C₁ Metabolism in *Corynebacterium glutamicum*: an Endogenous Pathway for Oxidation of Methanol to Carbon Dioxide

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Methanol is considered an interesting carbon source in “bio-based” microbial production processes. Since *Corynebacterium glutamicum* is an important host in industrial biotechnology, in particular for amino acid production, we performed studies of the response of this organism to methanol. The *C. glutamicum* wild type was able to convert ¹³C-labeled methanol to ¹³CO₂. Analysis of global gene expression in the presence of methanol revealed several genes of ethanol catabolism to be upregulated, indicating that some of the corresponding enzymes are involved in methanol oxidation. Indeed, a mutant lacking the alcohol dehydrogenase gene *adhA* showed a 62% reduced methanol consumption rate, indicating that AdhA is mainly responsible for methanol oxidation to formaldehyde. Further studies revealed that oxidation of formaldehyde to formate is catalyzed predominantly by two enzymes, the acetaldehyde dehydrogenase Ald and the mycothiol-dependent formaldehyde dehydrogenase AdhE. The $\Delta ald \Delta adhE$ and $\Delta ald \Delta mshC$ deletion mutants were severely impaired in their ability to oxidize formaldehyde, but residual methanol oxidation to CO₂ was still possible. The oxidation of formate to CO₂ is catalyzed by the formate dehydrogenase FdhF, recently identified by us. Similar to the case with ethanol, methanol catabolism is subject to carbon catabolite repression in the presence of glucose and is dependent on the transcriptional regulator RamA, which was previously shown to be essential for expression of *adhA* and *ald*. In conclusion, we were able to show that *C. glutamicum* possesses an endogenous pathway for methanol oxidation to CO₂ and to identify the enzymes and a transcriptional regulator involved in this pathway.

Methylophilic microorganisms utilize reduced C₁ compounds, such as methane, methylamines, or methanol, as a sole carbon and energy source and play an important role in the global carbon cycle (1, 2). A key step in methylophilic metabolism is the oxidation of methanol to formaldehyde. Whereas this oxidation step is catalyzed by flavin adenine dinucleotide (FAD)-dependent methanol oxidases with concomitant reduction of oxygen to hydrogen peroxide in methylophilic yeast and fungi (3), Gram-negative methylophilic bacteria, such as *Methylobacterium extorquens*, use pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenases to oxidize methanol in the periplasm (4). Gram-positive thermotolerant *Bacillus* strains usually contain NAD⁺-dependent cytoplasmic methanol dehydrogenases (5). In methylophilic metabolism, cytotoxic formaldehyde represents a crucial central metabolic intermediate. The toxicity of formaldehyde arises from nonenzymatic reactions with biological macromolecules such as proteins and DNA, leading to irreversible alkylations and cross-linkages (6–8). Formaldehyde detoxification can occur either by assimilation into cell material or by oxidation to carbon dioxide (9, 10). Pathways for formaldehyde oxidation to CO₂ are widely distributed and are not limited to methylophilic, since virtually all organisms have to cope with toxic formaldehyde as a by-product of numerous environmental processes and various cellular demethylation and oxidation reactions. Hence, nature developed diverse strategies for its detoxification by efficient capture of formaldehyde by cofactors, such as glutathione, mycothiol, tetrahydrofolate, or tetrahydromethanopterin, and subsequent oxidation of the products (9, 10).

The soil bacterium *Corynebacterium glutamicum* can grow on various carbon sources, such as sugars, organic acids, alcohols, or aromatics, but presumably not on C₁ compounds since none of the known assimilation pathways is encoded in the genome. In agreement, it was reported that *C. glutamicum* strain R does not grow on methanol (11). In industrial biotechnology, highly pro-

ductive strains of *C. glutamicum* serve to produce several million tons of amino acids annually, in particular the flavor enhancer L-glutamate and the feed additive L-lysine. In addition, strains have been developed for a variety of other commercially interesting compounds (12), such as organic acids (13–15), diamines (16–18), or alcohols (19–21). Moreover, *C. glutamicum* is also an efficient host for the production of heterologous proteins (see reference 22 and references therein).

Since methanol can be produced from renewable carbon sources (23), it is an interesting carbon source for “bio-based” production of chemicals or proteins. As a first step toward the goal of making *C. glutamicum* a methylophilic, we studied the response of this organism to methanol at the metabolic level and at the gene expression level. As a result, we describe the identification and characterization of an endogenous pathway for the oxidation of methanol to CO₂.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids used or constructed in the course of this work are listed in Table 1, and oligonucleotides are listed in Table S1 in the supplemental material. *C. glutamicum* was routinely cultivated aerobically in 500-ml baffled shake flasks with 50 ml medium on a rotary shaker

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> DH5 α	F [−] ϕ 80dlac Δ (<i>lacZ</i>)M15 Δ (<i>lacZYA-argF</i>) U169 <i>endA1 recA1 hsdR17</i> (r _K [−] m _K ⁺) <i>deoR</i> <i>thi-1 phoA supE44</i> λ [−] <i>gyrA96 relA1</i>	Invitrogen (Karlsruhe, Germany)
<i>C. glutamicum</i> ATCC 13032	Biotin-auxotrophic wild-type strain	24
<i>C. glutamicum</i> Δ <i>fdhF</i> strain	Derivative of ATCC 13032 with in-frame deletion of <i>fdhF</i> gene	25
<i>C. glutamicum</i> Δ <i>adhA</i> strain	Derivative of ATCC 13032 with in-frame deletion of <i>adhA</i> gene	This work
<i>C. glutamicum</i> Δ <i>adhC</i> strain	Derivative of ATCC 13032 with in-frame deletion of <i>adhC</i> gene	This work
<i>C. glutamicum</i> Δ <i>adhE</i> strain	Derivative of ATCC 13032 with in-frame deletion of <i>adhE</i> gene	This work
<i>C. glutamicum</i> Δ <i>ald</i> strain	Derivative of ATCC 13032 with in-frame deletion of <i>ald</i> gene	This work
<i>C. glutamicum</i> Δ cg2714 strain	Derivative of ATCC 13032 with in-frame deletion of <i>cg2714</i>	This work
<i>C. glutamicum</i> Δ cg0273 strain	Derivative of ATCC 13032 with in-frame deletion of <i>cg0273</i>	This work
<i>C. glutamicum</i> Δ <i>ald</i> Δ <i>adhE</i> strain	Derivative of Δ <i>ald</i> strain with additional in-frame deletion of <i>adhE</i> gene	This work
<i>C. glutamicum</i> Δ <i>ald</i> Δ cg0388 strain	Derivative of Δ <i>ald</i> strain with additional in-frame deletion of <i>cg0388</i>	This work
<i>C. glutamicum</i> Δ <i>ald</i> Δ cg2193 strain	Derivative of Δ <i>ald</i> strain with additional in-frame deletion of <i>cg2193</i>	This work
<i>C. glutamicum</i> RG2	Derivative of ATCC 13032 with partial deletion of <i>ramA</i> gene	26
<i>C. glutamicum</i> Δ <i>mshC</i> strain	Derivative of ATCC 13032 with in-frame deletion of <i>mshC</i> gene	27
<i>C. glutamicum</i> Δ <i>ald</i> Δ <i>mshC</i> strain	Derivative of Δ <i>ald</i> strain with additional in-frame deletion of <i>mshC</i> gene	This study
<i>C. glutamicum</i> Δ <i>adhA</i> /pAN6- <i>adhA</i> strain	Plasmid-based complementation of <i>adhA</i> deletion	This work
<i>C. glutamicum</i> Δ <i>adhA</i> /pAN6 strain	Control strain	This work
<i>C. glutamicum</i> Δ <i>ald</i> /pAN6- <i>ald</i> strain	Plasmid-based complementation of <i>ald</i> deletion	This work
<i>C. glutamicum</i> Δ <i>adhE</i> /pAN6- <i>adhE</i> strain	Plasmid-based complementation of <i>adhE</i> deletion	This work
<i>C. glutamicum</i> Δ <i>ald</i> Δ <i>adhE</i> /pAN6- <i>ald-adhE</i> strain	Plasmid-based complementation of <i>ald-adhE</i> deletion	This work
Plasmids		
pK19 <i>mobsacB</i>	Kan ^r ; mobilizable <i>E. coli</i> vector used for allelic exchange in <i>C. glutamicum</i> (pK18 <i>oriV_{E.c.}</i> <i>oriT</i> <i>sacB</i> <i>lacZ</i>)	28
pK19 <i>AdhA</i>	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 1,010-bp PCR product covering upstream and downstream regions of <i>adhA</i>	This work
pK19 <i>AdhC</i>	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 966-bp PCR product covering upstream and downstream regions of <i>adhC</i>	This work
pK19 <i>Adald</i>	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 1,017-bp PCR product covering upstream and downstream regions of <i>ald</i>	This work
pK19 <i>AdhE</i>	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 1,042-bp PCR product covering upstream and downstream regions of <i>adhE</i>	This work
pK19 Δ cg0273	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 1,037-bp PCR product covering upstream and downstream regions of <i>cg0273</i>	This work
pK19 Δ cg2714	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 1,084-bp PCR product covering upstream and downstream regions of <i>cg2714</i>	This work
pK19 Δ cg0388	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 1,089-bp PCR product covering upstream and downstream regions of <i>cg0388</i>	This work
pK19 Δ cg2193	Kan ^r ; pK19 <i>mobsacB</i> derivative containing 1,063-bp PCR product covering upstream and downstream regions of <i>cg2193</i>	This work
pK18 <i>mobsacB</i> Δ ncgl1457	Kan ^r ; pK18 <i>mobsacB</i> derivative containing PCR product covering upstream and downstream regions of <i>ncgl1457</i> (<i>mshC</i>)	27
pAN6	Kan ^r ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector; derivative of pEKEx2 (<i>Ptac</i> , <i>lacI</i> ^q , pBL1 <i>oriV_{C.g.}</i> , pUC18 <i>oriV_{E.c.}</i>)	29
pAN6- <i>adhA</i>	Kan ^r ; pAN6 derivative containing <i>adhA</i> under control of <i>Ptac</i>	This work
pAN6- <i>ald</i>	Kan ^r ; pAN6 derivative containing <i>ald</i> under control of <i>Ptac</i>	This work
pAN6- <i>adhE</i>	Kan ^r ; pAN6 derivative containing <i>adhE</i> under control of <i>Ptac</i>	This work
pAN6- <i>ald-adhE</i>	Kan ^r ; pAN6 derivative containing <i>ald</i> and <i>adhE</i> under control of <i>Ptac</i>	This work

(120 rpm) at 30°C. Either LB medium (30) or modified CGXII minimal medium (29) containing 28 to 222 mM glucose and/or 100 mM ethanol as a carbon and energy source was used. Methanol was added to the culture medium in concentrations from 50 mM to 3,000 mM using a 5 M stock solution. For strain construction and maintenance, either LB or BHIS agar plates (brain heart infusion [BHI] agar [Difco, Detroit, MI, USA] supplemented with 0.5 M sorbitol) were used. Media used in the course of gene

deletion using pK19*mobsacB* were described before (31). *Escherichia coli* DH5 α was used for cloning purposes and was grown aerobically on a rotary shaker (170 rpm) at 37°C in 5 ml LB medium or on LB agar plates (LB medium with 1.8% [wt/vol] agar). If appropriate, kanamycin was added to final concentrations of 25 μ g ml^{−1} (*C. glutamicum*) or 50 μ g ml^{−1} (*E. coli*). Growth was determined by measuring of the optical density at 600 nm (OD₆₀₀).

Recombinant DNA techniques. The enzymes for recombinant DNA work were obtained from Fermentas (St. Leon-Rot, Germany) and Merck Millipore (Billerica, Massachusetts, USA). Chromosomal DNA from *C. glutamicum* ATCC 13032 was prepared as described previously (32). *E. coli* was transformed by the RbCl method (33) and *C. glutamicum* ATCC 13032 by electroporation (34). Routine methods like PCR, restriction, or ligation were carried out according to standard protocols (30). The oligonucleotides used for cloning were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table S1 in the supplemental material. The in-frame gene deletion mutants of *C. glutamicum* ATCC 13032 were constructed via a two-step homologous recombination procedure as described previously (31). The deletions in the chromosome were verified by PCR analysis using oligonucleotides hybridizing approximately 500 bp upstream and 500 bp downstream of the target gene (see Table S1). The coding regions of *adhA* (cg3107), *ald* (cg3096), and *adhE* (cg0387) were amplified by PCR using the oligonucleotide pairs P_*adhA*_fw_NdeI/P_*adhA*_rev_NheI, P_*ald*_fw_NdeI/P_*ald*_rev_NheI, and P_*adhE*_fw_NdeI/P_*adhE*_rev_NheI, respectively (see Table S1), digested with NdeI and NheI, and cloned into the expression vector pAN6 for complementation studies. The cloned regions were checked by DNA sequencing using plasmid-specific primers (see Table S1).

Determination of glucose and formate by HPLC. The concentrations of glucose and formate in cell-free culture supernatants were determined by HPLC analysis using an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) equipped with a cation exchange column (organic acid refill column, 300 by 8 mm [CS-Chromatographie Service GmbH, Langerwehe, Germany]) as described previously (35).

Determination of methanol by gas chromatography. The quantitative detection of methanol in the cell-free supernatant of *C. glutamicum* cultures was performed via capillary gas chromatography (GC) using an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an HP-5 column [(5% phenyl)-methylpolysiloxane, 30 m, 0.32 mm, 0.25 μ m (Agilent Technologies Waldbronn, Germany)]. Helium was used as a carrier gas with a flow rate of 2.8 ml/min. The sample (1 μ l) was injected via split injection (1:50), employing a Combi PAL GC autosampler (CTC Analytics GmbH, Zwingen, Germany). The injector temperature was set to 250°C, and the column temperature was kept constant at 40°C. Detection was performed using a flame ionization detector (FID) at a detector temperature of 250°C. Calibration was performed with methanol as an external standard. Butanol (60 mM) was used as an internal standard and was added to the samples in the same volume ratio. Concentrations were calculated from peak areas using calibration with external methanol and internal butanol standards.

Determination of formaldehyde. Formaldehyde concentrations were determined via a colorimetric photometric assay as described by Nash (36). This assay is based on the condensation of acetylacetone and free formaldehyde in the presence of excess ammonium salt to the chromophore diacetyldihydrolutidine (Hantzsch reaction), which is characterized by an absorption maximum at 412 nm and an extinction coefficient of 7.7 mM⁻¹ cm⁻¹. For the determination of formaldehyde concentrations, samples of 0.5 ml cell-free culture supernatant were taken at various time points during cultivation, mixed with 0.5 ml Nash reagent (2 M ammonium acetate, 50 mM acetic acid, and 20 mM acetylacetone), and incubated for 10 min at 60°C. Subsequently, the absorption at 412 nm was measured for each sample. A calibration curve showed that the assay was linear between 20 μ M and 1.5 mM formaldehyde.

DNA microarray analysis. Whole-genome DNA microarray analyses were performed to monitor changes in the global gene expression of the *C. glutamicum* wild type in response to the presence of methanol. Cells of a preculture in LB medium were used to inoculate a second preculture in CGXII minimal medium with 111 mM glucose. To test the influence of methanol in the presence of glucose, two parallel main cultures in CGXII minimal medium with 111 mM glucose were inoculated with the second preculture to an OD₆₀₀ of 0.5. After reaching an OD₆₀₀ of 5, 100 mM

methanol was added to one culture, whereas water was added to the second one as a control. After continuing incubation for 30 min, cells of both cultures were harvested in precooled (−20°C) ice-filled tubes via centrifugation (6,900 $\times$ g, 10 min, 4°C) and used for RNA isolation. To test the influence of methanol in the absence of glucose, a preculture in CGXII medium with 111 mM glucose was incubated until an OD₆₀₀ of 8 was reached. Cells were washed with CGXII minimal medium without a carbon source and resuspended in this medium to an OD₆₀₀ of 5. The cell suspensions were incubated for 2 h, and then 100 mM methanol was added to one of the suspensions and water to the second one. After incubation for another 30 min, cells were harvested and used for RNA isolation.

RNA isolation and synthesis of fluorescently labeled cDNA were carried out as described previously (37). For cDNA synthesis, 25 μ g total RNA of each sample was used. Custom-made DNA microarrays for *C. glutamicum* ATCC 13032 printed with 70-er oligonucleotides were obtained from Operon (Cologne, Germany) and were based on the genome sequence entry NC_006958 (38). Processing of DNA microarrays and evaluation of the obtained data were performed as previously described (29). The normalized and processed data were saved in the in-house microarray database (39). All DNA microarray experiments were performed in three replicates.

Determination of alcohol dehydrogenase activities in crude extracts. For the determination of alcohol dehydrogenase activity *in vitro*, the *C. glutamicum* wild type was cultivated in CGXII minimal medium either with 56 mM glucose, with 56 mM glucose and 100 mM ethanol, or with 56 mM glucose and 100 mM methanol. Cells were grown until the early stationary phase (OD₆₀₀ of ~22), and 50-ml culture samples were harvested by centrifugation for 15 min at 4,500 $\times$ g and 4°C. After washing in 25 ml buffer (100 mM Tris-HCl and 30% [vol/vol] glycerol, pH 7.5), cells were resuspended in 2 ml 100 mM Tris-HCl, 30% (vol/vol) glycerol, and 1 mM dithiothreitol, pH 7.5. Cells were disrupted by sonication (6 min; cycle, 0.5; amplitude, 50) using a UP200S sonifier (Hielscher GmbH, Stuttgart, Germany) with cooling on ice. After centrifugation (30 min, 16,000 $\times$ g, 4°C), the supernatant was used for enzyme assays. The protein concentration of the cell extracts was determined by the method of Bradford (40) using bovine serum albumin as the standard. The assay was performed as described previously (11) with slight modifications. The assay mixtures (1 ml total volume) contained 50 mM Tris-HCl (pH 8.6), 4 mM NAD⁺, and 10 μ l cell extract. The reaction was initiated by the addition of 340 mM ethanol and monitored by following the increase in absorption at 340 nm over 2 min using an Ultraspec 3100pro photometer (GE Healthcare Life Sciences, Freiburg, Germany) at 30°C. One unit of alcohol dehydrogenase activity is defined as the reduction of 1 μ mol NAD⁺ per minute.

Determination of ¹³CO₂ and ¹²CO₂. The conversion of methanol to CO₂ by *C. glutamicum* was monitored in ¹³C labeling experiments. Cells were grown in 200 ml CGXII medium with 56 mM glucose in a Dasgip parallel bioreactor system (Dasgip AG, Jülich, Germany), and 100 mM ¹³C-labeled methanol (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium immediately before inoculation. During cultivation, pH, dissolved oxygen concentration, and off-gas (CO₂ and O₂) were monitored online via the Dasgip monitoring system (Dasgip Control 4.0). Whereas the pH was not controlled during cultivation, the dissolved O₂ saturation was kept at >30%, varying the stirrer speed between 400 and 1,200 rpm at a constant airflow rate of 4 standard liters (sl)/h. If required, antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA) was added. Growth was monitored offline by OD₆₀₀ measurements. ¹³CO₂ and ¹²CO₂ off-gas analysis and quantification were performed using an FT-IR gas analyzer (Gasmeter CR-2000i [Ansyco, Karlsruhe, Germany]) and the Calcmnet software program, version 10 (Gasmeter Technologies Oy, Helsinki, Finland).

Microarray data accession number. The microarray data were deposited in the GEO database with the accession number GSE49936.

FIG 1 (A) Methanol consumption (■) and growth (■, in gray) of the *C. glutamicum* wild type in CGXII minimal medium supplemented with 111 mM glucose and 120 mM methanol. Four independent experiments were performed in shake flasks, always accompanied by a methanol evaporation control without cells (○). (B) ¹³CO₂ formation as ¹³CO₂/total CO₂ ratio (■, in gray), methanol consumption (■), and glucose consumption (▼) during growth of the *C. glutamicum* wild type in bioreactors with 56 mM glucose in the presence of 100 mM ¹³C-labeled methanol. The *C. glutamicum* wild type showed a growth rate of 0.27 ± 0.01 h⁻¹ and a final OD₆₀₀ of 20.4 ± 1.8 .

RESULTS

Methanol is metabolized by *C. glutamicum* in stationary growth phase.

Initial cultivation experiments of the *C. glutamicum* wild type in CGXII minimal medium containing 111 mM glucose and methanol at concentrations ranging from 0.05 M to 3 M showed that the growth rate (μ) decreased with increasing methanol concentrations. The following values were obtained: 0.41 h⁻¹ at 0 mM, 0.33 h⁻¹ at 0.05 M, 0.28 h⁻¹ at 0.1 M, 0.24 h⁻¹ at 0.2 M, 0.21 h⁻¹ at 0.7 M, 0.19 h⁻¹ at 1 M, 0.17 h⁻¹ at 1.3 M, and 0.09 h⁻¹ at 1.5 M (see Fig. S1 in the supplemental material). At 3 M methanol, growth was almost completely inhibited. Cultivation experiments in minimal medium with 111 mM glucose and 120 mM methanol revealed that the *C. glutamicum* wild type consumes methanol against the background of methanol evaporation with a rate of 63.2 μ mol h⁻¹ (g cell dry weight [CDW])⁻¹ (Fig. 1A). After 72 h of cultivation, only 41 mM methanol remained in the minimal medium, whereas 73 mM methanol could be detected in the control flask without *C. glutamicum* cells. Since *C. glutamicum* was not able to grow on methanol as a sole carbon source and does not possess one of the known pathways for methanol assimilation (data not shown), we assumed that methanol is oxidized to CO₂. To test this assumption, cultivation experiments were performed in bioreactors using CGXII minimal medium with 56 mM glucose and 100 mM ¹³C-labeled methanol. During these experiments, ¹³C-labeled CO₂ was detected by Fourier transform infrared spectroscopy (FTIR) as soon as methanol consumption started in the stationary growth phase, when glucose had been completely consumed (Fig. 1B).

FdhF is responsible for oxidation of formate to carbon dioxide. Oxidation of methanol to CO₂ is catalyzed stepwise by specific dehydrogenases via the intermediates formaldehyde and formate (10). Whereas no genes coding for methanol- or formaldehyde dehydrogenase have been annotated in the genome of *C. glutamicum* (41, 42), we recently identified genes coding for a molybdenum-containing formate dehydrogenase (25). To test the involvement of this enzyme in methanol oxidation, growth experiments similar to those described for Fig. 1A were conducted with a *C. glutamicum* Δ fdhF mutant (25), which resulted in a slightly reduced methanol consumption (27 mM $\pm$ 4 mM) compared to that of the wild type (34 mM $\pm$ 6 mM) after 72 h against an

evaporation background. Most important, the mutant formed an equimolar concentration of formate (27 mM $\pm$ 2 mM) in the culture supernatant, whereas the wild type formed only 18 mM $\pm$ 4 mM. This result shows that FdhF is responsible for formate oxidation to CO₂ in the wild type and confirms that methanol or formaldehyde is not assimilated by *C. glutamicum*. Additionally, the formate accumulation during growth of the wild type in methanol-containing medium indicated that the oxidation of formate to CO₂ is the limiting factor in the endogenous methanol oxidation pathway in *C. glutamicum*.

Search for genes involved in methanol oxidation to CO₂ by global gene expression analysis. In order to identify genes that might be involved in the oxidation of methanol and formaldehyde, we tested the influence of methanol on global gene expression using DNA microarray analysis. Gene expression was considered to be influenced by methanol if mRNA levels in at least two out of three biological replicates differed from those of the control without methanol. In total, 20 genes were up- and 19 genes were downregulated in the presence of 100 mM methanol (see Table S2 in the supplemental material). Interestingly, two genes known to be essential for the oxidation of ethanol to acetate in *C. glutamicum* were among the upregulated genes: *adhA* (cg3107; 4.4-fold), encoding an alcohol dehydrogenase (11, 43), and *ald* (cg3096; 2.1-fold), encoding acetaldehyde dehydrogenase (44).

Besides *adhA*, four other genes have been annotated as putative alcohol dehydrogenase genes in the genome of *C. glutamicum* (43): cg0273, cg0387 (*adhE*), cg0400 (*adhC*), and cg2714. Their expression levels were not altered in the presence of methanol. To determine the relevance of *adhA*, *ald*, and the other putative alcohol dehydrogenase genes for methanol or formaldehyde oxidation in *C. glutamicum*, in-frame deletion mutants lacking these genes were constructed and characterized.

AdhA is mainly responsible for oxidation of methanol to formaldehyde. The Δ adhA, Δ adhC, Δ adhE, Δ cg2714, and Δ cg0273 *C. glutamicum* deletion mutants were analyzed regarding their ability to oxidize methanol. All deletion mutants showed the same growth behavior in CGXII medium containing 111 mM glucose and 120 mM methanol (see Fig. S2 in the supplemental material). However, whereas the methanol oxidation rate of the Δ adhC, Δ adhE, Δ cg2714, and Δ cg0273 strains (70.0 ± 20 , 73 ± 4 , 70 ± 4 ,

FIG 2 (A) Methanol oxidation of *C. glutamicum* wild type (■), $\Delta adhA$ strain (●), $\Delta adhC$ strain (▲), $\Delta adhE$ strain (▼), $\Delta cg2714$ strain (◆), and $\Delta cg0273$ strain (◐) in CGXII minimal medium supplemented with 111 mM glucose and 120 mM methanol. Two to four independent experiments in shake flasks were performed, always accompanied by a methanol evaporation control (○). (B) $^{13}\text{CO}_2$ formation as $^{13}\text{CO}_2$ /total CO_2 ratio of *C. glutamicum* wild type (■) and $\Delta adhA$ strain (●) during cultivation in bioreactors in the presence of 100 mM ^{13}C -labeled methanol. Experiments to determine $^{13}\text{CO}_2$ generation in the *C. glutamicum* wild type were performed in triplicate and those for the $\Delta adhA$ mutant in duplicate. Mean values are shown.

and $61 \pm 3 \mu\text{mol h}^{-1} [\text{g CDW}]^{-1}$, respectively) were similar to that of the wild type ($63.3 \pm 10 \mu\text{mol h}^{-1} [\text{g CDW}]^{-1}$), the rate for the $\Delta adhA$ mutant was reduced by about 62%, to $26.1 \pm 6.1 \mu\text{mol h}^{-1} (\text{g CDW})^{-1}$ (Fig. 2A). Complementation of the $\Delta adhA$ strain with the expression plasmid pAN6-*adhA* (plus 1.5 mM isopropyl- β -D-thiogalactopyranoside [IPTG]) restored the wild-type methanol oxidation rate (data not shown). ^{13}C Methanol labeling experiments confirmed the participation of AdhA in methanol oxidation, since the $\Delta adhA$ mutant generated 60% less $^{13}\text{CO}_2$ than the wild type (Fig. 2B). These results indicate that the first step in methanol oxidation by *C. glutamicum* is predominantly catalyzed by AdhA but that at least one additional dehydrogenase of hitherto unknown identity is also involved.

Enzyme assays with *C. glutamicum* crude extracts revealed that cells cultivated in minimal medium with 56 mM glucose and 100 mM methanol possessed a 3-fold higher alcohol dehydrogenase activity ($280 \pm 22 \text{ U/g protein}$) in stationary phase than cells grown solely with 56 mM glucose ($96 \pm 60 \text{ U/g}$), suggesting that *adhA* expression is induced by methanol. Previous studies with *C. glutamicum* strain R are in agreement with this result (11).

Ald oxidizes formaldehyde to formate. In preliminary experiments, we tested growth of the *C. glutamicum* wild type in the presence of various concentrations of formaldehyde. In the pres-

ence of 2 mM formaldehyde, it showed a slight growth inhibition, and it showed a strong growth defect at 4 mM formaldehyde (see Fig. S3 in the supplemental material). As displayed in Fig. 3A, growth of the Δald mutant was slightly more inhibited by 2 mM formaldehyde than growth of the wild type, indicating a decreased ability of the mutant to metabolize formaldehyde. This was confirmed by the finding that the rate of formaldehyde disappearance as measured by the Nash assay was only $0.66 \pm 0.1 \text{ mM formaldehyde h}^{-1}$ for the Δald mutant, but it was $1.17 \text{ mM} \pm 0.18 \text{ mM h}^{-1}$ for the wild type (Fig. 3B). Complementation of the Δald mutant with the expression plasmid pAN6-*ald* (plus 0.3 mM IPTG) led to a formaldehyde oxidation rate of $2.2 \text{ mM} \pm 0.3 \text{ mM h}^{-1}$. These results clearly indicate that Ald plays an important role in formaldehyde oxidation to formate but that another enzyme involved in formaldehyde consumption must be present in *C. glutamicum*.

Mycothioli-dependent oxidation of formaldehyde via AdhE.

Inspection of the genome sequence of *C. glutamicum* for further candidates involved in formaldehyde oxidation led to the identification of Cg0387 (AdhE). As mentioned above, AdhE is annotated as alcohol dehydrogenase but shares 66 to 69% amino acid sequence identity to mycothiol-dependent formaldehyde dehydrogenases of *Amycolatopsis methanolica* (45), *Mycobacterium*

FIG 3 Growth (A) or formaldehyde oxidation (B) of *C. glutamicum* wild type (■) and Δald (●), $\Delta adhE$ (▲, in gray), $\Delta cg0273$ (▼, in gray), and $\Delta ald \Delta adhE$ (◆) mutant strains in CGXII minimal medium with 111 mM glucose. After cultivation for 4 h, 2 mM formaldehyde was added (indicated by an arrow in panel A). Mean values and standard deviations for three biological replicates are shown, always accompanied by an evaporation control (○).

FIG 4 Growth (A) or formaldehyde oxidation (B) of *C. glutamicum* wild type (■) and $\Delta adhE$ (▲), $\Delta mshC$ (▲, in gray), $\Delta ald \Delta adhE$ (◆), and $\Delta ald \Delta mshC$ (◆, in gray) mutant strains in CGXII minimal medium with 111 mM glucose. After cultivation for 4 h, 2 mM formaldehyde was added (indicated by an arrow in panel A). Mean values and standard deviations for three biological replicates are shown.

smegmatis (46), and *Rhodococcus erythropolis* (47). These proteins catalyze the NAD⁺-dependent oxidation of a spontaneously formed product of mycothiol and formaldehyde, S-hydroxymethyl-mycothiol, to S-formyl-mycothiol, which can subsequently be hydrolyzed to formate and mycothiol (48–50). Similar to the case with the *C. glutamicum* Δald strain, the deletion of *adhE* resulted in slightly impaired growth in the presence of 2 mM formaldehyde (Fig. 3A), and the rate of formaldehyde disappearance was reduced to $0.74 \pm 0.06 \text{ mM h}^{-1}$, compared to $1.17 \text{ mM} \pm 0.18 \text{ mM h}^{-1}$ observed for the wild type (Fig. 3B). Complementation of the $\Delta adhE$ mutant with the expression plasmid pAN6-*adhE* (plus 0.3 mM IPTG) led to a formaldehyde oxidation rate of $1.7 \text{ mM} \pm 0.7 \text{ mM h}^{-1}$. Thus, besides Ald, AdhE is also involved in formaldehyde oxidation in *C. glutamicum*.

To test for additional formaldehyde oxidation enzymes, the *C. glutamicum* $\Delta ald \Delta adhE$ double mutant was constructed and characterized. As shown in Fig. 3A, growth of the $\Delta ald \Delta adhE$ strain stopped shortly after addition of 2 mM formaldehyde to the minimal medium, and formaldehyde consumption was almost completely prevented (Fig. 3B). Plasmid-based expression of *ald* and *adhE* in the *C. glutamicum* $\Delta ald \Delta adhE$ strain with pAN6-*ald-adhE* (plus 0.3 mM IPTG) restored the ability to oxidize formaldehyde ($2.3 \text{ mM} \pm 0.1 \text{ mM h}^{-1}$). [¹³C]Methanol labeling experiments further confirmed the participation of Ald and AdhE in the methanol oxidation pathway, since the $\Delta ald \Delta adhE$ strain generated 75% less ¹³CO₂/total CO₂ than the wild type (data not shown). However, ¹³CO₂ was still generated, and although the methanol oxidation rate of the $\Delta ald \Delta adhE$ strain was lower than that of the wild type ($32.9 \pm 3.3 \mu\text{mol h}^{-1} [\text{g CDW}]^{-1}$ versus $63.3 \pm 10 \mu\text{mol h}^{-1} [\text{g CDW}]^{-1}$), *C. glutamicum* possesses another possibility for detoxifying formaldehyde besides using Ald and AdhE.

To verify the mycothiol-dependence of AdhE in *C. glutamicum*, a strain deficient in mycothiol biosynthesis (*C. glutamicum* $\Delta mshC$ [27]) was investigated regarding its capability to oxidize formaldehyde. The deletion of *mshC* resulted in an impaired growth in the presence of 2 mM formaldehyde (Fig. 4A), and similar to the case with the *C. glutamicum* $\Delta adhE$ strain, the formaldehyde oxidation rate was reduced ($0.54 \text{ mM} \pm 0.11 \text{ mM h}^{-1}$) in comparison to that of the wild type ($1.7 \text{ mM} \pm 0.7 \text{ mM h}^{-1}$) (Fig. 4B). In addition, the *C. glutamicum* $\Delta ald \Delta mshC$ double deletion mutant was constructed. It showed a phenotype similar

to that of the *C. glutamicum* $\Delta ald \Delta adhE$ strain, with respect to growth and its capability to oxidize formaldehyde; growth stopped shortly after addition of 2 mM formaldehyde to the minimal medium, and formaldehyde consumption was almost completely prevented (Fig. 4B). These results clearly support that formaldehyde oxidation by AdhE is strictly dependent on mycothiol.

Three possible pathways for conversion of S-formyl-mycothiol were reported: spontaneous hydrolysis to formate and mycothiol, hydrolysis to formate and mycothiol by an S-formyl-mycothiol hydrolase (FMH), or oxidation by a molybdoprotein aldehyde dehydrogenase to S-carboxy-mycothiol, which spontaneously decomposes to carbon dioxide and mycothiol (46, 51). The latter possibility appears unlikely for *C. glutamicum*, since the $\Delta fdhF$ mutant converted methanol stoichiometrically to formate. The gene located immediately downstream of *adhE* in *C. glutamicum*, cg0388, is annotated as Zn-dependent hydrolase, and the derived protein sequence has 65% sequence identity to that of the proposed *R. erythropolis* FMH (47). In addition, the putative lysophospholipase (cg2193) shows 38% sequence identity to the *Mycobacterium tuberculosis* Rv2854 protein, which is expected to be an FMH. It is located in an operon with the NADPH-dependent mycothiol reductase gene *mtr*, and the organization of these genes is conserved in most mycobacteria and corynebacteria, as well as in *C. glutamicum* (49).

To test the role of the genes cg0388 and cg2193 in methanol oxidation, both genes were individually deleted in the *C. glutamicum* Δald background. Growth experiments in CGXII minimal medium containing 56 mM glucose and 120 mM methanol revealed that the *C. glutamicum* $\Delta ald \Delta cg0388$ strain and the *C. glutamicum* $\Delta ald \Delta cg2193$ strains accumulate as much formate ($15.1 \pm 0.5 \text{ mM}$ and $17.5 \pm 0.5 \text{ mM}$, respectively) within 60 h as the wild type ($16.7 \pm 0.4 \text{ mM}$) and significantly more than the $\Delta ald \Delta adhE$ mutant ($6.1 \pm 0.7 \text{ mM}$), suggesting that neither Cg0388 nor Cg2193 is essential for conversion of S-formyl-mycothiol to mycothiol and formate, but this does not exclude that they possess such an activity. These results hint at a spontaneous hydrolysis of S-formyl-mycothiol to formate and mycothiol.

Methanol consumption by *C. glutamicum* is subject to catabolite repression. As shown above (Fig. 1), methanol oxidation is subject to carbon catabolite repression by glucose, since it starts in the stationary growth phase after glucose has been consumed.

FIG 5 Influence of methanol on expression of selected genes in *C. glutamicum*. DNA microarray experiments showed that genes participating in ethanol catabolism and methanol oxidation are upregulated in the presence of methanol (AdhA, alcohol dehydrogenase; Ald, acetaldehyde dehydrogenase; AK, acetate kinase; PTA, phosphotransacetylase; MS, malate synthase; ICL, isocitrate lyase). The influence of methanol was more pronounced when methanol was added to resting cells in the absence of glucose (black numbering) than after the addition of methanol to cells grown with glucose (gray numbers).

Such a behavior is quite unusual for *C. glutamicum*, since the majority of carbon sources, such as acetate (52) and gluconate (29), are consumed in parallel by this bacterium. Two known exceptions are glutamate (53) and ethanol (11, 54). In the case of ethanol, *adhA* expression was shown to be subject to a complex transcriptional control involving RamA as an essential activator and RamB and SucR as repressors (43, 55). RamA and RamB are global transcriptional regulators in *C. glutamicum* and control genes for enzymes of the central metabolism (26, 56, 57). SucR is now termed AtlR (58). In the case of *ald*, RamA was described as an essential activator and RamB as a weak repressor (44). Since AdhA was shown to be the major enzyme responsible for methanol oxidation to formaldehyde, we tested methanol consumption by a *C. glutamicum* $\Delta ramA$ mutant (26) and observed that its capability to oxidize methanol was strongly reduced (see Fig. S4A in the supplemental material) and was comparable to that of a $\Delta adhA$ mutant. Further support for the methanol oxidation defect of the $\Delta ramA$ mutant was obtained by [^{13}C]methanol labeling experiments. They revealed that the $\Delta ramA$ mutant produced 86% less $^{13}\text{CO}_2$ /total CO_2 than the wild type (see Fig. S4B). These results are in agreement with the essential role of RamA for *adhA* expression.

To determine the methanol oxidation capacity of *C. glutamicum* under optimal conditions, i.e., in the absence of carbon catabolite repression (which in the case of *adhA* is seen not only with glucose but also with acetate [43]), we analyzed cells cultivated in CGXII minimal medium containing 100 mM methanol and 100 mM ethanol. Under these conditions, the specific methanol consumption rate was $366 \pm 19 \mu\text{mol h}^{-1} (\text{g CDW})^{-1}$, compared to $170 \pm 60 \mu\text{mol h}^{-1} (\text{g CDW})^{-1}$ in medium containing 100 mM methanol and 28 mM glucose.

The DNA microarray experiments reported above were performed with cells cultivated in the presence of 111 mM glucose and 100 mM methanol. To test whether methanol alone provokes a more pronounced up- or downregulation of relevant genes, another set of DNA microarray experiments was performed in which RNA was prepared from resting cells incubated with or without methanol as a sole carbon source. In this case, an about 5-fold-

higher expression of genes involved in methanol, ethanol, and acetate metabolism was observed than in the previous DNA microarray experiment, confirming the negative effect of glucose on these genes (Fig. 5). The total number of genes showing altered expression in these experiments was much larger due to the usage of resting cells (see Table S3 in the supplemental material).

DISCUSSION

In this study, the capability of *C. glutamicum* for oxidation of methanol to CO_2 was demonstrated for the first time, and the key enzymes involved in this endogenous pathway were identified (Fig. 6). The dissimilation of C_1 compounds is widespread in nature and has also been found in other members of the *Actinomyetales*, such as *Amycolatopsis methanolica*, *R. erythropolis*, or *M. smegmatis* (59–61).

Our results revealed that the NAD^+ - and zinc-dependent alcohol dehydrogenase AdhA is primarily responsible for methanol oxidation. This is in line with the finding that AdhA of *C. glutamicum* strain R, which has 98% sequence identity to AdhA of strain ATCC 13032, is active not only with ethanol but also with methanol, propanol, and butanol (11). Besides AdhA, one or more additional dehydrogenase(s) capable of oxidizing methanol must be present in *C. glutamicum*. In contrast to the $\Delta adhA$ strain, the $\Delta adhC$, $\Delta adhE$, $\Delta cg2714$, and $\Delta cg0273$ deletion mutants did not show a reduction of the methanol oxidation rate, arguing against an important role of the encoded putative alcohol dehydrogenases in methanol catabolism.

The oxidation of formaldehyde by *C. glutamicum* was shown to be predominantly catalyzed by the enzymes Ald (Cg3096) and AdhE (Cg0387). Similar to AdhA, Ald is essential for growth on ethanol, since it catalyzed the oxidation of acetaldehyde to acetate (44). Interestingly, Ald of *C. glutamicum* shows 70% sequence identity to an NAD^+ -dependent aldehyde dehydrogenase (AldhR) from *R. erythropolis* UPV-1 (62). This enzyme is characterized by broad substrate specificity for aliphatic aldehydes, such as *n*-hexanal, *n*-octanal, and formaldehyde. Hence, it is no surprise that Ald of *C. glutamicum* also accepts formaldehyde as the substrate.

FIG 6 Overview of the methanol oxidation pathway in *C. glutamicum*. Methanol is oxidized to formaldehyde by the alcohol dehydrogenase AdhA and at least one additional unknown dehydrogenase. Formaldehyde is then either directly oxidized to formate by the acetaldehyde dehydrogenase Ald or spontaneously forms S-(hydroxymethyl)mycothiol with mycothiol (MSH). Subsequently, AdhE oxidizes S-(hydroxymethyl)mycothiol to S-formylmycothiol, which hydrolyzes to mycothiol and formate. Finally, formate is oxidized to carbon dioxide by the formate dehydrogenase FdhF, which is dependent on a yet-unknown redox cofactor.

Sequence similarity analyses revealed that AdhE belongs to the class III zinc-dependent alcohol dehydrogenases but also exhibits high sequence identity to the MSH-dependent formaldehyde dehydrogenase AdhC of *R. erythropolis*. In this organism, AdhC oxidizes aliphatic alcohols ranging from ethanol to octanol but not methanol in an MSH-independent manner in addition to the MSH-dependent oxidation of formaldehyde (60). The mycothiol dependence of formaldehyde oxidation by AdhE of *C. glutamicum* was confirmed by the fact that a mycothiol-defective mutant ($\Delta mshC$) showed the same phenotype as a $\Delta adhE$ mutant and a $\Delta ald \Delta mshC$ double mutant behaved similarly to a $\Delta ald \Delta adhE$ mutant. In *C. glutamicum*, mycothiol was reported to contribute to resistance to alkylating agents, glyphosate, ethanol, antibiotics, heavy metals, and aromatic compounds (63). Our findings indicate that *C. glutamicum*, similar to *R. erythropolis* (47), can oxidize formaldehyde in an MSH-dependent manner via AdhE and in an NAD-dependent manner via Ald.

In the case of MSH-dependent oxidation of formaldehyde, the intermediate S-formylmycothiol is formed. In *M. tuberculosis*, a gene (Rv2854) is found which might code for an S-formylmycothiol hydrolase (FMH). However, no thiol esterase activity converting S-formylmycothiol to formate was detected in *M. tuberculosis*, suggesting that S-formylmycothiol might be oxidatively converted by a molybdoprotein aldehyde dehydrogenase to an unstable carbon dioxide ester, which then spontaneously decomposes to CO_2 and MSH (51). A *C. glutamicum* Δald mutant still accumulates the same amount of formate as the wild type, indicating that S-formylmycothiol generated by AdhE is not oxidized to CO_2 but is hydrolyzed to formate and mycothiol either spontaneously or by an FMH. The genes cg0388 and cg2193 show sequence similarity to putative FMHs of *R. erythropolis* and *M. tuberculosis*. However, the $\Delta ald \Delta cg0388$ and $\Delta ald \Delta cg2193$ double mutants were unaltered with respect to formate accumulation, indicating that the Cg0388 and Cg2193 proteins are not required for hydrolysis of S-formyl-mycothiol and that this step may occur spontaneously.

The last step in the methanol oxidation pathway, the oxidation of formate to CO_2 , was shown to be catalyzed by the previously identified molybdenum cofactor-dependent formate dehydrogenase FdhF (25). The electron acceptor of this enzyme has not yet been identified.

During this study, we observed that methanol oxidation in *C. glutamicum* is subject to catabolite repression by glucose. Repression of *adhA* and *ald* expression by glucose has been described first for studies of ethanol catabolism in *C. glutamicum* (44). Expression of both genes is strictly dependent on the transcriptional regulator RamA, since a *ramA*-deficient strain was no longer able to grow on ethanol as a carbon source (26, 44, 54, 56). Our DNA microarray experiments confirmed the upregulation of *adhA* and *ald* in the presence of methanol, and enzyme assays with cell extracts revealed that the NAD-dependent ethanol dehydrogenase activity of cells cultivated in minimal medium with glucose and methanol was 3-fold higher than that in cells grown solely with glucose. Thus, not only ethanol but also methanol apparently is capable of triggering transcription of RamA-activated genes. The deletion mutant *C. glutamicum* $\Delta ramA$ was strongly impaired in its ability to oxidize methanol, in line with the fact that RamA is essential for transcriptional activation of *adhA* and *ald*. Until now, it has not been clear how RamA activity is controlled at the protein level. Our results clearly indicate that not only C_2 metabolites but also C_1 metabolites are capable of activating RamA.

In summary, we identified four enzymes (AdhA, Ald, AdhE, and Fdh) and one transcriptional regulator (RamA) that are involved in the endogenous oxidation of methanol to CO_2 by *C. glutamicum*. As indicated in the introduction, methanol could be an interesting carbon source for the microbial production of food and feed additives or fine chemicals (23). To date, only a few methylotrophic bacteria, such as *Bacillus methanolicus* or *Methylophilus methylotrophus*, were engineered for the production of amino acids from methanol (64, 65). As an alternative approach, it might be possible to establish the ability to utilize methanol as a carbon source in naturally nonmethylotrophic *C. glutamicum* production strains by introducing suitable heterologous pathways, such as the ribulose monophosphate pathway or the serine pathway. The elucidation of the route for methanol dissimilation to carbon dioxide and its regulation achieved in this study represents a first step toward this goal.

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