# Metabolic engineering of *Bacillus methanolicus* and *Corynebacterium glutamicum* for the production of cadaverine from methanol

**DISSERTATION**

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# Abstract

The $C_1$ compound methanol represents a promising alternative carbon source for microbial fermentation processes in the biotechnological industry due to its high grade of purity and high availability. Current industrial fermentations rely on sugars like molasses, but there is a high demand for cheap alternative carbon sources that do not compete with human nutrition, in order to reduce the fermentation costs and allow for a profitable process. The polyamine cadaverine (1,5-diaminopentane) is of high commercial interest because of its use as a monomeric building block for the synthesis of polyamides. Cadaverine is a product of the aspartate pathway and formed by the decarboxylation of L-lysine. The natural thermophilic methylotroph *Bacillus methanolicus* MGA3 is a promising host for the production from methanol, especially for products based on the amino acids L-lysine and L-glutamate, as well as the industrial well-established amino acid producer *Corynebacterium glutamicum* that has already been engineered for the use of a variety of alternative carbon sources. Both organisms lack enzymes for the conversion of L-lysine into cadaverine. Therefore, genes of *Escherichia coli* were used to enable them for the production of cadaverine. Since *C. glutamicum* is only able to oxidize methanol via formaldehyde to carbon dioxide, a strategy for the implementation of a synthetic methanol assimilation pathway using enzymes of the ribulose monophosphate (RuMP) pathway of *B. methanolicus*.

The main objectives of this work were to evaluate the potential of *B. methanolicus* MGA3 for the production of cadaverine and genetically engineer strains for high production levels. Further, *C. glutamicum* was rationally engineered for the co-utilization of the alternative carbon source methanol, using methanol dehydrogenase (Mdh), 3-hexulose-6-phosphate synthase (Hps), and 6-phospho-3-hexuloisomerase (Phi) of *B. methanolicus* MGA3 for an efficient conversion and assimilation of methanol.

The results of this work can be summarized as follows:

1. *B. methanolicus* MGA3 is a suitable host for the production of the polyamine cadaverine as it can tolerate 200 mM (35 g/L) of cadaverine, accompanied by only minor growth inhibition. Additionally, *B. methanolicus* MGA3 is neither able to use cadaverine as carbon or nitrogen source nor able to degrade it, for example, via acetylation.

2. Both the L-lysine overproducing *B. methanolicus* strain M168-20 and the wild type strain MGA3 were genetically engineered for the production of cadaverine by heterologous expression of the lysine decarboxylase genes *ldcC* and *cadA* of *Escherichi coli*. The highest production level was obtained using CadA. Interestingly, for LdcC the cadaverine production could be increased about twofold by raising the pH from 7.2 to 7.6-8.5.

3. A high production of cadaverine was achieved during high-cell-density fed-batch methanol fermentation using *B. methanolicus* MGA3(pTH1mp-*cadA*) leading to a volume-corrected production of 11.3 g/L.

4. The ability for methanol and formaldehyde detoxification could be restored in a *C. glutamicum* strain, lacking the endogenous linear formaldehyde dissimilation pathway, by heterologous expression of the RuMP pathway key genes *hps* and *phi*.

5. $^{13}$C-labeling experiments in this *C. glutamicum* strain, additionally expressing *mdh*, using $^{13}$C-methanol showed successful conversion of methanol by Mdh and assimilation into the central metabolites by Hps and Phi. Furthermore, the finding of two- and threefold-labeled hexoses indicated a successful regeneration of ribulose 5-phosphate (Ru5P), required for formaldehyde fixation.

6. *C. glutamicum* was engineered for the production of the non-native product cadaverine, using methanol as co-substrate. The successful conversion of methanol into the desired compound was confirmed by $^{13}$C-labeling experiments.

7. The transaldolase (Ta) of the alternative *B. methanolicus* wild type strain PB1 was biochemically characterized and an attempt was made to identify the putative coding sequence of Ta in MGA3.

In this thesis, the microbial production of the polyamine cadaverine from methanol could be shown for the first time, using the natural methylotroph *B. methanolicus* MGA3. Further, the non-methylotroph *C. glutamicum* was engineered for the co-utilization of methanol for the production of the non-native compound cadaverine. Thus, this study represents a major step towards a synthetic methylotroph as a production host.

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# Nomenclature

## General abbreviations

| | |
|---|---|
| ATCC | American Type Culture Collection |
| BLAST | Basic Local Alignment Search Tool |
| CDW | cell dry weight |
| $Clm^R$ | resistance to chloramphenicol |
| $Kan^R$ | resistance to kanamycin |
| $OD_{600}$ | optical density at 600 nm |
| PA | polyamide |
| PPP | pentose phosphate pathway |

## Enzymes

| | |
|---|---|
| Adh | alcohol dehydrogenase |
| Ald | acetaldehyde dehydrogenase |
| FadH | (mycothiol-dependent) formaldehyde dehydrogenase |
| Fdh | formate dehydrogenase |
| Fba | fructose-1,6-bisphosphate aldolase |
| Fbp | fructose-1,6-bisphosphatase |
| Fdh | formate dehydrogenase |
| GlpX | fructose-1,6-bisphosphatase |
| Hps | 3-hexulose-6-phosphate synthase |
| Mdh | methanol dehydrogenase |
| Pc | pyruvate carboxylase |
| Pfk | 6-phosphofructokinase |
| Pgi | phosphoglucose isomerase |
| Phi | 6-phospho-3-hexuloisomerase |
| Rpe | ribulose-5-phosphate 3-epimerase |
| Rpi | ribose-5-phosphate isomerase |
| Sba | sedoheptulose 1,7-bisphosphate aldolase |
| SBPase | sedoheptulose-1,7-bisphosphatase |
| Ta | transaldolase |
| Tkt | transketolase |
| Zwf | glucose-6-phosphate dehydrogenase |

**Metabolites**

| | |
|---|---|
| DHA | dihydroxyacetone |
| DHAP | dihydroxyacetone phosphate |
| E4P | erythrose 4-phosphate |
| F6P | fructose 6-phosphate |
| FBP | fructose 1,6-bisphosphate |
| G6P | glucose 6-phosphate |
| GAP | glyceraldehyde 3-phosphate |
| H6P | hexulose 6-phosphate |
| OAA | oxaloacetate |
| PEP | phosphoenolpyruvate |
| R5P | ribose 5-phosphate |
| Ru5P | ribulose 5-phosphate |
| RuBP | ribulose bisphosphate |
| RuMP | ribulose monophosphate |
| S7P | sedoheptulose 7-phosphate |
| SBP | sedoheptulose 1,7-bisphosphate |
| Xu5P | xylulose 5-phosphate |
| XuMP | xylulose monophosphate |

**Cofactors**

| | |
|---|---|
| PLP | pyridoxal-5'-phosphate |
| PQQ | pyrroloquinoline quinone |
| THF | tetrahydrofolate |

Common (biological) abbreviations, units, chemical formulas, and gene names are not included. Abbreviations are introduced in parentheses the first time they are used within the text and abbreviations only used in the figures are explained in each figure, but not included here.

# 1. Introduction

This chapter covers on the one hand methylotrophy with a focus on the model strain *Bacillus methanolicus* MGA3, and on the other hand *B. methanolicus* and *Corynebacterium glutamicum* as promising hosts for the production of the polyamine cadaverine.

## 1.1. Methanol as future feedstock

We are heading towards an economy in which methanol could play an important role as energy storage material, fuel, and feedstock, as for example proposed by Nobel laureate George A. Olah (Olah, 2005). With increasing raw oil prices and limitations in gas, oil, and coal deposits, there is a high demand for alternative resources. Today, these fossil fuels are the main energy source and are also used for the production of various chemical products (Offermanns *et al.*, 2014). Also in biotechnology, where sugars are the main carbon sources for bacterial fermentations, there is a need for an alternative carbon supply that is cheap, sustainable, and is not competing with human nutrition (Schrader *et al.*, 2009). Therefore, one future alternative might be methanol. Methanol is a $C_1$ compound that is of high interest as a carbon source for the biotechnological industry, because it is pure, a non-food feedstock, and can be completely utilized during microbial fermentations (Müller *et al.*, 2015; Ochsner *et al.*, 2015). It can be obtained either from fossil raw materials, such as natural gas and coal, or from renewable sources like biogas. Other alternatives include the formation of methanol using hydrogen derived from water electrolysis and $CO_2$. However, alternative regenerative power sources, including wind power and solar energy, could provide electricity for electrolysis and the derived hydrogen could be used for direct conversion of $CO_2$ into methanol, whilst lowering $CO_2$ pollution (Keim, 2014; Wernicke *et al.*, 2014).

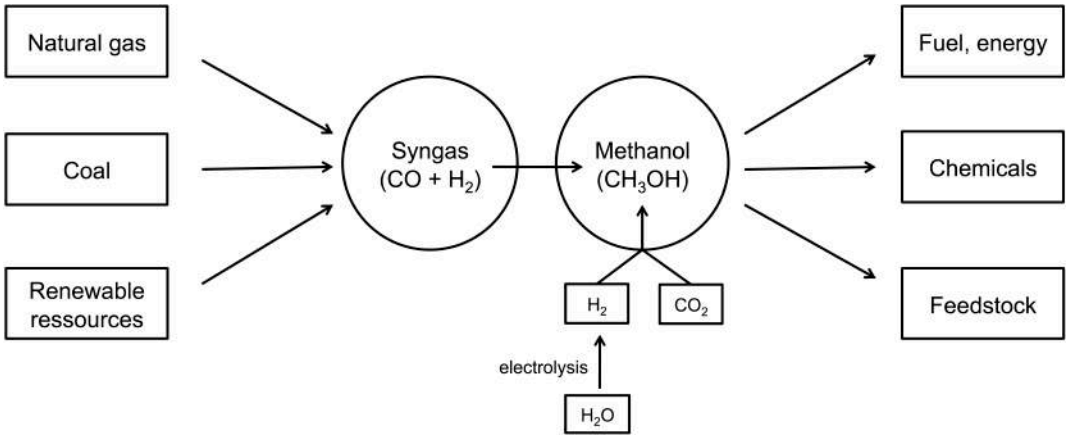


**Figure 1.1.: Main routes from raw materials to methanol** (adapted from (Offermanns *et al.*, 2014)).

Technical routes for methanol synthesis are based on the conversion into synthesis gas (syngas), which is a mixture of $H_2$ and CO. The main routes from raw materials to methanol and the applications of methanol are shown in Figure 1.1. Furthermore, the catalytic conversion of methane ($CH_4$) into methanol is progressing rapidly (Whitaker *et al.*, 2015). The current production of methanol is nearly 60 million tons per year (Wernicke *et al.*, 2014).

Data from Methanex (Methanex, 2015) and the US Department of Agriculture (US Department of Agriculture, 2015), showing the market trends for methanol and raw sugar from 2001 to 2015 (Figure 1.2.), indicates that the current raw sugar price (about 300 USD/MT) is lower than the price for methanol (about 400 USD/MT). Future perspectives for methanol prices predict a decrease to a level of about 100 USD/MT due to the construction of mega-methanol production facilities (5000 tons per day) in regions that are rich in natural gas deposits, such as the Caribbean, Latin America, and the Middle East (Brautaset *et al.*, 2007; Schrader *et al.*, 2009). This could lead to low and stable prices for methanol. Further, since methanol can be produced in various ways, it is less subject to market price fluctuations of raw materials compared to sugar, which depends on weather conditions, crop yields, as well as agricultural politics (Brautaset *et al.*, 2007; Ochsner *et al.*, 2015).

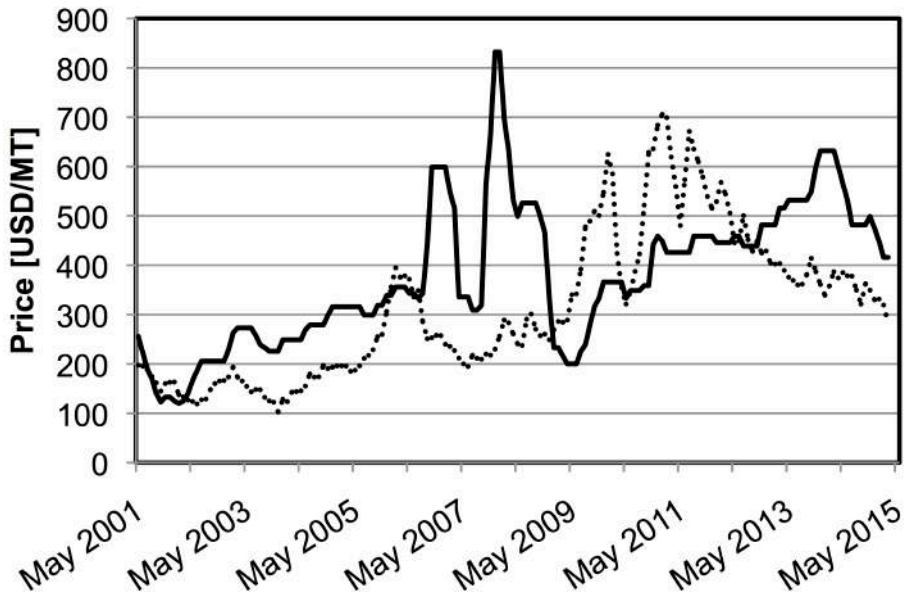


**Figure 1.2.: Comparison of the recent monthly price fluctuations of methanol (*solid line*) and raw sugar (*dashed line*).** Data shows the non-discounted reference price for North America from Methanex and the world raw sugar price (ICE contract 11) from the United States Department of Agriculture. Prices are shown in USD/MT for the years 2001-2015 (adapted from (Brautaset *et al.*, 2007)).

## 1.2. Methylotrophs

Methylotrophs have been defined as microorganisms that are able to use reduced carbon compounds, containing no carbon-carbon bonds, for biomass and energy formation (Anthony, 1982). Methylotrophy has been reported for prokaryotes as well as for eukaryotes (Anthony, 1986; Yurimoto *et al.*, 2005; Chistoserdova *et al.*, 2009). Methylotrophy in eukaryotes is restricted to some yeast species, such as *Pichia methanolica, Komagataella* (*Pichia*) *pastoris*, and *Candida boidinii* (Hagenson, 1991; Komagata, 1991; Yurimoto, 2009), which are limited to methanol as a carbon source (Yurimoto *et al.*, 2011). In contrast, methylotrophy is widespread among bacteria and has been found in *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Verrucomicrobia* (Anthony, 1982; Chistoserdova & Lidstrom, 2013; McTaggart *et al.*, 2015). Prokaryotic methylotrophs are able to use a variety of carbon sources, including methane, methanol, and formaldehyde (Anthony, 1982).

Due to their ability to use methanol as a carbon source, natural methylotrophs are a highly promising alternative to classical production strains for the industrial production of different compounds, such as polyhydroxyalkanoates and amino acids (Lee, 1996; Steinbüchel *et al.*, 1996; Brautaset *et al.*, 2010). Although methylotrophs are known for decades and have been used for the production of single-cell protein at industrial scale, their potential as production hosts has only been explored in recent years (Tannenbaum & Wang, 1975; Schrader *et al.*, 2009).

### 1.2.1. Methanol metabolism in methylotrophs

In nature, methanol is derived by demethylation of pectin and lignin, that are found in plants (Dijkhuizen *et al.*, 1992; von Dahl *et al.*, 2006). Methanol can also be formed by oxidation of methane in the troposphere by hydroxyl radicals, biomass burning, and plant decay (Large & Bamforth, 1988; Jacob *et al.*, 2005).

The first step of utilizing methanol is the oxidation to formaldehyde by methanol dehydrogenase (Mdh) (Anthony, 1982; Dijkhuizen *et al.*, 1992; Reid & Fewson, 1994; Hektor *et al.*, 2000; Kloosterman *et al.*, 2002). The Mdhs of methylotrophic bacteria belong to two groups. The first one are pyrroloquinoline quinone (PQQ)-containing and cytochrome-dependent periplasmic Mdhs that show a $\alpha_2\beta_2$ conformation and exist in Gram-negative bacteria (Anthony, 1982; Chistoserdova *et al.*, 2009). The second group form $NAD(P)^+$-dependent cytoplasmic Mdhs, consisting of one type of subunit, that are found in Gram-positive methylotrophic bacteria, such as *Bacillus methanolicus* (Anthony, 1982; Arfman *et al.*, 1992b; Bystrykh *et al.*, 1993; Anthony, 2004).

Formaldehyde represents a key intermediate in the utilization of methanol. Methanol toxicity is assumed to be caused by the accumulation of formaldehyde (Al-Awadhi *et al.*, 1990; Brooke *et al.*, 1990; Marx *et al.*, 2003). Due to non-specific reactions with proteins and nucleic acids, this intermediate is highly toxic to the cells (Merk & Speit, 1998; Kalapos, 1999; Quievryn & Zhitkovich, 2000). Therefore, a fast conversion into non-toxic intermediates is crucial. There are two different options for formaldehyde conversion: the oxidation to $CO_2$, leading to the generation of energy, and the assimilation into biomass. Formaldehyde can be used in both routes or only in the dissimilatory pathways (Vorholt, 2002).

### 1.2.1.1. Carbon assimilation pathways in methylotrophs

Nature contains a variety of assimilation pathways, showing the diversity among methylotrophs. The carbon compounds are oxidized either to formaldehyde or $CO_2$ prior to assimilation via a certain pathway. Hitherto, four cyclic assimilation pathways have been discovered (Anthony, 1991; Dijkhuizen *et al.*, 1992). These are the xylulose monophosphate (XuMP) pathway, the ribulose monophosphate (RuMP) pathway, the serine pathway, and the ribulose bisphosphate (RuBP) pathway (Figure 1.3.). These pathways have in common that a $C_1$ compound is condensed with a metabolite, which is regenerated by completing one round of the pathway, and that one $C_3$ compound is formed from three $C_1$ compounds.

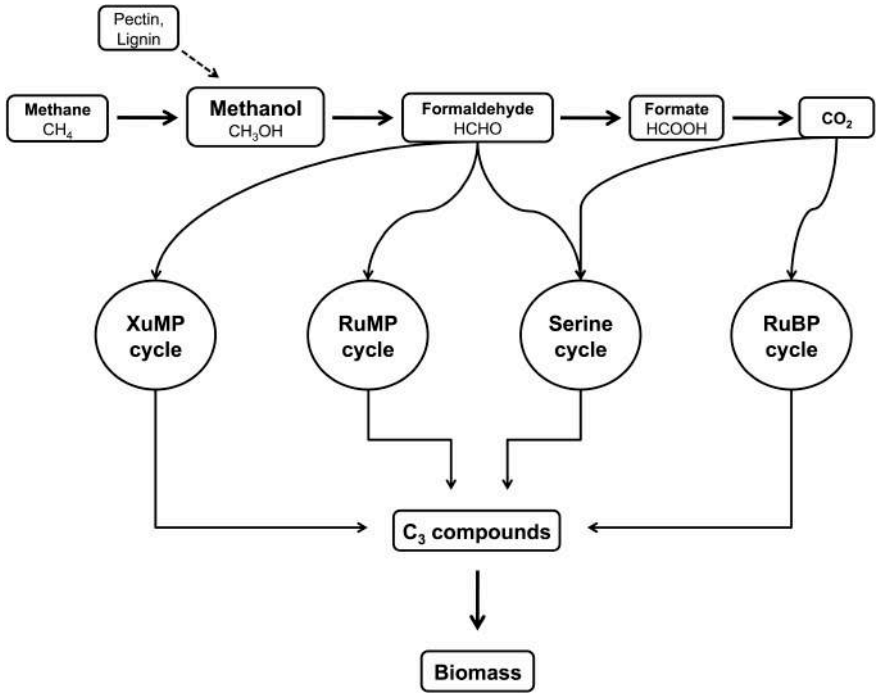


**Figure 1.3.: Assimilation pathways of methylotrophs.** The oxidation of methane and methanol to formaldehyde and $CO_2$, as well as the assimilation via the xylulose monophosphate (XuMP), ribulose monophosphate (RuMP), serine, or ribulose bisphosphate (RuBP) cycle are shown. The assimilation of three $C_1$ compounds leads to the formation of one $C_3$ compound that is used for biomass formation (adapted from (Dijkhuizen *et al.*, 1992).

The XuMP pathway, also referred to as dihydroxyacetone (DHA) pathway, is used for formaldehyde assimilation solely by yeasts (van Dijken *et al.*, 1978; Babel & Loffhagen, 1979; Kato *et al.*, 1979; O'Connor & Quayle, 1979). Methylotrophic bacteria that use the RuMP pathway for assimilation of formaldehyde belong to three groups: i) Gram-negative obligate methylotrophs, ii) Gram-positive facultative methylotrophs, and iii) thermotolerant *Bacillus* spp. (Anthony, 1982; de Vries *et al.*, 1990; Schendel *et al.*, 1990; Arfman *et al.*, 1992a). This pathway is used for the assimilation of carbon at the level of formaldehyde (Johnson & Quayle, 1965; Kemp & Quayle, 1966; Kemp & Quayle, 1967; Hanson *et al.*, 1996). In the RuBP pathway, also known as Calvin cycle, which is mainly used by chemolithotrophic (autotrophic) bacteria, carbon is assimilated from $CO_2$ (Kelly, 1971; Dijkhuizen & Harder, 1984; Anthony, 1991). The serine cycle, in which the carbon is assimilated from formaldehyde and $CO_2$, is the most distinct pathway since carboxylic acids and amino acids are used as intermediates, while the other three pathways involve carbohydrates (Large *et al.*, 1961; Anthony, 1982).

#### 1.2.1.2. Linear and cyclic formaldehyde dissimilation pathway

Dissimilatory pathways for formaldehyde detoxification are not only present in methylotrophic bacteria, but also in non-methylotrophs since formaldehyde can be formed in demethylation reactions (Yurimoto *et al.*, 2005). The most common formaldehyde dissimilation pathway is a linear pathway for oxidation to $CO_2$ via formate (Figure 1.4.), leading to the generation of reducing equivalents. In general, these reactions are catalyzed by formaldehyde dehydrogenase (Fadh) and formate dehydrogenase (Fdh). Fadh is normally NAD- and/or cofactor-linked, with tetrahydrofolate (THF) (Maden, 2000), tetrahydromethanopterin ($H_4$MPT) (Vorholt *et al.*, 1999), glutathione (GSH) (Harms *et al.*, 1996; Barber & Donohue, 1998), and mycothiol (MSH) (Duine, 1999) representing possible cofactors. The initial step of this pathway can be spontaneous or enzyme-catalyzed (Vorholt, 2002; Lidstrom, 2006).

The second pathway is a cyclic dissimilation pathway for formaldehyde oxidation to $CO_2$ that uses 3-hexulose-6-phosphate synthase (Hps) and 6-phospho-3-hexuloisomerase (Phi) together with enzymes of the oxidative pentose phosphate pathway (PPP) (Figure 1.4.). It generates reducing equivalents and the produced ribulose 5-phosphate (Ru5P) is reused for the fixation of formaldehyde (Vorholt, 2002).

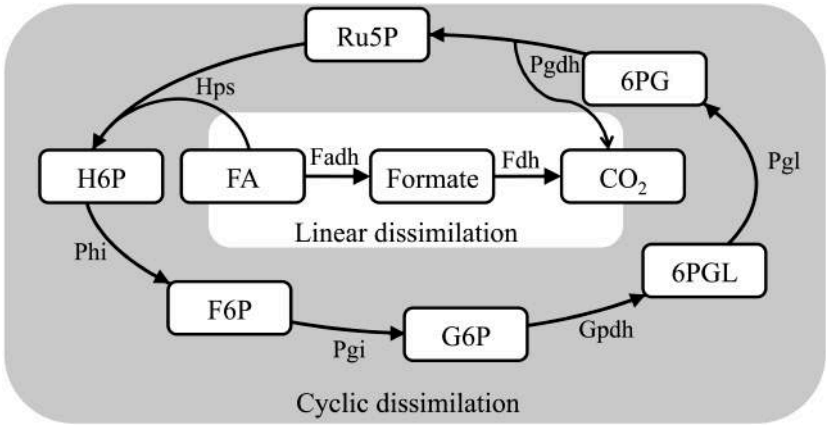


**Figure 1.4.: Linear and cyclic formaldehyde dissimilation pathway leading to the formation of $CO_2$.** Both pathways generate reducing equivalents. The linear pathway mainly consists of the two enzymes Fadh and Fdh, while the cyclic pathway is a combination of the fixation part of the RuMP pathway and the PPP (adapted from (Brautaset *et al.*, 2007)).

*Abbreviations of intermediates*: 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; F6P, fructose 6-phosphate; FA, formaldehyde; G6P, glucose 6-phosphate; H6P, hexulose 6-phosphate; Ru5P, ribulose 5-phosphate.
*Abbreviations of enzymes*: Fadh, formaldehyde dehydrogenase; Fdh, formate dehydrogenase; Gpdh, glucose-6-phosphate dehydrogenase; Hps, 3-hexulose-6-phosphate synthase; Pgdh, 6-phospho-gluconate dehydrogenase; Pgi, phosphoglucose isomerase; Pgl, 6-phosphogluconolactonase; Phi, 6-phospho-3-hexuloisomerase.

## 1.3. *Bacillus methanolicus* – a model strain for methylotrophy

*Bacillus methanolicus* is a Gram-positive, thermotolerant, and facultative methylotrophic bacterium that is able to use methanol as sole carbon and energy source (Schendel *et al.*, 1990). The model wild type strain MGA3 (ATCC 53907) has been isolated from soil samples in Minnesota (Schendel *et al.*, 1990) and the alternative wild type strain PB1 (ATCC 51375) from a sugar beet factory wastewater treatment system in Europe (Dijkhuizen *et al.*, 1988). *B. methanolicus* is a so-called restricted methylotroph, which means that this organism is able to utilize some multi-carbon sources, such as glucose or mannitol, for energy and biomass formation (Schendel *et al.*, 1990; Arfman *et al.*, 1992a; Heggeset *et al.*, 2012). The two wild type strains MGA3 and PB1 show differences with regard to amino acid production, growth behaviour, and respiration profiles (Heggeset *et al.*, 2012).

Methylotrophy in *B. methanolicus* MGA3 is well studied, since its genome has been fully sequenced (Heggeset *et al.*, 2012; Irla *et al.*, 2014) and a variety of enzymes has been identified and characterized (Heggeset *et al.*, 2012; Krog *et al.*, 2013; Stolzenberger *et al.*, 2013a; Stolzenberger *et al.*, 2013b; Irla *et al.*, 2014; Markert *et al.*, 2014; Ochsner *et al.*, 2014). Methylotrophy in *B. methanolicus* MGA3 is plasmid-dependent (Brautaset *et al.*, 2004) and genes encoding enzymes of the RuMP pathway are present on the plasmid pBM19 as well as on the chromosome (Figure 1.5.). Two sets of the genes *pfk* (encoding 6-phosphofructokinase), *fba* (encoding fructose-1,6-bisphosphate aldolase), *tkt* (encoding transketolase), *glpX* (encoding fructose-1,6-bisphosphatase), and *rpe* (encoding ribulose-5-phosphate 3-epimerase) exist in *B. methanolicus* MGA3 (Brautaset *et al.*, 2004; Brautaset *et al.*, 2007) with different specific functions in the metabolism (Krog *et al.*, 2013; Stolzenberger *et al.*, 2013a; Stolzenberger *et al.*, 2013b).

*B. methanolicus* MGA3 possesses three Mdhs (encoded by *mdh*, *mdh2*, and *mdh3*) (Figure 1.5.), which belong to the type III Fe-$NAD^+$-dependent alcohol dehydrogenase superfamily (de Vries *et al.*, 1992), and one Mdh-activity enhancing activator protein Act (Arfman *et al.*, 1991; Krog *et al.*, 2013; Ochsner *et al.*, 2014). Mdh is supposed to function as the main methanol dehydrogenase for the oxidation of methanol, leading to the formation of formaldehyde (Müller *et al.*, 2014; Bozdag *et al.*, 2015), which can be used in the RuMP pathway or dissimilatory routes.

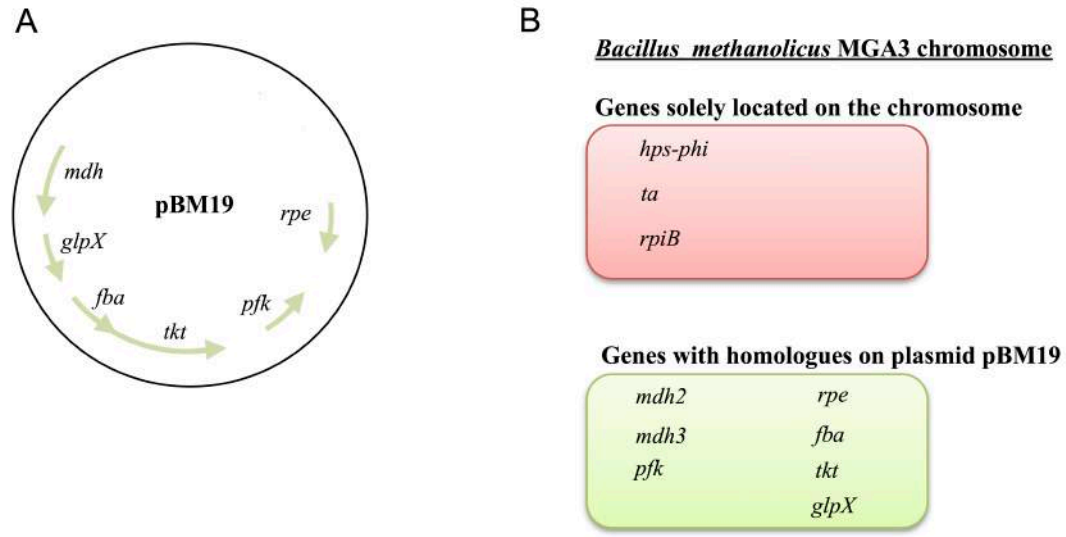


**Figure 1.5.: Schematic map of plasmid pBM19 of *Bacillus methanolicus* MGA3 and overview of known chromosomal RuMP pathway genes.** (A) the plasmid pBM19, (B) known RuMP genes on the chromosome. The red box shows genes only present on the chromosome and the green box shows genes on the chromosome, which have homologues on pBM19.

*Abbreviations of genes*: *fba*, fructose-1,6-bisphosphate aldolase; *glpX*, fructose-1,6-bisphosphatase; *hps*, 3-hexulose-6-phosphate synthase; *mdh*, methanol dehydrogenase; *pfk*, 6-phosphofructokinase; *phi*, 6-phospho-3-hexuloisomerase; *rpe*, ribulose-5-phosphate-3-epimerase; *rpiB*, ribose-5-phosphate isomerase; *ta*, transaldolase; *tkt*, transketolase.

### 1.3.1. The RuMP pathway for formaldehyde assimilation in *B. methanolicus* MGA3

The RuMP pathway can be divided into three parts: fixation, cleavage, and rearrangement (Figure 1.6.) (Brautaset *et al.*, 2007). Formaldehyde is condensed with Ru5P to hexulose 6-phosphate (H6P) for fixation, which is then converted to fructose 6-phosphate (F6P). These two steps are catalyzed by Hps and Phi respectively (Kato *et al.*, 2006).

The cleavage part consists of enzymes of the glycolysis, where the chromosomal fructose-1,6-bisphosphate aldolase ($Fba^C$) functions as the major glycolytic Fba (Stolzenberger *et al.*, 2013b), and the PPP leading to the formation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). DHAP is further used in the glycolysis for the formation of pyruvate (Anthony, 1991).

In the rearrangement part of the RuMP pathway, the eponymous metabolite Ru5P is regenerated for the fixation of formaldehyde involving transketolase (Tkt), ribose-5-phosphate isomerase (Rpi), and ribulose-5-phosphate 3-epimerase (Rpe) (Anthony, 1982). *B. methanolicus* MGA3 uses the sedoheptulose-1,7-bisphosphatase (SBPase) variant for Ru5P regeneration via sedoheptulose 1,7-bisphosphate (SBP) and sedoheptulose 7-phosphate (S7P) (Stolzenberger *et al.*, 2013a). Both Fba enzymes catalyze the reaction of erythrose 4-phosphate (E4P) and DHAP to SBP, and thus, act as a sedoheptulose-1,7-bisphosphate aldolase (Sba) (Stolzenberger *et al.*, 2013a), whereas the plasmid-encoded fructose-1,6-bisphosphatase ($GlpX^P$) has been found to function as the only SBPase, catalyzing the dephosphorylation of SBP to S7P (Figure 1.6.) (Stolzenberger *et al.*, 2013a). In summary, three formaldehyde molecules are converted into three F6P molecules, of which one is converted to pyruvate and GAP. Ru5P is regenerated from GAP and the two remaining F6P molecules.

A putative transaldolase gene (*ta*) has been identified in the genome of *B. methanolicus* MGA3 (Heggeset *et al*., 2012; Irla *et al*., 2014) and its function and possible role in a energetically favorable Fba/Ta variant of the RuMP pathway (Dijkhuizen *et al.*, 1992; Kato *et al.*, 2006) was unknown at the beginning of this thesis.

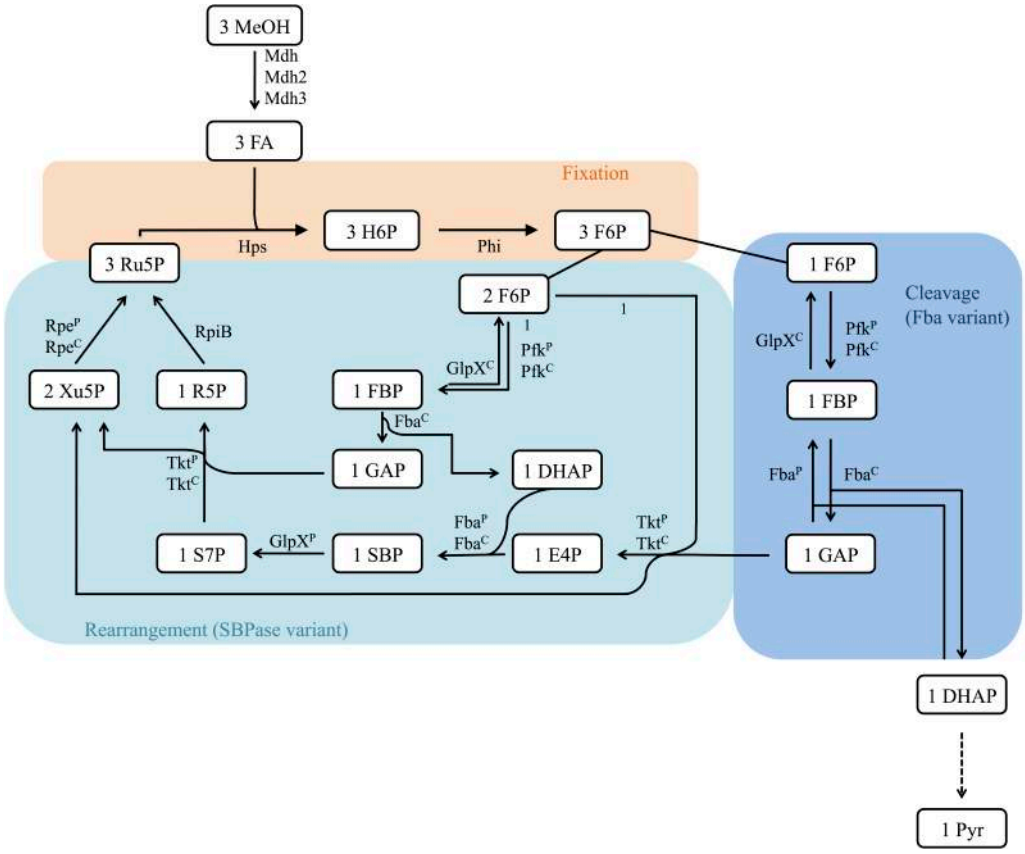


**Figure 1.6.: The RuMP pathway for formaldehyde assimilation of *Bacillus methanolicus* MGA3.** The three parts : fixation, cleavage (Fba variant), and rearrangement (SBPase variant) are shown. Three molecules of methanol are converted to one molecule of pyruvate, while the remaining molecules are used for regeneration of Ru5P. The solid arrows indicate a one-step reaction and the dashed arrows a multi-step reaction. The small numbers relate to the stoichiometry of the reaction. Enzymes encoded on the chromosome (C) or on the plasmid pBM19 (P) are indicated.

*Abbreviations of intermediates*: DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FA, formaldehyde; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; MeOH, methanol; H6P, hexulose 6-phosphate; Pyr, pyruvate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; X5P, xylulose 5-phosphate.
*Abbreviations of enzymes*: Fba, fructose-1,6-bisphosphate aldolase; GlpX, fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase; Hps, 3-hexulose-6-phosphate synthase; Mdh, methanol dehydrogenase, Pfk, 6-phosphofructokinase; Phi, 6-phospho-3-hexuloisomerase; Rpe, ribulose-5-phosphate 3-epimerase; Rpi, ribose-5-phosphate isomerase; Ta, transaldolase; Tkt, transketolase.

### 1.3.1.4. Formaldehyde dissimilation in *Bacillus methanolicus* – the linear tetrahydrofolate pathway and the cyclic RuMP pathway

It has been shown by $^{13}C$-experiments that *B. methanolicus* MGA3 possesses a linear formaldehyde dissimilation pathway via formate to $CO_2$ (Pluschkell & Flickinger, 2002). Based on the genome sequence a putative linear dissimilatory tetrahydrofolate pathway for the conversion of formaldehyde into $CO_2$ has been proposed (Figure 1.7.) (Heggeset *et al.*, 2012). This pathway is found in a variety of methylotrophic bacteria and, besides its function for formaldehyde detoxification and energy generation, it plays an important role in providing $C_1$ units for biosynthetic reactions (Vorholt, 2002).

In addition, *B. methanolicus* MGA3 also possesses a cyclic dissimilatory RuMP pathway for formaldehyde detoxification (Figure 1.7.). This pathway uses enzymes of the oxidative PPP together with Hps and Phi for formaldehyde oxidation and regeneration of Ru5P. The cyclic dissimilatory pathway is important for the generation of reducing power (NAD(P)H) (Chistoserdova *et al.*, 2009).

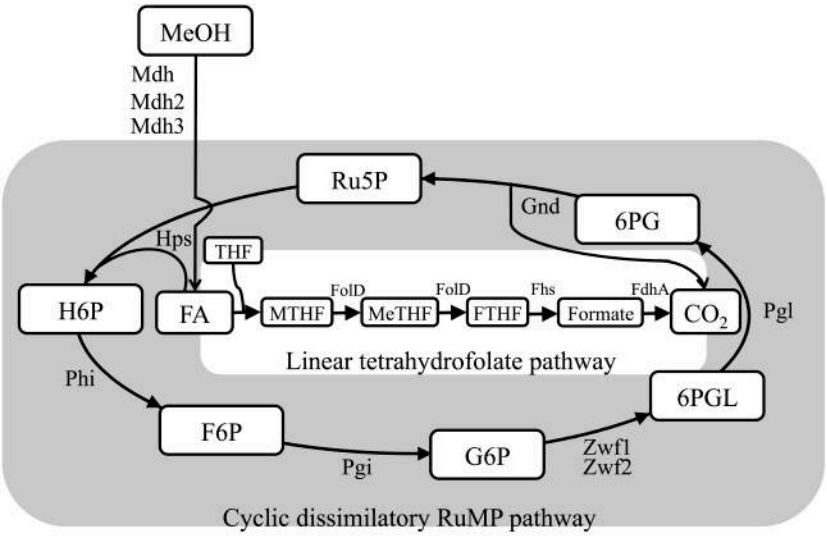


**Figure 1.7.: Linear tetrahydrofolate pathway and cyclic dissimilatory RuMP pathway for the dissimilation of formaldehyde in *B. methanolicus* MGA3.** Both pathways lead to the generation of reducing equivalents and the formation of $CO_2$.

*Abbreviations of intermediates:* 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; F6P, fructose 6-phosphate; FA, formaldehyde; FTHF, formate tetrahydrofolate; G6P, glucose 6-phosphate; H6P, hexulose 6-phosphate; MeOH, methanol; MeTHF, methenyl tetrahydrofolate; Ru5P, ribulose 5-phosphate; THF, tetrahydrofolate.
*Abbreviations of enzymes:* FdhA, formate dehydrogenase; FolD, 5,10-methylenetetrahydrofolate dehydrogenase/5,10-methenyltetrahydrofolate cyclohydrolase; Fhs, formate-tetrahydrofolate ligase; Gnd, 6-phosphogluconate dehydrogenase; Hps, 3-hexulose-6-phosphate synthase; Mdh, methanol dehydrogenase; Pgi, phosphoglucose isomerase; Pgl, 6-phosphogluconolactonase; Phi, 6-phospho-3-hexuloisomerase; Zwf, glucose-6-phosphate dehydrogenase.

## 1.4. Engineering of *C. glutamicum* for the access to alternative carbon sources

*Corynebacterium glutamicum* is a Gram-positive soil bacterium (Kinoshita, 2005), which is used in the biotechnological industry for many decades (Eggeling & Bott, 2015). *C. glutamicum* can use a variety of sugars as carbon source for growth and its energy supply (Blombach & Seibold, 2010). Further, it is able to utilize some alcohols, organic acids, but as well some amino acids or sugar alcohols (Cocaign *et al.*, 1993; Lee *et al.*, 1998; Claes *et al.*, 2002; Gerstmeir *et al.*, 2003; Stansen *et al.*, 2005; Krings *et al.*, 2006; Arndt *et al.*, 2008; Kato *et al.*, 2010; Laslo *et al.*, 2012). *C. glutamicum* is capable of co-utilizing different carbon sources, when supplemented as a mixture (Blombach & Seibold, 2010). In contrast, diauxic growth and sequential utilization of carbon sources is rare in C. glutamicum and reported, for example, for glucose plus L-glutamate or ethanol, where glucose is the preferred carbon source (Kronemeyer *et al.*, 1995; Arndt & Eikmanns, 2007; Leßmeier *et al.*, 2014).

Currently, industrial fermentation processes using *C. glutamicum* are based on sugars derived from starch and molasses (Kelle *et al.*, 2005; Kimura, 2005). But there is a high demand for alternative cheap non-food feedstocks (Chapter 1.1.). Thus, *C. glutamicum* has been genetically engineered for the use of a variety of different alternative carbon sources, like pentoses, starch, (hemi)cellulose, glycerol, and glucosamine, to broaden its substrate spectrum (reviewed by (Zahoor *et al.*, 2012; Leßmeier *et al.*, 2014; Peters-Wendisch & Wendisch, 2014). However, complex carbon sources have some disadvantages, like containing salts or inhibitors (Rumbold *et al.*, 2010). Thus, to further reduce the fermentation costs and overcome these potential drawbacks, the utilization of methanol by *C. glutamicum* in industrial fermentations opens up new possibilities for the future.

### 1.4.1. Formaldehyde dissimilation pathway in *C. glutamicum*

$^{13}$C-labeling studies using $^{13}$C-methanol have shown that methanol can be converted into $CO_2$ by *C. glutamicum*, leading to the identification of a linear formaldehyde dissimilation pathway via formate to $CO_2$ (Leßmeier *et al.*, 2013; Witthoff *et al.*, 2013). Several genes of the ethanol catabolism were found to be upregulated in the presence of methanol (Witthoff *et al.*, 2013). The alcohol dehydrogenase AdhA catalyzes the oxidation of methanol to formaldehyde, which is then converted to formate by the mycothiol-dependent formaldehyde dehydrogenase FadH and the acetaldehyde dehydrogenase Ald

(Figure 1.8.). The last step to $CO_2$ is catalyzed by the molybdenum-dependent formate dehydrogenase Fdh (Witthoff *et al.*, 2012; Leßmeier *et al.*, 2013; Witthoff *et al.*, 2013). It has been shown that methanol catabolism is subject to catabolite repression in the presence of glucose and that the transcriptional regulator RamA is required for expression of *ald* and *adhA* (Witthoff *et al.*, 2013). However, *C. glutamicum* lacks enzymes for the fixation of formaldehyde, the key step in the assimilation of methanol.

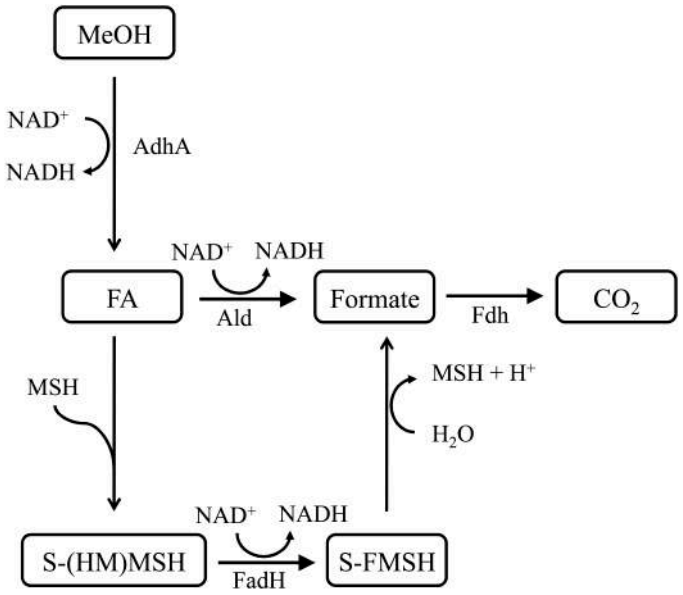


**Figure 1.8.: Linear formaldehyde dissimilation pathway of *C. glutamicum*.** This pathway, consisting of the enzymes Ald, FadH, and Fdh, leads to the generation of NADH and formation of $CO_2$ (adapted from (Witthoff *et al.*, 2013)).

*Abbreviations of intermediates:* FA, formaldehyde; MeOH, methanol; MSH, mycothiol; S-FMSH, S-formylmycothiol; S-(HM)MSH, S-(hydroxymethyl)mycothiol.
*Abbreviations of enzymes:* AdhA, alcohol dehydrogenase; Ald, acetaldehyde dehydrogenase; FadH, formaldehyde dehydrogenase; Fdh, formate dehydrogenase.

## 1.5. Polyamines

Polyamines are defined as low molecular weight aliphatic polycations with at least two primary amino groups (Tabor *et al.*, 1961; Takahashi & Kakehi, 2010) and were discovered in 1678 by Antoni van Leeuwenhoek (van Leeuwenhoek, 1678).

### 1.5.1. The physiological role of polyamines

Polyamines are present in nearly all living organisms and play an important role in gene expression, translation, cell proliferation, and membrane stabilization, most probably due to their positive charge at physiological pH (Tabor & Tabor, 1984; Tabor & Tabor, 1985; Cohen, 1998; Igarashi & Kashiwagi, 2000; Wallace *et al.*, 2003). Besides, bacterial polyamines have different additional functions: they i) are part of the outer membrane of Gram-negative bacteria (Takatsuka & Kamio, 2004), ii) play a role in the biosynthesis of siderophores (Griffiths *et al.*, 1984; Burrell *et al.*, 2012), iii) are part of the acid stress response system (Lin *et al.*, 1996; Richard & Foster, 2004; Fritz *et al.*, 2009; Kanjee & Houry, 2013), iv) protect the cell from oxygen toxicity (Chattopadhyay *et al.*, 2003), v) play a role in signaling for cellular differentiation (Sturgill & Rather, 2004), and vi) are required for plaque biofilm formation (Patel *et al.*, 2006; Kusano *et al.*, 2008).

Polyamines can be found in high concentrations of about 1 mM in the cell (Miyamoto *et al.*, 1993). Bacteria primarily synthesize putrescine (1,4-diaminobutane) and spermidine. In contrast, other polyamines, like cadaverine, are less abundant (Wortham *et al.*, 2007). Further, thermophilic bacteria synthesize additional polyamines, including long-chain and branched polyamines, that play a role in stabilizing nucleic acids (Terui *et al.*, 2005; Oshima, 2007). Although it was assumed that polyamines are essential for life, an *E. coli* strain lacking polyamines was constructed that was still able to grow (Chattopadhyay *et al.*, 2009).

### 1.5.2. Biosynthesis and export of cadaverine

Cadaverine is a linear aliphatic diamine. The name derives from its contribution to the foul odor of the putrefying flesh of a cadaver, since it is a bacterial decomposition compound (Brieger, 1885; Kusano *et al.*, 2008).

The biosynthesis of cadaverine starts from L-lysine and involves a one-step reaction (Figure 1.9.) catalzyed by a pyridoxal-5'-phosphate (PLP)-dependent lysine decarboxylase (Tabor & Tabor, 1985; Sandmeier *et al.*, 1994; Foster, 2004).

Cadaverine is only found in low concentrations in *E. coli* under normal growth conditions (Tabor & Tabor, 1985). *E. coli* possesses two isozymes for the decarboxylation of L-lysine. The first one is the lysine dcarboxylase CadA that is induced at acidic pH in the presence of L-lysine (Sabo *et al.*, 1974), and the second one is the constitutive LdcC (Kikuchi *et al.*, 1997; Lemonnier & Lane, 1998). LdcC is responsible for a constant production of cadaverine, which is required for growth and other cellular processes, whereas CadA is part of the acid stress response system (Meng & Bennett, 1992; Neely & Olson, 1996; Samartzidou *et al.*, 2003; Kanjee & Houry, 2013).

In *E. coli*, cadaverine is secreted into the medium by the exporter CadB, which is a member of the amino acid-polyamine-organocation (APC) family. CadB functions as cadaverine:proton symporter at neutral pH, taking up cadaverine. In contrast, at acidic pH it functions as cadaverine:lysine antiporter, leading to the secretion of cadaverine (Soksawatmaekhin *et al.*, 2004).

In *C. glutamicum*, overexpression of the putative permease gene *cgmA* led to an increased cadaverine production. Thus, CgmA is supposed to be involved in the secretion of cadaverine (Kind *et al.*, 2011; Nguyen *et al.*, 2015). Further, it has been also reported that the lysine exporter LysE might contribute to the export of cadaverine in *C. glutamicum* (Stäbler *et al.*, 2011), but has been excluded as a major cadaverine exporter (Kind *et al.*, 2011).

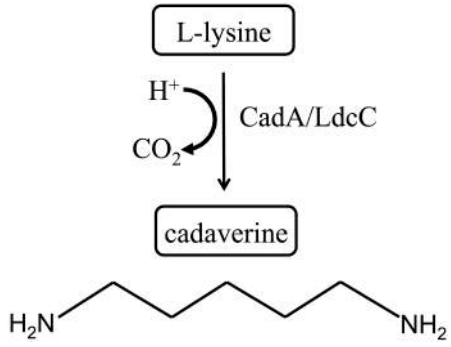


**Figure 1.9.: Formation of cadaverine by decarboxylation of L-lysine in *E. coli*.** The reaction is catalyzed by the lysine decarboxylase (CadA and LdcC). The structure of cadaverine is given.

### 1.5.3. Polyamines as building blocks for bio-polyamides

Today, nearly all conventional plastics productions are based on non-renewable natural gas and petroleum resources (Adkins *et al.*, 2012). Since the natural resources are limited and the global plastics production is increasing, surpassing a production of 297 million tons by 2015, there is a high demand for plastics production from renewable and sustainable resources, so called "bioplastics" (Adkins *et al.*, 2012).

In general, polyamines find application as intermediates for the production of polymers, dyes, pharmaceuticals, and agrochemicals (Imm *et al.*, 2011). The focus here is on the use of diamines, in particular cadaverine, and their function as monomeric building blocks for the production of bio-polyamides (Schaffer & Haas, 2014). The chemical synthesis of diamines relies on petrol-based raw materials and they are mainly produced by reductive amination of the corresponding carbonyl compounds (Imm *et al.*, 2011).

Polyamides, often referred to as nylons, are high polymers that consist of a polymer backbone containing amide repeat linkages. Two classes of polyamides can be defined based on the regularity of the amide linkages: i) type AB is formed in a polycondensation reaction from ω-amino acids, where the amide linkages are in the same orientation and ii) type AABB is formed from diacids and diamines in a polycondensation reaction yielding a polymer with alternating amide linkages (Weber, 2000). Polyamides can be synthesized in different ways. First, by direct amidation, second, by acid chloride reaction, and third, by ring-opening polymerization that is used for the conversion of lactams into polyamides (Weber, 2000). Synthetic polyamides are characterized by a high mechanical strength, durability, and stability (Zeng & Guan, 2011). The most common

polyamides are polyamide (PA)-6,6 and PA-6, which account for 85% to 90% of the polyamide market (Adkins *et al.*, 2012). The global polyamide market makes up for about 6.6 million tons per year (Kind *et al.*, 2014).

Cadaverine can be used, for example, for the production of PA-5,6 and PA-5,10, which are polymerized from cadaverine plus adipic acid and sebacic acid, respectively. Polyamides derived from cadaverine feature a high melting point and a low water absorption (Kind & Wittmann, 2011). Hitherto, there has been no example for a production of diamines by bacterial fermentation in the industry. But there are several companies that produce bio-polyamides by using alternative sources, such as glucose, castor oil, and butadiene or propene for the production process.

In 2007, BASF produced the first castor oil-based bio-polyamide, PA-6,10, for which sebacic acid is used that is derived from castor oil. Other companies, like Evonik Industries, DuPont, and DSM, Netherlands, also produce PAs from natural and renewable sources, marketed for example as Vestamid® Terra HS, as part of the Zytel® family, and as Stanyl™ and EcoPaXX™, respectively (Weber, 2000). Cathay Industrial Biotech produces 1,5-pentamethylenediamine (C-BIO N5) in a patented process from 100% sugar plants. Based on C-BIO N5, several polyamides, such as PA-5,6, are produced. PA-5,6, trademarked as Terryl®, has some unique properties, like higher moisture absorbance, dyeability, and elasticity, compared to polyester and nylon, making it a new and promising target, especially for the textile market (Cathay Biotech, 2015).

### 1.6. *C. glutamicum* and *B. methanolicus* as production platforms for cadaverine

Since L-lysine-overproducing strains of *C. glutamicum* (Becker *et al.*, 2011; Becker & Wittmann, 2012; Eggeling & Bott, 2015) as well as *B. methanolicus* (Brautaset *et al.*, 2007; Jakobsen *et al.*, 2009; Nærdal *et al.*, 2011) are available, it is reasonable to construct cadaverine-producing strains by metabolic engineering. In the last years, studies have been carried out showing the potential of bacteria, including *E. coli* and *C. glutamicum*, for the production of polyamines (Schneider & Wendisch, 2011). However, both *B. methanolicus* and *C. glutamicum* lack a lysine decarboxylase for the formation of cadaverine.

### 1.6.1. L-lysine biosynthesis and overproduction in *B. methanolicus* MGA3

The *B. methanolicus* MGA3 wild type only produces about 0.2 g/L of L-lysine during fed-batch fermentation, but several mutants have been constructed leading to a secretion of up to 47 g/L of L-lysine for classical mutants (Hanson *et al.*, 1996; Brautaset *et al.*, 2007; Brautaset *et al.*, 2010), including the *hom1* (encoding homoserine dehydrogenase I) mutant M168-20 that produces 11 g/L of L-lysine (Jakobsen, 2008; Brautaset *et al.*, 2010), and up to 11 g/L of L-lysine for recombinant *B. methanolicus* strains (Jakobsen *et al.*, 2009).

L-lysine is a product of the aspartate pathway, derived from the precursor oxaloacetate (OAA). The aspartate pathway branches into three routes leading to i) the synthesis of the amino acids L-threonine and L-methionine via homoserine, ii) the formation of dipicolinate, and iii) the synthesis of L-lysine via *meso*-diaminopimelate (Chen *et al.*, 1993; Müller *et al.*, 2015). *B. methanolicus* MGA3 possesses three aspartokinases (AK I-III), encoded by the genes *dapG*, *lysC*, and *yclM* (Schendel & Flickinger, 1992; Jakobsen *et al.*, 2009), catalyzing the first step of the aspartate pathway. In the following, eight enzymatic steps lead to the formation of L-lysine (Brautaset *et al.*, 2007). Analysis of the genome sequence has revealed a putative lysine exporter, encoded by *lysE*, but the biological function of its gene product is unknown up to now (Heggeset *et al.*, 2012). The identification of *dapH* (encoding tetrahydrodipicolinate *N*-acetyltransferase), *patA* (encoding acetyl-diaminopimelate aminotransferase), and *dapL* (encoding *N*-acetyl-diaminopimelate deacetylase) indicates that *B. methanolicus* MGA3, like most *Bacillus* species (Paulus, 1993), uses the acetylase variant of the L-lysine pathway (Brautaset *et al.*, 2007; Heggeset *et al.*, 2012).

### 1.6.2. L-lysine biosynthesis and overproduction in *C. glutamicum*

*Corynebacterium glutamicum* is best known for its use in industrial fermentations for the production of the amino acids L-glutamate and L-lysine, with 2.96 million tons/year and 2.1 million tons/year, respectively (Ajinomoto, 2015).

The *C. glutamicum* wild type strain ATCC 13032 is not able to secrete significant amounts of L-lysine (Ikeda *et al.*, 2006). Classical *C. glutamicum* strains for L-lysine production have been obtained by random mutagenesis (Nakayama *et al.*, 1978), leading to production titers of more than 100 g/L of L-lysine (Leuchtenberger, 1996; Ikeda, 2003).

Further, the development of a wide range of methods for directed gene deletion and gene overexpression has led to the rational construction of L-lysine-producing strains by metabolic engineering (Sahm *et al.*, 2000). Additionally, different *–omics* technologies for studies of the transcriptome and proteome are available, leading to a wealth of knowledge of *C. glutamicum* as a production host (Wendisch, 2003; Wendisch *et al.*, 2006a; Wendisch *et al.*, 2006b).

*C. glutamicum* has been genetically engineered for the production of various industrial relevant compounds, like amino acids, biofuel alcohols, organic acids, carotenoids, and the diamines putrescine and cadaverine, from glucose as well as from alternative non-natural carbon sources (reviewed by (Zahoor *et al.*, 2012; Peters-Wendisch & Wendisch, 2014; Wendisch, 2014).

In the aspartate pathway, *C. glutamicum* harbors only one aspartokinase, LysC, for the conversion of L-aspartate to aspartate 4-phosphate. LysC is feedback-inhibited by L-lysine and L-threonine (Park & Lee, 2010). L-lysine is produced via a split pathway from tetrahydrodipicolinate (Schrumpf *et al.*, 1991; Sonntag *et al.*, 1993). The first pathway is the succinylase branch, where the enzymes DapD (tetrahydrodipicolinate succinylase), DapC (*N*-succinyl-diaminopimelate aminotransferase), DapE (succinyl-diaminopimelate desuccinylase), and DapF (diaminopimelate epimerase) are involved, and the second variant is the dehydrogenase branch via the diaminopimelate dehydrogenase (Ddh). Finally, the export of L-lysine is catalyzed by the lysine exporter LysE (Vrljic *et al.*, 1999).

## 1.7. Objectives

The first part of this thesis deals with the use of the natural methylotroph *B. methanolicus* for the production of the industrial relevant polyamine cadaverine from the cheap and renewable carbon source methanol. The second part of this thesis includes the transfer of methylotrophy from *B. methanolicus* MGA3 to the important production host *C. glutamicum* and the production of cadaverine by a synthetic *C. glutamicum* strain co-utilizing methanol. The RuMP pathway of *B. methanolicus* MGA3 was chosen for engineering methylotrophy in *C. glutamicum.* The main reason is that this pathway is more efficient than the serine and the RuBP pathway.

The Fba/Ta variant of the RuMP pathway yields 1 mol of NAD(P)H and 1 mol of ATP per 1 mol of pyruvate produced, whereas the serine cycle leads to 2 mol of $NAD(P)^+$ and 2 mol of ADP and the RuBP pathway even requires 7 mol of ATP plus 1 mol of $NAD(P)^+$ (Quayle & Ferenci, 1978; Kato *et al.*, 2006; Whitaker *et al.*, 2015).

The detailed aims of this study are i) to evaluate the potential of *B. methanolicus* as a production host for cadaverine, ii) to biochemically characterize the Ta of *B. methanolicus*, iii) to identify the role of Ta for the regeneration of Ru5P in the RuMP pathway, iv) to transfer the RuMP pathway into *C. glutamicum*, and v) to evaluate the potential of a methanol-utilizing *C. glutamicum* strain for the production of cadaverine.

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# 2. Results

The main objective of this thesis was the production of the polyamine cadaverine from methanol by engineered *Bacillus methanolicus* and *Corynebacterium glutamicum* strains. The results of this thesis are summarized in three manuscripts.

The first manuscript „Methanol-based cadaverine production by genetically engineered *Bacillus methanolicus* strains“ describes the construction of cadaverine-producing strains of the wild type *B. methanolicus* MGA3 as well as the L-lysine-overproducing mutant M168-20 by heterologous expression of the lysine decarboxylase genes *ldcC* and *cadA* of *E. coli*. Cadaverine tolerance studies and cadaverine utilization/degradation experiments revealed a promising potential of *B. methanolicus* as a production platform for cadaverine. Further, production levels of up to 500 mg/L of cadaverine were obtained in shake flask cultivations and the potential of a high-level production of cadaverine was shown in fed-batch methanol fermentations leading to a volume-corrected production of 11.3 g/L of cadaverine.

The second manuscript „Production of cadaverine by engineered *Corynebacterium glutamicum* using methanol as co-substrate” deals with the construction of a *C. glutamicum* strain that is able to convert methanol as a co-substrate into the non-natural product cadaverine. A *C. glutamicum* strain, lacking the linear formaldehyde dissimilatory pathway to $CO_2$, was engineered for formaldehyde fixation by heterologous expression of *hps* (encoding 3-hexulose-6-phosphate synthase) and *phi* (encoding 6-phospho-3-hexuloisomerase) of *B. methanolicus* MGA3 and their homologues *hxlA* and *hxlB* of *B. subtilis*, respectively. This could partially restore methanol and formaldehyde detoxification in *C. glutamicum*. The additional expression of *mdh* (encoding methanol dehydrogenase) of *B. methanolicus* MGA3 led to an increased methanol oxidation to formaldehyde. The incorporation of the carbon from methanol into metabolic intermediates by recombinant *C. glutamicum* strains, expressing *mdh* and *hxlAB*, could be successfully shown in $^{13}$C-labeling experiments using $^{13}$C-methanol. The implementation of the methanol utilization module into a cadaverine-producing *C. glutamicum* strain, constructed by overexpression of $lysC^{fbr}$ and heterologous expression of *ldcC*, led to the co-utilization of methanol for the formation of cadaverine. The conversion of methanol into cadaverine was confirmed by $^{13}$C-labeling experiments with $^{13}$C-methanol plus glucose and ribose respectively.

The third manuscript “Characterization of transaldolase from *Bacillus methanolicus*” covers on the one hand the biochemical characterization of the transaldolase of *B. methanolicus* PB1, and on the other hand the identification of a possible transaldolase coding sequence in *B. methanolicus* MGA3. Further, its putative function and role in the RuMP pathway, especially for the regeneration of ribulose 5-phosphate (Ru5P), was addressed.

## 2.1. Methanol-based cadaverine production by genetically engineered *Bacillus methanolicus* strains

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### 2.1.1. Summary

Methanol is regarded as an attractive substrate for biotechnological production of value-added bulk products, such as amino acids and polyamines. In the present study, the methylotrophic and thermophilic bacterium *Bacillus methanolicus* was engineered into a microbial cell factory for the production of the platform chemical 1,5-diaminopentane (cadaverine) from methanol. This was achieved by the heterologous expression of the *Escherichia coli* genes *cadA* and *ldcC* encoding two different lysine decarboxylase enzymes, and by increasing the overall L-lysine production levels in this host. Both CadA and LdcC were functional in *B. methanolicus* cultivated at 50 °C and expression of *cadA* resulted in cadaverine production levels up to 500 mg/L during shake flask conditions. A volume-corrected concentration of 11.3 g/L of cadaverine was obtained by high-cell density fed-batch methanol fermentation. Our results demonstrated that efficient conversion of L-lysine into cadaverine presumably has severe effects on feedback regulation of the L-lysine biosynthetic pathway in *B. methanolicus*. By also investigating the cadaverine tolerance level, *B. methanolicus* proved to be an exciting alternative host and comparable to the well-known bacterial hosts *E. coli* and *Corynebacterium glutamicum*. This study represents the first demonstration of microbial production of cadaverine from methanol.

### 2.1.2. Introduction

There is a high societal demand for – and scientific interest in – more environmental-friendly and sustainable production processes for large quantity bulk products. As examples, amino acids and polyamines find applications as food/feed additives as well as in the pharmaceutical, plastics and polymer industry (Wendisch, 2014). The polyamine monomer 1,5-diaminopentane, commonly known as cadaverine, is a sought-after platform chemical used for production of various polyamides and is currently mainly fabricated by petroleum-based chemical synthesis. With the increasing focus on bio-economy and low-carbon footprints in the industry, efforts have been made to develop biotechnological production processes for several polyamines (Adkins *et al.*, 2012; Buschke *et al.*, 2013; Meiswinkel *et al.*, 2013a). Applying bacteria as microbial production hosts, certain polyamines can be obtained from amino acids including L-lysine, L-arginine and L-ornithine by thermodynamically favourable decarboxylation reactions (Schneider & Wendisch, 2011). These amino acids can be obtained by microbial fermentation processes and the worldwide production of the feed amino acid L-lysine amounts to almost 2 million tons per year (Wendisch, 2014). The common approach has been to establish L-lysine overproducing hosts for the concomitant engineering towards efficient production of cadaverine, as this compound is formed by a one-step conversion of L-lysine catalysed by lysine decarboxylase (Kind *et al.*, 2010; Kind & Wittmann, 2011; Qian *et al.*, 2011) (Figure 2.1.1.). In particular, the genes of the lysine decarboxylases found naturally in *Escherichia coli*, encoded by *cadA* and *ldcC*, have been applied and overexpressed. Also cadaverine secretion has been a target for optimization of production (Kind *et al.*, 2011; Li *et al.*, 2014). Typically, these production processes rely on *E. coli* and *Corynebacterium glutamicum* as microbial hosts using sugars from molasses or from starch hydrolysis as carbon and energy substrates leading to an unwanted competition with human food supply, and consequently nutrition prices are rising worldwide (Schrader *et al.*, 2009). As an alternative, e.g. recombinant *C. glutamicum* strains have been developed to accept alternative carbon sources such as glycerol from the biodiesel process (Meiswinkel *et al.*, 2013a), amino sugars derived from chitin (Uhde *et al.*, 2013; Matano *et al.*, 2014) and pentoses present in lignocellulosic hydrolysates (Gopinath *et al.*, 2011; Meiswinkel *et al.*, 2013b). More generally, the possibility to produce polyamines, amino acids and other bulk products and biofuels from alternative non-food carbon sources has been in the research focus of biotechnology in recent years. The one-carbon substrate methanol has long been regarded as a convenient fuel

and raw material for manmade hydrocarbon-based products (Olah, 2005). It occurs abundantly throughout nature, it is a pure raw material that can be completely utilized in microbial fermentation processes, and the price is expected to remain independent from and lower than sugar prices (Brautaset *et al.*, 2007; Schrader *et al.*, 2009). Based on all this, methanol is regarded as a highly attractive non-food substrate for microbial bio-processes.

The Gram-positive and facultative methylotrophic bacterium *Bacillus methanolicus* is able to utilize methanol as sole carbon and energy source for growth (Müller *et al.*, 2015). As methanol growth is characterized by high oxygen demands leading to an increased heat output, it is an advantage that *B. methanolicus* has a growth optimum at 50–55 °C, reducing the process cooling costs. The genome sequences of two wild-type *B. methanolicus* strains MGA3 and PB1 were recently published (Heggeset *et al.*, 2012; Irla *et al.*, 2014) and its transcriptome characterized (Irla *et al.*, 2015) serving as a solid basis for increased understanding of methylotrophy and product formation, e.g. L-glutamate and L-lysine, in this industrially relevant bacterium. It has been well documented that *B. methanolicus* has great potential for L-lysine overproduction through classical mutagenesis studies and selection of strains resistant to the L-lysine analog S-2-aminoethylcysteine (Hanson *et al.*, 1996; Brautaset *et al.*, 2010). Several key genes and enzymes of the aspartate pathway of *B. methanolicus* have been characterized, and insight into genetic repression and feedback inhibition has been established (Jakobsen *et al.*, 2009; Brautaset *et al.*, 2010). Furthermore, metabolic engineering of central metabolism and the aspartate pathway towards L-lysine in the MGA3 wild-type strain yielded significant L-lysine overproduction during shake flask experiments and fed-batch fermentations (Nærdal *et al.*, 2011). The theoretical maximum L-lysine yield from methanol has been calculated to 0.82 g/g in this bacterium (Brautaset *et al.*, 2007) which is comparable to the estimated maximum L-lysine yield from glucose in *C. glutamicum* (de Graaf, 2000; Wittmann & Becker, 2007). Thus, *B. methanolicus* was regarded as a potential promising host for production of cadaverine from methanol.

In the present study, we have investigated the potential of methanol-based biotechnological production of cadaverine at elevated temperature using wild-type and mutant *B. methanolicus* strains as hosts. Since inspection of the genome sequence did not reveal a gene putatively encoding a lysine decarboxylase (Figure 2.1.1.), synthetic cadaverine production modules based on the lysine decarboxylase isozymes LdcC and CadA

from Gram-negative *E. coli* were constructed and heterologously expressed in *B. methanolicus* strains. Both enzymes proved functional and resulted in cadaverine production in *B. methanolicus*, and *cadA* overexpression provided the highest cadaverine production levels. This is to our knowledge the first demonstration of microbial cadaverine production from methanol.

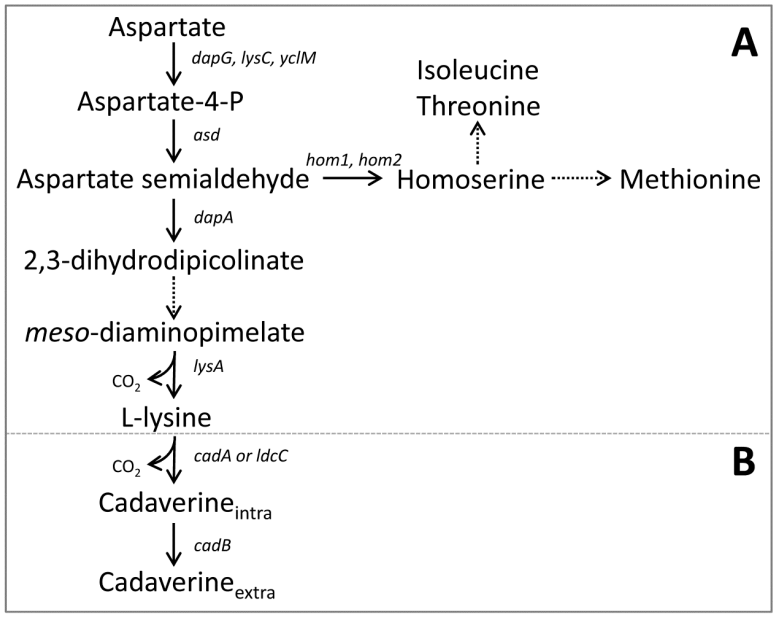


**Figure 2.1.1.: Pathway for L-lysine and cadaverine biosynthesis in *B. methanolicus*.** Gene names are indicated next to arrows representing reactions. Dotted lines summarize several reactions. Reactions present in box A are endogenous in *B. methanolicus*, whereas reactions in box B are only present in recombinant *B. methanolicus* strains due to heterologous expression of *E. coli* genes.

## 2.1.3. Materials & Methods

### 2.1.3.1. Biological materials, DNA manipulations and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1.1. *E. coli* DH5α was used as a general cloning host. *E. coli* strains were cultivated in liquid and on solid lysogeny broth medium at 37 °C and standard recombinant deoxyribonucleic acid (DNA) procedures were performed as described elsewhere (Sambrook *et al.*, 2001). *B. methanolicus* strains were cultivated at 50 °C and 200 rpm in methanol ($MeOH_{200}$) medium (Jakobsen *et al.*, 2006) containing salt buffer (4.1 g/L $K_2HPO4$, 1.3 g/L $NaH_2PO_4$, 2.1 g/L $(NH_4)_2SO_4$) and 0.025% yeast extract (Difco) adjusted to pH 7.2 unless stated otherwise. After autoclavation, the medium was supplemented with 1 mM

$MgSO_4$, vitamins, trace metals and 200 mM methanol as described elsewhere (Schendel *et al.*, 1990; Jakobsen *et al.*, 2006). The transformation of *B. methanolicus* was performed by electroporation as described previously (Jakobsen *et al.*, 2006). For classical *B. methanolicus* mutant strain M168-20 (Brautaset *et al.*, 2010) the growth medium was supplemented with D,L-methionine (1.5 mM). Recombinant *E. coli* and *B. methanolicus* strains were cultivated in media supplemented with chloramphenicol (15 and 5 mg/ml respectively). Bacterial growth was monitored by measuring optical density at 600 nm ($OD_{600}$). Tolerance of *B. methanolicus* to cadaverine was investigated by monitoring bacterial growth in the presence of different cadaverine concentrations. Cadaverine dihydrochloride (Sigma Aldrich Biochemie GmbH, Hamburg, Germany) was dissolved in $MeOH_{200}$ medium, and the solution was pH adjusted to 7.2 and pre-warmed before cadaverine was supplemented in different concentrations to the growing cell cultures. Control cultures without cadaverine were included.

**Table 2.1.1.: Bacterial strains and plasmids used in this study.**

| Strain or plasmid | Description | Reference |
|---|---|---|
| *E. coli* | | |
| DH5α | General cloning host | Stratagene |
| MG1655 | Wild-type strain | ATCC 47076 |
| *B. methanolicus* | | |
| MGA3 | Wild-type strain | ATCC 53907 |
| M168-20 | AEC-resistant *hom1* MGA3 mutant | (Brautaset *et al.*, 2010) |
| Plasmids | | |
| pHP13 | *E. coli-B. subtilis* shuttle vector, $Clm^r$ | (Haima *et al.*, 1987) |
| pTH1mp-*lysC* | pHP13 derivate with *lysC* under control of *mdh* promoter | (Brautaset *et al.*, 2010) |
| pTH1mp-*lysA* | pHP13 derivate with *lysA* under control of *mdh* promoter | (Nærdal *et al.*, 2011) |
| pTH1mp-*ldcC* | pHP13 derivate with *ldcC* under control of *mdh* promoter | This study |
| pTH1mp-*cadA* | pHP13 derivate with *cadA* under control of *mdh* promoter | This study |
| pTH1mp-*ldcC-lysC* | pTH1mp-*ldcC* with *lysC* downstream of the *ldcC* gene | This study |
| pTH1mp-*ldcC-lysA* | pTH1mp-*ldcC* with *lysA* downstream of the *ldcC* gene | This study |
| pTH1mp-*cadA-lysA* | pTH1mp-*cadA* with *lysA* downstream of the *cadA* gene | This study |

$Clm^r$, chloramphenicol resistance

### 2.1.3.2. Construction of expression vectors

The *ldcC* gene of *E. coli* MG1655 was polymerase chain reaction (PCR) amplified from genomic DNA using primers ldcC-PciI-Fwd: 5'- GCTGCACATGTGAACAT-CATTGCCATTATGG-3' and ldcC-XbaI-Rev 5'-GCTGCTCTAGATTATCCCGCCATTTTTAGGAC-3'. The resulting 2162 bp PCR product was digested with *Pci*I and *Xba*I (restriction sites underlined) and ligated into corresponding sites of pTH1mp-*lysC* (replacing *lysC*) resulting in plasmid pTH1mp-*ldcC*. Vector pTH1mp-*lysC* was digested with *Spe*I and *Nco*I and the 2017 bp fragment containing *lysC* was ligated into the *Xba*I (compatible with *Spe*I) and *Nco*I sites of pTH1mp-*ldcC* resulting in plasmid pTH1mp-*ldcC*-*lysC*. Vector pTH1mp-*lysA* was digested with *Spe*I and *Nco*I, and the 1834 bp fragment containing *lysA* was ligated into the *Xba*I (compatible with *Spe*I) and *Nco*I sites of pTH1mp-*ldcC* resulting in plasmid pTH1mp-*ldcC*-*lysA*. The *cadA* gene (2148 bp) was PCR amplified from genomic DNA isolated from *E. coli* MG1655 using the following primer pair: cadA-fw: 5'-AGGAGGTAGTACATGTGAACGTTATTGCAATATTGAATC-3' and cadA-rv: 5'-CCTATGGCGGGTACCTTATTTTTTGCTTTCTTCTTTCAA-3'. The obtained PCR product was ligated into the vector pTH1mp-*lysC*, digested with *Pci*I/*Kpn*I (replacing the *lysC* gene), using the isothermal DNA assembly method (Gibson *et al.*, 2009) yielding expression vector pTH1mp-*cadA*. Vector pTH1mp-*lysA* was digested with *Spe*I and *Nco*I, and the 1834 bp fragment containing *lysA* was ligated into the *Xba*I (compatible with *Spe*I) and *Nco*I sites of pTH1mp-*cadA* resulting in plasmid pTH1mp-*cadA*-*lysA*.

### 2.1.3.3. Lysine decarboxylase activity assays in *B. methanolicus* crude extracts

The lysine decarboxylase activity was determined in *B. methanolicus* crude cell extracts. The preparation of crude cell extracts was performed as described elsewhere previously (Brautaset *et al.*, 2004). The cells were inoculated from a glycerol stock and grown in $MeOH_{200}$ medium overnight before they were transferred to fresh $MeOH_{200}$ medium and grown to an $OD_{600}$ of 1.5 to 2.0. Forty millilitre of the culture was harvested by centrifugation (4000 x g, 30 min, 4 °C), washed in 100 mM sodium citrate buffer (pH 7.5) and stored at -20 °C. The cells were disrupted by sonication (Brautaset *et al.*, 2003). The cell debris was removed by centrifugation (14.000 x g, 60 min, 4 °C) and the supernatant was used as crude extract for measuring the lysine decarboxylase activity.

Lysine decarboxylase activity was calculated by measuring the conversion of lysine to cadaverine over time using HPLC as described elsewhere (Kind *et al.*, 2010). The assays were carried out at 50 °C, and one unit of lysine decarboxylase activity was defined as the amount of enzyme that formed 1 μmol of cadaverine per min at 50 °C. Protein concentration was determined using the assay of Bradford (Bradford, 1976).

### 2.1.3.4. Cadaverine and L-lysine shake flask production studies

Production experiments were performed in 500 ml baffled shake flasks (Belco) containing 100 ml $MeOH_{200}$ medium (Jakobsen *et al.*, 2006). *B. methanolicus* strains were cultivated in triplicate cultures using inoculum made from exponentially growing cells (Brautaset *et al.*, 2010; Nærdal *et al.*, 2011). Samples for amino acid measurements were collected during the late exponential and stationary growth phases as described previously (Jakobsen *et al.*, 2009; Nærdal *et al.*, 2011), and measurements of cadaverine and amino acids were performed by using 9-fluorenylmethyl chloroformate (FMOC) or o-phthaldialdehyde (OPA) derivatization and reverse-phase high-performance liquid chromatography (Jakobsen *et al.*, 2009; Brautaset *et al.*, 2010; Schneider & Wendisch, 2010). Concentrations for cadaverine are reported for the free base (MW of 102.18 g/mol).

### 2.1.3.5. High-cell-density fed-batch methanol fermentation

Fed-batch fermentation was performed at 50 °C in UMN1 medium in Applikon 3-L fermentors with an initial volume of 0.75 litre essentially as described previously (Jakobsen *et al.*, 2009; Brautaset *et al.*, 2010). Chloramphenicol (5 μg/ml) was added to the initial batch growth medium, the pH was maintained at 6.5 by automatic addition of 12.5% (wt/vol) $NH_3$ solution, and the dissolved oxygen level was maintained at 30% saturation by increasing the agitation speed and using enriched air (up to 60% $O_2$). The methanol concentration in the fermentor was monitored by online analysis of the headspace gas with a mass spectrometer (Balzers Omnistar GSD 300 02). The headspace gas was transferred from the fermentors to the mass spectrometer in insulated heated (60 °C) stainless steel tubing. The methanol concentration in the medium was maintained at a set point of 150 mM by automatic addition of methanol feed solution containing methanol, trace metals and antifoam 204 (Sigma), as described in (Brautaset *et al.*, 2010). The inoculum preparation protocol, the fermentation conditions and fermen-

tation progress was as described previously (Brautaset *et al.*, 2010). All fermentations were run until the carbon dioxide content of the exhaust gas was close to zero (no cell respiration). Bacterial growth was monitored by measuring the optical density at 600 nm ($OD_{600}$). Dry cell weight was calculated using a conversion factor of one $OD_{600}$ unit corresponding to 0.24 g dry cell weight per litre (calculated based on multiple measurements of dry cell weight and $OD_{600}$ during the fermentation trial). Due to the significant increase in culture volume throughout the fermentation, the biomass, cadaverine and amino acid concentrations were corrected for the increase in volume and subsequent dilution. The volume correction factor of 1.8 was used for values presented in Table 2.1.5. The actual concentrations measured in the bioreactors were therefore accordingly lower as described previously (Jakobsen *et al.*, 2009). Samples for determination of volumetric cadaverine and amino acid yields were collected from early exponential phase and throughout the cultivation (10-47 h) and analyzed as described above.

### 2.1.4. Results

#### 2.1.4.1. *B. methanolicus* lacks cadaverine biosynthetic and degradation genes and tolerates up to 200 mM cadaverine before growth is severely affected

The genome sequencing of the wild-type *B. methanolicus* strains MGA3 and PB1 (Heggeset *et al.*, 2012; Irla *et al.*, 2014) has identified all genes of the aspartate pathway leading to L-lysine, while genes putatively encoding L-lysine decarboxylases for conversion of L-lysine to cadaverine were not found. Furthermore, no putative cadaverine exporter genes were identified in the *B. methanolicus* genomes. Hence, heterologous expression of a lysine decarboxylase gene in *B. methanolicus* is a requirement for cadaverine production, which has never been reported for this species.

To test the tolerance of *B. methanolicus* to cadaverine, this compound was added to exponentially growing cells and growth was monitored. For this purpose, the *B. methanolicus* strain M168-20 was used and cultivated in shake flasks containing methanol ($MeOH_{200}$) medium. The cells were grown to an $OD_{600}$ of 0.4 before different concentrations of cadaverine dihydrochloride (0-200 mM, corresponding to 0-35 g/L) were added to triplicate cultures for each concentration. The control cultures without cadaverine supplementation grew with a specific growth rate ($\mu$) of $0.46 \pm 0.01$ $h^{-1}$ to an $OD_{600}$ of $8.7 \pm 0.14$. With the addition of 50 mM, 100 mM and 200 mM of cadaverine dihydrochloride, the maximum $OD_{600}$ values obtained were $7.5 \pm 0.18$, $6.2 \pm 0.20$ and

5.5 ± 0.22, respectively, and the accompanied specific growth rates were also reduced (0.40 ± 0.02 $h^{-1}$, 0.39 ± 0.01 $h^{-1}$ and 0.36 ± 0.01 $h^{-1}$).

Thus, a minor growth inhibition by cadaverine was observed since addition of 200 mM (35 g/L) cadaverine dihydrochloride reduced the growth rate by about 20%.

Cadaverine may be degraded by certain bacteria and the involved genes have been identified (Schneider & Wendisch, 2011). However, inspection of the *B. methanolicus* MGA3 and PB1 genomes did not indicate that this bacterium is capable of catabolizing cadaverine. This was also experimentally confirmed in shake flask cultures by substituting methanol and ammonium sulphate, as carbon and nitrogen source, respectively, with cadaverine. Cadaverine did not support bacterial growth, and the cadaverine concentration did not decrease throughout the cultivation as analyzed by reverse-phase high-performance liquid chromatography (data not shown).

### 2.1.4.2. Heterologous expression of L-lysine decarboxylase genes enabled methanol-based cadaverine production by *B. methanolicus* classical mutant strain M168-20

Since *B. methanolicus* lacks a lysine decarboxylase gene, the lysine decarboxylase genes *ldcC* and *cadA* from *E. coli* MG1655 were cloned into a pHP13 derivative expression vector carrying the strong *mdh* promoter for overexpression and used to transform the L-lysine overproducing classical mutant *B. methanolicus* strain M168-20. To assay for functional expression of *ldcC* and *cadA*, respectively, crude extracts of strains M168-20(pTH1mp-*ldcC*) and M168-20(pTH1mp-*cadA*) were prepared and the specific L-lysine decarboxylase activities were determined (Table 2.1.2.). The protein concentrations of the crude extracts of M168-20(pHP13), M168-20(pTH1mp-*ldcC*), and M168-20(pTH1mp-*cadA*) were 7.0 ± 0.3 mg, 7.8 ± 0.5 mg and 12.0 ± 1.5 mg, respectively. L-lysine decarboxylase activity could not be detected in the empty vector control (< 1 nmol/min/mg), whereas expression of *ldcC* and of *cadA* resulted in L-lysine decarboxylase activities of 7 ± 1 nmol/min/mg in M168-20(pTH1mp-*ldcC*) and of 88 ± 11 nmol/min/mg in M168-20(pTH1mp-*cadA*) (Table 2.1.2.).

**Table 2.1.2.: Specific L-lysine decarboxylase activities, cadaverine and L-lysine production levels in recombinant *B. methanolicus* M168-20 strains.**

| Plasmid | L-lysine decarboxylase specific activity | Cadaverine | L-lysine | Cadaverine + L-lysine |
|---|---|---|---|---|
| | nmol/min/mg protein | mg/L | mg/L | mg/L |
| pHP13 | < 1 ± 0.2 | 0.0 | 140 ± 10 | 140 |
| pTH1mp-*ldcC* | 7.0 ± 1.0 | 130 ± 10 | 40 ± 5 | 170 |
| pTH1mp-*cadA* | 88.0 ± 11.0 | 420 ± 25 | 10 ± 2 | 430 |

The results shown are from triplicate (cadaverine and L-lysine) and duplicate (lysine decarboxylase activity) cultures. Activity was measured using crude extracts from exponentially growing cells whereas the production levels were found from late stationary cultures, ca. 20 hours after inoculation.

Subsequently, production experiments were carried out with *B. methanolicus* strains M168-20(pTH1mp-*ldcC*) and M168-20(pTH1mp-*cadA*) at 50 °C in 500 ml shake flask cultures with $MeOH_{200}$ medium pH 7.2, and samples were harvested and analyzed by HPLC, as described in Materials & Methods. As experimental control, the M168-20 strain transformed with the empty vector pHP13 was included. In accordance with previously reported data, the M168-20(pHP13) strain produced 140 ± 10 mg/L of L-lysine under these conditions (Nærdal *et al.*, 2011) and, as expected, no cadaverine production was detected. The heterologous expression of *ldcC* in *B. methanolicus* M168-20 resulted in production of 130 ± 10 mg/L cadaverine and a L-lysine level of 40 ± 5 mg/L (Table 2.1.2.), confirming that the *ldcC* encoded lysine decarboxylase functions *in vivo* in *B. methanolicus* at 50 °C. Similarly, heterologous expression of *cadA* entailed a surprisingly high cadaverine production level of 420 ± 25 mg/L and only 10 ± 2 mg/L L-lysine could be detected as side-product (Table 2.1.2.). Thus, methanol-based production of cadaverine by *B. methanolicus* was achieved. Notably, combined formation of cadaverine and L-lysine by the *cadA* and *ldcC* expressing strains was above 3-fold higher than L-lysine formation by the parent strain (Table 2.1.2.), which might indicate feedback deregulation by L-lysine as consequence of a metabolic pull by lysine decarboxylase.

### 2.1.4.3. Effect of the medium pH on cadaverine production by recombinant *B. methanolicus*

Since LdcC and CadA function in pH homeostasis in *E. coli*, the effect of varying the pH of the production media on cadaverine production was investigated. *B. methanolicus* strains M168-20(pHP13), M168-20(pTH1mp-*ldcC*) and M168-20(pTH1mp-*cadA*) were cultivated in $MeOH_{200}$ medium adjusted to different pH values ranging from pH 6.5 to 8.5 prior to autoclaving. The standard $MeOH_{200}$ medium pH of 7.2 was included as control in these shake flask experiments for direct comparison. The control strain M168-20(pHP13) was included to test for any potential pH effects on L-lysine production. L-lysine production by M168-20(pHP13) was reduced to about 50 ± 5 mg/L at slightly acidic pH (pH 6.5), but remained stable (130-140 mg/L) at slightly alkaline pH (pH 7.2 to 8.5). Cadaverine production by M168-20(pTH1mp-*ldcC*) was lower at pH 6.5 (52 ± 5 mg/L) than at pH 7.2 (135 ± 10 mg/L), but about twofold higher at pH values between 7.6 and 8.5 (about 300 mg/L; Table 2.1.3.). However, the productivity was maximal at pH 7.6 since the growth rate decreased at higher pH values (data not shown). Strain M168-20(pTH1mp-*cadA*) accumulated similar concentrations of cadaverine (430 to 520 mg/L) at all tested pH values except at pH 6.5 (45 ± 5 mg/L), a condition also characterized by reduced production of the immediate precursor L-lysine (Table 2.1.3.).

**Table 2.1.3.: Production of cadaverine and L-lysine by recombinant *B. methanolicus* M168-20 strains cultivated at different medium pH.**

| | M168-20(pHP13) | | M168-20(pTH1mp-*ldcC*) | | M168-20(pTH1mp-*cadA*) | |
|---|---|---|---|---|---|---|
| pH | Cadaverine | L-lysine | Cadaverine | L-lysine | Cadaverine | L-lysine |
| 6.5 | 0 | 50 ± 10 | 52 ± 5 | < 15 | 45 ± 5 | < 15 |
| 7.2 | 0 | 130 ± 10 | 135 ± 10 | 40 ± 5 | 430 ± 20 | < 30 |
| 7.6 | 0 | 140 ± 10 | 315 ± 20 | < 30 | 450 ± 20 | < 30 |
| 8.0 | 0 | 140 ± 10 | 305 ± 30 | < 15 | 500 ± 30 | < 30 |
| 8.5 | 0 | 140 ± 10 | 305 ± 30 | < 15 | 520 ± 30 | < 30 |

The mean values (mg/L) and standard deviation of triplicate cultures is presented. The production levels were found from late stationary cultures, from 20-30 hours after inoculation.

As lysine decarboxylase activity is reported to depend on pyridoxal-5-phosphate (PLP) as cofactor, addition of pyridoxal-5-phosphate hydrate (1 mg/L) to $MeOH_{200}$ medium at pH 7.6 was tested. However, PLP supply in *B. methanolicus* was not limiting cadaverine production under the chosen conditions since production did not increase upon addition of pyridoxal phosphate (data not shown).

### 2.1.4.4. Construction of cadaverine overproducing strains by using the wild-type *B. methanolicus* MGA3 as a host

We have previously achieved L-lysine overproduction by engineering of the aspartate pathway and using wild-type *B. methanolicus* strain MGA3 as host. For example, overexpression of the genes *lysC* and *lysA*, encoding aspartokinase II and meso-diaminopimelate decarboxylase, respectively, resulted in L-lysine overproduction (Nærdal *et al.*, 2011). We hypothesized that coupled overexpression of these two genes together with the *ldcC* and *cadA* genes in MGA3 could result in effective cadaverine production. The recombinant strains MGA3(pTH1mp-*ldcC*-*lysC*), MGA3(pTH1mp-*ldcC*-*lysA*) and MGA3(pTH1mp-*cadA*-*lysA*) were therefore constructed. To investigate if heterologous expression of *ldcC* and *cadA* alone entails cadaverine production in MGA3, strains MGA3(pTH1mp-*ldcC*) and MGA3(pTH1mp-*cadA*) were also established. Expression of *ldcC* alone resulted in only minor cadaverine production (20 ± 4 mg/L), while coupled overexpression with endogenous *lysC* and *lysA* improved cadaverine production (140 ± 10 and 190 ± 10 mg/L), and these strains produced 10 mg/L of L-lysine (Table 2.1.4.). Interestingly, L-lysine production was in each case lower (7, 55 and 150 mg/L, respectively; Table 2.1.4.) for the three isogenic strains that do not express *ldcC*, i.e. MGA3(pHP13), MGA3(pTH1mp-*lysC*) and MGA3(pTH1mp-*lysA*), respectively, indicating that LdcC exerts a metabolic pull deregulating flux through the L-lysine biosynthesis pathway. This notion is supported by the finding that heterologous expression of *cadA* alone in MGA3 resulted in 450 ± 30 mg/L cadaverine production (Table 2.1.4.). The coupled overexpression of *cadA* with the endogenous *lysA* gene did not significantly increase cadaverine production further as 480 ± 30 mg/L was measured.

**Table 2.1.4.: Cadaverine and L-lysine production by recombinant *B. methanolicus* MGA3 strains.**

| Plasmid | Cadaverine mg/L | L-lysine mg/L |
|---|---|---|
| pHP13 | 0 | 7 ± 1[a] |
| pTH1mp-*lysC* | 0 | 55 ± 5[a] |
| pTH1mp-*lysA* | 0 | 150 ± 10[a] |
| pTH1mp-*ldcC* | 20 ± 4 | 7 ± 1 |
| pTH1mp-*ldcC*-*lysC* | 140 ± 10 | < 10 |
| pTH1mp-*ldcC*-*lysA* | 190 ± 10 | < 10 |
| pTH1mp-*cadA* | 450 ± 30 | < 10 |
| pTH1mp-*cadA*-*lysA* | 480 ± 30 | < 10 |

a. Data imported from (Nærdal *et al.*, 2011).
The production levels were found from late stationary cultures, ca. 20 hours after inoculation.

### 2.1.4.5. Fed-batch methanol cultivation of strain MGA3(pTH1mp-*cadA*) leads to the substantial volumetric production level of 11.3 g/L

We chose to investigate the promising cadaverine production strain MGA3(pTH1mp-*cadA*) during high-cell-density fed-batch methanol fermentation conditions. This strain was tested in duplicates and samples for cadaverine and amino acid analysis, cell dry weight and $OD_{600}$ were taken throughout the cultivation. Due to the significant increase in culture volume, all values were volume corrected by multiplying with the respective correction factor. We have previously cultivated strain MGA3(pHP13) at the same fed-batch conditions and reported volume corrected values as published in (Brautaset *et al.*, 2010). From these data we know that L-glutamate accumulates throughout the cultivation (59 g/L), whereas the L-lysine level remains low (0.4 g/L), and no cadaverine can be detected (Table 2.1.5.). As also observed in shake flask studies, cadaverine accumulated during the fed-batch cultivation, but during fed-batch conditions MGA3(pTH1mp-*cadA*) reached a high volumetric yield, i.e. a volume-corrected concentration of 11.3 g/L cadaverine (Table 2.1.5.). At the same time, no L-lysine could be detected. Despite of the high cadaverine production, high levels of L-glutamate and biomass were still measured indicating that the cadaverine production did not negatively affect these parameters. However, a slight reduction of the specific growth rate was observed (Table 2.1.5.). The MGA3(pTH1mp-*cadA*) production levels of L-aspartate and L-alanine were similar to previously reported values for MGA3(pHP13).

**Table 2.1.5.: Fed-batch methanol fermentation production data of strains MGA3(pTH1mp-*cadA*) and MGA3(pHP13).**

| Strain | CDW | μ[a] | Asp[b] | Glu[b] | Ala[b] | Lys[b] | Cad[b] |
|---|---|---|---|---|---|---|---|
| | g/L | $h^{-1}$ | g/L | g/L | g/L | g/L | g/L |
| MGA3(pTH1mp-*cadA*) | 65.5 | 0.45 | 1.5 | 71.8 | 10.2 | 0.0 | 11.3 |
| MGA3(pHP13) | 45.0 | 0.49 | 1.1 | 59.0 | 12.0 | 0.4 | 0.0 |

a. Specific growth rates are maximum values calculated from the exponential growth period.
b. CDW, cadaverine and amino acid concentrations are maximum values and volume corrected (see Materials & Methods section).
The maximum mean values from early stationary (CDW) or late stationary growth phase are presented for the MGA3(pTH1mp-*cadA*) duplicate cultures and the deviation never exceed ten per cent.
The MGA3(pHP13) data were imported from (Brautaset *et al.*, 2010). CDW, cell dry weight; μ, specific growth rate; Asp, L-aspartate; Glu, L-glutamate; Ala, L-alanine; Lys, L-lysine, Cad, cadaverine.

### 2.1.5. Discussion

Methanol-based cadaverine production was shown here for the first time. The tolerance level of the thermophilic methylotroph *B. methanolicus* towards the end-product cadaverine was found to be similar to that of the natural cadaverine producer *E. coli*. 200 mM cadaverine added to the growth medium resulted in reduced growth rates by *B. methanolicus* and *E. coli* by 20% and 35% respectively (Qian *et al.*, 2011). Reports using agar plate assays suggested a slightly higher cadaverine tolerance of *C. glutamicum* (Mimitsuka *et al.*, 2007). Due to its tolerance to cadaverine and its proven inability to degrade this compound, *B. methanolicus* appears to be a suitable host for the production of cadaverine. Heterologous expression of both *ldcC* and *cadA* resulted in cadaverine production in *B. methanolicus*. Cadaverine production level was higher with *cadA* than with *ldcC* in both *B. methanolicus* host strains MGA3 and M168-20. Production of L-lysine as significant by-product was observed in a *ldcC* expressing strain (40 mg/L by M168-20(pTH1mp-*ldcC*) at pH 7.2). The *in vitro* pH optima of LdcC and CadA are reported to be 7.6 (Yamamoto *et al.*, 1997; Lemonnier & Lane, 1998) and 5.7 (Moreau, 2007) respectively. The low pH optimum of CadA fits to its role in L-lysine dependent acid stress response of *E. coli* where *cadA* expression is induced at low pH and in the presence of L-lysine by the positive regulator CadC (Küper & Jung, 2005). The intracellular pH of *B. methanolicus* has not yet been experimentally tested. A slightly acidic pH of the cultivation medium reduced L-lysine production, and as consequence lower cadaverine production was observed (Table 2.1.3.). At slightly alkaline medium pH reduced L-lysine synthesis did not limit cadaverine production.

Notably, in each isogenic strain pair analyzed, cadaverine production due to heterologous L-lysine decarboxylase production was higher than L-lysine production by the respective parent strain. We propose that intracellular L-lysine concentrations are low as result of LdcC or CadA activity and that key aspartate pathway enzymes are relieved from feedback inhibition by L-lysine and/or their synthesis is relieved from repression by L-lysine. Indeed, AKII and DAP decarboxylase are known to be feedback inhibited by L-lysine (Mills & Flickinger, 1993; Jakobsen *et al.*, 2009).

Expression of *cadA* in *B. methanolicus* strains led to higher cadaverine production than expression of *ldcC* (Table 2.1.2., 2.1.3., and 2.1.4.). Two factors may explain this finding. First, *cadA* expression led to higher L-lysine decarboxylase activities in crude extracts as compared with *ldcC* expression (Table 2.1.2.). Second, CadA is reported to display a higher affinity to L-lysine than LdcC with Km values for L-lysine of 0.84 mM and 0.27 mM respectively (Krithika *et al.*, 2010).

We could demonstrate high-level cadaverine production during high-cell-density fed-batch methanol fermentation of strain MGA3(pTH1mp-*cadA*). Whereas no L-lysine accumulated during the fermentation, the volume-corrected production level of cadaverine reached 11.3 g/L after 30 h and remained stable throughout the cultivation time of 47 h. The volume-corrected concentrations of biomass (65.5 g/L) and L-glutamate (71.8 g/L) obtained for MGA3(pTH1mp-*cadA*) were slightly higher than previously reported values for MGA3(pHP13) (Table 2.1.5.). The finding that cadaverine could accumulate to higher concentrations in the fermenter than in shake flasks may in part be explained by the fact that the fermenter was pH-controlled and that the shake flask cultures acidified with time (data not shown). Moreover, higher cadaverine concentrations were tolerated by *B. methanolicus* since only minor negative effects on biomass and specific growth rate were observed upon addition of up to 35 g/L (200 mM) pH-adjusted cadaverine. It was observed that the cadaverine concentration increased throughout the growth phase until the early stationary phase, as also reported previously for *E. coli* and *C. glutamicum* (Kind *et al.*, 2011; Qian *et al.*, 2011). Due to the significant accumulation of L-glutamate in strain MGA3(pTH1mp-*cadA*) during fed-batch fermentation, there should be a great potential to increase cadaverine production further, especially by co-expression of the 2-oxoglutarate dehydrogenase (OGDH) from *B. methanolicus* recently found to reduce L-glutamate production 5-fold and increase L-lysine production twofold in *B. methanolicus* M168-20 (Krog *et al.*, 2013).

An improved understanding of both L-lysine and cadaverine secretion in *B. methanolicus* and heterologous expression of relevant known exporter or permease genes like *cadB* from *E. coli* (Li *et al.*, 2014) and *cg2893* from *C. glutamicum* (Kind *et al.*, 2011) could certainly be valuable for future high-level methanol-based cadaverine production in *B. methanolicus*.

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## 2.1.7. References

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## 2.2. Production of cadaverine by engineered *Corynebacterium glutamicum* using methanol as co-substrate

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### 2.2.1. Summary

Methanol, a $C_1$ 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 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 $^{13}$C-methanol added as co-substrate to sugars was followed and the isotopomer labeling pattern indicated incorporation into central metabolites and *in vivo* activity of the RuMP pathway. In addition, $^{13}$C-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.

### 2.2.2. Introduction

Methanol, a $C_1$ compound, is present in most ecosystems (Yang *et al.*, 2013) and is produced in large scale from natural gas as a carbon feedstock in chemical industry. Besides the industrial application as carbon source, methanol could also be used 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 $C_1$ 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 & Williams, 2003). The well studied enzymes of Gram-negative methylotrophs like *Methylobacterium extorquens* are localized in the periplasm of the cells, contain the prosthetic group pyrroloquinoline quinone (PQQ) and use cytochrome $c_L$ as electron acceptor, which is unique for methylotrophic bacteria (Cox *et al.*, 1992; Afolabi *et al.*, 2001; Williams *et al.*, 2006). 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; Hektor *et al.*, 2002; Krog *et al.*, 2013; Ochsner *et al.*, 2014).

Formaldehyde produced in the oxidation of methanol is the branching point of several different dissimilatory and assimilatory pathways. Non-methylotrophic organisms only harbor dissimilatory pathways in which formaldehyde is oxidized to $CO_2$. In contrast, assimilatory and dissimilatory pathways coexist in methylotrophic bacteria (Chistoserdova, 2011; Müller *et al.*, 2015a; Ochsner *et al.*, 2015). While assimilatory pathways give rise to biomass, the dissimilatory pathways play an important role in the regeneration of reducing equivalents and contribute to the regulation of toxic formaldehyde levels (Gutheil *et al.*, 1997; Vorholt, 2002; Müller *et al.*, 2015a).

Probably the most widespread dissimilatory pathways, particularly in non-methylotrophs, are linear pathways involving the sequential oxidation to $CO_2$ (Figure 2.2.1.). These pathways can differ in their cofactor dependence, as some enzymes oxidize formaldehyde directly (Ando *et al.*, 1979; Tsuru *et al.*, 1997) while others use a $C_1$ carrier like glutathione (Gutheil *et al.*, 1992), mycothiol (Misset-Smits *et al.*, 1997; Vogt *et al.*, 2003; Lessmeier *et al.*, 2013; Witthoff *et al.*, 2013), bacillithiol (Newton *et al.*, 2009), tetrahydrofolate (THF) (Kallen & Jencks, 1966) or tetrahydromethanopterine (Maden, 2000) before it is sequentially oxidized to $CO_2$ (Pomper *et al.*, 1999).

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 (Quayle, 1980; Anthony, 1982). 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 (Figure 2.2.1.). The fixation part is characterized by the key enzymes 3-hexulose-6-phosphate synthase (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 can be converted by enzymes of the Entner-Doudoroff pathway resulting in dihydroxyacetone 3-phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). Three molecules of formaldehyde result in one molecule pyruvate used for the production of cell constituents. DHAP, GAP, and F6P are subsequently converted in the rearrangement part to regenerate Ru5P (Strom *et al.*, 1974), which can involve transaldolase, transketolase, ribulose-5-phosphate 3-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 be used for a cyclic dissimilation of methanol to $CO_2$ (Pluschkell & Flickinger, 2002; Müller *et al.*, 2014). Like the assimilatory RuMP pathway, this cyclic dissimilatory RuMP pathway is initiated by fixation of formaldehyde by Hps and Phi. However, the carbon moiety is not converted into biomass, since Ru5P

is regenerated by reactions of the oxidative part of the PPP with concomitant release of $CO_2$ and gain of energy (Chistoserdova *et al.*, 2000) (Anthony, 1982). This pathway is not restricted to methylotrophic organisms using the assimilatory RuMP pathway (Reizer *et al.*, 1997). Homologues 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), where the cyclic dissimilatory RuMP pathway plays an important role in the detoxification of formaldehyde (Kato *et al.*, 2006).

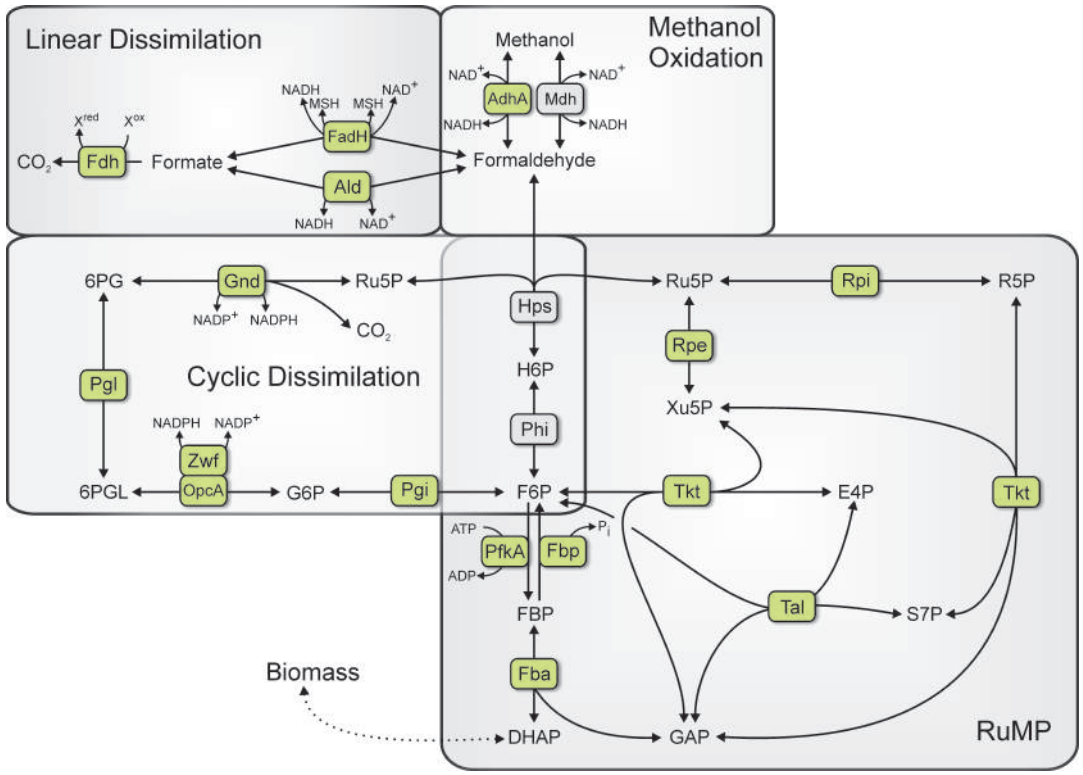


**Figure 2.2.1.: Overview of methanol oxidation and linear formaldehyde dissimilation in *C. glutamicum* and the synthetic RuMP pathway for formaldehyde assimilation as well as cyclic dissimilation.** Enzymes highlighted in green are present in *C. glutamicum*, heterologous enzymes are depicted in grey.

*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, 6-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.

The Gram-positive *Corynebacterium glutamicum* belongs to the mycolic acid-containing actinomycetes and is particularly known for its use in the million-ton-scale production of the amino acids L-glutamate and L-lysine (Peters-Wendisch *et al.*, 2014; Eggeling & Bott, 2015). *C. glutamicum* has been engineered 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* has also been engineered for access to alternative carbon sources (Wendisch, 2014; Leßmeier *et al.*, 2015). *C. glutamicum* is a non-methylotrophic organism and not able to utilize methanol as a carbon source, but it oxidizes methanol to $CO_2$ by means of linear dissimilation. The oxidation of methanol to formaldehyde is mainly performed by an alcohol dehydrogenase encoded by *adhA*, but at least one additional enzyme of hitherto unknown identity is also involved (Witthoff *et al.*, 2013). Formaldehyde can be oxidized to formate by two distinct enzymes: the acetaldehyde dehydrogenase Ald and the mycothiol-dependent formaldehyde dehydrogenase FadH (Lessmeier *et al.*, 2013; Witthoff *et al.*, 2013). Oxidation of formate to $CO_2$ is catalyzed by the formate dehydrogenase FdhF (encoded by 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.

### 2.2.3. Materials & Methods

#### 2.2.3.1. Microorganisms and cultivation conditions

Strains and plasmids used in this study are listed in the Appendix (Table A.1.1.). The *E. coli* strain DH5α 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 $\mu g\ ml^{-1}$); pVWEx1: kanamycin (25 $\mu g\ ml^{-1}$)). For induction of vector-based gene expression, the medium was supplemented with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG).

#### 2.2.3.2. DNA preparation, manipulation, and transformation

Plasmid isolation, molecular cloning, and transformation of *E. coli*, as well as electrophoresis, were performed using standard procedures (Sambrook & Russell, 2001). Electroporation was used for transformation of *C. glutamicum* as described previously (Tauch *et al.*, 2002). Polymerase chain reaction (PCR) experiments were performed using GoTaq DNA polymerase (Promega) or KOD Hot start polymerase (Novagen) with oligonucleotides obtained from Metabion (listed in Table A.1.2.). All restriction enzymes and phosphonucleokinase were obtained from Fermentas and used according to the manufacturer's instructions. The Rapid DNA Dephos & 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).

### 2.2.3.3. 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 A.1.2. 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 A.1.1. were controlled by DNA sequencing.

### 2.2.3.4. 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 $MgSO_4$, 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).

### 2.2.3.5. 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 Ru5P 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 Ru5P 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 $MgCl_2$, 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 Ru5P, the mixture was preincubated at 30 °C for 6 min before the reaction was started by addition of 5 mM formaldehyde.

### 2.2.3.6. Measurement of formaldehyde concentrations

A colorimetric method was used for the measurement of formaldehyde concentrations as described previously (Nash, 1953; Lessmeier *et al.*, 2013). *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 were inoculated to an $OD_{600}$ 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 were supplemented with 100 mM ribose and 1 mM IPTG. The medium was inoculated to an $OD_{600}$ of 1 from a culture cultivated in LB medium with 1 mM IPTG. The assay was started by addition of 0.5 mM formaldehyde.

### 2.2.3.7. Determination of $^{13}$C-labeled intracellular metabolites

The assimilation of methanol in recombinant *C. glutamicum* strains was monitored in a kinetic $^{13}$C-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 $^{13}$C-methanol (99% $^{13}$C-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.* (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 $^{13}$C-enrichment error using this methodology are 0.5% and 1.5% respectively (Millard *et al.*, 2012).

### 2.2.3.8. Determination of $^{13}$C-labeled cadaverine

*C. glutamicum* ΔΔ(pEKEx3-*mdh,hxlAB*)(pVWEx1-*lysC*$^{fbr}$-*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 $^{13}$C-methanol (99%, Sigma-Aldrich, Germany). Supernatants were taken after 30 h, 56 h and 100 h and analyzed on a Bruker Ascend 800MHz and Avance II 500MHz magnets. Concentration of cadaverine in each sample was measured using 1D $^{1}$H-NMR experiment and $^{13}$C-decoupled DANTE-Z pulse was applied to measure $^{13}$C-enrichments (Nicolas *et al.*, 2008).

## 2.2.4. Results

### 2.2.4.1. 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 aldehyde dehydrogenase Ald (Arndt *et al.*, 2008) and mycothiol-dependent formaldehyde dehydrogenase FadH (Lessmeier *et al.*, 2013; Witthoff *et al.*, 2013) were deleted. *C. glutamicum* oxidizes methanol to formaldehyde by alcohol dehydrogenase AdhA (Arndt & 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 pyrroloquinoline quinone (PQQ) (Shen *et al.*, 2012) or cytochrome $c_L$ (Williams *et al.*, 2006). Therefore, the three NAD$^+$-dependent methanol dehydrogenases from the thermotolerant methylotroph *Bacillus 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 (Figure 2.2.1.).

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 homologues *hxlA* and *hxlB* from the mesophilic *B. subtilis* (Yasueda *et al.*, 1999) were also tested.

### 2.2.4.2. 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 2.2.1.). 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 comparable activities in *C. glutamicum* (Table 2.2.1.). Co-expression of two methanol dehydrogenase genes led to lower *in vitro* activities (Table 2.2.1.). 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 2.2.1.). 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 2.2.1.). However, Mdh2_S97G and Mdh3_S97G revealed no detectable methanol dehydrogenase activity.

**Table 2.2.1.: *In vitro* and *in vivo* activities of methanol dehydrogenases heterologously expressed in *C. 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. |

n. a.: Data not available

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 Δ*ald*Δ*fadH*(pEKEx3) using the vector pVWEx1 for heterologous expression. Means and experimental imprecision of two independent cultures are shown.

Methanol oxidation *in vivo* was assayed by monitoring formaldehyde accumulation in the supernatant. The control strain Δ*ald*Δ*fadH*(pEKEx3)(pVWEx1) showed no significant accumulation of formaldehyde. High methanol oxidation to formaldehyde was observed in strains derived from *C. glutamicum* Δ*ald*Δ*fadH* overproducing Mdh (9.0 ± 0.5 nmol $min^{-1}$ mg $CDW^{-1}$) and to lesser extents overproducing Mdh2 (1.3 ± 0.0 nmol $min^{-1}$ mg $CDW^{-1}$) or Mdh3 (0.8 ± 0.1 nmol $min^{-1}$ mg $CDW^{-1}$) (Figure 2.2.2). Additional expression of *act*, cg1607 or introduction of the S97G mutation

into methanol dehydrogenases did not increase formaldehyde accumulation *in vivo* although *in vitro* activities were higher (Table 2.2.1.). Taken together, heterologous expression of *mdh* from *B. methanolicus* in *C. glutamicum* Δ*ald*Δ*fadH* showed the highest methanol oxidation activities *in vitro*, and thus, was chosen for further experiments.

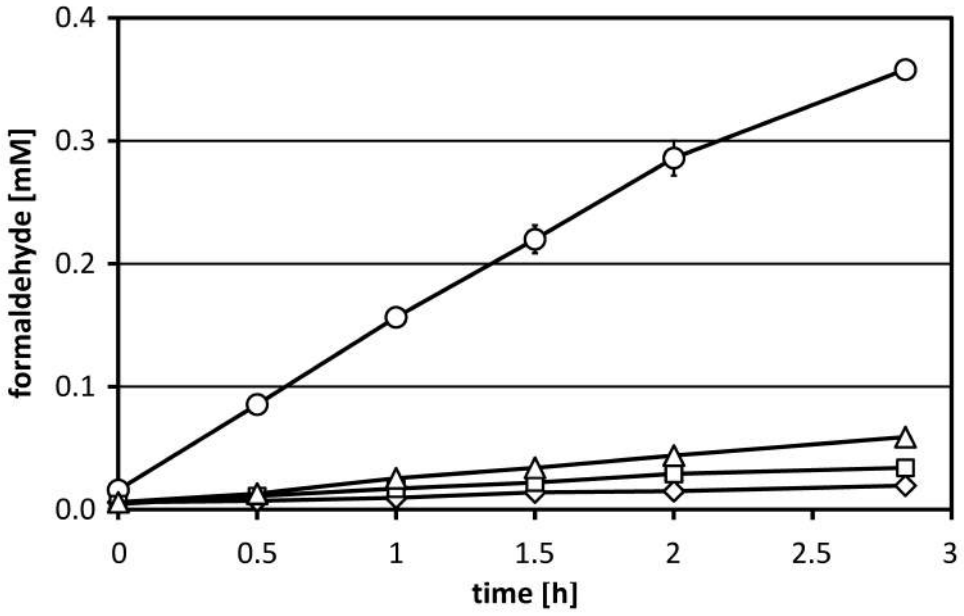


**Figure 2.2.2.: *In vivo* activities of methanol dehydrogenases in *C. glutamicum* strains expressing heterologous *mdh* genes.** An *in vivo* assay was used to measure methanol oxidation via the accumulation of formaldehyde from 240 mM methanol. *C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3) strains were used 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.

### 2.2.4.3. 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* Δ*ald*Δ*fadH*. The combined enzyme activity of Hps and Phi was analyzed *in vitro* in crude extracts. While *C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3) lacked detectable Hps and Phi activity (< 0.005 U/mg), activities of 0.27 ± 0.02 U/mg and 0.53 ± 0.12 U/mg, respectively, were observed in crude extracts of *C. glutamicum* Δ*ald*Δ*fadH* expressing *hps* and *phi* from *B. methanolicus* or *hlxA* and *hxlB* 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 Ru5P 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. While the control strain showed no significant formaldehyde degradation (< 2 nmol $min^{-1}$ mg $CDW^{-1}$), the strain expressing *hps* and *phi* degraded formaldehyde with a rate of 9.0 ± 0.2 nmol $min^{-1}$ mg $CDW^{-1}$, which is about 25% lower as compared to expression of *B. subtilis hxlA* and *hxlB* (12 ± 0.5 nmol $min^{-1}$ mg $CDW^{-1}$) (Figure 2.2.3.). Since HxlA and HxlB from *B. subtilis* showed the highest activities *in vitro* as well as *in vivo*, these enzymes were used for further experiments.

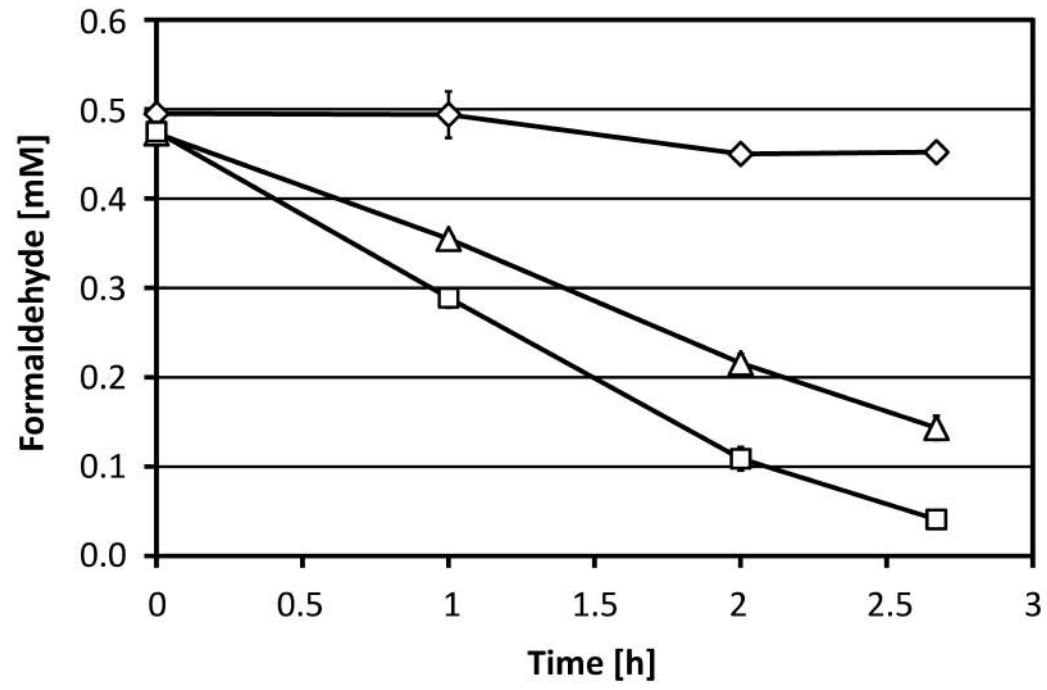


**Figure 2.2.3.: Formaldehyde degradation by *C. glutamicum* strains expressing heterologous RuMP genes.** *In vivo* formaldehyde degradation assay of *C. glutamicum* Δ*ald*Δ*fadH* carrying the empty vector pEKEx3 (diamonds), pEKEx3-*hps,phi* (triangles) or pEKEx3-*hxlAB* (squares). Means and experimental imprecision of two independent cultures are shown.

### 2.2.4.4. 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 Δ*ald*Δ*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 *hxlAB* allowed growth with a growth rate of 0.18 ± 0.00 $h^{-1}$ (Figure 2.2.4.A).

Growth experiments with different methanol concentrations showed that the strain expressing *hxlA* and *hxlB* was able to grow in the presence of higher methanol concentrations than the control strain (Figure 2.2.4.B). Accumulation of formaldehyde was monitored for strains expressing *mdh* and/or *hxlAB* cultivated in the presence of methanol (Figure 2.2.5.A). The empty vector carrying control strain accumulated 0.34 ± 0.01 mM formaldehyde within 6 hours of cultivation, whereas expression of *hxlA* and *hxlB* resulted in growth with a growth rate of 0.21 ± 0.00 $h^{-1}$ and no significant formaldehyde accumulation (< 0.06 mM) could be observed irrespective if in addition *mdh* was expressed (Figure 2.2.5.B). Taken together, heterologous expression of *hxlA* and *hxlB* allowed growth in the presence of formaldehyde and improved tolerance to methanol by reducing formaldehyde accumulation.

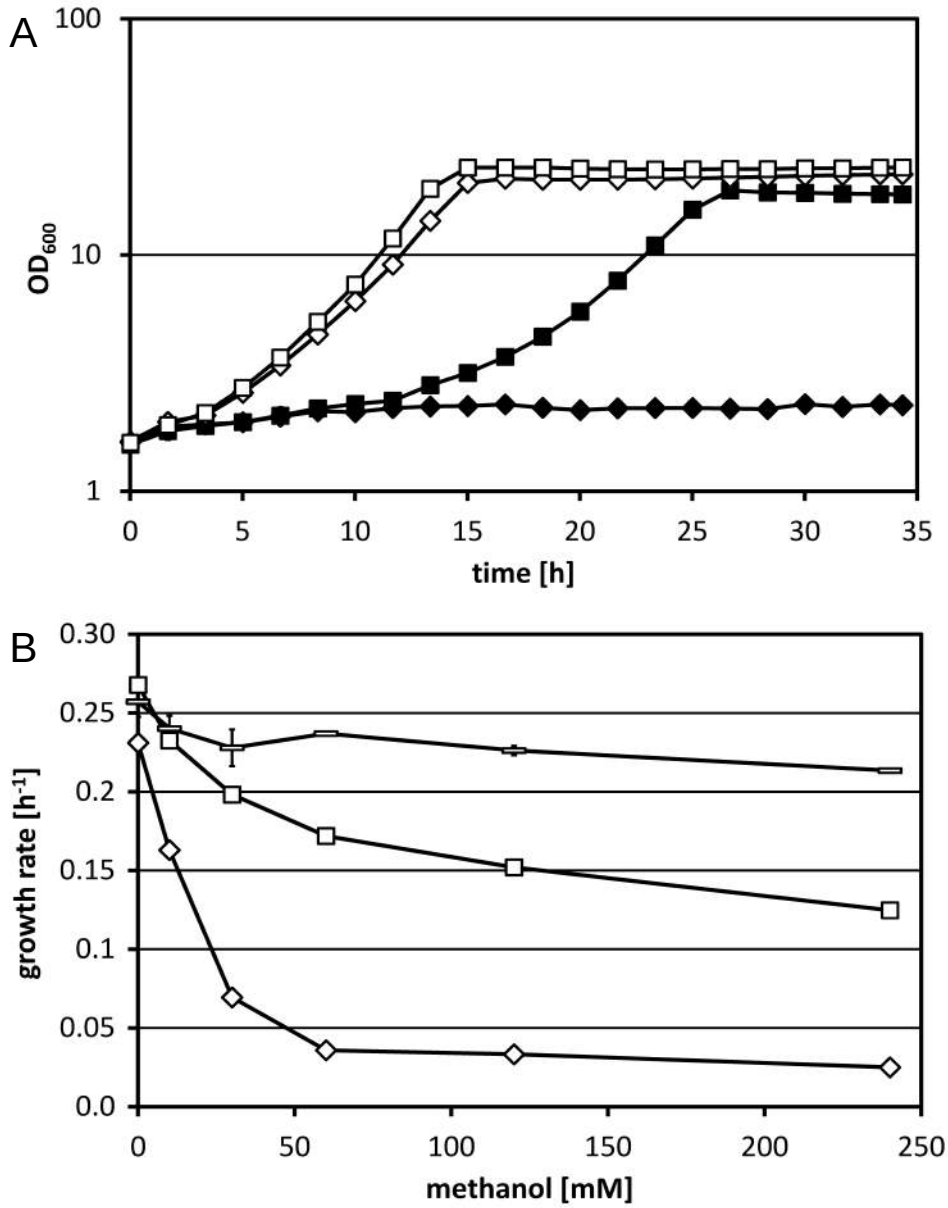


**Figure 2.2.4.: Growth of recombinant *C. glutamicum* strains in the presence of formaldehyde (A) or methanol (B).** (A) Growth of *C. glutamicum* strains Δ*ald*Δ*fadH*(pEKEx3) (diamonds) and Δ*ald*Δ*fadH*(pEKEx3-*hxlAB*) (squares) cultivated in minimal medium with 100 mM ribose and no (open symbols) or 5 µM formaldehyde (closed symbols). (B) Dependence of the growth rate of *C. glutamicum* wild type (bars) and strains Δ*ald*Δ*fadH*(pEKEx3) (diamonds) and Δ*ald*Δ*fadH*(pEKEx3-*hxlAB*) (squares) on methanol added to minimal medium with 100 mM ribose. Means and experimental imprecision of two independent cultures are shown.

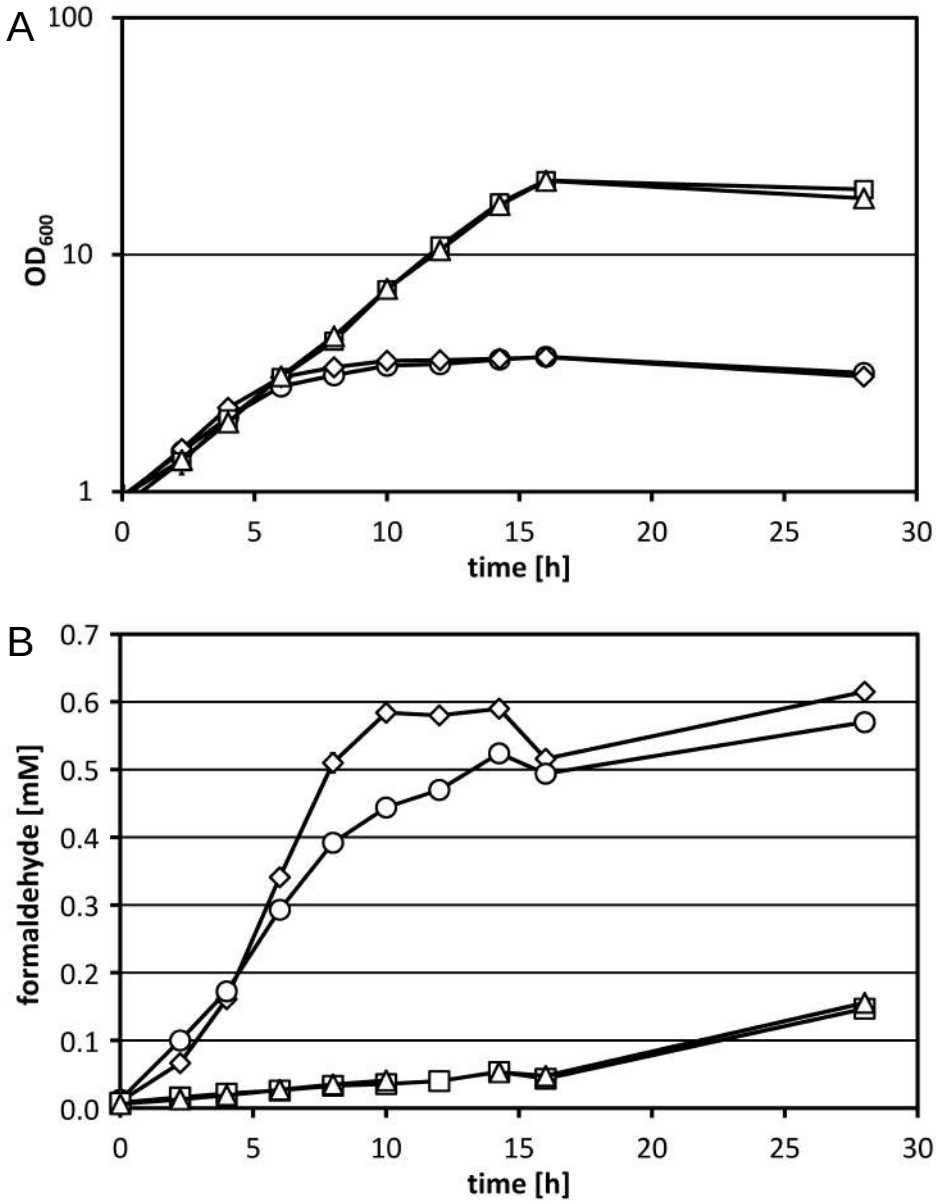


**Figure 2.2.5.: Growth (A) and formaldehyde accumulation (B) by *C. glutamicum* strains in the presence of methanol.** (A) Growth of and (B) formaldehyde concentrations in the supernatants of *C. glutamicum* strains Δ*ald*Δ*fadH*(pEKEx3) (diamonds), Δ*ald*Δ*fadH*(pEKEx3-*mdh*) (circles), Δ*ald*Δ*fadH* (pEKEx3-*hxlAB*) (squares) and Δ*ald*Δ*fadH*(pEKEx3-*mdh*,*hxlAB*) (triangles) on minimal medium with 100 mM ribose and 30 mM methanol. Means and experimental imprecision of two independent cultures are shown.

### 2.2.4.5. $^{13}$C-labeling patterns of metabolic intermediates in recombinant *C. glutamicum* grown in the presence of $^{13}$C-methanol

In order to analyze how methanol is converted by recombinant *C. glutamicum,* labeling experiments with $^{13}$C-methanol were performed. Strains *C. glutamicum*(pEKEx3), Δ*ald*Δ*fadH*(pEKEx3), Δ*ald*Δ*fadH*(pEKEx3-*hxlAB*) and Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*) were labeled in M9 low salt medium with $^{13}$C-methanol as sole carbon source as described in the Materials & Methods section. 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 (pEKEx3) and Δ*ald*Δ*fadH*(pEKEx3) showed low or no labeling of glucose 6-phosphate (G6P), respectively, while significant $^{13}$C incorporation was observed for the strains expressing *hxlAB* and *mdh* as well as *hxlAB* (Figure 2.2.6.). The labeling kinetics are in good accordance with the theoretical carbon flow via the RuMP cycle as the hexoses G6P and fructose 6-phosphate (F6P) were labeled before the trioses 2/3-phosphoglycerate (PG) and phosphoenolpyruvate (PEP) (Figure 2.2.6.).

Observation of the M2 and M3 isotopomers 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. For F6P up to 40% of M1 and 45% of M2 were found showing a high incorporation of carbon from methanol into the hexoses (Figure 2.2.7.). These results show that assimilation of methanol in *C. glutamicum* is functional by the heterologous expression of *hxlA* and *hxlB*. Methanol oxidation to formaldehyde by endogenous AdhA is optimized by expression of heterologous *mdh*.

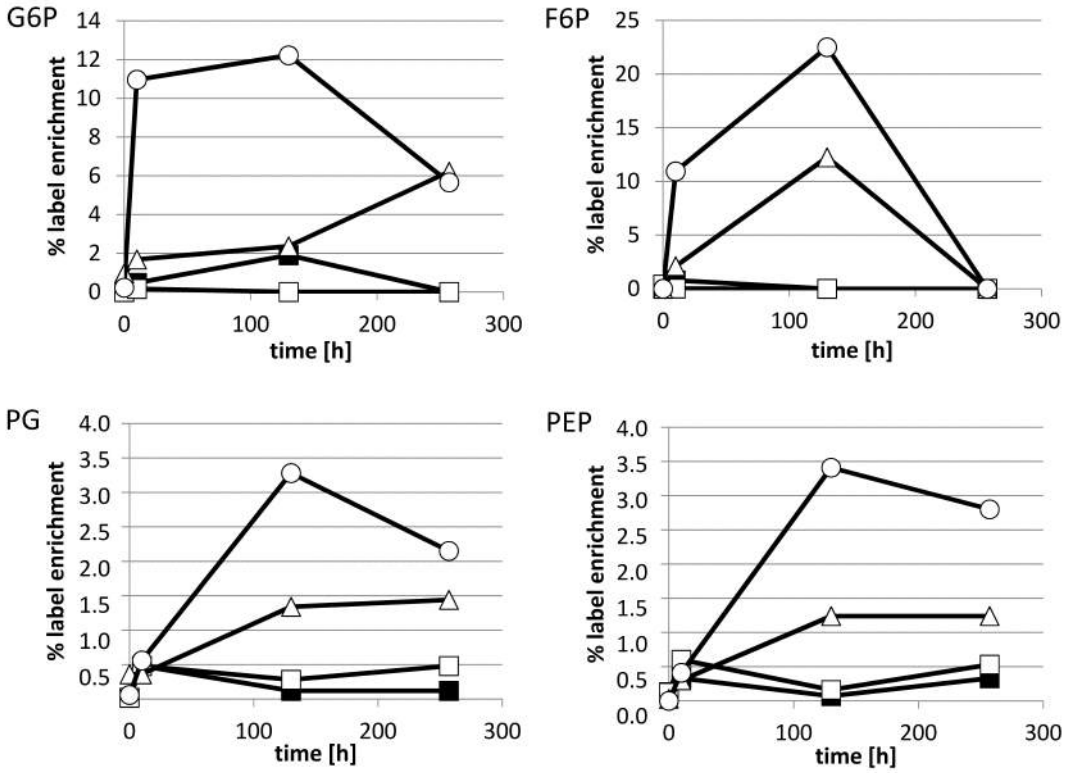


**Figure 2.2.6.: Label enrichment in different metabolites from $^{13}$C-methanol.** The *C. glutamicum* strains WT(pEKEx3) (closed squares), Δ*ald*Δ*fadH*(pEKEx3) (open squares), Δ*ald*Δ*fadH*(pEKEx3-*hxlAB*) (open triangles) and Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*) (open circles) were cultivated in M9 minimal medium with $^{13}$C-methanol as sole carbon source. G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; PG: combination of 2-phosphoglycerate and 3-phosphoglycerate; PEP: phosphoenolpyruvate.

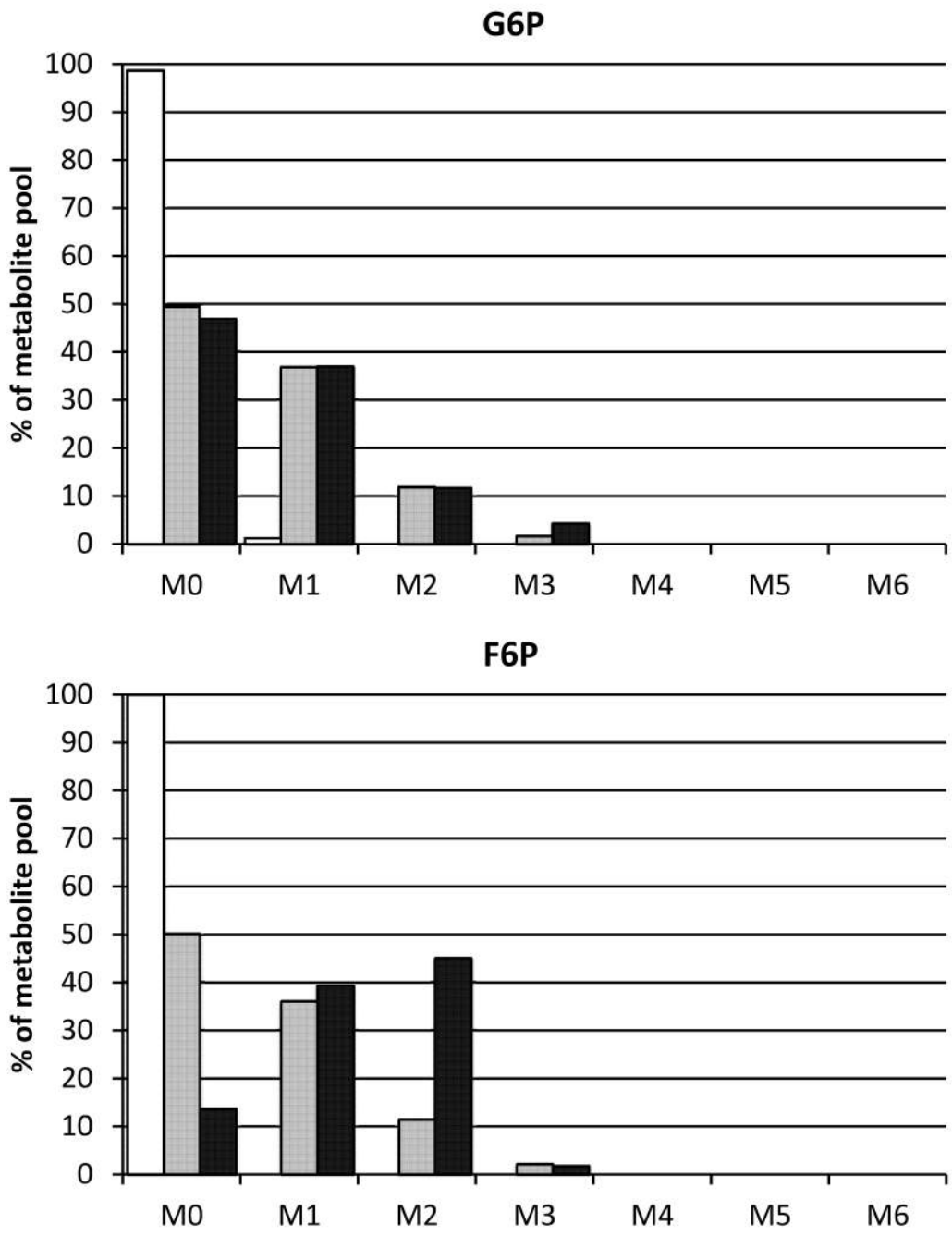


**Figure 2.2.7.: Mass isotopolog distribution of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) from $^{13}$C-methanol.** *C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*) was cultivated in M9 minimal medium with $^{13}$C-methanol as sole carbon source. Mass isotopolog distribution after 0 minutes (white), 10 minutes (grey) and 130 minutes (black) is shown.

### 2.2.4.6. $^{13}$C-labeling patterns of the product cadaverine in recombinant *C. glutamicum* grown in the presence of $^{13}$C-methanol

*C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*) was genetically engineered for the production of cadaverine by expression of genes for endogenous, feedback-resistant aspartokinase (*lysC*$^{fbr}$) and lysine decarboxylase LdcC from *E. coli*. The strain *C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*)(pVWEx1-*lysC*$^{fbr}$-*ldcC*) produced up to 1.5 g/L of cadaverine in shake flask cultivation. After growing this strain in CgXII minimal medium containing 20 mM glucose or 20 mM ribose and 200 mM $^{13}$C-methanol, the supernatant was analyzed with respect to cadaverine labeling patterns by NMR. The symmetric molecule cadaverine showed the highest $^{13}$C-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 2.2.2.). Notably, position C3 only revealed the natural $^{13}$C abundance. With glucose used in the pre-culture, the labeling was significantly lower with about 5% labeled carbon at positions C2 and C4. Only natural $^{13}$C abundance was observed at positions C1, C3 and C5.

**Table 2.2.2.: Production of $^{13}$C-labeled cadaverine from $^{13}$C-methanol.** Production was performed by strain *C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*)(pVWEx1-*lysC*$^{fbr}$-*ldcC*) in CGXII medium with 20 mM glucose or ribose and 200 mM $^{13}$C-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 |

Labeling of cadaverine at positions C2 and C4 indicates that $^{13}$C-methanol was converted to pyruvate labeled at position C3 in the reactions of the cyclic RuMP and onwards to cadaverine (Figure 2.2.8.A). Labeling from $^{13}CO_2$, 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 (Figure 2.2.8.B). Thus, labeling of cadaverine at positions C1/C5 and C2/C4, but not at position C3 indicates that $^{13}$C-methanol was converted to cadaverine via the RuMP pathway by the engineered *C. glutamicum* strain.

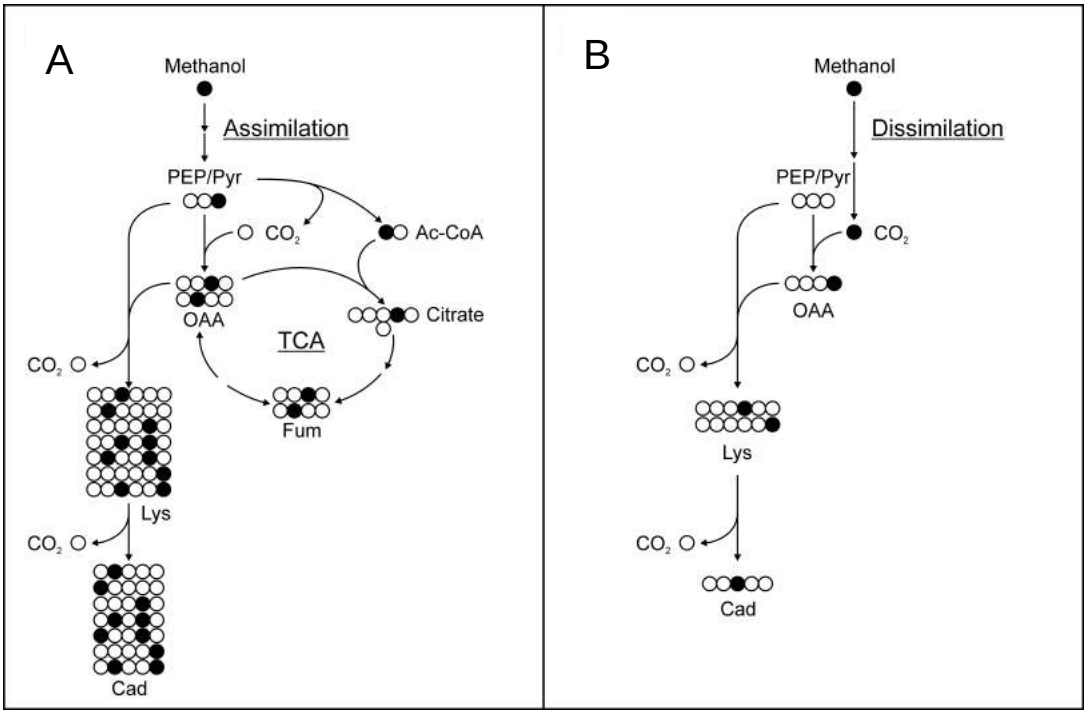


**Figure 2.2.8.: Transition of carbon from $^{13}$C-methanol to cadaverine.** Label incorporation into cadaverine and metabolites when methanol is assimilated in the RuMP pathway (A) or is oxidized to $^{13}CO_2$ and fixed as $CO_2$ by anaplerotic carboxylases (B). PEP, phosphoenolpyruvate; Pyr, pyruvate; Ac-CoA, acetyl-Coenzyme A; Fum, fumarate; OAA, oxaloacetate; Lys, lysine; Cad, cadaverine.

### 2.2.5. 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. The strategy for methanol metabolization has been followed in *Pseudomonas putida* (Koopman *et al.*, 2009), *Escherichia 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*, $^{13}$C-labeling from $^{13}$C-methanol was observed in metabolic intermediates, but not in a secreted product (Witthoff *et al.*, 2015). To the best of our knowledge, conversion of $^{13}$C-methanol as non-native substrate and secretion of a $^{13}$C-labeled, non-native product by a non-methylotrophic host has not been reported previously.

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*. 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 2.2.1. and (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 2.2.1.).

Hps and Phi from *B. methanolicus* and 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 an order of magnitude (Witthoff *et al.*, 2015).

In comparison to the *in vitro* Hps and Phi activities, the *in vivo* rates of formaldehyde degradation in *E. coli* (Müller *et al.*, 2015b) and *C. glutamicum* (Figure 2.2.3. and Table 2.2.1.) 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). However, Hps and Phi activities due to expression of *hxlA* and *hxlB* were sufficient to partially restore formaldehyde and methanol detoxification in *C. glutamicum* Δ*ald*Δ*fadH*, which lacks the endogenous formaldehyde oxidation pathway (Figure 2.2.4. and 2.2.5.). This suggested that the endogenous linear formaldehyde dissimilation pathway could be replaced by the cyclic RuMP pathway. Expression of heterologous *mdh* was not required since methanol is oxidized to formaldehyde by endogenous AdhA, but it improved methanol conversion.

$^{13}$C-labeling of central metabolites such as sugar phosphates of up to about 25% label enrichment was observed here using M9 minimal medium with 60 mM $^{13}$C-methanol (Figure 2.2.6.) as well as in CGXII minimal medium with 55 mM glucose and 120 mM $^{13}$C-labeled methanol (Witthoff *et al.*, 2015). As shown here, up to 40% of F6P were M1 and 45% M2 indicating a high incorporation of carbon from methanol into the hexoses (Figure 2.2.7.). Most importantly, M2 and M3 labeled hexoses were detected here for the first time (Figure 2.2.7.), which indicated repeated cyclic regeneration of Ru5P involving RuMP key enzymes Hps and Phi.

Cadaverine secreted as metabolic end-product by *C. glutamicum* strain Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*)(pVWEx1-*lysC*$^{fbr}$-*ldcC*) showed $^{13}$C-labeling at positions C1/C5 and C2/C4, but not C3, after adding $^{13}$C-methanol to cultures pre-grown either in glucose or ribose medium. This $^{13}$C-labeling pattern arose by methanol assimilation via the RuMP pathway and was not due to re-assimilation of $^{13}CO_2$ formed by linear or cyclic oxidation of $^{13}$C-methanol (Figure 2.2.8.). Cadaverine is a non-native metabolic end-product of *C. glutamicum* occurring only as 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 & 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 (Tateno *et al.*, 2009; Buschke *et al.*, 2011; Buschke *et al.*, 2013).

Our report on engineering non-methylotroph *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.

### 2.2.6. Acknowledgements

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## 2.3. Characterization of transaldolase from *Bacillus methanolicus*

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### 2.3.1. Summary

The Gram-positive facultative methylotrophic bacterium *Bacillus methanolicus* uses the ribulose monophosphate (RuMP) pathway for the growth on the $C_1$ carbon source methanol. The enzymes required for this pathway are encoded by genes located on the natural plasmid pBM19 and the chromosome. Several of the RuMP pathway enzymes of *B. methanolicus* MGA3 have been studied and characterized so far. In this study, the focus was on the characterization of transaldolase and its possible role in the RuMP pathway. Transaldolase of *B. methanolicus* PB1 was purified from recombinant *Escherichia coli* and characterized. It was found to be active as a homodimer with a molecular weight of 54 kDa and it showed kinetic parameters for $K_M$ of 0.74 mM and for $V_{max}$ of 16.33 U/mg. Since it has been shown before that *B. methanolicus* MGA3 uses the SBPase variant of the RuMP pathway, the finding of an active transaldolase in the genome of *B. methanolicus* indicates that this organism might use both variants in parallel, maybe preferring one. Further, it could be shown in this study that the annotated transaldolase coding sequence of *B. methanolicus* MGA3 is not translated into an active protein. The finding of an alternative start codon within the reading frame of the annotated stop codon was identified (*ta*$^{MGA3put}$). It could be shown by an amino acid sequence alignment with transaldolases from other bacilli that the annotated version lacks about 80 N-terminal amino acids present in all other transaldolases as well as in the alternative transaldolase sequence. Although the third codon of *ta*$^{MGA3put}$ would be a TAA stop codon, additional data from RNA-sequencing and proteome data indicated that this sequence encodes a protein responsible for transaldolase activity in *B. methanolicus* MGA3.

### 2.3.2. Introduction

Methylotrophic bacteria that use the ribulose monophosphate (RuMP) pathway for the fixation of formaldehyde consist of three groups: Gram-negative obligate methylotrophs, Gram-positive facultative methylotrophs, and thermotolerant *Bacillus* spp. (Anthony, 1982; Arfman *et al.*, 1992a).

*Bacillus methanolicus* MGA3 is a facultative methylotroph, which belongs to the third group. It utilizes methanol as its sole carbon and energy source and assimilates formaldehyde via the RuMP cycle (Kemp & Quayle, 1967; Schendel *et al.*, 1990). In *B. methanolicus*, methanol dehydrogenase (Mdh) catalyzes the initial step of methanol utilization, the oxidation to formaldehyde (Dijkhuizen *et al.*, 1992; Arfman *et al.*, 1997; Hektor *et al.*, 2000). Recently, it has been shown that this bacterium has three genes, all encoding active Mdhs (Heggeset *et al.*, 2012; Krog *et al.*, 2013).

The RuMP pathway can be divided into three parts: fixation, cleavage, and rearrangement. The fixation part includes aldol condensation of formaldehyde with ribulose 5-phosphate (Ru5P) by 3-hexulose-6-phosphate synthase (Hps) and the subsequent isomerization of the product, hexulose 6-phosphate (H6P), to fructose 6-phosphate (F6P), by 6-phospho-3-hexuloisomerase (Phi). While Mdh, Hps, and Phi are specific for the RuMP cycle, the other enzymes also occur in other pathways, for example, glycolysis, pentose phosphate pathway, and Calvin cycle. In the cleavage part of the RuMP cycle fructose 1,6-bisphosphate (FBP), generated from F6P by 6-phosphofructokinase (Pfk), is cleaved into the triosephosphates glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by fructose-1,6-bisphosphate aldolase (Fba). *B. methanolicus* possesses one chromosomally and one plasmid encoded Fba ($Fba^P$ and $Fba^C$ respectively), which catalyze opposite reactions, since $Fba^C$ is the major glycolytic Fba, whereas $Fba^P$ catalyzes the aldol condensation in gluconeogenesis (Stolzenberger *et al.*, 2013b). *B. methanolicus* encodes enzymes for both variants of the rearrangement part of the RuMP pathway for the conversion of the triosephosphates and F6P to Ru5P: the sedoheptulose-1,7-bisphosphatase (SBPase) variant and the transaldolase (Ta) variant. In the SBPase variant, sedoheptulose 7-phosphate (S7P) is generated in two subsequent reactions. The condensation of erythrose 4-phosphate (E4P) and DHAP to sedoheptulose 1,7-bisphosphate (SBP) is catalyzed by either $Fba^P$ or $Fba^C$, which thus are also sedoheptulose-1,7-bisphosphate aldolases (Sbas) (Stolzenberger *et al.*, 2013a). Afterwards, SBPase dephosphorylates SBP to yield S7P. Whereas $GlpX^C$ and $GlpX^P$ are

both active as bisphosphatases with FBP, only the plasmid encoded $GlpX^P$ showed activity as SBPase (Stolzenberger *et al.*, 2013a). Both variants of the RuMP cycle involve transketolases (Markert *et al.*, 2014), ribose-5-phosphate isomerase (Rpi), and ribulose-phosphate 3-epimerases (Rpe). In the Ta variant, S7P is generated by direct conversion of E4P and F6P to GAP and S7P.

With the exception of Ta and Rpi that are only present on the chromosome, the enzymes of the rearrangement part are encoded either on the naturally occurring plasmid pBM19 or on the chromosome (Brautaset *et al.*, 2004; Heggeset *et al.*, 2012). However, the proof of an active transaldolase in *B. methanolicus* and hence an additional active regeneration route to the SBPase variant was part of this study.

Transaldolase is an ubiquitous enzyme catalyzing the transfer of a dihydroxyacetone (DHA) moiety from a ketose donor onto an aldehyde acceptor. The enzyme requires no known cofactors and performs a base-catalyzed aldol cleavage reaction, in which a Schiff base intermediate is formed. Tas have been purified and characterized from various sources, such as bacteria (Sprenger *et al.*, 1995), archaea (Soderberg & Alver, 2004), yeasts (Fiki *et al.*, 2007), fungi (Kourtoglou *et al.*, 2008), plants (Moehs *et al.*, 1996), and mammals, including humans (Banki *et al.*, 1994). The Ta of *Methanocaldococcus jannaschii* is the only thermostable Ta characterized to date (Soderberg & Alver, 2004). Based on the phylogeny of Ta, five subfamilies can be distinguished (Samland & Sprenger, 2009). Subfamily 1 is considered as the classical Ta, which occurs in all domains of life, for example, TalB of *E. coli* (Sprenger *et al.*, 1995), but not in plants. Subfamily 2 and subfamily 3 Tas occur in plants and in the case of subfamily 3 also in bacteria (Samland *et al.*, 2012). Subfamily 4 comprises small Tas from bacteria and archaea. To the best of our knowledge, biochemical evidence for Ta enzyme activity has not been demonstrated yet for enzymes belonging to either subfamily 2 or 5 (Samland *et al.*, 2009; Caillau & Quick, 2005). Notably, enzymes of subfamily 5 are active as F6P aldolases as demonstrated for respective enzymes of *E. coli* (Schürmann & Sprenger, 2001). However, certain organisms, like *Entamoeba histolytica* (Susskind *et al.*, 1982), *Zymomobas mobilis* (Feldmann *et al.*, 1992), and certain mammalian tissues do not express Ta (Grossman *et al.*, 2004) and the non-oxidative branch can function without this enzyme (McIntyre *et al.*, 1989; Perl, 2007).

Since it is unknown whether *B. methanolicus* possesses an active transaldolase, the proteins encoded by the putative subfamily 4 Ta gene of *B. methanolicus* strains MGA3 and PB1 were investigated in this study. Ta of PB1 was produced with a C-terminal His-tag in recombinant *E. coli*, purified and tested for activity. The aim of this study was on the one hand to characterize Ta of *B. methanolicus* PB1 to extend our knowledge of methylotrophy and the RuMP pathway in this organism, and on the other hand to identify the coding sequence of Ta in *B. methanolicus* MGA3, as the annotated coding sequence revealed no Ta activity.

### 2.3.3. Materials & Methods

#### 2.3.3.1. Microorganisms and cultivation conditions

*Bacillus methanolicus* strains were grown at 50 °C in the following media. SOBsuc medium is SOB medium (Difco) supplemented with 0.25 M sucrose. Methanol growth of *B. methanolicus* was performed in $MeOH_{200}$ medium, containing salt buffer, 1 mM $MgSO_4$, vitamins, trace metals, 0.025% yeast extract, and 200 mM methanol (Schendel *et al.*, 1990; Jakobsen *et al.*, 2006). The medium pH was adjusted to 7.2 unless stated otherwise. Bacterial growth was performed in shake flasks (500 ml) in 100 ml medium at 200 rpm and monitored by measuring the optical density at 600 nm ($OD_{600}$). Chloramphenicol (5 µg/ml) was added to the medium when appropriate. The inoculation of the precultures for all growth experiments with *B. methanolicus* strains was performed with frozen ampoules of *B. methanolicus* as a starter culture. Ampoules of *B. methanolicus* cells were prepared from exponentially growing cultures ($OD_{600}$ of 1.0 to 1.5) and stored at -80 °C in 15% (vol/vol) glycerol. Transformation of this bacterium was performed by electroporation as described previously (Jakobsen *et al.*, 2006). The *Escherichia coli* strain DH5α was used as a standard cloning host. Recombinant cells were grown in lysogeny broth (LB) medium at 37 °C supplemented with kanamycin (25 µg/ml), spectinomycin (100 µg/ml), chloramphenicol (15 µg/ml), and 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) when appropriate and standard recombinant DNA procedures were performed as described elsewhere (Sambrook & Russell, 2001). *Corynebacterium glutamicum* cells were grown in lysogeny broth (LB) medium or CgXII minimal medium (Keilhauer *et al.*, 1993) and incubated at 30 °C. Recombinant protein production was carried out using *E. coli* BL21 (DE3) as host. Bacterial strains and plasmids used in this work are listed in table 2.3.1. and oligonucleotides for polymerase chain reaction (PCR) and cloning are listed in table 2.3.2.

**Table 2.3.1.: List of bacterial strains and plasmids used in this study.**

| Strain, plasmid | Function and relevant characteristics | Reference |
|---|---|---|
| *B. methanolicus* strains | | |
| MGA3 | Wild type strain | (Schendel *et al.*, 1990) |
| PB1 | Wild type strain | (Schendel *et al.*, 1990) |
| *E. coli* strains | | |
| DH5α | $F^-$ *thi-1 endA1 hsdR17(*$r^-$ $m^-$*) supE44* ΔlacU169 (80lacZΔM15) *recA1 gyrA96 relA1* | Bethesda Research Laboratories |
| BL21 | *ompT hsdSB(*$rB^-$ $mB^-$*) gal dcm* (DE3) | Novagen |
| *C. glutamicum* strains | | |
| ATCC 13032 | WT strain, auxotrophic for biotin | (Abe *et al.*, 1967) |
| *Δtal* | In-frame deletion of the *tal* gene of WT | This study |
| Plasmids | | |
| pVWEx1 | $Kan^R$; *C. glutamicum-E. coli* shuttle vector ($P_{tac}$ $lacI^q$ $oriV_{C.g}$ $oriV_{E.c}$) | (Peters-Wendisch *et al.*, 2001) |
| pVWEx1-$ta^{PB1}$ | derived from pVWEx1, for regulated expression of *ta* of *B. methanolicus* PB1 | This study |
| pVWEx1-$ta^{MGA3rec}$ | derived from pVWEx1, for regulated expression of modified *ta* of *B. methanolicus* MGA3 | This study |
| pVWEx1-$tal^{CG}$ | derived from pVWEx1, for regulated expression of *tal* (cg1776) of *C. glutamicum* ATCC 13032 | This study |
| pHP13 | *B. subtilis-E coli* shuttle vector; $Clm^R$ | (Haima *et al.*, 1987) |
| pTH1mp-$ta^{PB1}$ | pHP13 derivate with *ta* of *B. methanolicus* PB1 under control of *mdh* promoter | This study |
| pET28b | $Kan^R$; T7*lac*; vector for his-tagged protein overproduction | (Novagen) |
| pET28b-$ta^{PB1}$ | purification of his-tagged *B. methanolicus* PB1 Ta from *E. coli* BL21(DE3) | This study |
| pET28b-$ta^{MGA3rec}$ | purification of his-tagged *B. methanolicus* MGA3 Ta from *E. coli* BL21(DE3) | This study |
| pET28b-$ta^{MGA3}$_M1 | derived from pET28b, for expression of variant M1 of $ta^{MGA3}$ from *B. methanolicus* MGA3, see table 2.3.2. | This study |
| pET28b-$ta^{MGA3}$_M2 | derived from pET28b, for expression of variant M2 of $ta^{MGA3}$ from *B. methanolicus* MGA3, see table 2.3.2. | This study |
| pET28b-$ta^{MGA3}$_M3 | derived from pET28b, for expression of variant M3 of $ta^{MGA3}$ from *B. methanolicus* MGA3, see table 2.3.2. | This study |
| pET28b-$ta^{MGA3}$_M4 | derived from pET28b, for expression of variant M4 of $ta^{MGA3}$ from *B. methanolicus* MGA3, see table 2.3.2. | This study |
| pET28b-$ta^{MGA3}$_M5 | derived from pET28b, for expression of variant M5 of $ta^{MGA3}$ from *B. methanolicus* MGA3, see table 2.3.2. | This study |
| pET28b-$ta^{MGA3}$_M6 | derived from pET28b, for expression of variant M6 of $ta^{MGA3}$ from *B. methanolicus* MGA3, see table 2.3.2. | This study |
| pET28b-$ta^{MGA3}$ | derived from pET28b, for expression of variant of the annotated $ta^{MGA3}$ from *B. methanolicus* MGA3, see table 2.3.2. | This study |
| pK19mobsacB | $Kan^R$; vector for gene deletions (RP4 *mob*; *sacB B. subtilis*; *lacZ;* $OriV_{E.c}$) | (Schäfer *et al.*, 1994) |
| pK19mobsacB-Δ*tal* | derived from pK19mobsacB for in-frame deletion of *C. glutamicum tal* | This study |

Abbreviations: $Clm^R$, chloramphenicol resistance; $Kan^R$ kanamycin resistance.

**Table 2.3.2.: List of oligonucleotides used in this study.**

| Name | Sequence (5´-3´) |
|---|---|
| pET28b_Fw | GACTCACTATAGGGGAATTGTGAGCG |
| pET28b_Rv | AGATCCGGCTGCTAACAAAGCCCGA |
| pVWEx1_fw | CACTCCCGTTCTGGATAATG |
| pVWEx1_rv | GCTACGGCGTTTCACTTCTG |
| pTH1_fw | CTGCCCTTCCACCTTAACC |
| pTH1_rv | ATGTCACTAACCTGCCCCG |
| *ta*_MGA3rec-RBS-fw | GCGC**GGATCC**GAA*AGGAGG*CCCTTCAGATGGATGATT-CAAAACAGTT |
| *ta*_MGA3rec-rv | GCGC**GGTACC**TTATTTCCCGCGTTTATTCC |
| *ta*_PB1-RBS-fw | GCGC**GGATCC**GAA*AGGAGG*CCCTTCAGATGATTCAAAA-CAGCATCCA |
| *ta*_PB1-rv | GCGC**GGTACC**TTATTGCCCGCGTTTTTTCC |
| *tal*_CG-RBS-fw | GCGC**GGATCC**GAA*AGGAGG*CCCTTCAGATGTCTCACATT-GATGATCT |
| *tal*_CG-rv | GCGC**GGTACC**CTACTTCAGGCGAGCTTCCA |
| *ta*_PB1-TH-fw | GCGC**ACATGT**GATTCAAAACAGCATCCAACGAAAT |
| *ta*_PB1-TH-rv | ATGC**GGTACC**TTATTGCCCGCGTTTTTTCC |
| *ta*_PB1_NcoI-fw | AGAG**CCATGG**ATGATTCAAAACAGCATCCA |
| *ta*_PB1_Xho-rv | AGAG**CTCGAG**TTGCCCGCGTTTTTTCCAATCTG |
| *ta*_MGA3rec_NcoI-fw | AGAG**CCATGG**ATGATTCAAAACAGTTTCCAACCAAATA-AAG |
| *ta*_MGA3rec_Xho-rv | AGAG**CTCGAG**TTTCCCGCGTTTATTCCAGTC |
| *tal*_cg_del_A | GCGCGGATCCGGCTCCGGCTCCGAGGTTCA |
| *tal*_cg_del_B | *CCCATCCACTAAACTTAAACA*GAGCTGTGCAAGATCATCAA |
| *tal*_cg_del_C | *TGTTTAAGTTTAGTGGATGGG*CTTGAGTCCATGGAAGCTCG |
| *tal*_cg_del_D | GCGCGGATCCGCGGGTTTTGTCGATGCGCT |
| *tal*_cg_del_E | CTGCGTCCTGCAGATGCGAA |
| *tal*_cg_del_F | GGTCGATGCGGAACACAGAA |
| *ta*_MGA3,M1_BamHI-fw | CGC**GGATCC**GTGTCATATTTTAATACAGTT |
| *ta*_MGA3,M2_BamHI-fw | CGC**GGATCC**GTGTCAAAATTTAATACAGTT |
| *ta*_MGA3,M3_BamHI-fw | CGC**GGATCC**GTGTCATTATTTAATACAGTT |
| *ta*_MGA3,M4_BamHI-fw | CGC**GGATCC**GTGTCATCATTTAATACAGTT |
| *ta*_MGA3,M5_BamHI-fw | CGC**GGATCC**GTGTCACAATTTAATACAGTT |
| *ta*_MGA3,M6_BamHI-fw | CGC**GGATCC**GTGTCAGAATTTAATACAGTT |
| *ta*_MGA3,M_BamHI-rv | CCCC**GGATCC**TTATTTCCCGCGTTTATTCC |
| *ta*_MGA3_fw | GCGC**CCATGG**ATGATCAAGGAAGGAAAAGA |
| *ta*_MGA3_rv | GCGC**CTCGAG**TTTCCCGCGTTTATTCCAGT |

Restriction sites are highlighted in bold, linker sequences for crossover PCR and ribosomal binding sites are shown in italics, stop and start codons are underlined.

The *C. glutamicum* wild type (WT) strain (ATCC 13032) and the derived Δ*tal* mutant, lacking transaldolase activity, were used for the heterologous expression of the Ta genes $ta^{PB1}$ and $ta^{MGA3rec}$ from *B. methanolicus* PB1 and MGA3 respectively. The vector pVWEx1 was used for IPTG-inducible expression of $ta^{PB1}$. For growth experiments, *C. glutamicum* cells were harvested from cultures grown in LB medium overnight by centrifugation (4000 × g for 10 min), washed in CgXII minimal medium, and used to inoculate fresh CgXII minimal medium. All growth experiments with *C. glutamicum* were carried out with 50 ml medium in 500 ml baffled shake flasks at 30 °C and 120 rpm. Growth was monitored by determination of the optical density at 600 nm ($OD_{600}$) until the stationary phase was reached.

#### 2.3.3.2. Construction of *tal* (cg1776) deletion mutant of *C. glutamicum*

PCR products from chromosomal *C. glutamicum* DNA were obtained using primer pairs *tal*_cg_del_A; *tal*_cg_del_B and *tal*_cg_del_C; *tal*_cg_del_D. The resulting PCR products were used as template-DNA in a crossover PCR using primer pair *tal*_cg_del_A; *tal*_cg_del_D. The resulting PCR product with a shortened *tal* gene was then phosphorylated and ligated into the *Sma*I restricted vector pK19mobsacB (Schäfer *et al.*, 1994). Deletion was performed as described elsewehere (Eggeling & Bott, 2005) and confirmed by sequencing using primers *tal*_cg_del_E; *tal*_cg_del_F.

#### 2.3.3.3. Heterologous expression of $ta^{PB1}$ and $ta^{MGA3rec}$ of *B. methanolicus* and homologous expression of $tal^{CG}$ in *C. glutamicum* Δ*tal*

The PCR products from $ta^{PB1}$ and $ta^{MGA3rec}$ were generated from genomic DNA of *B. methanolicus* PB1 and MGA3 by PCR, using the oligonucleotide primer pairs *ta*_PB1-RBS-fw; *ta*_PB1-rv and *ta*_MGA3rec-RBS-fw; *ta*_MGA3rec-rv (Table 2.3.2.). The PCR product from $tal^{CG}$ was generated from genomic DNA of *C. glutamicum* ATCC 13032 by PCR using the oligonucleotide primer pair *tal*_CG-RBS-fw; *tal*_CG-rv (Table 2.3.2.). The amplified PCR products were cut with *BamH*I and *Kpn*I and then ligated with the *BamH*I and *Kpn*I restricted vector pVWEx1 (Peters-Wendisch *et al.*, 2001). The resulting vectors were named pVWEx1-$ta^{PB1}$, pVWEx1-$ta^{MGA3rec}$, and pVWEx1-$tal^{CG}$. The vector pVWEx1 allows IPTG-inducible gene expression in *C. glutamicum* and *E. coli*. All resulting vector inserts were sequenced using the primer pair pVWEx1_fw; pVWEx1_rv to confirm their sequence integrity.

### 2.3.3.4. Heterologous expression of different *ta*$^{MGA3}$ variants in *E. coli*

For the heterologous expression of the different *ta*$^{MGA3put}$ variants M1-M6 in *E. coli* DH5α, the primers *ta*_MGA3,M1_BamHI-fw to *ta*_MGA3,M6_BamHI-fw; *ta*_MGA3,M_BamHI-rv (Table 2.3.2.) were used for amplification by PCR from chromosomal DNA of *B. methanolicus* MGA3. The PCR product of the annotated *ta*$^{MGA3}$ gene was generated using primers *ta*_MGA3_fw; *ta*_MGA3_rv. The resulting PCR products were cut with *BamH*I and ligated into the *BamH*I restricted vector pET28b (Table 2.3.1.). Ta activity in crude cell extracts of recombinant *E. coli* DH5α strains was determined as described in 2.3.3.7.

### 2.3.3.5. Overexpression of *ta*$^{PB1}$ in *B. methanolicus* MGA3

The expression vector pTH1mp was used to allow methanol-inducible expression of the *B. methanolicus* PB1 Ta gene. This vector is analogous to the plasmid pHP13, in which the strong *mdh* promoter was cloned in-frame with the *mdh* ribosome-binding site (RBS) region to allow methanol-inducible expression in *B. methanolicus*. The DNA fragment of the *ta*$^{PB1}$-coding region was amplified from DNA of *B. methanolicus* using primer pair *ta*_PB1-TH-fw; *ta*_PB1-TH-rv (Table 2.3.2.). The resulting PCR product was digested with *Pci*I/*Kpn*I and ligated with the *Pci*I/*Kpn*I digested vector pTH1mp. The resulting vector was named pTH1mp-*ta*$^{PB1}$ and the correct insert was verified by sequencing using the primer pair pTH1_fw; pTH1_rv. Crude cell extracts were prepared based on the protocol described elsewhere (Brautaset *et al.*, 2004). The cells were inoculated from a glycerol stock and grown in $MeOH_{200}$ medium overnight before they were transferred to fresh $MeOH_{200}$ medium and grown to an $OD_{600}$ of 1.5 to 2.0. 40 ml of the cell culture was harvested by centrifugation (4000 × g, 30 min, 4 °C), washed in 50 mM potassium phosphate buffer (pH 7.8), and stored at -20 °C. The cells were disrupted by sonication. Cell debris was removed by centrifugation (14,000 x g, 60 min, 4 °C) and the supernatant was collected as crude extract. Ta activity was measured according to the conditions of the assay described in 2.3.3.7.

### 2.3.3.6. Purification and molecular mass determination of Ta proteins

For protein production in *E. coli* BL21 (DE3) (Studier *et al.*, 1990), $ta^{PB1}$ and $ta^{MGA3rec}$ were amplified by PCR using the primer pairs *ta*_PB1_NcoI-fw; *ta*_PB1_Xho-rv and *ta*_MGA3rec_NcoI-fw; *ta*_MGA3rec_Xho-rv (Table 2.3.2.). After restriction with *Nco*I and *Xho*I the resulting PCR products were ligated into *Nco*I and *Xho*I restricted pET28b (Novagen, Madison, Wisconsin, USA), resulting in pET28b-$ta^{PB1}$ and pET28b-$ta^{MGA3rec}$. The pET28b vector allows the production of a C-terminal hexahistidine (His)-tagged Ta in *E. coli* BL21 (DE3). Protein production and purification were performed as described previously (Lindner *et al.*, 2007). The enzyme was purified to homogenity. The protein purification was analyzed by a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The protein concentration was measured according to the method of Bradford using the Sigma Bradford reagent with bovine serum albumin (BSA) as a standard. The dimeric structures of the Ta proteins were determined by gel filtration as described previously (Lindner *et al.*, 2007) using 1 mg Ta dissolved in 2 ml of 20 mM Tris-HCl, pH 7.8.

### 2.3.3.7. Enzyme assays for the purified Ta proteins

To study the thermal stability of the Ta proteins, the assay mixture described below was prepared in 1.5 ml reaction tubes and incubated for up to 2 h at 30 °C to 80 °C. Samples were taken periodically and the residual enzyme activity was measured under standard conditions in a separate reaction mixture.

The Ta activity in the direction of S7P and GAP from E4P and F6P was determined. The standard reaction mixture (final volume 1 ml) contained 50 mM Tris-HCl buffer (pH 7.8), 0.25 mM nicotinamide adenine dinucleotide (NADH), triosephosphate isomerase (TIM), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and purified Ta protein, which was preheated for 3 min at 50 °C. NADH oxidation was followed at 340 nm ($\varepsilon_{340nm}$ = 6.22 $mM^{-1}$ $cm^{-1}$) on a Shimadzu UV1700 spectrophotometer. The reaction was initiated by the addition of E4P or F6P respectively (final concentration varied between 0.05-10 mM).

### 2.3.3.8. Tryptic digestion of proteins and mass spectrometry analysis

Proteins were separated using a SDS-PAGE and the bands were excised from the gel and put in clean reaction tubes. The tubes were treated with an aqueous solution containing 60% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA) for removal of plasticizers and dried over night. The protein bands were treated two times with an aqueous solution containing 0.1 M ammonia carbonate and 30% (v/v) acetonitrile for 10 min each with soft shaking. The supernatant was removed and the fragments were dried in a Eppendorf SpeedVac. The dried fragments were treated with a trypsin solution (1 µL trypsin + 14 µL 10 mM ammonia carbonate, Promega) for 30 min at 21 °C. After addition of 20 µL 10 mM ammonia carbonate solution, the samples were incubated for 12 h at 37 °C. The trypsin-digested samples were dried in the SpeedVac for 30 min. After the addition of 10 µL 50% (v/v) acetonitrile and 0.1% (v/v) TFA the samples were spotted onto a 800 µm Burker Anchor Chip, following the Burker Daltronics protocol. An Ultraflex matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) (Bruker, Bremen, Germany) was used to obtain the corresponding peptide mass fingerprints, using the standard manufacturer's parameters. The proteins were identified from a primary sequence database of *B. methanolicus* MGA3 using the Mascot (Matrix Science, London) search engine.

### 2.3.3.9. Computational analysis

Sequence comparisons were carried out using protein sequences obtained from the NCBI database (http://www.ncbi.nlm.nih.gov) and using BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997).

## 2.3.4. Results

### 2.3.4.1. Bioinformatical analysis of transaldolase genes from *B. methanolicus* PB1 and MGA3

The amino acid sequences of the annotated transaldolases from *B. methanolicus* strains MGA3 ($Ta^{MGA3}$) and PB1 ($Ta^{PB1}$) (Heggeset *et al.*, 2012) were aligned with different Ta proteins from other bacilli (Figure 2.3.1.). This revealed that the annotated $Ta^{MGA3}$ lacks about 80 N-terminal amino acids present in $Ta^{PB1}$ and Tas from other bacilli. Importantly, $Ta^{MGA3}$ lacks the aspartyl residue (D20 in $Ta^{PB1}$) of the catalytic triad (D20-E84-K107 in $Ta^{PB1}$) (Schörken *et al.*, 2001; Samland & Sprenger, 2009), indicating that $Ta^{MGA3}$ will likely not show Ta activity.

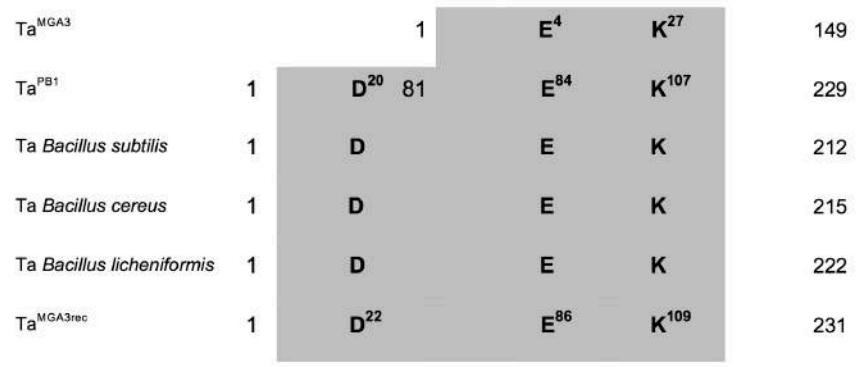


**Figure 2.3.1.: Schematic comparison of transaldolases from *B. methanolicus* strains MGA3 and PB1 with transaldolases from *Bacillus subtilis, Bacillus cereus,* and *Bacillus licheniformis*.** The shaded box represents the protein sequences, the number in front of and behind the shaded box represent the first and last amino acid. Residues of the catalytic active center are given in one letter code with their position in the protein in superscript. D, E, and K represent the amino acids aspartate, glutamate, and lysine.

Inspection of the genome sequences of strains MGA3 and PB1 revealed a mutation of the translation initiation codon ATG, present in PB1, to ATA in *B. methanolicus* MGA3 (Figure 2.3.2.). There is an alternative GTG start codon that is in-frame with the annotated stop codon, but the third codon would be a TAA stop codon ($Ta^{MGA3put}$). An error in the annotation of the sequence can be ruled out, as this part was sequenced by us several times (data not shown).

In order to recreate a full-length version of Ta$^{MGA3}$, ATA was changed to ATG and, in addition, the "T" downstream (indicated in pink) was deleted to correct for a reading-frame shift (Figure 2.3.2.). The corresponding protein was named Ta$^{MGA3rec}$. An alternative way to restore activity of Ta$^{MGA3}$ was based on GTG as start codon and six versions, where the TAA stop codon was changed to different amino acids (named M1 to M6), were constructed as detailed in figure 2.3.2.

```
PB1:              ATG ATT CAA AAC AGC ATC CAA CGA AAT AAA ...
MGA3put.:   GTG TC-TA A-- T-- T-- --TTT-- --- -C- --- --- ...
MGA3rec:          ATG --- --- --- --T T-- --- -C- --- --- ...
M1:         GTG TC--A T-- T-- T-- --TTT-- --- -C- --- --- ...
M2:         GTG TC-AA --- T-- T-- --TTT-- --- -C- --- --- ...
M3:         GTG TC--T --- T-- T-- --TTT-- --- -C- --- --- ...
M4:         GTG TC--C --- T-- T-- --TTT-- --- -C- --- --- ...
M5:         GTG TC-CA --- T-- T-- --TTT-- --- -C- --- --- ...
M6:         GTG TC-GA --- T-- T-- --TTT-- --- -C- --- --- ...
```

**Figure 2.3.2.: Construction of different variants of the transaldolase of MGA3. The nucleotide sequences are given.** MGA3$^{put}$ represents an alternative Ta coding sequence of MGA3. For MGA3$^{rec}$ the ATA was changed to ATG and adjusted to that of PB1. For the variants M1-M6, the third codon of the sequence upstream of *ta*$^{MGA3}$ was changed from a stop TAA codon to different codons. The dashes indicate the same nucleotide as for PB1 and the dots show that the sequence continues. The red letters indicate a stop codon and the pink letter shows an additional nucleotide at that position.

### 2.3.4.2. Heterologous expression of different constructed Ta variants in *E. coli*

To test for Ta activity in crude cell extracts of *E. coli* DH5α, different Ta variants (M1-M6, *ta*$^{MGA3rec}$, and *ta*$^{MGA3}$) were constructed (Figure 2.3.2.). As suggested based on the bioinformatical analysis, the annotated Ta of *B. methanolicus* MGA3 showed no activity due to the missing N-terminus, whereas all of our constructed Ta variants were found to be active.

### 2.3.4.3. Overexpression and purification of Ta proteins from *B. methanolicus*

In the next step, we focused on the characterization of Ta of *B. methanolicus* PB1. The $Ta^{PB1}$-coding sequence was PCR-amplified and cloned into the vector pET28b for production of the corresponding enzyme with a C-terminal His-tag. *E. coli* BL21 (DE3) was transformed with the resulting plasmid and recombinant protein production was induced by the addition of IPTG to exponentially growing cell cultures. Cells were harvested, crude extracts were prepared, and after Ni-NTA chromatography the enzyme was buffered in 50 mM Tris–HCl (pH 7.8). Protein purification from 500 ml of culture broth led to average concentrations of about 2.5 mg/ml and a total amount of about 7.5 mg per purification. Gel filtration and Ta activity assay of the eluted fractions showed that the protein eluted in a single fraction indicating that it is active as a homodimer with a molecular weight of 54 kDa. Further, also the $Ta^{MGA3rec}$ protein was purified by the same method and activity was verified. However, since this protein was not synthesized from the original $ta^{MGA3}$ nucleotide sequence, the biochemical characterization was limited to $Ta^{PB1}$.

### 2.3.4.4. Optimal conditions for $Ta^{PB1}$ activity

The optimal assay conditions for $Ta^{PB1}$ were determined by using a coupled spectrometric assay for measuring the formation of GAP from F6P and E4P (as described in the Materials & Methods section). The activity of the auxiliary enzymes TIM and GAPDH were first checked under the different conditions and added in excess.

The pH optimum of $Ta^{PB1}$ was found to be at 7.8. The highest activity was measured in the range of 60 °C to 65 °C (data not shown). For determination of the kinetic parameters, the physiological temperature of 50 °C was chosen.

### 2.3.4.5. Kinetic parameters of $Ta^{PB1}$

The kinetic parameters of $Ta^{PB1}$ were determined for the conversion of F6P and E4P to S7P and GAP (Table 2.3.3.). The assays were performed at 50 °C and pH 7.8 in 50 mM Tris-HCl. For F6P, a $K_M$ of 740 µM and a $V_{max}$ of 16.33 U/mg could be determined. The catalytic efficiency for F6P was calculated to be 9.98 $s^{-1}$ $mM^{-1}$. The following affinities were observed for E4P: $K_M$ 2,5 mM and $V_{max}$ 8.9 U/mg. The corresponding catalytic efficiency was 1.6 $s^{-1}$ $mM^{-1}$.

**Table 2.3.3.: Biochemical properties of $Ta^{PB1}$.**

| Parameter | | $Ta^{PB1}$ |
|---|---|---|
| Molecular weight | | 54 kDa |
| Optimal conditions: | | 50 mM Tris-HCl, pH 7.8, 50 °C |
| Optimal pH | | 7.2-7.4 |
| Optimal temperature | | 60 °C |
| Temperature stability | | < 60°C |
| Kinetic parameters | | |
| F6P | $K_M$ | 0.74 mM |
| | $V_{max}$ | 16.33 U/mg |
| | $K_{cat}$ | 7.35 $s^{-1}$ |
| | $K_{cat}/K_M$ | 9.98 $s^{-1}$ $mM^{-1}$ |
| E4P | $K_M$ | 2.5 mM |
| | $V_{max}$ | 8.9 U/mg |
| | $K_{cat}$ | 4.01 $s^{-1}$ |
| | $K_{cat}/K_M$ | 1.6 $s^{-1}$ $mM^{-1}$ |

Values for $K_M$ (mM), $V_{max}$ (U/mg), and catalytic efficiency ($K_{cat}/K_M$ = $s^{-1}$ $mM^{-1}$) were determined for two independent protein purifications and mean values and arithmetric deviations from the mean are given.

### 2.3.4.6. Heterologous expression of *ta* from *B. methanolicus* in *C. glutamicum* Δ*tal*

*C. glutamicum* possesses a Ta (encoded by *tal*), which functions in the pentose phosphate pathway. Since methods for gene deletion in *B. methanolicus* are lacking, a defined *tal* deletion mutant of *C. glutamicum* was constructed. Comparative growth analysis revealed that *C. glutamicum* requires Ta for normal growth in minimal medium with ribose as sole carbon source. The defined *C. glutamicum tal* deletion mutant could be complemented by expression of the endogenous *tal* from plasmid pVWEx1, since *C. glutamicum* Δ*tal*(pVWEx1-$tal^{CG}$) grew with an even slightly higher specific growth

rate than the wild type in CGXII minimal medium supplemented with 2% ribose. In contrast, the strain carrying the empty vector showed a lower specific growth rate (Figure 2.3.3.). This confirmed the absence of secondary effects, such as polar effects, of the deletion of *tal*, which is part of the *tkt-tal-zwf-opcA-pgl* operon in this bacterium. In order to test if Ta from *B. methanolicus* PB1 is active in *C. glutamicum*, $ta^{PB1}$ was heterologously expressed in the Δ*tal* strain. Expression of $ta^{PB1}$ could complement *C. glutamicum* Δ*tal*, as this strain showed a similar specific growth rate as *C. glutamicum* Δ*tal*(pVWEx1-$tal^{CG}$) (Figure 2.3.3.). Further, the same effect was observed when heterologously expressing $ta^{MGA3rec}$. Thus, Ta of *B. methanolicus* PB1 and the reconstructed version of the *B. methanolicus* MGA3 Ta protein are functional as active Ta in *C. glutamicum*.

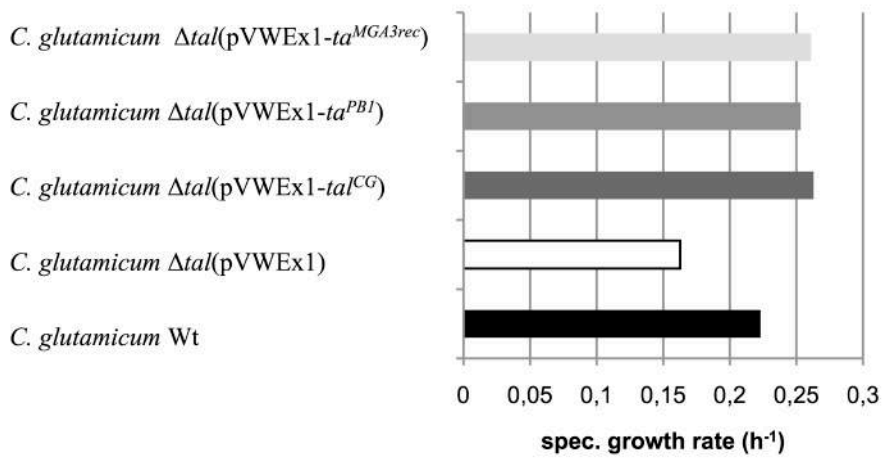


**Figure 2.3.3.: Complementation of *C. glutamicum* Δ*tal* by $ta^{PB1}$, $ta^{MGA3rec}$, and $tal^{CG}$.** The mean values and standard deviations for the specific growth rate of three replicates are given for growth in CGXII minimal medium supplemented with 2% ribose.

### 2.3.4.7. Physiological effect of overexpression of $ta^{PB1}$ in *B. methanolicus* MGA3

After characterization of $Ta^{PB1}$, we investigated the effect of overexpressing $ta^{PB1}$ in *B. methanolicus* MGA3 during growth on methanol. We were interested in the question if MGA3 has an advantage through expression of $ta^{PB1}$, when exposed to different methanol concentrations. Therefore, the strains *B. methanolicus* MGA3(pHP13) and *B. methanolicus* MGA3(pTH1mp-$ta^{PB1}$) were constructed. The strains were grown in MeOH medium supplemented with different methanol concentrations. We could not detect any significant differences in the growth behavior (data not shown). To make

sure that *ta*$^{PB1}$ is transcribed correctly and active, we tested for Ta activity in crude cell extracts of *B. methanolicus*. To our surprise, Ta activity for the empty vector strain MGA3(pHP13) was measured (57 mU/mg). To exclude background activity, crude cell extracts of *C. glutamicum* Δ*tal* (no detectable activity) were used for comparison. We also wanted to exclude potential bifunctional enzymes with transaldolase activity, such as glucose-6-phosphate isomerase/transaldolase described for *Gluconobacter oxydans* (Sugiyama *et al.*, 2003). Thus, we used the nucleotide and amino acid sequences of Ta from *B. methanolicus* PB1 for a BLAST search against the genome of MGA3. This search revealed no other enzymes with a putative Ta function. In addition, no enzymes that might use substrates of the Ta enzyme assay, like fructose-6-phosphate aldolase, were found. After finding Ta activity in the *B. methanolicus* MGA3 strain, we tried to investigate what causes this activity and how the coding sequence might look like.

#### 2.3.4.8. Size exclusion chromatography and MALDI-TOF analysis

First, Ta activity was measured in a crude cell extract of *B. methanolicus* MGA3 (0.284 U), which then was used for size exclusion chromatography (gel filtration), in order to determine if Ta activity can be assigned to a particular fraction. Ta activity was confirmed for fractions 2 (0.174 U) and 8 (0.110 U) of a total of 18 fractions received, leading to 100% of the initial activity found in the crude cell extract. The two fractions showing Ta activity were separated using a SDS-PAGE and every band was extracted from the gel and subsequently used for analysis by MALDI-TOF. However, the search against a *B. methanolicus* MGA3 database revealed no findings of Ta for any fragment measured.

#### 2.3.4.9. Transcriptome and proteome data indicates expression of *ta*$^{MGA3put}$

Since the annotated Ta of *B. methanolicus* MGA3 (Ta$^{MGA3}$) showed no activity when expressed in *E. coli*, but activity was found in crude extracts of MGA3, we used available transcriptome data from RNA-sequencing (Irla *et al.*, 2015) to search for a possible transcription start site (TSS) upstream of the start codon of our proposed Ta coding sequence *ta*$^{MGA3put}$ (Figure 2.3.2.). With this data, a putative TSS as well as a -10 region of TTTCAA(T) and a -35 region of TTGAAA were identified. The -10 region shares only a low similarity with the *B. methanolicus* MGA3 -10 region consensus sequence (TATAAT), but the -35 region represents the consensus sequence (Irla *et al.*, 2015).

We also used the amino acid sequence of $Ta^{MGA3put}$ for a search against a database including proteome data of *B. methanolicus* MGA3 (Müller *et al.*, 2014). With this, we were able to identify peptides upstream of the annotated Ta (Müller, personal communication), indicating transcription and translation of $ta^{MGA3put}$. Summing up, analysis of the available transcriptome and proteome data indicates transcription of the proposed $ta^{MGA3put}$-coding sequence, leading to the translation of a functional Ta protein, responsible for the measured Ta activity in *B. methanolicus* MGA3.

### 2.3.5. Discussion

The focus of this study was on the biochemical characterization of Ta from *B. methanolicus* and its possible role in the RuMP pathway. Since the annotated Ta of *B. methanolicus* MGA3 showed no activity, only Ta of PB1 was purified and characterized.

Ta of *B. methanolicus* is a putative subfamily 4 gene, belonging to the same subfamily as Ta of *Bacillus subtilis.* Other well characterized Ta proteins are TalB from *E. coli* and Ta from *C. glutamicum*, which belong to subfamily 1 and 3 respectively.

The kinetic parameters of Ta from PB1 were measured at the physiological conditions of 50 °C and pH 7.8. The kinetic parameters of Ta from *E. coli*, *B. subtilis*, and *C. glutamicum* have been determined at 30 °C and pH 8.5 (Samland *et al.*, 2012). The measured $K_M$ and $V_{max}$ for F6P (0.74 mM and 16 U/mg) and E4P (2.5 mM and 8.9 U/mg) are in the same range as those determined for Ta of *B. subtilis* (1.4 mM and 28 U/mg; 1.2 mM and 19 U/mg respectively) (Schürmann, 2001). While the $K_M$ values for F6P of TalB from *E. coli* (1.2 mM) and Ta from *C. glutamicum* (1.3 mM) are in the same range as of Ta from *B. methanolicus* PB1, $V_{max}$ is slightly higher (60 U/mg and 110 U/mg respectively) (Sprenger *et al.*, 1995; Samland *et al.*, 2012). For E4P, TalB and Ta have a lower $K_M$ (0.09 mM and 0.7 mM respectively), whereas $V_{max}$ is slightly higher (80 U/mg and 84 U/mg respectively) (Sprenger *et al.*, 1995; Samland *et al.*, 2012).

Ta of PB1 was found to have a temperature optimum of 60 °C, which is higher compared to TalB from *E. coli* (40 °C) (Sprenger *et al.*, 1995) and Ta from *C. glutamicum* (40 °C) (Samland *et al.*, 2012). This was expected, since *B. methanolicus* is a thermophilic bacterium with an optimal growth temperature of 50 °C to 55 °C (Schendel *et al.*,

1990; Arfman *et al.*, 1992b). Further, TalB and Ta of *C. glutamicum* lost the activity above 50 °C, whereas Ta of *B. subtilis* showed a steady increase in activity from 20 °C to 55 °C (Sprenger *et al.*, 1995; Schürmann, 2001; Samland *et al.*, 2012). The thermostability is a feature of Tas of subfamily 4 and 5 and is not only restricted to thermophilic organisms (Samland *et al.*, 2012). It has been proposed that a compact structure and a tight packing of the subunits leads to a higher thermostability of subfamily 4 and 5 Ta proteins (Thorell *et al.*, 2002). In sum, Ta of *B. methanolicus* PB1 shows similar $K_M$ and $V_{max}$ values to Ta of *B. subtilis* and both proteins show highest activity at temperatures above 50 °C.
In contrast, Ta of PB1 slightly differs from TalB of *E. coli* and Ta of *C. glutamicum* with respect to $K_M$ for E4P, $V_{max}$, and the temperature optimum. Ta of *B. methanolicus* PB1 was identified to form a homodimer, whereas Ta of *B. subtilis* forms a decamer (dimer of pentamers) (Schürmann, 2001). TalB of *E. coli* exists as homodimer and Ta of *C. glutamicum* as monomer (Samland *et al.*, 2012).

The active site is well conserved among the Ta proteins. Thus, it has also been proposed that the catalytic mechanism is highly conserved (Samland *et al.*, 2012). The important and highly conserved amino acid aspartate (Figure 2.3.1.) acts as follows. The aspartate residue assists in the deprotonation of the C4 hydroxyl group of the enzyme-bound imine during the reaction catalyzed by Ta. This leads to the cleavage of the imine and release of the first product, glyceraldehyde 3-phosphate (GAP). The resulting Schiff base intermediate is stabilized by resonance until the acceptor molecule is bound at the active site (Jia *et al.*, 1996; Jia *et al.*, 1997; Schörken *et al.*, 2001; Schneider *et al.*, 2008; Samland & Sprenger, 2009).

Since the annotated Ta of *B. methanolicus* MGA3 lacks the aspartate residue responsible for the deprotonation and release of GAP, this explains why no activity was detected. However, Ta activity was found in *B. methanolicus* MGA3 crude cell extracts, although the annotated Ta gene showed no activity when heterologously expressed in *E. coli*. An alternative GTG start codon was found upstream of the annotated start codon, which is within the reading frame of the stop codon. For this coding sequence ($ta^{MGA3put}$), the third codon would be a TAA stop codon, but RNA-sequencing and proteome data of *B. methanolicus* MGA3 indicates that this sequence is transcribed and translated into a functional Ta protein. However, a clear evidence for the start codon and coding sequence is missing. Different strategies for changing the coding sequence

to the start of *ta* of PB1 (*ta*$^{MGA3rec}$) and replacement of the TAA stop codon by different amino acids (Figure 2.3.2.), all led to functional Ta proteins in recombinant *E. coli* strains. When comparing the nucleotide sequences of MGA3 and PB1, it might be possible that a point mutation in MGA3 has changed the original ATG start codon to ATA (Figure 2.3.2.). But then also an insertion of a ”T” has had to be occurred to match the reading frame of the annotated stop codon. Assuming that the GTG functions now as start codon, the following third codon would be a TAA stop codon.
In this context, the term “recoding”, which refers to alternative translational decoding that is utilized for gene expression, is also important (Gesteland *et al.*, 1992; Sharma *et al.*, 2011). There are three different classes of recoding in nature: i) programmed-frameshifting, ii) translational bypassing, and iii) translational redefinition of codons (Gesteland & Atkins, 1996; Atkins *et al.*, 2001). Recoding is caused by special signals on the mRNA and is characterized by an unchanged genetic code as well as the synthesis of more than one protein from one mRNA (Atkins & Baranov, 2010).

In case of translational bypassing, the ribosome stops translation at a certain point, skips some nucleotides, and then continues translation downstream (Baranov *et al.*, 2002). This can either lead to a frameshift or can occur in-frame. Readthrough of a stop codon, leading to redefiniton, has been reported for genes of *Drosophila melanogaster* (Xue & Cooley, 1993; Bergstrom *et al.*, 1995; Steneberg *et al.*, 1998), the bacteriophage Qβ (Weiner & Weber, 1973; Hofstetter *et al.*, 1974), and a phage-derived gene of enterotoxigenic *E. coli*, which encodes several proteins that are produced by readthrough of a UAG stop codon (Jalajakumari *et al.*, 1989). Further, in *Bacillus firmus* two open reading frames (ORFs) have been found that are separated by a UGA stop codon and might be subject to readthrough (Ivey *et al.*, 1992).

The general mechanisms of translational bypassing or stop codon readthrough might serve as an explanation for the translation of an active Ta protein in MGA3, but do not match the definition of recoding. However, based on the definition, reassignment would be the fitting mechanism, as it is characterized by an alternative genetic code and only one protein translated from one mRNA (Atkins & Baranov, 2010). For eukaryotes, it has been shown that they can use TAA and TGA stop codons to encode for other amino acids (stop codon “reassignment”), but for bacteria this strategy has only been reported for TGA stop codons (Ivanova *et al.*, 2014).

We also searched for enzymes capable of using metabolites of the enzyme assay, like fructose-6-phosphate aldolase (Schürmann & Sprenger, 2001), and therefore, leading to a false positive result. Since bioinformatical analysis revealed no such enzymes and also no potential bifunctional enzymes, like glucose-6-phosphate isomerase/transaldolase, the measured activity was most probably due to a functional Ta protein in *B. methanolicus* MGA3. The results from bioinformatical analysis as well as data from our measurements, RNA-sequencing, and proteome analysis, indicate that $ta^{MGA3put}$ might be the coding sequence for an active Ta in *B. methanolicus* MGA3. It has to be tested if an increased Ta activity can be measured in *B. methanolicus* MGA3 crude cell extracts, when this sequence, including the possible promoter and RBS sequence upstream of the GTG codon, is expressed on a plasmid lacking a promoter.

As we were able to show that both *B. methanolicus* strains MGA3 and PB1 possess an active Ta protein, the potential relevance for the RuMP pathway in this organism will be discussed here. It has been shown that *B. methanolicus* uses the SBPase variant of the RuMP pathway for regeneration of Ru5P (Stolzenberger *et al.*, 2013a). Maybe *B. methanolicus* uses in addition the Ta variant, allowing a high methanol tolerance due to a fast and efficient regeneration of Ru5P. Data from metabolic flux analysis in this organism has shown that both variants are used in parallel (Carnicer, personal communication). However, Ta has been neither upregulated on the transcriptome nor the proteome level during growth on methanol (Heggeset *et al.*, 2012; Müller *et al.*, 2014). But this may be due to the wrongly annotated coding sequence. So far, no bacterium has been reported that uses the Ta and SBPase variant of the RuMP pathway in parallel, but plants use Ta besides SBPase (Raines, 2003).

Summing up, we biochemically characterized Ta of *B. methanolicus* PB1, which showed kinetic parameters, generally matching those reported for *B. subtilis*, *E. coli*, and *C. glutamicum.* This shows, as reported before, that the biochemical properties are highly conserved among Tas of different organisms (Samland *et al.*, 2012). Further, we identified an alternative Ta coding sequence ($ta^{MGA3put}$) in *B. methanolicus* MGA3 that might lead to the translation of a functional Ta protein. Its possible role in the RuMP pathway remains unclear and needs to be further tested.

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## 2.3.7. References

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# 3. Discussion

This thesis outlines two different aspects of the utilization of methanol as an alternative carbon source: i) the partial conversion of methanol into the polyamide building block cadaverine by a synthetic *Corynebacterium glutamicum* strain and ii) the production of cadaverine by genetically engineered strains of the natural methylotroph *Bacillus methanolicus.* Further, the analysis of approaches and accomplishments in synthetic methylotrophy may shed light on future developments of this establishing field. Lastly, the potential for an industrial application of both hosts is examined.

## 3.1. The way towards a synthetic methylotrophic *C. glutamicum* strain

To date, no non-methylotroph has been successfully genetically engineered for growth on methanol as sole carbon source due to the difficulties of implementing a $C_1$-pathway and adjusting it to the host's central metabolism. This work represents a major step towards synthetic methylotrophic bacteria by demonstrating, for the first time, the conversion of methanol into a non-natural product by *C. glutamicum* (Chapter 2.2.). The long-term goal is to engineer a *C. glutamicum* strain that is able to use solely methanol for biomass and product formation, since synthetic methylotrophs possess some advantages over natural methylotrophs. This includes for instance, a higher production of small metabolites, more efficient genetic-transfer systems, and a higher efficiency with regard to electron conservation and product yields (Fei *et al.*, 2014; Haynes & Gonzalez, 2014; Whitaker *et al.*, 2015).

As described in the introduction part, the ribulose monophosphate (RuMP) pathway seems to be most suitable for the construction of a synthetic methanol assimilation pathway in bacteria due to its energy efficiency (Quayle & Ferenci, 1978; Kato *et al.*, 2006; Whitaker *et al.*, 2015). Up to now, *C. glutamicum, Escherichia coli*, and *Pseudomonas putida* have been engineered for methanol conversion by using enzymes of the RuMP pathway (Koopman *et al.*, 2009; Müller *et al.*, 2015b; Witthoff *et al.*, 2015). Similar strategies have been followed for *E. coli* and *C. glutamicum*, using methanol dehydrogenases (Mdhs), 3-hexulose-6-phosphate synthase (Hps), and 6-phospho-3-hexuloisomerase (Phi) of *B. methanolicus*.

In this work, the $NAD^+$-dependent Mdhs of *B. methanolicus* MGA3 and PB1 were heterologously expressed in *C. glutamicum* for the oxidation of methanol to formaldehyde (Chapter 2.2.). Mdh of *B. methanolicus* MGA3 showed the highest activity, confirming previous results (Witthoff *et al.*, 2015). The importance of Mdhs has been noted in prior studies, for example, Mdh2 and Mdh3 of *B. methanolicus* MGA3 have shown significant *in vivo* activities in *E. coli* (Brautaset *et al.*, 2013; Müller *et al.*, 2015b). Moreover, Mdhs have been claimed in a patent to enhance the production of 1,4-butanediol from methanol by increasing the pool of reducing equivalents, although no data has been provided (Burgard *et al.*, 2014). However, Mdh activity was found to be low in *C. glutamicum* and is limited by its low affinity to methanol, indicated by the high $K_m$ value of 170 mM (Krog *et al.*, 2013). Thus, Mdhs represent future targets to ensure an efficient oxidation of methanol and capture of electrons, which can also be used to increase the maximum metabolite yields (Whitaker *et al.*, 2015). The low substrate affinity and selectivity could be improved by protein engineering (Song *et al.*, 2002; Bastian *et al.*, 2011). Further, Mdhs from other methylotrophs, such as *Bacillus stearothermophilus* (Dowds *et al.*, 1988) or mesophilic *Amycolatopsis methanolica* (Bystrykh *et al.*, 1993) constitute alternatives. However, further research is needed to assess their implications in synthetic methylotrophy (Hektor *et al.*, 2000).

Formaldehyde is the key intermediate in the utilization of methanol and represents the branching point of the dissimilatory and assimilatory routes (Vorholt, 2002; Lidstrom, 2006). Towards a synthetic methylotroph, the oxidation of formaldehyde is an additional obstacle, as the carbon is lost in form of $CO_2$. Formaldehyde dissimilation pathways are ubiquitous and these endogenous pathways could compete with the engineered assimilatory pathway. Hence, in this work a *C. glutamicum* strain lacking the linear formaldehyde dissimilation pathway as a consequence of deleting *ald* (encoding acetaldehyde dehydrogenase) and *fadH* (encoding formaldehyde dehydrogenase) was used for engineering methylotrophy in this host (Leßmeier *et al.*, 2013). However, due to the high toxicity of formaldehyde, methanol concentrations and a synthetic methanol assimilation pathway have to be tailored to the metabolism. The *C. glutamicum* wild type strain is able to tolerate up to 1.3 M of methanol (Witthoff *et al.*, 2013), but the deletion of the endogenous formaldehyde dissimilation route removed the tolerance nearly completely (Chapter 2.2.).

A key issue is the fixation of the toxic intermediate formaldehyde, catalyzed by Hps and Phi. In this work, Hps and Phi of *B. methanolicus* MGA3 as well as their homologues HxlA and HxlB of *Bacillus subtilis* (Yasueda *et al.*, 1999) were tested for activity in *C. glutamicum*. HxlA and HxlB displayed the highest *in vivo* activities, most probably due to their origin from a mesophilic organism, and thus, are more suitable for the metabolism of *C. glutamicum* (Chapter 2.2.). Both the Hps-Phi and HxlA-HxlB system partially restored tolerance and growth in *C. glutamicum* Δ*ald*Δ*fadH* in the presence of methanol (Chapter 2.2.). Several studies investigating the impact of the heterologous expression of *hps* and *phi* on the formaldehyde tolerance have been carried out. For example, it has been demonstrated that expression of *hps* and *phi* from *Mycobacterium gastri* in *E. coli* led to an increased formaldehyde tolerance (Orita *et al.*, 2007). Further, a *hps*- and *phi*-expressing *Pseudomonas putida* S12 strain showed higher biomass yields in a "chemostat" fermentation on glucose with the addition of formaldehyde (Koopman *et al.*, 2009). Due to the fact that fixation of formaldehyde is a critical step in methylotrophy, a future objective for an enhanced Hps-Phi activity and more efficient conversion of formaldehyde, could be the construction of a Hps-Phi fusion protein (Kato *et al.*, 2006; Orita *et al.*, 2007). One strategy to further increase methanol tolerance in a strain lacking the linear formaldehyde dissimilation pathway, is the identification of additional enzymes involved in methanol and formaldehyde tolerance. A random mutagenesis approach resulted in a *C. glutamicum* strain, named Tol1, with an increased growth rate in the presence of up to 1 M of methanol. This strain carries two point mutations. One leading to an amino acid exchange in the O-acetylhomoserine sulfhydrolase MetY and the other leading to a shortened CoA transferase Cat (Leßmeier & Wendisch, 2015).

Another challenge is the regeneration of ribulose 5-phosphate (Ru5P) for the fixation of formaldehyde. The engineered *C. glutamicum* Δ*ald*Δ*fadH* strain, heterologously expressing *mdh* and *hxlAB*, showed significant label incorporation into central metabolites and the functional regeneration of Ru5P was proven by the finding of up to threefold-labeled hexoses in labeling experiments using $^{13}$C-methanol (Chapter 2.2.). In engineered *E. coli* strains, even 6-fold-labeled hexoses have been observed in similar experiments (Müller *et al.*, 2015b). Interestingly, the label incorporation was higher in the *E. coli* wild type background than in Δ*frmA* (encoding formaldehyde dehydrogenase) (Müller *et al.*, 2015b). In *B. methanolicus*, all genes and enzymes required for the regeneration of Ru5P are upregulated in the presence of methanol (Heggeset *et al.*, 2012;

Müller *et al.*, 2014). Both *C. glutamicum* and *E. coli* possess the required enzymes as part of the pentose phosphate pathway (PPP). Yet, an increase of enzyme activity is suggested to accelerate regeneration of Ru5P. The finding of a transaldolase (Ta) in MGA3 opened up the question if this organism use Ta in addition to the sedoheptulose-1,7,-bisphosphatase (SBPase) variant for an efficient formaldehyde fixation and regeneration of Ru5P (Chapter 2.3.). Ta was not upregulated on the transcriptome or proteome level (Heggeset *et al.*, 2012; Müller *et al.*, 2014), but this might be related to the wrong annotation of *ta* in MGA3 (Chapter 2.3.). Therefore, it can be inferred that Ta might support the SBPase in Ru5P regeneration under high methanol conditions. *C. glutamicum* possesses a Ta but the activity maybe has to be increased either by heterologous expression of a Ta gene from a methylotroph or by overexpression of the endogenous *tal*.

The most interesting finding of this work was the proof of methanol utilization for product formation of the non-natural compound cadaverine in *C. glutamicum* (Chapter 2.2.). This was shown in $^{13}$C-labeling experiments, where $^{13}$C-methanol was used as carbon source in addition to the sugars glucose and ribose respectively. The results indicated significant labeling at the C2/C4 position of cadaverine, and thus, demonstrated the successful conversion of methanol (Chapter 2.2.). However, no biomass formation from methanol was obtained in *C. glutamicum*, as also reported before for *E. coli* (Müller *et al.*, 2015b). The $^{13}$C-labeling results, showing only low labeling of the trioses and high glucose labeling, indicated a high metabolic flux through the oxidative PPP, where the carbon from methanol might be emitted as $CO_2$. Thus, it was reasoned that the deletion of the phosphoglucose isomerase gene (*pgi*) and consequently blocking the carbon flux from fructose 6-phosphate via the cyclic dissimilatory RuMP pathway might be beneficial (Chapter 2.2., Figure 2.2.1.). This could help to enable *C. glutamicum* for methanol assimilation into biomass, since the deletion of *pgi* only allows regeneration of Ru5P via the assimilatory RuMP pathway.

Hence, an experiment investigating the effect of deleting *pgi* was carried out within this work. The growth of the triple mutant strain *C. glutamicum* Δ*ald*Δ*fadH*Δ*pgi*(pEKEx3-*mdh,hxlAB*) was compared with the double mutant strain *C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*) in the presence of different methanol concentrations. In general, the deletion of *pgi* caused a reduced growth rate ($0.23 \pm 0.02$ h$^{-1}$ vs. $0.37 \pm 0.01$ h$^{-1}$) but the biomass ($oD_{600}$: $36.1 \pm 0.5$) was slightly

higher compared to the double mutant ($oD_{600}$: 33.5 ± 0.6) (Figure 3.1.). Upon addition of 200 mM methanol, the growth rate of strain ΔΔ was decreased by about 30% (0.27 ± 0.00 $h^{-1}$), while the growth rate of strain ΔΔΔ*pgi* was reduced by 40% (0.14 ± 0.00 $h^{-1}$). Although the Δ*pgi* mutant was more sensitive to methanol, the detoxification was functional in this strain, showing that methanol detoxification via the RuMP pathway is not restricted to the cyclic dissimilation via the oxidative PPP. The addition of methanol caused a similar biomass reduction in both strains, indicating that the deletion of *pgi* did not lead to an increased methanol-based biomass formation.

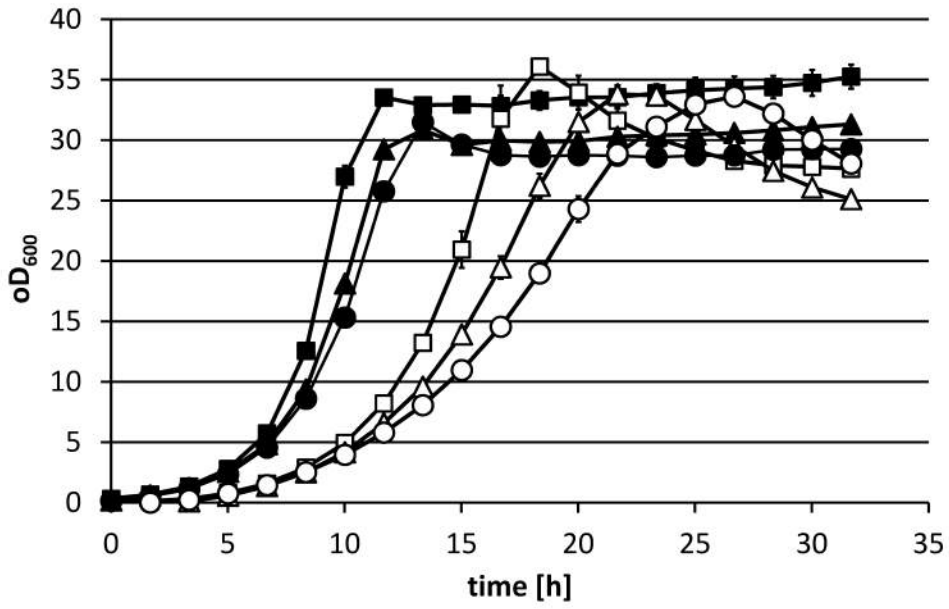


**Figure 3.1.: Growth behavior of *C. glutamicum* ΔΔΔ*pgi* in the presence of methanol.** Growth of *C. glutamicum* ΔΔ (closed symbols) and ΔΔΔ*pgi* (open symbols) in CgXII minimal medium supplemented with 50 mM ribose and 50 mM glucose and additional 0 mM (squares), 50 mM (triangles) and 200 mM (circles) methanol. Means and standard deviations of two independent cultures are shown.

Although the RuMP enzymes are well characterized in *B. methanolicus* (Krog *et al.*, 2013; Stolzenberger *et al.*, 2013a; Stolzenberger *et al.*, 2013b; Markert *et al.*, 2014; Ochsner *et al.*, 2014), their essentiality for methylotrophy remains unclear (Müller *et al.*, 2015a). It is known that methylotrophy in *B. methanolicus* is plasmid-dependent (Brautaset *et al.*, 2004) and that the RuMP enzymes encoded on plasmid pBM19 plus Hps and Phi are upregulated on the transcriptome and proteome level during growth on methanol compared to mannitol (Jakobsen *et al.*, 2006; Heggeset *et al.*, 2012; Müller *et*

*al.*, 2014). Therefore, it has been proposed that only these enzymes play an important role in methylotrophy in *B. methanolicus* (Heggeset *et al.*, 2012). Since the enzymes of *B. methanolicus* show different biochemical properties compared to *C. glutamicum* and might be more adapted to methanol utilization, all known RuMP pathway genes from plasmid pBM19 and *rpiB* from the chromosome of *B. methanolicus* MGA3 were heterologously expressed in *C. glutamicum* Δ*ald*Δ*fadH*(pEKEx3-*mdh,hxlAB*) and Δ*ald*Δ*fadH*Δ*pgi*(pEKEx3-*mdh,hxlAB*). Additionally, the gene *ywlG* that forms an operon with *rpiB* in the genome of *B. methanolicus* MGA3 and therefore might play a role in methylotrophy was heterologously expressed. This approach initiated the construction of the vector pVWEx1-*glpX*$^{P}$-*fba*$^{P}$-*tkt*$^{P}$-*pfk*$^{P}$-*rpe*$^{P}$-*rpiB-ywlG* (named pVWEx1-RuMP). The additional use of pVWEx1-RuMP led to a slower growth and a lower final $oD_{600}$ due to the expression of a large number of genes and the related metabolic burden, but no increased biomass formation from methanol could be observed (data not shown). A comparable approach in *B. subtilis*, overexpressing the genes *glpX*, *fba*, *tkt*, *pfk*, and *rpe* from *B. methanolicus*, has increased the assimilation of methanol into central metabolites (Brautaset *et al.*, 2013). It has to be tested if the genes are expressed correctly and are successfully translated into active proteins in *C. glutamicum*. Furthermore, the order of the genes in the expression vector may be reconsidered depending on the importance and activity of each enzyme in the RuMP pathway in *B. methanolicus*. Also, the introduction of additional promoters might be helpful to ensure a sufficient expression. Future studies might also focus on how the metabolism of recombinant *C. glutamicum* strains responds to methanol as carbon source, for example, by measuring mRNA levels using RNA-sequencing or microarrays, as performed before for the *C. glutamicum* wild type (Witthoff *et al.*, 2013).

### 3.2. The potential of *B. methanolicus* and *C. glutamicum* as industrial production hosts for cadaverine

Current production processes for monomeric polyamide building blocks are characterized by high production costs and so far only a few bio-polyamides, like PA-6,10, are commercialized (Weber, 2000). Therefore, the fermentative production of polyamines using bacteria provides a promising approach, especially with regard to the alternative and sustainable carbon source methanol.

Up to today, only a few methylotrophs have been used in industrial fermentations, like the yeast *Pichia* (*Komagataella*) *pastoris* for recombinant protein production (Zhang *et al.*, 2000; Dikicioglu *et al.*, 2014) and methanol-utilizing microorganisms as a source of single-cell protein (Tannenbaum & Wang, 1975). Different working groups have also explored the potential of methylotrophs and methanol as carbon source for the production of polyhydroxyalkanoates (PHAs). Suzuki *et al.* reported a production of 136 g/L of polyhydroxybutyrate (PHB) with 66% of the CDW using *Pseudomonas* sp. K, that is to date the highest yield for any methylotrophic organism (Suzuki *et al.*, 1986).

This work contributes to the growing field of (synthetic) methylotrophy by demonstrating a methanol-based production of the polyamine cadaverine by the natural methylotroph *B. methanolicus* (Chapter 2.1.), and the construction of a synthetic *C. glutamicum* strain that is able to partially convert methanol into cadaverine (Chapter 2.2.).

### 3.2.1. Strain development: current status and future targets for cadaverine production

Prior to the application in industrial fermentation processes, the development of a high-level-producing strain is required. Important factors are a high tolerance of the host to the produced compound and its inability to degrade it. For *B. methanolicus*, it could be shown in this work that this organism tolerates cadaverine concentrations of up to 200 mM (35 g/L) with a reduced growth rate of 20% (Chapter 2.1.). Based on these results, *B. methanolicus* represents a promising production host for cadaverine, although the tolerance level could lead to problems at very high production titers. Similar cadaverine tolerance studies have been performed with *E. coli* and *C. glutamicum*. For *E. coli*, the specific growth rate was reduced by 35% upon supplementation with 200 mM cadaverine (Qian *et al.*, 2011), whereas *C. glutamicum* seems slightly more robust tolerating up to 300 mM cadaverine on agar plate (Mimitsuka *et al.*, 2007), and still showing exponential growth in the presence of 1 M cadaverine in defined liquid medium, although accompanied by a reduced growth rate (Kind *et al.*, 2010a).

Cadaverine degradation and utilization pathways have been reported for both *C. glutamicum* and *E. coli* and several studies aiming at their deletion have been conducted. In *C. glutamicum*, cadaverine can be acetylated leading to the undesired by-product *N*-acetylcadaverine (Kind *et al.*, 2010a). To increase the production level and purity of cadaverine, the responsible enzyme, a cadaverine acetyltransferase, has been

identified and deletion of its corresponding gene (cg1722) has led to an 11% higher cadaverine yield (Kind *et al.*, 2010b). Degradation of cadaverine in *E. coli* has been prevented by deletion of *speE*, encoding a putrescine/cadaverine aminopropyltransferase, and *speG*, encoding a putative diamine acetyltransferase (Haywood & Large, 1985; Qian *et al.*, 2011) (Figure 3.2.). In addition, genes of the two putrescine degradation pathways YgjG-YdcW (Samsonova *et al.*, 2003; Samsonova *et al.*, 2005) and Puu pathway (Kurihara *et al.*, 2005; Kurihara *et al.*, 2006; Kurihara *et al.*, 2008; Kurihara *et al.*, 2009; Kurihara *et al.*, 2010) have been deleted as they may also use the similar diamine cadaverine as a substrate (Qian *et al.*, 2011). *ygjG* encodes a putrescine/cadaverine transaminase (Samsonova *et al.*, 2003) and the gene product of *puuA* catalyzes the γ-glutamylation of putrescine/cadaverine (Kurihara *et al.*, 2008) (Figure 3.2.). This work showed no formation of acetylcadaverine or other by-products in *B. methanolicus* (data not shown) and since this host was not able to degrade cadaverine (Chapter 2.1.), deletion of cadaverine utilization pathways is not required.

For engineering of an efficient and high-level cadaverine-producing strain, the genes and enzymes of the aspartate pathway, leading to the precursor L-lysine, represent future targets to improve the *B. methanolicus* and *C. glutamicum* strains that were constructed within this work (Chapter 2.1. & 2.2.). Thus, overexpression of the aspartokinase genes *dapG*, *lysC*, and *yclM* of *B. methanolicus*, which have been shown to increase L-lysine production 2-, 10-, and 60-fold respectively, could be a promising approach (Jakobsen *et al.*, 2009) (Figure 3.2.). In addition, combined overexpression of the three aspartate pathway genes *dapA*, *yclM*, and *lysA* has led to an 80-fold increased L-lysine production in shake flask experiments (Nærdal *et al.*, 2011). For a sufficient supply of L-lysine, the release of the aspartate pathway enzymes from feedback inhibition is also advantageous. In this work, a feedback-resistant variant of the aspartokinase LysC (Kalinowski *et al.*, 1991) was used to facilitate L-lysine production in *C. glutamicum* (Chapter 2.2.). Further targets in *C. glutamicum* are the homoserine dehydrogenase gene, where a mutation has led to increased L-lysine levels (Ohnishi *et al.*, 2002; Georgi *et al.*, 2005) and the enzymes of the dehydrogenase and succinylase branch of the aspartate pathway (Figure 3.2.), since it has been shown that a higher flux through these routes resulted in an enhanced formation of L-lysine (Eggeling *et al.*, 1998; Kelle *et al.*, 2005; Becker *et al.*, 2011). Moreover, the deletion of *lysE*, encoding the lysine exporter LysE, provides another strategy for a high precursor level (Vrljic *et al.*, 1996; Vrljic *et al.*, 1999; Bellmann *et al.*, 2001; Kind *et al.*, 2011). Today, every gene of the L-lysine

biosynthesis pathway of *C. glutamicum* is covered with at least one patent, showing the importance of the involved enzymes for the production of L-lysine (Wittmann & Becker, 2007), and thus, also cadaverine.

Besides regulating the activity of enzymes of the aspartate pathway, the supply of the ubiquitous key metabolites pyruvate, phosphoenolpyruvate (PEP), acetyl-CoA, oxaloacetate (OAA), and α-ketoglutarate (Alber, 2011), which are essential for the production of different amino acids and other compounds (Heggeset *et al.*, 2012), could be addressed. Hence, regulation of the carbon flow at the PEP–pyruvate–OAA node, represents an interesting target for metabolic engineering (Sauer & Eikmanns, 2005). Genome sequencing of *B. methanolicus* revealed a pyruvate carboxylase (Pc) gene (*pyc*), but no PEP carboxylase gene has been identified (Heggeset *et al.*, 2012). Since no increased L-lysine levels were obtained upon overexpression of *pyc* in *B. methanolicus*, the supply of OAA does not represent a critical factor in this organism (Brautaset *et al.*, 2010). In contrast, Pc was identified as a major bottleneck for the production of L-lysine in *C. glutamicum* (Peters-Wendisch *et al.*, 2001) and as an effect of overexpressing its corresponding gene *pyc*, the formation of L-lysine was increased by 50% (Peters-Wendisch *et al.*, 2001). Increased OAA and L-aspartate levels were reported in consequence of deleting *pck*, encoding phosphoenolpyruvate carboxykinase, which led to an increased flux towards L-lysine and resulted in a higher L-lysine accumulation (Petersen *et al.*, 2001; Riedel *et al.*, 2001).

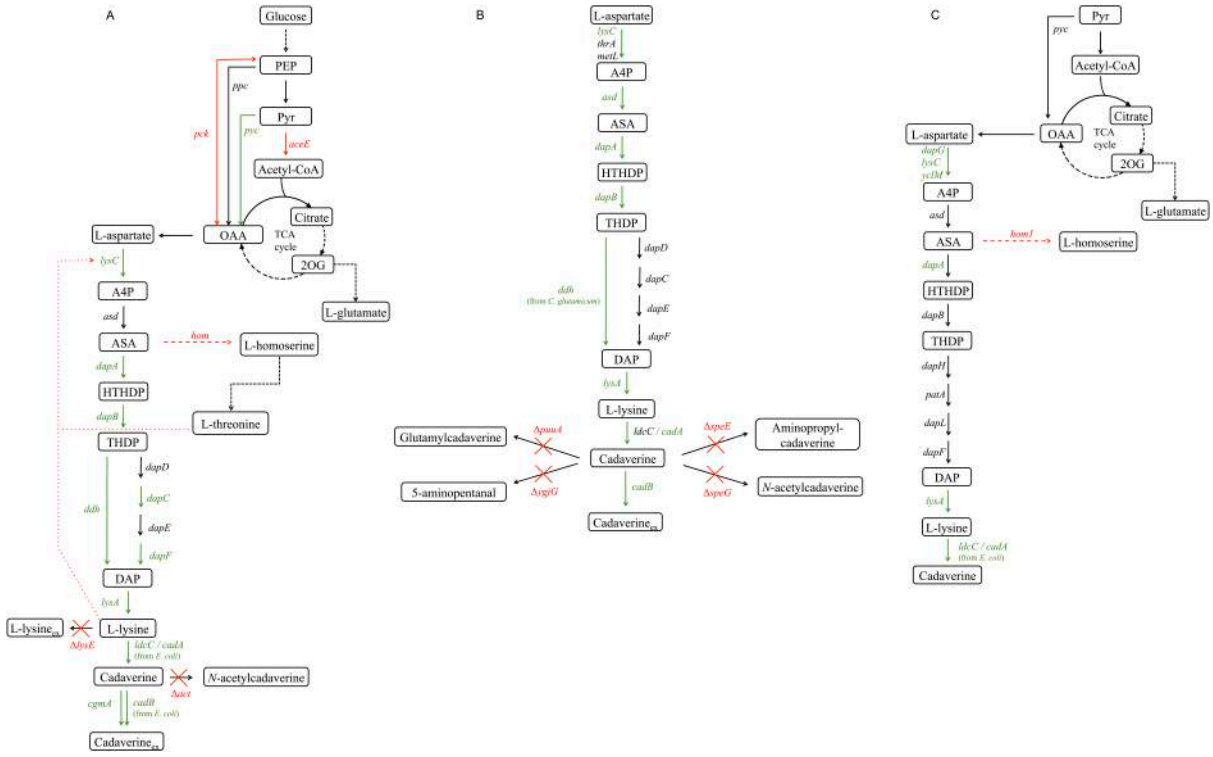


**Figure 3.2.: Metabolic engineering of (A) *C. glutamicum,* (B) *E. coli,* and (C) *B. methanolicus* for the production of L-lysine and cadaverine.** Modifications leading to an increased L-lysine and cadaverine formation used in this work and reported in literature are presented. Green color indicates homologous or heterologous expression, whereas red color plus the symbol "X" refers to gene deletion. Red color in combination with a dashed line indicates gene attenuation and in combination with a dotted line release from feedback inhibition of the corresponding enzyme.

*Abbreviations of intermediates*: 2OG, 2-oxoglutarate; A4P, aspartate 4-phosphate; acetyl-CoA, acetyl coenzyme A; ASA, aspartate semialdehyde; DAP, *meso*-2,6-diaminopimelate; HTHDP, (2S,4S)-4-hydroxy-2,3,4,5-tetrahydrodipicolinate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; THDP, 2,3,4,5-tetrahydrodipicolinate.
*The enzymes encoded by the corresponding genes are: aceE*, E1p enzyme of the pyruvate dehydrogenase complex; *act*, cadaverine acetyltransferase; *asd*, aspartate-semialdehyde dehydrogenase; *cadA*, lysine decarboxylase; *cadB*, cadaverine:lysine antiporter; *cgmA*, major facilitator permease; *dapA*, 4-hydroxy-tetrahydrodipicolinate synthase; *dapB*, 4-hydroxytetrahydrodipicolinate reductase; *dapC*, *N*-succinyl-diaminopimelate aminotransferase; *dapD*, tetrahydrodipicolinate succinylase; *dapE*, succinyl-diaminopimelate desuccinylase; *dapF*, diaminopimelate epimerase; *dapG*, aspartokinase; *dapH*, tetrahydrodipicolinate *N*-acetyltransferase; *dapL*, *N*-acetyldiaminopimelate deacetylase; *ddh*, diaminopimelate dehydrogenase; *hom*, homoserine dehydrogenase; *ldcC*, lysine decarboxylase; *lysA*, diaminopimelate decarboxylase; *lysC*, aspartokinase; *lysE*, lysine exporter; *metL*, aspartokinase; *pck*, phosphoenolpyruvate carboxykinase; *ppc*, phosphoenolpyruvate carboxylase; *puuA*, glutamate–putrescine/glutamate–cadaverine ligase; *pyc*, pyruvate carboxylase; *speE*, putrescine/cadaverine aminopropyltransferase; *speG*, spermidine acetyltransferase; *thrA*, aspartokinase; *yclM*, aspartokinase; *ygjG*, putrescine/cadaverine aminotransferase.

Apart from the supply of the precursor L-lysine, the decarboxylation step from L-lysine to cadaverine could be optimized. In this work, cadaverine production in *C. glutamicum* (Chapter 2.2.) and *B. methanolicus* (Chapter 2.1.) was realized by heterologous expression of the well studied lysine decarboxylase genes *ldcC* and *cadA* of *E. coli*. A recent study reported a 30% higher cadaverine production using LdcC instead of CadA in *C. glutamicum* (Kind *et al.*, 2010a). Based on these findings, LdcC was the only considered isozyme in this work. For *B. methanolicus* both variants were tested, resulting in an up to threefold higher cadaverine production by CadA in the L-lysine overproducing strain M168-20 and wild type MGA3, which was supported by a 12-fold higher *in vitro* activity of CadA in crude cell extracts of M168-20 (Chapter 2.1.). Although L-lysine did not occur as a significant by-product in the majority of the constructed strains, the conversion of L-lysine into cadaverine might be accelerated. The decarboxylation of L-lysine by engineered bacteria covers a broad spectrum of different lysine decarboxylases. The two lysine decarboxylases LdcC and CadA from *E. coli* have already been used for the cadaverine production in *E. coli* and *C. glutamicum* (Mimitsuka *et al.*, 2007; Tateno *et al.*, 2009; Kind *et al.*, 2010a). Besides, Ldc from *Hafnia alvei* (Li *et al.*, 2014a) and LdcC from *Klebsiella oxytoca* (Li *et al.*, 2014b) have been used in *C. glutamicum*.

High-level-producing strains are characterized by an efficient secretion of the product. It has been observed that overexpression of the putative cadaverine exporter *cgmA* has led to an increased cadaverine production, whereas its deletion has reduced the secretion by 90% (Kind *et al.*, 2011) (Figure 3.2.). In *E. coli*, cadaverine is exported by CadB, a lysine:cadaverine antiporter (Figure 3.2.). At acidic pH and in the presence of lysine in the extracellular medium, lysine is imported and cadaverine is exported, helping to maintain the intracellular pH of the cell (Soksawatmaekhin *et al.*, 2004). It has been shown that by heterologous expression of *cadB*, the cadaverine secretion in *C. glutamicum* could be increased by 22% (Li *et al.*, 2014a). For *B. methanolicus*, the co-expression of *cadB* with *cadA* and *ldcC* respectively was investigated within this work. The effect of CadB on the cadaverine production was measured at different pH conditions but no significantly increased cadaverine formation could be observed (Table 3.1.). Either CadB is not functional in *B. methanolicus* or some fine-tuning of the expression is needed, since heterologous expression of membrane proteins is known to be challenging (Miroux & Walker, 1996; Schulz, 2002; Terpe, 2006). Besides the fermentative production by microorganisms, whole-cell biocatalysis is an alternative ap-

proach. For whole-cell biocatalysis using *E. coli*, *cadB* has been fused to the *pelB* signal peptide that controls translocation and increases both transcription and translation efficiencies of the fused gene (Lei *et al.*, 1987; Sletta *et al.*, 2007). The use of CadA together with CadB resulted in a 12% higher production of cadaverine than using CadA alone. A production of 221 g/L of cadaverine with a molar yield of 92% from L-lysine has been obtained (Ma *et al.*, 2014).

**Table 3.1.: Cadaverine production by recombinant *B. methanolicus* M168-20 strains.**

| | M168-20(pTH1mp-*ldcC*)* | M168-20(pTH1mp-*ldcC-cadB*) | M168-20(pTH1mp-*cadA*) | M168-20(pTH1mp-*cadA-cadB*) |
|---|---|---|---|---|
| pH | Cadaverine (mg/L) | Cadaverine (mg/L) | Cadaverine (mg/L) | Cadaverine (mg/L) |
| < 7.0 | 52 ± 5 (pH 6.5) | < 5 (pH 6.6) | 50 ± 10 (pH 6.5) | 45 ± 5 (pH 6) |
| 7.2 | 135 ± 10 | 30 ± 10 | 465 ± 140 | 290 ± 15 |
| 8.5 | 305 ± 30 | 190 ± 10 | 480 ± 30 | 500 ± 65 |

*data imported from (Nærdal *et al.*, 2015). The mean values (mg/L) and standard deviation of triplicate shake flask cultures are presented.

When evaluating the potential as production hosts, the maximum yield has to be considered. Similar maximum theoretical yields of L-lysine and thus cadaverine can be obtained by *B. methanolicus* from methanol (0.71/0.81 g L-lysine/g methanol) and by *C. glutamicum* from glucose (0.68/0.82 g L-lysine/g glucose). For *C. glutamicum* the maximum yield depends on whether the succinylase branch (0.68 g/g) or the dehydrogenase branch (0.82 g/g) is used. For *B. methanolicus*, the yield depends on the formation of a NAD(P)H upon formaldehyde oxidation (Large & Bamforth, 1988; Brautaset *et al.*, 2007).

The relevance of both *C. glutamicum* and *B. methanolicus* as production hosts for cadaverine is supported by the underlying findings and the availability of L-lysine overproducing strains (Chapter 2.1. & 2.2.). *C. glutamicum* has the advantage that methods for directed gene deletion are available, whereas *B. methanolicus* lacks those tools and metabolic engineering is therefore limited to heterologous and homologous gene expression. Additionally, current vector systems allow regulation of gene expression in both organisms. However, transformation efficiency of *B. methanolicus* is rather low, indicated by $10^3$-$10^4$ transformants per 1 μg DNA (Brautaset, unpublished).

### 3.2.2. Methylotrophs in biotechnological fermentation processes

Compared to conventional microbial fermentations, there are differences regarding the bioprocess technology using methylotrophs (Dijkhuizen *et al.*, 1985). An important aspect is the possibility of using cheap mineral media, as there is no need for adding the carbon source as a complex medium component, like starch hydrolysates or molasses (Linton & Niekus, 1987; Bertau *et al.*, 2014). For example, *B. methanolicus* is able to grow in cheap seawater-based medium with methanol as carbon source (Komives *et al.*, 2005). This is an important factor regarding fermentation economics, as the choice of raw materials, especially the carbon source, and media composition account for a major part of the fermentation costs (Hermann, 2003; Todaro & Vogel, 2014). The use of mineral media also leads to reduced costs for the downstream processing of products that are secreted into the medium (Schrader *et al.*, 2009). Methanol is easy to transport and store, pure, and can be completely utilized by bacteria during fermentation (Schendel *et al.*, 1990). In contrast, molasses, which is mainly used in the fermentation industry, cannot be stored over a long period of time, the composition varies, and components have to be removed from the product in the downstream processing (Linton & Niekus, 1987). The low methanol prices expected in future (Chapter 1.) could allow for profitable fermentation processes (Ugalde & Castrillo, 2002; Bertau *et al.*, 2014).

However, methanol fermentations go hand in hand with a high oxygen demand, because methanol is more reduced than sugars, leading to a higher heat output, which is an issue regarding fermentation economics (Snedecor & Cooney, 1974). One solution to this is the application of thermophilic methylotrophs, since most of the bacteria used for fermentation in industry belong to the group of mesophiles. This aspect also has to be taken into account when evaluating the potential of a synthetic methylotrophic

*C. glutamicum* strain. The construction of mutants either by random or directed mutagenesis, leading to strains with an increased thermotolerance, can be a way to overcome this drawback. As an example, a *C. glutamicum* L-lysine producing strain has been developed by "genome breeding", which is able to grow and produce with a 20% increased L-lysine yield during fermentation at 40 °C (Ohnishi *et al.*, 2003).

The key advantages of high-temperature fermentation can be discussed under the following aspects: i) process costs and ii) risk of contamination. For fermentation processes, the cooling costs represent an important factor, as the produced metabolic heat has to be dissipated (Bailey & Ollis, 1986). Maybe it would be possible to eliminate or at least drastically reduce the costs, when *B. methanolicus* is used and fermentation processes are performed at 50 °C (Thummer (BASF), personal communication). The cooling requirements for fermentations at 50 °C can be lowered by about 18% to 40% compared to fermentations at 30 °C (Lee *et al.*, 1996). For example, it has been calculated that a fermentation temperature rise of 5 °C would amount to 30.000 USD/year for a 30.000-kL scale ethanol plant (Abdel-Banat *et al.*, 2010). However, it has been reported that for a 200 $m^3$ reactor the cooling water requirements are the same for an organism that grows at 35 °C on glucose and one growing at 50 °C on methanol (Komives *et al.*, 2005). High-temperature fermentations also have a lower risk of contaminations. L-lactic acid production has been successfully performed at 50 °C with *Bacillus* sp. 2-6 and *Bacillus coagulans* WCP10-4, leading to a reduced risk of microbial contaminations even under non-sterilized conditions (Qin *et al.*, 2009; Zhou *et al.*, 2013). If no sterilization of the medium or fermenter is needed, this also reduces the costs for the fermentation process. But it has to be tested if this is also feasible at industrial scale. One main downside concerning high-temperature fermentation is the about 20% lowered oxygen solubility between 50 °C and 30 °C, leading to a higher demand for oxygen supply (Brautaset *et al.*, 2007).

The three main fermentation techniques are batch, fed-batch, and continuous fermentation. Whereas in batch fermentation all nutrients for growth and production are present before inoculation, the nutrients in fed-batch fermentation are fed continuously or intermittently with or without feedback control. For continuous fermentation ("chemostat"), the feed medium is continuously added to the fermenter at a constant rate and the culture medium is removed at the same rate (Todaro & Vogel, 2014). Advantages of fed-batch fermentation are the possibility of controlling the growth rate and oxygen

demand of the production host and preventing negative effects such as substrate inhibition or catabolite repression (McNeil & Harvey, 2008; Todaro & Vogel, 2014). Further, a high-cell-density can be achieved by using fed-batch, since there are no limitations in carbon supply. A disadvantage is the prerequisite for detailed knowledge of the organism's growth and production behavior. Batch fermentations are, for example, characterized by the simplicity of performing the experiment, production of non-growth related secondary metabolites, and a lower risk of contaminations (McNeil & Harvey, 2008; Todaro & Vogel, 2014).

For cadaverine production by *B. methanolicus*, a fed-batch fermentation was performed in this work (Chapter 2.1.), providing the opportunity of controlling and maintaining methanol at a limited and defined concentration (Schendel *et al.*, 1990; Lee *et al.*, 1996). Although methanol reduces the risk of contaminations, a negative effect on the production host has to be taken into consideration (Bourque *et al.*, 1992; Kim *et al.*, 2003). The feeding strategy prevents formaldehyde detoxification, due to high methanol levels, and partial loss of carbon from methanol as $CO_2$ that would be accompanied by an increased oxygen demand (Pluschkell & Flickinger, 2002; Brautaset *et al.*, 2007). *B. methanolicus* can tolerate up to 1 M of methanol, making this organism a suitable candidate for the industry (Jakobsen *et al.*, 2006). For the use as a production host in industrial fermentation processes, high production levels are a requirement. The highest production of cadaverine by *B. methanolicus* resulted in a volume-corrected 11.3 g/L during high-cell-density fed-batch methanol fermentation with a productivity of 0.28 g/L $h^{-1}$ (Chapter 2.1.). Engineered *E. coli* and *C. glutamicum* strains were able to produce up to 9.6 g/L and 88 g/L of cadaverine in fed-batch cultivation from glucose with a productivity of 0.32 g/L $h^{-1}$ and 2.2 g/L $h^{-1}$ respectively (Qian *et al.*, 2011; Kind *et al.*, 2014). In comparison, *B. methanolicus* showed a similar productivity to *E. coli*, provides the advantage of using methanol as sole carbon source and a high-production titer was obtained, showing the potential as a production host for cadaverine.

### 3.2.3. Downstream processing

Besides an efficient cadaverine-producing strain and alternative carbon sources, the recovery of the product, also called downstream processing, is an important factor of fermentation processes. Downstream processing is a limiting step in the commercialization of biotechnological processes and the costs can account for up to 50-80% of the total fermentation costs, depending on the product (Hacking, 1987; Sieberz *et al.*, 2014). The fermentative production of cadaverine and other diamines depends on aqueous solvents and final product concentrations are much lower compared to chemical processes. Further, as cadaverine is used as monomer for the production of polyamides, a high grade of purity (> 99.5%) is required (Kind *et al.*, 2011).

Recently, a method for fast and efficient isolation of cadaverine from the fermentation broth at industrial scale has been proposed. In the first step, cells are removed by filtration, separation, or flocculation. Then, the pH is adjusted to alkaline conditions. A thermal treatment, for example, to remove *N*-acetylcadaverine as a by-product and increase cadaverine production titers, is optional since this step leads to higher energy costs. The extraction is performed using an organic solvent that is stable at a high pH, such as *n*-butanol. Alkaline conditions are used to facilitate the transfer of cadaverine into the organic phase, since cadaverine is a strong base with a positive charge at physiological pH. In the last step, cadaverine is isolated from the cadaverine-comprising phase by distillation, chromatography, or precipitation. Interestingly, using dicarboxylic acids for precipitation of cadaverine is also functional and the salt can be directly used for polymerization, leading to the formation of polyamides (Völkert *et al.*, 2010; Kind *et al.*, 2011).

## 3.3. Conclusions and perspectives

The main purpose of this work was to determine the potential of *B. methanolicus* and *C. glutamicum* as hosts for the methanol-based production of the industrial relevant compound cadaverine. *B. methanolicus* only lacked a lysine decarboxylase for the formation of cadaverine, whereas in *C. glutamicum* a L-lysine and cadaverine production module as well as a module for the utilization of methanol had to be implemented.

The results of this work support the idea that the construction of a synthetic methylotroph can be achieved. Besides the strategy to enable methanol assimilation in non-methylotrophic bacteria, there are several other concepts. So far, different approaches in

the field of synthetic methylotrophy have been carried out. For example, a biocatalytic cycle, named methanol condensation cycle, for methanol conversion to higher-chain alcohols has been constructed. This pathway combines the RuMP pathway and the non-oxidative glycolysis and has been successfully used for the conversion of methanol into ethanol and *n*-butanol in a cell-free system (Bogorad *et al.*, 2014). Another approach is the implementation of a synthetic pathway based on computational design. This has been shown for the assimilation of formate in *E. coli* using the computationally designed enzyme formolase, which catalyzes the reaction from three formaldehyde molecules to one dihydroxyacetone (DHA) molecule. However, no cell growth has been observed due to the low efficiency of the formolase (Siegel *et al.*, 2015; Tai & Zhang, 2015).
A key strength of this work was the engineering of *C. glutamicum* for the conversion of methanol into cadaverine and the second major finding was to show, for the first time, a methanol-based production of cadaverine by the use of *B. methanolicus*. Except the fermentative production of cadaverine using microorganisms as an alternative to the current chemical synthesis, a method for the conversion of α,ω-diols to α,ω-diamines has been recently reported (Sattler *et al.*, 2012). Using a purified $NAD^+$-dependent alcohol dehydrogenase, a ω-transaminase, and an alanine dehydrogenase in an *in vitro* biotransformation setup, 1,8-octanediol and 1,10-decanediol have been converted into the corresponding diamines with product yields of 99% under optimal conditions (Sattler *et al.*, 2012; Schaffer & Haas, 2014). An advantage of this approach is its self-sufficiency regarding the redox equivalents (Sattler *et al.*, 2012).

On the one hand, future work will focus on the further understanding of methanol assimilation in natural methylotrophs, and on the other hand will be characterized by overcoming the current challenges of implementing a synthetic methanol assimilation pathway into non-methylotrophic bacteria. Further, *B. methanolicus* MGA3, which is able to secrete about 60 g/L of L-glutamate (Brautaset *et al.*, 2003; Brautaset *et al.*, 2007), opens up future perspectives as a production host for L-glutamate and its derived decarboxylation product γ-aminobutyric acid. In addition, L-lysine-producing strains of *C. glutamicum* and *B. methanolicus* could be engineered for other products, such as 5-aminovalerate, which has been produced by engineered *E. coli* strains, heterologously expressing genes of *P. putida* (Adkins *et al.*, 2013; Park *et al.*, 2013), and can be utilized for the synthesis of PA-6,5 (Park *et al.*, 2014).

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# A. Appendix

## A.1. Supplementary material to "Production of cadaverine by engineered *Corynebacterium glutamicum* using methanol as co-substrate "

**Table A.1.1.: Strains and plasmids used in this study.**

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| *E. coli* DH5α | F¯ *thi-1 endA1 hsdR17*(r¯m¯) *supE44* Δ*lacU169* (Φ80*lacZ*ΔM15) *recA1 gyrA96 relA1* | (Hanahan, 1983) |
| *C. glutamicum* strains | | |
| ATCC 13032 (WT) | Wild type strain | American Type Culture Collection |
| Δ*ald*Δ*fadH* (ΔΔ) | in-frame deletion of the genes *ald* and *fadH* of *C. glutamicum* WT | (Lessmeier *et al.*, 2013) |
| Plasmids | | |
| pEKEx3 | Spec$^r$; *C. glutamicum*/*E. coli* shuttle vector ($P_{tac}$ *lacI*$^q$; pBL1, $OriV_{C.g.}$, $OriV_{E.c.}$) | (Stansen *et al.*, 2005) |
| pEKEx3-*mdh* | derived from pEKEx3, for regulated expression of *mdh* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh2* | derived from pEKEx3, for regulated expression of *mdh2* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh3* | derived from pEKEx3, for regulated expression of *mdh3* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh*_PB1 | derived from pEKEx3, for regulated expression of *mdh* from *B. methanolicus* PB1 | This study |
| pEKEx3-*mdh1*_PB1 | derived from pEKEx3, for regulated expression of *mdh1* from *B. methanolicus* PB1 | This study |
| pEKEx3-*mdh2*_PB1 | derived from pEKEx3, for regulated expression of *mdh2* from *B. methanolicus* PB1 | This study |
| pEKEx3-*mdh*_S97G | derived from pEKEx3, for regulated expression of mutated *mdh* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh2*_S97G | derived from pEKEx3, for regulated expression of mutated *mdh2* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh3*_S97G | derived from pEKEx3, for regulated expression of mutated *mdh3* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh, act* | derived from pEKEx3, for regulated expression of *mdh* and *act* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh2, act* | derived from pEKEx3, for regulated expression of *mdh2* and *act* from *B. methanolicus* MGA3 | This study |

| | | |
|---|---|---|
| pEKEx3-*mdh3, act* | derived from pEKEx3, for regulated expression of *mdh3* and *act* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*hps,phi* | derived from pEKEx3, for regulated expression of *hps* and *phi* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*hxlAB* | derived from pEKEx3, for regulated expression of *hxlA* and *hxlB* from *B. subtilis* 168 | This study |
| pEKEx3-*mdh,hxlAB* | derived from pEKEx3, for regulated expression of *mdh* from *B. methanolicus* MGA3 and *hxlA* and *hxlB* from *B. subtilis* 168 | This study |
| pEKEx3-*mdh, mdh2* | derived from pEKEx3, for regulated expression of *mdh* and *mdh2* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh, mdh3* | derived from pEKEx3, for regulated expression of *mdh* and *mdh3* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh2, mdh3* | derived from pEKEx3, for regulated expression of *mdh2* and *mdh3* from *B. methanolicus* MGA3 | This study |
| pEKEx3-*mdh, mdh2, mdh3* | derived from pEKEx3, for regulated expression of *mdh, mdh2* and *mdh3* from *B. methanolicus* MGA3 | This study |
| pVWEx1 | $Km^r$; *C. glutamicum*/*E. coli* shuttle vector ($P_{tac}$ *lacI*$^q$-*ori*$V_{C.g}$. *ori*$V_{E.c}$.) | (Peters-Wendisch *et al.*, 2001) |
| pVWEx1-*adhA* | derived from pVWEx1, for regulated expression of *adhA* from *C. glutamicum* | This study |
| pVWEx1-*mdh* | derived from pVWEx1, for regulated expression of *mdh* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh2* | derived from pVWEx1, for regulated expression of *mdh2* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh3* | derived from pVWEx1, for regulated expression of *mdh3* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh*_S97G | derived from pVWEx1, for regulated expression of mutated *mdh* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh2*_S97G | derived from pVWEx1, for regulated expression of mutated *mdh2* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh3*_S97G | derived from pVWEx1, for regulated expression of mutated *mdh3* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh, act* | derived from pVWEx1, for regulated expression of *mdh* and *act* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh2, act* | derived from pVWEx1, for regulated expression of *mdh2* and *act* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*mdh3, act* | derived from pVWEx1, for regulated expression of *mdh3* and *act* from *B. methanolicus* MGA3 | This study |
| pVWEx1-*lysC*$^{fbr}$-*ldcC* | production of cadaverine; feedback-resistant aspartokinase (*lysC*$^{fbr}$) and lysine decarboxylase LdcC from *E. coli* | This study |

**Table A.1.2.: Oligonucleotides used in this study.**

| Name | Sequence (5'→ 3')[a] | Purpose, restriction site |
|---|---|---|
| *adhA* fw *Sbf*I | TTT**CCTGCAGG***GAAAGGAGGCCCTTCAG*ATGACCACT GCTGCACC | expression of *adhA*, *Sbf*I |
| *adhA* rev *Sal*I | CCG**GTCGAC**TTAGAAACGAATCGCCACAC | expression of *adhA*, *Sal*I |
| *mdh* fw *Sbf*I | GG**CCTGCAGG***GAAAGGAGGCCCTTCAG*ATGACAACA AACTTTTTCATTCCACC | expression of *mdh*, *Sbf*I |
| *mdh* rev *Bam*HI | GGC**GGATCC**TTACATAGCGTTTTTGATGATTTGTGCA AT | expression of *mdh*, *Bam*HI |
| *mdh2* fw *Sbf*I | AA**CCTGCAGG***GAAAGGAGGCCCTTCAG*ATGACAAAC ACTCAAAGTGCAT | expression of *mdh2*, *Sbf*I |
| *mdh2* rev *Bam*HI | GGC**GGATCC**TTACATCGCATTTTTAATAATTTGGATG AC | expression of *mdh2*, *Bam*HI |
| *mdh3* fw *Sbf*I | GG**CCTGCAGG***GAAAGGAGGCCCTTCAG*ATGAAAAAC ACTCAAAGTGC | expression of *mdh3*, *Sbf*I |
| *mdh3* rev *Bam*HI | CC**GGATCC**TTACATAGCATTTTTAATAATTTGGATGA | expression of *mdh3*, *Bam*HI |
| *act* fw *Bam*HI | CCG**GGATCC***GAAAGGAGGCCCTTCAG*ATGGGAAAAT TATTTGAGGAAAAAACAAT | expression of *act*, *Bam*HI |
| *act* rev *Bam*HI | GGG**GGATCC**TCATTTATTTTTGAGAGCCTCTTGAAG C | expression of *act*, *Bam*HI |
| *mdh*_PB1 fw *Sbf*I | GCG**CCTGCAGG***GAAAGGAGGCCCTTCAG*ATGACTAA AACAAAATTTTTCATTCCATC | expression of *mdh*_PB1, *Sbf*I |
| *mdh*_PB1 rev *SalI* | GC**GTCGAC**TCACATAGCATTTTTAATAATTTGTATAA CTTCTTC | expression of *mdh*_PB1, *SalI* |
| *mdh1*_PB1 fw *Sbf*I | GCG**CCTGCAGG***GAAAGGAGGCCCTTCAG*ATGACTAA AACAAAATTTTTCATTCCATC | expression of *mdh1*_PB1, *Sbf*I |
| *mdh1*_PB1 rev *SalI* | GC**GTCGAC**TCACAGAGCGTTTTTGATGATTTGTTG | expression of *mdh1*_PB1, *SalI* |
| *mdh2*_PB1 fw *Sbf*I | GCG**CCTGCAGG***GAAAGGAGGCCCTTCAG*ATGACAAA CACTCAAAGTATATTTTAC | expression of *mdh2*_PB1, *Sbf*I |
| *mdh2*_PB1 rev *SalI* | GC**GTCGAC**TCACATAGCATTTTTAATAATTTGTATAA CTTCTTC | expression of *mdh2*_PB1, *SalI* |
| *hps* fw *Bam*HI | CCT**GGATCC***GAAAGGAGGCCCTTCAG*ATGGAACTTCA ATTAGCTCT | expression of *hps*, *Bam*HI |
| *hps* rev *Sac*I | GCC**GAGCTC**TCATAACCCTTGTTTAACTA | expression of *hps*, *Sac*I |
| *phi* fw *Sac*I | CGC**GAGCTC***GAAAGGAGGCCCTTCAG*ATGCTGACAA CTGAATTTTT | expression of *phi*, *Sac*I |
| *phi* rev *Eco*RI | CGC**GAATTC**CTACTCGAGATTGGCATGTC | expression of *phi*, *Eco*RI |
| *hxlA* fw | *GAAAGGAGGCCCTTCAG*ATGGAATTACAGCTTGCATT AGACCTCG | expression of *hxlA* |
| *hxlA* rev | GGGTTATCCTTGGACAATCAGCTGC | expression of *hxlA* |
| *hxlB* fw | *GAAAGGAGGCCCTTCAG*ATGAAAACGACTGAATACGT AGCGG | expression of *hxlB* |
| *hxlB* rev | GGGCTATTCAAGGTTTGCGTGGTG | expression of *hxlB* |

[a] Restriction sites in the oligonucleotides are bold, start codons are underlined, and ribosome binding sites including spacer are italicized.

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# Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation eigenständig und ohne unerlaubte Hilfe angefertigt habe. Ich versichere, dass ich alle in Anspruch genommenen Quellen und Hilfsmittel angegeben, sowie Zitate kenntlich gemacht habe. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form nicht anderweitig als Prüfungsarbeit eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Bielefeld, den 02.07.2015. (Johannes Pfeifenschneider)

ANOTHER QUEST WILL START FROM HERE