

# L-Valine Production with Pyruvate Dehydrogenase Complex-Deficient *Corynebacterium glutamicum*<sup>∇</sup>

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*Corynebacterium glutamicum* was engineered for the production of L-valine from glucose by deletion of the *aceE* gene encoding the E1p enzyme of the pyruvate dehydrogenase complex and additional overexpression of the *ilvBNCE* genes encoding the L-valine biosynthetic enzymes acetohydroxyacid synthase, isomeroreductase, and transaminase B. In the absence of cellular growth, *C. glutamicum*  $\Delta aceE$  showed a relatively high intracellular concentration of pyruvate (25.9 mM) and produced significant amounts of pyruvate, L-alanine, and L-valine from glucose as the sole carbon source. Lactate or acetate was not formed. Plasmid-bound overexpression of *ilvBNCE* in *C. glutamicum*  $\Delta aceE$  resulted in an approximately 10-fold-lower intracellular pyruvate concentration (2.3 mM) and a shift of the extracellular product pattern from pyruvate and L-alanine towards L-valine. In fed-batch fermentations at high cell densities and an excess of glucose, *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE) produced up to 210 mM L-valine with a volumetric productivity of 10.0 mM h<sup>-1</sup> (1.17 g l<sup>-1</sup> h<sup>-1</sup>) and a maximum yield of about 0.6 mol per mol (0.4 g per g) of glucose.

*Corynebacterium glutamicum* is an aerobic, gram-positive organism that grows on a variety of sugars and organic acids and is used for the production of amino acids, e.g., L-glutamate, L-lysine, and L-valine (15, 17). The latter amino acid is essential for vertebrates and is used for infusion solutions and as a precursor for the chemical synthesis of herbicides (6, 14). Approximately 500 tons of L-valine is produced annually by fermentation or by extraction from acidic hydrolysates of proteins (6). Due to the growing world market for amino acids, there is an increasing interest in the further development and optimization of efficient fermentative L-valine production processes.

As shown in Fig. 1, L-valine is synthesized from pyruvate in a pathway comprising four reactions, catalyzed by acetohydroxyacid synthase (AHAS; *ilvBN* gene product), acetohydroxyacid isomeroreductase (AHAIR; *ilvC* gene product), dihydroxyacid dehydratase (*ilvD* gene product), and transaminase B (TA; *ilvE* gene product). In most organisms, these enzymes also catalyze the biosynthesis of L-isoleucine from pyruvate and 2-ketobutyrate. The latter is formed from L-threonine by threonine dehydratase (TD; *ilvA* gene product). Ketoisovalerate, the last intermediate of L-valine synthesis, is also the precursor for L-leucine and D-pantothenate biosynthesis.

AHAS is the key enzyme of branched-chain amino acid biosynthesis, and the regulation of the *C. glutamicum* enzyme as well as the regulation of the AHAS genes has been studied in detail (5, 9, 13, 16, 19). Only one AHAS was found in *C.*

*glutamicum* (13), and this enzyme was shown to be subject to feedback inhibition by L-valine, L-isoleucine, and L-leucine (5, 9, 19). Expression of the *ilvBN* genes, which form an operon with *ilvC*, is controlled by an attenuation mechanism, and it increases about twofold in response to shortage of branched-chain amino acids (19). On the other hand, expression of the operon is induced by 2-ketobutyrate (5, 19).

L-Valine-excreting strains of *C. glutamicum* have been generated by plasmid-bound overexpression of the L-valine biosynthesis genes *ilvBNCD* or *ilvBNCE* in combination with a deletion of the *ilvA* gene encoding TD (23, 24). Improved production of L-valine was achieved by the introduction of the genes encoding a feedback-resistant AHAS enzyme into L-valine-producing *C. glutamicum* strains (9). Moreover, Radmacher et al. (23) showed that inactivation of the D-pantothenate biosynthesis by deleting the *panBC* genes led to increased L-valine production of *C. glutamicum* cultivated under D-pantothenate-limiting conditions. The authors speculated that D-pantothenate limitation leads to an increased drain-off of pyruvate towards the L-valine biosynthetic pathway due to reduced coenzyme A availability for the reaction of the pyruvate dehydrogenase complex (PDHC) (23). In fact, *Escherichia coli* pyruvate-overproducing strains have been obtained by inactivation of the PDHC (27, 31). Increasing the intracellular availability of the precursor pyruvate by decreasing the PDHC activity thus represents an attractive approach for the optimization of L-valine production by *C. glutamicum*.

Recently, we identified and functionally characterized the E1p subunit of the PDHC in *C. glutamicum* and showed that this complex is essential for the growth of this organism on glucose, pyruvate, or lactate (26). In contrast, the PDHC activity was found to be dispensable for the growth of *C. glutamicum* in medium containing acetate. This growth phenotype opened the possibility to study the effect of PDHC deficiency on metabolite formation from glucose (and acetate) by grow-

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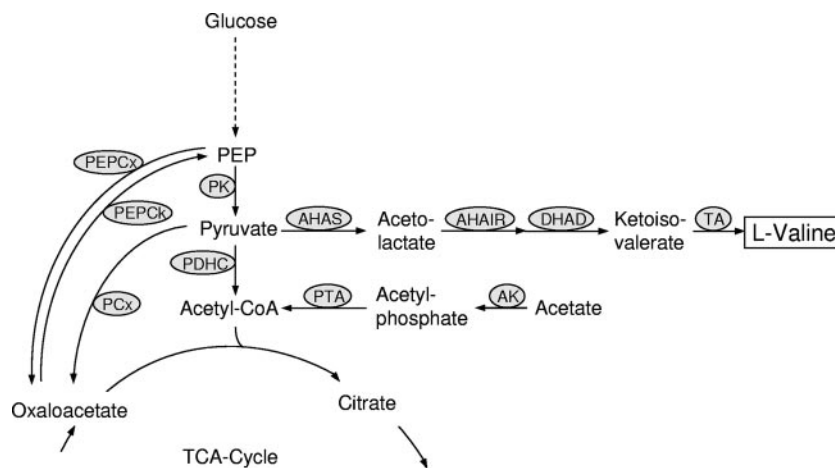


FIG. 1. The enzymes at the phosphoenolpyruvate-pyruvate-oxaloacetate node with the biosynthetic pathway of L-valine in *C. glutamicum*. Abbreviations: PK, pyruvate kinase; PCx, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCx, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; AK, acetate kinase; PTA, phosphotransacetylase; DHAD, dihydroxyacid dehydratase; CoA, coenzyme A; TCA, tricarboxylic acid.

ing and by nongrowing cells of *C. glutamicum*. In the present study, we investigate the effect of PDHC inactivation in *C. glutamicum* on the formation of organic acids and amino acids. We found that resting cells of the PDHC-deficient mutant of *C. glutamicum* wild type (WT) produce significant amounts of pyruvate, L-valine, and L-alanine. This prompted us to direct the carbon flux more efficiently from glucose to L-valine by additional overexpression of L-valine biosynthetic genes.

#### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The strains used in this study were *E. coli* DH5 $\alpha$  (10), *C. glutamicum* WT (i.e., ATCC 13032 from the American Type Culture Collection), and its derivative, *C. glutamicum*  $\Delta$ aceE (26). Plasmid pJC4ilvBNCE (23) was obtained from L. Eggeling (FZ Jülich).

**DNA preparation and transformation.** The isolation of plasmid pJC4ilvBNCE from *E. coli* and *C. glutamicum* was performed as described before (8). Plasmid DNA transfer into *C. glutamicum* was carried out by electroporation, and the recombinant strains were selected on LB-brain heart infusion (BHI) agar plates containing 0.5 M sorbitol and kanamycin (50  $\mu$ g ml<sup>-1</sup>) (29).

**Culture conditions.** *E. coli* was grown aerobically in 2 $\times$  TY complex medium (25) at 37°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Precultures of *C. glutamicum* WT, *C. glutamicum*  $\Delta$ aceE, and *C. glutamicum*  $\Delta$ aceE(pJC4ilvBNCE) were grown in 2 $\times$  TY medium containing 0.5% (wt/vol) potassium acetate. For amino acid fermentations in shake flasks, the cells of an overnight preculture were washed with 0.9% (wt/vol) NaCl and inoculated into CGXII minimal medium (7) to give an initial optical density at 600 nm (OD<sub>600</sub>) of about 1. The medium contained 4% (wt/vol) glucose and 1% or 1.5% (wt/vol) potassium acetate. Where indicated in Results, 0.5% (wt/vol) BHI powder (Merck) was added to the medium. The plasmid-carrying strains were grown in the presence of kanamycin (50  $\mu$ g ml<sup>-1</sup>). *C. glutamicum* was grown aerobically at 30°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm or as 300-ml cultures in a glass bioreactor (see below).

The batch and fed-batch fermentations were performed at 30°C in a 1-liter glass bioreactor (Biostat B; Braun, Melsungen, Germany). The pH was maintained at 7.0 by online measurement using a standard pH electrode (Mettler Toledo, Giessen, Germany) and the addition of 4 M KOH and 4 M HCl. Foam development was prohibited by manual injection of small amounts (about 20  $\mu$ l) of silicon antifoam (Roth, Karlsruhe, Germany). The dissolved oxygen was measured online by use of a polarimetric oxygen electrode (Mettler Toledo, Giessen, Germany), and it was adjusted to 30% of saturation in a cascade by stirring at 100 to 1,200 rpm and aerating with 1 to 3 liters of air per min. During the fed-batch processes, adequate amounts of 50% (wt/vol) glucose and 50% (wt/vol) potassium acetate were injected (at the time points indicated below [see Fig. 4]).

**Analytics.** One milliliter of the culture was harvested by centrifugation (13,000 rpm, 10 min, room temperature), and the supernatant was used for determination of amino acid, glucose, and/or organic acid concentrations in the culture fluid. The amino acid concentration was determined by reversed-phase high-pressure liquid chromatography (on an HP 1100 instrument; Hewlett-Packard, Waldbronn, Germany) with fluorimetric detection (excitation at 230 nm and emission at 450 nm) after automatic precolumn derivatization with *ortho*-phthalaldehyde (18). Separation was carried out at 40°C on a Multohyp octyldodecyl silane column (particle size, 5  $\mu$ m; 125 by 4 mm; CS-Chromatographie, Langerwehe, Germany). The elution buffer consisted of a polar phase (0.1 M sodium acetate, pH 7.2) and a nonpolar phase (methanol). Quantification was done by calculation of the concentration by use of an internal standard (L-ornithine at 100  $\mu$ M) and by a 10-point calibration curve for each amino acid. Glucose, acetate, and lactate concentrations were determined by enzymatic tests (Roche Diagnostics, Penzberg, Germany). The pyruvate concentrations were determined enzymatically according to the method of Bergmeyer (1).

For the measurement of intracellular pyruvate concentrations, the cells were cultivated in 50 ml CGXII medium. Samples of 5 ml were taken for measurements during growth and production phases, at about 9 h and 31 h after inoculation, respectively. All following steps were performed below -10°C to prevent changes in the metabolome (4, 12). After centrifugation of the samples at 10,400  $\times$  g for 5 min at -20°C, the cell pellets were resuspended in 5 ml ice-cold NaCl (0.9% [wt/vol]) and centrifuged again. The supernatants were discarded and the cells resuspended in 1 ml methanol (-80°C) and 1 ml Tris-EDTA buffer (pH 7.0). For cell extraction, 2 ml chloroform was added, and the suspension was shaken for 2 h at -20°C. After extraction, the pyruvate in the methanol phase was determined by liquid chromatography-tandem mass spectrometry (Finnigan TSQ Quantum Ultra; Thermo) according to the method of Luo et al. (18a). Quantification was done by external calibration due to the absence of matrix effects in the appropriate dilutions. For the calculation of the intracellular pyruvate concentration, a specific cell volume of 1.95  $\mu$ l/mg was assumed (21). All intracellular concentrations are based on data from three independent experiments with two replicates of each analytical measurement.

#### RESULTS

***C. glutamicum*  $\Delta$ aceE forms pyruvate, L-alanine, and L-valine.** Previously, we analyzed the *C. glutamicum* *aceE* gene, encoding the E1p subunit of the PDHC, and constructed and characterized an E1p-deficient mutant, *C. glutamicum*  $\Delta$ aceE (26). This mutant carries a deletion in the chromosomal *aceE* locus, shows no PDHC activity, and is unable to grow on minimal medium containing glucose as the carbon and energy source unless supplemented with acetate. To test *C. glutami-*

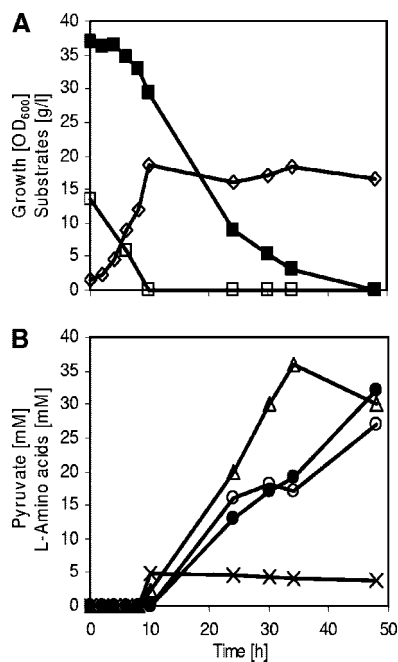


FIG. 2. Representative batch fermentation of *C. glutamicum*  $\Delta aceE$  on CGXII medium initially containing glucose (4%), acetate (1.5%). (A) ◇, growth; ■, glucose; □, acetate. (B) △, pyruvate; ○, L-alanine; ●, L-valine; ×, L-threonine. Three independent fermentations were performed, with all three showing comparable results.

*cum*  $\Delta aceE$  for its ability to form organic acids and/or amino acids from glucose and acetate and to compare its metabolite formation with that of the parental *C. glutamicum* WT, both strains were grown in a 1-liter bioreactor in CGXII medium containing 4% (wt/vol) glucose and 1.5% (wt/vol) potassium acetate, and growth, glucose, and acetate utilization and pyruvate, lactate, and amino acid accumulation were monitored over the course of the experiment. *C. glutamicum* WT grew with maximal growth rates of  $0.36 \text{ h}^{-1}$  to a final  $OD_{600}$  of about 65, metabolized glucose and acetate simultaneously, and did not form any of the tested metabolites to concentrations higher than 1 mM (not shown). For the first 6 to 8 hours, *C. glutamicum*  $\Delta aceE$  also grew exponentially, with a maximal growth rate of  $0.36 \text{ h}^{-1}$ , and simultaneously used acetate and glucose. During this growth phase, the strain also did not excrete any of the metabolites tested into the culture broth (Fig. 2). As expected, the PDHC-deficient mutant stopped growth as soon as the acetate concentration dropped to  $<1 \text{ mM}$  and the culture reached a maximal  $OD_{600}$  of about 19. However, the mutant continued to metabolize glucose and, in contrast to *C. glutamicum* WT, started to produce significant amounts of pyruvate (30 to 35 mM), L-alanine (25 to 30 mM), L-threonine (2 to 5 mM), and L-valine (30 to 35 mM) (Fig. 2). Lactate, acetate, and amino acids other than L-alanine, L-threonine, and L-valine were not formed in the course of the cultivation of *C. glutamicum*  $\Delta aceE$ . These results show that PDHC deficiency is an ideal condition to breed *C. glutamicum* strains producing either pyruvate, L-alanine, or L-valine. Furthermore, the results indicate that inactivation of the competing pathways of L-isoleucine biosynthesis and of D-pantothenate synthesis, e.g., by

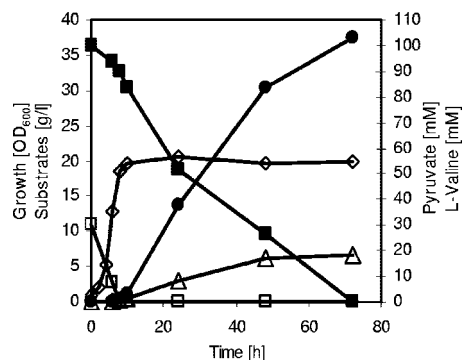


FIG. 3. L-Valine accumulation during a representative batch cultivation of *C. glutamicum*  $\Delta aceE(pJC4ilvBNCE)$  on CGXII medium initially containing glucose (4%), acetate (1%), and BHI (0.5%). ◇, growth; ■, glucose; □, acetate; △, pyruvate; ●, L-valine. Three independent fermentations were performed, with all three showing comparable results.

deleting the *ilvA* and/or *panB* gene(s), is not necessary for efficient L-valine formation.

**L-Valine production by *C. glutamicum*  $\Delta aceE$  strains overexpressing *ilv* genes.** In order to force the carbon flux in *C. glutamicum*  $\Delta aceE$  from pyruvate towards L-valine production, we transformed this strain with plasmid pJC4ilvBNCE. This plasmid carries the genes encoding three of the four enzymes involved in L-valine (and L-isoleucine) biosynthesis (see the introduction) and has previously been used to construct L-valine-producing derivatives of *C. glutamicum* WT, *C. glutamicum*  $\Delta ilvA$ , and *C. glutamicum*  $\Delta ilvA \Delta panBC$  (reference 23; also see the introduction). L-Valine fermentations with *C. glutamicum*  $\Delta aceE(pJC4ilvBNCE)$  were carried out in CGXII medium with 0.5% (wt/vol) BHI, 4% (wt/vol) glucose, and 1% (wt/vol) potassium acetate. Figure 3 shows the course of a typical fermentation. The culture grew within 8 h to an  $OD_{600}$  of about 20, consuming acetate completely (1%) and glucose to a minor part (0.5%). The cells then stopped growth, and they further consumed glucose and accumulated up to about 105 mM L-valine within 64 h. Although *C. glutamicum*  $\Delta aceE(pJC4ilvBNCE)$  produced relatively high amounts of L-valine, it nevertheless excreted nearly 20 mM pyruvate into the medium, indicating that there is still precursor available for L-valine production. No L-alanine was formed in *C. glutamicum* carrying  $\Delta aceE$  in combination with plasmid pJC4ilvBNCE. These results show that pyruvate supply on one hand and the enzymes for the L-valine biosynthetic pathway on the other are major bottlenecks for L-valine overproduction by *C. glutamicum*.

**Intracellular pyruvate concentrations in *C. glutamicum* and its derivatives.** The production pattern of *C. glutamicum*  $\Delta aceE$  and *C. glutamicum*  $\Delta aceE(pJC4ilvBNCE)$  suggested that the PDHC deficiency brought about by deletion of the *aceE* gene might have led to a high intracellular accumulation of pyruvate. To test for this hypothesis, we determined the intracellular pyruvate concentrations in *C. glutamicum* WT and its derivatives, the  $\Delta aceE$  and  $\Delta aceE(pJC4ilvBNCE)$  strains, during exponential growth and at the stationary phase, the latter being the production phase for *C. glutamicum*  $\Delta aceE$  and *C. glutamicum*  $\Delta aceE(pJC4ilvBNCE)$ . All three strains showed

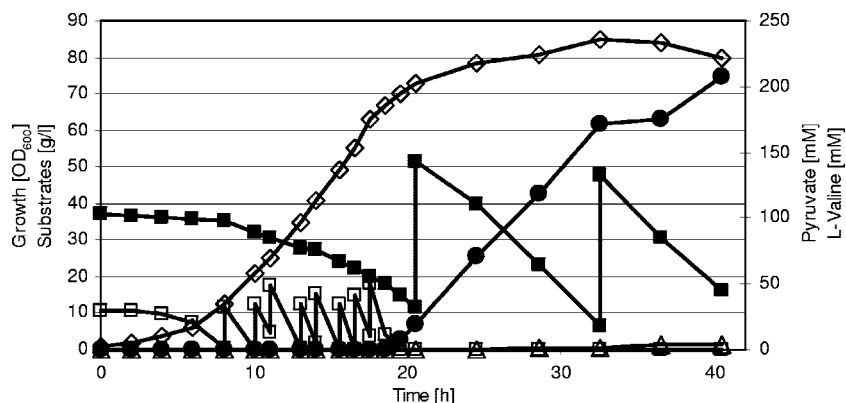


FIG. 4. L-Valine accumulation during a representative fed-batch fermentation of *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE) on CGXII medium initially containing glucose (4%), acetate (1%), and BHI (0.5%).  $\diamond$ , growth;  $\blacksquare$ , glucose;  $\square$ , acetate;  $\triangle$ , pyruvate;  $\bullet$ , L-valine. Three independent fed-batch fermentations were performed, with all three showing comparable results.

very low intracellular pyruvate concentrations ( $<0.05$  mM) in the exponential growth phase. In the stationary/production phase, however, *C. glutamicum* WT,  $\Delta aceE$ , and  $\Delta aceE$ (pJC4ilvBNCE) showed intracellular pyruvate concentrations of about 1.9 mM, 25.9 mM, and 2.3 mM, respectively. These results indicate that in the absence of acetate as the carbon and energy source, the *aceE* deletion in *C. glutamicum* leads to an intracellular accumulation of pyruvate. Furthermore, these results indicate that overexpression of the genes encoding L-valine biosynthetic enzymes leads to a drain-off of the pyruvate towards L-valine.

**Fed-batch fermentations with *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE).** In order to test the suitability of *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE) for an improved L-valine production process, we established a fed-batch fermentation based on mixed substrate (Fig. 4). These fermentations were carried out in CGXII medium containing 0.5% (wt/vol) BHI, 4% glucose, and an initial potassium acetate concentration of 1% (wt/vol). To allow growth to a high cell density, adequate amounts of a 50% (wt/vol) acetate stock solution were repeatedly added (eight times) to the growing cells (Fig. 4). Using this technique, an  $OD_{600}$  of about 70 was obtained after 19 h (Fig. 4). During this growth period, about 20 g glucose/liter was consumed in addition to acetate; however, neither L-valine nor pyruvate was excreted into the medium. After having consumed the last pulse of acetate, the cells started to excrete L-valine, and at time points 20 h and 33 h, we added pulses of 4% (wt/vol) glucose. As shown in Fig. 4, the cells accumulated about 210 mM L-valine within 21 h with a volumetric productivity of  $10.0 \text{ mM h}^{-1}$  ( $1.17 \text{ g liter}^{-1} \text{ h}^{-1}$ ) and a maximal yield of 0.6 mol L-valine per mol of glucose (between 19 h and 33 h in Fig. 4). The overall yield in the production phase (between 19 h and 40 h) was about 0.5 mol per mol of glucose. In addition to L-valine, the cells also still excreted small amounts of pyruvate (5 mM) into the medium, indicating that L-valine production by *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE) can be further increased. Taken together, all these results demonstrate that *C. glutamicum*  $\Delta aceE$  with plasmid-bound overexpression of L-valine biosynthetic genes is a very useful platform for optimizing L-valine production with *C. glutamicum*.

## DISCUSSION

L-Valine-producing strains of *C. glutamicum* have previously been obtained by undirected mutagenesis (20, 28) and by overexpression of genes encoding (deregulated) L-valine biosynthetic enzymes, in combination with the deletion of *ilvA* and/or of the D-pantothenate biosynthetic genes *panBC* (9, 23, 24). The knockout of the *ilvA* gene eliminates TD activity and thus the formation of 2-ketobutyrate, which together with pyruvate is a substrate for L-isoleucine biosynthesis via AHAS, AHAI, dihydroxyacid dehydratase, and TA. This leads to an isoleucine auxotrophy on the one hand and to an enhanced pyruvate availability on the other. Both these effects have been proposed to be responsible for enhanced L-valine production, since the former should lead to a suspension of the allosteric AHAS inhibition and of the attenuation control of the *ilvBNC* genes by L-isoleucine (5, 19) and the latter to an enhanced pyruvate flux into the L-valine biosynthetic pathway. As shown here, *ilvA* deletion (or TD inactivation) is not a prerequisite for effective L-valine production with *C. glutamicum*, indicating that the allosteric regulation of AHAS and the *ilvBNC* expression control by intracellular L-isoleucine are not very critical for the L-valine production process. Deletion of the *panBC* genes in *C. glutamicum* has been shown to result in pantothenate auxotrophy (23). Due to the finding that a *panC* mutant excreted pyruvate and alanine into the growth medium and due to the hypothesis that reduced coenzyme A availability in pantothenate-auxotrophic *C. glutamicum* strains should lead to reduced PDHC activity, Radmacher et al. (23) speculated that in this mutant increased quantities of precursor are directly available for L-valine synthesis. In accordance with this hypothesis, we here show that a PDHC-deficient mutant of *C. glutamicum* WT without any further genetic engineering is able to produce significant amounts of L-valine from glucose. In addition, *C. glutamicum*  $\Delta aceE$  excreted significant amounts of pyruvate and L-alanine, indicating that L-valine production in this strain is limited by the reactions from pyruvate to L-valine and not by pyruvate availability. This hypothesis is corroborated by our results obtained with *C. glutamicum*  $\Delta aceE$  carrying the *ilvBNC* genes on plasmid pJC4. In batch fermentations, this

strain produced much more L-valine, less pyruvate, and no L-alanine, and in fed-batch fermentations, the product spectrum was almost completely shifted towards L-valine. Since *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE) in shake flask batch fermentation produced as much L-valine as or even more L-valine than did previously constructed L-valine-producing strains (references 3, 9, and 23; also see below), our results indicate that effective L-valine production with *C. glutamicum* can easily be attained by optimization of the pyruvate availability and increasing the intracellular content of L-valine biosynthetic enzymes AHAS, AHAI, and TA. Furthermore, the results show that effective L-valine production neither relies on isoleucine and/or pantothenate auxotrophy nor requires the presence of deregulated L-valine biosynthetic enzymes.

Independently of the presence or absence of plasmid pJC4ilvBNCE and independently of the fermentation process, L-valine production in *C. glutamicum*  $\Delta aceE$  was decoupled from growth, i.e., took place only after cessation of the growth, or in other words, after depletion of the acetate required for growth of the mutant. This phenotype is surprising in view of the fact that L-valine (and also L-lysine, L-leucine, L-alanine, and L-threonine) production with *C. glutamicum* generally occurs in the course of the growth (2, 9, 11). One explanation for the production phenotype of *C. glutamicum*  $\Delta aceE$  might be that the presence of acetate may have a negative effect on glucose uptake and/or on one or several enzymes involved in glycolysis; thus, the pyruvate availability in the growth phase might be restricted to the requirements for cell mass biosynthesis. In favor of this argument, we previously showed that glucose consumption and the carbon flux from external glucose to pyruvate was reduced when *C. glutamicum* grows on glucose plus acetate instead of on glucose alone (30). Moreover, we here show that in fact the intracellular pyruvate concentrations in *C. glutamicum* WT as well as in the  $\Delta aceE$  deletion mutants were very low in the course of the growth and much higher in the stationary/production phase. Here it is interesting to note that AHAS, the first enzyme in L-valine synthesis from pyruvate, has a relatively weak affinity for pyruvate ( $K_m = 8.3$  mM) (5, 16) compared to those of enzymes competing with AHAS for pyruvate, e.g., that of pyruvate carboxylase ( $K_m = 1.3$  mM) (22).

Brik Ternbach et al. (2) recently used fed-batch experiments with *C. glutamicum*  $\Delta ilvA \Delta panBC$ (pJC4ilvBNCD) for model development and improvement of the L-valine fermentation process. In an experiment designed to maximize the total volumetric productivity, the final product concentration was almost 200 mM, and the maximal volumetric productivity was 6.2 mM L-valine per h. However, the cells produced several by-products, such as L-alanine, L-glycine, and 2-ketoglutarate (2). Leuchtenberger et al. (15) reported on a *C. glutamicum* mutant (VR3) which was obtained by classical mutagenesis and which produced up to 850 mM L-valine with a yield of 0.3 to 0.4 g per g of sucrose, which is equivalent to 0.45 to 0.6 mol per mol of glucose. Comparing these numbers with the data presented here for the fed-batch fermentation with *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE), i.e., a final L-valine concentration of 210 mM, a volumetric productivity of 10.0 mM L-valine per h and a yield of 0.5 to 0.6 mol L-valine per mol of glucose within 21 h of production, the performance of our simple model strain becomes strikingly evident. However, the maximal theoretical

yield amounts to 1 mol L-valine per mol of glucose (i.e., 0.65 g per g), indicating that L-valine production with *C. glutamicum* can be further optimized. Moreover, taking the numbers given above into account, we neglect that *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE) does not produce L-valine in the growth phase. With respect to L-valine process development, the opportunity for a simple growth-decoupled production is a significant advantage allowing for more efficient use of the biocatalyst.

Further approaches to improve L-valine production with *C. glutamicum*  $\Delta aceE$ (pJC4ilvBNCE) will aim at unraveling and overcoming the metabolic or regulatory reason for the non-production phenotype during the growth phase to allow both growth-coupled and -decoupled product formation. However, although not yet maximized as L-valine production strains, derivatives of *C. glutamicum*  $\Delta aceE$  may prove useful as cost-effective biocatalysts for the production of pyruvate itself and/or of products derived from this intermediate.

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