

Linking Central Metabolism with Increased Pathway Flux: L-Valine Accumulation by *Corynebacterium glutamicum*

Eva Radmacher,¹ Adela Vaitsikova,² Udo Burger,¹† Karin Krumbach,¹ Hermann Sahn,¹ and Lothar Eggeling^{1*}

Institut für Biotechnologie, Forschungszentrum Jülich, D-52425 Jülich, Germany,¹
and BIOTIKA, 976 13 Slovenská Lupča, Slovakia²

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Mutants of *Corynebacterium glutamicum* were made and enzymatically characterized to clone *ilvD* and *ilvE*, which encode dihydroxy acid dehydratase and transaminase B, respectively. These genes of the branched-chain amino acid synthesis were overexpressed together with *ilvBN* (which encodes acetohydroxy acid synthase) and *ilvC* (which encodes isomeroreductase) in the wild type, which does not excrete L-valine, to result in an accumulation of this amino acid to a concentration of 42 mM. Since L-valine originates from two pyruvate molecules, this illustrates the comparatively easy accessibility of the central metabolite pyruvate. The same genes, *ilvBNCD*, overexpressed in an *ilvA* deletion mutant which is unable to synthesize L-isoleucine increased the concentration of this amino acid to 58 mM. A further dramatic increase was obtained when *panBC* was deleted, making the resulting mutant auxotrophic for D-pantothenate. When the resulting strain, *C. glutamicum* 13032Δ*ilvA*Δ*panBC* with *ilvBNCD* overexpressed, was grown under limiting conditions it accumulated 91 mM L-valine. This is attributed to a reduced coenzyme A availability and therefore reduced flux of pyruvate via pyruvate dehydrogenase enabling its increased drain-off via the L-valine biosynthesis pathway.

Metabolites of the aspartate family like L-lysine, L-threonine, the branched-chain amino acids, and D-pantothenate are of particular industrial interest since they are essential for vertebrates. We have already developed L-isoleucine-producing strains from *Corynebacterium glutamicum* by redirecting the carbon flux in an L-lysine producer to L-isoleucine (11, 21, 22). This was done by the use of the biosynthesis genes *thrB*, *thrC* (13, 30), *ilvBN*, and *ilvC* (5, 15) together with feedback-resistant variants of the key enzymes encoded by *hom* (29) and *ilvA* (19). In a further development we used the *ilvBNC* genes together with *panBC* to make D-pantothenate (32). This is possible because the acetohydroxy acid synthase encoded by *ilvBN* catalyzes not only the conversion of ketobutyrate plus pyruvate but also that of pyruvate plus pyruvate to make ketomethyl valerate and ketoiso valerate, respectively (Fig. 1). Whereas the former keto acid is the precursor for L-isoleucine only, the latter is the precursor for D-pantothenate, L-leucine, and L-valine as well. This therefore represents an opportunity to use the existing information and genes to study L-valine formation.

Wild-type isolates of various bacteria accumulating L-valine have already been described (4, 28, 39). By undirected mutagenesis, strains of *C. glutamicum* excreting up to 10 g of L-valine liter⁻¹ have been obtained (23). A mutant of *C. glutamicum* subsp. *lactofermentum* accumulates as much as 31 g liter⁻¹ (38). Through an optimum supply of oxygen, accumulation with this strain is increased to about 40 g liter⁻¹ (1). Mutants excreting L-valine are also known from *Serratia mar-*

censens (16). In general, it is poorly understood why the excretion of L-valine is possible since all strains described so far have been made by undirected mutagenesis. However, at least in some cases L-valine excretion seems to be correlated to acetohydroxy acid synthase activity (37) or the lack of allosteric control of this enzyme (16).

The aim of this work was to study L-valine formation with *C. glutamicum*. To this end the as yet not available biosynthesis genes of the branched-chain amino acid synthesis *ilvD* (dihydroxy acid dehydratase) and *ilvE* (transaminase) needed to be made available. Since the carbon skeleton of L-valine originates exclusively from pyruvate, we were furthermore very interested in discovering whether an increased availability of this precursor, important for many metabolites, can be achieved intracellularly and in testing the effect this would have on L-valine formation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise specified, *C. glutamicum* was cultured at 30°C in complex medium CGIII or salt medium CGIX (15). *Escherichia coli* was grown at 30°C in Luria-Bertani medium. When appropriate chloramphenicol (5 mg liter⁻¹) or kanamycin (25 mg liter⁻¹) was used.

For accumulation of amino acids, cells were pregrown overnight in CGIII. After washing, they were inoculated into CGXII (22) to give an initial optical density at 600 nm of 1.0. In the case of strains with *ilvA* deleted, CGXII contained 2 mM L-isoleucine.

Cloning of genes. The restriction-deficient strain R-127 was mutagenized as described by Vrljić et al. (41), and auxotrophic mutants were isolated by replica plating on minimal medium CGIX with or without amino acids or keto acids (each 300 mg liter⁻¹). The gene bank of the genomic DNA of wild-type *C. glutamicum* ATCC 13032 is also described by Vrljić et al. (41). It was used to transform strain R127-7 and R127-12 to kanamycin resistance (18). Prototrophic clones were identified after replica plating on CGIX without supplements.

Plasmid constructions. In previous work focusing on D-pantothenate accumulation we had already constructed vectors to enable the overexpression of dif-

* Corresponding author. Mailing address: Institut für Biotechnologie, Forschungszentrum Jülich, D-52425 Jülich, Germany. Phone: 49 2461 61 5132. Fax: 49 2461 61 2710. E-mail: leggeling@fz-juelich.de.

† Present address: Institut für Biochemie, Universität zu Köln, D-57674 Cologne, Germany.

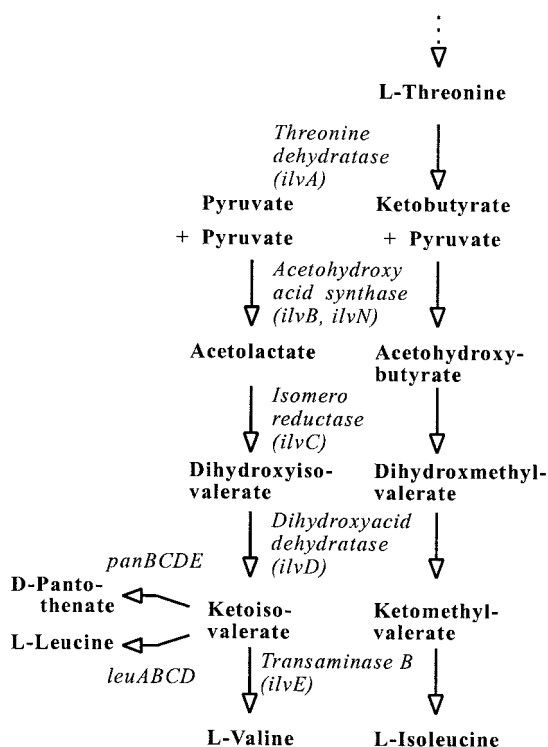


FIG. 1. Linked synthesis of L-valine with those of D-pantothenate, L-leucine, and L-isoleucine.

ferent combinations of *ilv* genes (32). To include studies on the influence of *ilvE* on L-valine accumulation, plasmid pJC4ilvBNCE was constructed. For this purpose, plasmid pJC4 was cleaved with *Xba*I and blunted. *ilvBNC* was isolated as a 5.9-kb *Eco*RI-*Hind*III fragment from pCC2-42 (5) and, after treatment with Klenow enzyme, used for ligation. The resulting vector pJC4ilvBNC was restricted with *Bam*HI, blunted, and ligated with the *ilvE*-carrying fragment derived from pUC18ilvE. The *ilvE* fragment was 1.57 kb in size (*Bgl*I-*Nae*I) and was Klenow treated before ligation. The final plasmid pJC4ilvBNCE of 13.4 kb was characterized by restriction analysis and transaminase activity determination.

Enzyme assays. The transaminase activity was determined with crude extract passed over a PD10 column, with 20 mM Tris-HCl, pH 8, used for equilibration. The assay system contained (in 1 ml) 200 mM Tris-HCl (pH 8), 0.25 mM pyridoxal-5'-phosphate, 4 mM ketoisocaproate, 50 mM sodium glutamate, and crude extract. Samples (50 μ l) were taken at several time points over a period of 20 min, and the reaction was terminated by addition of 30 μ l of stop reagent consisting of 40 ml of ethanol, 6.7 ml of perchloric acid (70%), and 43 ml of water. Samples were subsequently neutralized, and L-leucine formation was quantified by high-performance liquid chromatography.

The dihydroxy acid dehydratase activity was determined in a system containing (per milliliter) 50 mM Tris-HCl (pH 8), 10 mM MgCl₂, 10 mM dihydroxymethyl valerate (pH 4), and 50 μ l of extract. Samples (200 μ l) were taken at several time points to quantify the ketomethylvalerate formed according to the method of Hara et al. (14). The samples were mixed with 200 μ l 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 and afterwards were separated by reverse-phase chromatography and quantified by fluorescence detection. The mobile phase consisted of a gradient of 20 mM phosphate buffer (pH 3.2) and methanol.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers of the sequences derived are AJ012293 for *ilvD* and AF424637 for *ilvE*.

RESULTS

Identification of *ilvD*. We first isolated 10 mutants of *C. glutamicum* R127 requiring the three branched-chain amino acids for growth. Enzyme tests showed that two of these strains

did not have any detectable dihydroxy acid dehydratase activity, whereas with extracts of the initial strain a specific activity of 0.001 μ mol min⁻¹ mg (dry weight)⁻¹ was obtained. However, the acetohydroxy acid synthase and isomeroeductase activities were unchanged (not shown). Using the gene bank of genomic DNA of the wild type described by Vrljić et al. (41), we transformed one of the mutants (strain R127-7) to kanamycin resistance. In this way, a prototrophic clone was obtained which contained a recombinant plasmid with an insert of 4.2 kb. This was subcloned as a 2.9-kb *Sca*I-*Xho*I fragment. Sequencing revealed that the open reading frame (ORF) termed *ilvD* encodes a polypeptide of 611 aminoacyl residues ($M_r = 64,899$). It exhibits high identities over its entire length with similar peptides present in many bacteria and archaea. For instance, that of *S. coelicolor* shares 63% identical amino acyl residues, that of *Neisseria meningitidis* shares 62% identical amino acyl residues, and that of *Methanococcus jannaschii* share 42% identical amino acyl residues. To check for the function of the gene a 2.9-kb *Xba*I fragment was subcloned into the wild-type ATCC 13032. If ATCC 13032 had a specific activity of 0.008 μ mol min⁻¹ mg of protein⁻¹, then through pJCilvD and pECM3ilvD, activities of 0.051 and 0.126 μ mol min⁻¹ mg of protein⁻¹ resulted. This shows that the cloned fragment codes for active dehydratase.

Identification of *ilvE*. Mutants obtained as above and requiring the three branched-chain amino acids but unable to grow upon supply of ketomethylvalerate plus ketoisovalerate plus ketoisocaproate were assayed for transaminase B activity. Mutant R127-12 showed the expected very low specific transaminase activity of less than 0.0005 μ mol min⁻¹ mg of protein⁻¹, in comparison to 0.017 μ mol min⁻¹ mg of protein⁻¹ in R127. The growth defect of the mutant was complemented by plasmid pJCilvE1. With this plasmid, the resulting specific activity

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
R127	Restriction deficient	18
R127-7	Dehydratase negative	This work
R127-12	Transaminase negative	This work
13032	Wild type	ATCC
13032 Δ ilvA	Wild type with <i>ilvA</i> deleted	32
13032 Δ panBC	Wild type with <i>panBC</i> deleted	This work
13032 Δ ilvA Δ panBC	Wild type with <i>ilvA</i> and <i>panBC</i> deleted	This work
13032pk18mob'panC'	Wild type with <i>panC</i> disrupted	This work
pRV	pJC1::4.2-kb genomic <i>Sau</i> 3A fragment of wild type	41
pJCilvE1	pJC1::6.2-kb genomic <i>Sau</i> 3A fragment of wild type	41
pUC18ilvE	pUC18::3.8-kb <i>Nae</i> I fragment with <i>ilvE</i>	This work
pJC1ilvD	pJC1::2.9-kb <i>Xba</i> I fragment	This work
pJC1ilvBNCD	pKK5::2.6-kb <i>Xba</i> I fragment with <i>ilvD</i>	32
pECM3ilvD	pECM3::2.9-kb <i>Xba</i> I fragment	This work
pECM3ilvBNCD	pECM3::5.7-kb <i>Xba</i> I fragment with <i>ilvBNC</i> and 3.1-kb <i>Xba</i> I fragment with <i>ilvD</i>	32
pJC4	Shuttle vector, derived from pJC1; Kan ^r	6
pJC4ilvBNC	pJC4::5.9-kb <i>Eco</i> RI- <i>Hind</i> III fragment with <i>ilvBNC</i> from pCC2-42	This work
pJC4ilvBNCE	pJC4ilvBNC::1.6-kb <i>Bgl</i> I- <i>Nae</i> I fragment with <i>ilvE</i>	This work
pk18mob'panC'	Mobilizable vector with 168-bp internal <i>panC</i> fragment	32

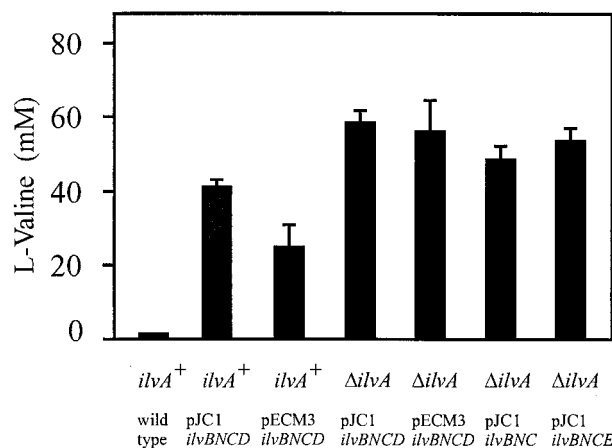


FIG. 2. L-Valine accumulation with isogenic *C. glutamicum* strains. Below the columns the genotype of each strain, either *ilvA*⁺ or $\Delta ilvA$, is given. The strains additionally carry the plasmid pECM3 or pJC1 carrying *ilvBNC*, *ilvBNCD*, or *ilvBNCE*. Means are from three independent experiments. Error bars show standard deviations.

in R127-12 was $0.21 \mu\text{mol min}^{-1} \text{mg (dry weight)}^{-1}$. Sequencing the corresponding insert of 6,185 bp yielded an ORF with the expected identities to several transaminase B genes. The gene *ilvE* encodes a polypeptide of 367 aminoacyl residues ($M_r = 40,363$). A 1.57-kb *BglI-NaeI* fragment of pUCilvE was subcloned in pJC4ilvBNC. With the resulting vector pJC4ilvBNCE, the specific activity in strain R127 was $0.27 \mu\text{mol min}^{-1} \text{mg of protein}^{-1}$, thus confirming the functional identity and the integrity of the cloned gene.

L-Valine accumulation with overexpressed *ilv* genes. In previous work focusing on D-pantothenate accumulation we had already found L-valine accumulation upon overexpression of *ilvBNC* (32). Furthermore, we had found that the deletion of *ilvA*, which codes for threonine dehydratase, favorably influences L-valine formation, possibly due to an increased pyruvate availability (Fig. 1). In order to study the effect of overexpressing *ilvD* and *ilvE* in combination with other genes, plasmid pJC1ilvBNCE was used. This plasmid was transferred into the wild-type ATCC 13032 as well as into strain 13032 $\Delta ilvA$. The resulting strains were subsequently cultivated in minimal medium CGXII together with controls (22). Three independent fermentations were carried out, with the results shown in Fig. 2. Obviously, with *ilvA* deleted, L-valine accumulates in higher concentrations than when *ilvA* is present. Moreover, the use of pJC1 and the *ilvBNCD* genes is superior to the same genes in pECM3, at least in the *ilvA*⁺ background. A further advantage of the genes in pJC1 is the reduced variation of L-valine which accumulated in the different experiments.

In the $\Delta ilvA$ background a possible effect of *ilvE* was studied (Fig. 2). With plasmid pJC1ilvBNCE an increased L-valine accumulation of 53.7 mM was obtained compared to that obtained with pJC1ilvBNC (48.0 mM). However, the effect of pJC1ilvBNCE is not as high as that of pJC1ilvBNCD. We did not attempt to assay for a combined effect of *ilvE* and *ilvD* due to the large size of the vectors already made and the absence of appropriate cloning sites.

D-Pantothenate-dependent product formation. Since L-valine originates from two pyruvate molecules we looked for

ways to increase pyruvate availability. It is known that by limiting a *C. glutamicum* subsp. *lactofermentum* mutant for D-pantothenate its L-lysine synthesis (which requires one pyruvate molecule) can be increased (2). Since D-pantothenate is a constituent of coenzyme A (CoA) it might be possible to attribute this effect to its reduced availability and thus to the reduced flux from pyruvate to acetyl-CoA. In order to verify this directly we inactivated *panC* in the wild-type ATCC 13032 with an internal fragment of this pantothenate biosynthesis gene (32). Salt medium CGXII with or without 0.001 mM D-pantothenate was inoculated with the resulting strain, 13032pk18mob'panC', and after 30 h the cell density was determined. The optical density without D-pantothenate was 18, compared to 26 in the control. As shown in independent experiments, growth observed without vitamin addition is apparently due to sufficient D-pantothenate still being present in the cells used for inoculation, since growth depends on inoculation density and the number of washing steps (not shown). Without any D-pantothenate addition, 6.3 mM pyruvate, 11.3 mM L-alanine, and 5.6 mM L-valine accumulated, whereas in the culture with the vitamin added, no pyruvate and only a maximum of 0.1 mM amino acid in each case were formed.

Construction of a *panBC* deletion mutant. Based on this promising information, we constructed a strain with no *panBC* sequences present to have the kanamycin resistance gene available for plasmid selection. For this purpose, the 1,293-bp *EcoRV-NruI* fragment, encompassing *panBC* sequences, was deleted from plasmid pUR1.2 (32). After religation PCR was used to amplify the deletion fragment. This fragment, with *EcoRI* and *SalI* sites attached during the PCR, was isolated and ligated with pK19mobsacB. Taking advantage of intergeneric gene transfer (33), homologous recombination, and two rounds of positive selection, the wild-type derivative *C. glutamicum* 13032 $\Delta panBC$ was obtained, and the chromosomal deletion of this derivative was verified by PCR. *C. glutamicum* 13032 $\Delta ilvA \Delta panBC$ was made in a similar manner, using 13032 $\Delta ilvA$ as a recipient during gene transfer. These two strains were transformed with the pJC-based vectors containing *ilv* genes and then used to assay for product accumulation.

L-Valine accumulation with $\Delta panBC$ strains. The fermentations were carried out in salt medium CGXII (22), with the $\Delta panBC$ strains receiving 0.001 mM D-pantothenate, and the $\Delta ilvA$ strains receiving 150 mg of L-isoleucine liter⁻¹. As expected from the previous experiment, the D-pantothenate limitation led to a considerable accumulation of L-valine and L-alanine synthesized directly from pyruvate (Table 2). More L-alanine than L-valine is obtained, which may be due to the

TABLE 2. Amino acid accumulation with recombinant wild-type derivatives of *C. glutamicum*

Strain	Amino acid concn (mM) at time			
	24 h		48 h	
	L-Ala	L-Val	L-Ala	L-Val
13032	9.7	1.2	0	0
13032 $\Delta panBC$	27.2	6.7	50.1	7.6
13032 $\Delta panBC \Delta ilvA$	19.9	4.0	29.1	20.5
13032 $\Delta panBC \Delta ilvA$ pJC1ilvBNCD	3.8	43.1	1.3	91.9
13032 $\Delta panBC \Delta ilvA$ pJC1ilvBNCE	9.7	49.4	7.1	81.2

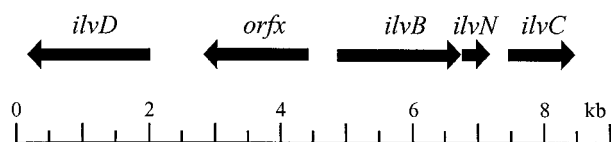


FIG. 3. The *ilvBNCD* gene locus in *C. glutamicum* ATCC 13032. Within the locus of about 9 kb is located the *ilvBNC* operon (5, 15), as well as *ilvD*. These are separated by *orfx*, an ORF of unknown function.

one-step synthesis pathway for forming L-alanine. Interestingly, with simultaneous *ilvA* deletion the product spectrum shifts toward L-valine. This is probably due to increased aceto-hydroxy acid synthase and isomeroreductase activity as a consequence of *ilvA* deletion, since the *ilvBNC* operon encoding these enzymes (20) is controlled by a translationally coupled attenuation mechanism involving L-isoleucine (7). If *ilvBNCD* was overexpressed in the strain with the double deletion, then after 48 h a very high L-valine concentration of more than 90 mM was obtained. L-Alanine could only be detected in low concentrations. In comparison to the strain overexpressing *ilvBNCD*, the strain overexpressing *ilvBNCE* displayed a reduced accumulation of L-valine. This is in line with an elevated concentration of L-alanine. Together with the analysis of product formation in strains without *panBC* deletion (Fig. 2), this leads one to assume that the major factor for the high L-valine accumulation in the constructed wild-type derivatives of *C. glutamicum* is the D-pantothenate limitation.

DISCUSSION

The gene *ilvE* is the only transaminase in *C. glutamicum* of relevance for the transamination step within the biosynthesis of the three branched-chain amino acids. This has to be concluded from the *ilvE* deletion mutant's requirement for the branched-chain amino acids and confirms previous results obtained with strains derived by undirected mutagenesis (7). This is therefore different from *E. coli*, an *ilvE* mutant of which requires L-isoleucine only (3). This is attributed to an overlapping specificity of the *avtA*-encoded alanine valine transaminase, which might supply L-valine and L-leucine and, in the case of its overexpression, might even make the *ilvE* mutant independent of L-isoleucine. Inspection of the genome sequence of *C. glutamicum* suggests that *ilvE*, like *ilvA* encoding the threonine dehydratase (5), is not part of an operon or clustered with other genes of the pathway. However, *ilvD* (dihydroxy acid dehydratase) is separated by just one ORF from the *ilvBNC* operon of *C. glutamicum* (Fig. 3). As in the case of *ilvBNC* (15), there are no other known genes or corresponding enzyme activities for *ilvE* (transaminase B) either. Therefore, in contrast to the *Enterobacteriaceae*, for instance, *C. glutamicum* does not have isoenzymes. Thus, together with the genes of the L-leucine synthesis (25, 26), all biosynthesis genes of the branched-chain amino acids are known.

Upon overexpression of *ilvBNCD* the wild type already accumulates 42 mM L-valine. Therefore, the known inhibition of the *ilvBN*-encoded aceto-hydroxy acid synthase is not a major problem, probably due to the only partial inhibition of the enzyme (7). A further increase in the L-valine concentration up

to 58 mM is present in the case of the strain from which *ilvA*, the threonine dehydratase-encoding gene, is deleted and which, therefore, requires L-isoleucine for growth. This could be due to several effects. First, since aceto-hydroxy acid synthase and isomeroreductase are involved in L-isoleucine and L-valine synthesis too (Fig. 1), the entire catalytic activity is now available for the initial steps of L-valine synthesis. Second, since *ilvBNC* expression is increased upon L-isoleucine shortage by an attenuation mechanism (20), an increased enzyme level might also result in increased pathway flux. A third possibility would be a general growth limitation introduced by the L-isoleucine auxotrophy. Several amino acid producer strains have auxotrophies (2, 23, 37, 39), often for an amino acid whose pathway is linked to that of the amino acid synthesized (17). It is therefore difficult to separate regulatory effects from others, such as, for instance, an increased precursor availability. However, in the case of L-lysine formation with *C. glutamicum*, graded growth limitation introduced by graded gene expression has demonstrated that improved lysine formation is based on increased precursor availability (12).

Whereas the effects discussed so far focus on the biosynthesis genes of the branched-chain amino acids, *panBC* deletion does not. Although the general principles of engineering the biosynthesis paths for increased pathway flux are comparatively well established (8), considerably less information is available to link further cellular activities like central metabolism (31), energy metabolism (24), or even cell wall structures to cellular flux properties (9, 10, 35). One example in which the precursor availability was engineered is the increased availability of erythrose-4-phosphate and phosphoenolpyruvate for aromatic amino acid synthesis (27). As shown by the *panC* inactivation mutant in this study, reduced D-pantothenate availability leads to pyruvate excretion, meaning that increased quantities of precursor are directly available for L-valine synthesis. Reduced CoA formation and thus a reduced flux through the pyruvate dehydrogenase is probably due to the D-pantothenate limitation, because there is no acceptor for the dihydrolipoyl transacetylase activity of the pyruvate dehydrogenase complex. If the pyruvate dehydrogenase activity in *E. coli* is controlled by lipoic acid deficiency, pyruvate excretion may result (42). Pyruvate is also a precursor of L-lysine. In successful attempts to influence L-lysine formation by reduced pyruvate dehydrogenase activity, an additional formation of the amino acid L-alanine, formed directly from pyruvate, was observed (36). Furthermore, L-lysine formation can be influenced with a *C. glutamicum* mutant requiring D-pantothenate through the availability of D-pantothenate (2).

Engineering pyruvate dehydrogenase activity itself is an attractive proposition. Due to its large number of coenzymes and domains (34, 40), this enzyme offers a wide range of approaches to its engineering. On the whole, the decisive factor is naturally the proper integration of activities in the central metabolism and the biosynthesis pathway. Since the strain currently available still excretes some L-alanine at the start of the fermentation, this strain has the potential for improving the L-valine accumulation by further increasing the flux via subsequent "bottlenecks," such as the synthesis pathway or the cell wall.

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