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

Bolten, Christoph J.,* Hartwig Schröder, Jeroen Dickschat, and Christoph Wittmann

Biochemical Engineering Institute, Technische Universität Braunschweig, 38106 Braunschweig, Germany

Organic Chemistry Institute, Technische Universität Braunschweig, 38106 Braunschweig, Germany

BASF SE, Research Fine Chemicals and Biotechnology, 67056 Ludwigshafen, Germany

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

In the present work, methanethiol and dimethylsulfide 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 dimethylsulfide as sole sulfur sources. Isotopic 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 dimethylsulfide. Both sulfur compounds are incorporated as an entire molecule, adding the terminal S-CH$_2$ group to O-acetylsulfhydrylase. 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 auxotropic strains grown on methanethiol or dimethylsulfide as sole sulfur sources. Plasmid-based overexpression of metY in the *ΔmetY* background restored the capacity to grow on methanethiol or dimethylsulfide 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 dimethylsulfide (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-acetylsulfhydrylase, 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$_3$SH) 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-acetylsulfhydrylase, 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*. 
In the present study, we analyzed the ability of \textit{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.

\textbf{MATERIALS AND METHODS}

\textbf{Microorganisms}

In the current work, the wild type of \textit{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). \textit{Escherichia coli} DH5\textalpha{} and NM522 were obtained from Invitrogen (Karlsruhe, Germany) and used for plasmid amplification and DNA methylation, respectively.

\textbf{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 \textit{Escherichia coli} NM522 and DH5\textalpha{} 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.

\textbf{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 \textit{meF} are given in Table 2. The obtained DNA fragment was subsequently inserted into the vector pClik int

![Fig. 1. Methionine biosynthesis in \textit{Corynebacterium glutamicum}, including the supply of sulfur and C\textsubscript{1} metabolism. THF, tetrahydrofolate.](image-url)