Adaptation of *Corynebacterium glutamicum* to Ammonium Limitation: a Global Analysis Using Transcriptome and Proteome Techniques†

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The response of *Corynebacterium glutamicum* to ammonium limitation was studied by transcriptional and proteome profiling of cells grown in a chemostat. Our results show that ammonium-limited growth of *C. glutamicum* results in a rearrangement of the cellular transport capacity, changes in metabolic pathways for nitrogen assimilation, amino acid biosynthesis, and carbon metabolism, as well as a decreased cell division. Since transcription at different growth rates was studied, it was possible to distinguish specific responses to ammonium limitation and more general, growth rate-dependent alterations in gene expression. The latter include a number of genes encoding ribosomal proteins and genes for F₀F₁-ATP synthase subunits.

Corynebacterium glutamicum was isolated in 1957 by Kinoshita and coworkers in a screening program for L-glutamate-producing bacteria from a soil sample collected at Ueno Zoo in Tokyo, and at that time it was designated as *Micrococcus glutamicus* (10, 21, 33). Less than 50 years later, enormous amounts of L-glutamate (more than 1,500,000 tons per year) and L-lysine (more than 560,000 tons per year) are produced by use of different *C. glutamicum* strains, in addition to smaller amounts of some less industrially important amino acids (L-alanine, L-isoleucine, and L-proline) and in addition to different nucleotides (15). In contrast to closely related pathogenic species, such as *Corynebacterium diphtheriae*, *Mycobacterium leprae*, and *Mycobacterium tuberculosis*, *C. glutamicum* is generally recognized as a nonhazardous organism which is safe to handle. Furthermore, based on its extremely well-investigated central metabolism and well-established molecular biology tools, *C. glutamicum* is suitable as a model organism for high G+C content gram-positive bacteria in general and mycolic-acid-containing *Actinomycetales* in particular.

The regulation of nitrogen metabolism in the *Actinomycetales* was the subject of research mainly in the last few years (for a review, see references 7 and 8). For *C. glutamicum*, detailed information of transport and assimilation of nitrogen sources as well as nitrogen regulation is available on a molecular level (for a review, see references 7 and 8). Uptake systems for ammonium, creatinine, and glutamate were studied, and assimilatory enzymes and pathways were investigated. Additionally, the key components of nitrogen control were identified; namely, AmtR, the master regulator of nitrogen control in *C. glutamicum*, GlnK, the sole P_{II}-type signal transduction

protein in this organism, and two modifying enzymes, a putative adenylyltransferase and the GlnD protein (32).

While previous studies focused on specific genes or enzymes, here we present a global analysis of the *C. glutamicum* ammonium limitation response by transcriptional profiling and two-dimensional gel electrophoresis. In this study these two global approaches were combined with the continuous cultivation of cells. In contrast to shaking flask experiments, this technique allows us to establish highly defined growth conditions over a long period of time, i.e., over days or weeks. As a consequence, cells which are optimally adapted to a specific environment or nutrient supply can be investigated.

MATERIALS AND METHODS

Bacterial strains and growth. *C. glutamicum* type strain ATCC 13032 (1) was grown at 30°C in a modified MM1 minimal medium (20). For continuous, ammonium-limited fermentations the concentration of nitrogen sources in the starting medium and in the substrate feed was decreased compared to the original medium. Urea was omitted from both media, and in the substrate feed ammonium sulfate was reduced from 5 g liter⁻¹ to 2.75 g liter⁻¹. In addition, the glucose content was reduced from 40 g liter⁻¹ to 20 g liter⁻¹. The process was started in the batch mode by inoculation of 1.3 liters starting medium to an optical density at 600 nm (OD₆₀₀) of 1 using a minimal medium overnight culture. The fermentation was carried out at 30°C and an aeration rate of 1 vvm (volume per volume and min). The pH was automatically adjusted to 7.0 with 3 M NaOH. During the batch phase, the stirrer speed was increased from an initial 600 rpm to a final value of 1,000 rpm. At an OD₆₀₀ of 18, 30 ml cell broth was harvested for proteome analysis and 1 ml for transcriptome analysis. Then the process was switched to continuous fermentation mode by starting the steady supplementation of substrate feed with a dilution rate of 0.075 h⁻¹. To guarantee a constant filling volume during the whole fermentation, the surplus of culture broth was removed. After the steady state was reached, samples for transcriptome and proteome analyses were taken (see above) and the dilution rate was increased to a value of 0.15 h⁻¹. After the new steady state was reached, again samples were taken.

Total RNA preparation from *C. glutamicum*. The frozen aliquots of *C. glutamicum* cell material were suspended in 700 µl RA1 buffer (NucleoSpinRNA II kit; Macherey-Nagel, Düren, Germany) and immediately disrupted using glass beads and a Q-BIOgene FastPrep FP120 instrument (Q-BIOgene, Heidelberg, Germany). Disruption was performed by two 30-s cycles at a speed of 6.5 m s⁻¹. After the cell debris was separated, the RNA was isolated using the NucleoSpin-RNA II kit following the supplier's recommendations. If necessary, a second

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DNase digestion was performed with DNase I (Amersham Biosciences, Freiburg, Germany) to completely remove the chromosomal DNA. RNA samples were finally stored at -80°C .

Transcriptome analyses. For transcriptome analyses, 5 μg of total *C. glutamicum* RNA was used for cDNA synthesis. During reverse transcription of the RNA, aminoallyl-modified dUTP (aa-dUTP; Sigma-Aldrich, Taufkirchen, Germany) was incorporated to prepare the samples for indirect labeling. Afterwards, Cy3 or Cy5 monofunctional NHS-esters (Amersham Biosciences, Freiburg, Germany) were coupled with the aa-dUTPs, excess NHS-esters were removed, and the samples were purified using a MinElute PCR purification kit (QIAGEN, Hilden, Germany). For detailed information concerning cDNA synthesis and fluorescent labeling see reference 16. For hybridization, Cy3- and Cy5-labeled samples were combined and vacuum dried. Microarray slides covering more than 93% of all *C. glutamicum* genes in four replicates were prehybridized and prepared as described elsewhere (16). The vacuum-dried sample was suspended in 70 μl DIG-Easy Hyb hybridization solution (Roche Diagnostics, Mannheim, Germany). This mixture was used for hybridization under a coverslip inside an in situ hybridization chamber (TeleChem International, Sunnyvale, CA). After washing and drying the microarrays (16), the signal acquisition was performed with a ScanArray 4000 microarray scanner (Perkin-Elmer, Boston, MA). The Image 5.0 software (Biodiscovery, Los Angeles, CA) was used for spot finding, signal-background segmentation, and intensity quantification (16). Ratios were calculated and normalization as well as *t* test statistics were performed using the EMMA microarray data analyses software (9). All signal intensities with ratios above 1.74 were regarded as significant, if a *P* value below 0.05 was assigned. For each comparison of interest, two independent experiments were performed.

Protein sample preparation and 2-D PAGE. *C. glutamicum* cells were disrupted using glass beads and a Q-BIOgene FastPrep FPI20 instrument (Q-BIOgene, Heidelberg, Germany) by lysing the cells four times for 30 s and 6.5 m s^{-1} in the presence of the proteinase inhibitor Complete as recommended by the supplier (Roche, Basel, Switzerland). Proteins were separated by ultracentrifugation in cytoplasmic and membrane-associated protein fractions (13, 14). In this study, only the cytoplasmic proteins were further analyzed. Protein concentrations were determined with Amido Black (28). For isoelectric focusing, 24-cm precast IPG strips, pI 4 to 7, and an IPGphor isoelectric focusing unit (Amersham Biosciences, Freiburg, Germany) were used as described previously (14). One hundred micrograms of protein was focused for 68,000 $\text{V} \cdot \text{h}$ in a sample buffer containing 6 M urea, 2 M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.5% Phormalyte (pH 3 to 10), and 0.4% dithiothreitol. The run for the second dimension was carried out using precast 12 to 14% polyacrylamide linear gradient gels (ExcelGel Gradient XL 12-14; Amersham Biosciences, Freiburg, Germany) in a Multiphor II apparatus as described previously (14). After electrophoresis, two-dimensional (2-D) gels were stained with Coomassie brilliant blue (27). The Coomassie-stained gels were aligned using the Delta2D software, version 3.2 (Decodon, Greifswald, Germany). All samples were separated at least twice by 2-D polyacrylamide gel electrophoresis (PAGE) to minimize irregularities (technical replicates). To validate the results, each comparison of interest was performed using samples from two independent experiments (biological replicates). The Delta2D software (version 3.2) also was used for spot quantification. Proteins were regarded as regulated if (i) the corresponding ratios referring to the relative volumes of the spots were changed more than twofold and if (ii) this regulation pattern was found in all biological and technical replicates. All other proteins were classified as "not regulated."

MALDI-TOF MS. Protein spots of interest were excised from Coomassie-stained 2-D gels for peptide mass fingerprinting via matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS). All MALDI-MS experiments were conducted in the reflectron mode (resolution, full width at half maximum of $\geq 10,000$) on a Voyager-DE STR reflectron TOF mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a N_2 -UV laser (337-nm, 3-ns pulse length). The gel pieces were washed twice for 5 min with 500 μl of 50 mM NH_4HCO_3 and once for 30 min with 500 μl of 50 mM NH_4HCO_3 to remove all contaminants. Subsequently, the gel pieces were destained twice for 30 min with 50 mM NH_4HCO_3 in 50% acetonitrile, shrunk with 100 μl acetonitrile for 5 min, and dried under vacuum for 30 min (Concentrator 5301; Eppendorf, Hamburg, Germany). Tryptic in-gel digestion was started by rehydration of the gel matrix by the addition of 1 to 2 μl of 25 mM NH_4HCO_3 containing 10 $\mu\text{g}/\text{ml}$ trypsin (sequence grade; Promega, Madison, WI). After 30 min, 25 mM NH_4HCO_3 was added to cover the sample and digestion was continued overnight at room temperature. Another 2 μl of 25 mM NH_4HCO_3 and the following incubation for 90 min at room temperature were used for additional peptide extraction. This peptide solution (0.5 μl) was mixed with 0.5 μl of 5 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile and

0.1% trifluoroacetic acid on a standard 100-spot stainless steel sample plate (Applied Biosystems, Darmstadt, Germany). All chemicals were of analytical grade and used as purchased from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Steinheim, Germany). Data acquisition and subsequent analysis was done by Voyager Instrument Control Panel software and Voyager Data Explorer software (version V5.1; Applied Biosystems, Darmstadt, Germany). External mass calibration was done close to each sample spot by using calibration mixtures 1 and 2 of the Sequazyme peptide mass standard kit (Applied Biosystems, Darmstadt, Germany). Samples were analyzed manually in the positive reflectron mode with delayed extraction of ions (150 ns), 20 kV acceleration voltage, and 66% acceleration grid voltage.

All database searches were performed using the GPMW software, version 6.0 (Lighthouse Data, Odense, Denmark). The resulting peptide mass lists were compared with a local in-house database of *C. glutamicum* proteins (Institut für Genomforschung, Universität Bielefeld). The search criteria were set to a mass accuracy of ≤ 100 ppm and preferably no and maximally one miscleaved peptide per protein. Proteins were considered as identified when more than 30% amino acid sequence was covered by the identified peptides and four or more peptides matching the search criteria with a deviation of mass accuracy based on an incorrect calibration equation.

RESULTS

Ammonium limitation stimulated drastic changes in the expression of 285 genes in *C. glutamicum* wild-type cells (for a complete list of genes with altered expression depending on ammonium supply, see Table S1 in the supplemental material; for genes discussed in this study, see Table 1). These genes include open reading frames encoding transport proteins, proteins involved in nitrogen metabolism and regulation, energy generation, and protein turnover (Fig. 1). Additionally, 74 genes coding for proteins of unknown function or hypothetical proteins are altered in their transcriptional profile. The latter will not be discussed in the following sections.

Transport. Transport systems play a crucial role for the adaptation of cells to a situation of limiting resources, since uptake of a nutrient into the cell is a prerequisite for its utilization. Drastic changes in the transcription of genes encoding different membrane proteins were observed as a response to ammonium limitation in *C. glutamicum*. Especially, expression of genes encoding a creatinine permease, the two ammonium transporters AmtA and AmtB, and an ABC-type urea uptake system was strongly enhanced by factors between 25 and 75. Only one other gene, *glnK* (see below), showed similar increases in transcription. Obviously, scavenging for nutrients is a major response of *C. glutamicum* to ammonium limitation.

Genes encoding other transport systems were less affected. The transcription of NCgl2051 which codes for the secreted component of an ABC-type amino acid transport system was moderately enhanced by a factor of 1.97. Expression of *ptsG*, encoding a protein involved in glucose uptake, NCgl1968, coding for a putative di- and tricarboxylate transporter, *gntP*, encoding a putative gluconate uptake system, and NCgl1915-1918, coding for a putative peptide transporter, was moderately increased in response to ammonium limitation (with factors between approximately 2 and 3). The upregulation of the *gluABCD* cluster might be attributed to the uptake of carbon sources as well, although glutamate can be utilized as a carbon and a nitrogen source in *C. glutamicum*, since this gene cluster is also moderately enhanced in expression, while transporters working exclusively for the uptake of nitrogen sources show much higher factors of upregulation (see above).

The transcription of *betP* and *putP* which code for compat-

TABLE 1. Alterations in transcription in response to long-term ammonium limitation^a

NCBI no.	Acc. no.	Gene name	b vs c _{low}	b vs c _{high}	Annotation
Transport related					
NCgl0074	cg0103	<i>cmT^b</i>	76.32	44.35	Creatinine permease
NCgl0893	cg1061	<i>urtA^c</i>	20.93	20.73	ABC-type urea uptake system, secreted component
NCgl0894	cg1062	<i>urtB^c</i>	36.73	33.01	ABC-type urea uptake system, permease component
NCgl0895	cg1064	<i>urtC^c</i>	47.71	48.79	ABC-type urea uptake system, permease component
NCgl0896	cg1065	<i>urtD^c</i>	19.61	14.19	ABC-type urea uptake system, ATPase component
NCgl0897	cg1066	<i>urtE^c</i>	38.93	36.25	ABC-type urea uptake system, ATPase component
NCgl1305	cg1537	<i>ptsG</i>	2.85	2.21	Phosphotransferase system IIC component, glucose specific
NCgl1521	cg1785	<i>amtA</i>	49.89	50.29	Ammonium transporter AmtA
NCgl1875	cg2136	<i>gluA</i>	2.82	2.78	Glutamate transport ATP-binding protein
NCgl1876	cg2137	<i>gluB</i>	2.56	1.95	Glutamate-binding protein GluB precursor
NCgl1877	cg2138	<i>gluC</i>	2.61	2.16	Glutamate transport system permease protein
NCgl1878	cg2139	<i>gluD</i>	2.87	2.18	Glutamate transport system permease protein GluD
NCgl1915	cg2181		2.89	3.77	ABC-type peptide transport system, periplasmic component
NCgl1917	cg2183		2.20	2.80	ABC-type peptide transport system, permease component
NCgl1918	cg2184		1.87	2.38	ATPase component of peptide ABC-type transport system, contains duplicated ATPase domains
NCgl1968	cg2243		1.77	n.r.	Di- and tricarboxylate transporters
NCgl1983	cg2261	<i>amtB</i>	25.47	32.17	Low-affinity ammonium uptake protein AmtB
NCgl2051	cg2340		1.97		ABC-type amino acid transport system, secreted component
NCgl2808	cg3216	<i>gntP</i>	n.r.	1.91	Putative gluconate permease
NCgl0856	cg1016	<i>betP</i>	0.20	0.26	Glycine betaine transporter
NCgl1116	cg1314	<i>putP</i>	n.r.	0.57	Na ⁺ /proline symporter
NCgl2933	cg3365	<i>rpmC</i>	0.38	n.r.	Putative ribitol transport membrane protein
NCgl2934	cg3366	<i>rmpA</i>	0.32	0.54	Putative ribitol-specific enzyme II of PTS system
Nitrogen metabolism and amino acid biosynthesis					
NCgl0075	cg0104	<i>codA^b</i>	8.86	11.17	Creatinine deaminase
NCgl0083	cg0113	<i>ureA</i>	8.74	9.57	Urease γ subunit
NCgl0084	cg0114	<i>ureB</i>	n.r.	2.10	Urease β subunit
NCgl0085	cg0115	<i>ureC</i>	5.15	5.71	Urease α subunit
NCgl0086	cg0116	<i>ureE</i>	8.06	10.35	Urease accessory protein
NCgl0087	cg0117	<i>ureF</i>	6.43	9.72	Urease accessory protein
NCgl0088	cg0118	<i>ureG</i>	6.88	8.07	Ni ²⁺ -binding GTPase
NCgl0089	cg0119	<i>ureD</i>	5.47	5.85	Urease-associated protein
NCgl0181	cg0229	<i>glbB</i>	10.42	16.53	Glutamine 2-oxoglutarate aminotransferase, large subunit
NCgl0182	cg0230	<i>glbD</i>	25.13	24.59	Glutamine 2-oxoglutarate aminotransferase, small subunit
NCgl1061	cg1256	<i>dapD</i>	2.10	2.83	Tetrahydronicotinate- <i>N</i> -succinyltransferase
NCgl2133	cg2429	<i>gluA</i>	5.47	5.07	Glutamine synthetase I
NCgl0245	cg0303	<i>leuA</i>	0.20	0.17	2-Isopropylmalate synthase
NCgl0730	cg0873	<i>aroA</i>	0.56	n.r.	5-Enolpyruvylshikimate-3-phosphate synthase
NCgl0950	cg1129	<i>aroF</i>	0.26	0.15	Phospho-2-dehydro-3-deoxyheptonate aldolase
NCgl1132	cg1333	<i>argS</i>	0.52	0.56	Arginyl-tRNA synthetase
NCgl1133	cg1334	<i>lysA</i>	0.46	0.50	Diaminopimelate decarboxylase
NCgl1137	cg1338	<i>thrB</i>	n.r.	0.52	Homoserine kinase
NCgl1340	cg1580	<i>argC</i>	0.35	0.26	Acetylglutamate semialdehyde dehydrogenase
NCgl1341	cg1581	<i>argJ</i>	0.33	0.24	Glutamate <i>N</i> -acetyltransferase/amino-acid <i>N</i> -acetyltransferase
NCgl1342	cg1582	<i>argB</i>	n.r.	0.13	<i>N</i> -Acetylglutamate kinase
NCgl1343	cg1583	<i>argD</i>	0.29	0.18	PLP-dependent aminotransferase
NCgl1344	cg1584	<i>argF</i>	0.30	0.20	Ornithine carbamoyltransferase
NCgl1345	cg1585	<i>argR</i>	0.30	0.23	Arginine repressor
NCgl1346	cg1586	<i>argG</i>	0.26	0.23	Argininosuccinate synthase
NCgl1347	cg1588	<i>argH</i>	0.35	0.33	Argininosuccinate lyase
NCgl1446	cg1697	<i>aspA</i>	n.r.	0.56	Aspartate ammonia-lyase
NCgl2098	cg2391	<i>aroG</i>	0.47	n.r.	Phospho-2-dehydro-3-deoxyheptonate aldolase
NCgl2528	cg2900	<i>ddh</i>	0.55	0.41	D-2-Hydroxyisocaproate dehydrogenase surface polysaccharide
Carbon and energy metabolism					
NCgl0359	cg0445	<i>sdhCD</i>	2.57	3.05	Succinate dehydrogenase CD
NCgl0360	cg0446	<i>sdhA</i>	3.20	3.15	Succinate dehydrogenase A
NCgl0361	cg0447	<i>sdhB</i>	2.41	2.98	Succinate dehydrogenase B
NCgl0427	cg0527	<i>ccsB</i>	1.82	2.10	Cytochrome <i>c</i> assembly protein
NCgl1526	cg1791	<i>gap</i>	1.84	n.r.	Glyceraldehyde-3-phosphate dehydrogenase
NCgl2110	cg2404	<i>qcrA1</i>	2.27	2.55	Rieske Fe-S protein
NCgl2111	cg2405	<i>qcrC</i>	n.r.	2.14	Cytochrome <i>c</i>

Continued on following page

TABLE 1—Continued

NCBI no.	Acc. no.	Gene name	b vs c_{low}	b vs c_{high}	Annotation
NCgl2112	cg2406	<i>ctaE</i>	2.71	2.49	Cytochrome <i>c</i> oxidase subunit III
NCgl2437	cg2780	<i>ctaD</i>	2.64	2.85	Heme/copper-type cytochrome/quinol oxidase, subunit 1
NCgl2673	cg3068	<i>fda</i>	1.84	2.01	Fructose-bisphosphate aldolase
NCgl2247	cg2559	<i>aceB</i>	0.37	n.r.	Malate synthase
NCgl2248	cg2560	<i>aceA</i>	0.51	0.29	Isocitrate lyase
NCgl2709	cg3107	<i>adhA</i>	0.54	0.43	Zn-dependent alcohol dehydrogenase
Protein stability and turnover					
NCgl0572	cg0690	<i>groES</i>	1.93	2.45	Co-chaperonin GroES
NCgl0573	cg0693	<i>groEL</i>	1.77	2.05	Chaperonin GroEL
NCgl2621	cg3011	<i>groEL</i>	n.r.	1.97	Chaperonin GroEL
NCgl0274	cg0336		0.54	n.r.	Membrane carboxypeptidase
NCgl0440	cg0538		0.55	n.r.	Putative serine protease (ClpP class)
NCgl2124	cg2419	<i>pepB</i>	0.46	0.50	Leucyl aminopeptidase
NCgl2737	cg3138		0.23	0.27	Putative membrane protease subunit
Cell division and cell wall synthesis					
NCgl0337	cg0414	<i>wzz</i>	0.52	n.r.	Cell surface polysaccharide biosynthesis/chain length determinant protein
NCgl0344	cg0421	<i>wzx</i>	0.42	0.49	Putative translocase involved in export of a cell
NCgl1366	cg1610	<i>parA2</i>	n.r.	0.53	ATPase involved in chromosome partitioning
NCgl2084	cg2375	<i>ftsI</i>	0.54	0.49	Cell division protein FtsI
NCgl2086	cg2377	<i>mraW</i>	n.r.	0.46	S-Adenosylmethionine-dependent methyltransferase involved in cell envelope biogenesis
Regulation					
NCgl0275	cg0337	<i>whiB4</i>	2.69	n.r.	Putative regulatory protein (WhiB-related protein)
NCgl0734	cg0878	<i>whiB1</i>	1.75	n.r.	Transcription factor WhiB
NCgl1317	cg1552		n.r.	1.77	Predicted transcriptional regulator
NCgl1887	cg2152		2.02	n.r.	Predicted transcriptional regulator
NCgl1981	cg2258	<i>glnD</i>	17.11	24.32	Uridyltransferase
NCgl1982	cg2260	<i>glnK</i>	31.78	36.12	P ₁₁ -type signal transduction protein GlnK
NCgl2199	cg2500		1.95	1.80	Predicted transcriptional regulator
NCgl2518	cg2888	<i>cgtR3</i>	1.88	n.r.	Putative two-component response regulator
NCgl2684	cg3082		1.74	3.03	Predicted transcriptional regulator
NCgl0911	cg1083	<i>cgtS10</i>	n.r.	0.53	Two-component system sensory transduction histidine kinase
NCgl1345	cg1585	<i>argR</i>	0.30	0.23	Arginine repressor
NCgl1856	cg2115		0.53	0.43	Transcriptional regulators of sugar metabolism
NCgl2941	cg3373		n.r.	0.33	Predicted transcriptional regulator

^a Factors of transcriptional changes are shown for RNA isolated from batch phase cells versus continuous phase cells grown with low (b vs c_{low}) and high (b vs c_{high}) dilution rates. For each comparison of interest, two independent biological experiments were performed and two microarrays with a fourfold genome coverage were used for hybridization. The qualitative results are indicated with the concrete ratios or "n.r." (not regulated, $0.58 \geq \text{ratio} \leq 1.74$). For each functional category, genes with increased transcription are shown first (gene order according to NCgl annotation). Acc. no., accession number according to reference 19.

^b According to reference 5.

^c According to reference 3.

ible solute uptake systems is decreased in response to ammonium limitation. Compatible solutes are accumulated inside the cell during hyperosmotic stress (for a recent review, see reference 23) and cold shock. They do not interfere with the cellular metabolism and cannot serve as a nitrogen or carbon source. Under the osmotically strictly controlled conditions of continuous fermentation, their transport is dispensable. Additionally, two genes coding for putative ribitol-specific transport proteins, *mpmA* and *mpmC*, were downregulated.

Nitrogen metabolism and amino acid biosynthesis. To cope with the situation of ammonium limitation and in order to utilize the alternative nitrogen sources taken up by newly synthesized transport systems, cellular pathways used for ammonium assimilation were rearranged and those for the metabolism of other nitrogen sources were activated. In response to ammonium limitation, an enhanced transcription of the *gltBD*

operon encoding glutamate synthase (GOGAT) and the *glnA* gene, encoding glutamine synthetase (GS), was observed by DNA microarray analyses. The expression of these genes allows the assimilation of ammonium via the high-affinity GS/GOGAT pathway rather than by the low-affinity glutamate dehydrogenase reaction. Since *glnA* is the only gene encoding an active glutamine synthetase in *C. glutamicum* and already shows an expression level in nitrogen-rich medium that is enough to satisfy the cellular glutamine demand (25), transcription of *glnA* is upregulated by a factor of 5 only. In contrast, *gltBD* expression, which is almost not detectable when cells are grown in nitrogen-rich minimal medium (2, 31), is increased up to 25-fold. Additionally, an up to 10-fold increase in expression of the urease-encoding genes, the *ureABCEFGD* operon, supports the utilization of urea as an alternative nitrogen source. This result is also in accord with the recently

FIG. 1. Functional categories of genes with altered transcription profiles and their part of total regulated genes. For a complete list of genes with altered expression depending on ammonium supply, see Table S1 in the supplemental material.

published regulation of *ureABCDEFGF* expression by the master regulator of nitrogen control AmtR (3). As in the case of *glnA*, this operon also shows a considerable background expression in nitrogen-rich medium. A drastic increase in transcription upon ammonium limitation is not necessary. The enhanced expression of the *codA* gene, which codes for creatinine deaminase, allows degradation of creatinine to 1-methylhydantoin and ammonia (5). The latter can then be assimilated to produce glutamate or glutamine.

Besides an increase in transcription, a downregulation of genes was observed as well. Especially genes encoding proteins of the L-aspartate (*aspA*), L-arginine (*argB*, *argC*, *argD*, *argF*, *argG*, *argH*, *argJ*, *argR*, and *argS*), L-leucine (*leuA*), L-threonine (*thrB*), L-lysine (*lysA*), and aromatic L-amino acids biosynthesis pathways (*aroA*, *aroF*, and *aroG*) showed a decreased expression in ammonium-limited chemostat cultures.

Interestingly, expression of the *ddh* gene coding for meso-diaminopimelate dehydrogenase is downregulated, while transcription of *dapD* coding for tetrahydrodipicolinate-*N*-succinyltransferase is increased. These two enzymes are part of the split diaminopimelate pathway (30, 34), which is important for biosynthesis of cell wall precursors and of L-lysine. Downregulation of *ddh* expression impairs the low-ammonium-affinity branch of this pathway, while upregulation of *dapD* transcription increases flux via the high-ammonium-affinity branch, allowing synthesis of the cell wall building block diaminopimelate even under ammonium limitation. To avoid a drain of ammonium into L-lysine biosynthesis, which diverts from this pathway, transcription of the *lysA* gene encoding diaminopimelate decarboxylase is repressed in response to ammonium limitation.

Carbon and energy metabolism. Active transport of nitrogen sources and ammonium assimilation via the GS/GOGAT pathway needs a higher amount of energy than passive diffusion of ammonia across the cytoplasmic membrane and assimilation by glutamate dehydrogenase. As a consequence, another group of genes exhibiting increased transcription during ammonium limitation are those encoding proteins involved in energy metabolism. Examples are *fda* and *gap*, which code for the glyco-

lysis pathway enzymes fructose-1,6-diphosphate aldolase and glyceraldehyde dehydrogenase. Additionally, transcription of a number of genes encoding respiratory chain components is enhanced. These include the *sdhA*, *sdhB*, and *sdhCD* genes encoding succinate dehydrogenase proteins, *ccsB* coding for cytochrome *c* assembly protein, *qcrA1* encoding a Rieske Fe-S protein, *qcrC* coding for cytochrome *c*, *ctaE* for cytochrome oxidase subunit III, and *ctaD* for heme/copper-type cytochrome/quinol oxidase subunit I. Upregulation of expression of these genes is moderate, with factors observed between 2 and 4. This is in accord with factors observed for the increased transcription of carbon source uptake systems.

Genes with downregulated transcription in response to ammonium limitation are *aceA* and *aceB*, which code for isocitrate lyase and malate synthase, as well as *adhA*, coding for alcohol dehydrogenase.

Protein stability and turnover. The increased transcription of the *groEL* genes, coding for the GroEL chaperonin, and *groES*, coding for the GroES co-chaperonin might indicate a trend towards protein stabilization in ammonium-limited fermentation. In accord with this hypothesis, a downregulation of transcription of protease-encoding genes *pepB*, NCgl0274, NCgl0440, and NCgl2737 was observed.

Cell division and cell wall synthesis. Since ammonium limitation impairs growth and the growth rate is restricted to values of 0.075 and 0.15 h⁻¹ by the dilution rate of continuous fermentation mode, the cell division machinery of *C. glutamicum* might be at least partially dispensable under these conditions. We observed that transcription of *ftsI*, encoding the cell division protein FtsI, *wzz*, coding for a cell surface polysaccharide biosynthesis protein, *wzx*, coding for a putative translocase involved in export of a cell surface polysaccharide, *mraW*, coding for a *S*-adenosylmethionine-dependent methyltransferase involved in cell envelope biogenesis, and *parA2*, coding for a putative ATPase involved in chromosome partitioning, was decreased moderately by a factor of approximately 0.5.

Regulatory systems. Based on the strongest increases in transcription observed in ammonium-limited cells, the most important genes in this category are *glnD* and *glnK*, with factors

TABLE 2. Growth rate-dependent gene expression^a

NCBI no.	Acc. no.	Gene name	C _{low} vs C _{high}	Annotation
Transport-related				
NCgl0856	cg1016	<i>betP</i>	2.13	Choline-glycine-betaine transporter
Nitrogen metabolism				
NCgl2715	cg3114	<i>cysN</i>	0.43	Sulfate adenylate transferase subunit 1
NCgl2716	cg3115	<i>cysD</i>	0.40	Sulfate adenylate transferase subunit 2
NCgl2717	cg3116	<i>cysH</i>	0.55	Phosphoadenosine phosphosulfate reductase
NCgl2718	cg3118	<i>cysI</i>	0.42	Putative nitrite reductase
NCgl2719	cg3119	<i>cysJ</i>	0.36	Putative ferredoxin/ferredoxin-NADP reductase
Carbon and energy metabolism				
NCgl0356	cg0442	<i>galU2</i>	1.96	UDP-glucose pyrophosphorylase
NCgl0817	cg0973	<i>pgi</i>	1.83	Glucose-6-phosphate isomerase
NCgl1161	cg1364	<i>atpF</i>	2.06	F ₀ F ₁ -type ATP synthase b subunit
NCgl1162	cg1365	<i>atpH</i>	2.32	F ₀ F ₁ -type ATP synthase δ subunit
NCgl1163	cg1366	<i>atpA</i>	2.02	F ₀ F ₁ -type ATP synthase α subunit
NCgl1164	cg1367	<i>atpG</i>	1.85	F ₀ F ₁ -type ATP synthase γ subunit
NCgl1165	cg1368	<i>atpD</i>	1.76	F ₀ F ₁ -type ATP synthase β subunit
NCgl1166	cg1369	<i>atpC</i>	1.82	F ₀ F ₁ -type ATP synthase ε subunit
NCgl1482	cg1737	<i>acn</i>	3.92	Aconitase A
NCgl1512	cg1774	<i>tkt</i>	1.88	Transketolase
NCgl2579	cg2954	<i>cynT</i>	1.99	Carbonic anhydrase
NCgl2904	cg3335	<i>mez</i>	0.22	Malic enzyme
Protein stability and turnover				
NCgl0477	cg0582	<i>rpsG</i>	1.76	Ribosomal protein S7
NCgl0515	cg0628	<i>rpsH</i>	2.09	Ribosomal protein S8
NCgl0516	cg0629	<i>rplF</i>	1.82	Ribosomal protein L6
NCgl0556	cg0673	<i>rplM</i>	1.89	Ribosomal protein L13
NCgl0557	cg0674	<i>rpsI</i>	2.10	Ribosomal protein S9
NCgl2261	cg2573	<i>rpsT</i>	2.06	Ribosomal protein S20
NCgl1075	cg1271	<i>sigE</i>	0.29	DNA-directed RNA polymerase specialized sigma subunits
NCgl1844	cg2102	<i>sigB</i>	0.40	DNA-directed RNA polymerase sigma subunit SigB
NCgl2520	cg2890		0.55	Amino acid processing enzyme
NCgl2682	cg3079	<i>clpB</i>	0.55	ATPase with chaperone activity, ATP-binding subunit
Cell division and cell wall synthesis				
NCgl0885	cg1052	<i>cmt3</i>	1.77	Corynomycyl transferase
NCgl0987	cg1170	<i>cmt5</i>	2.09	Corynomycyl transferase
NCgl2101	cg2394	<i>cmt4</i>	1.81	Corynomycyl transferase

^a Factors of transcriptional changes are shown for RNA isolated from cells grown with low and high dilution rates in continuous fermentations (C_{low} vs C_{high}). For each comparison of interest, two independent biological experiments were performed and two microarrays with a fourfold genome coverage were used for hybridization. For each functional category, genes with increased transcription are shown first (gene order according to NCgl annotation). Acc. no., accession number according to reference 22.

of increase of about 17 and 32. This is in accord with the function of the corresponding gene products, since *glnD* and *glnK* encode essential proteins for the *C. glutamicum* nitrogen starvation response (26).

Besides these drastic changes, moderate alterations (with factors between 0.3 and 3.0) in the transcription of genes coding for various regulators (*argR*, *whiB4*, *whiB1*, NCgl1317, NCgl1856, NCgl1887, NCgl2199, NCgl2684, and NCgl2941) and two different proteins of two-component signal transduction systems (the *cgtR3* gene encoding a response regulator and the *cgtS10* gene coding for a sensor kinase) were observed (Table 1), indicating a modulation of other metabolic pathways not directly connected to nitrogen metabolism.

Growth rate-dependent effects on transcription. The chemostat experiments carried out allowed not only insights into ammonium limitation-dependent expression of genes but also the first data on growth rate-dependent transcription. When ammonium-limited fermentations with different dilution rates

were compared, several genes were identified that showed a growth rate-dependent expression pattern (Table 2). Transcription of the *atpFHAGDC* genes encoding ATP synthase F₀ subunit b and F₁ subunits δ, α, γ, β, and ε as well as expression of the *cmt3*, *cmt4*, and *cmt5* genes coding for corynomycyl transferases, the *tkt* gene coding for transketolase, the *galU2* gene for UDP-glucose pyrophosphatase, the *pgi* gene for glucose-6-phosphate isomerase, *cynT* encoding carbonic anhydrase, *betP* coding for an osmoregulated glycinebetaine uptake system, and six genes encoding ribosomal proteins are increased in faster growing cells. In contrast to this upregulated gene expression, transcription of the *cysD*, *cysH*, *cysI*, *cysJ*, and *cysN* genes, coding for enzymes involved in sulfur metabolism, the *mez* gene, coding for malic enzyme, the sigma factor-encoding *sigB* and *sigE* genes, as well as *clpB*, coding for an ATPase with chaperone activity, is decreased in fast-growing cells.

TABLE 3. Proteins identified by 2-D PAGE and peptide mass fingerprint analysis^a

Spot	NCgl no.	Acc. no.	Gene name	Annotation	MW (kDa)	pI	Ratio
Nitrogen metabolism							
26	NCgl0084	cg0114	<i>ureB</i>	Urease β subunit	17.6	4.48	Up
40	NCgl2133	cg2429	<i>glnA</i>	Glutamine synthetase I	53.3	4.90	Up
49	NCgl1520	cg1784	<i>ocd</i>	Putative ornithine-cyclodecarboxylase Ocd	41.0	4.71	Up
61	NCgl0088	cg0118	<i>ureG</i>	Ni ²⁺ -binding GTPase	22.4	4.58	Up
73	NCgl0083	cg0113	<i>ureA</i>	Urea amidohydrolase (urease) γ subunit	11.2	5.18	Up
74	NCgl0085	cg0115	<i>ureC</i>	Urea amidohydrolase (urease) α subunit	61.5	5.14	Up
79	NCgl0075	cg0104	<i>codA^b</i>	Creatinine deaminase	46.3	5.21	Up
81	NCgl0075	cg0104	<i>codA^b</i>	Creatinine deaminase	46.3	5.21	Up
94	NCgl0086	cg0116	<i>ureE</i>	Urease accessory protein	17.6	5.28	Up
105	NCgl1061	cg1256	<i>dapD</i>	Tetrahydrodipicolinate- <i>N</i> -succinyltransferase	31.2	5.43	Up
114	NCgl0089	cg0119	<i>ureD</i>	Urease-associated protein	31.6	5.82	Up
13	NCgl1224	cg1437	<i>ilvC</i>	Ketol-acid reductoisomerase	36.2	4.62	n.r.
16	NCgl0075	cg0104	<i>codA^b</i>	Cytosine deaminase or related metal-dependent hydrolase	46.3	5.21	n.r.
43	NCgl1235	cg1451	<i>serA</i>	Phosphoglycerate dehydrogenase	55.3	4.70	n.r.
57	NCgl1520	cg1784	<i>ocd</i>	Putative ornithine-cyclodecarboxylase	41.0	4.71	n.r.
89	NCgl1519	cg1783	<i>soxA</i>	Sarcosine oxidase soxA	31.0	5.10	n.r.
95	NCgl1520	cg1784	<i>ocd</i>	Putative ornithine-cyclodecarboxylase	41.0	4.71	n.r.
102	NCgl1999	cg2280	<i>gdh</i>	Glutamate dehydrogenase	49.0	5.57	n.r.
17	NCgl1224	cg1437	<i>ilvC</i>	Ketol-acid reductoisomerase	36.2	4.40	Down
41	NCgl0245	cg0303	<i>leuA</i>	2-Isopropylmalate synthase	68.2	4.79	Down
68	NCgl1341	cg1581	<i>argJ</i>	Glutamate <i>N</i> -acetyltransferase/amino-acid <i>N</i> -acetyltransferase	39.7	4.28	Down
Carbon and energy metabolism							
14	NCgl1513	cg1776	<i>tal</i>	Transaldolase	38.3	4.47	Up
27	NCgl2167	cg2466	<i>aceE</i>	Pyruvate dehydrogenase, decarboxylase component	102.8	5.26	Up
36	NCgl2167	cg2466	<i>aceE</i>	Pyruvate dehydrogenase, decarboxylase component	102.8	5.26	Up
77	NCgl2521	cg2891	<i>poxB</i>	Pyruvate:quinone oxidoreductase	62.0	5.34	Up
86	NCgl1526	cg1791	<i>gap</i>	Glyceraldehyde-3-phosphate dehydrogenase	36.0	5.16	Up
90	NCgl1162	cg1365	<i>atpH</i>	F ₀ F ₁ -type ATP synthase δ subunit	28.9	5.37	Up
97	NCgl2167	cg2466	<i>aceE</i>	Pyruvate dehydrogenase, decarboxylase component	102.8	5.26	Up
98	NCgl2167	cg2466	<i>aceE</i>	Pyruvate dehydrogenase, decarboxylase component	102.8	5.26	Up
99	NCgl2167	cg2466	<i>aceE</i>	Pyruvate dehydrogenase, decarboxylase component	102.8	5.26	Up
1	NCgl1482	cg1737	<i>acn</i>	Aconitase A	102.2	4.53	n.r.
44	NCgl0935	cg1111	<i>eno</i>	Enolase	44.9	4.65	n.r.
54	NCgl2673	cg3068	<i>fda</i>	Fructose-bisphosphate aldolase	37.2	5.04	n.r.
58	NCgl2297	cg2613	<i>mdh</i>	Malate/lactate dehydrogenase	34.9	4.79	n.r.
76	NCgl2521	cg2891	<i>poxB</i>	Pyruvate:quinone oxidoreductase	62.0	5.34	n.r.
83	NCgl1526	cg1791	<i>gap</i>	Glyceraldehyde-3-phosphate dehydrogenase	36.0	5.16	n.r.
84	NCgl0795	cg0949	<i>gltA</i>	Citrate synthase	48.9	5.19	n.r.
100	NCgl2008	cg2291	<i>pyk</i>	Pyruvate kinase	51.6	5.43	n.r.
101	NCgl0954	cg1133	<i>glyA</i>	Glycine hydroxymethyltransferase	46.5	5.42	n.r.
107	NCgl0666	cg0798	<i>prpC1</i>	(Methyl) citrate synthase	42.6	5.51	n.r.
59	NCgl2582	cg2958	<i>butA</i>	L-2,3-Butanediol dehydrogenase	27.1	4.62	Down
78	NCgl2167	cg2466	<i>aceE</i>	Pyruvate dehydrogenase, decarboxylase component	102.8	5.26	Down
103	NCgl2008	cg2291	<i>pyk</i>	Pyruvate kinase	51.6	5.43	Down
Protein stability and turnover							
21	NCgl0902	cg1072	<i>rplY</i>	Ribosomal protein L25	21.8	4.29	Up
29	NCgl2340	cg2662	<i>pepN</i>	Aminopeptidase N	96.1	4.64	Up
7	NCgl2702	cg3100	<i>dnaK</i>	70-kDa heat shock chaperonin	66.3	4.55	n.r.
33	NCgl2915	cg3346	<i>leuS</i>	Leucyl-tRNA synthetase	108.2	4.63	n.r.
34	NCgl0480	cg0587	<i>tuf</i>	Elongation factor EF-Tu	43.9	4.92	n.r.
50	NCgl2227	cg2536		PLP-dependent aminotransferase	40.7	4.76	n.r.
53	NCgl0480	cg0587	<i>tuf</i>	Elongation factor Tu	43.9	4.92	n.r.
63	NCgl0033	cg0048	<i>ppiA</i>	Peptidyl-prolyl <i>cis-trans</i> isomerase (rotamase)	18.5	4.70	n.r.
8	NCgl1304	cg1531	<i>rpsA</i>	Ribosomal protein S1	54.0	4.56	Down
9	NCgl2329	cg2647	<i>tig</i>	Putative trigger factor	49.7	4.40	Down
10	NCgl2631	cg3021		Peptidase, M20/M25/M40 family	48.5	4.40	Down
11	NCgl0540	cg0655	<i>rpoA</i>	DNA-directed RNA polymerase α subunit	36.7	4.33	Down
19	NCgl0902	cg1072	<i>rplY</i>	Ribosomal protein L25	21.8	4.29	Down
70	NCgl0469	cg0573	<i>rplL</i>	Ribosomal protein L7/L12	13.3	4.40	Down
92	NCgl0573	cg0693	<i>groEL</i>	Chaperonin GroEL	45.3	4.72	Down
96	NCgl0468	cg0572	<i>rplJ</i>	Ribosomal protein L10	17.9	5.50	Down
104	NCgl1950	cg2222	<i>rpsB</i>	Ribosomal protein S2	30.1	5.35	Down
109	NCgl0480	cg0587	<i>tuf</i>	Elongation factor EF-Tu	43.9	4.92	Down

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TABLE 3—Continued

Spot	NCgl no.	Acc. no.	Gene name	Annotation	MW (kDa)	pI	Ratio
Regulation							
62	NCgl1385	cg1630		Putative signal transduction protein, FHA-domain-containing protein	15.4	4.76	Up
64	NCgl2440	cg2783		Transcriptional regulator, GntR family	27.2	4.91	Up
65	NCgl1982	cg2260	<i>glnK</i>	PII-type signal transduction protein-GlnK	12.2	5.01	Up
66	NCgl1982	cg2260	<i>glnK</i>	PII-type signal transduction protein-GlnK	12.2	4.86	Up
Others							
48	NCgl2450	cg2796		Hypothetical protein	49.3	4.77	Up
106	NCgl0620	cg0750	<i>folD</i>	Methylenetetrahydrofolate dehydrogenase	30.1	5.51	Up
115	NCgl1099	cg1295		Predicted hydrolase	28.8	5.73	Up
116	NCgl2602	cg2983	<i>folE</i>	GTP cyclohydrolase I	22.0	6.08	Up
2	NCgl2499	cg2862	<i>purL</i>	Phosphoribosylformylglycinamide synthase, synthetase domain	80.8	4.48	n.r.
25	NCgl1316	cg1551	<i>uspA1</i>	Universal stress protein UspA	15.4	4.56	n.r.
46	NCgl1012	cg1203		Mg-chelatase subunit ChII	50.1	4.72	n.r.
69	NCgl1731	cg2025		Hypothetical protein	11.3	5.20	n.r.
85	NCgl2955	cg3389		Putative dehydrogenase	39.6	4.94	n.r.
88	NCgl2449	cg2795		Putative Zn-NADPH:quinone dehydrogenase	35.8	5.16	n.r.
93	NCgl2826	cg3237	<i>sod</i>	Superoxide dismutase	22.1	5.14	n.r.
112	NCgl0578	cg0699	<i>guaB2</i>	Inosine monophosphate dehydrogenase	53.4	5.98	n.r.
113	NCgl1529	cg1794		Predicted P-loop-ATPase protein	34.7	6.01	n.r.
6	NCgl0134	cg0173		Hypothetical protein	62.2	4.45	Down
23	NCgl2439	cg2782	<i>ftn</i>	Ferritin-like protein	18.1	4.42	Down
24	NCgl0727	cg0870		Predicted hydrolase (HAD superfamily)	14.5	4.29	Down
71	NCgl2287	cg2603	<i>ndk</i>	Nucleoside diphosphate kinase	14.8	4.97	Down
91	NCgl1824	cg2079		Hypothetical protein	27.0	5.10	Down
108	NCgl0620	cg0750	<i>folD</i>	Methylenetetrahydrofolate dehydrogenase	30.1	5.51	Down
Multiple protein containing spots (2 or more proteins per spot)							
18	NCgl0937	cg1113		Hypothetical protein	20.2	4.58	Up
	NCgl1316	cg1551	<i>uspA1</i>	Universal stress protein UspA	15.4	4.56	
20	NCgl0902	cg1072	<i>rplY</i>	Ribosomal protein L25	21.8	4.29	Up
	NCgl0385	cg0475		Hypothetical protein	17.8	4.30	
28	NCgl2340	cg2662	<i>pepN</i>	Aminopeptidase N	96.1	4.64	Up
	NCgl1336	cg1575	<i>pheT</i>	Phenylalanyl-tRNA synthetase β subunit	89.4	4.65	
37	NCgl1607	cg1880	<i>thrS</i>	Threonyl tRNA ligase	78.1	5.04	Up
	NCgl1094	cg1290	<i>metE</i>	Methionine synthase II	81.3	4.78	
	NCgl2150	cg2450		Putative pyridoxine biosynthesis enzyme	67.5	4.87	
	NCgl1471	cg1725	<i>mcmB</i>	Methylmalonyl-CoA mutase, N-terminal domain/subunit	80.1	4.94	
38	NCgl1900	cg2166	<i>gpsI</i>	Putative polyribonucleotide nucleotidyltransferase	81.3	5.04	Up
	NCgl2037	cg2323	<i>treY</i>	Maltooligosyl trehalose synthase	90.5	5.03	
39	NCgl1900	cg2166	<i>gpsI</i>	Putative polyribonucleotide nucleotidyltransferase	81.3	5.04	Up
	NCgl1384	cg1629	<i>secA2</i>	Preprotein translocase subunit SecA	83.5	4.95	
	NCgl0478	cg0583	<i>fusA</i>	Elongation factor G	77.9	4.81	
56	NCgl0390	cg0482	<i>gpmA</i>	Phosphoglycerate mutase I	27.2	4.91	Up
	NCgl2451	cg2797		Hypothetical protein	30.4	4.86	
60	NCgl2582	cg2958	<i>butA</i>	L-2,3-Butanediol dehydrogenase	27.1	4.62	Up
	NCgl0242	cg0299		Predicted glutamine amidotransferase	26.4	4.67	
3	NCgl2133	cg2429	<i>glnA</i>	Glutamine synthetase I	53.3	4.90	n.r.
	NCgl1071	cg1267		β -Fructosidase (levanase/invertase)	54.6	4.34	
12	NCgl0540	cg0655	<i>rpoA</i>	DNA-directed RNA polymerase α subunit	36.7	4.33	n.r.
	NCgl1525	cg1790	<i>pgk</i>	3-Phosphoglycerate kinase	42.7	4.40	
15	NCgl1525	cg1790	<i>pgk</i>	3-Phosphoglycerate kinase	42.7	4.40	n.r.
	NCgl0540	cg0655	<i>rpoA</i>	DNA-directed RNA polymerase α subunit	36.7	4.33	
	NCgl0794	cg0948	<i>serC</i>	Phosphoserine aminotransferase	40.0	4.53	
22	NCgl0902	cg1072	<i>rplY</i>	Ribosomal protein L25	21.8	4.29	n.r.
	NCgl2439	cg2782	<i>ftn</i>	Ferritin-like protein	18.1	4.42	
35	NCgl1512	cg1774	<i>tkt</i>	Transketolase	75.1	4.80	n.r.
	NCgl1094	cg1290	<i>metE</i>	Methionine synthase II	81.3	4.78	
42	NCgl2217	cg2523	<i>malQ</i>	4- α -Glucanotransferase	78.5	4.58	n.r.
	NCgl0478	cg0583	<i>fusA</i>	Elongation factor G	77.9	4.81	
	NCgl1094	cg1290	<i>metE</i>	Methionine synthase II	81.3	4.78	

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TABLE 3—Continued

Spot	NCgl no.	Acc. no.	Gene name	Annotation	MW (kDa)	pI	Ratio
47	NCgl1012	cg1203		Mg-chelatase subunit ChII	50.1	4.72	n.r.
	NCgl1235	cg1451	<i>serA</i>	Phosphoglycerate dehydrogenase	55.3	4.70	
51	NCgl2070	cg2361		Cell division initiation protein	38.7	4.87	n.r.
	NCgl10730	cg0873	<i>aroA</i>	5-Enolpyruvylshikimate-3-phosphate synthase	45.5	4.86	
	NCgl1548	cg1814	<i>carA</i>	Carbamoylphosphate synthase, small subunit	42.1	4.87	
52	NCgl0967	cg1145	<i>fum</i>	Fumarase	49.8	4.92	n.r.
	NCgl2153	cg2453		Exoribonucleases	54.2	4.85	
	NCgl2698	cg3096		NAD-dependent aldehyde dehydrogenase	55.1	4.82	
55	NCgl0390	cg0482	<i>gpmA</i>	Phosphoglycerate mutase I	27.2	4.91	n.r.
	NCgl2451	cg2797		Hypothetical protein	30.4	4.86	
80	NCgl1442	cg1693	<i>pepC</i>	Aspartyl aminopeptidase	44.9	5.10	n.r.
	NCgl0625	cg0755	<i>metY</i>	O-Acetylhomoserine (thiol)-lyase	46.8	5.16	
	NCgl0345	cg0422	<i>murA</i>	UDP-N-acetylglucosamine enolpyruvyl transferase	46.9	5.06	
75	NCgl1926	cg2192	<i>mgo</i>	Malate:quinone oxidoreductase	54.8	5.18	n.r.
	NCgl0827	cg0984	<i>purH</i>	5'-Phosphoribosyl-5-aminoimidazole-4-carboxamide formyl-transferase	55.8	5.26	
4	NCgl2133	cg2429	<i>glnA</i>	Glutamine synthetase I	53.3	4.90	Down
	NCgl1071	cg1267		β -Fructosidase (levanase/invertase)	54.6	4.34	
5	NCgl2133	cg2429	<i>glnA</i>	Glutamine synthetase I	53.3	4.90	Down
	NCgl1071	cg1267		β -Fructosidase (levanase/invertase)	54.6	4.34	
72	NCgl2826	cg3237	<i>sod</i>	Superoxide dismutase	22.1	5.14	Down
	NCgl2471	cg2830	<i>pduO</i>	Adenosylcobalamin-dependent diol dehydratase	21.0	5.01	
82	NCgl1502	cg1763	<i>sufB</i>	Predicted iron-regulated ABC-type transporter SufB	42.3	5.04	Down
	NCgl0075	cg0104	<i>codA^b</i>	Creatinine deaminase	45.5	5.04	
87	NCgl2716	cg3115	<i>cysD</i>	Sulfate adenyltransferase subunit 2	34.3	5.09	Down
	NCgl0754	cg0898		Pyridoxine biosynthesis enzyme	31.7	5.09	
No identification							
110				No similar protein found			Up
111				No similar protein found			Up
30				No similar protein found			n.r.
31				No similar protein found			n.r.
32				No similar protein found			n.r.
67				No similar protein found			n.r.
45				No similar protein found			Down

^a Cytoplasmic proteins were isolated from cells which were cultivated in batch (nitrogen excess) and continuous mode of a fermentation at a low dilution rate (nitrogen limitation, 0.075 h^{-1}). Accession numbers according to the NCBI data base and to reference 19 (Acc. no.) are given. Molecular mass (MW) and isoelectric point (pI) correspond to the NCBI database. Delta2D software (version 3.2; Decodon, Greifswald, Germany) was used for protein quantification. Proteins which showed two or more-fold changes in their expression pattern in all biological and technical replicates were regarded as regulated. The ratio refers to the relative volume of the corresponding protein spot in continuous versus batch mode of fermentation. The qualitative results are indicated with the marks "up" (ratio above 2.0), "down" (ratio below 0.5), and "n.r." (not regulated, $0.5 > \text{ratio} < 2.0$) in the ratio column.

^b According to reference 5.

Proteome analysis. While transcriptome analyses reveal changes in the amount of mRNA transcribed, proteome analyses allow us to compare the actual amount of proteins present under different environmental conditions. Previously carried out proteome analyses of *C. glutamicum* shaking flask cultures revealed that differences in the protein profile of cells grown under nitrogen-rich conditions and nitrogen-starved cells are hardly detectable when conventional staining techniques (i.e., Coomassie or silver staining) are used. To circumvent this problem, in different studies in vivo, [³⁵S]methionine labeling and autoradiography have been used to improve the sensitivity of two-dimensional gel electrophoresis (26, 29). Besides these methods relying on the use of radio-nucleotides, the use of continuous fermentation is an interesting alternative. Continuous fermentation offers the possibility to obtain a well-defined and highly reproducible growth state of the cells. The cell population can adjust over a long period of time to a constant environment, which significantly improves the sensitivity of the proteome approach as shown in this study.

Spots detected with different intensities and sizes depending

on the nitrogen supply were excised from the gel, and the proteins were identified by peptide mass fingerprint analyses using tryptic in-gel digest and MALDI-TOF MS (Table 3; Fig. 2). Protein spots with increased size on gels loaded with cell extract from nitrogen-deprived cultures were identified as six of the seven urease subunits (the *ureA*, *ureB*, *ureC*, *ureE*, *ureG*, and *ureD* gene products), a putative ornithine cyclodeaminase (encoded by the *ocd* gene), creatinine deaminase (*codA* gene product), the GlnK protein, tetrahydrodipicolinate-N-succinyltransferase (encoded by *dapD*), and the *glnA* gene product glutamine synthetase. Besides these proteins involved in nitrogen metabolism, proteins of carbon and energy metabolism were found, namely, glyceraldehyde-3-phosphate dehydrogenase (*gap* gene product) and the ATP synthetase $F_1 \delta$ subunit (encoded by *atpH*). Additionally, a putative L-2,3-butanediol dehydrogenase (*butA*) and two hypothetical proteins (NCgl2450 and NCgl2451) were found to be present in higher amounts during nitrogen limitation.

Proteins with decreased concentration in response to nitrogen starvation were 2-isopropylmalate synthase (*leuA*), gluta-

FIG. 2. Protein profiles of *C. glutamicum* cells grown with different nitrogen supply. Two-dimensional protein profiles of cytoplasmic proteins of batch (nitrogen-rich) phase (A) and continuous (nitrogen-limited) phase at a low dilution rate (0.075 h^{-1}) (B). One hundred micrograms of protein was separated on each gel; staining was carried out with Coomassie brilliant blue. For each condition, at least two independent gels were analyzed and only spots which showed the same pattern in all gels were subjected to MALDI-TOF MS. Positions of molecular weight markers and pH values are indicated. Numbers indicate the positions of proteins in Table 3.

mate *N*-acetyltransferase (*argJ*), and sulfate adenylyl transferase subunit 2 (*cysD*), which are involved in the biosynthesis of different amino acids.

An interesting aspect of proteome analyses is the possibility to identify protein modifications. In this study, we were able to identify the modification of the central nitrogen signal transduction protein GlnK, which is an AMP group protein (32). Spot 65 corresponds to the unmodified GlnK protein, while the protein of spot 66 carries an adenylylation as indicated by MALDI-TOF MS (data not shown). Additionally, a pI shift of glutamine synthesis from approximately 4.6 to 5.1 was observed when cells were grown under nitrogen limitation. This is in accord with a deadenylylation of the GS enzyme in response to nitrogen deprivation, which was reported previously (17, 18).

DISCUSSION

Transcriptome analyses by DNA microarrays and proteome analyses allow a global view of the responses of a cell induced by changes in environmental conditions. In this study, the response of the biotechnologically important mycolic acid-containing actinomycete *C. glutamicum* to ammonium limitation

was tested in chemostat experiments. A major advantage of the cultivation in continuous fermentation mode is the possibility to establish highly defined long-term conditions of nutrient limitation, while in shaking flasks only a nutrient starvation can be initiated by a total lack of nutrients. Moreover, by comparing different dilution rates in ammonium-limited chemostats, we were able to distinguish between specific and general cellular responses to ammonium limitation and to separate these from growth rate-dependent regulatory mechanisms.

The core response of ammonium-limited cells is characterized by the highest factors of increases in transcription. Our transcriptome analyses indicated that scavenging of nitrogen sources, an adaptation strategy which was first observed for the gram-negative model organism *Escherichia coli* (36), is a major response to nitrogen starvation in the gram-positive *C. glutamicum* as well. In this bacterium, synthesis of new transport systems includes ammonium uptake systems, a creatinine permease, an uptake system for urea, and a putative peptide transport system.

An adaptation of similar importance might be the rearrangement of metabolic pathways. As indicated by our transcriptome data, the GS/GOGAT pathway is recruited for ammo-

B

FIG. 2—Continued.

nium assimilation during transcription. Interestingly, the factor of upregulation of *gltBD* expression observed in this study is much higher than previously reported (31). This result indicates an extremely high sensitivity of the DNA microarray approach applied here. This view is supported by the fact that the *ureABCEFGD* cluster was found to be highly upregulated as well. Most likely due to low mRNA abundance, a transcriptional upregulation of these genes was not detected previously although urease activity increased significantly in response to nitrogen limitation in shaking flask experiments (24) and only very recently the mechanism of transcription control of the urease-encoding genes has been solved (3).

Besides the need for sufficient nitrogen sources, a bacterial cell is challenged by an extra demand of energy when subjected to nitrogen limitation. It has already been published that *C. glutamicum* has an extremely high energy demand in case of a complete fixation of ammonium via the GS/GOGAT pathway in comparison for example to *E. coli* (29). These calculations were based on the nitrogen-assimilating enzymes only and did not include the extra demand of energy necessary for enhanced transport processes or synthesis of new proteins. As a consequence, increasing the amount of glycolytic enzymes and redox chain components as indicated by the transcriptome analyses is a reasonable response to nitrogen starvation. The results obtained by transcriptome analyses are supported by the experiments of Schmid and coworkers

(29) who observed an enhanced oxygen consumption of cells deprived of nitrogen sources. In contrast to this situation in *C. glutamicum*, a decreased respiration rate during nutrient starvation was observed for the closely related pathogen *M. tuberculosis* (6). However, in this case cells were suspended in phosphate-buffered saline and therefore deprived of nitrogen and carbon sources as well as various other nutrients. In addition to the changes in expression of genes encoding glycolytic enzymes and respiratory chain components, carbon uptake systems were induced. The factors of upregulation observed for the increase in transcription of genes encoding components of carbon metabolism and respiration were quite moderate, indicating a more general or indirect response to the situation of ammonium limitation.

During nitrogen limitation in continuous fermentation mode, the growth rate is restricted to very low values. The decreased transcription of two genes encoding proteins with a function in cell division as well as three genes coding for cell envelope biosynthesis proteins indicated a reduction of the cell division machinery of *C. glutamicum*. Additionally, the overall protein stability was increased by positive regulation of three chaperonins on the one hand and the decreased expression of five different protease- and peptidase-encoding genes.

The comparison of different dilution rates in ammonium-limited chemostats allowed us to detect even more indirect effects. Some genes, e.g., those encoding ATP synthase subunits, we reg-

ulated in parallel to the growth rate of *C. glutamicum*, which is of course also influenced by nitrogen availability but also by other factors (trace elements, temperature, etc.). In shaking flasks experiments, it is impossible to distinguish between specific or indirect effects of nitrogen limitation, since a starvation can only be realized by complete lack of any nitrogen source.

The transcriptome approach used in this study was combined with and validated by proteome analyses, a combination used by other groups for different bacteria (11, 12, 22, 35) as well. The lack of a suitable method for the two-dimensional gel electrophoresis of membrane proteins with more than one transmembrane helix is a clear drawback of proteome analyses. This is also the reason why only cytoplasmic proteins were analyzed by 2-D PAGE in this study although a considerable number of transporter-encoding genes were identified by transcriptome analyses. However, the advantage of this combination results from the fact that proteome techniques allow us to analyze not only the synthesis but also the modification of proteins (4), which might be of major importance for regulatory networks. As shown in this study, the usefulness of these global analysis techniques can be further improved by their combination with the use of continuous cultivation techniques. The longer adaptation time for the cells allows a better analysis of differences in protein profiles.

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