance of excreted metabolites showed that the carbon flow in this strain was completely redirected from the lysine pathway into the isoleucine pathway, it also showed that more pyruvate was diverted into amino acid synthesis (Fig. 7). This increase in pyruvate supply is also indicated by the maximum specific production rate of total amino acids from the aspartate family. The maximum specific production rate increased from around 0.4 mmol (g dry cell weight)$^{-1}$ h^{-1} (wild-type strain and the strains harboring pAPE19 and pAPE20) to 0.55 mmol (g dry cell weight)$^{-1}$ h^{-1} in the strain expressing the *tdcB* gene (pAPE18) (Fig. 6).

We noticed that the strain harboring the pAPE18 plasmid started growing linearly after 16 h of fermentation. As we found in our previous study (Guillouet et al. 1999), the addition of an amino acid mixture into the medium of this strain returned exponential growth, specific growth rate and yield to levels resembling those of the controls (*C. glutamicum* harboring pAPE19). These data are in agreement with the explanation given previously (Guillouet et al. 1999). The redirection of carbon flow from lysine into threonine or isoleucine leads to a shortage in the methionine and/or valine supply due to competition among the threonine, isoleucine, valine and methionine pathways for the precursors pyruvate and homoserine. This shortage then leads to a marked growth limitation in the strain harboring pAPE18, characterized by a linear growth phase, and as a result to a higher availability of precursor (pyruvate) for amino acid synthesis. Similar examples have been reported previously in which increased metabolite precursor concentrations are available to enter the biosynthetic pathways due to the introduction of growth limitations (Eggeling et al. 1998; Patek et al. 1994).

We also determined that addition of pantothenate to the growth medium could restore exponential growth to the *C. glutamicum* (pAPE18) cultures. Valine and pantothenate are both derived from keto-isovalerate (Fig. 1). It is possible that pantothenate and valine can be interconverted in this strain. In *E. coli*, the steps leading from keto-isovalerate to pantothenate are all reversible, except

for the final step catalyzed by pantothenate synthetase (Cronan et al. 1982). If the same holds true in *Corynebacterium*, conversion of pantothenate back into keto-isovalerate would require a pantothenase, as has been seen in *Pseudomonas* (Airas 1988). However, there is as yet no evidence that *Corynebacterium* can catalyze such an interconversion. One alternate explanation for pantothenate's observed effects on growth might be that, since both pantothenate synthesis and valine synthesis draw from the same pool of keto-isovalerate, addition of pantothenate relieves the demand for this precursor allowing the cell to convert the keto-isovalerate it has into valine. By the same argument, adding valine to the medium may be relieving a pantothenate limitation. Finally, it is also possible that adding pantothenate to the growth medium increases the amount of coenzyme-A, a derivative of pantothenate, which benefits the cell. However, it is not clear why these cells would require abnormally high levels of coenzyme-A nor how this relates specifically to *tdcB* overexpression.

By expressing the catabolic threonine dehydratase in combination with homoserine kinase and a deregulated homoserine dehydrogenase, we increased isoleucine production with an integral molar yield of 0.15 mol isoleucine per mol glucose and a maximum specific productivity reached 0.55 mmol (g dry cell weight^{-1}) h^{-1} during the exponential growth phase. In comparison, the best isoleucine producer (obtained by overexpressing deregulated homoserine dehydrogenase and threonine dehydratase) reported in the literature achieved 20 g l^{-1} with a maximum specific productivity of 0.35 mmol (g dry cell weight^{-1}) h^{-1} (Kelle et al. 1996). Although it is difficult to compare production performances of two different strains obtained by different strategies, our results highlight the interesting potential of the *tdcB*-expressing strain. Experiments with fed-batch cultivations are underway to quantitate possible gains in high-scale isoleucine production.

By expressing the catabolic threonine dehydratase in a threonine-producing strain, we not only succeeded in completely redirecting carbon flow from the lysine pathway into isoleucine, but we also induced a total metabolic readjustment. We are currently using DNA microarrays to assess the extent and effects of the metabolic perturbation caused by the expression of *tdcB* in this strain.

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References

Airas RK (1988) Pantothenases from pseudomonads produce either pantoyl lactone or pantoic acid. Biochem J 250:447–51

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–54

Colon GE, Jetten MS, Nguyen TT, Gubler ME, Follettie MT, Sinskey AJ, Stephanopoulos G (1995a) Effect of inducible *thrB* expression on amino acid production in *Corynebacterium lactofermentum* ATCC 21799. Appl Environ Microbiol 61:74–8

Colon GE, Nguyen TT, Jetten MS, Sinskey AJ, Stephanopoulos G (1995b) Production of isoleucine by overexpression of *ilvA* in a *Corynebacterium lactofermentum* threonine producer. Appl Microbiol Biotechnol 43:482–8

Cronan JE, Jr. Littel KJ, Jackowski S (1982) Genetic and biochemical analyses of pantothenate biosynthesis in *Escherichia coli* and *Salmonella typhimurium*. J Bacteriol 149:916–22

Eggeling L, Oberle S, Sahm H (1998) Improved L-lysine yield with *Corynebacterium glutamicum*: use of *dapA* resulting in increased flux combined with growth limitation. Appl Microbiol Biotechnol 49:24–30

Follettie MT, Peoples OP, Agoropoulou C, Sinskey AJ (1993) Gene structure and expression of the *Corynebacterium flavum* N13 ask-asd operon. J Bacteriol 175:4096–103

Guillouet S, Rodal AA, An G, Lessard PA, Sinskey AJ (1999) Expression of the *Escherichia coli* catabolic threonine dehydratase in *Corynebacterium glutamicum* and its effect on isoleucine production. Appl Environ Microbiol 65:3100–7

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61–8

Kelle R, Hermann T, Weuster-Botz D, Eggeling L, Kraemer R, Wandrey C (1996) Glucose-controlled L-isoleucine fed-batch production with recombinant strains of *Corynebacterium glutamicum*. J Biotechnol 50:123–126

Lessard PA, Guillouet S, Willis LB, Sinskey AJ (1999) Corynebacteria, Brevibacteria. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation. Wiley, New York, pp 729–740

Patek M, Krumbach K, Eggeling L, Sahm H (1994) Leucine synthesis in *Corynebacterium glutamicum*: enzyme activities, structure of *leuA*, and effect of *leuA* inactivation on lysine synthesis. Appl Environ Microbiol 60:133–40

Zhang Y, Praszkier J, Hodgson A, Pittard AJ (1994) Molecular analysis and characterization of a broad-host-range plasmid, pEP2. J Bacteriol 176:5718–28