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Global Expression Profiling and Physiological Characterization of *Corynebacterium glutamicum* Grown in the Presence of L-Valine

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Addition of L-valine (50 to 200 mM) to glucose minimal medium had no effect on the growth of wild-type *Corynebacterium glutamicum* ATCC 13032 but inhibited the growth of the derived valine production strain VAL1 [13032 Δ*ilvA* Δ*panBC*(pJC1*ilvBNCD*)] in a concentration-dependent manner. In order to explore this strain-specific valine effect, genomewide expression profiling was performed using DNA microarrays, which showed that valine caused an increased *ilvBN* mRNA level in VAL1 but not in the wild type. This unexpected result was confirmed by an increased cellular level of the *ilvB* protein product, i.e., the large subunit of acetohydroxyacid synthase (AHAS), and by an increased AHAS activity of valine-treated VAL1 cells. The conclusion that valine caused the limitation of another branched-chain amino acid was confirmed by showing that high concentrations of L-isoleucine could relieve the valine effect on VAL1 whereas L-leucine had the same effect as valine. The valine-caused isoleucine limitation was supported by the finding that the inhibitory valine effect was linked to the *ilvA* deletion that results in isoleucine auxotrophy. Taken together, these results implied that the valine effect is caused by competition for uptake of isoleucine by the carrier BrnQ, which transports all branched-chained amino acids. Indeed, valine inhibition could also be relieved by supplementing VAL1 with the dipeptide isoleucyl-isoleucine, which is taken up by a dipeptide transport system rather than by BrnQ. Interestingly, addition of external valine stimulated valine production by VAL1. This effect is most probably due to a reduced carbon usage for biomass production and to the increased expression of *ilvBN*, indicating that AHAS activity may still be a limiting factor for valine production in the VAL1 strain.

Corynebacterium glutamicum was isolated as an L-glutamate-excreting bacterium (1) and is used today for the large-scale biotechnological production of two amino acids, i.e., L-glutamate (800,000 tons per year), a flavor enhancer, and L-lysine (400,000 tons per year), which is mainly used as a feed additive (17). In contrast to glutamate, lysine can be produced only by an appropriate mutant strain of *C. glutamicum*, e.g., MH20-22B (50), which accumulates 230 mM L-lysine in the culture medium. The use of *C. glutamicum* for lysine production is particularly advantageous, since it is a "GRAS" (generally regarded as safe) organism, and therefore, downstream processing of lysine does not require separation from the biomass.

For a number of reasons, *C. glutamicum* is very well suited for the production of additional amino acids. (i) The regulation of biosynthetic pathways is often less complex in *C. glutamicum* than in many other bacteria. For example, *C. glutamicum* possesses only one acetohydroxyacid synthase (AHAS) (28), while *Escherichia coli* contains three differently regulated isoenzymes (3, 56). (ii) Central carbon metabolism, anaplerotic reactions, and several amino acid biosynthesis pathways, as well as many transport processes, have been studied in great detail in *C. glutamicum* (45). (iii) The genome sequence is known, and effective tools for genetic manipulation are available.

By metabolic engineering, *C. glutamicum* strains that produce L-valine have been created (41). This branched-chain amino acid is essential for vertebrates, and its production is of commercial interest because of its use as a feed additive, for infusion solutions, and as a precursor for the chemical synthesis of herbicides (17, 34). Currently, ~500 tons of L-valine is produced per year by fermentation or extraction from acidic hydrolysates of proteins (17). As shown in Fig. 1, valine is synthesized from two molecules of pyruvate in a pathway involving four reactions which are catalyzed by AHAS (the *ilvBN* gene product), isomeroreductase (the *ilvC* gene product), dihydroxyacid dehydratase (the *ilvD* gene product), and transaminase B (the *ilvE* gene product). As in other organisms, the same enzymes also catalyze the synthesis of L-isoleucine from pyruvate and 2-ketobutyrate. The latter is formed from L-threonine by threonine dehydratase (the *ilvA* gene product). AHAS is the key enzyme of branched-chain amino acid synthesis. This enzyme is feedback inhibited by valine, leucine, and isoleucine, but even in the presence of all three amino acids, the activity is inhibited maximally to ~50% (16, 19). In *E. coli*, the effect of valine on its three AHAS isoenzymes has been described (54). Valine causes feedback inhibition of AHAS I, encoded by *ilvBN*, and AHAS III, encoded by *ilvIH* (13), and their small regulatory subunits, IlvN and IlvH, were shown to be necessary for valine sensitivity (6, 7, 20). In contrast, AHAS II, encoded by *ilvGM*, is resistant to valine (4, 13, 24, 54). In the presence of valine, the lack of *ilvGM* expression in *E. coli* K-12 causes a growth defect due to 2-ketobutyrate toxicity and leucine and isoleucine limitation (13, 24, 31, 54, 59). Valine-resistant mutants of *E. coli* K-12 showed restored *ilvGM* expression (31). In *E. coli*, valine regulates the expression of *ilvBN*, as well as *ilvGMEDA*, by an attenuation mechanism (14, 21, 25, 32, 54), whereas *ilvIH* expression is controlled by the leucine-responsive protein Lrp (43, 54, 55). In *C. glutamicum,* the synthesis of AHAS is regulated as well. Expression of *ilvBN* is altered about twofold in response to the

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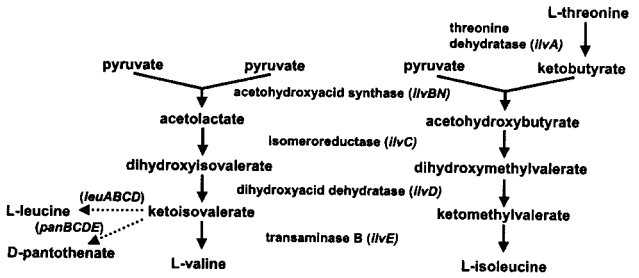

FIG. 1. Biosynthetic pathways of L-valine, L-isoleucine, L-leucine, and D-pantothenate in *C. glutamicum*. The solid arrows indicate reactions catalyzed by the indicated enzymes, and the dotted arrows indicate multistep pathways. Relevant gene names are given in parentheses.

branched-chain amino acid concentration via an attenuation mechanism (38). In contrast to a direct amino acid deficiency, a valine, leucine, and pantothenate shortage due to ketobutyrate addition led to ~10-fold-increased AHAS activity, probably caused by an additional as-yet-unknown control (16, 38).

The *C. glutamicum* valine production strain 13032 Δ*ilvA* Δ*panBC*(pJC1*ilvBNCD*) (called VAL1 hereafter), derived from the wild type by metabolic engineering, excretes up to 90 mM L-valine (41). This was made possible by a number of defined genetic alterations. (i) The valine biosynthesis genes *ilvB*, *ilvN*, *ilvC*, and *ilvD* were overexpressed from a plasmid. (ii) The chromosomal *ilvA* gene was deleted in order to avoid the formation of isoleucine as a major by-product. In addition, cultivation of the strain under isoleucine limitation could increase *ilvBN* expression by the attenuation mechanism (38). (iii) The *panBC* genes encoding two steps in the D-pantothenate synthesis pathway were deleted. Pantothenate is a constituent of coenzyme A (CoA) (27) and thus is required for the oxidative decarboxylation of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex. Cultivation under pantothenate limitation will limit this reaction and thereby increase the availability of the valine precursor pyruvate.

Within a project aiming to improve valine production by further metabolic engineering of the VAL1 strain, the question arose as to what effects the presence of valine itself would have on growth and valine production. This question was triggered by the observation that addition of 30 mM L-valine or L-isoleucine to the culture medium significantly reduced the growth rate of the lysine producer *C. glutamicum* MH20-22B (18). It was speculated that the hydrophobicity of these branched-chain amino acids was somehow responsible for the deleterious effect, because the hydrophilic amino acids L-alanine and L-lysine caused no inhibition. In addition, mischarging of tRNA has been discussed as an alternative explanation (18). The inhibitory effect could also be responsible for the generally lower levels of production by *C. glutamicum* of nonpolar amino acids like valine compared to polar amino acids like lysine (34). Since the mechanism of growth inhibition of *C. glutamicum* MH20-22B by valine and isoleucine remained unexplained, we decided to study the influence of valine on wild-type *C. glutamicum* ATCC 13032 and on the valine production strain VAL1 by combining growth studies with transcriptome and proteome analyses.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. For the first preculture, 5 ml of CGIII medium (37) was inoculated from a fresh Luria-Bertani (47) agar plate and incubated overnight at 30°C and 170 rpm. The cells were harvested and used to inoculate 60 ml of CGXII medium (28) containing 0.03 g of protocatechuic acid/liter and 0.2 M glucose. This second preculture was grown overnight at 30°C and 120 rpm in a 500-ml baffled shake flask. The main culture was inoculated with cells from the second preculture and grown under the same conditions. Strains with the *panBC* deletion were supplemented with 3 μM sodium D-pantothenate, and strains with the *ilvA* deletion were supplemented with 3.4 mM L-isoleucine. Strains carrying pJC1-derived plasmids were cultivated in the presence of 50 μg of kanamycin/ml. When desired, branched-chain amino acids were added to CGXII medium before the pH was titrated to 7.0, and then the medium was autoclaved.

For transcriptome analysis, proteome analysis, and determination of AHAS activity, the CGXII preculture contained the same supplements as the main culture to ensure full adaptation. Main cultures were harvested in the exponential growth phase. Altogether, the bacteria were cultivated for at least 10 generations under equivalent conditions.

Generation of *C. glutamicum* DNA microarrays. DNA microarrays based on the PCR products of *C. glutamicum* genes were used for global gene expression analysis. The genes were amplified in 96-well plates with genomic DNA of *C. glutamicum* ATCC 13032 as a template and gene-specific primers purchased from degussa (Frankfurt, Germany). The sizes and quantities of the PCR products were checked by gel electrophoresis. Then, the PCR products were precipitated with isopropanol, resuspended in 3× SSC (20× SSC is 3 M NaCl and 0.3 M sodium citrate, pH 7.0), and transferred to 384-well plates as described previously (57, 61; http://cmgm.stanford.edu/pbrown/mguide/index.html). The PCR products were printed onto poly-L-lysine-coated glass slides using an arraying robot (http://cmgm.stanford.edu/pbrown/mguide/index.html). The DNA microarrays were rehydrated in a humidity chamber containing 1× SSC, UV cross-linked (650 μJ), and blocked in 230 ml of methylpyrrolidinone containing 15 ml of 1 M boric acid (titrated to pH 8.0 with sodium hydroxide) and 4.4 g of succinic anhydride (57, 61; http://cmgm.stanford.edu/pbrown/mguide/index.html). Depending on the series, the DNA microarrays contained PCR products for up to 3,530 of 3,567 predicted *C. glutamicum* open reading frames. Most

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference
C. glutamicum strains		
ATCC 13032	Wild type	1
13032 Δ*ilvA*	*ilvA* deletion mutant	44
13032 Δ*panBC*	*panBC* deletion mutant	41
13032 Δ*ilvA* Δ*panBC*	*ilvA* and *panBC* deletion mutant	41
13032 Δ*ilvA* Δ*panBC*(pJC1*ilvBNCD*) (VAL1)	*ilvA* and *panBC* deletion mutant with plasmid pJC1*ilvBNCD*	41
Plasmids		
pJC1	*E. coli-C. glutamicum* shuttle vector; Kan^r $oriV_{E.c.}$ $oriV_{C.g.}$	11
pJC1*ilvBNCD*	pJC1 with restriction fragments encompassing *ilvBNC* and *ilvD* with their native promoters	44

[a] Subscripts: E.c., *E. coli*; C.g.; *C. glutamicum*.

genes were represented by a single spot, but 506 genes were represented by two spots. Up to 100 spots of *C. glutamicum* genomic DNA were present as a quality control and for normalization. As negative controls, λ DNA, *E. coli* genomic DNA, and the *E. coli aceK* gene were included.

Total RNA preparation and cDNA synthesis. Aliquots (~25 ml) of exponentially growing *C. glutamicum* cultures (optical density at 600 nm [OD_{600}], between 3 and 5) were added to 25 g of crushed ice precooled to −20°C and immediately harvested by centrifugation (5 min; 3,500 × *g*; 4°C) as previously described for *E. coli* (57). The cells were resuspended in 350 μl of RNeasy RLT buffer (Qiagen, Hilden, Germany) and mechanically disrupted by 30 s of bead beating with 0.5 g of 0.1-mm-diameter zirconium-silica beads (Roth, Karlsruhe, Germany) using a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (2 min; 14,500 × *g*), the supernatant was processed using the RNeasy system (Qiagen) with DNase on-column treatment according to the manufacturer's instructions for RNA extraction. The quantity and quality of the extracted total RNA were determined by UV spectroscopy (at 260, 280, and 230 nm) and denaturing formaldehyde agarose gel electrophoresis (47).

Identical amounts (20 to 25 μg) of total RNA were used for random hexamer-primed synthesis of fluorescently labeled cDNA by reverse transcription with Superscript II (GibcoBRL-Life Technologies, Gaithersburg, Md.) using the fluorescent nucleotide analogue FluoroLink Cy3-dUTP (green) or Cy5-dUTP (red) (Amersham Pharmacia, Little Chalfont, United Kingdom) as described before (30, 57). The labeled cDNA probes were purified and concentrated using Microcon YM-30 filter units (Millipore, Bedford, Mass.) (30; http://cmgm.stanford.edu/pbrown/mguide/index.html).

DNA microarray hybridization and washing. Combined Cy5- and Cy3-labeled cDNA probes containing 1.2 μg of poly(A) (Sigma, Taufkirchen, Germany)/μl as a competitor, 30 mM HEPES, and 0.3% sodium dodecyl sulfate (SDS) in 3× SSC, were hybridized to the arrays for 5 to 16 h at 65°C. After hybridization, the arrays were washed in 1× SSC containing 0.03% SDS and finally in 0.05× SSC. The DNA microarrays were dried by brief centrifugation (5 min; 45 × *g*). For detailed protocols, see reference 30 and http://cmgm.stanford.edu/pbrown/mguide/index.html.

Data normalization and gene expression analysis. Immediately after stringent washing of the arrays, fluorescence intensities at 635 and 532 nm were acquired using a GenePix 4000 laser scanner (Axon Inc., Union City, Calif.) and processed as TIFF images. Raw fluorescence data were analyzed quantitatively using GenePix version 3.0 software (Axon Inc.). Data were normalized to the average ratio of *C. glutamicum* genomic DNA. The normalized ratio of the median (GenePix) was taken to reflect the relative RNA abundance for spots whose green or red fluorescence signal was at least threefold above the fluorescence background. When both fluorescence signals were less than threefold above background, the signals were considered too weak to be analyzed quantitatively. For statistical analysis (2, 42), *P* values from independent replicate experiments were calculated based on Student's *t* test using log-transformed gene ratios and genomic DNA ratios which were normalized to zero (33). Only genes showing significantly changed RNA levels (*P* values of <0.05) were considered for further analysis. Analysis of gene expression data was performed by selecting genes showing at least twofold-increased or -decreased average RNA levels. All genes belonging to a putative operon were listed if at least one gene of the operon showed significant expression changes.

Proteome analysis. Proteome analysis of the soluble protein fraction was performed by two-dimensional (2-D) gel electrophoresis essentially as described previously (48). Cells were cultivated as described above and harvested at an OD_{600} of 3 to 5 by centrifugation (5 min; 3,500 × *g*; 4°C). After being washed in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), the cells were resuspended in either 1 or 10 ml of TE buffer containing Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany), 100 μg of RNase A, and 25 μg of DNase I, depending on whether disruption was performed by bead beating (1 ml of cell suspension was added to 1 g of zirconium-silica beads and subjected to four cycles [30 s] of bead beating) or by passage through a French pressure cell (10 ml of cell suspension was passed five times through a French pressure cell [SLM AMINCO Spectronic Instruments, Rochester, N.Y.] at 172 MPa). Intact cells and cell debris were removed by centrifugation (20 min; 27,000 × *g*; 4°C), and the soluble protein fraction was separated from the membrane protein fraction by ultracentrifugation (1 h; 150,000 × *g*; 4°C). Protein concentrations were determined using the BC assay kit (Pierce Chemical Company, Rockford, Ill.), and 300 μg of protein was precipitated with acetone. After solubilization, isoelectric focusing was performed using an IPGphor electrophoresis unit (Amersham Pharmacia) and 18-cm-long Immobiline DryStrips (Amersham Pharmacia) with a pH range of 4 to 7 or 4.5 to 5.5. After being focused, the Immobiline DryStrips were equilibrated and 2-D separation was performed using a Multiphor II electrophoresis unit and Excel SDS gradient gels (12 to 14%; Amersham Pharmacia). The gels were fixed, Coomassie stained, destained, and dried as described previously (48).

The gels were scanned with a JX-330 scanner (Sharp, Tokyo, Japan), and the images were analyzed using ProteomeWeaver 2-D gel analysis software version 1.1.3 (Definiens Imaging GmbH, Munich, Germany). Spots of interest were excised and digested with trypsin, and the peptide masses were determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry as described previously (48). Peptide mass lists were used to search a tryptic digest database of 3,746 *C. glutamicum* proteins provided by degussa.

Determination of amino acid concentrations by HPLC. L-Valine concentrations were determined by automatic precolumn derivatization with *ortho*-phthaldialdehyde (35) and reversed-phase high-performance liquid chromatography (HPLC) (HP1100 series; Hewlett-Packard, Waldbronn, Germany) with fluorimetric detection (excitation at 230 nm; emission at 450 nm). Hypersil ODS 5-μm columns were used (precolumn, 40 by 4 mm; column, 120 by 4 mm; Chromatographie Service GmbH, Langerwehe, Germany). The buffer gradient consisted of 0.1 M sodium acetate, pH 7.2 (with 0.03% sodium azide), as the polar phase and methanol as the nonpolar phase. Quantification was performed with L-asparagine as an internal standard and by comparison of the sample peaks with an external standard.

Determination of AHAS activity. Cells (50 ml) were harvested at an OD_{600} of 3 to 5 by centrifugation, washed with 50 ml of 100 mM potassium phosphate buffer (pH 7.5), and suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 26% (vol/vol) glycerol. The cells were disrupted by sonification with a microtip-equipped Branson Sonifier W-250 (Branson-Emerson, Danbury, Conn.) for 10 min on ice (intensity, 20%; pulse length, 20%). Cell debris and intact cells were separated from the cell extract by centrifugation (1 h; 14,500 × *g*; 4°C). The enzyme assay was performed as described previously (16) for 15 min at 30°C. Acetolactate formed from pyruvate was decarboxylated to acetoin and detected by the colorimetric method of Westerfeld (58), which is based on a reaction between acetoin and the guanidino group of creatine in the presence of α-naphthol and alkali. The absorbance at 530 nm was compared with an acetoin standard. Protein concentrations were determined using the biuret method (22). Specific AHAS activities are given as mU per milligram (nanomoles of product formed per minute and milligram of protein).

Determination of ornithine carbamoyltransferase activity. Cultivation and crude extract preparation were performed as described for the AHAS activity measurement. The reaction mixture contained cell extract, 15 mM L-ornithine, and 10 mM lithium carbamoylphosphate in potassium phosphate buffer (100 mM; pH 7.5). The enzyme assay was performed for 15 min at 30°C and stopped by addition of HCl to a concentration of 0.2 M. The L-citrulline formed during the reaction was detected by reversed-phase HPLC with an L-citrulline standard as described for the determination of amino acid concentrations.

RESULTS

Growth of wild-type *C. glutamicum* and the VAL1 strain in the presence of valine. Since the presence of 30 mM valine inhibited the growth of the lysine producer *C. glutamicum* MH20-22B (18), we were interested in examining the effects on the growth of the *C. glutamicum* wild-type strain ATCC 13032 and the valine production strain VAL1. Cells were cultivated in CGXII minimal medium with 0.2 M glucose and different valine concentrations, and the growth rates were determined. The medium for strain VAL1 was supplemented with 3 μM sodium D-pantothenate and 3.4 mM L-isoleucine. As shown in Fig. 2, L-valine at concentrations up to 200 mM had no effect on the growth rate of the wild type. In contrast, the VAL1 strain derived from this wild type showed decreasing growth rates at increasing L-valine concentrations. Half-maximal inhibition was found at a concentration of 250 mM L-valine (data not shown). These results indicate that the effect of external L-valine on the growth of *C. glutamicum* is strain specific. In the context of these studies, it was also shown that wild type *C. glutamicum* is unable to use L-valine as a sole carbon or a sole nitrogen source (data not shown). This clearly indicates that *C. glutamicum* lacks one or several enzyme activities required for L-valine degradation.

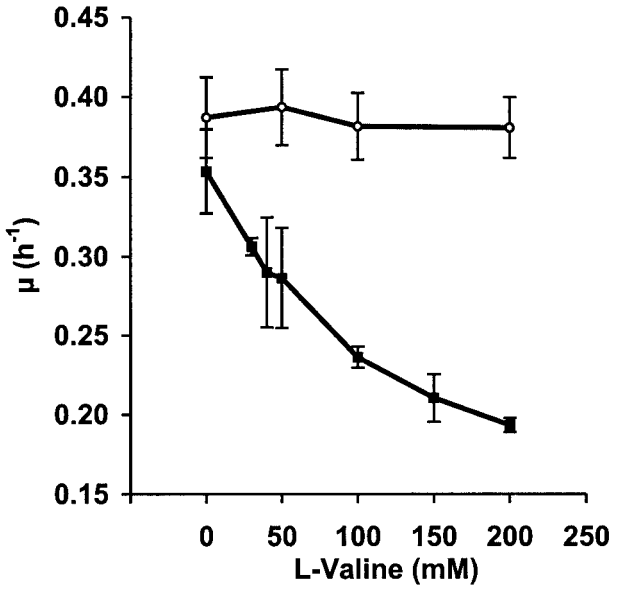

FIG. 2. Effect of L-valine in the culture medium on growth rates (μ) of wild-type *C. glutamicum*, ATCC 13032 (○), and the valine production strain, VAL1 (■). The average growth rates and standard deviations of at least two independent cultivations are given.

Influence of L-valine on global gene expression in wild type *C. glutamicum* and the VAL1 strain. In order to find the cause of the different effects of valine on growth of the wild type and the VAL1 strain, its influence on global gene expression was studied by transcriptome analysis. In the case of the wild type, parallel cultures were grown in CGXII glucose minimal medium either with or without 50, 100, or 300 mM L-valine. The cells were kept in the exponential growth phase by repeated dilution and harvested for RNA extraction after at least 10 generations. Relative RNA levels were determined by hybridization to DNA microarrays representing up to 3,530 different open reading frames (ORFs) of *C. glutamicum* ATCC 13032 (see Materials and Methods). Table 2 lists statistically significant gene expression changes (P value, <0.05) of 23 ORFs with at least twofold-decreased RNA levels and 16 genes with at least twofold-increased RNA levels (in all cases, the RNA levels represent the ratio with valine/without valine). Additionally, genes that showed changes of less than twofold but that formed an operon with one of the 39 genes that had at least twofold-altered mRNA levels were included.

In the case of the VAL1 strain, parallel cultures were grown in CGXII glucose minimal medium either without L-valine (growth rate, 0.35 h^{-1}) or with 40 mM L-valine (growth rate, 0.29 h^{-1}) and used for RNA isolation and global gene expression analysis as described for the wild type. The use of 40 mM valine was a compromise, because at that concentration the effect of valine on the growth of VAL1 was already clearly detectable but presumably not so strong that all gene expression changes were due to the reduced growth rate. In this context, it has to be mentioned that the VAL1 strain is capable of producing ~90 mM L-valine by itself. However, due to the dilution steps, there was <5 mM valine produced by VAL1 in the medium at the time of RNA isolation. As summarized in Table 3, 11 and 10 ORFs showed significantly changed RNA levels ($P < 0.05$) that were at least twofold decreased or increased, respectively, after growth in the presence of valine. Table 3 also includes those genes whose RNA levels changed less than twofold but which presumably are cotranscribed with one of the 21 genes showing at least twofold changes.

A comparison of the gene expression changes upon valine addition in the wild type and the VAL1 strain identified common as well as strain-specific expression changes. Three genes showed increased expression in the presence of valine, i.e., *leuD*, *ileS*, and its adjacent ORF (Tables 2 and 3). The *leuD* gene encodes the small subunit of isopropylmalate dehydratase, an enzyme involved in the biosynthesis of L-leucine from 2-oxoisovalerate (39). The levels of RNA of *leuC* (ORF 2737; NCgl1262), encoding the large subunit of this enzyme, were also increased in both strains but showed P values of >0.05. The *ileS* gene encodes isoleucyl tRNA synthetase, i.e., the enzyme that charges isoleucine tRNA.

Expression of several genes or operons, e.g., that of the *prpD2B2C2* operon, which is essential for growth on propionate (10) and encodes enzymes involved in the methylcitrate cycle, i.e., 2-methylcitrate synthase (*prpC*), 2-methylcitrate dehydratase (*prpD*), and 2-methylisocitrate lyase (*prpB*), was increased in the presence of valine only in the wild type (Tables 2 and 3). Expression of the homologous *prpD1B1C1* operon, which is not essential for growth on propionate (10), was also increased. Similarly, increased expression was found for the putative *narKGHJI* operon that encodes a nitrate-nitrite transport protein and the four subunits of nitrate reductase (*narG*, with a ratio of 2.0 and a P value of 0.07) and for nearly all genes involved in arginine biosynthesis (*argC*, *argB*, *argD*, *argF*, *argG*, *argH*, and *argR*) (8, 9, 46). The increased RNA level of the *argF* gene during growth in the presence of valine correlated with increased activities of ornithine carbamoyltransferase (the *argF* gene product). Wild-type cells grown in the presence or absence of valine had ornithine carbamoyltransferase activities of 210 and 95 mU/mg of protein, respectively. In the VAL1 strain, the ornithine carbamoyltransferase activities were 145 and 120 mU/mg of protein in the presence or absence of valine, respectively.

Expression of the putative *oppABCD* operon encoding an oligopeptide ABC transport system was significantly increased only in strain VAL1 (Tables 2 and 3). Finally, it was obvious that the levels of mRNA of *ilvBN*, which encodes AHAS, were increased in the presence of valine only in the VAL1 strain but decreased or almost unaltered in the wild type (Tables 2 and 3).

Influence of L-valine on the protein profile of wild-type *C. glutamicum* and the VAL1 strain. The influence of valine in the growth medium on protein abundances in the wild type and the VAL1 strain was examined using 2-D gels. The strains were cultivated in CGXII glucose minimal medium with or without 300 mM L-valine in the case of the wild type and with or without 40 mM L-valine in the case of the VAL1 strain. After growth for at least 10 generations, the cells were harvested in the exponential phase, and crude extracts were prepared. For each of the four different conditions, three independent cultivations were performed and used for protein profiling. For each of the 12 samples, 2-D gel electrophoresis was carried out both in a pH range of 4 to 7 and in a range of 4.5 to 5.5. The Coomassie-stained gels were analyzed quantitatively using

TABLE 2. ORFs showing altered relative mRNA levels in response to L-valine in the wild-type *C. glutamicum* ATCC 13032

ORF	NCBI[a]	Annotation[b]	Gene[c]	Relative mRNA level +/− valine[d]					
				50	100a	100b	300a	300b	Average
237	NCgl2749	Similarity to probable membrane protein SCQ11.10c (*Streptomyces coelicolor*)		0.5			0.6	0.3	0.5
585	NCgl0190	Weak similarity to putative two-component system histidine kinase SCE25.03c (*S. coelicolor*)					0.5	0.5	0.5
860	NCgl0385	Hypothetical protein		0.5			0.7	0.5	0.5
1495	NCgl0891	Similarity to hypothetical protein Rv1128c (*Mycobacterium tuberculosis*)					0.6	0.5	0.5
1606	NCgl0986	Strong similarity to sodium-dependent proline transporter SLC6A12 (*Rattus norvegicus*)			0.6	0.5	0.6		0.5
1623	NCgl1004	Strong similarity to morphine 6-dehydrogenase *mor A* (*Pseudomonas putida*)					0.5	0.5	0.5
1906	NCgl2919	Similarity to hypothetical protein SCC54.19 (*S. coelicolor*)					0.4	0.5	0.4
1953	NCgl1770	Weak similarity to UMP synthase and OMP decarboxylase *ump* (*Naegleria gruberi*)					0.4	0.5	0.5
2136	NCgl1952	Weak similarity to zoocin A endopeptidase (*Streptococcus zooepidemicus*)					0.5	0.6	0.5
2552	NCgl1405	Similarity to cell division ATP-binding protein *ftsE* (*E. coli* [putative frameshift])		0.5			0.5	0.7	0.5
2792	NCgl1223	Acetolactate synthase small-chain *ilvN* (*C. glutamicum*)	*ilvN*	0.4	0.4	0.3	0.8	0.7	0.5
2827	(2778481–2778807)	Questionable ORF		0.4		0.4	0.6	0.8	0.5
3312	NCgl0018	Similarity to 27-kDa outer membrane protein (*Coxiella burnetii*)					0.5	0.6	0.5
3384	NCgl1669	Similarity to putative primase (*Lactococcus* bacteriophage phi31)					0.5	0.5	0.5
3396[e]	NCgl1678	Weak similarity to hypothetical protein *yciE* (serovar Typhimurium)					0.4	0.4	0.4
3397[e]	NCgl1679	Weak similarity to hypothetical protein (*C. glutamicum*)					0.6	0.7	0.6
3398[e]	NCgl1680	Weak similarity to class A beta-lactamase precursor *tla-I* (*E. coli* plasmid RZA9)					0.3	0.4	0.3
3431	NCgl2953	Strong similarity to D-xylose proton symporter *xylT* (*Lactobacillus brevis*)					0.4	0.3	0.3
3443	NCgl2960	Weak similarity to pyrroline-5-carboxylate reductase *proC* (*E. coli*)					0.5	0.5	0.5
3481	NCgl2433	Strong similarity to ATP-dependent DNA helicase *dinG* (*E. coli*)			0.6	0.5	0.5		
3501[e]	NCgl2417	Hypothetical protein					0.4	0.4	0.4
3503[e]	NCgl2415	Strong similarity to RNase PH *rph* (*E. coli*)					0.8	0.8	0.8
3514	NCgl2407	Hypothetical protein					0.5	0.4	0.4
3549	NCgl0482	Strong similarity to ferric enterobactin ABC-type transport system ATP-binding protein *fepC* (*E coli*)					0.4	0.3	0.3
66065	NCgl2320	Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases (*C. glutamicum*)					0.4	0.4	0.4
675	NCgl0254	Branched-chain amino acid exporter *brnF* (*C. glutamicum*)	*brnF*	1.6		2.6	1.7	2.4	2.1
676	NCgl0254	Branched-chain amino acid exporter *brnF* (*C. glutamicum*)	*brnF*	1.5		1.5	0.9	1.5	1.4
830	NCgl0359	Succinate dehydrogenase C or D *sdhCD* (*C. glutamicum*)	*sdhCD*	1.7	1.1	1.0	2.3	4.2	2.1
1160[e]	NCgl0627; NCgl0628	Methylaconitase *prpD2* (*C. glutamicum*)	*prpD2*	6.4	9.5	7.4	3.5	4.6	6.3
1161[e]	NCgl0629	Methylisocitrate lyase *prpB2* (*C. glutamicum*)	*prpB2*	4.1	8.8	5.7	3.9	3.7	5.2
1162[e]	NCgl0630	Methyl citrate synthase *prpC2* (*C. glutamicum*)	*prpC2*	4.0	6.7	7.5	4.6	4.2	5.4
1204[e]	NCgl0664	Methylaconitase *prpD1* (*C. glutamicum*)	*prpD1*	1.4	1.9	1.4	1.1	1.1	1.4
1205[e]	NCgl0665	Methylisocitrate lyase *prpB1* (*C. glutamicum*)	*prpB1*	1.1	1.7	1.5	1.4		1.4
1206[e]	NCgl0666	Methyl citrate synthase *prpC1* (*C. glutamicum*)	*prpC1*	4.3	4.0	4.1	2.2	4.0	3.7
1451	NCgl0856	Glycine betaine transporter *betP* (*C. glutamicum* [putative frameshift])	*betP*	1.6	1.6	1.5	2.0	3.9	2.1
1783[e]	NCgl1139	Strong similarity to nitrate reductase gamma chain *narI* (*Bacillus subtilis*)					2.2	2.2	2.2
1784[e]	NCgl1140	Strong similarity to nitrate reductase delta chain *narJ* (*B. subtilis*)				1.0	1.0		1.0
1785[e]	NCgl1141	Strong similarity to nitrate reductase beta chain *narH* (*B. subtilis*)		1.6	1.1	1.1	3.0	4.6	2.3
1787[e]	NCgl1143	Strong similarity to nitrite extrusion protein *narK2* (*Thermus thermophilus* [putative frameshift])		1.2	1.2	1.1	1.6	2.5	1.5
1788[e]	NCgl1143	Strong similarity to nitrite extrusion protein *narK* (*E. coli* [putative frameshift])		1.5	1.2	1.2	2.4	2.0	1.7
2280[a]	NCgl2086	Strong similarity to isoleucine-tRNA ligase *ileS*		1.9	1.4	1.4	3.1	2.6	2.1
2281[a]	(2274263–2274442)	Hypothetical protein		1.4	1.5	1.2	1.6	2.7	1.7
2622[e]	NCgl1347	Argininosuccinate lyase *argH* (*C. glutamicum*)	*argH*	1.2	1.5	1.1	1.1	1.8	1.3
2623[e]	NCgl1346	Argininosuccinate synthase *argG* (*C. glutamicum*)	*argG*	1.4	1.5	1.3	2.3	1.6	1.6
2624[e]	NCgl1345	Arginine repressor *argR* (*C. glutamicum*)	*argR*	1.4	1.8	1.7	3.8	1.4	2.0
2625[e]	NCgl1344	Ornithine carbamoyltransferase *argF* (*C. glutamicum*)	*argF*	2.1	2.4	2.4	3.5	2.0	2.5
2626[e]	NCgl1343	Acelylornithine aminotransferase *argD* (*C. glutamicum*)	*argD*	1.4	1.8	1.5	2.1	2.1	1.8
2627[e]	NCgl1342	Acetylglutamate kinase *argB* (*C. glutamicum*)	*argB*	1.7	1.9	1.9	2.4	1.4	1.9
2628[e]	NCgl1341	Glutamate *N*-acetyltransferase *argJ* (*C. glutamicum*)	*argJ*	1.3		1.4	1.3	1.9	1.5
2629[e]	NCgl1340	*N*-Acetyl-gamma-glutamyl-phosphate reductase *argC* (*C. glutamicum*)	*argC*	2.0	2.2	1.6	2.7	2.5	2.2
2736	NCgl1263	3-Isopropylmalate dehydratase small-chain *leuD* (*C. glutamicum*)	*leuD*	2.2	1.6	1.6	5.1	3.9	2.9
2850	NCgl2504	Weak similarity to hypothetical protein (*C. glutamicum*)					1.8	2.1	2.0
66055	NCgl2319	Protocatechuate 3,4-dioxygenase beta subunit (*C. glutamicum*)					2.1	2.5	2.3

[a] Numbers for the corresponding ORFs of the the National Center for Biotechnology information (NCBI) revised *C. glutamicum* genome published by (http://www.ncbi.nlm.nih.gov/egi-bin/Entrez/framik?db=genome&gi=232). If no corresponding ORF exists, the position is given in parentheses.

[b] Most similar gene in public databases.

[c] Only characterized *C. glutamicum* genes are given.

[d] Relative mRNA levels with and without valine addition; and the average mRNA level from all experiments. Only ORFs with P values of < 0.05 in Student's t test are listed; in addition to ORFs showing twofold changes of RNA levels, all ORFs of putative operons are shown. Replicate experiments are indicated by letters behind the valine millimolar concentration.

[e] ORF belonging to putative operon.

TABLE 3. ORFs showing altered relative mRNA levels in response to L-valine in the valine production strain VAL1[a]

ORF	NCBI	Annotation	Gene	Relative mRNA level +/− valine					
				40a	40b	40c	40d	40e	Average
638	NCgl0232	Similarity to ABC transporter *strW* (*Streptomyces glaucescens*)		0.4				0.5	0.4
732[b]	NCgl0296	Strong similarity to hypothetical protein Rv3658c (*Mycobacterium tuberculosis*)		0.5	0.6			0.5	0.5
733[b]	NCgl0298	Strong similarity to hypothetical protein Rv3657c (*M. tuberculosis*)		0.6	0.8			0.6	0.7
910	NCgl0415	Hypothetical protein		0.4	0.5				0.4
1035	NCgl0528	Weak similarity to pyruvate, water dikinase *ppsA* (*E. coli*)		0.4	0.4				0.4
1155	NCgl0626	questionable ORF		0.5				0.5	0.5
1156	NCgl0626	Carbon starvation protein A *cstA* (*C. glutamicum*)		0.8	0.5			0.7	0.6
1935[b]	NCgl2942	Strong similarity to xenobiotic reductase A (*Pseudomonas putida* [putative frameshift])		0.6	0.7	0.7		0.5	0.6
1936[b]	NCgl2942	Strong similarity to xenobiotic reductase B (*P. putida* [putative frameshift])		0.3	0.9	0.4		0.5	0.5
2400	NCgl1464	Strong similarity to transposase (*Pseudomonas syringae*)		0.5	0.7	0.4		0.5	0.5
2789	(1341778–1341957)	Hypothetical protein		0.6	0.5				0.5
3032	(2631147–2631479)	Hypothetical protein		0.5	0.5				0.5
3211	(2371029–2371466)	Questionable ORF		0.5	0.9	0.3		0.3	0.5
3543	NCgl0381	Similarity to hypothetical protein SCC75A. 17c (*Streptomyces coelicolor*)		0.5	0.4				0.5
1362	NCgl0785	Similarity to hypothetical protein Rv0867c (*M. tuberculosis*)			1.8	2.1	5.5	1.6	2.7
1497	NCgl0892	Strong similarity to peptide chain release factor *prfC* (*E. coli*)		1.4	1.3	1.7	3.3	2.2	2.0
2092[b]	NCgl1915	Similarity to oligopeptide-binding protein *oppA* (*E. coli* [putative frameshift])		1.2	1.5	2.2	1.9	2.0	1.8
2093[b]	NCgl1915	Similarity to oligopeptide-binding protein *oppA* (*E. coli* [putative frameshift])		0.9	1.9	2.5	4.1	1.9	2.3
2094[b]	NCgl1916	Strong similarity to oligopeptide transport system permease protein *oppB* (*Bacillus subtilis*)		1.1	1.5	2.0	2.2	1.7	1.7
2095[b]	NCgl1917	Strong similarity to oligopeptide ABC transporter (permease) *oppC* (*B. subtilis*)		0.9	1.9	2.3	2.7	1.6	1.9
2096[b]	NCgl1918	Similarity to oligopeptide transport ATP-binding protein *oppD* (*B. subtilis*)		1.1	1.4	1.8	1.7	1.3	1.5
2280[b]	NCgl2086	Strong similarity to isoleucine-tRNA ligase *ileS*		2.5	1.9	2.8	1.9	2.4	2.3
2281[b]	(2274263–2274442)	Hypothetical protein		1.1	2.0		4.1	1.8	2.3
2425	NCgl1482	Aconitase *acn* (*C. glutamicum*)	*acn*	4.0	2.3	1.8	1.5	1.9	2.3
2453[b]	NCgl1502	Strong similarity to hypothetical protein Rv1462 (*M. tuberculosis* [putative frameshift])		2.0	1.6	2.2	1.4	1.4	1.7
2455[b]	NCgl1503	Strong similarity to conserved hypothetical protein SCC22.07c (*S. coelicolor*)		1.3	1.7		3.5	1.6	2.0
2456[b]	NCgl1504	Strong similarity to putative transcriptional regulator Rv1460 (*M. tuberculosis*)		2.0	1.3	1.2	1.0	1.2	1.4
2736	NCgl1263	3-Isopropylmalate dehydratase small-chain *leuD* (*C. glutamicum*)	*leuD*	2.9	2.3	1.4		1.4	2.0
2790[b]	NCgl1224	Ketol-acid reductoisomerase *ilvC* (*C. glutamicum*)	*ilvC*	1.2	1.2			1.7	1.3
2791[b]	(1340543–1340725)	Hypothetical protein		1.6	1.5	1.4	1.0	1.6	1.4
2792[b]	NCgl1223	Acetolactate synthase small-chain *ilvN* (*C. glutamicum*)	*ilvN*	2.4	2.3	2.9	2.6	2.4	2.5
2793[b]	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	1.8	2.1	2.0		2.5	2.1

[a] Abbreviations, conditions, and statistical analysis are described in footnote to Table 2.
[b] ORF belonging to putative operon.

ProteomeWeaver software. Tables 4 and 5 summarize those proteins that showed at least twofold changes in abundance due to the presence of valine. Because of higher standard deviations observed between protein spot intensities in comparison to the DNA microarray results, the criterion for significant changes was set to a P value of <0.1 in Student's t test (in comparison to a P value of <0.05 for the relative RNA levels).

In the wild type (Table 4), 11 proteins showed decreased abundance in response to the presence of valine. However, none of the corresponding genes displayed altered relative RNA levels (all corresponding spots could be evaluated and showed ratios of ~1). Thus, the decrease in protein concentration should be due to regulation at the level of translation or protein stability rather than to transcriptional regulation. Three proteins showed increased abundance in response to valine, which in all cases correlated with increased RNA levels. The 2-methylcitrate dehydratase PrpD2 had an eightfold-increased level (mRNA level, sixfold), the arginine repressor ArgR had a fivefold-increased level (mRNA level, twofold), and *N*-acetylglutamate semialdehyde dehydrogenase, ArgC, had a fourfold-increased level (mRNA level, twofold).

In the VAL1 strain, two proteins showed reduced abundance in response to valine, one of which was the thiamine diphosphate-dependent pyruvate dehydrogenase (EC 1.2.4.1; component E1), which is encoded by *aceE* and is part of the pyruvate dehydrogenase complex (Table 5). The corresponding genes showed unaltered RNA levels under the same conditions (data not shown). Three proteins displayed increased levels in response to valine, i.e., the translation elongation factor EF-G (twofold increase); PurH (phosphoribosylaminoimidazolecarboxamide formyltransferase-IMP cyclohydrolase), a bifunctional enzyme involved in purine biosynthesis (fourfold increase); and IlvB, the large subunit of AHAS. Whereas the genes encoding EF-G and PurH showed no changes in the RNA level in response to valine, the *ilvB* mRNA level was increased twofold in the presence of valine (Table 3). Remarkably, eight different spots were identified as IlvB, all having quite similar masses of ~64 to 67 kDa but pIs ranging from ~4.7 to 5.4. This could be explained by successive deg-

TABLE 4. Proteins showing altered abundances on 2-D gels in response to L-valine in the wild-type *C. glutamicum* ATCC 13032

ORF	NCBI[a]	Annotation[b]	Gene[a]	Intensity		
				−Val	+Val	Ratio
16	NCgl2582	Identical to L-2.3-butanediol dehydrogenase (*Brevibacterium saccharolyticum*)		1.9	0.8	0.4
68	NCgl2621	Strong similarity to GroEL2 protein (*Mycobacterium tuberculosis*)		0.5	0.2	0.4
486	NCgl0099	Strong similarity to aryl-alcohol dehydrogenase ($NADP^+$) (*Phanerochaete chrysosporium*)		0.5	0.2	0.4
978	NCgl0469	Strong similarity to 50S ribosomal protein L12 (*Streptomyces coelicolor*)		20.0	7.5	0.4
1167	NCgl0634	Isocitrate dehydrogenase *icd* (*C. glutamicum*)	*icd*	1.0	0.4	0.4
1211	NCgl0670	accBC; acyl coenzyme A carboxylase (*C. glutamicum*)	*accBC*	0.6	0.2	0.3
1315	NCgl0754	Strong similarity to hypothetical protein Rv2606c (*M. tuberculosis*)		1.1	0.4	0.4
2071	NCgl1900	Strong similarity to guanosine pentaphosphate synthetase *gpsI* (*Streptomyces antibioticus*)		0.3	0.1	0.4
2149	NCgl1961	Similarity to thiamin-phosphate pyrophosphorylase *thiC* (*Bacillus subtilis*)		0.5	0.1	0.3
2470	NCgl1512	Transketolase *tkt* (*C. glutamicum*)	*tkt*	0.5	0.2	0.4
3065	NCgl2268	Similarity to phosphoglycerate mutase SCC123.14c (*S. coelicolor*)		0.8	0.3	0.3
1160	NCgl0627; NCgl0628	Methylaconitase *prpD2* (*C. glutamicum*)	*prpD2*	0.4	3.4	7.9
2624	NCgl1345	Arginine repressor *argR* (*C. glutamicum*)	*argR*	0.1	0.4	4.8
2629	NCgl1340	*N*-Acetyl-gamma-glutamyl-phosphate reductase *argC* (*C. glutamicum*)	*argC*	0.3	1.3	4.0

[a] Numbers for the corresponding ORFs of the revised *C. glutamicum* genome published by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/egi-bin/Eutrez/framikidb=genome&gi=232). If no corresponding ORF exists, the position is given in parentheses.
[b] Most similar gene in public databases.
[c] Only characterized *C. glutamicum* genes are given.
[d] Average spot intensities of Coomassie-stained 2-D gels from cells cultivated without valine (−Val) or in the presence of 300 mM L-valine (+Val) and relative protein abundances (with/without valine addition ratio). Only proteins with significant changes in abundance (P values of <0.1 in Student's t test) are listed.

radation of the C terminus (http://www.expasy.org/tools/pi_tool.html). The 30 C-terminal amino acids include 10 aspartate and glutamate residues, and their successive degradation would cause predicted shifts in pI from 4.82 to 5.14 and in mass from 66.8 to 63.8 kDa.

Influence of valine on the AHAS activity of the wild type and the VAL1 strain. The transcriptome studies revealed different effects of valine on the *ilvBN* mRNA levels in the wild type and the VAL1 strain, which at least partly correlated with the protein levels. Since AHAS is the key enzyme of branched-chain amino acid biosynthesis, it was important to complement the mRNA and protein data by activity measurements. As shown in Table 6, the AHAS activity in the wild type (20 mU/mg of protein) was not influenced by the presence of 300 mM valine in the growth medium. In the VAL1 strain, the activity of cells grown in the absence of externally added valine was 200 mU/mg of protein. This 10-fold increase compared to the wild type is most probably due to increased expression of *ilvBN* from the plasmid pJC1*ilvBNCD*. After growth in the presence of 40 mM added valine, the AHAS activity rose to 700 mU/mg. This 3.5-fold increase in AHAS activity correlated well with the 2.5-fold-increased RNA levels of the *ilvBN* genes and with the increased IlvB protein level.

Influence of branched-chain amino acids on growth and AHAS activity of the VAL1 strain. To examine whether the inhibitory effect of valine on growth and the stimulatory effect on AHAS activity of strain VAL1 are specific for valine and to gain more insight into the mechanism causing these effects, the influences of L-leucine and L-isoleucine and of all possible combinations of branched-chain amino acids (40 mM each) on growth and on AHAS activity were tested. The doubling times of the different cultures are shown in Fig. 3A, and the corresponding AHAS activities are shown in Fig. 3B. It is obvious that not only valine but also leucine inhibited the growth of VAL1 and stimulated AHAS activity. From the effects of the different combinations, it is very clear that only the mixture of valine and leucine had qualitatively the same effect as valine or leucine alone, whereas all combinations that included 40 mM

TABLE 5. Proteins showing altered abundances on 2-D gels in response to L-valine in the valine production strain VAL1[a]

ORF	NCBI	Annotation	Gene	Intensity[b]		
				−Val	+Val	Ratio
1504	NCgl0899	Similarity to 2-nitropropane dioxygenase (*Williopsis mrakii*)		0.4	0.2	0.5
3194	NCgl2167	Pyruvate dehydrogenase component E1 *aceE* (*C. glutamicum*)		0.6	0.2	0.3
990	NCgl0478	Strong similarity to translation elongation factor EF-G *fusA* (*E. coli*)		0.3	0.6	2.3
1414	NCgl0827	Strong similarity to PurH bifunctional enzyme (*E. coli*)		0.3	1.5	4.3
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	1.6	4.9	3.1
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	0.8	3.1	3.6
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	0.4	1.5	4.1
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	0.2	0.7	4.2
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	0.5	2.4	5.1
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	0.6	3.4	5.3
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	0.2	1.0	5.9
2793	NCgl1222	Acetolactate synthase large-chain *ilvB* (*C. glutamicum*)	*ilvB*	0.2	1.6	7.2

[a] Abbreviations, conditions, and statistical analysis are described in footnotes to Table 3.
[b] +Val, growth under addition of 40 mM L-valine.

TABLE 6. AHAS activity after cultivation of wild-type *C. glutamicum* ATCC 13032 and of the valine production strain VAL1 in the presence or absence of L-valine

Strain	Valine addition (mM)	AHAS activity (mU/mg of protein)[a]	Relative activity[b]
13032	0	20 ± 15%	1
	300	20 ± 20%	1
VAL1	0	200 ± 15%	10
	40	700 ± 25%	35

[a] AHAS activities are given with percent experimental imprecision.
[b] For relative AHAS activities, the activity in ATCC 13032 without valine addition was normalized to 1.

isoleucine had no effect on either growth or AHAS activity. Thus, the presence of 40 mM isoleucine (instead of 3.4 mM, always present as a supplement) was able to abolish the effects of valine and leucine. In contrast to the VAL1 strain, the wild type transformed with plasmid pJC1 showed doubling times of 1.6 to 2.1 h under all conditions mentioned above, slightly increased AHAS activity upon isoleucine addition (120% of the activity without isoleucine), and decreased activities in response to all other conditions mentioned (40 to 70% of the activity obtained without amino acid addition).

Correlation between valine inhibition and the *ilvA* deletion of strain VAL1. The results described above clearly indicated that the effects of valine and leucine on the growth and AHAS activity of the VAL1 strain were caused by a limitation of isoleucine, which is required by this strain as a supplement due to the deletion of the *ilvA* gene encoding threonine dehydratase. To confirm this conclusion, the effect of 200 mM valine on the growth of a number of isogenic derivatives of the wild type, ATCC 13032, was tested. As shown in Fig. 4, all six strains investigated had the same doubling time (T_d) of ~1.7 h in the absence of valine. In the presence of valine, there was a slight increase in the doubling time due to the presence of plasmid pJC1*ilvBNCD* (T_d, ~2 h), but a large increase was due to the *ilvA* deletion (T_d, ~2.7 h). The combination of plasmid pJC1*ilvBNCD* and the *ilvA* deletion led to an even greater increase of the doubling time (T_d, ~3.5 h), indicating that these effects were synergistic. Strains carrying the plasmid pJC1*ilvBNCD* produced more valine than the respective pJC1 control strains and thus were exposed to higher valine concentrations. The deletion of the *panBC* genes had no effect on growth in the presence of valine. These data clearly support the assumption that isoleucine limitation is responsible for growth inhibition by valine and leucine in the VAL1 strain.

Abolishment of valine-triggered growth inhibition by supplementation of the VAL1 strain with isoleucyl-isoleucine dipeptide. From the sum of the results described above, the conclusion was drawn that the isoleucine limitation in the presence of valine or leucine is caused by the competition of these amino acids with isoleucine uptake by the carrier protein BrnQ (15, 52). This carrier is responsible for the uptake of all three branched-chain amino acids, and therefore, high concentrations of valine or leucine compete for the uptake of the essential supplement isoleucine, which usually was present at an initial concentration of 3.4 mM in the medium. Consequently, the inhibitory effects of valine and leucine can be abolished by increased isoleucine concentrations (Fig. 3). Final proof of this explanation was obtained from an experiment in which the dipeptide isoleucyl-isoleucine was used instead of isoleucine as a supplement. In contrast to isoleucine, the dipeptide should not be taken up by BrnQ but by a dipeptide transport system, as in *E. coli* (12, 51), or by one of the peptide transport systems known from many other organisms (40). The putative OppABCD ABC transport system induced by valine in VAL1 (Table 3) encodes a homologue of a peptide transport system and thus could be induced because of the isoleucine limitation. As expected, growth of the VAL1 strain in the presence of 1.7 mM isoleucyl-isoleucine was not inhibited by valine concentrations of up to 200 mM, whereas the control supplemented with 3.4 mM isoleucine was strongly inhibited (Fig. 5).

As indicated in the introduction, the starting point of this

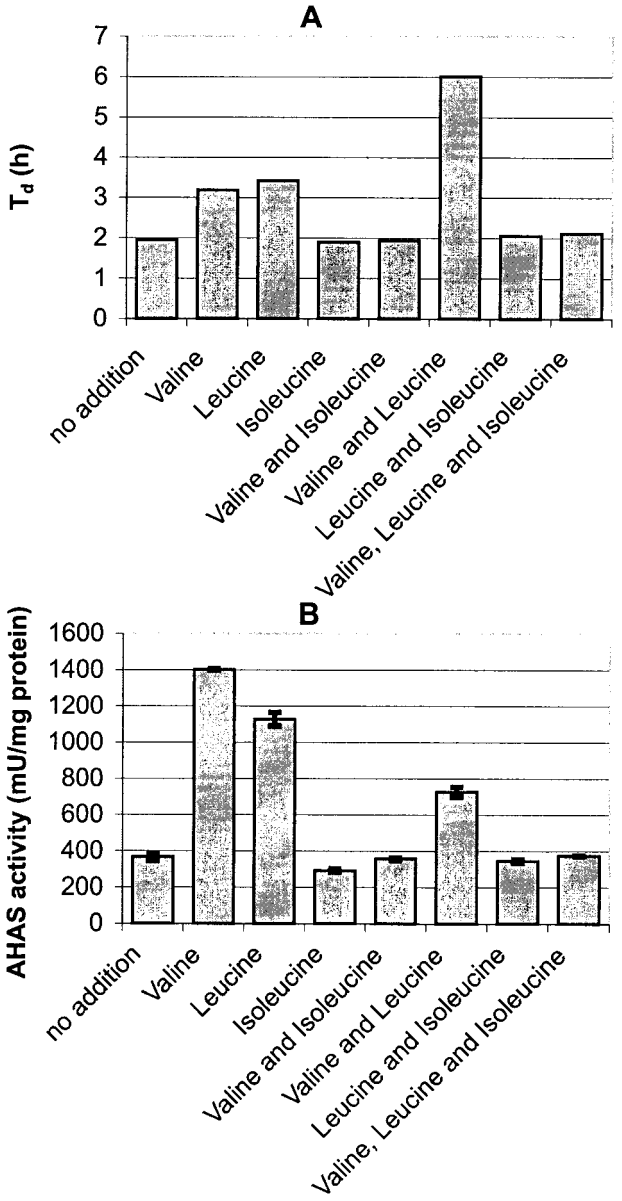

FIG. 3. Effects of L-valine, L-leucine, and L-isoleucine (40 mM each) addition to the culture medium on doubling time (A) and specific AHAS activity (B) of *C. glutamicum* VAL1. The error bars indicate standard deviations.

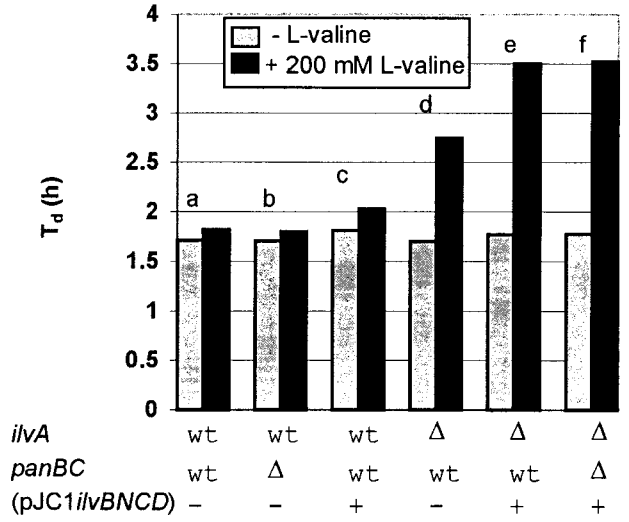

FIG. 4. Doubling times of different strains derived from *C. glutamicum* ATCC 13032 without (shaded bars) or with (solid bars) addition of 200 mM L-valine. The strains utilized were ATCC 13032 (a), 13032Δ*panBC* (b), 13032(pJC1*ilvBNCD*) (c), 13032Δ*ilvA* (d), 13032Δ*ilvA* (pJC1*ilvBNCD*) (e), and VAL1 [13032 Δ*ilvA* Δ*panBC*(pJC1*ilvBNCD*)] (f). Below the columns, it is indicated if the strains do (Δ) or do not (wt) carry a deletion of *ilvA* or *panBC* and if the strains carry plasmid pJC1*ilvBNCD* (+) or not (−).

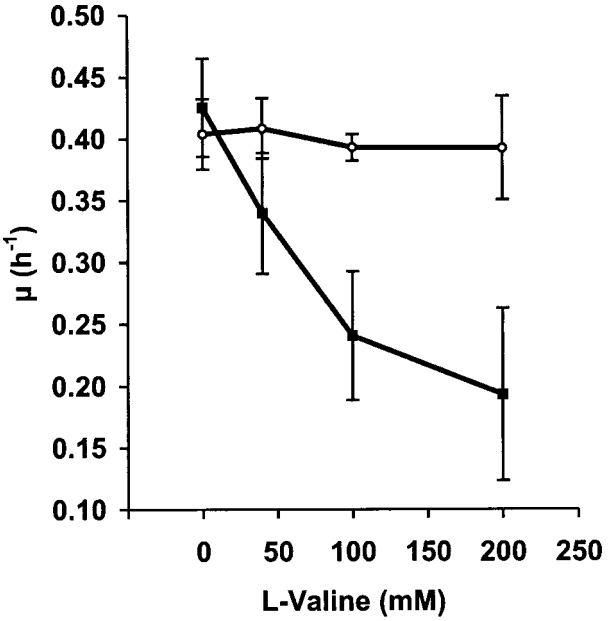

FIG. 5. Effects of different L-valine concentrations in the culture medium on growth rates (μ) of the *C. glutamicum* valine production strain VAL1 when supplemented with 3.4 mM isoleucine (■) or 1.7 mM isoleucine dipeptide (○). The average growth rates and standard deviations of two independent cultivations are given.

study was the inhibitory effect of valine on growth of the leucine-auxotrophic lysine production strain *C. glutamicum* MH20-22B (18). This effect could now also be explained by competition of valine for leucine uptake via BrnQ. Growth experiments confirmed that the inhibitory effect of valine was abolished when leucine was provided in the form of the dipeptide alanyl-leucine instead of leucine (data not shown).

Influence of externally added valine on valine production by the VAL1 strain. Since valine inhibited the growth and stimulated the AHAS activity of the VAL1 strain supplemented with isoleucine, it was interesting to examine the influence of externally added valine on valine production. The strain was cultivated three times independently with either 0, 40, or 175 mM valine, and the valine concentration was determined after 24, 48, and 72 h. After 48 h, no further increase occurred. As shown in Table 7, the initial addition of valine to the medium had a positive effect on valine production, leading to an increase of 33% (by addition of 40 mM valine) or even 50% (by addition of 175 mM valine).

DISCUSSION

Based on the observation that L-valine inhibited the growth of the lysine production strain *C. glutamicum* MH20-22B, the effects of valine on the *C. glutamicum* wild type, ATCC 13032, and a derived valine producer strain, VAL1, were studied in this work. Remarkably, the growth of the wild type was not influenced by valine, whereas growth of the VAL1 strain was inhibited. Using a variety of techniques, such as transcript profiling, proteome analysis, and enzymatic studies, we could finally ascribe the inhibitory effect of valine on the isoleucine-auxotrophic VAL1 strain to a competition of isoleucine with valine for uptake by BrnQ. This secondary carrier is responsible for the uptake of all branched-chain amino acids in *C. glutamicum* (52) and has only slightly different affinities for them (15). The isoleucine transport inhibition could be autoamplified by decreased BrnQ levels, since it has been reported that *brnQ* is expressed only if the internal isoleucine concentration exceeds 0.5 mM (5).

The first and most important hint of the occurrence of a limitation of either isoleucine or leucine in the VAL1 strain was obtained by transcriptome studies showing that the *ilvBN* mRNA level was increased by valine in the VAL1 strain but not in the wild type (the different levels of *ilvB*, *ilvN*, *and ilvC* RNAs are caused by the fact that three different transcripts are formed, i.e., the full-length *ilvBNC* transcript, an *ilvNC* transcript, and an *ilvC* transcript [28]). This is surprising, as valine was previously shown to cause decreased *ilvBNC* transcription due to an attenuation mechanism (38). The increased RNA level correlated with an increased IlvB protein level (IlvN was not present on the 2-D gels, which covered pH 4 to 7, due to its predicted pI of 9.15 [http://www.expasy.org/tools/pi_tool.html]) and an increased AHAS activity in the VAL1 strain. Growth studies with all branched-chain amino acids used singly or in combination then revealed that L-leucine had the same

TABLE 7. Effects of different starting concentrations of L-valine in the medium on L-valine production by VAL1[a]

Starting concn	Final concn (48 h)	Production
0 ± 0	60 ± 5	60 ± 5
40 ± 0	120 ± 5	80 ± 5
175 ± 5	265 ± 15	90 ± 10

[a] Average values (millimolar L-valine) and standard deviations from three independent parallel cultivations are given.

effect as L-valine and that this effect could be abolished by higher concentrations of L-isoleucine. Isoleucine, on the other hand, had no effect on growth or AHAS activity. Thus, the inhibitory effect was shown to be due to isoleucine limitation, and this was confirmed by the fact that valine inhibition was caused primarily by the *ilvA* deletion of the VAL1 strain. Final proof of transport competition between valine and isoleucine was obtained by a growth experiment using 1.7 mM isoleucylisoleucine as a supplement instead of 3.4 mM isoleucine. This dipeptide completely abolished valine-dependent growth inhibition, because it is taken up by a peptide transport system rather than by BrnQ and therefore valine does not compete with its uptake.

Besides showing the differential effects of valine on *ilvBN* expression in the wild type and the VAL1 strain, the transcriptome studies identified totals of 39 and 21 ORFs that showed at least twofold-changed mRNA levels in response to valine in the wild type and the VAL1 strain, respectively. Three ORFs showed similar increases of RNA levels in both strains, i.e., a putative isoleucine-tRNA ligase gene (*ileS*), an adjacent short hypothetical ORF (186 bp), and the *leuD* gene. In view of the fact that isoleucine-tRNA ligase from *E. coli* is derepressed under isoleucine starvation (23), the same could hold true in *C. glutamicum*. Whereas isoleucine limitation in the VAL1 strain has been unequivocally demonstrated and explained (see above), its occurrence and the reason for its occurrence in the wild type are not clear. One cause could be increased synthesis of the branched-chain amino acid exporter BrnEF (29). Alternatively, a shortage of charged isoleucyl-tRNA could be caused by a competition of valine and isoleucine for binding to isoleucyl-tRNA, as the isoleucyl-tRNA synthetase of *E. coli* also misactivates valine (49). The *leuD* gene encodes one subunit of isopropylmalate dehydratase, an enzyme involved in leucine biosynthesis, and the increased *leuD* mRNA level in the presence of valine might indicate starvation for this amino acid, similar to the case of *ileS*. The gene for the second subunit of isopropylmalate dehydratase, *leuC*, showed 6.6- and 1.7-fold-increased RNA levels in the wild type and the VAL1 strain, respectively, but the *P* values were above 0.05. In the wild type, leucine limitation again could be caused by an increased level of the exporter BrnEF. In the VAL1 strain, however, the reason for a possible leucine limitation is not obvious. Eventually, valine could have a direct effect on *leuCD* expression in *C. glutamicum*.

Interestingly, levels of RNAs of the *prpD2B2C2* operon and the *prpC1* gene of the *prpD1B1C1* operon were four- to sixfold increased in the wild type but unchanged or even reduced in the VAL1 strain. In the case of PrpD2, the increased RNA level correlated with an eightfold-increased protein level on 2-D gels. The spots for PrpB2, PrpC2, and PrpC1 could not be detected on the gels. The *prpD2B2C2* operon is essential for propionate utilization by *C. glutamicum* and is induced by propionate (10). The operon shows sequence similarity to the *prp* operons of *Salmonella enterica* serovar Typhimurium and *E. coli*, in which it was shown that the encoded proteins convert propionyl-CoA to pyruvate (26, 53). Since the degradation of valine leads to the formation of propionyl-CoA (36, 60), the induction of the *prp* genes by valine could indicate involvement in the conversion of valine-derived propionyl-CoA to pyruvate. However, we could show that *C. glutamicum* is unable to grow with L-valine as a sole carbon source or as a sole nitrogen source. Therefore, induction of the enzymes of the methylcitrate cycle for the purpose of valine degradation does not make sense. One could argue that the ability to catabolize valine was lost fairly recently, whereas the regulatory mechanisms involved remain unchanged. This appears to be unlikely, because isoleucine, which, like valine, can be catabolized via propionyl-CoA and therefore should also induce the *prp* genes, was found to inhibit expression of the *prpD2B2C2* operon (data not shown). In conclusion, the reason for the induction of the *prp* genes by valine (and their repression by isoleucine) remains unclear. The same holds true for the result showing that valine did not induce the *prp* genes in the VAL1 strain. The argument that valine induction and isoleucine repression counterbalance each other is unlikely because isoleucine was shown to be limiting.

A comparison of the results of transcriptome and proteome analyses revealed that 13 proteins had reduced levels in the presence of valine, but none of the corresponding genes showed decreased RNA levels. This might be explained by some kind of technical limitation and/or by the fact that a significant part of the regulation of protein synthesis does not occur at the RNA level but by other means, e.g., translational regulation or protein stability. In the cases of the proteins with increased abundance in the presence of valine, most of the corresponding genes (except those for PurH and EF-G) also showed increased mRNA levels.

An important result of our studies was the observation that the presence of valine in the medium stimulates its own production by the VAL1 strain. This effect is presumably due to the increased AHAS activity and to the growth inhibition, which may favor valine production at the expense of biomass formation. These aspects certainly should be considered in the further improvement of VAL1 strain valine production by metabolic engineering.

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REFERENCES

1. **Abe, S., K.-I. Takayama, and S. Kinoshita.** 1967. Taxonomical studies on glutamic acid-producing bacteria. J. Gen. Appl. Microbiol. **13:**279–301.
2. **Arfin, S. M., A. D. Long, E. T. Ito, L. Tolleri, M. M. Riehle, E. S. Paegle, and G. W. Hatfield.** 2000. Global gene expression profiling in *Escherichia coli* K12. The effects of integration host factor. J. Biol. Chem. **275:**29672–29684.
3. **Barak, Z., D. M. Chipman, and N. Gollop.** 1987. Physiological implications of the specificity of acetohydroxy acid synthase isozymes of enteric bacteria. J. Bacteriol. **169:**3750–3756.
4. **Barak, Z., N. Kogan, N. Gollop, and D. M. Chipman.** 1990. Importance of AHAS isozymes in branched chain amino acid biosynthesis, p. 91–107. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH Verlagsgesellschaft, Weinheim, Germany.
5. **Boles, E., H. Ebbighausen, B. J. Eikmanns, and R. Krämer.** 1993. Unusual regulation of the uptake system for branched-chain amino acids in *Corynebacterium glutamicum*. Arch. Microbiol. **159:**147–152.
6. **Chipman, D., Z. Barak, and J. V. Schloss.** 1998. Biosynthesis of 2-aceto-2-hydroxy acids: acetolactate synthases and acetohydroxyacid synthases. Biochim. Biophys. Acta **1385:**401–419.
7. **Chipman, D. M., N. Gollop, B. Damri, and Z. Barak.** 1990. Kinetics and mechanism of acetohydroxyacid synthases, p. 243–267. *In* Z. Barak, D. M.

Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH Verlagsgesellschaft, Weinheim, Germany.
8. **Chun, J. Y., E. J. Lee, H. S. Lee, C. I. Cheon, K. H. Min, and M. S. Lee.** 1998. Molecular cloning and analysis of the *argC* gene from *Corynebacterium glutamicum*. Biochem. Mol. Biol. Int. **46:**437–447.
9. **Chun, J. Y., and M. S. Lee.** 1999. Cloning of the *argF* gene encoding the ornithine carbamoyltransferase from *Corynebacterium glutamicum*. Mol. Cells **9:**333–337.
10. **Claes, W. A., A. Pühler, and J. Kalinowski.** 2002. Identification of two *prpDBC* gene clusters in *Corynebacterium glutamicum* and their involvement in propionate degradation via the 2-methylcitrate cycle. J. Bacteriol. **184:** 2728–2739.
11. **Cremer, J., L. Eggeling, and H. Sahm.** 1990. Cloning the *dapA dapB* cluster of the lysine-secreting bacterium *Corynebacterium glutamicum*. Mol. Gen. Genet. **220:**478–480.
12. **De Felice, M., J. Guardiola, A. Lamberti, and M. Iaccarino.** 1973. *Escherichia coli* K-12 mutants altered in the transport systems for oligo- and dipeptides. J. Bacteriol. **116:**751–756.
13. **De Felice, M., M. Levinthal, M. Iaccarino, and J. Guardiola.** 1979. Growth inhibition as a consequence of antagonism between related amino acids: effect of valine in *Escherichia coli* K-12. Microbiol. Rev. **43:**42–58.
14. **De Felice, M., T. Newman, and M. Levinthal.** 1978. Regulation of synthesis of the acetohydroxy acid synthase I isoenzyme in *Escherichia coli* K-12. Biochim. Biophys. Acta **541:**1–8.
15. **Ebbighausen, H., B. Weil, and R. Krämer.** 1989. Transport of branched-chain amino acids in *Corynebacterium glutamicum*. Arch. Microbiol. **151:**238–244.
16. **Eggeling, I., C. Cordes, L. Eggeling, and H. Sahm.** 1987. Regulation of acetohydroxy acid synthase in *Corynebacterium glutamicum* during fermentation of α-ketobutyrate to L-isoleucine. Appl. Microbiol. Biotechnol. **25:** 346–351.
17. **Eggeling, L.** 2001. Amino acids, p. 281–303. *In* C. Ratledge and B. Kristiansen (ed.), Basic bio/technology. Cambridge University Press, London, United Kingdom.
18. **Eggeling, L., S. Morbach, and H. Sahm.** 1997. The fruits of molecular physiology: engineering the L-isoleucine biosynthesis pathway in *Corynebacterium glutamicum*. J. Biotechnol. **56:**167–182.
19. **Eggeling, L., E. Scheer, C. Cordes, A. Nassenstein, I. Eggeling, and H. Sahm.** 1990. Isoleucine formation from hydroxybutyrate with *Corynebacterium glutamicum*: biochemistry, limiting reactions, genes, p. 179–191. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH Verlagsgesellschaft, Weinheim, Germany.
20. **Eoyang, L., and P. M. Silverman.** 1986. Role of small subunit (IlvN polypeptide) of acetohydroxyacid synthase I from *Escherichia coli* K-12 in sensitivity of the enzyme to valine inhibition. J. Bacteriol. **166:**901–904.
21. **Friden, P., T. Newman, and M. Freundlich.** 1982. Nucleotide sequence of the *ilvB* promoter-regulatory region: a biosynthetic operon controlled by attenuation and cyclic AMP. Proc. Natl. Acad. Sci. USA **79:**6156–6160.
22. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of biuret reaction. J. Biol. Chem. **177:**751–766.
23. **Grunberg-Manago, M.** 1996. Regulation of the expression of aminoacyl-tRNA synthetases and translation factors, p. 1432–1457. *In* F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
24. **Guardiola, J., M. De Felice, A. Lamberti, and M. Iaccarino.** 1977. The acetolactate synthase isoenzymes of *Escherichia coli* K-12. Mol. Gen. Genet. **156:**17–25.
25. **Hauser, C. A., and G. W. Hatfield.** 1984. Attenuation of the *ilvB* operon by amino acids reflecting substrates or products of the *ilvB* gene product. Proc. Natl. Acad. Sci. USA **81:**76–79.
26. **Horswill, A. R., and J. C. Escalante-Semerena.** 1999. *Salmonella typhimurium* LT2 catabolizes propionate via the 2-methylcitric acid cycle. J. Bacteriol. **181:**5615–5623.
27. **Jackowski, S., and C. O. Rock.** 1981. Regulation of coenzyme A biosynthesis. J. Bacteriol. **148:**926–932.
28. **Keilhauer, C., L. Eggeling, and H. Sahm.** 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J. Bacteriol. **175:**5595–5603.
29. **Kennerknecht, N., H. Sahm, M. R. Yen, M. Patek, M. H. Saier, Jr., and L. Eggeling.** 2002. Export of L-isoleucine from *Corynebacterium glutamicum*: a two-gene-encoded member of a new translocator family. J. Bacteriol. **184:** 3947–3956.
30. **Khodursky, A., J. A. Bernstein, B. J. Peter, V. Rhodius, V. F. Wendisch, and D. P. Zimmer.** *Escherichia coli* spotted double strand DNA microarrays: RNA extraction, labeling, hybridization, quality control and data management. Methods Mol. Biol., in press.
31. **Lawther, R. P., D. H. Calhoun, C. W. Adams, C. A. Hauser, J. Gray, and G. W. Hatfield.** 1981. Molecular basis of valine resistance in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **78:**922–925.
32. **Lawther, R. P., and G. W. Hatfield.** 1980. Multivalent translational control of transcription termination at attenuator of *ilvGEDA* operon of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **77:**1862–1866.
33. **Lehnen, D., C. Blumer, T. Polen, B. Wackwitz, V. F. Wendisch, and G. Unden.** 2002. LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in *Escherichia coli*. Mol. Microbiol. **45:**521–532.
34. **Leuchtenberger, W.** 1996. Amino acids—technical production and use, p. 465–502. *In* H.-J. Rehm, G. Reed, A. Pühler, and P. Stadler (ed.), Bio/technology: products of primary metabolism, vol. 6. Verlag Chemie, Weinheim, Germany.
35. **Lindroth, P., and K. Mopper.** 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. Anal. Chem. **51:**1667–1674.
36. **Massey, L. K., J. R. Sokatch, and R. S. Conrad.** 1976. Branched-chain amino acid catabolism in bacteria. Bacteriol. Rev. **40:**42–54.
37. **Menkel, E., G. Thierbach, L. Eggeling, and H. Sahm.** 1989. Influence of increased aspartate availability on lysine formation by a recombinant strain of *Corynebacterium glutamicum* and utilization of fumarate. Appl. Environ. Microbiol. **55:**684–688.
38. **Morbach, S., C. Junger, H. Sahm, and L. Eggeling.** 2000. Attenuation control of *ilvBNC* in *Corynebacterium glutamicum*: evidence of leader peptide formation without the presence of a ribosome binding site. J. Biosci. Bioeng. **90:**501–507.
39. **Patek, M., K. Krumbach, L. Eggeling, and H. Sahm.** 1994. Leucine synthesis in *Corynebacterium glutamicum*: enzyme activities, structure of *leuA*, and effect of *leuA* inactivation on lysine synthesis. Appl. Environ. Microbiol. **60:**133–140.
40. **Payne, J. W., and M. W. Smith.** 1994. Peptide transport by micro-organisms. Adv. Microb. Physiol. **36:**1–81.
41. **Radmacher, E., A. Vaitsikova, U. Burger, K. Krumbach, H. Sahm, and L. Eggeling.** 2002. Linking central metabolism with increased pathway flux: L-valine accumulation by *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **68:**2246–2250.
42. **Rhodius, V., T. K. Van Dyk, C. Gross, and R. A. LaRossa.** 2002. Impact of genomic technologies on studies of bacterial gene expression. Annu. Rev. Microbiol. **56:**599–624.
43. **Ricca, E., D. A. Aker, and J. M. Calvo.** 1989. A protein that binds to the regulatory region of the *Escherichia coli ilvIH* operon. J. Bacteriol. **171:**1658–1664.
44. **Sahm, H., and L. Eggeling.** 1999. D-Pantothenate synthesis in *Corynebacterium glutamicum* and use of *panBC* and genes encoding L-valine synthesis for D-pantothenate overproduction. Appl. Environ. Microbiol. **65:**1973–1979.
45. **Sahm, H., L. Eggeling, and A. A. de Graaf.** 2000. Pathway analysis and metabolic engineering in *Corynebacterium glutamicum*. Biol. Chem. **381:**899–910.
46. **Sakanyan, V., P. Petrosyan, M. Lecocq, A. Boyen, C. Legrain, M. Demarez, J. N. Hallet, and N. Glansdorff.** 1996. Genes and enzymes of the acetyl cycle of arginine biosynthesis in *Corynebacterium glutamicum*: enzyme evolution in the early steps of the arginine pathway. Microbiology **142:**99–108.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Schaffer, S., B. Weil, V. D. Nguyen, G. Dongmann, K. Gunther, M. Nickolaus, T. Hermann, and M. Bott.** 2001. A high-resolution reference map for cytoplasmic and membrane-associated proteins of *Corynebacterium glutamicum*. Electrophoresis **22:**4404–4422.
49. **Schmidt, E., and P. Schimmel.** 1994. Mutational isolation of a sieve for editing in a transfer RNA synthetase. Science **264:**265–267.
50. **Schrumpf, B., L. Eggeling, and H. Sahm.** 1992. Isolation and prominent characteristics of an L-lysine hyperproducing strain of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **37:**566–571.
51. **Smith, M. W., D. R. Tyreman, G. M. Payne, N. J. Marshall, and J. W. Payne.** 1999. Substrate specificity of the periplasmic dipeptide-binding protein from *Escherichia coli*: experimental basis for the design of peptide prodrugs. Microbiology **145:**2891–2901.
52. **Tauch, A., T. Hermann, A. Burkovski, R. Krämer, A. Pühler, and J. Kalinowski.** 1998. Isoleucine uptake in *Corynebacterium glutamicum* ATCC 13032 is directed by the *brnQ* gene product. Arch. Microbiol. **169:**303–312.
53. **Textor, S., V. F. Wendisch, A. A. De Graaf, U. Muller, M. I. Linder, D. Linder, and W. Buckel.** 1997. Propionate oxidation in *Escherichia coli*: evidence for operation of a methylcitrate cycle in bacteria. Arch. Microbiol. **168:**428–436.
54. **Umbarger, H. E.** 1996. Biosynthesis of the branched-chain amino acids, p. 442–457. *In* F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: molecular and cellular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
55. **Wang, Q., and J. M. Calvo.** 1993. Lrp, a global regulatory protein of *Escherichia coli*, binds co-operatively to multiple sites and activates transcription of *ilvIH*. J. Mol. Biol **229:**306–318.
56. **Wek, R. C., C. A. Hauser, and G. W. Hatfield.** 1985. The nucleotide sequence

of the *ilvBN* operon of *Escherichia coli*: sequence homologies of the acetohydroxy acid synthase isozymes. Nucleic Acids Res. **13:**3995–4010.

57. **Wendisch, V. F., D. P. Zimmer, A. Khodursky, B. Peter, N. Cozzarelli, and S. Kustu.** 2001. Isolation of *Escherichia coli* mRNA and comparison of expression using mRNA and total RNA on DNA microarrays. Anal. Biochem. **290:**205–213.
58. **Westerfeld, W. W.** 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. **161:**495–502.
59. **Williams, A. L., and L. S. Williams.** 1985. Control of isoleucine-valine biosynthesis in a valine-resistant mutant of *Escherichia coli* K-12 that simultaneously acquired azaleucine-resistance. Biochem. Biophys. Res. Commun. **131:**994–1002.
60. **Zhang, Y. X., L. Tang, and C. R. Hutchinson.** 1996. Cloning and characterization of a gene (*msdA*) encoding methylmalonic acid semialdehyde dehydrogenase from *Streptomyces coelicolor*. J. Bacteriol. **178:**490–495.
61. **Zimmer, D. P., E. Soupene, H. L. Lee, V. F. Wendisch, A. B. Khodursky, B. J. Peter, R. A. Bender, and S. Kustu.** 2000. Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. Proc. Natl. Acad. Sci. USA **97:**14674–14679.