J. Microbiol. Biotechnol. (2010), **20**(8), 1196–1203
doi: 10.4014/jmb.1002.02018
First published online 11 June 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Towards Methionine Overproduction in *Corynebacterium glutamicum* – Methanethiol and Dimethyldisulfide as Reduced Sulfur Sources

Bolten, Christoph J.[1], Hartwig Schröder[2], Jeroen Dickschat[3], and Christoph Wittmann[1]*

[1]*Biochemical Engineering Institute, Technische Universität Braunschweig, 38106 Braunschweig, Germany*
[2]*BASF SE, Research Fine Chemicals and Biotechnology, 67056 Ludwigshafen, Germany*
[3]*Organic Chemistry Institute, Technische Universität Braunschweig, 38106 Braunschweig, Germany*

Received: February 17, 2010 / Revised: April 13, 2010 / Accepted: April 14, 2010

In the present work, methanethiol and dimethyldisulfide were investigated as sulfur sources for methionine synthesis in *Corynebacterium glutamicum*. *In silico* pathway analysis predicted a high methionine yield for these reduced compounds, provided that they could be utilized. Wild-type cells were able to grow on both methanethiol and dimethyldisulfide as sole sulfur sources. Isotope labeling studies with mutant strains, exhibiting targeted modification of methionine biosynthesis, gave detailed insight into the underlying pathways involved in the assimilation of methanethiol and dimethyldisulfide. Both sulfur compounds are incorporated as an entire molecule, adding the terminal S-CH_3 group to *O*-acetylhomoserine. In this reaction, methionine is directly formed. MetY (*O*-acetylhomoserine sulfhydrylase) was identified as the enzyme catalyzing the reaction. The deletion of *metY* resulted in methionine auxotrophic strains grown on methanethiol or dimethyldisulfide as sole sulfur sources. Plasmid-based overexpression of *metY* in the Δ*metY* background restored the capacity to grow on methanethiol or dimethyldisulfide as sole sulfur sources. *In vitro* studies with the *C. glutamicum* wild type revealed a relatively low activity of MetY for methanethiol (63 mU/mg) and dimethyldisulfide (61 mU/mg). Overexpression of *metY* increased the *in vitro* activity to 1,780 mU/mg and was beneficial for methionine production, since the intracellular methionine pool was increased 2-fold in the engineered strain. This positive effect was limited by a depletion of the *metY* substrate *O*-acetylhomoserine, suggesting a need for further metabolic engineering targets towards competitive production strains.

Keywords: NADPH, *O*-acetylhomoserine sulfhydrylase, *metY*, metabolic engineering

**Corresponding author*
Phone: +49-531-391-7651; Fax: +49-531-391-7652;
E-mail: c.wittmann@tu-bs.de

Corynebacterium glutamicum is utilized for the industrial production of 1,500,000 metric tons of glutamate and 900,000 metric tons of lysine every year [12, 28]. It is also regarded as a promising candidate for biotechnological methionine production to replace current petrochemical-based methods that annually supply about 600,000 tons of racemic mixture [24, 25]. Despite initial efforts that have shown the feasibility of *C. glutamicum* for the overproduction of this compound, production titers and yields are still too low for industrial applicability [26].

In *C. glutamicum*, methionine biosynthesis is carried out through two parallel pathways, transulfuration or direct sulfhydrylation, and utilizing homoserine as a precursor stemming from the TCA cycle, as well as sulfur and C_1 carbon [11, 21, 22] (Fig. 1). However, using sulfate, the most common sulfur source for the cultivation of microorganisms, methionine synthesis requires 8 moles of nicotinamide adenine dinucleotide phosphate (NADPH) [23]. Even in the case of optimum flux distribution of sulfate at zero growth, the predicted maximum yield is only 54% [20], since the supply of the required NADPH is inherently linked to significant carbon loss as CO_2. Interestingly, this latter simulation study identified different strategies that may go towards an increased methionine yield. The most striking effect was predicted for the utilization of methanethiol (CH_3SH) as a reduced sulfur source, provided that it can be metabolized by *C. glutamicum*. The use of methanethiol with the incorporation of the entire –S-CH_3 moiety would allow the direct formation of methionine from *O*-acetylhomoserine, avoiding the need for an extra C_1 carbon [20]. The capability to utilize methanethiol is not known for *C. glutamicum*, but has been described for *Saccharomyces cerevisiae* [31] and marine bacterioplankton [16]. However, it is known that it can produce the desired sulfur compound under certain conditions [5]. The two central genes, *metB* [9, 14, 16] and *metY* [31], are also annotated for *C. glutamicum*.

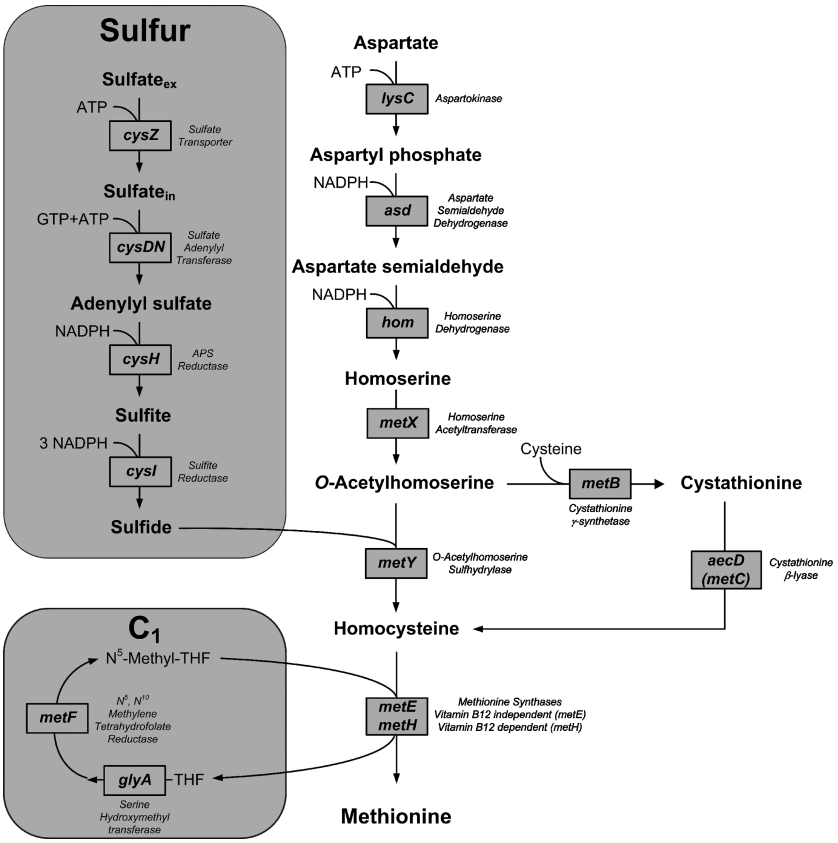

Fig. 1. Methionine biosynthesis in *Corynebacterium glutamicum*, including the supply of sulfur and C_1 metabolism.
THF, tetrahydrofolate.

In the present study, we analyzed the ability of *C. glutamicum* to grow on methanethiol, and its dimeric form dimethyldisulfide, as sole sulfur sources, and investigated the underlying pathways and enzymes. Based on a detailed characterization, it is hoped the gene for methanethiol utilization may be identifiable. Subsequent overexpression should result in improved methionine synthesis, which would itself be an important contribution towards future potential processes for methionine biosynthesis.

Materials and Methods

Microorganisms

In the current work, the wild type of *Corynebacterium glutamicum* ATCC 13032 (American Type and Culture Collection, Manassas, U.S.A.), and specifically designed mutants, constructed on the basis of the wild type, were investigated (Table 1). *Escherichia coli* DH5α and NM522 were obtained from Invitrogen (Karlsruhe, Germany) and used for plasmid amplification and DNA methylation, respectively.

Nucleic Acid Isolation

The cells were maintained at 30°C on agar plates, harvested after 2 days with a sterile inoculation loop, and resolved in 500 µl of sterile water. Cell disruption was performed for 1 min at 30 Hz in a ribolyzer (MM301; Retsch, Haan, Germany) after the addition of glass beads (0.1–0.25 mm; Retsch, Haan, Germany) and 700 µl of a mixture of phenol–chloroform–isoamyl-alcohol (Carl-Roth GmbH, Karlsruhe, Germany). After the separation of the aqueous and organic phases (10,000 ×*g*, 5 min; Eppendorf centrifuge 5415R; Hamburg, Germany), DNA from the aqueous phase was precipitated by the addition of 65 µl of sodium acetate (3 M, pH 5.5) and 1.3 ml of ethanol (100%), with a centrifugation step (10,000 ×*g*, 10 min; Eppendorf centrifuge 5415R; Hamburg, Germany). Subsequently, the supernatant was removed. The precipitated genomic DNA was dissolved in 100 µl of sterile water. Isolation of plasmid DNA from *Escherichia coli* NM522 and DH5α was performed using DNA isolation kits GFX Micro Plasmid Prep (GE Healthcare, Piscataway, NJ, U.S.A.) and HiSpeed Plasmid Midi Prep (Qiagen, Hilden, Germany), respectively, and according to the manufacturer's instructions.

Strain Construction

Gene deletion was achieved by the allelic replacement of the wild-type gene with a shortened DNA fragment, lacking about 300 bp. The knockout is usually carried out by a deletion of 300–500 bp to be able to distinguish between the wild-type and knockout genes in gel electrophoresis. The desired DNA fragment was obtained in three steps by PCR. Sequences of the respective site-specific primers P1–P4 for deletion of *metY* are given in Table 2. The obtained DNA fragment was subsequently inserted into the vector pClik int

Table 1. Strains of *Corynebacterium glutamicum* investigated in the present study.

Strain	Modification (s)	Reference
ATCC 13032	Wild type	ATCC
ATCC 13032$^{KanR\ epi}$	Kanamycin resistance	This work
ATCC 13032 Δ*metY*	Deletion of *O*-acetylhomoserine sulfhydrylase (MetY)	This work
ATCC 13032 P_{sod} *metY*$^{KanR\ epi}$	Kanamycin resistance and episomal overexpression of MetY	This work
ATCC 13032 Δ*metF*	Deletion of 5,10-methylenetetrahydrofolate reductase	This work
ATCC 13032 Δ*metF*Δ*metB*	Deletion of MetF and of cystathionine γ-synthase (MetB)	This work
ATCC 13032 Δ*metF*Δ*metY*	Deletion of MetF and MetY	This work
ATCC 13032 Δ*metF*Δ*metY* P_{sod} *metY*$^{KanR\ epi}$	Deletion of MetF and MetY; kanamycin resistance and episomal overexpression of MetY	This work

sacB via two restriction sites. The plasmids for deletion of *metB* and *metF* were donated by Omnigene Bioproducts (Woburn, MA, U.S.A.). For overexpression of *metY*, episomal plasmid pCZ4 P_{sod}*metY*, carrying kanamycin resistance, was used. Hereby, the native *metY* promoter was replaced by the strong constitutive promoter of superoxide dismutase (*sod*). It has been recently reported that this promoter enables a high expression level in *C. glutamicum* [1, 3]. Additionally, the *C. glutamicum* wild type was transformed with episomal plasmid pCZ4 to provide kanamycin resistance for comparative experiments. Vector amplification was performed in *E. coli* DH5α and *E. coli* NM522, carrying a plasmid-encoded copy of the *C. glutamicum* specific DNA methyltransferase gene, to enable the appropriate DNA methylation pattern. *C. glutamicum* was transformed by electroporation as described previously [3]. Gene deletion was validated by PCR analysis. As the genetic modification was restricted to the internal coding sequence of each enzyme, no other genes were affected.

Media and Growth Conditions

Cells were maintained at 30°C on agar plates containing complex medium with 10 g/l glucose, 2.5 g/l NaCl, 2 g/l urea, 5 g/l yeast extract, 5 g/l beef extract, 5 g/l polypeptone, and 20 g/l agar. For the first preculture, the same medium without agar was used. For the second preculture and main cultivation, a minimal medium (pH 7.0) was applied that contained (per liter) 10 g glucose, 16 g K_2HPO_4, 4 g KH_2PO_4, 4.05 g NH_4Cl, 300 mg 3,4-dihydroxybenzoic acid, 10 mg $CaCl_2$, 250 mg $MgCl_2 \cdot 6\ H_2O$, 10 mg $FeCl_2 \cdot 4\ H_2O$, 10 mg $MnCl_2 \cdot 4\ H_2O$, 2 mg $ZnCl_2$, 200 µg $CuCl_2$, 20 µg $NiCl_2 \cdot 6\ H_2O$, 20 µg $Na_2MoO_4 \cdot 2\ H_2O$, 100 µg cyanocobalamin, 300 µg thiamine, 4 µg pyridoxal phosphate, and 100 µg biotin. As sulfur sources, 40 mg/l of methionine, methanethiol, or dimethyldisulfide was added. Prior to its addition to the culture medium, methanethiol was fluidified in an ethanol–dry-ice bath at 0°C for accurate addition. Control cultivations were performed with 5 g/l of $(NH_4)_2SO_4$, or the addition of 40 mg l^{-1} of cysteine. In ^{13}C tracer experiments, 99% [U-^{13}C] glucose was used instead of naturally labeled glucose in equimolar amounts. All cultivations in liquid media were carried out in duplicate at 30°C on a rotary shaker (230 rpm, 5 cm shaking diameter; Multitron, Infors, Bottmingen, Switzerland). For growth studies on agar plates, the described minimal medium was supplemented with 10 g/l agar noble and sulfate in a final concentration of 40 mg/l. In selected studies, Amberlite XAD4 (Fluka, Buchs, Switzerland), a beaded macroporous polystyrene resin, was used as a delivery system for methanethiol, or dimethyldisulfide. For stability purposes of the episomal plasmids, kanamycin was added in a final concentration of 25 mg/l.

Chemicals

Beef extract, polypeptone, and yeast extract were purchased from Difco (Detroit, U.S.A.). Methanethiol and dimethyldisulfide, with a purity of higher than 99%, were obtained from Aldrich (Steinheim, Germany). All other chemicals were of analytical grade with a sulfur content of less than 0.005% and obtained from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), or Fluka (Buchs, Switzerland). The tracer substrate, 99% [U-^{13}C] glucose, was purchased from Cambridge Isotopes Inc. (Andova, MA, U.S.A.).

Analysis of Cell Concentration

Cell concentration was determined as optical density (OD_{660}) (Libra S11; Biochrome, Cambridge, U.K.), or gravimetrically as cell dry mass (CDM) [15]. The correlation factor between OD_{660} and CDM was determined as CDM [g/l]=0.255×OD_{660} [2].

Analysis of Intracellular Metabolites

For the quantification of intracellular amino acids and intermediates of the L-methionine pathway, sampling was performed *via* fast filtration and subsequent quenching and extraction in boiling water of the cells retained on the filter [30]. Cells were washed on the filter using a washing solution with an ionic strength equal (2.6% NaCl) to that of the medium [4]. Quantification was performed by HPLC with fluorescence detection (Agilent 1200, Waldbronn, Germany), applying

Table 2. Primer sequences used for deletion of *metY* in *C. glutamicum*.

Target gene	Primer sequence
metY	*metY*-Forward: 5'-GATCCTCGAGCGACAATTCCAATGCTG-3 *metY*-Fusion-Forward: 5'-TAGAAGGCCAACGCGAATCTCGCCGAAGAATGCT-3 *metY*-Fusion-Reverse: 5'-AGCATTCTTCGGCGAGATTCGCGTTGGCCTTCTA-3 *metY*-Reverse: 5'-GATCACGCGTAGATTGCAGCAAAGCCG-3'

pre-column derivatization, using *ortho*-phthalaldehyde and α-aminobutyrate, as an internal standard as described previously [18], except that a Gemini column (5 µm, 150×4.6 µm; Phenomenex, Aschaffenburg, Germany) was used and 3-mercaptopropionic acid was applied as a thiol reagent. The limit of detection was 0.05 µmol/g-CDM.

GC–MS Labeling Analysis of Methionine from Cell Protein

Mass isotopomer fractions of methionine from hydrolyzed and lyophilized cell proteins were measured by gas chromatography–mass spectrometry (Agilent, Waldbronn, Germany) [15, 29]. Hereby, the hydrolysis time was 4 h (6 M HCl, 105°C) in order to reduce the degradation of methionine [27]. Sample preparation and measurement were performed as described previously [3].

Analysis of Volatile Sulfur Compounds

Formation of volatile sulfur compounds including; dimethyldisulfide, dimethyltrisulfide, 2,3,5-trithiahexane, and *S*-methyl-methanethiosulfonate, by cell cultures of *C. glutamicum* were analyzed by closed loop stripping analysis (CLSA) [7] and GC–MS analysis as previously described [8].

***O*-Acetylhomoserine Sulfhydrylase (MetY) Enzyme Assay**

Crude cell extract was prepared from cells growing in a minimal medium as described above. Cells were harvested during the exponential phase by centrifugation (9,800 ×*g*, 5 min, 4°C; Biofuge Stratos, Kendro Laboratory Products, Langenselbold, Germany), washed with a disruption buffer (100 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.75 mM DTT), and disrupted by a ribolyzer [1]. Cell debris was removed by centrifugation (9,800 ×*g*, 5 min, 4°C; Biofuge Stratos). The obtained supernatant was used for the determination of the activity of *O*-acetylhomoserine sulfhydrylase [10]. Protein concentration was determined through the Bradford method [6]. The reaction mix for quantification of the *O*-acetylhomoserine sulfhydrylase activity contained 500 mM KH_2PO_4 (pH 7.8), 200 µM pyridoxal-phosphate, 5 mM *O*-acetylhomoserine, and 50 µl of cell extract in a total volume of 700 µl. After a pre-incubation period of 5 min at 30°C, methanethiol, dimethyldisulfide, or sulfide was added at a final concentration of 4 mM. Negative controls were carried out without sulfur compounds or without cell extract. The mixture was incubated for 15 min at 30°C. The reaction was then stopped by adding 100 µl of a 30% trichloroacetic acid solution. After centrifugation (10,000 ×*g*, 5 min; Eppendorf centrifuge 5415R), the supernatant was freeze-dried and resuspended in 160 µl of water. The assay products methionine (methanethiol and dimethyldisulfide), or homocysteine (sulfide), were quantified by HPLC (Agilent 1200; Agilent, Waldbronn, Germany) after pre-column derivatization with *O*-phthaldialdehyde [18].

Results

Growth of *C. glutamicum* on Methanethiol and Dimethyldisulfide

C. glutamicum was found to be able to grow on either methanethiol or dimethyldisulfide as sole sulfur sources (Fig. 2A). Cells reached a final cell concentration comparable to that on the positive control with methionine. Cells did not grow in a minimal medium without the addition of a sulfur compound. Using CLSA analysis, no typical oxidation products such as dimethyltrisulfide, 2,3,5-trithiahexane, or

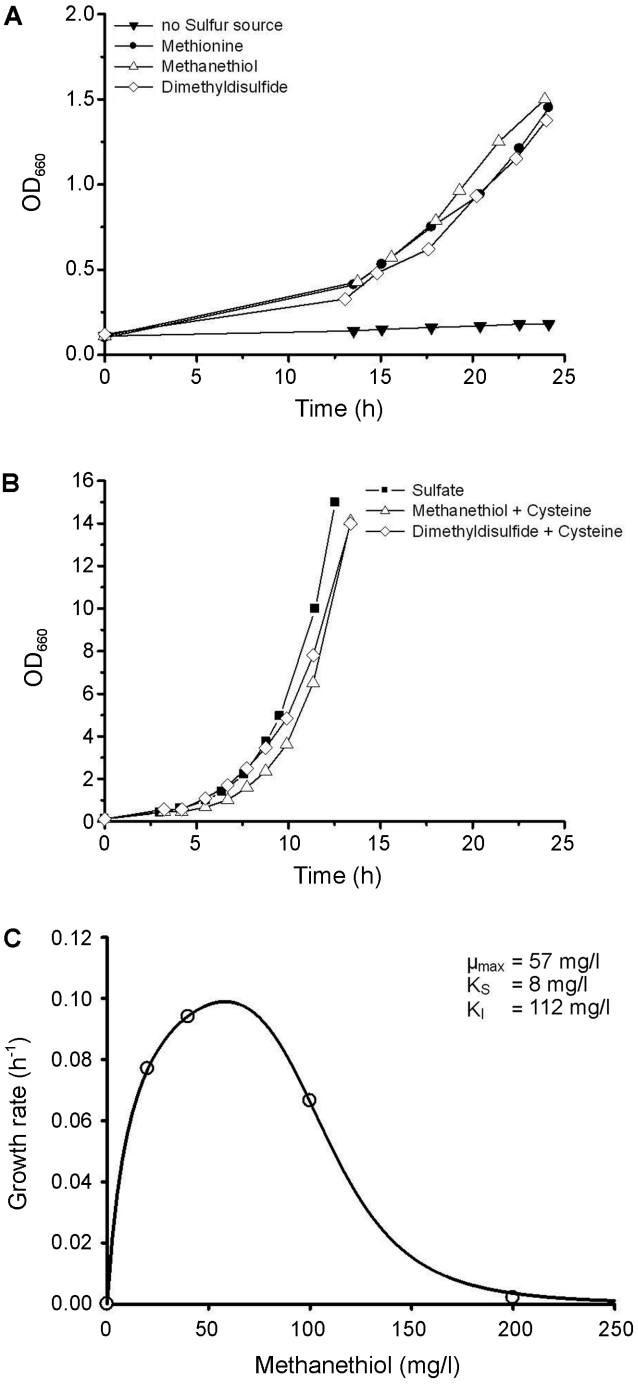

Fig. 2. Growth of *Corynebacterium glutamicum* on a minimal medium with methanethiol, dimethyldisulfide, methionine (positive control), or without a sulfur compound (negative control) (**A**); growth of *Corynebacterium glutamicum* on a minimal medium with sulfate, methanethiol plus cysteine, or dimethyldisulfide plus cysteine (**B**); and inhibition kinetics for growth on methanethiol as sulfur source (**C**).
The fitted curve displays a modified Yano model.

S-methyl-methanethiosulfonate, were detected. It therefore seemed likely that both compounds are assimilated in the reduced form. Compared with sulfate (μ=0.4 h^{-1}), the specific growth rate on methanethiol and dimethyldisulfide (μ= 0.1 h^{-1}) was lower. It is interesting to note that this was also observed for growth on methionine, suggesting that the slow growth is probably due to a limited supply of sulfur for biosynthesis of cysteine. This was confirmed by further experiments, in which cysteine was added in addition to either methanethiol and dimethyldisulfide, or methionine (Fig. 2B). Here, the growth rate was equal to that on sulfate. Increasing concentrations of methanethiol and dimethyldisulfide inhibited growth (Fig. 2C). At 200 mg/l, no growth could be observed for both compounds. The inhibition behavior could be well described by a modified Yano model [13] (Eq. 1).

$$\mu = \frac{\mu_{max} * S}{K_S + S + \frac{S^{6.5}}{K_I^2}} \quad (1)$$

The Michaelis–Menten constant (K_s) and the inhibition constant (K_I) were estimated at 8 mg/l and 112 mg/l, respectively. Optimal growth was observed at 57 mg/l. Further studies revealed that toxic effects could be reduced using a beaded macroporous polystyrene resin as a delivery system. This allowed for a higher initial concentration of up to 500 mg/l in both sulfur compounds without any inhibitory effects (data not shown).

Incorporation of Methanethiol and Dimethyldisulfide into Methionine

The optimal use of methanethiol and dimethyldisulfide, with respect to efficient methionine production, requires their incorporation as intact –S-CH_3 building blocks. Only in these conditions, as is the case for growth on sulfate, will the terminal methylation of the methionine pathway be dispensable. Methylation was blocked at the level of 5,10-methylene-tetrahydrofolate reductase, supplying the methyl group for the last step of methionine biosynthesis. As expected *C. glutamicum* Δ*metF*, lacking this enzyme, could not grow on sulfate. Growth was however possible with methionine in the medium (Fig. 3). *C. glutamicum* Δ*metF* could also grow on sulfate plus methanethiol, or on sulfate plus dimethyldisulfide, indicating that these compounds obviously served as both external donors of sulfur and of C_1 carbon, for methionine biosynthesis. The incorporation of the entire methyl group was further confirmed by an isotope study. *C. glutamicum* Δ*metF* was cultivated in a liquid medium with 99% [$^{13}C_6$] glucose and naturally labeled methanethiol (or dimethyldisulfide) as the sole sulfur source. In this experiment the labeling pattern of intracellular methionine allowed a clear conclusion to be drawn of the origin of the terminal side-chain carbon.

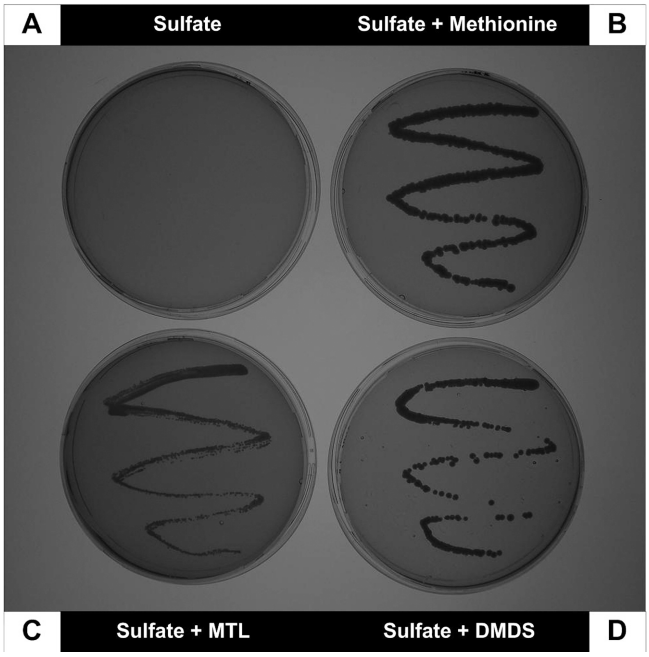

Fig. 3. Growth of *Corynebacterium glutamicum* Δ*metF* on different sulfur sources: sulfate (**A**), sulfate+methionine (**B**), sulfate+methanethiol (**C**), sulfate+dimethyldisulfide (**D**).
Abbreviations: methanethiol, MTL; dimethyldisulfide, DMDS.

Hereby, [$^{13}C_5$]-labeled methionine with a mass shift of m+5 can be attributed to complete synthesis from [$^{13}C_6$] glucose. By contrast, [$^{12}C_1$ $^{13}C_4$] methionine with a mass

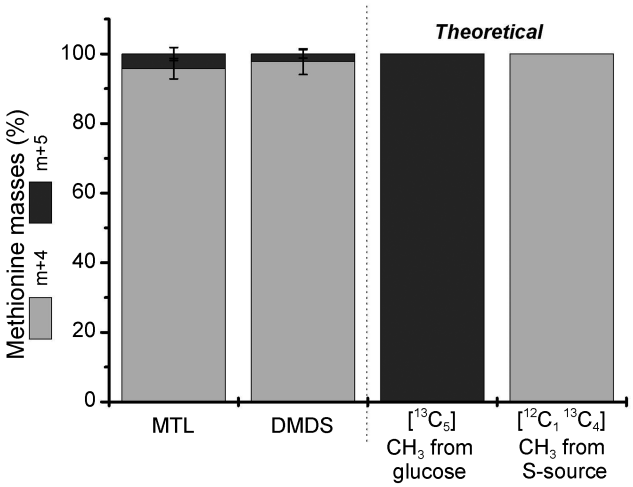

Fig. 4. Labeling pattern of proteinogenic methionine in *C. glutamicum* Δ*metF*: experimental labeling pattern formed during growth on a minimal medium with [$^{13}C_6$] glucose and naturally labeled methanethiol (MTL) or dimethyldisulfide (DMDS), and theoretical labeling pattern to be expected for exclusive origin of the terminal methyl group from [$^{13}C_6$] glucose or from the added sulfur source, respectively.
The data given display average values and deviation from four replicate measurements.

shift of m+4 represents molecules with four carbons from the labeled glucose, and the terminal methyl carbon from the non-labeled sulfur source. For *C. glutamicum* Δ*metF* growing on methanethiol or on dimethyldisulfide, more than 95% of the methionine pool was thus derived from the incorporation of the intact $-S-CH_3$ building blocks (Fig. 4).

Identification of the Catalyzing Enzyme

Further studies were performed to uncover the enzyme responsible for the incorporation of alternative sulfur sources into methionine. *C. glutamicum* Δ*metF*Δ*metB* and *C. glutamicum* Δ*metF*Δ*metY* were constructed to study the potential responsibility of *metB* and *metY* on the metabolization of methanethiol and dimethyldisulfide. Fig. 5 shows the growth of *C. glutamicum* Δ*metF* as well as the double knockouts of Δ*metF*Δ*metB* and Δ*metF*Δ*metY* on agar plates with different sulfur sources (sulfate in combination with methionine, methanethiol, or dimethyldisulfide). The double deletion of *metF* and *metB* could grow on sulfate plus methanethiol or dimethyldisulfide. By contrast, the knockout of *metY* resulted in methionine auxotrophy on sulfate plus methanethiol or dimethyldisulfide. None of the strains were able to grow on sulfate as the sole sulfur source (negative control), owing to the *metF* deletion. The positive control sulfate plus methionine provided growth in all strains. Complementation of the deletion strain *C. glutamicum* Δ*metF*Δ*metY*, by plasmid-based overexpression of *metY*, restored growth on sulfate plus methanethiol or dimethyldisulfide. This clearly demonstrated that MetY (*i.e.*, *O*-acetylhomoserine sulfhydrylase) is responsible for the incorporation of methanethiol and dimethyldisulfide into methionine in *C. glutamicum*.

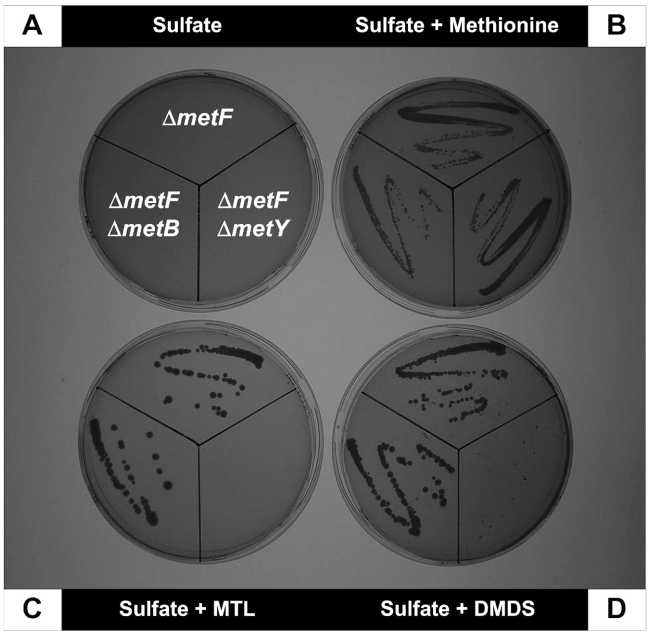

Fig. 5. Growth of *C. glutamicum* Δ*metF*, *C. glutamicum* Δ*metF*Δ*metB*, and *C. glutamicum* Δ*metF*Δ*metY* on different sulfur sources: sulfate (**A**), sulfate+methionine (**B**), sulfate+methanethiol (**C**), sulfate+dimethyldisulfide (**D**).
Abbreviations: methanethiol, MTL; dimethyldisulfide, DMDS.

***In Vitro* Activity of *O*-Acetylhomoserine Sulfhydrylase (MetY)**

The specific *in vitro* activity of *O*-acetylhomoserine sulfhydrylase for methanethiol as a substrate was only 63 mU/mg and thus rather low. A similar value of 61 mU/mg resulted for dimethyldisulfide. It seems likely that dimethyldisulfide is reductively cleaved into methanethiol prior to assimilation, thus yielding the same *in vitro* activity of MetY. For the deletion mutant of *metY*, no significant activity (<0.1 mU/mg) was observed, underlining that *metY* is responsible for the assimilation reaction. A significantly higher activity (480 mU/mg) was found when using sulfide as a substrate.

Overexpression of *O*-Acetylhomoserine Sulfhydrylase (MetY)

Owing to the relatively low endogeneous *metY* activity in *C. glutamicum*, this gene was overexpressed on an episomal plasmid under control of the strong constitutive *sod* promoter. For methanethiol and dimethyldisulfide, MetY activity was increased by a factor of 30 to 1,780 mU/mg. The control strain, carrying the empty plasmid, revealed an unchanged activity (62 U/mg). It was now interesting to see, to which extent the higher MetY activity influenced the formation of the target product, methionine, on the reduced sulfur sources. To this end, samples were taken

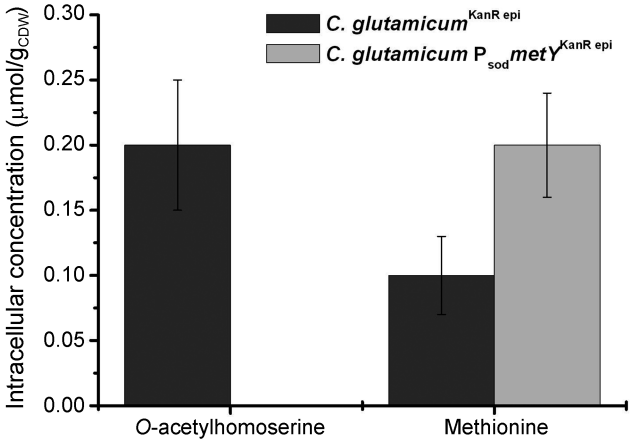

Fig. 6. Intracellular concentrations of *O*-acetylhomoserine and methionine in *C. glutamicum*$^{KanR\ epi}$ and *C. glutamicum* P_{sod}*metY*$^{KanR\ epi}$ during growth on dimethyldisulfide as sole sulfur sources.
The data are mean values of four biological samples. The *O*-acetylhomoserine concentration in *C. glutamicum* P_{sod}*metY*$^{KanR\ epi}$ was below the limit of detection at 0.05 µmol/g-CDM.

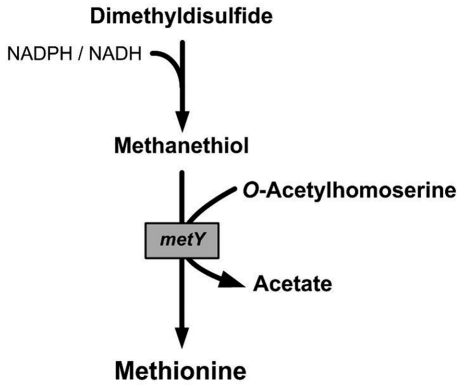

Fig. 7. The proposed pathway for assimilation of methanethiol and dimethyldisulfide into methionine in *Corynebacterium glutamicum*.
The reaction of methanethiol and *O*-acetylhomoserine to methionine is catalyzed by *metY* (*O*-acetylhomoserine sulfhydrylase).

from the *metY* mutant and the control strain during growth on glucose and dimethyldisulfide. The analysis of the cell extract, obtained by fast filtration and extraction in boiling water, revealed a more than 100% increase in the intracellular methionine pool (Fig. 6). This increase was clearly limited by the availability of the substrate of MetY, *O*-acetylhomoserine, which was completely depleted in the *metY* mutant. Methioine was, however, not secreted into the medium.

DISCUSSION

In this study, the potential utilization of methanethiol and dimethyldisulfide as reduced sulfur sources in *C. glutamicum* was explored. It was shown for both compounds that the S-CH_3 group is entirely added to *O*-acetylhomoserine, directly yielding methionine. This reaction is catalyzed by MetY, creating a shortened pathway for methionine biosynthesis (Fig. 7). The fact that the *in vitro* activity is almost the same for both sulfur sources illustrates that dimethyldisulfide might be easily cleaved into methanethiol. These findings open up new possibilities for the use of improved strains for methionine overproduction. In a first proof-of-concept, the amplification of MetY clearly revealed a positive effect on methionine synthesis (Fig. 6). It thus seems a relevant metabolic engineering target for the use of methanethiol or dimethyldisulfide. The beauty of these sulfur sources is their high degree of reduction, significantly reducing the NADPH demand required for the synthesis of methionine. From the view point of production capacity, methanethiol or dimethyldisulfide would theoretically enable highly efficient production with a predicted maximum yield of 90%, which encourages their future utilization in biotechnological methionine production. Since NADPH is a key factor limiting amino acid production in existing processes with *C. glutamicum*, this would be of immediate relevance. Additionally, the shortened pathway would not require the terminal methylation step, so that the difficulties linked to an increased supply of the C_1 carbon, or the undesired accumulation of homolanthionine, could be avoided [17, 19].

The *in vitro* activity of MetY in the engineered strain would be sufficient for a theoretical productivity of approximately 6 g/g/h, which seems competitive for industrial production. Despite this encouraging potential, the amplification of MetY activity can only be a first step towards rational *C. glutamicum* strains overproducing methionine. The depletion of *O*-acetylhomoserine, the substrate of MetY, suggests that other enzymes of the methionine biosynthesis have to be overexpressed. As shown by the present work, in-depth analyses of the metabolites and enzymes of the methionine pathway are crucial to the formation of an understanding of its functions and regulation, and may lead to the identification of further, potentially superior, strains.

Acknowledgments

Christoph J. Bolten and Christoph Wittman acknowledge financial support by the BASF SE. We further thank Stefanie Kind (Biochemical Engineering Institute, TU Braunschweig) for transformation of *C. glutamicum* with episomal plasmids.

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