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Production of carbon-13-labeled cadaverine by engineered *Corynebacterium glutamicum* using carbon-13-labeled methanol as co-substrate

Lennart Leßmeier • Johannes Pfeifenschneider • Marc Carnicer •
Stephanie Heux • Jean-Charles Portais • Volker F. Wendisch

Abstract Methanol, a one-carbon compound, can be utilized by a variety of bacteria and other organisms as carbon and energy source and is regarded as a promising substrate for biotechnological production. In this study, a strain of non-methylotrophic *Corynebacterium glutamicum*, which was able to produce the polyamide building block cadaverine as non-native product, was engineered for co-utilization of methanol. Expression of the gene encoding NAD⁺-dependent methanol dehydrogenase (Mdh) from the natural methylotroph *Bacillus methanolicus* increased methanol oxidation. Deletion of the endogenous aldehyde dehydrogenase genes *ald* and *fadH* prevented methanol oxidation to carbon dioxide and formaldehyde detoxification via the linear formaldehyde dissimilation pathway. Heterologous expression of genes for the key enzymes hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase of the ribulose monophosphate (RuMP) pathway in this strain restored growth in the presence of methanol or formaldehyde, which suggested efficient formaldehyde detoxification involving RuMP key enzymes. While growth with methanol as sole carbon source was not observed, the fate of ¹³C-methanol added as co-substrate to sugars was followed and the isotopologue distribution indicated incorporation into central metabolites and in vivo activity of the RuMP pathway. In addition, ¹³C-label from methanol was traced to the secreted product cadaverine. Thus, this synthetic biology approach led to a *C. glutamicum* strain that converted the non-natural carbon substrate methanol at least partially to the non-native product cadaverine.

Introduction

Methanol, a one-carbon-compound, is present in most ecosystems (Yang et al. 2013) and is produced in a large scale from natural gas as a carbon feedstock in chemical industry or as fuel and energy-storage material in the future (Olah 2005). It represents a promising carbon source for microbial fermentations, providing an alternative to common carbon sources such as sugars as it is not competing with human nutrition (Olah et al. 2013; Whitaker et al. 2015). Furthermore, methanol is a sustainable carbon source and its price is expected to remain stable. Methylotrophic bacteria are able to utilize methanol or other one-carbon compounds as carbon and energy sources. The different pathways of methanol metabolism are typically initialized by oxidation of methanol to the cytotoxic metabolite formaldehyde (Chistoserdova et al. 2009). Oxidation of methanol can be catalyzed by several different enzymes like the non-specific class I alcohol dehydrogenase, e.g., in humans (Wagner et al. 1983) or alcohol oxidase in yeasts like *Candida boidinii* (Roggenkamp et al. 1975). In methylotrophic bacteria, the oxidation of methanol is typically carried out by

methanol dehydrogenases (Mdh) (Anthony and Williams 2003). These may be localized in the periplasm, contain pyrroloquinoline quinone (PQQ) as prosthetic group, and use cytochrome c_L as electron acceptor (Afolabi et al. 2001; Cox et al. 1992; Williams et al. 2006) as in the Gram-negative methylotrophs like *Methylobacterium extorquens*. Gram-positive methylotrophs like *Bacillus methanolicus* harbor NAD(P)⁺-dependent cytoplasmic methanol dehydrogenases (Brautaset et al. 2013), which belong to the type III alcohol dehydrogenase superfamily (Hektor et al. 2000, 2002; Krog et al. 2013; Ochsner et al. 2014).

Formaldehyde produced in the oxidation of methanol may be dissimilated by oxidation to CO₂, in order to regenerate reducing equivalents and to regulate toxic formaldehyde levels (Gutheil et al. 1997; Müller et al. 2015a; Vorholt 2002). These pathways can also be found in non-methylotrophs, are often linear (Fig. 1), and may differ in their cofactor dependence, as some enzymes oxidize formaldehyde directly (Ando et al. 1979; Tsuru et al. 1997) while others use a carrier like glutathione (Gutheil et al. 1992), mycothiol (Lessmeier et al. 2013; Misset-Smits et al. 1997;

Vogt et al. 2003; Witthoff et al. 2013), bacillithiol (Newton et al. 2009), tetrahydrofolate (THF) (Kallen and Jencks 1966), or tetrahydromethanopterin (Maden 2000) before it is sequentially oxidized to CO₂ (Pomper et al. 1999).

In contrast, assimilatory and dissimilatory pathways coexist in methylotrophic bacteria (Chistoserdova 2011; Müller et al. 2015a; Ochsner et al. 2015). Assimilation of formaldehyde may occur via the serine cycle, e.g., in *M. extorquens* (Smejkalova et al. 2010), in which 5,10-methylene-THF is a central intermediate (Anthony 1982; Quayle 1980). Other pathways are the xylulose monophosphate cycle (Kato et al. 1982; Yurimoto et al. 2005) in methylotrophic yeasts like *Pichia pastoris* (Luers et al. 1998) and as the main assimilation pathway in bacteria the ribulose monophosphate (RuMP) pathway (Kato et al. 2006), which is active in, e.g., *Methylomonas aminofaciens* (Sakai et al. 1999) or *B. methanolicus* (Brautaset et al. 2004; Jakobsen et al. 2006; Stolzenberger et al. 2013).

The RuMP pathway consists of three parts: fixation, cleavage, and rearrangement (Fig. 1). The fixation part is characterized by the key enzymes 3-hexulose-6-phosphate synthase

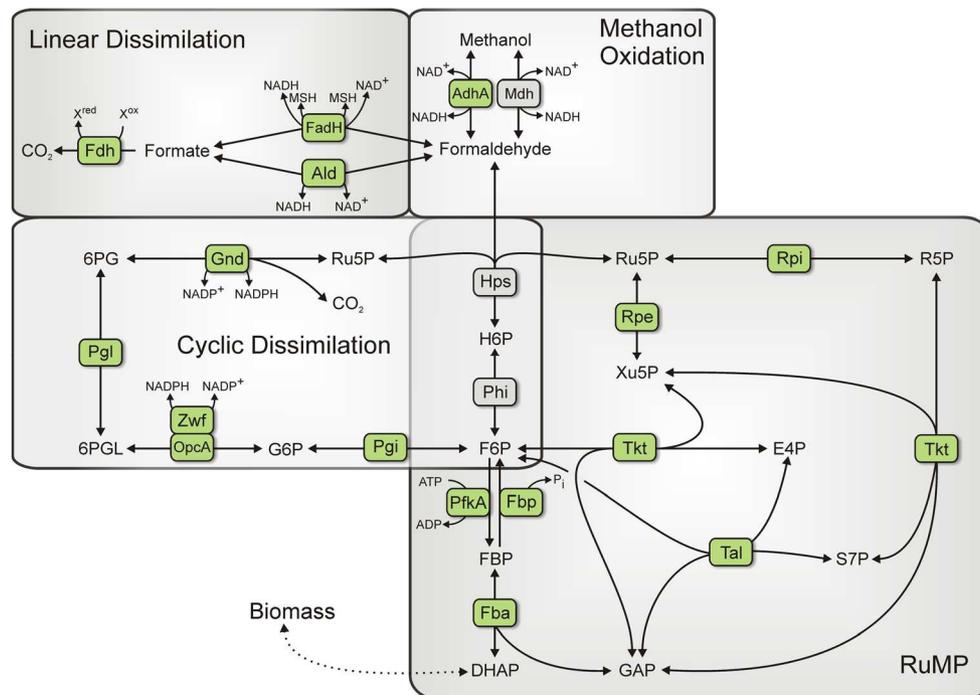


Fig. 1 Overview of methanol oxidation and linear formaldehyde dissimilation in *Corynebacterium glutamicum* and the synthetic RuMP pathway for formaldehyde assimilation as well as cyclic dissimilation. Enzymes highlighted in green are present in *C. glutamicum*; heterologous enzymes are depicted in gray. Abbreviations: 6PG 6-phosphogluconate, 6PGL 6-phosphogluconolactone, AdhA alcohol dehydrogenase, Ald acetaldehyde dehydrogenase, DHAP dihydroxyacetone-phosphate, E4P erythrose 4-phosphate, F6P fructose 6-phosphate, FadH MSH-dependent formaldehyde dehydrogenase, Fba fructose-1,6-bisphosphate aldolase, Fbp fructose-1,6-bisphosphatase, FBP fructose 1,6-bisphosphate, Fdh formate dehydrogenase, G6P

glucose 6-phosphate, GAP glyceraldehyde 3-phosphate, Gnd 6-phosphogluconate dehydrogenase, H6P hexulose 6-phosphate, Hps 3-hexulose-6-phosphate synthase, Mdh methanol dehydrogenase, MSH mycothiol, PfkA phosphofructokinase, Pgi phosphoglucose isomerase, Pgl 6-phosphogluconolactonase, Phi 6-phospho-3-hexuloisomerase, Ru5P ribulose 5-phosphate, R5P ribose 5-phosphate, Rpe ribulose-5-phosphate-3 epimerase, Rpi ribose-5-phosphate isomerase, S7P sedoheptulose 7-phosphate, Tal transaldolase, Tkt transketolase, X unknown cofactor, Xu5P xylulose 5-phosphate, Zwf and OpcA glucose-6-phosphate dehydrogenase

(Hps) and 6-phospho-3-hexuloisomerase (Phi), which introduce formaldehyde into the central carbon metabolism by condensing it with ribulose 5-phosphate (Ru5P) to hexulose 6-phosphate, and then converting it to fructose 6-phosphate (F6P) (Kato et al. 2006). In the cleavage part, F6P is converted to dihydroxyacetone 3-phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) by glycolytic enzymes. DHAP, GAP, and F6P can also be rearranged to regenerate Ru5P (Strom et al. 1974), involving transaldolase, transketolase, ribulose-5-phosphate epimerase (Rpe), and ribose-5-phosphate isomerase (Rpi) from the non-oxidative part of the pentose phosphate pathway (PPP) (Jakobsen et al. 2006). The RuMP pathway may also operate for a cyclic dissimilation of methanol to CO₂ (Müller et al. 2014; Pluschkell and Flickinger 2002), involving reactions of the oxidative part of the PPP with concomitant release of CO₂ and gain of energy (Anthony 1982; Chistoserdova et al. 2000). This pathway is not restricted to methylotrophic organisms (Reizer et al. 1997), and homologs of Hps and Phi have also been identified in non-methylotrophs like *Bacillus subtilis* (Yasueda et al. 1999) and *Burkholderia cepacia* (Mitsui et al. 2003).

The Gram-positive *Corynebacterium glutamicum* is known for its use in the million-ton-scale production of the amino acids L-glutamate and L-lysine (Eggeling and Bott 2015; Peters-Wendisch et al. 2014). *C. glutamicum* has been engineered for access to alternative carbon sources (Leßmeier et al. 2015; Wendisch 2014) and for the production of a variety of different products (Wendisch 2014), like the diamine 1,5-diaminopentane (Mimitsuka et al. 2007), a monomeric polyamide precursor commonly known as cadaverine (Kind et al. 2010; Zahoor et al. 2012). *C. glutamicum* is a non-methylotrophic organism and not able to utilize methanol as a carbon source, but it oxidizes methanol to CO₂ by means of linear dissimilation involving alcohol dehydrogenase encoded by *adhA* and at least one additional enzyme for oxidation of methanol to formaldehyde (Witthoff et al. 2013). Formaldehyde can be oxidized to formate either by acetaldehyde dehydrogenase (Ald) or by mycothiol-dependent formaldehyde dehydrogenase (FadH) (Lessmeier et al. 2013; Witthoff et al. 2013). Subsequently, formate is oxidized to CO₂ by formate dehydrogenase FdhF (cg0618), involving a currently unknown electron acceptor and the gene products encoded by cg0616 and cg0617 (Witthoff et al. 2012). Recently, assimilation of methanol into central metabolites by engineered bacteria has been described for *Pseudomonas putida* (Koopman et al. 2009), *Escherichia coli* (Müller et al. 2015b), and also *C. glutamicum* (Witthoff et al. 2015). Based on this engineering strategy, the study described here aimed one step further by engineering conversion of methanol into the industrially relevant non-native product cadaverine while retaining (some) carbon from methanol in the secreted product.

Materials and methods

Microorganisms and cultivation conditions

Strains and plasmids used in this study are listed in Table S1. The *E. coli* strain DH5 α was used as a standard host for cloning (Hanahan 1983). Cultivation of *E. coli* strains was performed aerobically in lysogeny broth complex medium (LB) on a rotary shaker (120 rpm) at 37 °C. *C. glutamicum* was cultivated aerobically on a rotary shaker (120 rpm) at 30 °C. Growth experiments with *C. glutamicum* were also performed in the microbioreactor system BioLector (m2p labs; Baesweiler, Germany) using FlowerPlate microtiter plates (m2p labs; Baesweiler, Germany). The growth conditions were set to 1100 rpm, 30 °C, and 85 % humidity. LB medium supplemented with 50 mM glucose was used for precultures and the expression of proteins for enzyme assays. If ribose was used as carbon source, the precultures were grown in LB medium without glucose. Growth experiments with *C. glutamicum* were performed in the minimal medium mCGXII (Lessmeier et al. 2013), a modification of the CGXII medium (Keilhauer et al. 1993). The medium has been supplemented with ribose as carbon and energy source. For selection of clones carrying plasmids and their derivatives, appropriate antibiotics were used (pEKEx3, spectinomycin (100 μ g ml⁻¹); pVWEx1, kanamycin (25 μ g ml⁻¹)). For induction of vector-based gene expression, the medium was supplemented with 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG).

DNA preparation, manipulation, and transformation

Plasmid isolation, molecular cloning, and transformation of *E. coli*, as well as electrophoresis, were performed using standard procedures (Sambrook and Russell 2001). Electroporation was used for transformation of *C. glutamicum* as described previously (Tauch et al. 2002). PCR experiments were performed using GoTaq DNA polymerase (Promega) or KOD hot start polymerase (Novagen) with oligonucleotides obtained from Metabion (listed in Table S2). All restriction enzymes and phosphonucleokinase were obtained from Fermentas and used according to the manufacturer's instructions. The Rapid DNA Dephos and Ligation Kit from Roche was used for dephosphorylation and ligation of DNA. Plasmids were isolated from *E. coli* with the QIAprep miniprep kit (Qiagen, Hilden, Germany).

Construction of expression vectors

The vectors pEKEx3 (Stansen et al. 2005) and pVWEx1 (Peters-Wendisch et al. 2001) were used for IPTG-induced expression in *C. glutamicum*. Genes were amplified via PCR from genomic DNA using the corresponding forward (fw) and reverse (rev) primers listed in Table S2. The PCR products of

the genes were cloned into the particular vector using the denoted restriction enzymes. If no restriction enzyme is mentioned for the corresponding primers, a blunt end cloning strategy was used in which the PCR product was phosphorylated with polynucleotide kinase and ligated into *Sma*I digested vector. All inserts of the plasmid constructs listed in Table S1 were controlled by DNA sequencing.

Methanol dehydrogenase enzyme assay

Methanol dehydrogenase activities in crude cell extracts were measured at 30 °C using a Shimadzu UV-1800 spectrophotometer by following the formation of NADH at 340 nm. Crude cell extracts were prepared using sonication treatment (Lessmeier et al. 2013). The reaction mix (1 ml) contained 100 mM Glycine-KOH pH 9.5, 5 mM MgSO₄, 1 mM NAD⁺ and was started by addition of 500 mM methanol (Krog et al. 2013). Protein concentration was measured according to the method of Bradford via the Bio-Rad Protein-Assay using bovine serum albumin as a standard (Bradford 1976).

Assay of Hps and Phi activities

The combined enzyme activity of Hps and Phi in crude cell extracts was assayed spectrophotometrically at 30 °C as described previously (Yasueda et al. 1999), following the formation of NADPH at 340 nm. Fructose 6-phosphate formed by these two enzymes from formaldehyde and ribulose 5-phosphate is converted to glucose 6-phosphate by glucose-6-phosphate isomerase. This conversion was coupled to NADPH formation via glucose-6-phosphate dehydrogenase, which converts glucose 6-phosphate and NADP⁺ to glucono-1,5-lactone 6-phosphate and NADPH. The substrate ribulose 5-phosphate was produced by phosphoriboisomerase from ribose 5-phosphate. The reaction mixture of 1 ml contained 50 mM potassium phosphate pH 7.6, 2.5 mM NADP⁺, 5 mM ribose 5-phosphate, 5 mM MgCl₂, 10 U phosphoriboisomerase from spinach (Sigma), 10 U phosphoglucoisomerase from yeast (Sigma), and 10 U glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma). For the conversion of ribose 5-phosphate to ribulose 5-phosphate, the mixture was preincubated at 30 °C for 6 min before the reaction was started by addition of 5 mM formaldehyde.

Measurement of formaldehyde concentrations

A colorimetric method was used for the measurement of formaldehyde concentrations as described previously (Lessmeier et al. 2013; Nash 1953). In vivo methanol dehydrogenase activity assays were performed measuring the formaldehyde formation by resting *C. glutamicum* Δ ald Δ fadH cells, which show no significant degradation of formaldehyde (Lessmeier

et al. 2013). LB medium supplemented with 50 mM glucose and 1 mM IPTG was used for precultures. For the assays, 50 ml mCGXII medium without carbon source was inoculated to an OD₆₀₀ of 1 and supplemented with 1 mM IPTG. Incubation was performed in 500 ml baffled Erlenmeyer flasks at 30 °C and 120 rpm. The reaction was started by addition of 240 mM methanol. In vivo activity assays for formaldehyde assimilation were performed following the decrease of the formaldehyde concentration. For this purpose, 50 ml mCGXII medium without nitrogen sources was supplemented with 100 mM ribose and 1 mM IPTG. The medium was inoculated to an OD₆₀₀ of 1 from a culture cultivated in LB medium with 1 mM IPTG. The assay was started by addition of 0.5 mM formaldehyde.

Determination of ¹³C-labeled intracellular metabolites

The assimilation of methanol in recombinant *C. glutamicum* strains was monitored in a kinetic ¹³C-methanol-labeling experiment. Cells were cultivated in LB medium supplemented with spectinomycin and 1 mM IPTG. Cells were transferred to low salt content M9 medium (Millard et al. 2014) with 1 mM IPTG, 0.2 µg/l biotin, and 60 mM ¹³C-methanol (99 % ¹³C-methanol from Euroisotop, Paris, France). Methanol was added as unique pulse after subtracting the time 0 sample. IC-MS/MS analysis was used to analyze the isotopic enrichment of each metabolite as described by Kiefer et al. 2007. Specifically, the Dionex ICS 2000 system (Dionex, Sunnyvale, USA) coupled to a triple quadrupole QTrap 4000 (Applied Biosystems, Foster City, USA) mass spectrometer was used. Integration of all peaks was performed manually using the Analyst 1.5.2 software (Sierra Analytics, USA). After peak integration, the raw peak areas were corrected for the contribution of all naturally abundant isotopes using the IsoCor software (Millard et al. 2012). Carbon isotopologue distributions (CIDs) and ¹³C-enrichment error using this methodology are 0.5 and 1.5 %, respectively (Millard et al. 2012). The percent label enrichment refers to the total number of ¹³C carbons divided by the total number of carbons in a molecule ($M1 \times 1 + M2 \times 2 + M3 \times 3$ /total number of carbons). Individual isotopologues are labeled M0, M1, M2, etc. if they contain 0, 1, 2, etc. ¹³C atoms.

Determination of ¹³C-labeled cadaverine

C. glutamicum Δ ald Δ fadH(pEKEx3-mdh,hxlAB)(pVWEx1-lysC^{br}-ldcC) was cultivated in LB medium supplemented with 1 mM IPTG and antibiotics. Cells were subsequently transferred to CgXII minimal medium supplemented with 20 mM ribose and 200 mM ¹³C-methanol (99 %, Sigma-Aldrich, Germany) or 50 mM glucose and 50 mM ¹³C-methanol. Supernatants were taken after 30, 56, and 100 h and analyzed on a Bruker Ascend 800 MHz and Avance II

500 MHz magnets. Concentration of cadaverine in each sample was measured using 1D ^1H -NMR experiment, and ^{13}C -decoupled DANTE-Z pulse was applied to measure ^{13}C -enrichments (Nicolas et al. 2008).

Results

Engineering strategy

A similar engineering strategy as used very recently for engineering utilization of methanol as co-substrate in the non-methylotrophs *E. coli* (Müller et al. 2015b) and *C. glutamicum* (Witthoff et al. 2015) was applied here. To block formaldehyde oxidation to carbon dioxide in the linear formaldehyde detoxification pathway of *C. glutamicum*, the genes encoding Ald (Arndt et al. 2008) and FadH (Lessmeier et al. 2013; Witthoff et al. 2013) were deleted. *C. glutamicum* oxidizes methanol to formaldehyde by alcohol dehydrogenase AdhA (Arndt and Eikmanns 2007; Witthoff et al. 2013). To increase methanol oxidation, PQQ-dependent methanol dehydrogenases from Gram-negative methylotrophs seemed not suitable as *C. glutamicum* is not known to synthesize PQQ (Shen et al. 2012) or cytochrome c_L (Williams et al. 2006). Therefore, the three NAD^+ -dependent methanol dehydrogenases from the thermotolerant methylotroph *B. methanolicus* (Arfman et al. 1997; Brautaset et al. 2004; Heggeset et al. 2012; Krog et al. 2013) and the activator protein Act, which increases the in vitro activity of the three methanol dehydrogenases significantly, were chosen (Arfman et al. 1991; Krog et al. 2013; Ochsner et al. 2014). A mutation in the active site serine (S97G) was shown to activate methanol dehydrogenase in the absence of Act (Hektor et al. 2002). *C. glutamicum* only lacks two enzymes of the RuMP, 3-hexulose-6-phosphate synthase (Hps), and 6-phospho-3-hexuloisomerase (Phi) since it possesses the pentose phosphate pathway and the Embden-Meyerhof-Parnas pathway of glycolysis (Fig. 1). In the same way, the cyclic dissimilation is completed by enzymes of the oxidative PPP. Besides the Hps and Phi encoding genes from thermophilic *B. methanolicus* MGA3, the homologs Hx1A and Hx1B from the mesophilic *B. subtilis* (Yasueda et al. 1999) were also tested.

Heterologous expression and functional analysis of different methanol dehydrogenases in vitro and in vivo

In vitro enzyme activity assays with crude extracts showed that the three methanol dehydrogenase genes of *B. methanolicus* MGA3 were functionally expressed in *C. glutamicum* (Table 1). Mdh was the most active in *C. glutamicum* (3.7 ± 0.9 mU/mg), while Mdh2 (1.2 ± 0.1 mU/mg) and Mdh3 (1.7 ± 0.5 mU/mg) showed only low in vitro activities. The enzymes from *B. methanolicus* strain PB1 possessed

Table 1 In vitro and in vivo activities of methanol dehydrogenases heterologously expressed in *Corynebacterium glutamicum*

	Specific in vitro activity (mU/mg)	In vivo activity (nmol min ⁻¹ mg CDW ⁻¹)
Empty vector	<0.5	<0.5
Mdh	3.7 ± 0.9	9.0 ± 0.5
Mdh2	1.2 ± 0.1	1.3 ± 0.0
Mdh3	1.7 ± 0.5	0.8 ± 0.1
Mdh_PB1	3.5 ± 0.8	n.a.
Mdh2_PB1	1.9 ± 0.3	n.a.
Mdh3_PB1	1.6 ± 0.4	n.a.
Mdh + Act	9.6 ± 0.1	9.4 ± 0.0
Mdh2 + Act	3.9 ± 0.5	2.5 ± 0.0
Mdh3 + Act	2.8 ± 0.5	1.4 ± 0.1
Mdh_S97G	11.2 ± 0.9	2.5 ± 0.2
Mdh2_S97G	<0.5	<0.5
Mdh3_S97G	<0.5	<0.5
Mdh + cg1607	9.5 ± 1.7	10
AdhA	1.7 ± 0.2	1.2 ± 0.0
Mdh + Mdh2	2.2 ± 0.2	n.a.
Mdh + Mdh3	2.6 ± 0.3	n.a.
Mdh2 + Mdh3	2.2 ± 0.3	n.a.
Mdh + Mdh2 + Mdh3	1.5 ± 0.5	n.a.

Expression for in vitro activity measurements was performed using the vector pEKEx3 (AdhA activity was measured using pVWEx1). The in vivo activities were measured in strain $\Delta\text{ald}\Delta\text{fadH}$ (pEKEx3) using the vector pVWEx1 for heterologous expression. Means and experimental imprecision of two independent cultures are shown

n.a. data not available

comparable activities in *C. glutamicum* (Table 1). Co-expression of two methanol dehydrogenase genes led to lower in vitro activities (Table 1). Overexpression of the endogenous alcohol dehydrogenase gene *adhA* led to similar activities as those resulting from overproduction of Mdh2 or Mdh3 (1.65 ± 0.17 mU/mg). When *act* from *B. methanolicus* MGA3 was expressed in addition to methanol dehydrogenase genes, the in vitro activities increased about 2–3-fold in crude extracts of the respective *C. glutamicum* strains (Table 1). Overexpression of cg1607, encoding a putative homolog of *act* in *C. glutamicum*, increased the Mdh in vitro enzyme activity similar to Act (9.5 ± 1.7 mU/mg). Introducing the S97G mutation (Hektor et al. 2002) in Mdh increased in vitro methanol dehydrogenase activities to similar values as observed when Act was co-expressed (Table 1). However, Mdh2_S97G and Mdh3_S97G revealed no detectable methanol dehydrogenase activity.

Methanol oxidation in vivo was assayed by monitoring formaldehyde accumulation in the supernatant. The control strain $\Delta\text{ald}\Delta\text{fadH}$ (pEKEx3)(pVWEx1) showed no significant accumulation of formaldehyde. High methanol oxidation to formaldehyde was observed in strains

derived from *C. glutamicum* $\Delta ald\Delta fadH$ overproducing Mdh ($9.0 \pm 0.5 \text{ nmol min}^{-1} \text{ mg CDW}^{-1}$) and to lesser extents overproducing Mdh2 ($1.3 \pm 0.0 \text{ nmol min}^{-1} \text{ mg CDW}^{-1}$) or Mdh3 ($0.8 \pm 0.1 \text{ nmol min}^{-1} \text{ mg CDW}^{-1}$) (Fig. 2). Additional expression of *act* or *cgl607* or introduction of the S97G mutation into methanol dehydrogenases did not increase formaldehyde accumulation in vivo although in vitro activities were higher (Table 1). Taken together, heterologous expression of *mdh* from *B. methanolicus* in *C. glutamicum* $\Delta ald\Delta fadH$ showed the highest methanol oxidation activities in vitro and, thus, was chosen for further experiments.

Heterologous expression and functional analysis of formaldehyde-assimilating enzymes in vitro and in vivo

Genes encoding the RuMP key enzymes 3-hexulose-6-phosphate synthase (Hps) and 6-phospho-3-hexuloisomerase (Phi) from *B. methanolicus* MGA3 or *B. subtilis* were expressed heterologously in *C. glutamicum* $\Delta ald\Delta fadH$. The combined enzyme activity of Hps and Phi was analyzed in vitro in crude extracts. While *C. glutamicum* $\Delta ald\Delta fadH$ (pEKEx3) lacked detectable Hps and Phi activity ($<0.005 \text{ U/mg}$), activities of $0.27 \pm 0.02 \text{ U/mg}$ and $0.53 \pm 0.12 \text{ U/mg}$, respectively, were observed in crude extracts of *C. glutamicum* $\Delta ald\Delta fadH$ expressing *hps* and *phi* from *B. methanolicus* or *hxA* and *hxB* from *B. subtilis*, respectively.

To assay the effect of these RuMP key enzymes in vivo, formaldehyde degradation was monitored. Since these enzymes require the pentose ribulose-5-phosphate as a second substrate, the medium was supplemented with 100 mM ribose. Cell growth was prevented by nitrogen limitation to determine a degradation rate of formaldehyde. The control

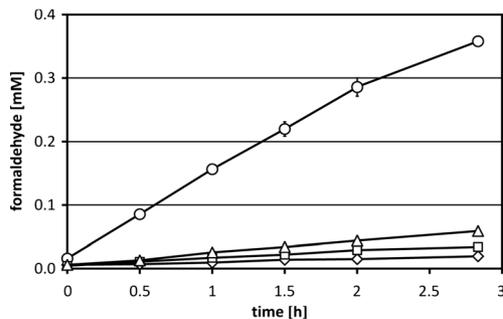


Fig. 2 In vivo activities of methanol dehydrogenases in *Corynebacterium glutamicum* strains expressing heterologous *mdh* genes. An in vivo assay was used to measure methanol oxidation via the accumulation of formaldehyde from 240 mM methanol. The Nash assay to determine formaldehyde concentrations was performed using resting cells (about 0.25 g cell dry weight/l) in minimal medium without additional carbon source. *C. glutamicum* $\Delta ald\Delta fadH$ (pEKEx3) strains were used carrying additional vectors pVWEx1 (diamonds), pVWEx1-*mdh* (circles), pVWEx1-*mdh2* (triangles), and pVWEx1-*mdh3* (squares). Means and experimental imprecision of two independent cultures are shown

strain showed (almost) no significant formaldehyde degradation ($<2 \text{ nmol min}^{-1} \text{ mg CDW}^{-1}$). The observed decrease after 2 h may be due to spontaneous dissociation of methanol to methanediol in water. The strain expressing *hps* and *phi* degraded formaldehyde with a rate of $9.0 \pm 0.2 \text{ nmol min}^{-1} \text{ mg CDW}^{-1}$, which is about 25 % lower as compared with expression of *B. subtilis* *hxA* and *hxB* ($12 \pm 0.5 \text{ nmol min}^{-1} \text{ mg CDW}^{-1}$) (Fig. 3). Since HxA and HxB from *B. subtilis* showed the highest activities in vitro as well as in vivo, these enzymes were used for further experiments.

Growth of recombinant *C. glutamicum* strains in the presence of methanol or formaldehyde

To test if expression of genes for the RuMP key enzymes affected tolerance of *C. glutamicum* strain $\Delta ald\Delta fadH$ to methanol or formaldehyde, growth in minimal medium containing 100 mM ribose was assayed in the presence of formaldehyde or methanol. In the presence of 5 μM formaldehyde, the empty vector carrying strain did not grow, however, heterologous expression of *hxA* allowed growth with a growth rate of $0.18 \pm 0.00 \text{ h}^{-1}$ (Fig. 4a).

Growth experiments with 100 mM ribose and different methanol concentrations showed that the strain expressing *hxA* and *hxB* was able to grow in the presence of higher methanol concentrations than the control strain (Fig. 4b). Accumulation of formaldehyde was monitored for strains expressing *mdh* and/or *hxA* cultivated with 100 mM ribose in the presence of 30 mM methanol (Fig. 5a). The empty vector carrying control strain accumulated $0.34 \pm 0.01 \text{ mM}$ formaldehyde within 6 h of cultivation, whereas expression of *hxA* and *hxB* resulted in growth with a growth rate of $0.21 \pm 0.00 \text{ h}^{-1}$, and no significant formaldehyde

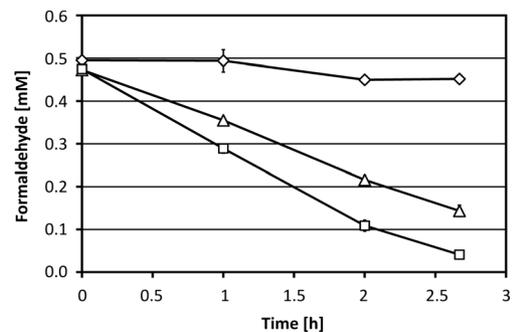


Fig. 3 Formaldehyde degradation by *Corynebacterium glutamicum* strains expressing heterologous RuMP genes. In vivo formaldehyde degradation assay of *C. glutamicum* $\Delta ald\Delta fadH$ carrying the empty vector pEKEx3 (diamonds), pEKEx3-*hps.phi* (triangles), or pEKEx3-*hxA.hxB* (squares). The assay was performed using resting cells (about 0.25 g cell dry weight/l) in minimal medium with 100 mM ribose as carbon source but without nitrogen source. Formaldehyde (0.5 mM) was added at 0 h and formaldehyde concentrations were determined using the Nash test. Means and experimental imprecision of two independent cultures are shown

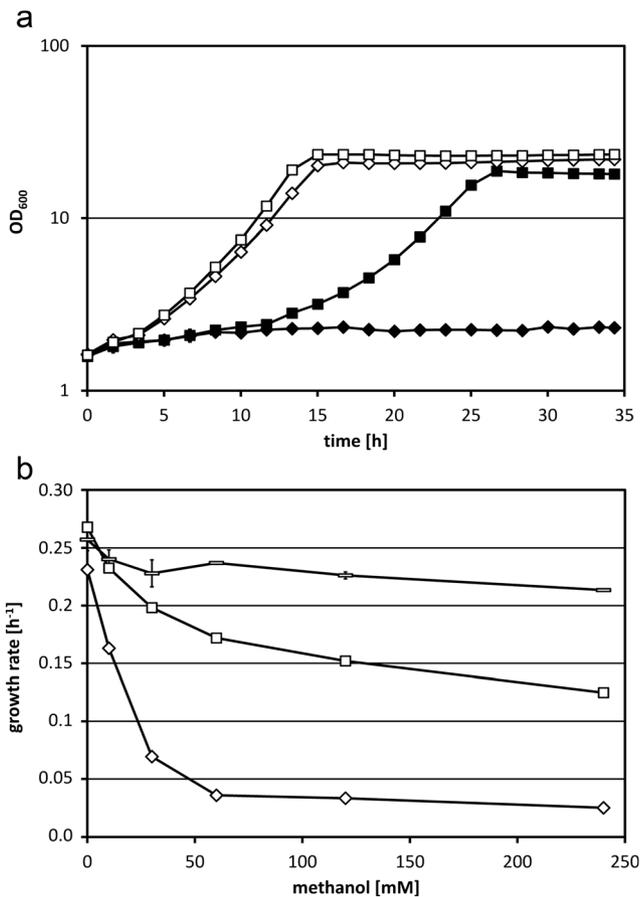


Fig. 4 Growth of recombinant *Corynebacterium glutamicum* strains in the presence of formaldehyde (a) or methanol (b). a Growth of *C. glutamicum* strains $\Delta ald \Delta fadH$ (pEKEx3) (diamonds) and $\Delta ald \Delta fadH$ (pEKEx3-hxlAB) (squares) cultivated in minimal medium with 100 mM ribose and no (open symbols) or 5 μ M formaldehyde (closed symbols). b Dependence of the growth rate of *C. glutamicum* wild type (bars) and strains $\Delta ald \Delta fadH$ (pEKEx3) (diamonds) and $\Delta ald \Delta fadH$ (pEKEx3-hxlAB) (squares) on methanol added to minimal medium with 100 mM ribose. The cultures were inoculated with an OD₆₀₀ of about 1.6, which is equivalent to about 0.4 g cell dry weight/l. Means and experimental imprecision of two independent cultures are shown

accumulation (<0.06 mM) could be observed irrespective if in addition *mdh* was expressed (Fig. 5b). Taken together, heterologous expression of *hxlA* and *hxlB* allowed growth in the presence of formaldehyde and improved tolerance to methanol by reducing formaldehyde accumulation.

¹³C-labeling patterns of metabolic intermediates in recombinant *C. glutamicum* from ¹³C-methanol

In order to analyze how methanol is converted by recombinant *C. glutamicum*, labeling experiments with ¹³C-methanol were performed. Strains *C. glutamicum* WT (pEKEx3), $\Delta ald \Delta fadH$ (pEKEx3), $\Delta ald \Delta fadH$ (pEKEx3-hxlAB), and $\Delta ald \Delta fadH$ (pEKEx3-*mdh,hxlAB*) were labeled in M9 low salt medium with

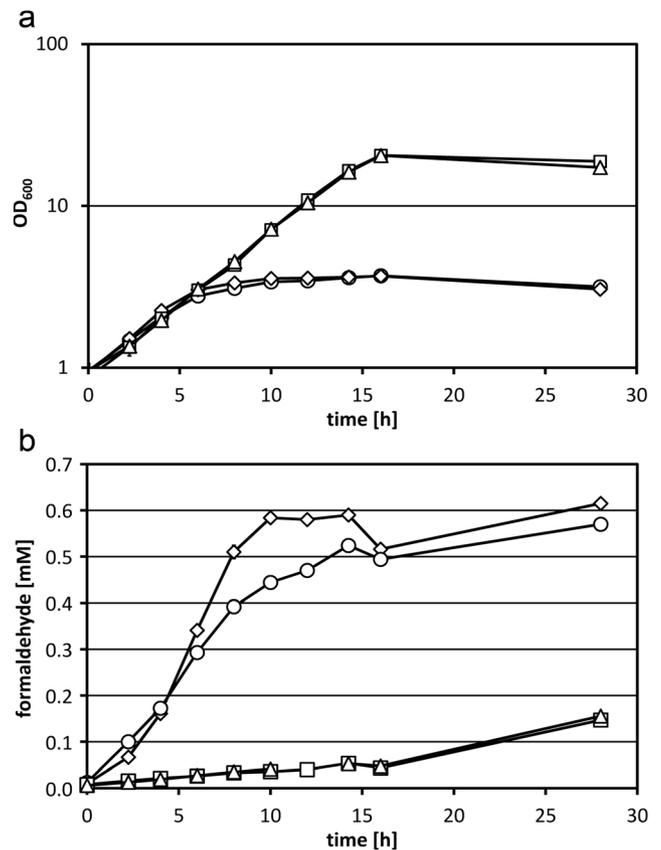
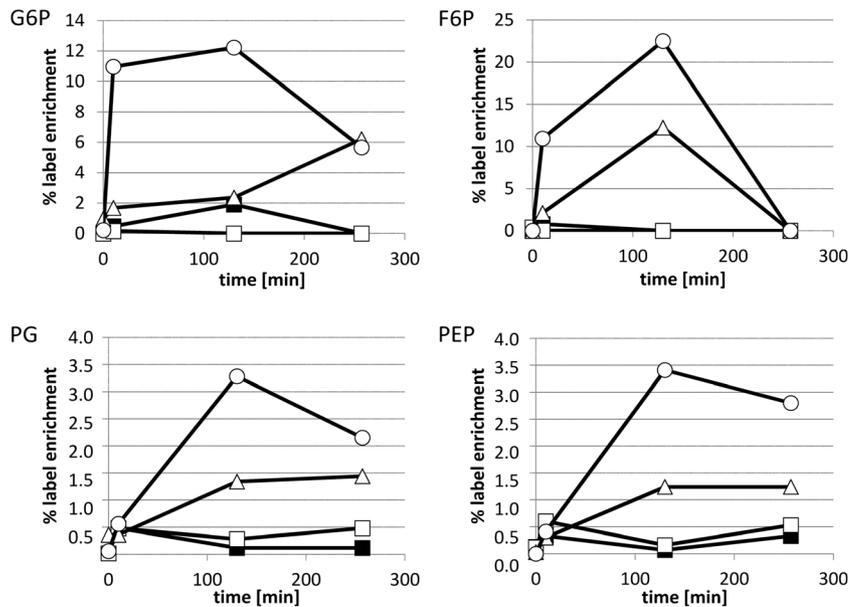


Fig. 5 Growth (a) and formaldehyde accumulation (b) by *Corynebacterium glutamicum* strains in the presence of methanol. a Growth of and (b) formaldehyde concentrations in the supernatants of *C. glutamicum* strains $\Delta ald \Delta fadH$ (pEKEx3) (diamonds), $\Delta ald \Delta fadH$ (pEKEx3-*mdh*) (circles), $\Delta ald \Delta fadH$ (pEKEx3-hxlAB) (squares), and $\Delta ald \Delta fadH$ (pEKEx3-*mdh,hxlAB*) (triangles) on minimal medium with 100 mM ribose and 30 mM methanol. The cultures were inoculated with an OD₆₀₀ of about 1, which is equivalent to 0.25 g cell dry weight/l. Means and experimental imprecision of two independent cultures are shown

60 mM ¹³C-methanol as sole carbon source as described in the [Materials and methods](#).[^] M9 medium was used for this experiment because the original medium mCGXII contains MOPS, which is incompatible with the ionic chromatography used to analyze the intracellular metabolites. No growth was observed under these conditions (data not shown). *C. glutamicum* strains WT(pEKEx3) and $\Delta ald \Delta fadH$ (pEKEx3) showed low or no labeling of glucose 6-phosphate (G6P), respectively, while significant ¹³C incorporation was observed for the strains expressing *hxlAB* and *mdh* as well as *hxlAB* (Fig. 6). Within 10 min after addition of ¹³C-methanol, about 12 % of the carbon atoms in G6P and fructose 6-phosphate (F6P) were labeled (Fig. 6). Labeling of the three-carbon-compounds 2/3-phosphoglycerate (PG) and phosphoenolpyruvate (PEP) was lower and occurred later (Fig. 6). Labeling of F6P and G6P prior to labeling of the three-carbon-compounds is in accordance with assimilation in the RuMP cycle. The highest label incorporation (25 %)

Fig. 6 Label enrichment in different metabolites from ^{13}C -methanol. The *Corynebacterium glutamicum* strains WT(pEKEx3) (closed squares), $\Delta\text{ald}\Delta\text{fadH}$ (pEKEx3) (open squares), $\Delta\text{ald}\Delta\text{fadH}$ (pEKEx3-*hxlAB*) (open triangles), and $\Delta\text{ald}\Delta\text{fadH}$ (pEKEx3-*mdh,hxlAB*) (open circles) were cultivated in M9 minimal medium with 60 mM ^{13}C -methanol as sole carbon source. *G6P* glucose 6-phosphate, *F6P* fructose 6-phosphate, *PG* combination of 2-phosphoglycerate and 3-phosphoglycerate, *PEP* phosphoenolpyruvate. The data are from a representative experiment from duplicates



was observed for F6P 120 min after ^{13}C -methanol addition (Fig. 6). Net generation of a three-carbon molecule from methanol via the RuMP cycle requires assimilation of three molecules of methanol and cyclic operation of the RuMP cycle (Müller et al. 2015b). To assess if multiply labeled F6P and G6P molecules were formed, the isotopologue distribution of G6P and F6P was determined (Fig. 7). Observation of the M2 and M3 isotopologues indicated functional regeneration of Ru5P via the RuMP cycle. The finding of M3 for the hexoses G6P and F6P shows that these molecules have passed the complete RuMP cycle three times (Müller et al. 2015b). For F6P, up to 40 % of M1 and 45 % of M2 were found showing a high incorporation of carbon from methanol into the hexoses (Fig. 7). These results revealed that assimilation of methanol in *C. glutamicum* is functional by the heterologous expression of *hxlA* and *hxlB*. However, none of the metabolites was fully labeled which indicated that operation of the RuMP cycle is limited and is commensurate with the finding that methanol cannot be utilized as sole carbon source for growth.

^{13}C -labeling patterns of the product cadaverine in recombinant *C. glutamicum* grown in the presence of ^{13}C -methanol

The finding that ^{13}C -methanol is metabolized to intermediates of central carbon metabolism involving assimilation via the RuMP cycle led us to assess if the non-natural carbon substrate methanol can be converted at least partially to a non-native product by a recombinant *C. glutamicum* strain. Cadaverine was chosen as non-native product, since it can be derived from lysine by decarboxylation if a heterologous gene for lysine decarboxylase is expressed. *C. glutamicum* $\Delta\text{ald}\Delta\text{fadH}$ (pEKEx3-*mdh,hxlAB*) was

genetically engineered for the production of cadaverine by expression of genes for endogenous, feedback-resistant

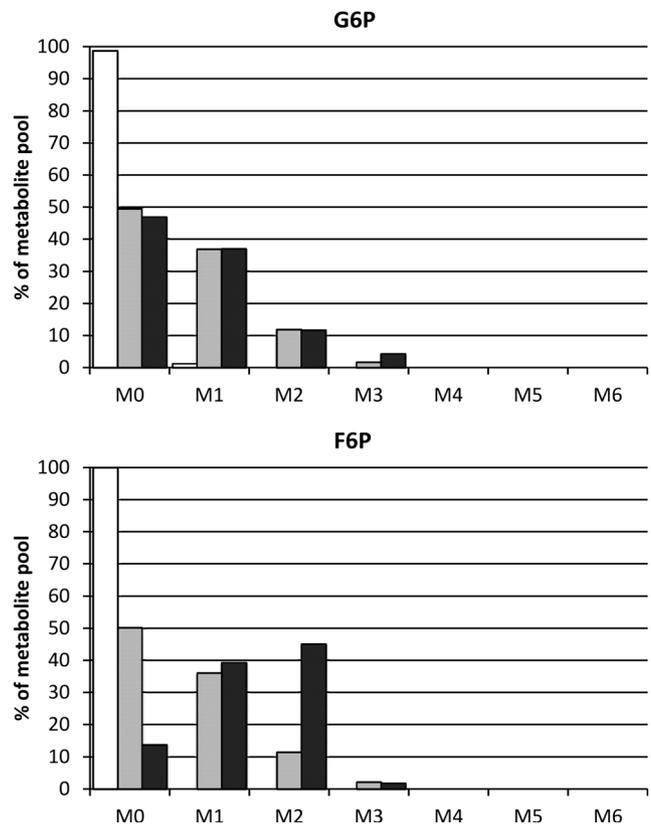


Fig. 7 Mass isotopologue distribution of glucose 6-phosphate (*G6P*) and fructose 6-phosphate (*F6P*) from ^{13}C -methanol. *Corynebacterium glutamicum* $\Delta\text{ald}\Delta\text{fadH}$ (pEKEx3-*mdh,hxlAB*) was cultivated in M9 minimal medium with 60 mM ^{13}C -methanol as sole carbon source. Mass isotopologue distribution after 0 min (white), 10 min (gray), and 130 min (black) is shown. The data are from a representative experiment from duplicates

aspartokinase (*lysC^{br}*) and lysine decarboxylase LdcC from *E. coli*. The strain *C. glutamicum* Δ ald Δ fadH(pEKEx3-*mdh*, *hxlAB*)(pVWEx1-*lysC^{br}*-*ldcC*) produced up to 1.5 g/l of cadaverine in shake flask cultivation. After growing this strain in CgXII minimal medium containing 50 mM glucose and 50 mM ¹³C-methanol or 20 mM ribose and 200 mM ¹³C-methanol, the supernatant was analyzed with respect to cadaverine-labeling patterns by NMR. The symmetric molecule cadaverine showed the highest ¹³C-labeling of up to 5 % at positions C1 and C5 and about 15 % at positions C2 and C4 when cultivated with the RuMP pathway precursor ribose (Table 2). Notably, position C3 only revealed the natural ¹³C abundance. With glucose used in the cultivation medium, the labeling was significantly lower with about 5 % labeled carbon at positions C2 and C4. Only natural ¹³C abundance was observed at positions C1, C3, and C5.

Labeling of cadaverine at positions C2 and C4 indicates that ¹³C-methanol was converted to pyruvate labeled at position C3 in the reactions of the cyclic RuMP and onwards to cadaverine (Fig. 8a). Labeling from ¹³CO₂, which may have formed by decarboxylation reactions such as catalyzed by pyruvate dehydrogenase or 6-phosphogluconate dehydrogenase and may have led to labeled oxaloacetate by anaplerotic carboxylation reactions, would lead to cadaverine labeled at the C3 position (Fig. 8b). Thus, labeling of cadaverine at positions C1/C5 and C2/C4, but not at position C3 indicates that ¹³C-methanol was converted to cadaverine via the RuMP pathway by the engineered *C. glutamicum* strain.

Discussion

In this study, a *C. glutamicum* strain was engineered that metabolized the non-natural carbon substrate methanol and converted methanol at least partially to the non-native product cadaverine. The engineered strain expressed heterologous genes for (a) lysine decarboxylase from *E. coli*, (b) methanol dehydrogenase from *B. methanolicus*, and (c) hexulose-6-

phosphate synthase and 6-phospho-3-hexuloisomerase from *B. subtilis* but lacked endogenous (d) mycothiol-dependent formaldehyde dehydrogenase and (e) acetaldehyde dehydrogenase that are required in the linear methanol dissimilation pathway. Comparable strategies for methanol metabolization have been followed in *P. putida* (Koopman et al. 2009), *E. coli* (Müller et al. 2015b), and also *C. glutamicum* (Witthoff et al. 2015). As observed here, in no case growth with methanol as sole carbon source was observed (Whitaker et al. 2015). For *C. glutamicum*, ¹³C-labeling from ¹³C-methanol was observed in metabolic intermediates but not in a secreted product (Witthoff et al. 2015). To the best of our knowledge, conversion of ¹³C-methanol as non-native substrate and secretion of a ¹³C-labeled, non-native product by a non-methylotrophic host has not been reported previously.

Although *C. glutamicum* Adh oxidizes methanol, expression of heterologous Mdh genes was beneficial (Table 1). Among the methanol dehydrogenases from *B. methanolicus* strains MGA3 and PB1, Mdh from MGA3 with the lowest *K_m* value for methanol (170 ± 20 mM) (Krog et al. 2013) was found to have the highest activity in recombinant *C. glutamicum*, as observed by Witthoff et al. (2015). Interestingly, only Mdh from strain MGA3 and Mdh_PB1 showed high activity in *C. glutamicum*, although Mdh1_PB1 shares very high similarity to both enzymes (>90 %) while Mdh2, Mdh3, and Mdh2_PB1 belong to another group (Müller et al. 2015a). However, recent studies showed that Mdh2 from *B. methanolicus* MGA3 is most suitable for heterologous expression in *E. coli* (Müller et al. 2015b). The activation of methanol dehydrogenase by Act, a widely occurring nudix hydrolase able to hydrolyze NAD⁺ to AMP and NMN⁺ (Ochsner et al. 2014) in *E. coli* (Müller et al. 2015b) or *C. glutamicum* (Table 1; Witthoff et al. 2015) increased activity in vitro but not in vivo. The active site serine mutation S97G (Hektor et al. 2002) bypassing activation by Act increased activity in vitro and in vivo of Mdh from MGA3 in *E. coli* (Müller et al. 2015b). As shown here, this mutation only increased in vitro Mdh activity in *C. glutamicum* (Table 1). Apparently, Act is neither beneficial in vivo when expressed in *E. coli* nor in *C. glutamicum*, and

Table 2 Production of ¹³C-labeled cadaverine from ¹³C-methanol

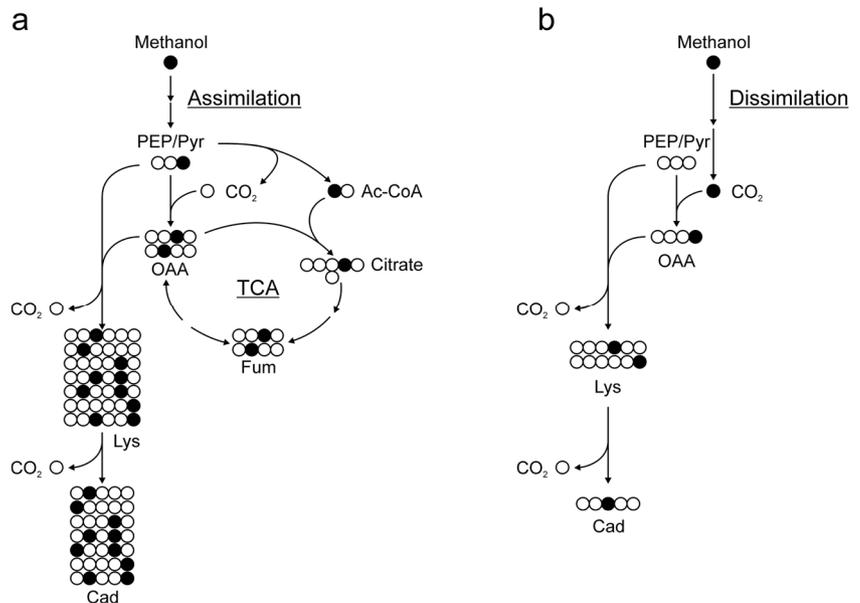
Carbon sources	Time (h)	% labeling at C2/C4	% labeling at C3	% labeling at C1/C5
Glucose + ¹³ C-methanol	30	6.2 ± 0.3	<3	<3
	56	5.1 ± 0.1	<3	<3
	100	4.7 ± 0.7	<3	<3
Ribose + ¹³ C-methanol	30	15.7 ± 1.4	<3	<3
	56	14.6 ± 0.7	<3	5.0 ± 0.3
	100	14.5 ± 1.6	<3	<3

Production was performed by strain *Corynebacterium glutamicum* Δ ald Δ fadH(pEKEx3-*mdh*,*hxlAB*)(pVWEx1-*lysC^{br}*-*ldcC*) in mCGXII medium with 50 mM glucose and 50 mM ¹³C-methanol or 20 mM ribose and 200 mM ¹³C-methanol

it is not clear whether it is required or beneficial in the donor *B. methanolicus* since a gene deletion system is not available for *B. methanolicus*. Future studies on the physiological significance of the methanol dehydrogenase and Act proteins in *B. methanolicus* are very relevant for engineering transfer of methylotrophy from this donor to *E. coli* or *C. glutamicum*.

The RuMP cycle enzymes Hps and Phi from *B. methanolicus* as well as HxlA and HxlB from *B. subtilis* were functionally active in *C. glutamicum*, and in vitro and in vivo analyses showed that they represent suitable enzymes for the construction of a synthetic RuMP pathway in this host. By contrast, using the respective enzymes from *Mycobacterium gastri* led to in vitro activities, which were lower by more than

Fig. 8 Transition of carbon from ^{13}C -methanol to cadaverine. Label incorporation into cadaverine and metabolites when methanol is assimilated in the RuMP pathway (a) or is oxidized to $^{13}\text{CO}_2$ and fixed as CO_2 by anaplerotic carboxylases (b). *PEP* phosphoenolpyruvate, *Pyr* pyruvate, *Ac-CoA* acetyl-coenzyme A, *Fum* fumarate, *OAA* oxaloacetate, *Lys* lysine, *Cad* cadaverine



an order of magnitude (Witthoff et al. 2015). Hps and Phi activities due to expression of *hxlA* and *hxlB* were sufficient to partially restore formaldehyde and methanol detoxification in *C. glutamicum* $\Delta ald\Delta fadH$, which lacks the endogenous linear formaldehyde oxidation pathway (Figs. 4 and 5). This suggested that the endogenous linear formaldehyde dissimilation pathway could be replaced by the cyclic formaldehyde dissimilation pathway. Expression of heterologous *mdh* was not required since methanol is oxidized to formaldehyde by endogenous *AdhA*, but it improved methanol conversion. However, in comparison with the in vitro Hps and Phi activities, the in vivo rates of formaldehyde degradation in *E. coli* (Müller et al. 2015b) and *C. glutamicum* (Fig. 3; Table 1) were 30- to 60-fold lower. Since the in vivo analyses were dependent on addition of extracellular ribose, the intracellular pentose phosphate pool may limit formaldehyde degradation. These low activities may explain why growth with methanol as sole carbon source was not possible (Müller et al. 2015b).

The extent of ^{13}C -labeling from ^{13}C -methanol described here was higher than reported by Witthoff et al. (2015). Labeling in M1 of G6P and F6P were around 35–40 % in the present study (Fig. 7) as compared with 5–12 % in G6P and 22–27 % in F6P as observed and reported by Witthoff et al. (2015). Since the genetic makeup of the strains used was similar (deletions of *ald* and *fadH*, heterologous expression of *mdh*, *hps*, and *phi*), the different labeling efficiencies are likely due to different conditions of the labeling experiments. Incubation of non-growing cells with 60 mM ^{13}C -methanol as described here apparently led to higher label enrichment as compared with the growth experiment described by Witthoff et al. (2015), in which 120 mM ^{13}C -methanol was added to 55 mM glucose minimal medium and label incorporation was determined in growing cells. The labeling experiments

described here also provided evidence for cyclic operation of the RuMP cycle (occurrence of M2 and M3 isotopologues, Fig. 7), while Witthoff et al. (2015) did not report detection of M2 or higher isotopologues. However, we also did not observe F6P or G6P molecules with four-, five-, or all six-labeled carbon atoms (isotopologues M4, M5, and M6; Fig. 7), which indicates that operation of the cyclic RuMP cycle was limited. This finding may explain why the recombinant *C. glutamicum* strains reported here and by (Witthoff et al. 2015) could not grow with methanol as sole carbon source. In the recombinant *E. coli* strains described by Müller et al. (2015b), the percent of labeled carbon in hexose 6-phosphates was almost 40 % while we observed maximally 12 and 25 % labeled carbon in G6P and F6P, respectively (Fig. 6). In both studies, labeling of intermediates was determined in non-growing cells after incubation with ^{13}C -methanol as only carbon source. More importantly, Müller et al. (2015b) detected hexose 6-phosphates with five-labeled carbon atoms (M5) at 6 min and fully labeled hexose 6-phosphates (M6) at 10 min after adding ^{13}C -methanol. However, the fraction of fully labeled hexose 6-phosphates (M6) remained below 5 %. Thus, although incorporation of ^{13}C -methanol into intermediates of central carbon metabolism by the recombinant *E. coli* strains described by Müller et al. (2015b) was more efficient than by the recombinant *C. glutamicum* strains, cyclic operation of the RuMP cycle was insufficient to support growth with methanol as sole carbon source. Labeling of intermediates of lower glycolysis (triose phosphates, PG, PEP) observed here (Fig. 6) and for *E. coli* (Müller et al. 2015b) was lower than that of hexose 6-phosphates. This may be explained by dilution of the ^{13}C -label from ^{13}C -methanol due to mobilization of intracellular storage molecules, such as glycogen, which can account for

up to 90 mg/CDW (Seibold et al. 2007). Alternatively or in addition, ^{13}C -label may be lost from F6P to $^{13}\text{CO}_2$ due to cyclic formaldehyde dissimilation (Fig. 1). In fact, the high label incorporation into G6P may indicate high carbon flux via cyclic formaldehyde dissimilation. Therefore, enzymes like phosphoglucose isomerase which are important for cyclic formaldehyde dissimilation but not required for RuMP cycle operation might be future targets to enable *C. glutamicum* for growth on methanol. Besides targeted metabolic engineering approaches, experimental evolution strategies, e.g., as applied to improve tolerance against *n*-butanol of *E. coli* (Zhu et al. 2015), may help to increase ^{13}C -labeling from ^{13}C -methanol and, ultimately, to enable growth with methanol as sole carbon source.

Cadaverine secreted as metabolic endproduct by *C. glutamicum* strain $\Delta ald \Delta fadH$ (pEKE \times 3-*mdh*, *hxlAB*)(pVWEx1-*lysC^{br}*-*ldcC*) showed ^{13}C -labeling at positions C1/C5 and C2/C4, but not C3, when ^{13}C -methanol and glucose or ribose were added to the cultures. This ^{13}C -labeling pattern arose by methanol assimilation via the RuMP pathway and was not due to re-assimilation of $^{13}\text{CO}_2$ formed by linear or cyclic oxidation of ^{13}C -methanol (Fig. 8). Cadaverine is a non-native metabolic endproduct of *C. glutamicum* occurring only as a consequence of expression of heterologous lysine decarboxylase genes *cadA* (Mimitsuka et al. 2007) or *ldcC* (Kind et al. 2010). *C. glutamicum* is a suitable host for overproduction of diamines (Schneider and Wendisch 2011) such as cadaverine as it withstands high cadaverine concentrations (Kind et al. 2010). Cadaverine has also been produced from alternative carbon sources such as starch, xylose, or hemicellulose using appropriate recombinant *C. glutamicum* strains (Buschke et al. 2013; Heider and Wendisch 2015; Tateno et al. 2009). Our report on engineering non-methylotrophic *C. glutamicum* to partially convert methanol to the non-native product cadaverine may be considered a first step towards methanol-based production of cadaverine. However, future studies have yet to reveal if production of cadaverine from methanol by engineered natural methylotrophs such as recombinant *B. methanolicus* (Naerdal et al. 2015) or by synthetic methylotrophs such as *E. coli* or *C. glutamicum* will be more efficient regarding yields, productivities, and process design.

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