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Accumulation of Homolanthionine and Activation of a Novel Pathway for Isoleucine Biosynthesis in *Corynebacterium glutamicum* McbR Deletion Strains

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In the present work, the metabolic consequences of the deletion of the methionine and cysteine biosynthesis repressor protein (McbR) in *Corynebacterium glutamicum*, which releases almost all enzymes of methionine biosynthesis and sulfate assimilation from transcriptional regulation (D. A. Rey, A. Pühler, and J. Kalinowski, J. Biotechnol. 103:51–65, 2003), were studied. *C. glutamicum* ATCC 13032 Δ*mcbR* showed no overproduction of methionine. Metabolome analysis revealed drastic accumulation of a single metabolite, which was not present in the wild type. It was identified by isotopic labeling studies and gas chromatography/mass spectrometry as L-homolanthionine {*S*-[(3*S*)-3-amino-3-carboxypropyl]-L-homocysteine}. The accumulation of homolanthionine to an intracellular concentration of 130 mM in the Δ*mcbR* strain was accompanied by an elevated intracellular homocysteine level. It was shown that cystathionine-γ-synthase (MetB) produced homolanthionine as a side reaction. MetB showed higher substrate affinity for cysteine (K_m = 260 μM) than for homocysteine (K_m = 540 μM). The cell is able to cleave homolanthionine at low rates via cystathionine-β-lyase (MetC). This cleavage opens a novel threonine-independent pathway for isoleucine biosynthesis via 2-oxobutanoate formed by MetC. In fact, the deletion mutant exhibited an increased intracellular isoleucine level. Metabolic flux analysis of *C. glutamicum* Δ*mcbR* revealed that only 24% of the *O*-acetylhomoserine at the entry of the methionine pathway is utilized for methionine biosynthesis; the dominating fraction is either stored as homolanthionine or redirected towards the formation of isoleucine. Deletion of *metB* completely prevents homolanthionine accumulation, which is regarded as an important step in the development of *C. glutamicum* strains for biotechnological methionine production.

The gram-positive bacterium *Corynebacterium glutamicum* is an important organism for the industrial production of fine chemicals, such as the amino acids lysine and glutamate (3). Methionine, applied in large amounts for animal nutrition, is, however, produced by a chemical process yielding the racemic DL mixture and employing rather hazardous chemicals (19). In this regard, *C. glutamicum* appears as a promising candidate for future biotechnological production of L-methionine. Accordingly, the biosynthetic pathway for this amino acid has been the focus of research in recent years (9, 10, 26–28). In *C. glutamicum*, methionine biosynthesis is carried out by parallel pathways of transsulfuration and direct sulfhydrylation (10, 18). The corresponding genes were recently identified by targeted gene deletion and homologous complementation (28). Moreover, the regulation of the methionine pathway was studied by proteome (27) and transcriptome (26) analyses. Hereby, a transcriptional repressor (McbR) that controls 86 genes was identified. Among these genes are several of methionine and cysteine metabolism, including *hom* (homoserine dehydrogenase), *metX* (homoserine *O*-acetyltransferase), *metB* (cystathionine-γ-synthase), *metY* (*O*-acetylhomoserine sulfhydrylase), *metE* and *metH* (cysteine synthases I and II, respectively), *metK* (*S*-adenosylmethionine synthetase), *cysJ* and *cysI* (putative sulfite reductases), and *cysK* (*O*-acetylserine sulfhydrylase). Additionally, McbR regulates its own expression and that of at least two other putative transcriptional regulators, indicating a complex regulatory network with McbR as the master regulator (26). Different enzymes of the methionine pathway are further regulated by feedback inhibition (17). This involves homoserine dehydrogenase (controlled by threonine), homoserine *O*-acetyltransferase (methionine, *S*-adenosylmethionine, and *O*-acetylhomoserine), cystathionine-γ-synthase (*S*-adenosylmethionine), cystathionine-β-lyase (methionine, cysteine, and glycine), and *O*-acetylhomoserine sulfhydrylase (methionine and *O*-acetylserine). It has been suggested that the deletion of McbR might be an important step in constructing a methionine-overproducing organism. In the present work, the effect of deleting this repressor in *C. glutamicum* was investigated from a metabolic perspective.

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MATERIALS AND METHODS

Bacterial strains. The wild-type *Corynebacterium glutamicum* ATCC 13032 was obtained from the American Type Culture Collection (Manassas, VA). The *C. glutamicum* Δ*mcbR* strain was derived from the wild type. *C. glutamicum* Δ*mcbR* Δ*metB* was an McbR knockout strain in which cystathionine γ-synthase was also deleted. *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk* was a threonine and homoserine auxotrophic strain also derived from the wild type. The knockout mutants were constructed as follows. The deletion of *mcbR* in *C. glutamicum* ATCC 13032 was performed by using the primer pairs BK1987/BK1968 and BK1967/BK1988 for crossover PCR (Table 1). This resulted in a DNA construct containing the upstream and downstream sequences of the *mcbR* gene but not its coding sequence. The obtained PCR construct containing the deleted *mcbR*

TABLE 1. Primer sequences used for the construction of *C. glutamicum* Δ*mcbR*, *C. glutamicum* Δ*mcbR* Δ*metB*, and *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk*

Primer	Sequence
BK1987	GAGAGAGACTCGAGCTCTCCAATCTCCACTGAGG
BK1967	ACTCTTGCCTGAAGCGCTAGCAGCCACGTT
BK1968	GAGAGAGGCTAGCTAATCCTTGATGGTGGA
BK1988	CTCTCTCTACGCGTCAGCAACAACCTGTGGACGC
HS 605	BamHI GCGGGATCCATGACCTCAGCATCT
HS 606	CCCATCCACTAAACTTAAACACCGCTGCATCAGCAA
HS 607	TGTTTAAGTTTAGTGGATGGGGATGCTCGTGAGTCT
HS 608	BamHI GCGGGATCCATCTTCCAAACACGC

allele was then integrated into *C. glutamicum* ATCC 13032 for first and second recombinants. As vector, the plasmid pClik, which carried kanamycin resistance and the *sacB* gene as selection markers, was used. Since pClik cannot replicate in *C. glutamicum*, transformation of the organism with the plasmid and subsequent selection for the kanamycin resistance plasmid marker yielded transformants which had integrated the plasmid DNA into the genome via a single-crossover homologous-recombination event. Subsequently, each kanamycin-resistant integrant was grown for 1 day without kanamycin to allow a second recombination event to take place. The *sacB*-positive selection system (11) was used to select for the second recombination event. Since the expression of integrated plasmid-borne *sacB* in the presence of sucrose is lethal to *C. glutamicum*, cells can grow only on the selective plate if *sacB* has been deleted as a consequence of the second homologous recombination. By this recombination, either the wild-type *mcbR* gene or the shortened DNA fragment with the deleted coding region of *mcbR* remains in the genome. Clones positive for the deletion of *mcbR* were identified using primers BK1987 and BK1988. These two primers flank the 5′ and the 3′ regions of the insert. In the case of the *mcbR* knockout mutant, PCR amplification leads to a shortened PCR fragment length compared to that obtained for the wild type. The subsequent knockout of *metB* in *C. glutamicum* Δ*mcbR*, leading to *C. glutamicum* Δ*mcbR* Δ*metB*, was performed with plasmid pSL315 as described by Hwang et al. (10). For the construction of *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk*, the primer pairs HS605/HS606 and HS607/HS608 were used for crossover PCR on chromosomal DNA of the *C. glutamicum* wild type to delete approximately 1,780 internal nucleotides of the *hom* and *hsk* loci. The yielded PCR fragment contained a small 5′ fragment of *hom* and a small 3′ fragment of *hsk* together with the *hsk* downstream region. It was cloned into the pCLIK5A *sacB* vector using the BamHI sites. Subsequently, it was introduced into *C. glutamicum* Δ*mcbR* for first and second recombinants. Clones positive for the deletion of *hom* and *hsk* were identified, as described above, using primers HS605 and HS608, which yielded a shortened PCR product compared to that for the wild type.

Chemicals. L-Homocysteine was prepared from L-homocysteine thiolactone (1). Casamino Acids, beef extract, polypeptone, and yeast extract were supplied by Difco (Detroit, MI). The tracer substrates, 99% [$^{13}C_6$]glucose and 98% [$^{13}C_4$]threonine, were supplied by Cambridge Isotopes, Inc. (Andova, MA). Ammonium sulfate labeled with ^{15}N (99%) was purchased from Campro Scientific (Veenendaal, The Netherlands), and [^{34}S]sulfate was kindly provided by BASF AG (Ludwigshafen, Germany). All other chemicals were of analytical grade and purchased from Grüssing (Filsum, Germany), Acros Organics (Geel, Belgium), Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), or Fluka (Buchs, Switzerland).

Media and growth conditions. Cells for inoculation were grown at 30°C on agar plates with rich medium containing 10.0 g/liter glucose, 2.5 g/liter NaCl, 2.0 g/liter urea, 5.0 g/liter yeast extract, 5.0 g/liter beef extract, 5.0 g/liter polypeptone, 20.0 g/liter Casamino Acids, and 20.0 g/liter agar. Single colonies served as inoculum for the precultures, which were grown overnight in 250-ml baffled shake flasks with 25 ml rich medium as described above without agar. Cells were harvested by centrifugation (2 min, 10,000 × *g*, 4°C), washed twice with 0.9% NaCl, and used for inoculation of the second preculture on minimal medium. The second preculture growth was harvested as described above and used as inoculum for the main cultivations, which were carried out in 500-ml baffled shake flasks with 50 ml minimal medium. The minimal medium contained the following per liter: 20 g glucose, 16 g K_2HPO_4, 4 g KH_2PO_4, 20 g $(NH_4)_2SO_4$, 300 mg 3,4-dihydroxybenzoic acid, 10 mg $CaCl_2$, 250 mg $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$, 200 μg $CuSO_4 \cdot 5H_2O$, 20 μg $NiCl_2 \cdot 6H_2O$, 20 μg $Na_2MoO_4 \cdot 2H_2O$, 100 μg cyanocobalamin, 300 μg thiamine, 4 μg pyridoxal phosphate, and 100 μg biotin. Tracer experiments were performed with 5 ml minimal medium in 50-ml baffled shake flasks. Hereby, selected medium constituents were replaced by the corresponding compounds labeled with stable isotopes. All cultivations were carried out at 30°C on a rotary shaker (250 rpm; shaking diameter, 5 cm) (Multitron; Infors AG, Bottmingen, Switzerland).

Quantification of intermediates of the methionine pathway. Intracellular metabolites were extracted by quick filtration and subsequent boiling in water (15 min) as described previously (16). The obtained cell extract was used to quantify metabolites related to methionine biosynthesis in *C. glutamicum* (cysteine, homocysteine, homoserine, *O*-acetylhomoserine, cystathionine, and homolanthionine). For this purpose, high-performance liquid chromatography (HPLC) with precolumn derivatization by *o*-phthaldialdehyde was utilized (15). Quantification of homolanthionine, for which no external standard was commercially available, was based on the calibration factor for cystathionine, which had a highly similar structure. In this context, serine and homoserine differing by one methylene group, as did cystathionine and homolanthionine, showed only a 5% difference in the calibration factor. Due to this, the calibration via cystathionine should not significantly affect the quantification of homolanthionine.

GC/MS labeling analysis. Determination of labeling patterns of metabolites in cell extracts or amino acids in the cell protein was carried out by gas chromatography/mass spectrometry (GC/MS). For the analysis of amino acids from the cell protein, biomass (1 mg) was harvested from an exponentially growing culture and washed twice with H_2O. The washed cell pellet was hydrolyzed for 48 h (50 μl 6 M HCl, 105°C). The obtained hydrolysate was neutralized (6 M NaOH), clarified by centrifugation (5 min, 16,000 × *g*) (ultrafree-MC filter units, 0.22-μm Durapore membrane; Millipore, Bedford, MA), and subsequently freeze-dried. GC/MS analysis of the amino acids was performed after derivatization into the *t*-butyl-dimethylsilyl (TBDMS) derivate (31). For the analysis of intracellular metabolites, 400 μl cell extract was freeze-dried and subsequently derivatized as described above for the hydrolysate.

Overexpression and purification of cystathionine-γ-synthase and cystathionine-β-lyase. Cystathionine-γ-synthase (MetB) and cystathionine-β-lyase (MetC) of *C. glutamicum* were separately cloned and overexpressed in *Escherichia coli* XL1-Blue. For this purpose, the corresponding genes were cloned into vector pQE30 (QIAGEN, Inc., Chatsworth, CA) comprising the addition of a His tag to the N terminus of the expressed protein. The vector carrying either the *metB* or the *metC* gene was transformed into *Escherichia coli* using the $CaCl_2$ method. Transformed *E. coli* cells were cultivated at 37°C and 250 rpm on Terrific broth (20) with 100 mg/liter ampicillin. At a cell optical density at 600 nm of 1 unit, protein expression was induced by the addition of 1 mM isopropylthiogalactoside (final concentration). After 16 h of induced growth, cells (4 g wet weight) were harvested by centrifugation (4,225 × *g*, 15 min, 2°C) and resuspended in 16 ml phosphate buffer (100 mM; 100 μM pyridoxal phosphate, 1 g/liter DNase I [pH 7.4]; 4°C), including a washing step in the same buffer. Subsequently, cells were extracted by sonication on ice (five times for 15 s each time; 20 μm). The crude extracts were separated from cell debris by centrifugation (30 min, 2°C, 20,000 × *g*). The recombinant enzymes were finally purified by affinity chromatography (ÄKTA purifier 900; Amersham Biosciences, Little Chalfont, England) equipped with a chelating nickel-Sepharose column (5 ml) (HiTrap; Amersham Biosciences, Little Chalfont, England). The column was equilibrated with 0.02 M sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. After the cell extract was applied to the column, the column was washed with 10 volumes of 0.02 M sodium phosphate buffer (pH 7.4). Elution was carried out with a linear gradient of 0.02 M sodium phosphate buffer (pH 7.4; 0.5 M NaCl; 0.5 M imidazole). The fractions containing MetB or MetC were checked for purity with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then pooled together. Proteins were concentrated and separated from imidazole by three cycles of filtration (molecular weight cutoff, 20,000) (Centrisart; Sartorius, Göttingen, Germany). The protein content (Bio-Rad protein assay; Munich, Germany) finally obtained was 12 mg ml^{-1} for MetB and 0.3 mg ml^{-1} for MetC.

In vitro assay of cystathionine-γ-synthase. The in vitro activity of cystathionine-γ-synthase (MetB) was determined photometrically (Helios α; Thermo Electron, Dreieich, Germany). The assay was based on the quantification of free SH groups using Ellman's reagent with detection at 412 nm (4). The assay mixture (final volume, 1 ml) contained 1.25 mM cysteine (or homocysteine), 3 mM *O*-acetylhomoserine, and 10 μM pyridoxal-5-phosphate in phosphate buffer (100 mM, pH 7.5). The reaction was started by the addition of 1 μl MetB solution (12 mg ml^{-1}). During the incubation, samples (65 μl) were taken from the assay mixture and immediately injected into 935 μl of a stop solution (100 mM phosphate buffer (pH 7.5) with 38% ethanol and 1 mM dithionitrobenzoic acid. Dithionitrobenzoic acid formed a yellow complex with the remaining homocysteine or cysteine. The extinction of the yellow complex at 412 nm was linearly correlated with free SH groups, e.g., from cysteine or from homocysteine, up to

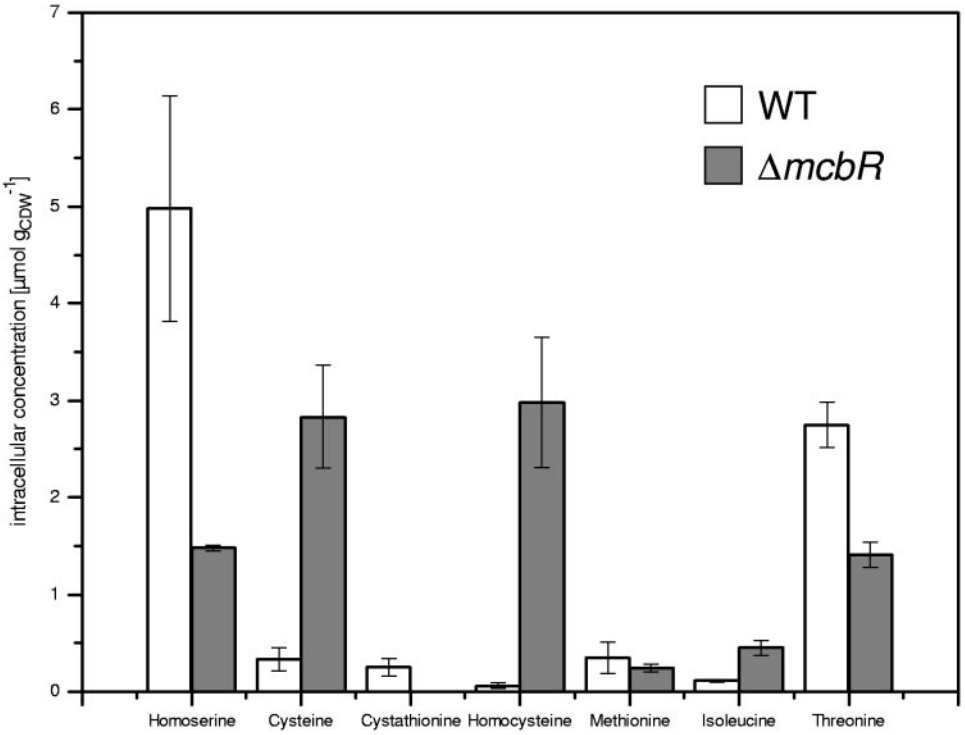

FIG. 1. Intracellular pools of methionine intermediates in exponentially growing *C. glutamicum* ATCC 13032 (wild type [WT]) and *C. glutamicum* ATCC 13032 Δ*mcbR*. The concentrations are given in μmoles per g_{CDW}. Mean values for four cell extracts from two different time points with corresponding standard deviations are shown. The level of cystathionine in the Δ*mcbR* strain was below the detection limit (around 0.1 μmol g_{CDW}^{-1}).

1.5 mM. The K_m values of MetB for cysteine and homocysteine were determined from double-reciprocal Lineweaver-Burk plots.

In vitro assay of cystathionine-β-lyase. The assay for cystathionine-β-lyase (MetC) was based on the same principle as that for MetB. Due to that fact that the reaction catalyzed by MetC leads to the generation of free SH groups, the activity of the enzyme was monitored photometrically (Helios α; Thermo Electron, Dreieich, Germany) by the increase of extinction at 412 nm using Ellman's reagent (4). The assay mixture (final volume, 1 ml) contained 1.25 mM cystathionine and 10 μM pyridoxal 5-phosphate in phosphate buffer (100 mM, pH 7.5). The reaction was started by the addition of 100 μl MetC solution (0.3 mg ml^{-1}). It should be noted that homolanthionine was not commercially available. Due to this, homolanthionine was added from the assay mixture of the MetB assay after removal of the MetB enzyme by three cycles of filtration (molecular weight cutoff, 20,000) (Centrisart; Sartorius, Göttingen, Germany). This solution contained primarily homolanthionine and to some extent the remaining homocysteine and *O*-acetylhomoserine. The lack of MetB activity in this solution was checked by the MetB assay. The exact concentration of homolanthionine in the MetC assay was determined by HPLC. During the incubation, samples (65 μl) were taken from the assay mixture and immediately injected into 935 μl of a stop solution as described above. The K_m value of MetC for cystathionine was determined from a double-reciprocal Lineweaver-Burk plot.

RESULTS

Metabolomic response to deletion of McbR. The deletion of the transcriptional repressor McbR in *Corynebacterium glutamicum* had a significant impact on the growth rate (μ). In contrast to that of the wild type (μ = 0. 40 h^{-1}), the specific growth of the mutant (μ = 0. 18 h^{-1}) was significantly decreased. Additionally, the intracellular level of intermediates of the methionine pathway was severely affected by the McbR deletion (Fig. 1). *C. glutamicum* ATCC 13032 Δ*mcbR* exhibited a decreased level of homoserine in comparison to that of the parent strain, *C. glutamicum* ATCC 13032, which indicated a higher flux into the methionine pathway. The intracellular level of homocysteine, however, was increased by a factor of 50, i.e., from 0.06 to 2.98 μmol g of cells (dry weight)$^{-1}$ (g_{CDW}^{-1}). The intracellular methionine pool was not affected by the deletion. *O*-Acetylhomoserine could not be detected in any of the strains. The most pronounced difference between the two strains, however, was the strong accumulation of a single unknown metabolite in the cell extract, which was observed by HPLC as well as by GC/MS analysis.

Identification of the novel metabolite as homolanthionine. The metabolite significantly accumulating as a consequence of *mcbR* deletion was identified from cell extracts of *C. glutamicum* by its GC/MS mass fragment pattern (Fig. 2A and B). After conversion into the TBDMS derivate, the metabolite eluted from the GC column after 44.0 min. The metabolite exhibited a mass spectrum with high similarity to that of cystathionine, which eluted a bit earlier (42.1 min). Characteristic ions typically observed for the performed derivatization, such as the molecular ion [M] at m/z = 692 and fragment ions [M-15] at m/z = 677, [M-57] at m/z = 635, or [M-85] at m/z = 607, all exhibited a mass shift of 14 compared to the corresponding fragments in cystathionine (Fig. 2A and B). The mass shift of 14 indicated the presence of an additional methylene group in the new metabolite compared to cystathionine. Additionally, fragments from the lower mass range, such as m/z = 170, m/z = 244, and m/z = 272, were also observed for cystathionine (Fig. 2A), homocysteine, and methionine (data not shown). These findings were the first indication that the

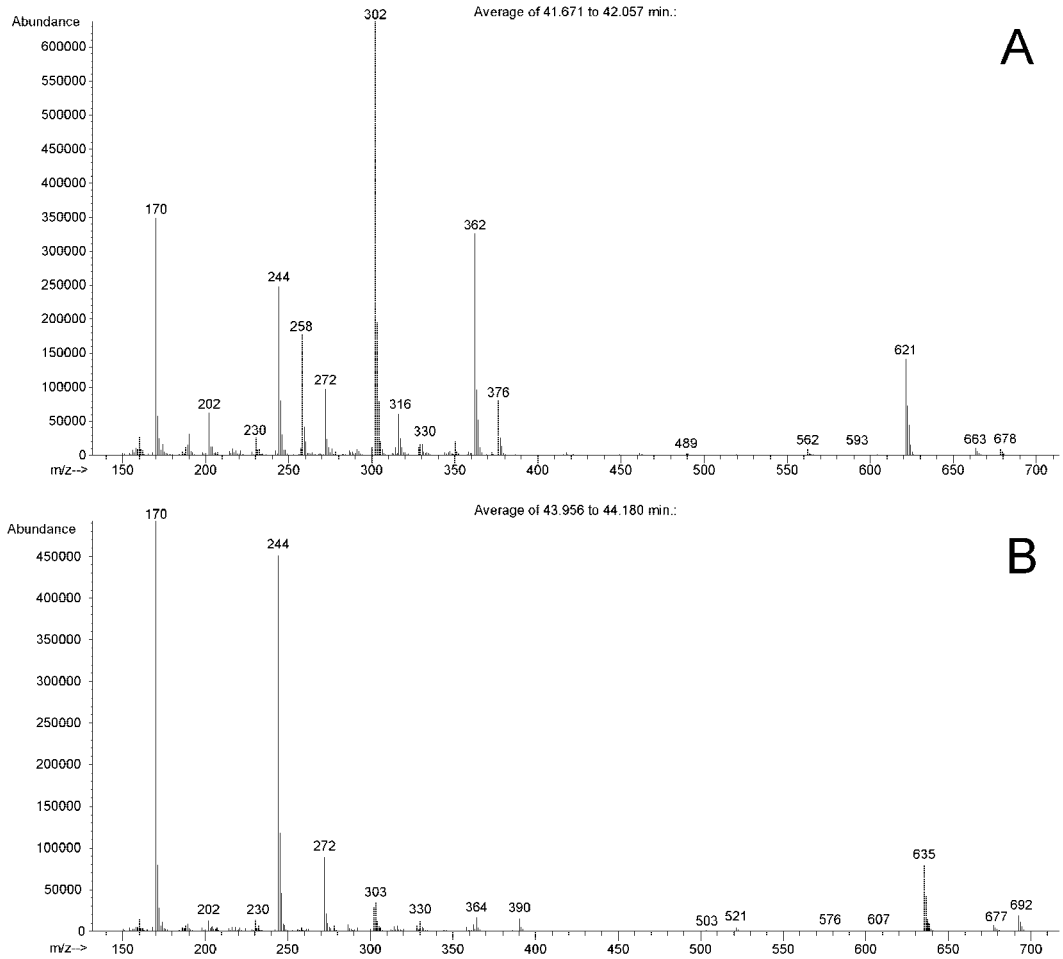

FIG. 2. GC/MS of TBDMS-derivatized L-cystathionine (A) and L-homolanthionine (B). The molecular ions have *m/z* values of 678 (cystathionine) and 692 (homolanthionine). The mass difference of 14 is due to the additional methylene group present in homolanthionine. Characteristic fragments at [M-15], [M-57], [M-85], and [M-302] typically found for TBDMS-derivatized compounds also exhibited a mass difference of 14. The ions at *m/z* 170, *m/z* 244, and *m/z* 272 are characteristic fragments of the homocysteine residue in both molecules. L-Cystathionine was applied as a pure compound, and L-homolanthionine was from a cell extract of *C. glutamicum* ATCC 13032 Δ*mcbR*.

new metabolite observed was linked to the methionine metabolism in *C. glutamicum* and that its accumulation was therefore a direct consequence of the deletion of the methionine repressor McbR. Subsequent tracer cultivations of *C. glutamicum* Δ*mcbR* with either [$^{13}C_6$]glucose, [$^{15}N_2$]ammonium sulfate, or [^{34}S]sulfate led to mass shifts in the molecular ion of the metabolite of 8, 2, and 2, respectively. This showed that the carbon, nitrogen, and sulfur contents of the observed metabolite were $C_8N_2S_1$. Based on these data, the new metabolite was identified as homolanthionine {*S*-[(3*S*)-3-amino-3-carboxypropyl]-L-homocysteine} (Fig. 3). Its mass isotopomer distribution determined by GC/MS showed excellent agreement with the theoretical value calculated from the natural abundance of stable isotopes present in the molecule. The homolanthionine structure is highly similar to that of cystathionine and differs by only the content of an additional methylene group. Quantification of homolanthionine in cell extracts of exponentially growing *C. glutamicum* Δ*mcbR* revealed a substantial accumulation of 250 μmol g_{CDW}^{-1}. With a cytoplasmic volume of *C. glutamicum* of 1.9 μl mg^{-1} (6), this equals an intracellular level of 130 mM. It should be noted that homolanthionine is not commercially available and that therefore its structure could not be confirmed by the analysis of the pure compound. The GC/MS data combined with feeding of labeled precursor substrates, however, provide a very strong indication for the identity of the compound. It is now interesting to see where homolanthionine originates in the metabolism of *C. glutamicum* and how this is related to the role of *mcbR*.

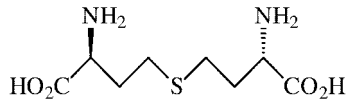

FIG. 3. Structure of L-homolanthionine {*S*-[(3*S*)-3-amino-3-carboxypropyl]-L-homocysteine; CAS 102044-65-5}.

Metabolic origin of homolanthionine. The first insight into the metabolic origin of homolanthionine was obtained via a triple-deletion mutant, *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk*. This mutant cannot synthesize homoserine from aspartate semialdehyde, and additionally, it cannot convert homo-

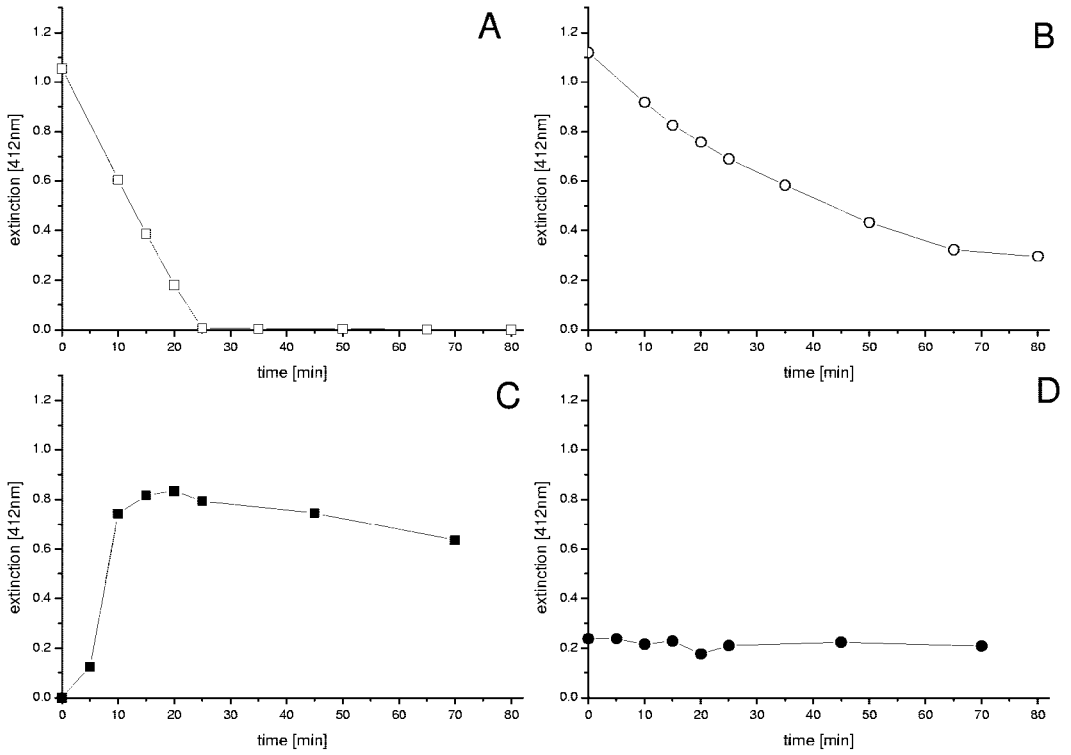

FIG. 4. Characterization of cystathionine-γ-synthase (MetB) and cystathionine-β-lyase (MetC) from *C. glutamicum* after overexpression as His-tagged proteins in *E. coli* and subsequent purification. The conversion of *O*-acetylhomoserine and L-cysteine (A) and *O*-acetylhomoserine and L-homocysteine (B) by MetB and the cleavage of cystathionine (C) and L-homolanthionine (D) by MetC were monitored by photometric measurement of free SH groups via Ellman's reagent at 412 nm. L-Homolanthionine was added from the assay mixture of the MetB assay after removal of the MetB enzyme by three cycles of filtration (molecular weight cutoff, 20,000) (Centrisart; Sartorius, Göttingen, Germany). This solution contained primarily homolanthionine and to some extent remaining homocysteine and *O*-acetylhomoserine, which explains the initial levels of free SH groups in the assay (D).

serine into homoserine phosphate. It was found that this mutant is auxotrophic for threonine and homoserine as expected. Subsequently, cultivation of *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk* was performed on minimal medium with 10 g liter^{-1} [$^{13}C_6$]glucose, 10 mM [$^{13}C_4$]threonine, and 10 mM nonlabeled homoserine. An additional cultivation on nonlabeled glucose, threonine, and homoserine was carried out in parallel. Homolanthionine was present in significant amounts in cell extracts of this strain. GC/MS labeling analysis of homolanthionine from both cultivations revealed identical mass spectra, i.e., that of the nonlabeled molecule. This clearly indicated that homoserine was exclusively the precursor of homolanthionine. Taking the number of carbons of homolanthionine (C_8) into account, this also indicated that two homoserine C_4 units are probably required to yield one molecule of homolanthionine. Thus, homolanthionine originates directly from the methionine pathway of *C. glutamicum*.

In additional experiments, *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk* was cultivated under the same conditions, except that methionine, cystathionine, or homocysteine was fed as an additional substrate instead of homoserine. The triple mutant grew on all of these substrates. However, no significant accumulation of homolanthionine was observed. Obviously, the formation of homolanthionine required one of the intermediates at the beginning of the methionine pathway, either homoserine or *O*-acetylhomoserine.

Combining all the findings, a potential route for the formation of homolanthionine in *C. glutamicum* could be postulated. It was very likely that an enzyme from the methionine pathway is involved. One possibility could be a side reaction of cystathionine-γ-synthase (MetB), which might accept homocysteine instead of the normal substrate cysteine and thus produces homolanthionine instead of cystathionine, via linkage to *O*-acetylhomoserine. To further address this question, MetB was overexpressed in *E. coli* and characterized.

Isolation and characterization of cystathionine-γ-synthase (MetB). MetB exhibited high activity when incubated with its normal substrates *O*-acetylhomoserine and cysteine (Fig. 4A). However, MetB could also use homocysteine instead of cysteine (Fig. 4B). Analysis of samples (0 min, 80 min) from the enzyme assays by HPLC and GC/MS showed that the product formed by MetB from *O*-acetylhomoserine and homocysteine was indeed homolanthionine. In the control incubation, MetB converted cysteine and *O*-acetylhomoserine into cystathionine. Additional studies on the kinetics of MetB yielded a K_m value for cysteine of 260 μM, whereas the K_m observed for homocysteine (540 μM) was significantly higher. The MetB activity in crude cell extracts of *C. glutamicum* Δ*mcbR* was about threefold higher than that of the wild type, showing that the expression of this enzyme is also probably controlled by McbR.

Isolation and characterization of cystathionine-β-lyase (MetC). The incubation of MetC with its natural substrate

TABLE 2. GC/MS labeling analysis of *t*-butyl-dimethylsilyl-derivatized amino acids from the cell protein of *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk* cultivated on 99% [$^{13}C_6$]glucose, 98% [$^{13}C_4$]threonine, and nonlabeled homoserine

Mass isotopomer	Relative mass fraction for[a]:		
	Isoleucine	Threonine	Alanine
M	0.5	1.1	0.7
M + 1	0.5	0.4	0.6
M + 2	13.3	1.1	5.2
M + 3	1.4	8.5	93.5
M + 4	8.0	88.8	
M + 5	76.3		

[a] The relative mass isotopomer fractions of the ion clusters of *m/z* 200 (isoleucine, C_2 to C_6), *m/z* 404 (threonine, C_1 to C_4), and *m/z* 260 (alanine, C_1 to C_3) are given as percentages. The nonlabeled mass isotopomer fraction is denoted as M and the single-labeled mass isotopomer fraction as M + 1; corresponding terms represent higher labeling.

cystathionine led to the effective release of free SH groups, i.e., via the accumulation of homocysteine (Fig. 4C). MetC revealed slight activity when incubated with homolanthionine, as indicated by the decrease of the homolanthionine level from 200 μM to 170 μM during the incubation and a corresponding increase of the level of homocysteine. This activity, however, was too weak to be observed in the photometric assay (Fig. 4D). Thus, we conclude that MetC is capable of cleaving homolanthionine at low rates. Analogous to the cleavage of cystathionine by MetC, which leads to homocysteine and pyruvate, the cleavage of homolanthionine should result in homocysteine and 2-oxobutanoate. The latter is a precursor of isoleucine. Due to this, further studies were performed to investigate the influence of McbR deletion on the isoleucine biosynthetic pathway in *C. glutamicum*.

Impact on isoleucine metabolism. In order to investigate the biosynthesis of isoleucine, *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk* was cultivated on [$^{13}C_6$]glucose, [$^{13}C_4$]threonine, and nonlabeled homoserine. During the exponential phase, cells were harvested and hydrolyzed for ^{13}C labeling analysis of amino acids from the cell protein, especially isoleucine, threonine, and alanine. For threonine and alanine, the fully labeled mass isotopomer displayed the major fraction. The threonine in the cell protein showed exactly the labeling pattern of the threonine added to the medium. The labeling degree of alanine agreed with that of the glucose added. In the performed tracer study, threonine and pyruvate, the two precursors of the known pathway for isoleucine biosynthesis, were thus completely labeled. The same was expected for isoleucine synthesized via this pathway. However, only 76.3% of the isoleucine in the cell protein was fully labeled. In contrast, the fraction of fully labeled threonine in the protein (88.8%) was significantly higher. This clearly showed that an alternative pathway for isoleucine formation was active in vivo. A closer inspection of the mass isotopomer distribution of isoleucine sheds light on this novel route. Obviously, 13% of the isoleucine formed contained two ^{13}C atoms (Table 2). This situation is possible only if the C_4 precursor is unlabeled and pyruvate is labeled, since the latter adds exactly two carbon atoms to the final isoleucine molecule. Due to the deletions present in *C. glutamicum* Δ*mcbR* Δ*hom* Δ*hsk*, it is clear that the nonlabeled C_4 precursor can come only from homoserine and thus via the methionine pathway. To summarize, *C. glutamicum* is able to generate isoleucine by a so-far-undescribed pathway which is independent from threonine. The findings are complemented by the fact that the intracellular isoleucine level was indeed significantly increased in *C. glutamicum* Δ*mcbR*. Obviously, the activation of the novel pathway leads to enhanced isoleucine supply. Combining previous knowledge of the methionine pathway and the new findings of the present work, we suggest an extended pathway for methionine and isoleucine biosynthesis in *C. glutamicum* (Fig. 5). In addition to the reactions known before, this extended scheme includes the newly found side reactions of MetB and MetC leading to the formation and cleavage of homolanthionine, respectively. Moreover, the novel biosynthetic pathway for isoleucine that branches off from the methionine pathway is shown.

Effect of MetB deletion on accumulation of homolanthionine. In contrast to *C. glutamicum* Δ*mcbR*, which accumulated high levels of homolanthionine as described above, homolanthionine was not present in cell extracts of *C. glutamicum* Δ*mcbR* Δ*metB*. This gives evidence that MetB is the only source for homolanthionine in *C. glutamicum*.

In vivo carbon flux through the methionine pathway in *C. glutamicum* Δ*mcbR*. The data from the present work provided information on the relative supply of isoleucine from threonine and from homolanthionine (Table 2). The intracellular homolanthionine pool was so large that the demand for its supply (250 μmol g^{-1}) was considered. In contrast, the demand for the other intermediates could be neglected due to their small intracellular levels (Fig. 1). Together with the anabolic demand for methionine (146 μmol g^{-1}), isoleucine (202 μmol g^{-1}), and threonine (275 μmol g^{-1}) in *C. glutamicum* (30) and with previous data on the relative flux through the parallel pathways of transsulfuration and direct sulfhydrylation (10), this study provides a detailed insight into the relative carbon flux through the methionine pathway in the McbR deletion mutant (Fig. 6). Hereby, the new findings, the side reaction of MetB leading to homolanthionine, and the novel route towards isoleucine are considered. At the homoserine node, the carbon flux is almost equally directed towards homoserine phosphate into the threonine biosynthesis and towards *O*-acetylhomoserine into the methionine pathway. It can be further seen that *O*-acetylhomoserine sulfhydrolase (MetZ) consumes only a small fraction of *O*-acetylhomoserine. The major flux consuming *O*-acetylhomoserine is catalyzed by MetB. MetB, however, shows two in vivo activities. A flux of 17.4% is assigned to its natural reaction leading to cystathionine, whereas an even higher flux of 24.1% is found for the formation of homolanthionine. Due to this latter reaction, a significant backflux results from homocysteine towards homolanthionine. In fact, the major fraction of homocysteine is not channeled towards methionine but is utilized for the formation of homolanthionine. It can be further seen that homolanthionine is to some extent cleaved and that its products are recycled back into the methionine and the isoleucine pathways. The major fraction of homolanthionine is accumulating inside the cell. Overall, from the flux of 59.8% entering the methionine pathway at the level of *O*-acetylhomoserine, only a relatively small flux (14.1%) is converted into methionine. The remaining fraction is either stored as homolanthionine or directed towards the synthesis of isoleucine.

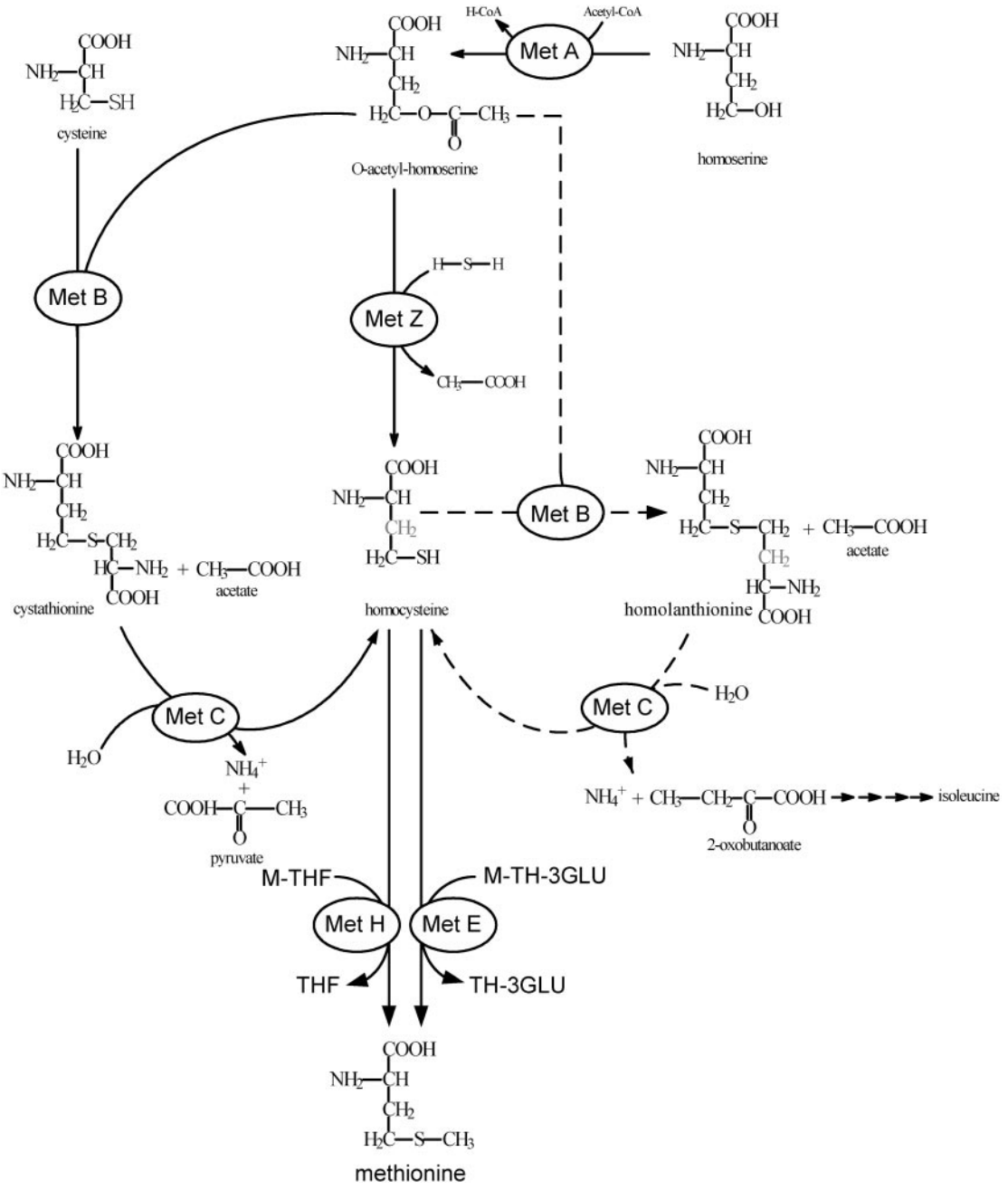

FIG. 5. Extended pathway for methionine and isoleucine biosynthesis in *C. glutamicum*. In addition to the reactions known before, the newly found side reactions of cystathionine-β-synthase (MetB) and cystathionine-β-lyase (MetC) leading to the formation and cleavage of homolanthionine, respectively, are shown. Further enzymes involved are MetA (homoserine transacetylase) and MetZ (*O*-acetylhomoserine sulfhydrolase).

DISCUSSION

Recently, the transcriptional repressor McbR, regulating the expression of a number of genes from methionine biosynthesis and sulfur assimilation, was identified in *C. glutamicum*. It was suggested that the deletion of McbR is an important step towards the development of *C. glutamicum* strains for the biotechnological production of methionine. In the present work, the effects of deleting McbR in *C. glutamicum* were investigated from a metabolic perspective. This included the comparison of the wild type, *C. glutamicum* ATCC 13032, and the corresponding deletion mutant, *C. glutamicum* ATCC 13032 Δ*mcbR*. As a response to the McbR deletion, almost all intracellular pools of intermediates from the methionine pathway were changed. This comprised a decreased homoserine level, which indicates an enhanced flux into the methionine pathway compared to that of the wild type. Homocysteine, however, was increased by a factor of 50. The elevated homocysteine level in *C. glutamicum* Δ*mcbR* is probably caused by the deregulated expression of homoserine dehydrogenase (Hom), *O*-acetylhomoserine sulfhydrolase (MetZ), and *S*-adenosylmethionine

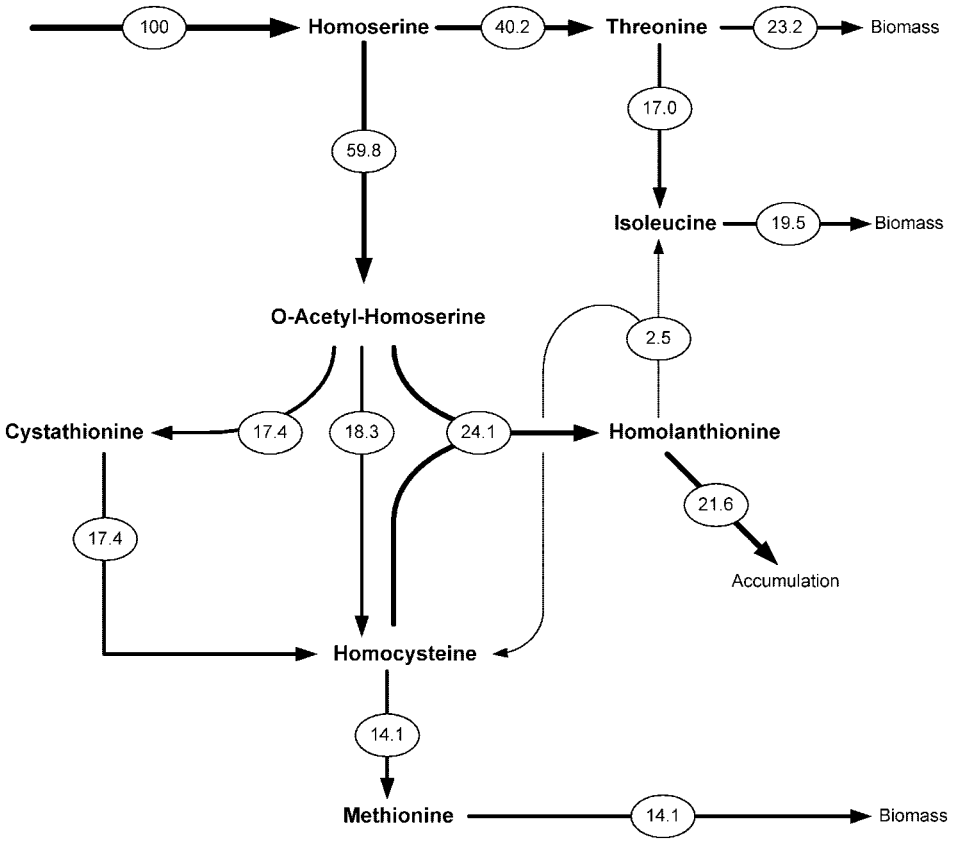

FIG. 6. Relative flux distribution in the methionine pathway of *C. glutamicum* Δ*mcbR*. Fluxes are given as relative fluxes with respect to the homoserine influx. Data on the split ratio between transsulfuration and direct sulfhydrylation are taken from the work of Hwang et al. (10).

synthase (MetK), which are all controlled by McbR (27). Homocysteine is the direct product of the reactions catalyzed by Hom and MetZ, whereas MetK converts methionine into *S*-adenosylmethionine, which is then recycled via *S*-adenosylhomocysteine back to homocysteine (26). Interestingly, the intracellular methionine titer remained constant in comparison to that of the *C. glutamicum* wild type. In combination with the increase of the homocysteine level in *C. glutamicum* Δ*mcbR*, this indicates that the preceding step, the reaction catalyzed by the two methionine synthases (MetE and MetH), is limiting. The increased expression of these two enzymes due to the deletion of McbR (26) is obviously not sufficient. In other organisms, homocysteine is directly involved in the regulation of the expression of methionine synthase on the transcriptional level (12, 22). Such a regulation, which additionally could help the cell to avoid undesired accumulation of homocysteine, however, has not been described for *C. glutamicum*. The limitation on the level of the methionine synthases could have different reasons. Possibly, methionine itself causes a feedback inhibition of both methionine synthases. Another possibility could be a limited supply with 5-methyltetrahydrofolate or 5-methyltetrahydropteroyltri-L-glutamate as the donor of the terminal methyl group in methionine.

The most pronounced change on the metabolic level was the drastic accumulation of a metabolite that could be identified as homolanthionine. Homolanthionine accumulation has been described for other organisms, such as *E. coli* and *Aspergillus nidulans* (8, 23). The strains showing this behavior were auxotrophic for methionine due to a knockout of methionine synthase. Methionine synthase, as the final enzyme of the methionine pathway, converts homocysteine into methionine. It seems likely that the lack of a functioning methionine synthase leads to an elevated intracellular homocysteine level, similar to the effects resulting from the deletion of McbR in the present study. The accumulation of homolanthionine observed in these mutants might therefore also be due to an increased pool of homocysteine, activating the side reaction of MetB. Also, cystathionase from human liver (29) and from *Streptomyces phaeochromogenes* (13) and cystathionine-γ-synthase from *Arabidopsis thaliana* (25) can catalyze the formation of homolanthionine from homocysteine and *O*-acetylhomoserine.

It was further shown in the present work that MetB is responsible for the formation of homolanthionine in *C. glutamicum*. Due to obviously low substrate specificity, MetB uses homocysteine instead of cysteine as a substrate. This reaction yields homolanthionine instead of cystathionine, the natural product of MetB. Although the substrate affinity of MetB for cysteine (K_m = 260 μM) is higher than that for homocysteine (K_m = 540 μM), the 50-fold increase in the intracellular homocysteine pool from 30 μM to 1,500 μM (calculated from the intracellular pool size and the cytoplasmic volume) (6) can explain the significant formation of homolanthionine in *C. glutamicum* Δ*mcbR*.

MetC from *C. glutamicum* was shown to cleave homolan-

TABLE 3. Affinity constants (K_m) for cystathionine-γ-synthase (MetB) and cystathonine-β-lyase (MetC) from *C. glutamicum* (this study) and from data for other bacteria and a plant taken from the literature

Enzyme	Substrate	K_m (μM) (organism or plant)	References
Cystathionine-γ-synthase	L-Cysteine	260 (*C. glutamicum*)	This study
	L-Cysteine	180 (Spinach)	24
	L-Cysteine	50 (*E. coli*)	7
	L-Homocysteine	540 (*C. glutamicum*)	This study
Cystathionine-β-lyase	L-Cystathionine	110 (*C. glutamicum*)	This study
	L-Cystathionine	40 (*E. coli*)	2
	L-Cystathionine	220 (Serovar Typhimurium[a])	2
	L-Cystathionine	70 (*Bordetella avium*)	5
	L-Homolanthionine	4,540 (*E. coli*)	2

[a] *Salmonella enterica* serovar Typhimurium.

thionine at a low rate. The natural reaction of MetC, the cleavage of cystathionine, was clearly the preferred route. The K_m of MetC for cystathionine was 110 μM. In comparison, this enzyme shows different affinities for cystathionine in other organisms (Table 3). The K_m value of the *E. coli* cystathionase for homolanthionine was previously determined to be 4,540 μM (Table 3). The affinity of the cystathionase from *E. coli* for homolanthionine is thus about 100-fold lower than that for cystathionine. Concerning the similarity of the K_m values of MetC for cystathionine, it appears likely that the affinity of MetC from *C. glutamicum* for homolanthionine is in the same range as that of MetC *E. coli*. The intracellular level of homolanthionine in *C. glutamicum* Δ*mcbR* was 130 mM. This concentration is still far above the probable K_m value, so the cleavage of homolanthionine is probably limited by the specific reaction rate of MetC but not by the availability of the substrate. It remains possible, however, that other enzymes are also involved in the cleavage of homolanthionine.

In *C. glutamicum* Δ*mcbR*, a novel route for the biosynthesis of isoleucine, branching off from the methionine pathway, was identified. This pathway accounted for about 13% of the total isoleucine supply. From the enzymatic studies with MetC, we conclude that the cleavage of homolanthionine by MetC supplies 2-oxobutanoate, a direct isoleucine precursor. Other enzymes, potentially producing 2-oxobutanoate from different intermediates of the methionine pathway, are methionine methanethiol lyase (EC 4.4.1.11), homocysteine hydrogen sulfide lyase (EC 4.4.1.2), and cystathionine cysteine lyase (EC 4.4.1.1). Feeding nonlabeled methionine, homocysteine, or cystathionine, the substrates of these enzymes, together with [$^{13}C_6$]glucose and [$^{13}C_4$]threonine, led to completely labeled isoleucine, so that their contribution to the formation of 2-oxobutanoate could be excluded (data not shown). Further evidence comes from the fact that none of these enzymes has been found in *C. glutamicum* (KEGG database, http://www.genome.jp/kegg/metabolism.html). On the basis of previous findings and results of the present work, we suggest an extended pathway for methionine and isoleucine biosynthesis in *C. glutamicum* (Fig. 5). Metabolic flux analysis of *C. glutamicum* Δ*mcbR* revealed that only 25% of the *O*-acetylhomoserine at the entry of the methionine pathway is utilized for methionine biosynthesis and that the dominating fraction of 75% is either stored inside the cell as homolanthionine or redirected towards the formation of isoleucine. It appears very likely that the effects observed here, i.e., the formation of homolanthionine and increased isoleucine synthesis via the methionine pathway, generally occur at increased levels of homocysteine. Therefore, we think that they are not restricted to Δ*mcbR* mutants. Also, other strains of *C. glutamicum* exhibiting an engineered methionine metabolism, e.g., targeted overexpression of enzymes of the methionine pathway, might show increased intracellular levels of homolanthionine and isoleucine. We can imagine that, under certain conditions, isoleucine could even be a secreted by-product in methionine-deregulated strains. The increased intracellular isoleucine level could trigger the export of this compound into the medium (14, 21).

The deletion of the transcriptional repressor McbR was previously suggested as the first important step towards the overproduction of methionine by *C. glutamicum* for a future biotechnological application (27). The deletion strain, however, does not secrete any methionine. The high homocysteine level caused by the deregulated expression of enzymes from the methionine pathway in the Δ*mcbR* mutant and the resulting enormous accumulation of homolanthionine are, however, highly undesired. *C. glutamicum* Δ*mcbR* Δ*metB* does not exhibit homolanthionine formation. The deletion of MetB in McbR strains could therefore be a promising strategy for the development and optimization of methionine-overproducing strains. In this regard, the amplification of flux through the direct sulfhydrylation pathway in *C. glutamicum* Δ*mcbR* Δ*metB* could be an interesting next step.

REFERENCES

1. **Duerre, J. A., and C. H. Miller.** 1966. Preparation of L-homocysteine from L-homocysteine thiolactone. Anal. Biochem. **17:**310–315.
2. **Dwivedi, C. M., R. C. Ragin, and J. R. Uren.** 1982. Cloning, purification, and characterization of beta-cystathionase from *Escherichia coli*. Biochemistry **21:**3064–3069.
3. **Eggeling, L., and H. Sahm.** 1999. L-Glutamate and L-lysine: traditional products with impetuous developments. Appl. Microbiol. Biotechnol. **52:**146–153.
4. **Ellman, G., and H. Lysko.** 1979. A precise method for the determination of whole blood and plasma sulfhydryl groups. Anal. Biochem. **93:**98–102.
5. **Gentry-Weeks, C. R., J. M. Keith, and J. Thompson.** 1993. Toxicity of *Bordetella avium* beta-cystathionase toward MC3T3-E1 osteogenic cells. J. Biol. Chem. **268:**7298–7314.
6. **Gutmann, M., C. Hoischen, and R. Krämer.** 1992. Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim. Biophys. Acta **1112:**115–123.
7. **Holbrook, E. L., R. C. Greene, and J. H. Krueger.** 1990. Purification and properties of cystathionine gamma-synthase from overproducing strains of *Escherichia coli*. Biochemistry **29:**435–442.
8. **Huang, H. T.** 1963. Accumulation of 1-homolanthionine by an *Escherichia coli* mutant. Biochemistry **2:**296–298.
9. **Hwang, B. J., Y. Kim, H. B. Kim, H. J. Hwang, J. H. Kim, and H. S. Lee.**

1999. Analysis of *Corynebacterium glutamicum* methionine biosynthetic pathway: isolation and analysis of metB encoding cystathionine gamma-synthase. Mol. Cells **9:**300–308.

10. **Hwang, B.-J., H.-J. Yeom, Y. Kim, and H.-S. Lee.** 2002. *Corynebacterium glutamicum* utilizes both transsulfuration and direct sulfhydrylation pathways for methionine biosynthesis. J. Bacteriol. **184:**1277–1286.
11. **Jäger, W., A. Schäfer, A. Pühler, G. Labes, and W. Wohlleben.** 1992. Expression of the *Bacillus subtilis sacB* gene leads to sucrose sensitivity in the gram-positive bacterium *Corynebacterium glutamicum* but not in *Streptomyces lividans*. J. Bacteriol. **174:**5462–5465.
12. **Kacprzak, M. M., I. Lewandowska, R. G. Matthews, and A. Paszewski.** 2003. Transcriptional regulation of methionine synthase by homocysteine and choline in *Aspergillus nidulans*. Biochem. J. **376:**517–524.
13. **Kanzaki, H., M. Kobayashi, T. Nagasawa, and H. Yamada.** 1986. Synthesis of *S*-substituted L-homocysteine derivatives by cystathionine γ-lyase of *Streptomyces phaeochromogenes*. Agric. Biol. Chem. **50:**391–397.
14. **Kennerknecht, N., H. Sahm, M.-R. Yen, M. Pátek, 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.
15. **Krömer, J. O., M. Fritz, E. Heinzle, and C. Wittmann.** 2005. In vivo quantification of intracellular amino acids and intermediates of the methionine pathway in *Corynebacterium glutamicum*. Anal. Biochem. **340:**171–173.
16. **Krömer, J. O., O. Sorgenfrei, K. Klopprogge, E. Heinzle, and C. Wittmann.** 2004. In-depth profiling of lysine-producing *Corynebacterium glutamicum* by combined analysis of the transcriptome, metabolome, and fluxome. J. Bacteriol. **186:**1769–1784.
17. **Lee, H. S.** 2005. Sulfur metabolism and its regulation, p. 351–376. *In* L. Eggeling and M. Bott (ed.), Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, Fla.
18. **Lee, H. S., and B. J. Hwang.** 2003. Methionine biosynthesis and its regulation in *Corynebacterium glutamicum*: parallel pathways of transsulfuration and direct sulfhydrylation. Appl. Microbiol. Biotechnol. **62:**459–467.
19. **Leuchtenberger, W.** 1996. Amino acids: technical production and use, p. 465–502. *In* H. J. Rehm, G. Reed, A. Pühler, and P. Stadler (ed.), Biotechnology, vol. 6. VCH, Weinheim, Germany.
20. **Losen, M., B. Frölich, M. Pohl, and J. Büchs.** 2004. Effect of oxygen limitation and medium composition on *Escherichia coli* fermentation in shake-flask cultures. Biotechnol. Prog. **20:**1062–1068.
21. **Morbach, S., H. Sahm, and L. Eggeling.** 1996. L-Isoleucine production with *Corynebacterium glutamicum*: further flux increase and limitation of export. Appl. Environ Microbiol. **62:**4345–4351.
22. **Ortiou, S., J. M. Alberto, J. L. Gueant, and M. Merten.** 2004. Homocysteine increases methionine synthase mRNA level in Caco-2 cells. Cell. Physiol. Biochem. **14:**407–414.
23. **Paszewski, A., and J. Grabski.** 1975. Homolanthionine in fungi: accumulation in the methionine-requiring mutants of *Aspergillus nidulans*. Acta Biochim. Pol. **22:**263–268.
24. **Ravanel, S., M. Droux, and R. Douce.** 1995. Methionine biosynthesis in higher plants. I. Purification and characterization of cystathionine gamma-synthase from spinach chloroplasts. Arch. Biochem. Biophys. **316:**572–584.
25. **Ravanel, S., B. Gakiere, D. Job, and R. Douce.** 1998. Cystathionine gamma-synthase from *Arabidopsis thaliana*: purification and biochemical characterization of the recombinant enzyme over expressed in *Escherichia coli*. Biochem. J. **331:**639–648.
26. **Rey, D. A., S. S. Nentwich, D. J. Koch, C. Rückert, A. Pühler, A. Tauch, and J. Kalinowski.** 2005. The McbR repressor modulated by the effector substance *s*-adenosylhomocysteine controls directly the transcription of a regulon involved in sulphur metabolism of *Corynebacterium glutamicum* ATCC 13032. Mol. Microbiol. **56:**871–887.
27. **Rey, D. A., A. Pühler, and J. Kalinowski.** 2003. The putative transcriptional repressor McbR, member of the TetR-family, is involved in the regulation of the metabolic network directing the synthesis of sulfur containing amino acids in *Corynebacterium glutamicum*. J. Biotechnol. **103:**51–65.
28. **Rückert, C., A. Pühler, and J. Kalinowski.** 2003. Genome-wide analysis of the L-methionine biosynthetic pathway in *Corynebacterium glutamicum* by targeted gene deletion and homologous complementation. J. Biotechnol. **104:**213–228.
29. **Tallan, H. H., T. A. Pascal, K. Schneidman, B. M. Gillam, and G. E. Gaull.** 1971. Homolanthionine synthesis by human liver cystathionase. Biochem. Biophys. Res. Commun. **43:**303–310.
30. **Wittmann, C., and A. de Graaf.** 2005. Metabolic flux analysis in *Corynebacterium glutamicum*, p. 277–304. *In* L. Eggeling and M. Bott (ed.), Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, Fla.
31. **Wittmann, C., M. Hans, and E. Heinzle.** 2002. In vivo analysis of intracellular amino acid labelings by GC/MS. Anal. Biochem. **307:**379–382.