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L-Isoleucine Production with *Corynebacterium glutamicum*: Further Flux Increase and Limitation of Export

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The synthesis of L-isoleucine with *Corynebacterium glutamicum* involves 11 reaction steps, in at least five of which activity or expression is regulated. We used four genes and alleles encoding feedback-resistant enzymes (Fbr) in various combinations to assay flux increase through the sequence. During strain construction, the order of genes overexpressed was important. Only when *ilvA*(Fbr) was first overexpressed could *hom*(Fbr) be introduced. This succession apparently prevents the toxic accumulation of biosynthesis intermediates. The best strain constructed (SM13) was characterized by high-level expression of *hom*(Fbr), *thrB*, and *ilvA*(Fbr). With this strain a yield of 0.22 g of L-isoleucine per g of glucose was obtained, with a maximal specific productivity of 0.10 g of L-isoleucine per g (dry weight) per h. In strain SM13, with the high metabolite flux through the reaction sequence, effects on (i) other enzyme levels, (ii) time-dependent variations with process time, and (iii) concentrations of cytosolic intermediates were quantified. Most importantly, the intracellular L-isoleucine concentration is always higher at all process times than the extracellular concentration. The intracellular concentration rises to 110 mM, whereas extracellularly only 60 mM is accumulated. Also the immediate L-isoleucine precursor 2-ketomethyl valerate accumulates in the cell. Therefore, in the high-level L-isoleucine producer SM13, the export of this amino acid is the major limiting reaction step and therefore is a new target of strain design for biotechnological purposes.

L-Isoleucine is of commercial interest as a food and feed additive, since mammals are not able to synthesize it. This branched-chain amino acid is currently produced on a scale of about 400 tons per year either by extraction of protein hydrolysates (25) or by fermentation with classically derived mutants of *Serratia marcescens* (15, 22, 23) or mutants of *Corynebacterium glutamicum* (8, 36). Recent success in engineering the L-lysine and L-threonine flux in *C. glutamicum* by recombinant DNA techniques has been achieved (3, 9, 35, 36). Thus there are prospects of further increasing the L-isoleucine synthesis with this bacterium by following this rational approach of strain construction.

A particular problem with L-isoleucine is its long biosynthetic pathway, consisting of 10 steps, with export as an additional 11th step when bioproduction is considered (Fig. 1). Further peculiarities are the facts that (i) L-isoleucine synthesis shares reactions with L-lysine and L-methionine synthesis, (ii) L-threonine is an intermediate, and (iii) the last four enzymes also carry out reactions involved in valine, leucine, and pantothenate synthesis (48). Therefore, it is not surprising that multiple regulatory steps identified in *C. glutamicum*, as in other bacteria, are required to ensure the balanced synthesis of all these metabolites for cellular demands. In *C. glutamicum* flux control is exerted by repression of the *homthrB* (12, 13, 32) and *ilvBNC* (19, 46) operons (Fig. 1). The activities of aspartate kinase (45), homoserine dehydrogenase (29), threonine dehydratase (31, 33), and acetohydroxy acid synthase (8, 19) are controlled by allosteric transitions of the proteins to provide feedback control loops, and homoserine kinase is inhibited in a competitive manner (3, 30).

In order to permit a detailed flux investigation of this complex pathway, we and others have cloned almost all genes of the pathway (reviewed in reference 11), analyzed in part their transcriptional control (19), and isolated (5, 40) or generated (34) alleles encoding feedback-resistant enzymes of the pathway. L-Isoleucine biosynthetic genes were overexpressed in L-lysine producers of *C. glutamicum* (4, 35, 36) to guarantee a high flux through the aspartate kinase reaction (Fig. 1). An increased flux up to L-threonine was obtained with chromosomally integrated *hom*(Fbr)*thrB* (41) or plasmid-encoded genes with additionally induced *thrB* expression (3). Also, the interesting possibility of using vectorless strains obtained by chromosomal replacement of wild-type *hom* by *hom*(Fbr) was demonstrated (35). A high copy number of *ilvA* was required to obtain further flux up to L-isoleucine (4, 35). Furthermore, of several *ilvA* alleles assayed, genes encoding feedback-resistant threonine dehydratases proved to be more advantageous than the wild-type allele. The current best strain constructed by us is characterized by three copies of *hom*(Fbr)*thrB* and a high copy number of *ilvA*(Fbr) (36). With this strain, the highest specific productivity was 0.052 g of L-isoleucine per g (dry weight) per h, with an instantaneous yield of 0.16 g of L-isoleucine per g of glucose. An overall yield of 0.19 g of L-isoleucine per g of glucose has been reported for a strain with induced *thrB* expression (4). Using the appropriate feeding strategy, we recently obtained a titer of 21 g of L-isoleucine per liter (20).

Interestingly, genetic and physiological analysis has revealed that plasmid-encoded *hom*(Fbr) expression is impossible in some *C. glutamicum* strains (1, 10) or results in plasmid instabilities (16). Also, in *Escherichia coli* strains, plasmid instabilities resulting from *hom*(Fbr) expression are a notorious problem (32), as is the case in response to overexpression of genes of aromatic amino acid synthesis (18). In our rational analysis of L-threonine synthesis, we have been able to trace this problem back to an extremely high intracellular concentration of this amino acid (41). We therefore concluded that the export of this amino acid, which is an actively driven process, is limiting (37). Consequently, export has to be considered as an entirely new type of reaction step, probably necessary for over-

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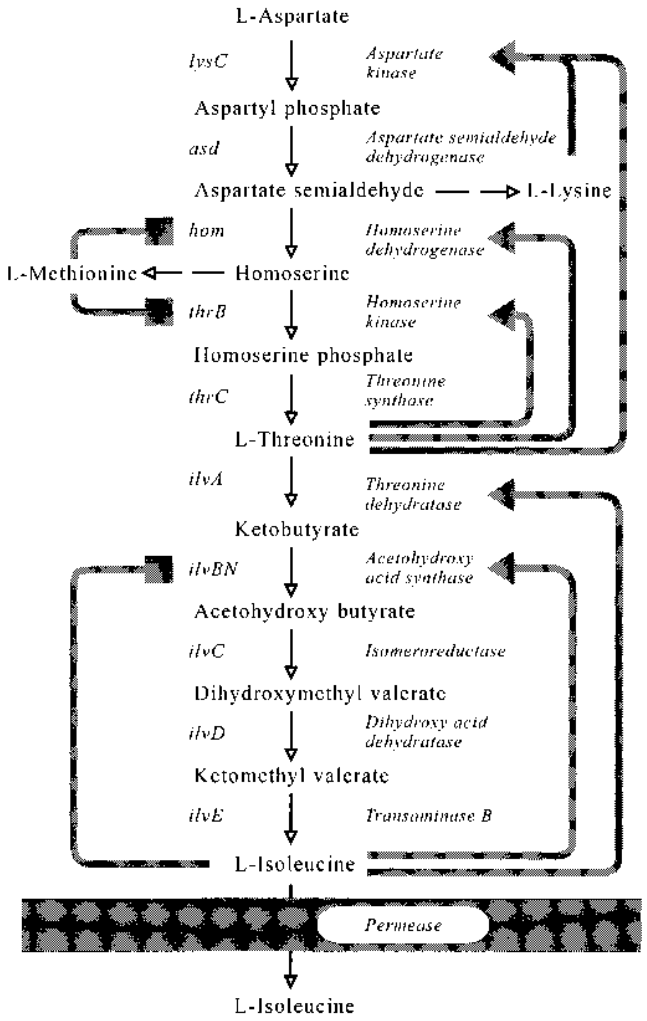

FIG. 1. Biosynthesis of L-isoleucine and its regulation in *C. glutamicum* by feedback control of enzyme activity (shaded lines with arrowhead ends) or regulation at the level of gene expression (shaded lines with square ends).

production of many amino acids (26). In fact, we recently obtained genetic evidence for an L-lysine exporter of *C. glutamicum* (49), which has high levels of activity in L-lysine overproducers (2, 44). The export of L-isoleucine from the cell is also carrier mediated but occurs by diffusion as well (52).

By using all this accumulated knowledge, further work on understanding and improving L-isoleucine oversynthesis must be directed to the following questions. (i) Is high *hom*(Fbr)*thrB* expression in any genetic background possible? (ii) Does the overexpression of further biosynthetic genes improve L-isoleucine accumulation? (iii) Can improved process characteristics be obtained? (iv) Is export of L-isoleucine limiting? The present work addressed these and additional questions.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The classically obtained L-lysine producer MH20-22B *lysC*(Fbr)*leuCD* was used (38, 44), as well as its threonine-producing derivatives DR1 together with DR17 (41) and the L-isoleucine-accumulating strains DR1/pECM3*ilvA*(V328A) [(V328A) indicates that V at position 328 was replaced by A] and DR17/pECM3*ilvA*(V328A) (36). The basic plasmid used was pECM3(Cm^r), a derivative of pECM2 (17), containing one of four different *ilvA* alleles: wild-type *ilv*, *ilvA*(H248R-L351S), *ilvA*(D378G), or *ilvA*(V328A) (35, 36). The other basic plasmid used was pEK0 (Km^r) (10). This plasmid was used to overexpress the *hom*(Fbr)*thrB* operon or *hom*(Fbr)*thrB* in combination with *thrC* (10). Cultures were pregrown overnight on complex medium CgIII (28) and then grown on salt medium CGXII with 4% glucose (19). For enzyme determinations, the cells were harvested at the end of the exponential growth phase, whereas for the determination of amino acid production, cultures were grown until all the glucose was consumed. When appropriate, the *C. glutamicum* cultures contained 25 μg of kanamycin or 3 μg of chloramphenicol per ml.

Genetic engineering. Transformations of *C. glutamicum* were made by conjugation (43) or electroporation (27) for plasmid pECM3 derivatives and pEK0-derivatives, respectively.

Analytical procedures. Glucose was assayed enzymatically in a coupled assay with hexokinase and glucose 6-phosphate dehydrogenase. Homoserine dehydrogenase activities were determined as NADPH-dependent aspartate semialdehyde reduction (10), and threonine dehydratase activities were determined by 2-ketobutyrate accumulation (33). Determination of acetohydroxy acid synthase activities was done as follows. The reaction mixture contained 65 mM potassium phosphate (pH 7.8), 100 mM sodium pyruvate, 0.2 mM thiamine PP_i, 10 mM $MgCl_2$, and crude extract in a final volume of 1 ml (47). The samples were incubated for 15 min at 37°C. The reaction was stopped by the addition of 100 μl of 6 N H_2SO_4. The acetolactate formed was subsequently decarboxylated to acetoin (15 min, 62°C), which was determined according to the method described by Westerfeld (51). For this purpose, a 0.25-ml sample (containing up to 0.07 μmol of acetoin) was mixed with 2.25 ml of H_2O, 0.5 ml of creatine (0.5%, in water), and 0.5 ml of α-naphthol (0.5% in 2.5 N NaOH). For this assay, the mixture was incubated for 60 min at room temperature in the dark, and the estimated A_{530} was compared with those of standards.

Determination of amino acid and keto acids. Cells were separated from the medium and inactivated by silica oil centrifugation, with further preparation as described previously (7). This yielded cellular and extracellular fractions. Amino acids were quantified as their *ortho*-phthaldialdehyde derivatives by automatic precolumn derivatization and separation by reversed-phase chromatography on a Hewlett-Packard model LC1090 ChemStation with fluorescence detection. The determination of α-keto acids was performed according to the method of Hara et al. (14). For derivatization, 200-μl samples (0.01 to 0.2 mM α-keto acids) were mixed with 200 μl of 4,5-dimethoxy-1,2-diaminobenzene solution (0.4 mM 4,5-dimethoxy-1,2-diaminobenzene and 0.21 mM β-mercaptoethanol in 0.5 mM hydrochloric acid). The samples were incubated for 2 h at 104°C, separated by reversed-phase chromatography, and quantified by fluorescence detection. The mobile phase was a gradient of 20 mM phosphate buffer (pH 3.2) and methanol.

RESULTS

Construction of strains. Our study with the graded series of *hom*(Fbr) expression revealed that a high cellular supply of L-threonine is necessary for the accumulation of high levels of L-isoleucine (41). Therefore, high-level expression, as obtainable by plasmids with high copy numbers, should be advantageous. We therefore tried to transform the L-lysine producer *C. glutamicum* MH20-22B (44) with the high-copy-number plasmid pEK*hom*(Fbr)*thrB* (10) but failed to obtain Km^r transformants. This finding had already been reported for several *C. glutamicum* strains (1, 10), although transformation had been successful with a strain not characterized further (4). We therefore first transformed *C. glutamicum* with pECM3*ilvA* (V323A). This yielded the expected Cm^r transformants with a high level of feedback-resistant threonine dehydratase activity. One transformant was chosen and subsequently used for electroporation with pEK*hom*(Fbr)*thrB*. Following this order, Km^r clones were then obtained. They were proven by plasmid isolations to contain both high-copy-number vectors. One transformant chosen was named SM13 (Table 1). With three additional *ilvA* alleles and following the same order of gene overexpression, strains SM10, SM11, and SM12 were constructed. These strains have high copy numbers of *hom*(Fbr), *thrB*, and *ilvA*. To assay the effect of *thrC* overexpression on L-isoleucine accumulation, four additional strains (SM14 to SM17) were constructed with plasmid pEK*hom*(Fbr)*thrBC* (10). Once again, the strains which overexpress *ilvA* were constructed first, and the plasmid with the three threonine-specific genes was subsequently introduced into them.

The eight constructed strains, as well as the ancestor strain MH20-22B and reference strains (36), were assayed for increased enzyme activities. For this purpose, the strains were grown on salt medium with 4% glucose containing the appro-

TABLE 1. Status of genes involved in L-isoleucine synthesis and their copy number in *C. glutamicum* strains derived from the L-lysine producer MH20-22B

Strain[a]	Gene used with pECM3[b]	Gene copy no.[c]						Enzyme activity (μmol/min/mg)[d]		
		hom(Fbr)	*thrB*	*thrC*	*ilvA*	*ilvA*(Fbr)	*ilvBN*	HDH	TDH	AHAS
MH20-22B			1	1	1		1	0.9 ± 0.16	0.04 ± 0.01	0.066 ± 0.005
DR1		1	2	1	1		1	1.6 ± 0.15	0.04 ± 0.01	0.057 ± 0.015
	ilvA(V328A)	1	2	1	1	High	1	1.1 ± 0.25	1.74 ± 0.18	0.085 ± 0.010
DR17		3	4	1	1		1	2.7 ± 0.38	0.05 ± 0.01	0.080 ± 0.010
	ilvA(V328A)	3	4	1	1	High	1	2.2 ± 0.45	1.3 ± 0.21	0.109 ± 0.017
SM10	*ilvA*wt	High	High	1	High		1	4.3 ± 0.67	2.1 ± 0.27	ND
SM11	*ilvA*(H248R-L351S)	High	High	1	1	High	1	2.5 ± 0.32	0.43 ± 0.08	ND
SM12	*ilvA*(D378G)	High	High	1	1	High	1	2.4 ± 0.32	1.2 ± 0.17	ND
SM13	*ilvA*(V328A)	High	High	1	1	High	1	7.6 ± 0.92	1.6 ± 0.12	0.124 ± 0.042
SM14	*ilvA*wt	High	High	High	High		1	5.4 ± 0.17	1.8 ± 0.55	ND
SM15	*ilvA*(H248R-L351S)	High	High	High	1	High	1	6.5 ± 0.40	0.34 ± 0.04	ND
SM16	*ilvA*(D378G)	High	High	High	1	High	1	3.3 ± 0.23	1.8 ± 0.20	ND
SM17	*ilvA*(V328A)	High	High	High	1	High	1	7.4 ± 0.40	1.0 ± 0.13	0.135 ± 0.015

[a] The DR strains contained copies of *hom*(Fbr)*thrB* integrated in their chromosome (41). Strains SM10 to SM13 contained plasmid pEK*hom*(Fbr)*thrB*, and strains SM14 to SM17 contained plasmid pEK*hom*(Fbr)*thrBC* (10).
[b] wt, wild type.
[c] High, high copy number of approximately 30 to 50 copies.
[d] Results are the means ± standard deviations for at least three independent experiments. HDH, homoserine dehydrogenase; TDH, threonine dehydratase; AHAS, acetohydroxy acid synthase; ND, not determined.

priate antibiotics and harvested during the late exponential growth phase. As shown in Table 1, homoserine dehydrogenase activity was increased three- to eightfold in the SM strains compared with that in the ancestor strain MH20-22B. This increase corresponds to that which was achieved for other enzymes with the same vector (10). It is, however, no longer proportional to the copy number as are those for DR1 or DR17. In all SM strains the feedback-resistant character of the homoserine dehydrogenase was confirmed by estimating the residual activity in the presence of 2 mM L-threonine. The activity was reduced by only about 25%, whereas the feedback-sensitive dehydrogenase was no longer active at this concentration of the heterotrophic effector L-threonine (29, 40). The threonine dehydratase activity was increased more than 25-fold, with the exception of that of the double mutant enzyme H278R-L351S. This level of activity is in accord with similar activities recently described in isogenic derivatives of the current strains (36). In selected strains we also determined the acetohydroxy acid synthase activity. As can be seen, the presence of high levels of homoserine dehydrogenase activity resulted in raised levels of synthase activity. This finding shows that the defined increase of expression levels also has unexpected consequences for other enzyme activities.

Comparative L-isoleucine accumulation. The influence of the increased enzyme activities on L-isoleucine accumulation was determined. Again, the salt medium with 4% glucose as a substrate was used. After 48 h when all of the substrate was consumed, the final L-isoleucine titers reached were estimated (Table 2). Compared with that on DR17/pECM3*ilvA*(V328A) (36), the L-isoleucine concentration with strains SM10, SM13, and SM14 increased about 15%. Overexpression of *thrC* apparently does not have a generally positive effect on the final L-isoleucine concentrations. Unexpectedly, use of the feedback-sensitive wild-type *ilvA* allele in SM10 and SM14 yielded concentrations comparable to or even higher than those obtained with the *ilvA*(Fbr) alleles. With the three SM strains with the highest L-isoleucine titers, maximum concentrations of 5 mM L-lysine, 1 mM L-alanine, and 0.2 mM L-homoserine were formed. L-Threonine was not excreted. Therefore, by-product formation was strongly reduced compared with that with previous strains (see Fig. 5).

Process characterization of SM13. For a batch culture of SM13, a detailed profile of glucose consumption, L-isoleucine formation, and growth was recorded (Fig. 2). Strain SM13 is isogenic to DR17/pECM3*ilvA*(V323A), which we have recently described (36), and differs only in the *hom*(Fbr) copy number. Roughly comparable results were obtained in two independent fermentations with strain SM13. The data from one experiment are shown in Fig. 2. The overall yield was 0.22 g of L-isoleucine per g of glucose. This is a strongly increased yield in comparison to that of 0.14 g/g obtained with DR17/pECM3*ilvA*

TABLE 2. Influence of different *ilvA* alleles in strains with increasing copy numbers of *hom*(Fbr)*thrB* or *hom*(Fbr)*thrBC* on L-isoleucine accumulation

Strain	Concn (mM)[a]
MH20-22B	≤1
DR1	ND
DR1/pECM3*ilvA*(V328A)	43 ± 2.5
DR17	13 ± 3.5
DR17/pECM3*ilvA*(V328A)	55 ± 2.9
SM10	61 ± 2.0
SM11	50 ± 3.5
SM12	48 ± 2.9
SM13[b]	63 ± 4.6
SM14	65 ± 4.3
SM15	52 ± 2.5
SM16	52 ± 2.0
SM17	50 ± 6.2

[a] The L-isoleucine concentration was determined in three independent batch fermentations. The means are given together with the standard deviations.
[b] The concentration is the average for five independent fermentations.

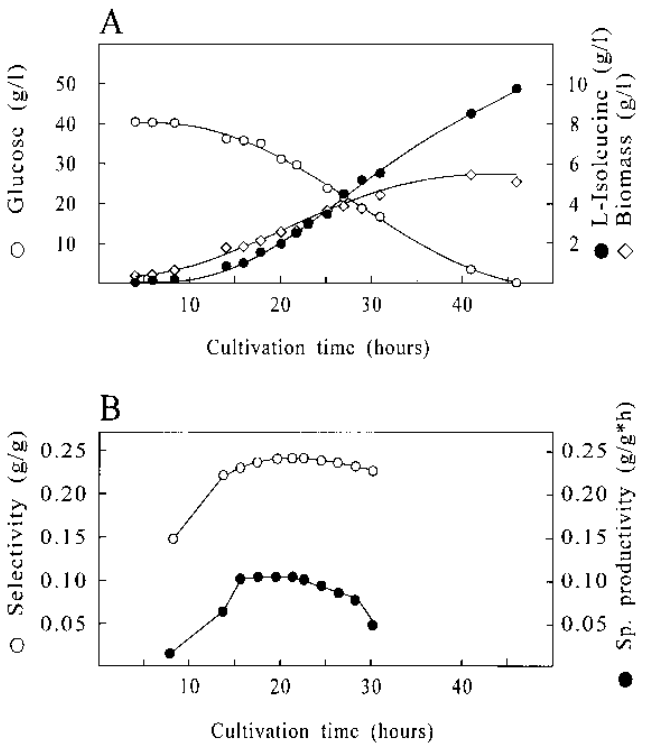

FIG. 2. L-Isoleucine formation from glucose by *C. glutamicum* SM13 in batch culture. (A) L-Isoleucine accumulation (●), glucose consumption (○), and biomass formation (◇). (B) Instantaneous selectivity (○) and specific (Sp.) productivity (●).

(V323A). A high instantaneous yield of up to 0.24 g of L-isoleucine per g of glucose was obtained during 14 and 30 h of the cultivation time. Therefore, a very high conversion rate was maintained from the end of the logarithmic growth phase to the middle of the stationary phase. This is different from the peak activity of DR17/pECM3*ilvA*(V323A) during exponential growth. On the basis of the cellular activity, the highest rate was 0.1 g of L-isoleucine per g (dry weight) per h, which lasted for 15 to 22 h of the cultivation time. At the later cultivation times, this overall cellular catalytic activity of L-isoleucine excretion decreased to a value of 0.05 g/g/h. This decrease could, in principle, be due to a reduced catalytic activity of enzymes within the biosynthetic reaction sequence. We therefore quantitated homoserine dehydrogenase, threonine dehydratase, and acetohydroxy acid synthase activities in *C. glutamicum* SM13 at 16, 23, and 30 h in parallel cultivations carried out in a manner identical to that for which the results are shown in Fig. 2. However, the activities were nearly unaltered at these times (data not shown).

Intracellular accumulation of metabolites within the reaction sequence. Of course, the export of L-isoleucine from the cell is an additional step in the production process of L-isoleucine (Fig. 1). This export is carrier mediated and also occurs by diffusion (52). To estimate whether limitations exist within the entire production sequence, we determined concentrations of metabolites within this sequence. For this purpose SM13 was grown on salt medium identical to that described above, and α-ketobutyrate, α-ketomethyl valerate, and L-isoleucine were quantitated intracellularly and extracellularly. An intracellular α-ketobutyrate concentration of 1.5 mM was found. This keto acid is undetectable in the ancestor strain MH20-22B (data not shown). Ketomethyl valerate was also present in SM13 but was

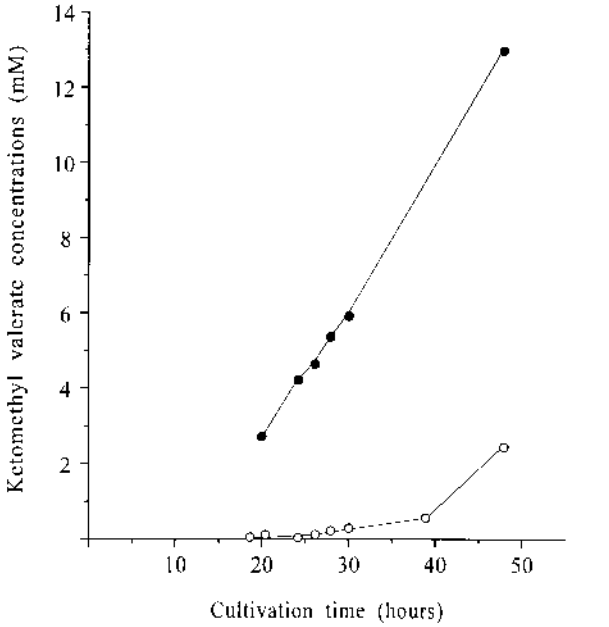

FIG. 3. Intracellular (●) and extracellular (○) concentrations of immediate L-isoleucine precursor α-ketomethyl valerate during L-isoleucine production with *C. glutamicum* SM13.

not detectable in MH20-22B. The immediate L-isoleucine precursor, α-ketomethyl valerate, was already present at the early stages of the cultivation (Fig. 3) and increased with time to the very high intracellular concentration of 13 mM. In addition, this keto acid was also excreted up to a concentration of 2.5 mM. The intracellular L-isoleucine concentrations were most informative. At all times of the cultivation they were higher than the external L-isoleucine concentrations (Fig. 4). At the end of the experiment, the cytosolic L-isoleucine concentration reached the extremely high value of 110 mM, whereas extra-

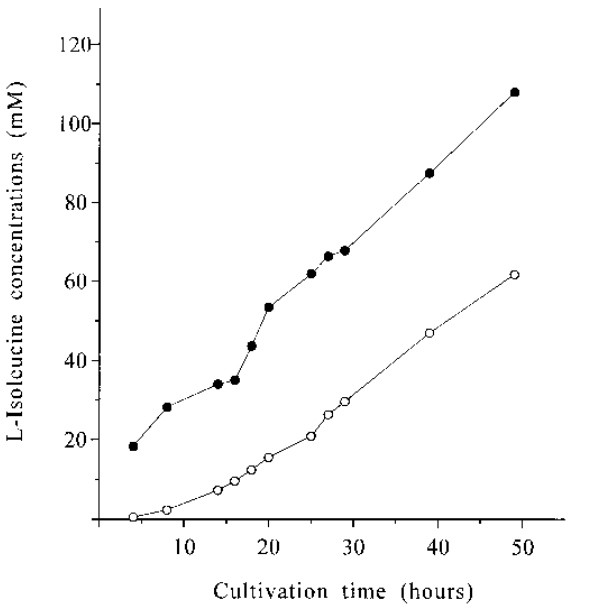

FIG. 4. Intracellular (●) and extracellular (○) concentrations of L-isoleucine with *C. glutamicum* SM13 during production.

cellularly a concentration of only 60 mM accumulated. In the ancestor strain MH20-22B the cytosolic L-isoleucine concentration is about 8 mM (data not shown). The existing gradient of high intracellular versus low extracellular L-isoleucine concentrations clearly shows that the export capacity in strain SM13 is limiting. Consequently, in the present constructed strain, the intracellular provision capacity of L-isoleucine is higher than its export capacity.

DISCUSSION

The present study adds to the large number of isogenic strains originating from the L-lysine producer *C. glutamicum* MH20-22B (20, 35, 36, 41, 44). The variety of strains constructed, as well as their productivities and flux characteristics, provides an exceptional opportunity to study the flux control within the aspartate family of amino acids of *C. glutamicum* and to draw general conclusions regarding strain constructions. High-level expression of *lysC*(Fbr) was problematic (5), as well as that of *hom*(Fbr) (1). To obtain flux up to extracellular L-threonine, both genes must be present together (10). In the *lysC*(Fbr) background, *hom*(Fbr) expression, compared with that of *ilvA*(Fbr), was particularly difficult (1, 10). This problem had already been indicated by the small numbers of clones obtained during replacement of the single chromosomal copy of *hom* by *hom*(Fbr) (35). From our detailed study using one, two, or three copies of *hom*(Fbr), we have been able to show that these problems during construction are due to an intracellular accumulation of extremely high levels of L-threonine (up to 100 mM), as well as of L-homoserine. Therefore, we assume that in the few documented cases of instability of overexpressed biosynthesis genes (18, 42), unphysiologically high cytosolic concentrations of metabolites also are the cause of the instability. Apparently, in the construction of *C. glutamicum* SM13, the overexpression of *ilvA*(Fbr) was first needed to enable further conversion to L-isoleucine, thereby avoiding accumulation of high levels of L-threonine and L-homoserine. Therefore, one general conclusion with respect to flux increase by overexpression would be to enable first increased flux at the latest steps within the reaction sequence of the pathway to be modified. Another general conclusion is to use different alleles of the steps to be modified and to reassay them under each new flux situation. This follows from the fact that in the background with three *hom*(Fbr) copies *ilvA*(Fbr)H278R-L351S gave the highest yield (36), whereas with *hom*(Fbr) in high copy numbers the wild-type *ilvA* allele gave the highest yield. Presumably, at lower intracellular L-threonine concentrations, removal of large amounts of this metabolite by threonine dehydratase activity is required, as is possible with *ilvA*(Fbr), whereas this is not necessary at higher intracellular L-threonine concentrations. However, it is currently impossible to link the well-characterized allosteric parameters of the different threonine dehydratases (34) to the flux performances of the strains, since wild-type *ilvA* does not result in measurable activity under in vitro conditions at the high L-isoleucine concentrations present in the SM strains. Therefore, the many predictions of flux increase based on the kinetic parameters of enzymes must be considered carefully (9). There is currently no known case in which such predictions have resulted in a strain with increased performance. Instead, the fact that wild-type *ilvA* is advantageous under a particular flux situation clearly shows that there are still unknown characteristics under relevant production conditions.

The success of strain generation for flux increase towards L-isoleucine is illustrated in Fig. 5, which shows the final accumulated concentrations of L-isoleucine and L-lysine in a selected set of isogenic strains constructed by us. As can be seen, in the final stages of strain construction, *hom*(Fbr)*thrB* expression is most significant for L-isoleucine accumulation. Furthermore, at elevated levels of the product, additional increases are more difficult to obtain. Interestingly, although both L-isoleucine and L-lysine result from the condensation of the precursor metabolites aspartate and pyruvate, the sum of the two metabolites is still somewhat lower in the L-isoleucine producer than in the starting strain. This is one argument indicating that the availability of precursors does not limit a further increase in L-isoleucine accumulation. This increase is different from the flux increase towards aromatic amino acids with *E. coli*, with which the supply of precursors derived from the pentose phosphate pathway is considered to result in a further accumulation (6). Most informative are the different process characteristics of L-isoleucine formation that we obtained with the molecularly generated strains. The highest concentration reported is 21 g/liter, which was obtained in a fed batch cultivation (20). Strain SM13 shows the highest integral yield of 0.24 g/g. The most important biological information is the specific productivity, since it gives the cellular flux rate through the reaction sequence. Here again an exceptionally high rate of 0.76 mmol/g (dry weight) per h is obtained with strain SM13, which prevails for a considerable fraction of the process time.

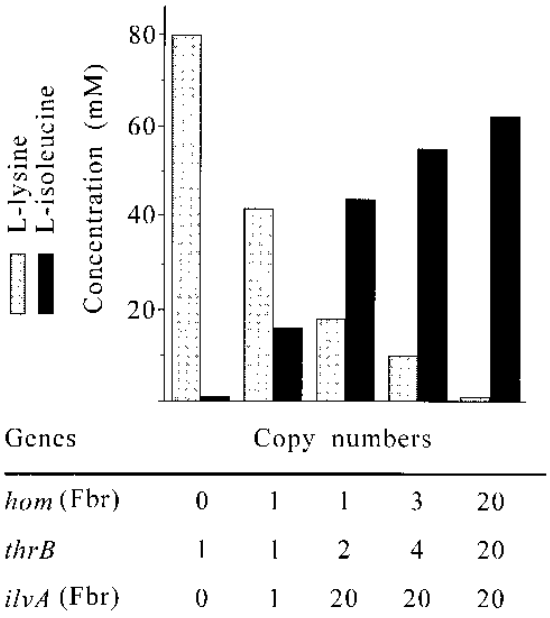

FIG. 5. Increase in accumulated L-isoleucine in isogenic *C. glutamicum* strains obtained from the L-lysine producer *C. glutamicum* MH20-22B. The lower part gives the gene status of the different strains used.

Current work on strain engineering for metabolite overproduction is nearly exclusively directed to the assembling pathway itself, neglecting secondary effects, or dynamic changes with fermentation time. Our detailed analyses revealed additional effects. The first is the consequence of increased synthesis of one enzyme (homoserine dehydrogenase) on the expression of another one (acetohydroxy acid synthase) of the biosynthesis sequence. The twofold increase of the synthase activity could well be advantageous for the process, as demonstrated in different experiments in which ketobutyric acid was fed (8), since all three branched-chain amino acids regulate both the expression and activity of the synthase. The high α-ketobutyrate concentration that we found confirms our pre-

vious model that increased cytosolic α-ketobutyrate concentrations trigger *ilvBN* expression (8, 19). Another effect which could specifically interfere with metabolism in L-isoleucine overproducers is the high α-ketomethyl valerate concentration that we found. Because of the known unspecificities of α-keto acid-metabolizing enzymes (39), a pantothenate or leucine analog could be synthesized from α-ketomethyl valerate with living cells (24). Since our L-isoleucine producer derives from MH20-22B, which is *leuCD* (38), only the pantothenate analog 2-amino-3-hydroxy-3-methyl-valerianic acid can be taken into consideration as a toxic metabolite. In this respect, it is interesting that for unexplained reasons a pantothenate auxotrophic *C. glutamicum* strain was chosen as a basis for the construction of one specific L-isoleucine producer (4). In addition to the biological effect on expression that we determined and that on toxic metabolites that we suggest, another effect is the time-dependent variation of metabolite concentrations and rates, which we quantitated. Although major alterations during metabolite production processes are known, and the chemical engineering approach involves the successful use of the extracellularly measurable consequences to trigger the overall performance of processes, the biological basis of this is extremely poorly defined. Thus, an observed decrease of L-lysine production with *C. glutamicum* could be due to a decrease in the aspartate kinase activity (21). We ruled out such a possibility for L-isoleucine production, since homoserine dehydrogenase, threonine dehydratase, and acetohydroxy acid synthase activities were not altered at decreasing levels of productivity. What we found using a different experimental setup (substrate feeding) was excretion of pyruvate and α-ketobutyrate, which indicates that also the supply of precursor metabolites is not limiting (35). Consequently, in addition to precursor supply and the assembling pathway, export has to be considered.

Although several amino acids are produced on a large scale with either *C. glutamicum* or *E. coli*, detailed investigations on the final export step leading to the extracellular accumulation of the desired product have been done only with *C. glutamicum* (Fig. 1). Considering either (i) diffusion, (ii) nonspecific leaks, (iii) inversion of uptake carriers, or (iv) export carriers, the biochemical characterization of the efflux process unequivocally demonstrated the presence of export carriers for L-lysine, L-glutamate, and L-isoleucine (26). We found an extremely high level of export activity of 0.57 mmol of L-lysine per g (dry weight) per h with *C. glutamicum* MH20-22B (44), the ancestor strain used in the present study. The fact that the carrier activity of this high-level producer is different from that of low-level producers (2) suggests the importance of the carrier for high-level L-lysine formation in a production process. For L-threonine production with actively growing cells, we had already found limitation of export within the entire reaction sequence (41). With SM13 at all stages of extracellular L-isoleucine accumulation, the intracellular concentration of this amino acid increased strongly compared with the extracellular concentration. Irrespective of the mechanism, this conclusively shows that export is limiting, since the rate of cellular synthesis is increased compared with that of export. Therefore, we propose that amplification of additional biosynthesis genes will result in only minor increases of extracellular L-isoleucine. The components involved in transport of hydrophobic L-isoleucine in *C. glutamicum* are passive diffusion, active export, and active import (52). When subtracting the diffusion component from the active transport components (see Fig. 5 in reference 52), even at a concentration of 70 mM intracellular L-isoleucine at which the export carrier is saturated, this carrier would export L-isoleucine at a rate of only about 0.21 mmol/g (dry weight) per h. These biochemical data are therefore consistent with limitation of the L-isoleucine export carrier capacity under producing conditions, with which up to 0.76 mmol of L-isoleucine/g (dry weight) per h is excreted. The view of constantly higher levels of activity of the biosynthetic part of the pathway compared with those for the export part is in full accord with the increasing concentrations of the α-keto acids that we found. Therefore, in the current strains, the biosynthesis-assembling sequence is no longer limiting, but the removal of L-isoleucine from the cell prevents its further accumulation.

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