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# Use of Feedback-Resistant Threonine Dehydratases of *Corynebacterium glutamicum* To Increase Carbon Flux towards L-Isoleucine

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**The biosynthesis of L-isoleucine proceeds via a highly regulated reaction sequence connected with L-lysine and L-threonine synthesis. Using defined genetic *Corynebacterium glutamicum* strains characterized by different fluxes through the homoserine dehydrogenase reaction, we analyzed the influence of four different *ilvA* alleles (encoding threonine dehydratase) in vectors with two different copy numbers on the total flux towards L-isoleucine. For this purpose, 18 different strains were constructed and analyzed. The result was that unlike *ilvA* in vectors with low copy numbers, *ilvA* in high-copy-number vectors increased the final L-isoleucine yield by about 20%. An additional 40% increase in L-isoleucine yield was obtained by the use of *ilvA* alleles encoding feedback-resistant threonine dehydratases. The strain with the highest yield was characterized by three *hom*(Fbr) copies encoding feedback-resistant homoserine dehydrogenase and *ilvA*(Fbr) encoding feedback-resistant threonine dehydratase on a multicopy plasmid. It accumulated 96 mM L-isoleucine, without any L-threonine as a by-product. The highest specific productivity was 0.052 g of L-isoleucine per g of biomass per h. This comparative flux analysis of isogenic strains showed that high levels of L-isoleucine formation from glucose can be achieved by the appropriate balance of homoserine dehydrogenase and threonine dehydratase activities in a strain background with feedback-resistant aspartate kinase. However, still-unknown limitations are present within the entire reaction sequence.**

L-Isoleucine, together with L-lysine, L-threonine, and L-methionine, belongs to the aspartate family of amino acids. These amino acids are of commercial interest, since mammals are not able to synthesize them. L-Lysine is manufactured by *Corynebacterium glutamicum* and its subspecies *flavum* and *lactofermentum* (31), and processes for the production of L-threonine (15) and L-isoleucine (45) from this organism are being developed. However, a particular problem with L-isoleucine overproduction is its long biosynthesis, which involves 10 steps starting from the central precursor metabolite, L-aspartate, with branches to L-lysine and L-methionine and additionally with L-threonine being produced as an intermediate (Fig. 1). A connection to L-valine and L-leucine synthesis also exists, since the last four enzymes are involved in the synthesis of valine and leucine as well (42). Therefore, it is not surprising that a multiplicity of regulatory steps are required in *C. glutamicum*, as in other bacteria, to ensure a balanced flux of these amino acids for cellular demands. In *C. glutamicum*, flux control is exerted by repression of the *hom-thrB* operon (8, 24) and that of *ilvBNC* (16, 41) (Fig. 1). The activities of aspartate kinase (38), homoserine dehydrogenase (24), threonine dehydratase (25, 27), and acetohydroxy acid synthase (4, 41) are controlled by allosteric transitions of the proteins to provide feedback control loops, and homoserine kinase is inhibited in a competitive manner (2, 23). Because of this tight regulation, fermentative processes for L-isoleucine are in general not as well developed as those for L-lysine, for which there seems to be rather simple types of flux control (3, 5, 36, 43).

To overcome some of these problems with the long and tightly controlled reaction sequence, processes have been used in which precursors are applied to channel the $C_4$ skeleton required for L-isoleucine synthesis directly into the final part of the pathway (4, 21, 40, 44). Some attempts by classical techniques to derive L-isoleucine directly from sugar have also been made, since that would be more economical. Protoplast fusion (9) or transduction (18) was used, which thereby enabled the regulatory features of strains to be combined. However, despite all these approaches, L-isoleucine yields and accumulations were low compared with those of L-lysine, and the relevance of each of the different reaction steps for flux increase is ill defined.

A rational approach to increasing and understanding L-isoleucine overproduction by *C. glutamicum* is offered by the use of recombinant DNA techniques. L-Lysine producers with their feedback-resistant aspartate kinase represent a basis for applying such strain construction techniques, since high levels of flux to the $C_4$ skeleton of aspartate semialdehyde is guaranteed (Fig. 1). As was expected, increased flux towards L-threonine was the result when feedback-resistant homoserine dehydrogenase was synthesized in *C. glutamicum* (10, 15, 34). However, in *C. glutamicum* and *Escherichia coli* as well (26, 32), delayed growth and plasmid instabilities are often observed. The concomitant accumulation of homoserine (10, 34) can be reduced by an adjusted increase of *thrB* expression (2, 30). In our rational approach, in which the flux in L-lysine producer *C. glutamicum* MH20-22B (36) was directed from aspartate semialdehyde towards L-threonine, we used graded low-level expression for flux analysis and attained stable expression of *hom*(Fbr) (34). We discovered that a high cytosolic accumulation of L-threonine already exists at three copies of *hom*(Fbr), showing that the export of L-threonine from the cell is limiting. The instabilities of the high-copy-number plasmids (15, 26) or those containing the *trp* promoter (11) for high-level expression of threonine biosynthetic genes are therefore likely to be due to high concentrations of cytosolic L-threonine or threonine-specific intermediates. However, in an L-threonine-

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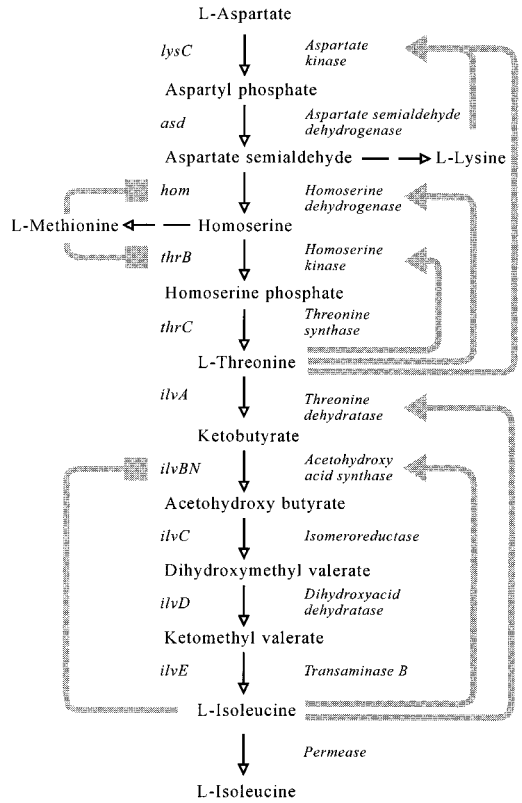


FIG. 1. The biosynthesis of L-isoleucine and its regulation in *C. glutamicum* by feedback control of enzyme activity (shaded lines with arrowhead ends) or at the level of gene expression (shaded lines with square ends).

accumulating strain, flux increase towards L-isoleucine by the use of an appropriate feedback-resistant threonine dehydratase is promising.

Using a molecular approach, we recently generated 19 different mutant threonine dehydratases with different insensitivities to negative heterotrophic effector L-isoleucine (28). We used a selected set of these threonine dehydratases to address the following questions. (i) Which allele is useful? (ii) How does copy number affect the flux towards L-isoleucine? (iii) Is plasmid stability a problem? (iv) What L-isoleucine titers and productivities can be obtained?

## MATERIALS AND METHODS

**Strains, plasmids, and culture conditions.** The classically obtained L-lysine producer, MH20-22B *lysC*(Fbr) *leuCD*, was used (33, 36), as were its Kan$^r$ derivatives, DR1, DR3, and DR17, containing one, two, or three copies of *hom*(Fbr) integrated in their chromosomes (34). The plasmids used were low-copy-number vector pKW0 carrying the Tet$^r$ gene (19) and high-copy-number vector pECM3 derived from pECM2 (12) carrying the Cm$^r$ gene. Precultures were grown on CGIII (22); cultivations for product formation were made in 22B minimal medium containing 10% glucose and $CaCl_2$ (36). For enzyme determinations, cells were grown to the end of the exponential growth phase in minimal medium CGXII (16). When appropriate, the *C. glutamicum* cultures received (per ml) 25 μg of kanamycin, 4 μg of tetracycline, and 3 μg of chloramphenicol and those of *E. coli* received (per ml) 25 μg of kanamycin, 15 μg of tetracycline, and 10 μg of chloramphenicol.

**Genetic engineering.** Plasmid pKW0 was cleaved with *Eco*RI and ligated with wild-type threonine dehydratase gene *ilvA* (27) as well as with the *ilvA*(V323A), *ilvA*(H278R-L351S), and *ilvA*(D378G) alleles which had been excised as 1.5-kb *Eco*RI fragments from the respective pBM20 derivatives (28). The presence of the insert and its orientation was confirmed by plasmid analysis. The orientation in pKW0::*ilvA*wt, pKW0::*ilvA*(V323A), is opposed to the direction of the Tet$^r$ gene, whereas in pKW0::*ilvA*(D378G) and pKW0::*ilvA*(H278R-L351S), it is in the same direction as the Tet$^r$ gene. To construct the high-copy-number plasmids carrying *ilvA*, pECM3 that had been *Sal*I cleaved and the *ilvA*-carrying *Eco*RI fragments were used, with the plasmid and fragments being made blunt before ligation. The *ilvA* gene in pECM3::*ilvA*(V323A) was in the direction of the Cm$^r$ gene, whereas in the other three plasmids, it was opposed to it. Transformations of *C. glutamicum* were done by electroporation (20), and plasmids containing the *mob* site were transferred by conjugation (35). Original constructs were made in *E. coli* by standard procedures.

**Analytical procedures.** Threonine dehydratase activity was assayed by the quantitation of ketobutyrate as described previously (27). Amino acids as their *ortho*-phthaldialdehyde derivatives were quantitated by automatic precolumn derivatization and reversed-phase liquid chromatography with a Hewlett-Packard LC1090 chromatograph with fluorescence detection. Glucose was assayed enzymatically in a coupled assay using hexokinase and glucose 6-P dehydrogenase.

## RESULTS

**Construction of plasmids and strains.** Because of the known instabilities of *lysC*(Fbr) (3) and *hom*(Fbr) (1, 29) on high-copy-number plasmids, a variety of constructs are required for a rational analysis of flux increase. The DR strains of *C. glutamicum* (derived from MH20-22B) carrying up to three *hom*(Fbr) copies in their chromosomes (Table 1) are kanamycin resistant because of the continued presence of vector sequences (34). We therefore had to use another resistance gene, which was available in pKW0. pKW0 carries the Tet$^r$ gene, and its derivatives had been successfully used for moderate expression of glutamate carrier *gluABCD* (19). As a second plasmid, pECM3 with a high copy number (12) was used, providing the Cm$^r$ gene. Threonine dehydratase wild-type gene *ilvA* and the feedback-resistant alleles were obtained from the respective pBM20 derivatives (28). Plasmid pKW0*ilvA*V323A was used to transform *C. glutamicum* strains DR1, DR3, and DR17 to tetracycline resistance. In addition, the plasmids carrying the wild-type *ilvA* allele as well as those carrying *ilvA*(H278R-L351S) and *ilvA*(D378G) were introduced into DR17. An overview of the genetic status of strains carrying *ilvA* alleles in the low-copy-number vector is given in Table 1. The high-copy-number plasmids carrying the *ilvA* alleles were introduced into the strains with the different *hom*(Fbr) statuses by conjugation (35), yielding a series of strains with high copy numbers of the different *ilvA* alleles (Table 1).

The 18 constructed strains as well as their ancestor strains were assayed for the degree of threonine dehydratase oversynthesis. For this purpose, they were grown on minimal medium CGXII (16) with the appropriate antibiotics and harvested during the late exponential growth phase for the determination of threonine dehydratase activity. As can be seen from Table 1, background activities in strain MH20-22B and the DR strains with the chromosomally encoded threonine dehydratase are consistently from 0.035 to 0.045 μmol/min/mg of protein. In the six strains constructed with the pKW0 derivatives, specific activities range from 0.22 to 0.37 μmol/min/mg of protein. This is a five- to eightfold oversynthesis, which is in accord with the fivefold oversynthesis for the glutamate uptake system obtained with the same vector (19) and the estimated copy number of the vector (39), for the glutamate uptake system. With the pECM3 derivatives, the oversynthesis of threonine dehydratase was always more than 30-fold, with the exception of that for the H278R-L351S enzyme, which yielded lower specific activities in extracts of the three DR strains. These lower activities are in accord with the lower catalytic activity of this mutant protein (28).

**Characterization of the threonine dehydratases.** The steady-state kinetics of wild-type and mutant threonine dehydratases had been determined at a constant low L-isoleucine concentration of 0.625 mM, and they served as criteria for the selection of the mutant enzymes from the set of 19 dehydratases available (28). To assay for the effect of higher concentrations of

TABLE 1. Status of genes involved in L-isoleucine synthesis and their copy numbers and the obtained oversynthesis of mutant threonine dehydratases in *C. glutamicum* strains originating from L-lysine producer MH20-22B

| Strain | Gene copy number[a] | | | | Threonine dehydratase activity (μmol/min/mg)wt |
|---|---|---|---|---|---|
| | *hom*(Fbr) | *thrB* | *ilvA*[b] | *ilvA*(Fbr) | |
| MH20-22B | | 1 | 1 | | 0.04 |
| DR1/pKW0::*ilvA*(V323A) | 1 | 2 | 1 | Low | 0.37 |
| DR3/pKW0::*ilvA*(V323A) | 2 | 3 | 1 | Low | 0.31 |
| DR17/pKW0::*ilvA*(V323A) | 3 | 4 | 1 | Low | 0.24 |
| DR17/pKW0::*ilvA*wt | 3 | 4 | Low | | 0.36 |
| DR17/pKW0::*ilvA*(H278R-L351S) | 3 | 4 | 1 | Low | 0.22 |
| DR17/pKW0::*ilvA*(D378G) | 3 | 4 | 1 | Low | 0.30 |
| DR1 | 1 | 2 | 1 | | 0.04 |
| DR1/pECM3::*ilvA*wt | 1 | 2 | High | | 2.12 |
| DR1/pECM3::*ilvA*(H278R-L351S) | 1 | 2 | 1 | High | 0.55 |
| DR1/pECM3::*ilvA*(D378G) | 1 | 2 | 1 | High | 2.00 |
| DR1/pECM3::*ilvA*(V323A) | 1 | 2 | 1 | High | 1.74 |
| DR3 | 2 | 3 | 1 | | 0.035 |
| DR3/pECM3::*ilvA*wt | 2 | 3 | High | | 2.29 |
| DR3/pECM3::*ilvA*(H278R-L351S) | 2 | 3 | 1 | High | 0.74 |
| DR3/pECM3::*ilvA*(D378G) | 2 | 3 | 1 | High | 2.32 |
| DR3/pECM3::*ilvA*(V323A) | 2 | 3 | 1 | High | 1.97 |
| DR17 | 3 | 4 | 1 | | 0.045 |
| DR17/pECM3::*ilvA*wt | 3 | 4 | High | | 2.54 |
| DR17/pECM3::*ilvA*(H278R-L351S) | 3 | 4 | 1 | High | 0.30 |
| DR17/pECM3::*ilvA*(D378G) | 3 | 4 | 1 | High | 2.67 |
| DR17/pECM3::*ilvA*(V323A) | 3 | 4 | 1 | High | 1.28 |

[a] Low, a copy number of 5 to 8; high, a copy number of ≥30 (see the text).
[b] Enzyme determinations for strain MH20-22B were made with cells from five independent cultivations, and those for each strain with low-copy-number plasmids were made with cells from two cultivations.

this heterotrophic allosteric effector on maximum velocity, extracts of DR1 with one of the four different oversynthesized dehydratases were used, and they were assayed at saturating L-threonine concentrations (40 mM). Figure 2 shows that the high initial activity of the wild-type enzyme readily decreases with increasing L-isoleucine concentration, with almost no activity above 2 mM L-isoleucine. This profile is in accord with previous measurements and also with that of the structurally different *E. coli* enzyme (7, 27). Mutant enzyme V323A is nearly unaffected by 10 mM L-isoleucine; it still has 22% activity at an inhibitor concentration of 50 mM. In contrast, both the mutant H278R-L351S enzyme and mutant D378G enzyme were activated at low L-isoleucine concentrations of about 0.5 mM, indicating an allosteric transition of the tetrameric enzyme

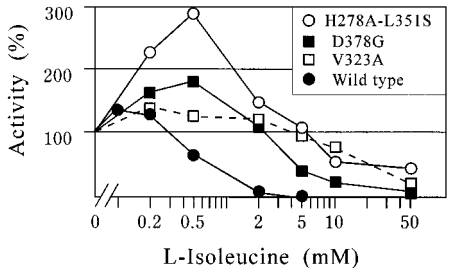


FIG. 2. Response of the activity of mutant threonine dehydratases to increasing L-isoleucine concentrations in vitro. Extracts were prepared from the DR1/pECM3 derivatives oversynthesizing the wild-type enzyme or mutant H278R-L351S, D378G, or V323A. The initial specific activities (in micromoles per minute per milligram of protein) were 1.2 for the wild-type enzyme, 0.55 for H278R-L351S, 1.5 for D378G, and 1.6 for the V323A enzyme.

(27), which is also known to occur for the activation of the enzyme by L-valine (28). The double mutant retained the highest activity at the highest L-isoleucine concentration assayed.

**Stability of constructed strains.** The stability of plasmids encoding feedback-resistant enzymes is a notorious problem in many processes (14, 29). We therefore grew the three DR strains with and without pECM3*ilvA*V323A and pECM3*ilvA*wt for about 70 generations in complex medium without chloramphenicol. In two independent experiments (involving the examination of at least 300 colonies of each of the nine strains), 0.4 to 10% of the clones had lost their plasmid, as judged by their sensitivity to chloramphenicol. No difference between the alleles was apparent, and stability was not increased with plasmid pECM3 alone. We therefore conclude that the loss of the plasmid is due to unknown vector-borne characteristics and that the presence of *ilvA* has no negative effects, as was the case with *hom*(Fbr) (34), but that the addition of chloramphenicol is essential for use of the pECM3 plasmids in fermentations.

**L-Isoleucine accumulation is dependent on the degree of dehydratase activity.** We chose the mutant *ilvA*(V323A) allele in either the low- or high-copy-number vector in the three DR strains to assay for flux increase towards L-isoleucine. Amino acids were determined after 72 h, when all the glucose was consumed (Table 2). The original strain, MH20-22B, did not accumulate significant amounts of L-threonine or L-isoleucine, whereas the DR strains accumulated L-threonine and threonine-derived amino acids homoserine and glycine because of feedback-resistant homoserine dehydrogenase (34). The DR strains also accumulated substantial amounts of L-isoleucine, which is indicative of partial activity by the wild-type enzyme

TABLE 2. Influence of feedback-resistant threonine dehydratase gene *ilvA*(V323A) in low- and high-copy-number plasmids on L-isoleucine accumulation in strains with different homoserine dehydrogenase activities

| Strain | Amino acid concn (mM) | | | | |
|---|---|---|---|---|---|
| | Hom | Gly | Thr | Lys | Ile |
| MH20-22B | | 7 | | 210 | 5 |
| DR1 | 15 | 22 | 33 | 69 | 13 |
| DR3 | 26 | 41 | 44 | 55 | 26 |
| DR17 | 26 | 28 | 40 | 39 | 19 |
| DR1/pKW0::*ilvA*(V323A) | 3 | 7 | | 99 | 53 |
| DR3/pKW0::*ilvA*(V323A) | 7 | 10 | | 60 | 69 |
| DR17/pKW0::*ilvA*(V323A) | 5 | 12 | | 39 | 64 |
| DR1/pECM3::*ilvA*(V323A) | 1 | 2 | | 64 | 63 |
| DR3/pECM3::*ilvA*(V323A) | 2 | 2 | | 47 | 85 |
| DR17/pECM3::*ilvA*(V323A) | 1 | 2 | | 32 | 79 |

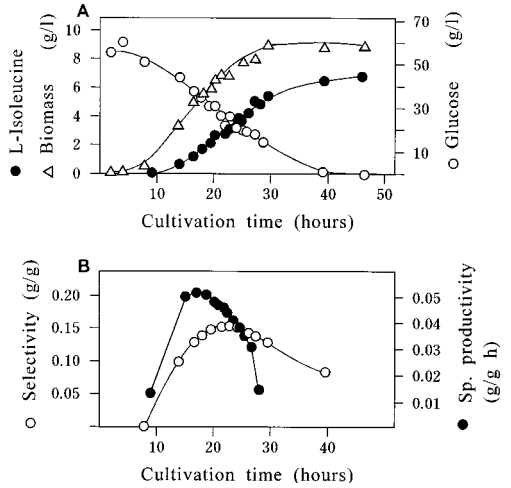


FIG. 3. L-Isoleucine formation from glucose by *C. glutamicum* DR17/pECM3::*ilvA*(V323A) in batch culture. (A) L-Isoleucine accumulation, glucose consumption, and biomass formation. (B) Selectivity and specific productivity.

under those conditions, in which a high cytosolic substrate concentration of threonine dehydratase prevails (28). With the roughly fivefold-oversynthesized, feedback-resistant threonine dehydratase, a higher final L-isoleucine concentration of up to 69 mM was obtained. No more L-threonine accumulated, but some homoserine and glycine did. With the approximately 30-fold-oversynthesized, feedback-resistant threonine dehydratase, the L-isoleucine concentrations were further increased and those of homoserine and glycine were reduced even further. It therefore follows that the threonine dehydratase gene on a high-copy-number vector is required for the increased direction of flux towards L-isoleucine.

**L-Isoleucine accumulation is dependent on selected dehydratase enzymes.** The DR strains with one of the four *ilvA* alleles in plasmid pECM3 (Table 3) were pregrown overnight in complex medium and then inoculated into the fermentation medium. The final optical density with the plasmid-carrying DR strains was always slightly reduced, with the strongest effect for DR17 (data not shown). Samples were taken after 72 h, when all the glucose was consumed, and subsequently the accumulated amino acids were determined. It can be seen from Table 3 that upon introduction of the four alleles on the multicopy plasmid, by-product formation (Thr, Gly, and Hom) was very low, with a shift towards L-isoleucine accumulation being obtained with all plasmids. Also, with oversynthesized, wild-type enzyme, a substantial amount of L-isoleucine was obtained. However, for increased L-isoleucine accumulation, the feedback-resistant threonine dehydratase enzymes were clearly advantageous compared with the wild-type enzyme for two- or threefold-oversynthesized, feedback-resistant homoserine dehydrogenase. The highest L-isoleucine concentrations were reached with the DR17 strains. This finding shows that each of the three selected dehydratase enzymes is suitable for maximum L-isoleucine accumulation (close to 100 mM) but that the degree of homoserine dehydrogenase activity is particularly important. Double mutant enzyme H278R-L351S could be beneficial in the case of two- and threefold oversynthesis of feedback-resistant homoserine dehydrogenase.

TABLE 3. Influence of different threonine dehydratase genes in a high-copy-number plasmid on L-isoleucine accumulation in strains with different homoserine dehydrogenase activities

| Strain | Amino acid concn (mM) | | | | |
|---|---|---|---|---|---|
| | Hom | Gly | Thr | Lys | Ile |
| MH20-22B | | 9 | | 210 | |
| DR1 | 15 | 22 | 33 | 69 | 13 |
| DR1/pECM3::*ilvA*wt | 3 | 3 | | 76 | 63 |
| DR1/pECM3::*ilvA*(H278R-L351S) | 1 | 1 | | 68 | 67 |
| DR1/pECM3::*ilvA*(D378G) | 2 | 2 | | 35 | 90 |
| DR1/pECM3::*ilvA*(V328A) | 2 | 2 | | 65 | 78 |
| DR3 | 27 | 41 | 44 | 55 | 26 |
| DR3/pECM3::*ilvA*wt | 4 | 4 | | 45 | 67 |
| DR3/pECM3::*ilvA*(H278R-L351S) | 2 | 2 | | 50 | 89 |
| DR3/pECM3::*ilvA*(D378G) | 4 | 3 | | 52 | 90 |
| DR3/pECM3::*ilvA*(V328A) | 2 | 2 | | 48 | 71 |
| DR17 | 26 | 28 | 40 | 39 | 19 |
| DR17/pECM3::*ilvA*wt | 3 | 3 | | 35 | 67 |
| DR17/pECM3::*ilvA*(H278R-L351S) | 1 | 2 | | 39 | 96 |
| DR17/pECM3::*ilvA*(D378G) | 3 | 3 | | 49 | 93 |
| DR17/pECM3::*ilvA*(V328A) | 2 | 2 | | 32 | 90 |

**Process characterization.** For a batch culture of strain DR17/pECM3::*ilvA*(V328A), a detailed profile for glucose consumption, L-isoleucine formation, and growth was recorded (Fig. 3). The final L-isoleucine concentration was 50 mM, corresponding to an overall yield of 0.14 g of L-isoleucine per g of glucose. The highest instantaneous yield (selectivity) was 0.16 g of L-isoleucine per g of glucose. A similar yield was obtained for the entire range at about 17 to 30 h, at the end of logarithmic growth. Thus, L-isoleucine formation is comparable to L-lysine synthesis with *C. glutamicum*, for which the highest instantaneous yields in batch culture also exist at the beginning of the stationary growth phase (17). The specific L-isoleucine productivity (grams of L-isoleucine per gram [dry weight] per hour) was shifted slightly towards the shorter cultivation times, with a maximum of 0.052 obtained at 17 h of cultivation (Fig. 3). This result indicates that the overall cellular catalytic activity towards L-isoleucine synthesis is highest at the end of exponential growth and that the activities of the kinase, dehydrogenase, and dehydratase in DR17/pECM3::*ilvA*(V328A) can, in principle, carry this high flux within the entire reaction sequence.

## DISCUSSION

The strains constructed in this study add to the family of strains derived from *C. glutamicum* MH20-22B. A comparison of these strains permits a rational flux analysis within the aspartate family of amino acids by defined genetic alterations. The original strain, MH20-22B, has feedback-resistant aspartate kinase and is an excellent L-lysine producer (22). Gene *lysC*(Fbr) encoding the feedback-resistant aspartate kinase had been replaced in this strain by the gene for the feedback-sensitive wild-type enzyme (36, 37). The resulting strain then no longer produces any L-lysine at all, which shows that flux deregulation at the aspartate kinase is absolutely essential for the provision of the $C_4$ unit aspartate semialdehyde. Increasing the copy number of *lysC*(Fbr) in different L-lysine producers of *C. glutamicum* has only marginal effects with respect to final L-lysine accumulation (3, 13). From this result, it follows that with respect to the provision of aspartate semialdehyde, there is practically no longer any flux control if one copy of *lysC*(Fbr) is present. Since L-isoleucine also results from the condensation of aspartate semialdehyde with pyruvate, an L-isoleucine accumulation comparable to that of L-lysine (around 200 mM) should also basically be possible with respect to the availability of the carbon skeletons in MH20-22B. The high availability of aspartate semialdehyde in MH20-22B has already been used in order to increase the flux in the L-threonine direction in this strain by *hom*(Fbr) expression (homoserine dehydrogenase) (34). However, it was apparent that the *hom*(Fbr) copy number was critical (6, 34), and the importance of the copy number was traced back to an extraordinarily high cytosolic L-threonine accumulation. Also, *thrB* expression is important to direct the flux towards L-threonine at this branching point (2, 29).

Against this background, an essential question concerned the copy number of feedback-resistant threonine dehydratase. What copy number is required for high accumulation (or is tolerated), and which allele from the range of different mutant enzymes is most effectively used? Surprisingly, the *ilvA* copy number is not so decisive as that of *hom*(Fbr), since with the 30-fold oversynthesis of *ilvA* compared with its fivefold oversynthesis, only 20% more L-isoleucine accumulates. This result points to the fact that in the DR strains, the threonine dehydratase reaction no longer plays such an important role, for example, as that involving homoserine dehydrogenase. This finding is in accordance with the finding that with regard to the various alleles, the degree of feedback resistance also only has a moderate influence on the accumulated L-isoleucine concentration. This conclusion is apparent not only from a comparison of the various mutant enzymes but in particular from the fact that L-isoleucine is already formed with the wild-type enzyme, thus showing that only limited inferences may be drawn from the in vitro studies on threonine dehydratase for the in vivo situation prevailing in the DR strains. A high concentration of homotropic effector L-threonine is found in vivo (34), and a high concentration of heterotropic effector L-isoleucine is also to be expected. These two effectors, together with the third effector, L-valine, regulate the enzyme activity (27).

Unexpectedly, the double mutant enzyme, H278R-L351S, is particularly suitable for L-isoleucine accumulation. This enzyme is unstable in vitro and requires the presence of L-isoleucine or pyridoxal-5′-phosphate to prevent a rapid loss of activity (28). The activity loss is probably of no significance in vivo, since sufficient cytosolic L-isoleucine should be present, which would also lead to increased basic activity in vivo (Fig. 2). The advantage of this enzyme could therefore consist in the slightly increased activity at very high L-isoleucine concentrations.

The stability of plasmids coding for feedback-resistant enzymes may be problematic, and instability has been associated with various causes. For example, *lysC*(Fbr) is unstable in high-copy-number vectors (3). In this case, a side activity of the aspartate kinase with the analogous substrate glutamate has been considered; this activity would lead to unnatural metabolites. In the case of *hom*(Fbr), analysis of the DR strains indicated an extremely high intracellular accumulation of homoserine and L-threonine, up to 100 mM each. The corresponding concentrations in original strain MH20-22B are below 10 mM (34). Since the expression of *hom*(Fbr) is problematic on high-copy-number vectors (1, 6), the unusual high concentrations of metabolites are undoubtedly a further general reason for plasmid instabilities. In the case of *ilvA*, the observed plasmid instability is due to the replicon itself, and we have not been able to make the feedback-resistant character of the *ilvA* allele responsible for this. This outcome is surprising in that the *ilvA* allele had been isolated, after all, because of its ability to poison *E. coli* with α-ketobutyrate (28). High α-ketobutyrate concentrations are also deleterious for *C. glutamicum* (4).

For this reason, it is conceivable that α-ketobutyrate, which is the substrate for the acetohydroxy acid synthase, does not accumulate to high levels in the cytosol of *C. glutamicum* DR17/pECM3::*ilvA*(V328A). This outcome would be in accord with the high affinity of the synthase of *C. glutamicum* for α-ketobutyrate (4) and the fact that this enzyme, as opposed to threonine dehydratase or homoserine dehydrogenase, is maximally inhibited to only 50% when allosteric effector L-isoleucine is present. However, the role of this enzyme for flux increase has to be investigated in detail. It is striking that upon expression of *ilvA*(Fbr), L-lysine accumulation is reduced within the DR1, DR3, and DR17 series, which points to the formation of unidentified products. The fact that this depends on *ilvA* greatly restricts the number of intermediates which can be considered. A detailed analysis of by-products, as was done for L-isoleucine synthesis from precursors (44), could help to define new limitations in current L-isoleucine producers and enable a new coordination of activities to be made between the metabolite-supplying and -consuming reactions within the entire reaction sequence of this industrially important amino acid.

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