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Acetohydroxyacid Synthase, a Novel Target for Improvement of L-Lysine Production by *Corynebacterium glutamicum*▽†

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The influence of acetohydroxy acid synthase (AHAS) on L-lysine production by *Corynebacterium glutamicum* was investigated. An AHAS with a deleted C-terminal domain in the regulatory subunit IlvN was engineered by truncating the *ilvN* gene. Compared to the wild-type AHAS, the newly constructed enzyme showed altered kinetic properties, i.e., (i) an about twofold-lower K_m for the substrate pyruvate and an about fourfold-lower V_{max}; (ii) a slightly increased K_m for the substrate α-ketobutyrate with an about twofold-lower V_{max}; and (iii) insensitivity against the inhibitors L-valine, L-isoleucine, and L-leucine (10 mM each). Introduction of the modified AHAS into the L-lysine producers *C. glutamicum* DM1729 and DM1933 increased L-lysine formation by 43% (30 mM versus 21 mM) and 36% (51 mM versus 37 mM), respectively, suggesting that decreased AHAS activity is linked to increased L-lysine formation. Complete inactivation of the AHAS in *C. glutamicum* DM1729 and DM1933 by deletion of the *ilvB* gene, encoding the catalytic subunit of AHAS, led to L-valine, L-isoleucine, and L-leucine auxotrophy and to further-improved L-lysine production. In batch fermentations, *C. glutamicum* DM1729 Δ*ilvB* produced about 85% more L-lysine (70 mM versus 38 mM) and showed an 85%-higher substrate-specific product yield (0.180 versus 0.098 mol C/mol C) than *C. glutamicum* DM1729. Comparative transcriptome analysis of *C. glutamicum* DM1729 and *C. glutamicum* DM1729 Δ*ilvB* indicated transcriptional differences for about 50 genes, although not for those encoding enzymes involved in the L-lysine biosynthetic pathway.

Corynebacterium glutamicum, a gram-positive soil bacterium that grows on a variety of sugars and organic acids, is the workhorse for the fermentative production of the amino acids L-glutamate (1.5×10^6 tons/year) and L-lysine (0.9×10^6 tons/year) (25, 27, 53). Due to the growing world market and steadily decreasing market prices (24), great efforts have been made to develop more powerful and efficient production strains (8, 20, 24, 42). Since the yields and productivities of the production strains still are below the expected theoretical values, there is a large interest to further improve the performance of bacterial production strains (53).

In *C. glutamicum*, L-lysine is synthesized from oxaloacetate and pyruvate to the branch of L-piperideine-2,6-dicarboxylate, which is converted to D,L-diaminopimelate either by diaminopimelate dehydrogenase, when ammonium is available in excess, or by the tetrahydrodipicolinate succinylase pathway, when ammonium availability is low (46, 52). The key enzyme for L-lysine synthesis is aspartate kinase, which in wild-type (WT) *C. glutamicum* catalyzes phosphorylation of aspartate and is strongly feedback inhibited by L-lysine plus L-threonine (33, 48). Overexpression of the respective *lysC* gene, and especially overexpressing alleles encoding feedback-resistant aspartate kinase, strongly improved L-lysine formation (7, 11, 47). Aside from tailoring the biosynthetic pathway for L-lysine overproduction, carbon flux analysis highlighted the importance of the NADPH supply for efficient L-lysine production with the pentose phosphate pathway (PPP) as the predominant route for NADPH supply during growth on glucose (28). Increased flux from glycolysis into the PPP was achieved by inactivation of phosphoglucose isomerase (29), by introduction of a mutant allele encoding a feedback-resistant 6-phosphogluconate dehydrogenase (34), or by overexpression of fructose 1,6-biphosphatase (1, 15). Also, the expression of the membrane-bound transhydrogenase genes from *Escherichia coli* in *C. glutamicum* increased the NADPH supply and thus improved L-lysine production (21). A number of studies indicated the extraordinary role of the pyruvate and/or oxaloacetate supply for L-lysine production by inactivation of the pyruvate dehydrogenase complex (PDHC) (4), by overexpression of the pyruvate carboxylase gene (38), by inactivation of the phosphoenolpyruvate (PEP) carboxykinase gene (41), or by disruption of malate:quinone oxidoreductase (31). Furthermore, the inactivation of citrate synthase and methylcitrate synthase was highly beneficial for L-lysine production due to an increased oxaloacetate supply (40). Other studies described a link between increased L-lysine formation and L-leucine auxotrophy in *C. glutamicum* MH20-22B, DG-52-5, and KK25 Δ*leuA* strains (36, 47, 51) or with a limited L-leucine supply in the defined L-lysine producer *C. glutamicum* ADL-3 (19). Acetohydroxy acid synthase (AHAS) is the key enzyme of the pathways for the synthesis of the branched-chain amino acids (BCAAs) L-valine, L-isoleucine, and L-leucine. It catalyzes the formation of either α-acetolactate from two molecules of pyruvate or the formation of α-acetohydroxybutyrate from pyruvate plus α-ketobutyrate. The *C. glutamicum* AHAS consists of two

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TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence	Source, reference, or purpose
Strains		
E. coli DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	17
WT *C. glutamicum*	WT strain ATCC 13032, biotin-auxotrophic	American Type Culture Collection
C. glutamicum DM1729	*pyc*(*P458S*) *hom*(*V59A*) *lysC*(*T311I*), derived from WT *C. glutamicum*	For construction, see Table S1 in the supplemental material
C. glutamicum DM1729 Δ*ilvB*	Strain DM1729 with deletion of *ilvB*, encoding the large subunit of the acetolactate synthase (AHAS)	This work
C. glutamicum DM1729 ΔC-T *ilvN*	Strain DM1729 with deletion of the last 83 amino acids of the C-terminal domain of *ilvN*, encoding the small subunit of the AHAS	This work
C. glutamicum DM1933	Δ*pck pyc*(*P458S*) *hom*(*V59A*), 2 copies of *lysC*(*T311I*), 2 copies of *asd*, 2 copies of *dapA*, 2 copies of *dapB*, 2 copies of *ddh*, 2 copies of *lysA*, 2 copies of *lysE* derived from WT *C. glutamicum*	For construction, see Table S1 in the supplemental material
C. glutamicum DM1933 Δ*ilvB*	Strain DM1933 with deletion of *ilvB*, encoding the large subunit of the AHAS	This work
C. glutamicum DM1933 ΔC-T *ilvN*	Strain DM1933 with deletion of the last 83 amino acids of the C-terminal domain of *ilvN*, encoding the small subunit of the AHAS	This work
Plasmids		
pK19*mobsacB*	Km^r, mobilizable (*oriT*), *oriV*	44
pK19*mobsacB* Δ*ilvB*	pK19*mobsacB* carrying a truncated *ilvB* gene	32
pK19*mobsacB* ΔC-T *ilvN*	pK18*mobsacB* carrying a truncated *ilvN* gene (shortened by 249 bp)	This work
Oligonucleotides		
P1	5′-CCCAAGCTTGCTGTTTCCAGATGACCAACC-3′	Primer for deletion of ΔC-T *ilvN*
P2	5′-GGCGATAGTGGTCTCTTCATCAAGTCGCACGACTTTGAGC-3′	Primer for deletion of ΔC-T *ilvN*; crossover overlap underlined
P3	5′-GAAGAGACCACTATCGCCACAGCAATTAATCTGATTGC-3′	Primer for deletion of ΔC-T *ilvN*; crossover overlap underlined
P4	5′-CGCGGATCCCGTTCAGGTTTGGCTCGATG-3′	Primer for deletion of ΔC-T *ilvN* and to verify *ilvB* deletion
PD1	5′-CCAAGATGGCTAATTCTGACGTCACC-3′	Primer to verify ΔC-T *ilvN* deletion
PD2	5′-GACTAGTCACATTTATGCAGCAGGTGC-3′	Primer to verify ΔC-T *ilvN* deletion
PilvB	5′-GCAACAGACATCTGTCGC-3′	Primer to verify *ilvB* deletion

catalytic and two regulatory subunits, which are encoded by *ilvB* and *ilvN*, respectively. Together with the acetohydroxy acid isomeroreductase gene *ilvC*, the two genes form the *ilvBNC* operon (6, 10). On the one hand, AHAS is subject to feedback inhibition by the three BCAAs (10, 14). On the other hand, expression of the *ilvBNC* operon is controlled by an attenuation mechanism, leading to an about twofold-higher expression in response to the shortage of BCAAs (32). Furthermore, it has been shown that α-ketobutyrate in the medium increases expression of the *ilvBNC* operon by about 10-fold by a so-far-unknown regulatory mechanism (10, 32). In contrast to *C. glutamicum*, *Escherichia coli* possesses three AHAS isoenzymes (I, II, and III), differing in their regulation and biochemical properties (54). AHAS III (encoded by *ilvIH*) exhibits the highest similarity to the AHAS of *C. glutamicum*. The regulatory subunit of *E. coli* (163 amino acids, encoded by *ilvH*) shares 39% identity with that of *C. glutamicum* (172 amino acids, encoded by *ilvN*) (35) and contains a characteristic N-terminal ATC domain, which has been shown to be responsible for L-valine binding (23). However, deletion of the C-terminal domain of *ilvH* in *E. coli* resulted in a functional AHAS III that has a higher V_{max}, has a higher catalytic efficiency, and is insensitive for L-valine inhibition (30). In a similar manner, the deletion of the C-terminal ATC domain of the L-serine-sensitive 3-phosphoglycerate dehydrogenase in *C. glutamicum* resulted in a fully functional enzyme that is insensitive to L-serine and that improved L-serine production (37, 39).

In the present work, we engineered a feedback-resistant AHAS, insensitive to the BCAAs by deletion of the C-terminal domain of IlvN. Originally we intended to use the modified enzyme for improvement of L-valine production by *C. glutamicum.* However, the kinetic properties of the newly constructed AHAS prompted us to test the enzyme for an effect on L-lysine production, leading to the finding that decreased AHAS activity is linked to increased L-lysine formation.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids and their relevant characteristics and sources are listed in Table 1. The oligonucleotides used and their sequences are also listed in Table 1.

DNA preparation and transformation. The isolation of plasmids from *E. coli* was performed as described previously (13). Plasmid DNA transfer into *C. glutamicum* was carried out by electroporation, and the recombinant strains were selected on LB brain heart infusion agar plates containing kanamycin (50 μg ml^{-1}) (55). The isolation of chromosomal DNA from *C. glutamicum* was performed as described previously (13). Electroporation of *E. coli* was carried out with competent cells according to the method of Dower et al. (9).

Culture conditions. *E. coli* was grown aerobically in 2× tryptone-yeast extract (TY) complex medium (43) at 37°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Precultures of WT *C. glutamicum* and *C. glutamicum* ΔC-T *ilvN* were grown in 2× TY medium. *C. glutamicum* DM1729, *C. glutamicum* DM1933, and their derivatives were grown in 3.7% (wt/vol) brain heart infusion medium (Merck). 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 (12) containing 4% (wt/vol) glucose to give an initial optical density at 600 nm (OD_{600}) of about 1. As indicated in Results, 0.5% (wt/wt) corn steep liquor (CSL; Roquette) or L-valine, L-isoleucine, and/or L-leucine (2 mM each) were added to the medium. *C. glutamicum* was grown aerobically at 30°C in 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Batch fermentations were performed at 30°C in 200-ml cultures in a fedbatch pro fermentation system from DASGIP (Jülich, Germany). The fermentation conditions for aeration and pH control were described by Blombach et al. (4).

Construction of *C. glutamicum* ΔC-T *ilvN* and *C. glutamicum* Δ*ilvB*. Chromosomal inactivation of the C-terminal domain of IlvN in *C. glutamicum* was performed using crossover PCR and the suicide vector pK19*mobsacB*. DNA fragments were generated using the primer pairs P1/P2 and P3/P4, respectively. The two fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primers P1 and P4. The resulting fusion product (containing the *ilvN* gene shortened by 249 bp) was ligated into BamHI/HindIII-restricted plasmid pK19*mobsacB* and transformed into *E. coli*. After isolation and sequencing (MWG Biotech), the recombinant plasmid was electroporated into WT *C. glutamicum*, *C. glutamicum* DM1729, and *C. glutamicum* DM1933. By application of the method described by Schäfer et al. (44), the intact chromosomal *ilvN* gene was replaced by the truncated *ilvN* gene via homologous recombination (double crossover). The screening of the *ilvN* mutants was done on 2× TY agar plates containing 10% (wt/vol) sucrose. The replacement at the chromosomal locus was verified by PCR using primers PD1/PD2.

Inactivation of the chromosomal *ilvB* gene in *C. glutamicum* DM1729 and *C. glutamicum* DM1933 was performed as described previously for WT *C. glutamicum* Δ*ilvB* (32), using the suicide vector pK19*mobsacB* Δ*ilvB*. The deletion at the chromosomal locus was verified by PCR using primers PilvB/P4.

Analytical methods. For quantification of substrate consumption and product formation, 1-ml samples were taken from the cultures and centrifuged at 15,000 × *g* (10 min), and the supernatant was used for determination of amino acid, glucose, and/or organic acid concentrations in the culture fluid. The amino acid concentrations were determined by reversed-phase high-pressure liquid chromatography as described previously (3). Glucose, acetate, and L-lactate concentrations were determined by enzymatic tests (Roche Diagnostics). The pyruvate concentrations were determined enzymatically according to Bergmeyer (2). α-Ketobutyrate concentrations were determined by reversed-phase high-pressure liquid chromatography with fluorimetric detection (excitation at 361 nm; emission at 448 nm) after precolumn derivatization with 1,2-diamino-4,5-dimethoxybenzene (18). Separation was carried out at 40°C on a Multohyp octadecyl silane column (particle size, 5 μm; 125 by 4 mm) (CS-Chromatographie, Langerwehe, Germany). The elution buffer consisted of a polar phase (water) and a nonpolar phase (methanol). Quantification was done by calculation of the concentration using an internal standard (α-ketovalerate at 100 μM) and by a five-point-calibration curve for α-ketobutyrate.

Online analysis of the oxygen and carbon dioxide contents of the exhaust gas was performed using the GA4 gas analyzer from DASGIP (Jülich, Germany). The carbon evolution rate [CER; given in moles/(liters · hours)] was determined by using the following equation:

$$\mathrm{CER} = \frac{v_{g,\mathrm{in}}}{V_R \times V_n} \times \left(Y^{\mathrm{out}}_{CO_2} \times \frac{(1 - Y^{\mathrm{in}}_{O_2} - Y^{\mathrm{in}}_{CO_2})}{(1 - Y^{\mathrm{out}}_{O_2} - Y^{\mathrm{out}}_{CO_2})} - Y^{\mathrm{in}}_{CO_2} \right)$$

V_n is the mole volume of the ideal gas (liters/moles) at standard conditions, V_R is the working volume of the bioreactor (liters), $v_{g,\mathrm{in}}$ is the volumetric inlet airflow (liters/hours) at standard conditions, and $Y^{\mathrm{in}}_{O_2}$, $Y^{\mathrm{in}}_{CO_2}$, $Y^{\mathrm{out}}_{O_2}$, and $Y^{\mathrm{out}}_{CO_2}$ are molecular fractions of oxygen and carbon dioxide in the inlet and outlet air, respectively. The total carbon dioxide concentration (moles/liters) was calculated by integration of the CER over the fermentation time.

Determination of AHAS activities and kinetic parameters. The standard assay for determination of AHAS activities was carried out using the colorimetric single-point method (26). *C. glutamicum* cells were cultivated in minimal medium containing 4% (wt/vol) glucose to an OD_{600} of about 5 and were harvested by centrifugation (4,200 × *g* for 10 min at 4°C). The cells were washed three times with ice-cold 2% (wt/vol) KCl at 4°C, resuspended in disruption buffer (100 mM potassium phosphate buffer [pH 7.3] containing 0.5 mM dithiothreitol and 20% [vol/vol] glycerol), and disrupted with a RyboLyser at 4°C. The reaction mixture (5 ml) contained 100 mM potassium phosphate buffer (pH 7.3), 10 mM $MgCl_2$, 100 μM flavin adenine dinucleotide, and 50 mM pyruvate. The reaction was started by adding 500 μl of diluted cell extract and was stopped by adding 100 μl of 50% (vol/vol) H_2SO_4 to 1 ml of the reaction mixture. Then, the assay solution was incubated for 30 min at 37°C to allow the conversion of α-acetolactate to acetoin. The concentration of acetoin was determined by the method of Westerfeld (56). The protein concentration was quantified with the BCA protein assay (Pierce) with bovine serum albumin as the standard. Assays were linear over time and proportional to the protein concentration.

For the determination of the Michaelis-Menten constant ($K_{m,P}$) and the maximal reaction rate ($V_{max,P}$) for pyruvate, 10 pyruvate concentrations ranging from 2.5 to 60 mM were used, and the formation of α-acetolactate was monitored as described above. For the determination of $K_{m,K}$ and $V_{max,K}$ for α-ketobutyrate, nine α-ketobutyrate concentrations ranging from 1 to 40 mM at a constant pyruvate concentration (100 mM) were used, and the decrease of α-ketobutyrate was measured as described above. K_m and V_{max} values were calculated using the Hanes-Wilkinson plot. One micromole of α-acetolactate formed or 1 μmol α-ketobutyrate converted per milligram of protein per minute corresponds to one unit.

RNA preparation and transcriptome analysis. For RNA isolation, *C. glutamicum* DM1729 and DM1729 Δ*ilvB* were grown in minimal medium containing 4% (wt/vol) glucose with L-valine, L-isoleucine, and L-leucine (2 mM each) harvested in the exponential growth phase (OD_{600} of about 20) and treated with 1 volume of ice-cold killing buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaN_3, 5 mM $MgCl_2$). The isolation procedure was performed as described previously (45), and aliquots of the RNA were stored at −70°C until use.

DNA microarray analysis, cDNA synthesis, fragmentation, and biotin labeling were carried out as described previously for samples of prokaryotes in the Affymetrix technical support manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx/). Labeled cDNA samples were hybridized to Affymetrix GeneChip Corynea520112F genome arrays (custom-specific design). This array consists of 3,571 probe sets which can be divided in genes and hypothetical open reading frames (3,221), intergenic probe sets (305), and control probe sets (45). Hybridized arrays were stained with streptavidin-phycoerythrin using the Affymetrix Fluidics station and scanned. The experiment was designed to minimize both false-positive and false-negative results for expressed genes. Two biological and two technical replicates were performed for the analysis of *C. glutamicum* DM1729 and DM1729 Δ*ilvB*. Statistical expression analysis was performed with Genedata Expressionist 5.0 software on the probe-level data from Affymetrix's CEL files condensed with the MAS 5.0 algorithm. The data quality *P* value threshold was set to 0.05. To test for significant differences in expression between the strains, one-way analysis of variance was performed at a significance level of 0.001; thus, for every 1,000 genes tested, only one false positive would be expected.

RESULTS

Impact of IlvN modification on growth of *C. glutamicum* and kinetic properties of the AHAS. For the *E. coli* AHAS III, it has been shown that deletion of the C-terminal 80 amino acids in the regulatory subunit led to a functional enzyme released from feedback inhibition by L-valine (30). To test for a similar effect, we deleted the last 249 bp of *ilvN* in WT *C. glutamicum*, resulting in *C. glutamicum* ΔC-T *ilvN*, and studied the effect of the IlvN modification on growth and AHAS properties. For comparative characterization of growth, we performed shake flask cultivations with WT *C. glutamicum* and *C. glutamicum* ΔC-T *ilvN* in minimal medium containing 4% (wt/vol) glucose with or without different combinations of the BCAAs L-valine, L-isoleucine, and L-leucine (2 mM each) (Fig. 1). Under all conditions tested, WT *C. glutamicum* showed a growth rate of 0.30 h^{-1} and reached a final OD_{600} of about 55. Figure 1 shows a representative growth curve of WT *C. glutamicum* in minimal medium containing glucose. *C. glutamicum* ΔC-T *ilvN* also grew under all conditions to a final OD_{600} of about 55. However, except when all three BCAAs were added to the medium, the growth rates of the mutant were lower than those of WT *C.*

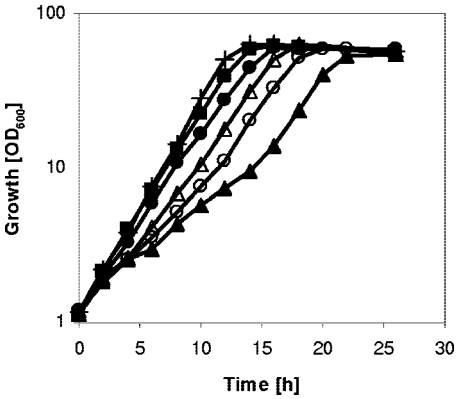

FIG. 1. Growth of WT *C. glutamicum* on CGXII medium containing glucose (4%) (+) and *C. glutamicum* ΔC-T *ilvN* on CGXII medium containing glucose (4%) (△) or with 2 mM L-isoleucine (▲), 2 mM L-leucine (○), 2 mM L-valine (●), or all three amino acids (2 mM each) (■).

glutamicum. In minimal medium with glucose, *C. glutamicum* ΔC-T *ilvN* grew with a growth rate of 0.22 h^{-1}, the addition of L-leucine resulted in a decreased growth rate of 0.19 h^{-1}, and the addition of L-isoleucine resulted in biphasic growth, with growth rates of 0.15 h^{-1} in the first and 0.27 h^{-1} in the second exponential growth phase (Fig. 1). After supplementation of L-valine, an increased growth rate of 0.27 h^{-1} was observed.

The observation that growth of *C. glutamicum* ΔC-T *ilvN* was impaired under certain conditions prompted us to compare selected kinetic parameters of AHAS ΔC-T *ilvN* and the WT AHAS (Table 2). For this purpose, cells were grown in minimal medium with glucose, and the kinetic parameters of the enzyme were determined in crude extracts. AHAS ΔC-T *ilvN* showed an about twofold-higher affinity for pyruvate ($K_{m,P}$, 4.7 mM versus 7.8 mM) and an about fourfold-lower $V_{max,P}$ (22.3 mU/mg versus 77.6 mU/mg) than that of the WT AHAS. With α-ketobutyrate as the substrate, AHAS ΔC-T *ilvN* exhibited a slightly higher $K_{m,K}$ (6.9 mM) and an about twofold-lower $V_{max,K}$ (56.8 mU/mg) than that of the WT enzyme ($K_{m,K}$, 5.6 mM; $V_{max,K}$, 113.9 mU/mg). In contrast to that of the WT AHAS, the specific activity of the modified AHAS was not affected by the presence of 10 mM L-valine, L-isoleucine, L-leucine, or all three BCAAs (10 mM each), showing that the C-terminal domain of IlvN in *C. glutamicum* is responsible for the feedback inhibition by the BCAAs.

IlvN modification improves L-lysine production with *C. glutamicum*. The assumption that the lower specific activity of the AHAS ΔC-T *ilvN* may lead to a reduction in the carbon flux toward the BCAAs, in combination with the previous findings that L-leucine limitation or a L-leucine auxotrophy seems to be beneficial for L-lysine biosynthesis (see Introduction), prompted us to test the relevance of the ΔC-T *ilvN* mutation for L-lysine production by *C. glutamicum*. For this purpose, we introduced the ΔC-T *ilvN* mutation into the chromosomal *ilvN* locus of the two L-lysine producers *C. glutamicum* DM1729 and *C. glutamicum* DM1933, resulting in *C. glutamicum* DM1729 ΔC-T *ilvN* and *C. glutamicum* DM1933 ΔC-T *ilvN*, and performed shake flask fermentations with the mutants and the parental strains in minimal medium containing glucose (Table 3). *C. glutamicum* DM1729 showed a growth rate of 0.25 h^{-1}, reached a final OD_{600} of 43, and accumulated 21.0 mM L-lysine. *C. glutamicum* DM1729 ΔC-T *ilvN* grew with a lower growth rate (0.17 h^{-1}) to about the same final OD_{600}; however, it produced 43% more L-lysine (29.7 mM). In the presence of L-valine, L-isoleucine, L-leucine, and 4% glucose, both *C. glutamicum* DM1729 and *C. glutamicum* DM1729 ΔC-T *ilvN* grew with identical growth rates of 0.24 h^{-1} to OD_{600} values of 31 and produced within 48 h about 25 mM and 29 mM L-lysine, respectively (Table 3). *C. glutamicum* DM1933 ΔC-T *ilvN* was unable to grow in minimal medium with glucose; therefore, we added 0.5% (wt/vol) CSL to the medium. Under these conditions, *C. glutamicum* DM1933 ΔC-T *ilvN* grew with a lower growth rate (0.19 h^{-1}) than strain DM1933; however, the mutant produced 36% more L-lysine (50.5 mM versus 37.0 mM) (Table 3).

Inactivation of the AHAS further improves L-lysine production with *C. glutamicum*. The results described above led us conclude that increased L-lysine formation might be linked to decreased AHAS activity. To test this hypothesis, we inactivated the AHAS completely by deletion of *ilvB* in *C. glutamicum* DM1729 and DM1933, resulting in *C. glutamicum* DM1729 Δ*ilvB* and DM1933 Δ*ilvB*. As expected, both of these mutants showed no AHAS activity (i.e., <0.2 mU [mg protein]$^{-1}$) and were unable to grow in minimal medium unless supplemented with L-valine, L-isoleucine, and L-leucine (data not shown). In minimal medium containing all three amino acids (2 mM each) and 4% glucose, *C. glutamicum* DM1729 grew with a growth rate of 0.24 h^{-1} to a maximal OD_{600} of 43, which then decreased to an OD_{600} of 31 after 48 h, and produced 24.7 mM L-lysine (Fig. 2). *C. glutamicum* DM1729 Δ*ilvB* showed a lower growth rate of 0.20 h^{-1} and reached a maximal OD_{600} of 43, which rapidly dropped after complete consumption of the glucose to a final value of 21 after 48 h. However, *C. glutamicum* DM1729 Δ*ilvB* produced more than twice as much L-lysine as the parental strain (51.3 mM versus 24.7 mM).

TABLE 2. $K_{m,P}$, $K_{m,K}$, $V_{max,P}$, $V_{max,K}$, and the residual activities in the presence of 10 mM L-valine, L-isoleucine, L-leucine, or all three BCAAs (10 mM each) of the WT AHAS or the modified AHAS ΔC-T *ilvN*

Enzyme	Values for indicated substrate				Residual activity in the presence of 10 mM inhibitor(s) (%)[a]			
	Pyruvate + pyruvate		α-Ketobutyrate + pyruvate					
	$K_{m,P}$ (mM)	$V_{max,P}$ (mU/mg)	$K_{m,K}$ (mM)	$V_{max,K}$ (mU/mg)	L-Valine	L-Isoleucine	L-Leucine	All three BCAAs
Wild-type AHAS	7.8	77.6	5.6	113.9	50	54	65	46
AHAS ΔC-T *ilvN*	4.7	22.3	6.9	56.8	104	104	104	107

[a] Activities were measured using 50 mM pyruvate as the substrate. A value of 100% corresponds to 73.5 mU/mg for the WT AHAS and 22.1 mU/mg for AHAS ΔC-T *ilvN*.

TABLE 3. Final OD_{600} values, growth rates, and L-lysine concentrations of *C. glutamicum* DM1729, *C. glutamicum* DM1933, and recombinant derivatives grown in shake flasks in minimal medium containing 4% glucose with or without different supplements after 48 h[a]

C. glutamicum strain	Supplement(s)	Final OD_{600}	μ (h^{-1})	Concn of L-lysine (mM)
DM1729		43 ± 4	0.25 ± 0.01	21.0 ± 0.9
DM1729 ΔC-T *ilvN*		44 ± 2	0.17 ± 0.01	29.7 ± 2.5
DM1729	2 mM L-valine, L-isoleucine, and L-leucine	31 ± 4	0.24 ± 0.01	24.7 ± 0.6
DM1729 ΔC-T *ilvN*	2 mM L-valine, L-isoleucine, and L-leucine	31 ± 2	0.24 ± 0.01	29.0 ± 1.0
DM1933		40 ± 4	0.23 ± 0.01	32.6 ± 2.3
DM1933 ΔC-T *ilvN*		NG[b]		
DM1933	0.5% (wt/vol) CSL	51 ± 2	0.25 ± 0.03	37.0 ± 2.0
DM1933 ΔC-T *ilvN*	0.5% (wt/vol) CSL	41 ± 2	0.19 ± 0.01	50.5 ± 0.4
DM1729 Δ*ilvB*	2 mM L-valine, L-isoleucine, and L-leucine	21 ± 3	0.20 ± 0.01	51.3 ± 1.3
DM1933	2 mM L-valine, L-isoleucine, and L-leucine	34 ± 2	0.19 ± 0.01	40.5 ± 1.3
DM1933 Δ*ilvB*	2 mM L-valine, L-isoleucine, and L-leucine	14 ± 3	0.18 ± 0.01	62.8 ± 4.2

[a] All values are means ± standard deviations of at least three independent experiments.
[b] NG, no growth.

For *C. glutamicum* DM1933, inactivation of the AHAS also increased L-lysine formation, although to a somewhat lesser extent of 55% (62.8 mM versus 40.5 mM) (Table 3). In glucose minimal medium containing 0.5% (wt/vol) CSL instead of 2 mM L-valine, L-isoleucine, and L-leucine, *C. glutamicum* DM1933 Δ*ilvB* grew only to an OD_{600} of about 6 (data not shown), indicating that 0.5% of CSL cannot completely substitute the three BCAAs.

To test for suitability of DM1729 Δ*ilvB* for improved L-valine production on a larger scale, we performed comparative batch cultivations in a parallel fermentation system. These fermentations with *C. glutamicum* DM1729 and DM1729 Δ*ilvB* were carried out in CGXII medium containing 0.5% (wt/vol) CSL, 7% (wt/vol) glucose, and L-valine, L-isoleucine, and L-leucine (4 mM each). Growth, substrate consumption, product and by-product accumulation, and carbon dioxide formation were monitored throughout the course of the experiment. Under these conditions, both strains grew with identical growth rates of 0.19 h^{-1} to maximal OD_{600} values of about 61 (DM1729) and 64 (DM1729 Δ*ilvB*). As shown in Table 4, *C. glutamicum* DM1729 Δ*ilvB* showed about 85%-higher L-lysine formation, an 85%-higher substrate-specific product yield ($Y_{P/S}$), and 10%-higher productivity than the parental strain DM1729. Neither strain secreted pyruvate, L-lactate, or acetate; however, *C. glutamicum* DM1729 Δ*ilvB* accumulated about 10 mM L-glutamate. Additionally, *C. glutamicum* DM1729 Δ*ilvB* showed a substrate-specific carbon dioxide yield ($Y_{CO2/S}$) of 0.424 mol C/mol C, which is about 20% lower than the $Y_{CO2/S}$ of *C. glutamicum* DM1729 (0.526 mol C/mol C) (Table 4). This result indicates that improved L-lysine formation of *C. glutamicum* DM1729 Δ*ilvB* is due to reduced carbon dioxide formation.

Comparison of the transcriptomes of *C. glutamicum* DM1729 and *C. glutamicum* DM1729 Δ*ilvB*. The finding that decreased or abolished AHAS activity leads to increased L-lysine formation might be due to a change in transcription of relevant genes in response to a diminished or abolished flux toward the BCAAs. To compare the genome-wide transcriptional profiles of *C. glutamicum* DM1729 Δ*ilvB* and *C. glutamicum* DM1729, both strains were cultivated in minimal medium with glucose plus L-valine, L-isoleucine, and L-leucine, total RNA was prepared, and labeled cDNA samples were hybridized to Affymetrix GeneChip arrays. The analysis revealed 49 genes with different mRNA levels (more than twofold) in *C. glutamicum* DM1729 Δ*ilvB* compared to the parental strain *C. glutamicum* DM1729 (Table 5). Among these, 18 genes showed a higher mRNA level, including genes of acetate metabolism (*aceA*, *aceB*, *pta*, *ack*) and two genes annotated as predicted transcriptional regulators (cg2320 and cg3303), as well as *ilvN* (about sevenfold) and *ilvC* (about threefold). The group of genes with significantly lower mRNA levels consisted of 31 candidates, including genes of L-leucine (*leuC*), L-arginine (*argBCDF*), and L-methionine (*metE*) biosynthesis, as well as sev-

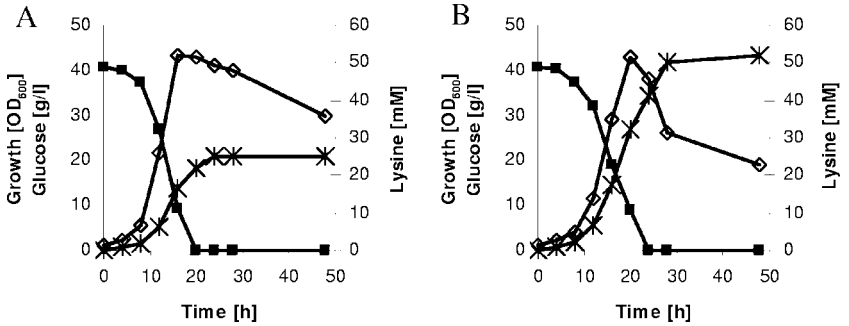

FIG. 2. Growth, substrate consumption, and product accumulation during a representative shake flask batch cultivation of *C. glutamicum* DM1729 (A) and *C. glutamicum* DM1729 Δ*ilvB* (B) on CGXII medium containing glucose (4%) and L-valine, L-isoleucine, and L-leucine (2 mM each). ◇, growth; ■, glucose; ×, L-lysine. Three independent fermentations were performed, all three showing comparable results.

TABLE 4. Maximal ODs, growth rates, L-lysine concentrations, substrate-specific product yields ($Y_{P/S}$), substrate-specific carbon dioxide yields ($Y_{CO2/S}$), and productivities of *C. glutamicum* DM1729 and DM1729 Δ*ilvB*[a]

C. glutamicum strain	Maximal OD_{600}	μ (h^{-1})	Concn of L-lysine (mM)	$Y_{P/S}$ (mol C/mol C)	$Y_{CO2/S}$ (mol C/mol C)	Productivity [mmol/(g CDW × h)][b]
DM1729	61	0.19	38	0.098	0.526	0.074
DM1729 Δ*ilvB*	64	0.19	70	0.180	0.424	0.082

[a] Two independent batch fermentations were performed, both showing comparable results. *C. glutamicum* DM1729 and DM1729 Δ*ilvB* were grown in batch culture in minimal medium containing 7% glucose, 0.5% CSL, and 4 mM each of L-valine, L-isoleucine, and L-leucine.
[b] Cell dry weight (CDW) was calculated from the OD_{600}, using a ratio of 0.3 g CDW $liter^{-1}$ per OD_{600} (4).

eral genes encoding enzymes involved in Fe-S cluster assembly (*sufBCDR*) and iron acquisition (cg0771, cg0924, cg0926, cg0927, cg0928, cg2445, and cg3404). Furthermore, the genes for a putative transcriptional regulator (cg0156), for SufR (probable regulator of the *suf* operon), for the L-arginine repressor ArgR, and for the L-leucine and L-tryptophan biosynthesis regulator LtbR showed lower mRNA levels in *C. glutamicum* DM1729 Δ*ilvB*. Interestingly, no significant changes were observed in the mRNA levels of genes encoding enzymes involved in the PPP, at the pyruvate-oxaloacetate node (e.g., pyruvate carboxylase, PEP carboxylase, PEP carboxykinase, and PDHC) or in L-lysine biosynthesis.

DISCUSSION

In the present work, we engineered a modified AHAS by deletion of the C-terminal domain in the regulatory subunit IlvN. The newly constructed enzyme showed a twofold-lower K_m for the substrate pyruvate, a slightly higher K_m for the substrate α-ketobutyrate, and a lower V_{max} for both pyruvate and pyruvate plus α-ketobutyrate. Furthermore, the modified AHAS was completely insensitive against the inhibitors L-valine, L-isoleucine, and L-leucine. These results indicate (i) that the C-terminal domain of the regulatory subunit IlvN of the *C. glutamicum* AHAS is responsible for inhibitor (L-valine, L-leucine, and L-valine) binding and/or inhibitor response and (ii) that the C-terminal domain is essential for maximal AHAS activity. The former result is in accordance with results obtained with the *E. coli* AHAS III, namely, with the findings that an AHAS with a deletion of 80 amino acids from the C terminus of the regulatory IlvH subunit is feedback resistant to L-valine and also shows an about twofold-higher affinity for the substrate pyruvate (30). However, in contrast to the *C. glutamicum* ΔC-T *ilvN* AHAS, the truncated AHAS III from *E. coli* even showed 40%-higher activity than the original AHAS III, indicating that in this enzyme, the C terminus of the regulatory subunit is not involved in recognition and activation of the catalytic subunit IlvI (30).

As an analogy to the above-mentioned findings with the *E. coli* AHAS III, the *C. glutamicum* ΔC-T *ilvN* AHAS originally was expected to be feedback resistant and also highly active. Since the AHAS reaction is one of the bottlenecks for efficient L-valine production (3, 5, 14), we therefore originally intended to use the ΔC-T *ilvN* AHAS for improvement of L-valine production strains (3, 5). Having tested the kinetic properties and having found the nearly fourfold-reduced $V_{max,P}$ values of the mutated AHAS, we realized that chromosomal introduction of the AHAS ΔC-T *ilvN* into L-valine producer strains most probably does not lead to improved L-valine production. It might be that multicopy introduction (plasmid-bound introduction) of the AHAS ΔC-T *ilvN* allele will lead to a higher carbon flux toward L-valine and/or the other BCAAs and their precursors; however, so far we did not test this possibility. Instead, we tested the newly constructed AHAS for its effect on L-lysine production. Chromosomal introduction of the AHAS ΔC-T *ilvN* into two L-lysine-producing strains of *C. glutamicum* and analysis of the resulting strains led us to conclude that a decrease in AHAS activity causes an increase in L-lysine formation. This conclusion is corroborated by the findings that (i) addition of the AHAS inhibitors L-valine, L-isoleucine, and L-leucine to the medium also resulted in increased L-lysine formation by both parental strains, *C. glutamicum* DM1729 (24.7 mM versus 21.0 mM) and DM1933 (40.5 mM versus 32.6 mM) (Table 3), and (ii) deletion of the *ilvB* gene (encoding the catalytic subunit of AHAS) and thus complete inactivation of the AHAS in both L-lysine producers resulted in even higher L-lysine production compared to only partial inactivation in *C. glutamicum* AHAS ΔC-T *ilvN*. Thus, this work identified the AHAS as a novel and promising target to improve L-lysine production with *C. glutamicum*.

The positive effect of decreased/inactivated AHAS activity for L-lysine production might at least partially be due to a reduced or abolished carbon flux toward the BCAAs and thus to an increase of the intracellular pyruvate availability. Pyruvate and oxaloacetate are central metabolic precursors for L-lysine formation, and their supply should be balanced for optimal L-lysine production. Several previous studies already indicated positive effects of increasing the pyruvate precursor supply on L-lysine production. Shiio et al. (49, 50) found that undefined mutants of *C. glutamicum* (formerly "*Brevibacterium flavum*") with either low citrate synthase or PDHC activity showed higher L-lysine production than their respective parental strains. Moreover, we recently showed that complete inactivation of the PDHC in *C. glutamicum* DM1729 led to improved L-lysine production (4). However, the PDHC-deficient *C. glutamicum* DM1729 BB1 did not accumulate as much L-lysine as *C. glutamicum* DM1729 Δ*ilvB* (30.0 mM versus 51.3 mM) and even excreted pyruvate and L-alanine (4). On the one hand, these observations indicate that L-lysine production in *C. glutamicum* DM1729 BB1 is not limited by pyruvate; on the other hand, they suggest that the positive effect of decreased/inactivated AHAS activity for L-lysine production cannot be explained exclusively by an increased pyruvate supply.

Hayashi et al. (19) recently showed that introduction of a *leuC* mutation, and thus introduction of a partial L-leucine auxotrophy in *C. glutamicum*, leads to an increased (14%)

TABLE 5. Comparison of gene expression in *C. glutamicum* DM1729 Δ*ilvB* to that in DM1729

Locus tag	mRNA ratio	Gene	Function(s)[a]
Amino acid biosynthesis			
cg1290	0.40	*metE*	Homocysteine methyltransferase
cg1436	7.43	*ilvN*	AHAS, small subunit
cg1437	2.68	*ilvC*	Acetohydroxy acid isomeroreductase
cg1487	0.35	*leuC*	3-Isopropylmalate dehydratase, large subunit
cg1580	0.47	*argC*	*N*-Acetyl-gamma-glutamyl-phosphate reductase
cg1582	0.38	*argB*	Acetylglutamate kinase
cg1583	0.43	*argD*	Acetylornithine aminotransferase
cg1584	0.32	*argF*	Ornithine carbamoyltransferase
cg1739	0.39		Glutamine amidotransferase domain
Central metabolism			
cg2559	3.25	*aceB*	Malate synthase
cg2560	2.30	*aceA*	Isocitrate lyase
cg3047	2.47	*ackA*	Acetate kinase
cg3048	2.27	*pta*	Phosphotransacetylase
Transcriptional			
cg0156	0.46		Bacterial regulatory protein, Crp family
cg1486	0.38	*ltbR*	L-Leucine and L-tryptophan biosynthesis regulator, IclR family
cg1585	0.27	*argR*	L-Arginine repressor
cg1765	0.44	*sufR*	Predicted transcriptional regulator for the *suf* operon
cg2320	2.20		Predicted transcriptional regulator
cg3303	2.35		Transcriptional regulator, PadR-like family
Iron metabolism			
cg0771	0.34		DtxR/iron-regulated lipoprotein
cg0924	0.20		ABC-type cobalamin/Fe_3^+-siderophore transport system
cg0926	0.39		ABC-type cobalamin/Fe_3^+-siderophore transport system
cg0927	0.29		ABC-type cobalamin/Fe_3^+-siderophore transport system
cg0928	0.37		ABC-type cobalamin/Fe_3^+-siderophore transport system
cg1762	0.46	*sufC*	Iron-regulated ABC transporter, ATPase subunit
cg1763	0.35	*sufD*	Component of an uncharacterized iron-regulated ABC-type transporter
cg1764	0.35	*sufB*	Component of an uncharacterized iron-regulated ABC-type transporter
cg2445	0.45		Probable heme oxygenase
cg3404	0.25		ABC-type cobalamin/Fe_3^+-siderophore transport system
Others			
cg0277	0.49		Sodium sulfate symporter transmembrane component
cg0503	0.45		Probable 3-dehydroquinate dehydratase
cg0569	3.30		Cation-transporting ATPase
cg0607	2.95		Hypothetical secreted protein
cg0963	2.38		Hypothetical protein
cg1043	2.09		Thiol-disulfide isomerase and thioredoxins
cg1049	2.15		Enoyl-coenzyme A hydratase/carnithine racemase
cg1085	2.68		Hypothetical protein predicted by Glimmer criteria
cg1087	2.21		Putative membrane protein
cg1090	2.71		Probable γ-glutamyltranspeptidase
cg1229	0.45		ABC-type cobalt transport system, permease component CbiQ
cg1279	0.44		Putative secreted protein
cg1365	0.47	*atpH*	H^+-ATPase, δ subunit
cg1367	0.47	*atpG*	ATP synthase, γ subunit
cg1419	0.49		Putative Na^+-dependent transporter
cg1998	0.49	*cglIIR*	Restriction endonuclease CGLIIR protein
cg2095	0.42		Putative membrane protein
cg2438	2.39		Hypothetical protein predicted by Glimmer criteria
cg2477	2.13		Conserved hypothetical protein
cg3256	0.43		Alkanal monooxygenase, alpha chain

[a] See reference 22.

L-lysine production. The authors also reported that in the *leuC* mutant (strain ADL-3), many different amino acid biosynthetic genes were upregulated, including the *lysC-asd* operon (encoding the L-lysine biosynthetic enzymes aspartate kinase and aspartate semialdehyde dehydrogenase, respectively), and they speculated that increased expression of *lysC-asd* is responsible for increased L-lysine production (19). As an analogy to these observations, we speculated that the positive effect of de-

creased or abolished AHAS activity (and thus, reduced or abolished carbon flux toward the BCAAs) on L-lysine formation might also at least partially be due to a change in transcription of relevant genes; e.g., of those coding for enzymes involved in the L-lysine biosynthetic pathway. To examine this possibility and to identify candidate genes, we carried out comparative transcriptome analysis of *C. glutamicum* DM1729 and *C. glutamicum* DM1729 Δ*ilvB*. This analysis revealed 49 genes with at least twofold-altered mRNA levels in *C. glutamicum* DM1729 Δ*ilvB*. However, in contrast to the *leuC* mutant *C. glutamicum* ADL-3, *C. glutamicum* DM1729 Δ*ilvB* showed no significant changes in the mRNA levels of L-lysine biosynthetic pathway genes. Also in contrast to the *leuC* mutant, *C. glutamicum* DM1729 Δ*ilvB* showed different mRNA levels of some central metabolic pathway genes, some (putative) regulator genes, and genes involved in iron metabolism (Table 5). These results clearly show that effects other than those observed in the *leuC* mutant *C. glutamicum* ADL-3 must be the reason for improved L-lysine production by *C. glutamicum* DM1729 Δ*ilvB*.

None of the genes with different mRNA levels in *C. glutamicum* DM1729 Δ*ilvB* is directly linked to the pathways from glucose to L-lysine, to NADPH supply, or to known expression regulation of L-lysine synthesis. It might be speculated that higher expression of the isocitrate lyase and malate synthase genes (*aceA* and *aceB*, respectively) (Table 5) may lead to a higher glyoxylate cycle flux and thus to a higher oxaloacetate availability for L-lysine formation. The 2.3- and 3.25-fold increases in *aceA* and *aceB* mRNA levels are rather low in comparison to the 28.6- and 8.4-fold increases in *aceA*- and *aceB*-specific mRNA observed when *C. glutamicum* cells were grown on acetate instead of on glucose (16). However, a higher glyoxylate cycle activity and thus an at least partial bypass of the CO_2-releasing reactions of the tricarboxylic acid cycle (i.e., isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase complex reactions) are in agreement with the low substrate-specific CO_2 yield of *C. glutamicum* DM1729 Δ*ilvB* compared to that of the parental strain DM1729. In fact, the lower substrate-specific CO_2 yield ($Y_{CO2/S}$ lowered by 0.102 mol C/mol C) approximately corresponds to the increase in substrate-specific product yield (0.082 mol C/mol C). The reduced respiration in combination with a lower tricarboxylic acid cycle activity possibly leads to more balanced oxaloacetate and pyruvate supplies for L-lysine production.

It should be kept in mind that all mRNA changes observed in *C. glutamicum* DM1729 Δ*ilvB* might be direct or indirect effects in response to abolished AHAS activity but might not be directly connected to L-lysine productivity. Further studies are necessary to mechanistically clarify why inactivation of *ilvB* in *C. glutamicum* obviously leads to different mRNA levels (different levels of expression) of so many genes (Table 5). The higher mRNA levels of the *ilvN* and *ilvC* genes can be explained by transcriptional activation of the truncated *ilvBNC* operon (*ilvB* deleted) by α-ketobutyrate (10, 32), which probably accumulates in *C. glutamicum* DM1729 Δ*ilvB*. However, it remains unclear whether α-ketobutyrate triggers expression of any of the other genes listed in Table 5.

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