ABSTRACT

BOZDAG, AHMET. Investigation of Methanol and Formaldehyde Metabolism in *Bacillus methanolicus*.(Under the direction of Prof. Michael C. Flickinger).

Bacillus methanolicus is a Gram-positive aerobic methylotroph growing optimally at 50-53 °C. Wild-type strains of *B. methanolicus* have been reported to secrete 58 g/l of L-glutamate in fed-batch cultures. Mutants of *B. methanolicus* created via classical mutangenesis can secrete 37 g/l of L-lysine, at 50 °C. The genes required for methylotrophyin *B. methanolicus* are encoded by an endogenous plasmid, pBM19 in strain MGA3, except for hexulose phosphate synthase (*hps*) and phosphohexuloisomerase (*phi*) which are encoded on the chromosome.It is a promising candidate for industrial production of chemical intermediates or amino acids from methanol. *B. methanolicus* employs the ribulose monophospate (RuMP) pathway to assimilate the carbon derived from the methanol, but enzymes that dissimilate carbon are not identified, although formaldehyde and formate were identified as intermediates by ^{13}C NMR. It is important to understand how methanol is oxidized to formaldehyde and then, to formate and carbon dioxide. This study aims to elucidate the methanol dissimilation pathway of *B. methanolicus*.

Growth rates of *B. methanolicus* MGA3 were assessed on methanol, mannitol, and glucose as a substrate. *B. methanolicus* achieved maximum growth rate, μ_{max}, when growing on 25 mM methanol, 0.65±0.007 h^{-1}, and it gradually decreased to 0.231±0.004 h^{-1} at 2Mmethanol concentration which demonstrates substrate inhibition. The maximum growth rates (μ_{max}) of *B. methanolicus* MGA3 on mannitol and glucose are 0.532±0.002 and

0.336±0.003 h^{-1}, respectively. Spiking with 100 mM methanol did not cause any growth perturbation of*B. methanolicus* MGA3 when grown on methanol, whereas, 50 mM methanol was enough to halt the growth when it was grown on mannitol. However, growth of *B. methanolicus* MGA3 on methanol was halted completely by a 1 mM formaldehyde spike, while mannitol grown *B. methanolicus* MGA3 can tolerate 2 mM formaldehyde addition. Formate did not have any apparent growth perturbations at tested levels (up to 2 mM).

B. methanolicus MGA3 has a small genome, ~3.3 Mb, and contains 2,773 ORFs larger than 100 bp. Its genome encodes two methanol dehydrogenase(*mdh*) genes beside the *mdh* on pBM19. All of the RuMP pathway genes encoded on the pBM19 have counterparts encoded on the chromosome also. The genome contains putative tetrahydrofolate (THF)-linked enzymes for formaldehyde oxidation besides putative aldehyde dehydrogenase(*aldh*) genes. Two putative formate dehydrogenase (*fdh*) genes were also identified in the genome as were the genes of the Wood-Ljungdahl pathway genes.*B. methanolicus* strain PB1 was also found to carry a plasmid which we named as pBM20. The difference in the formaldehyde dissimilation pathway of strain PB1 was shown via Southern Blot. The PB1 strain also has 2-fold higher formaldehyde dehydrogenase and 3-fold higher formate dehydrogenase activity compared to strain MGA3.

The methanol dissimilation pathway genes,- putative THF-linked formaldehyde oxidation genes, putative *aldh* genes, putative *fdh* gene- are all down regulated in response to spikes of methanol, formaldehyde and formate. However, the regulation of RuMP pathway genes were different in methanol vs. mannitol grown *B. methanolicus* MGA3 in that they were down regulated from already high levels in methanol grown cells whereas they were upregulated in mannitol grown cells. Formate is a more potent regulatory compound compared to methanol and formaldehyde.

Investigation of Methanol and Formaldehyde Metabolism in *Bacillus Methanolicus*

by
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DEDICATION

To my wife Turkan and daughter Vildan

BIOGRAPHY

Ahmet Bozdag was born in Besni, Southeast Turkey as the youngest of a large family. He moved to Gaziantep after elementary school, to seek a better education at the age of 11. He completed his high school and attended Middle East Technical University (METU) in Ankara. He taught science in middle and high schools in Adiyaman and Ankara while seeking a graduate degree at METU. Winning the Diversity Visa lottery, or Green Card, he moved to United States with his ambition to seek a doctoral degree in microbiology. He earned his Master's Degree at Georgia College & State University before attending the Microbiology department at NCSU during which he married to a beautiful woman, Turkan. He had his first child, a gorgeous baby girl Vildan just before completing his PhD degree.

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CHAPTER 1

INTRODUCTION

Methylotrophic microorganisms

Methylotrophs are those microorganisms able to grow at the expense of reduced carbon compounds containing one or more carbon atoms but no carbon-carbon bonds (Anthony, 1982). This is a broad definition and includes bacteria capable of growth on methane, methanol, methylated amines, halogenated methanes and methylated sulfur compounds. Obligate methylotrophs exclusively utilize C_1 compounds as a sole carbon and energy source while facultative methylotrophs can utilize multicarbon compounds as well. Although still considered to be methylotrophic bacteria, the group that is able to catalyze the conversion of methane to methanol is further characterized as methanotrophic bacteria. Although methylotrophic microorganisms were known to be Gram-negative, obligate aerobes until 1983 (Haber et. al., 1983), this statement has been broadened by the characterization of Gram-positive methylotrophs.

Habitats of methylotrophs

Methylotrophic bacteria are ubiquitous in the environment and have a significant contribution to the global carbon cycle. They can be isolated from a wide range of habitats. Nearly all samples taken from mud, swamps, rivers, rice paddies, oceans, and ponds, soils from meadows, and streams, sewage sludge, and several other habitats yielded methylotrophs in enrichments (Hanson, 1992). Although their presence may be attributed to the abundance of methane in these environments because of methanogens, methanol produced as the end product of pectin metabolism also offers a suitable niche for methylotrophs to thrive. Methanol is the major end product of pectin metabolism by pectinolytic microorganisms (Schink and Zeikus, 1980), although they do not consume it. Methanol concentrations are stoichiometrically coupled to pectin metabolism in pectinolytic microorganisms whether it is aerobic or anaerobic (Schink and Zeikus, 1980). Another compound that may serve as a source of methylotrophy is lignin. Formaldehyde, but not methanol, is the byproduct of lignin biodegradation (Kuwahara, H., 1980). Most methylotrophs, excluding organisms with RuBP, assimilate carbon at the level of formaldehyde. Methanol can be used as carbon, hydrogen and oxygen sources by these microorganisms.

Different techniques have been employed to detect methylotrophic microbial communities in various environments. Use of stable-isotope probing (SIP) demonstrated the presence of *Methylobacterium*, *Methylophaga* and *Bacillus spp*. in the methane, methanol and methylamine microcosms respectively, in Lonar Lake, which is a unique saline and alkaline ecosystem formed by a meteor impact some 52,000 years ago (Antony et. al., 2010). The same technique also detected a broad range of methylotrophs in samples from Lake Washington (Nercessian et. al., 2005 and Kalyuzhnaya et al., 2008), acidic forest soil (Radajewski et. al., 2002) marine samples (Neufeld et. al., 2008) and an alkaliphilic saline soda lake (Doronina et al., 2003). All these studies demonstrate the broad habitat of methylotrophs from fresh water lake sediments (Nercessian et. al., 2005) to soils and sediments from Antarctica (Azra et. al., 2005). Another habitat that was not thought to be associated with methylotrophic microorganisms, the human mouth, was also shown to contain methylotrophs. Methylotrophs from the human mouth were detected using 16S rRNA gene sequencing and primers designed for the *mxaF* gene of methanol dehydrogenase (Anesti et. al., 2005). The identification and isolation of methylotrophic microorganisms is not complete as new microorganisms are still being isolated (Boden et. al., 2008).

Carbon assimilation in methylotrophic microorganisms

The first reaction in methylotrophy is the oxidation of methanol to formaldehyde. Formaldehyde is formed from methanol via oxidation by methanol dehydrogenase or methanol oxidase. From this oxidation level most of the carbon is assimilated into cell biomass. There are four different pathways by which methylotrophic organisms can assimilate carbon substrates into cell material (Figure 1.1). One of the routes, the serine cycle, involves carboxylic acids and amino acids as intermediates. The other three routes, ribulosebisphosphate (RuBP), known as the Calvin cycle, ribulose monophosphate (RuMP), and xylulose monophosphate- also known as dihydroxyacetone pathway (XuMP), involve carbohydrate intermediates. The XuMP is found only in yeast grown on methanol. The C_1 carbon can be assimilated as different intermediates. RuMP and XuMP pathways incorporate carbon at the level of formaldehyde while the serine cycle can assimilate both formaldehyde and carbon dioxide. The distribution of assimilation pathways in methylotrophs is shown in Figure 1.2. In RuBP organisms the source of fixed carbon is the carbon dioxide. The methylotrophic bacteria are grouped according to the way they assimilate formaldehyde. The Type I microorganisms employ the ribulose monophosphate (RuMP) pathway whereas Type II microorganisms use the serine pathway for assimilation of formaldehyde. Some microorganisms like *M.capsulatus* (Bath), are classified as Type X since they also contain some enzymes of the serine cycle while carbon is assimilated via the RuMP pathway (Hanson and Hanson, 1996). The energy required for the assimilation reactions is obtained by oxidation of a portion of the formaldehyde to carbon dioxide which produces NADH.

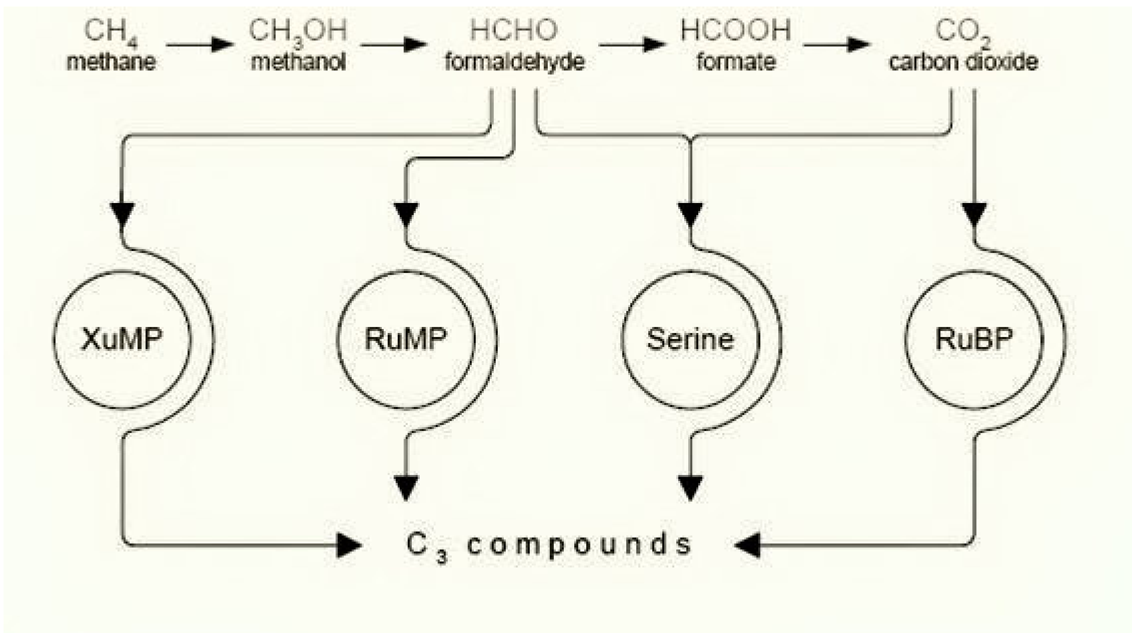


Figure 1.1. Four different pathways for one-carbon assimilation in methylotrophs (Dijkhuizen*et. al.*,1992).

Ribulosebiphosphate pathway, RuBP

The ribulosebisphosphate (RuBP) pathway, also known as the Calvin cycle, is the least common route for assimilation of carbon in methylotrophs. The carbon is assimilated at the level of carbon dioxide and is mainly employed by chemolithotrophic bacteria. Carbon dioxide is condensed to ribulose 1,5-bisphosphate to produce two molecules of 3-phosphoglycerate by the key enzyme of the cycle, ribulosebisphosphate carboxylase. This molecule is then reduced to glyceraldehyde-3-phosphate. One molecule of glyceraldehyde-3-phosphate is isomerized to dihydroxyacetone phosphate and then condensed with another glyceraldehyde-3-phosphate to yield a fructose-1,6-diphosphate (F6P). One molecule of F6P is gained from 6 molecules of carbon dioxide consuming 18 ATP and 12 NAD(P)H.

Table 1.1**.**Distribution of assimilation pathways in methylotrophs (Anthony, 1991).

Ribulose biphosphate (RuBP) Pathway

- *Paracoccus denitrificans*
- *Thiobacillus* sp.
- *Rhodopseudomonas acidophila*
- *Xanthobacter* sp.
- *Rhodobacter* sp.

Xylulose monophosphate (XuMP) Pathway

- *Hansenula* sp.
- *Candida* sp.
- *Pichia* sp.
- *Torulopsis* sp.

Ribulose monophoshpate (RuMP) Pathway

- *Methylococcus capsulatus*
- *Methylophilus methylotrophus*
- *Arthrobacter* P1
- *Bacillus* sp.
- *Acetobacter methanolicus* MB58

Serine Pathway

- *Methylosinus* sp.
- *Methylobacterium extorquens* AM1
- *Hyphomicrobium*

Xylulose monophosphate pathway, XuMP

The xylulose monophosphate cycle is a yeast specific pathway in which the net conversion of three formaldehyde molecules into a C_3 compound, dihydroxyacetone phosphate, is accomplished. Yeast was the first eukaryotic organism shown to grow on C_1 compounds in 1969 (Ogata et. al., 1969). The methylotrophic yeasts isolated from natural sources appear to belong to a limited number of genera. Screening of type-culture collections conducted by different research groups showed that only 15 species within the genera of *Candida*, *Hansenula*, *Pichia* and *Torulopsis* were capable of growth on methanol (Hazeu et. al., 1972, and Oki et. al., 1972). Lee and Komagata conducted a taxonomic study of 31 fresh isolates and 22 strains known to assimilate methanol (Lee and Komagata, 1980). Besides confirming the above results, they also observed that the majority of the strains examined were able to assimilate pectin, which yields methanol upon hydrolysis (Lee and Komagata, 1980).

The conversion of methanol into formaldehyde is catalyzed by alcohol oxidases and also yields hydrogen peroxide as a byproduct. While this reaction takes place in the peroxisomes, enzymes for the dissimilation of formaldehyde are located in the cytosol. The formaldehyde diffuses into the cytosol and condenses with reduced glutathione (GSH) non-enzymatically, to form S-hydroxymethylglutathione. The product S-glutathione is either hydrolyzed into formate and oxidized to carbon dioxide by formate dehydrogenase (Allais and Louktibi, 1983 and, Schütte et. al., 1976) or oxidized directly. Another enzyme, formaldehyde reductase, which reduces formaldehyde to methanol, has been detected in methylotrophic yeast which is thought to serve as a “safety valve” when the cytosolic formaldehyde concentration is too high (Trotsenko et. al., 1984).

Assimilation of formaldehyde is accomplished by the xylulose monophosphate (XuMP) cycle. Dihydroxyacetone (DHA) synthase was shown to be the first enzyme in the assimilation process which takes place in the peroxisome. This enzyme is a special transketolase that catalyzes the glycol-aldehyde transfer from xylulose-5-phosphate as a donor to the acceptor molecule formaldehyde in a reversible reaction (O'Connor and Quayle, 1980).

Ribulose monophosphate pathway, RuMP

In the ribulose monophosphate (RuMP) pathway, the carbon from methane or methanol is assimilated at the level of formaldehyde. In this pathway, one molecule of pyruvate or dihydroxyacetone is synthesized from three molecules of formaldehyde. The pathway can be divided into 3 parts; fixation, cleavage and rearrangement. In the first or fixation part, the formaldehyde is condensed with ribulose-5-phosphate to give hexulose phosphate which is then isomerized to fructose-6-phosphate. These steps are catalyzed by hexulose phosphate synthase (HPS) and hexulose phosphate isomerase (HPI), respectively. In the second part of the pathway, one molecule of F6P is converted either to 2-keto-3-deoxy 6-phosphogluconate (KDPG) or to fructose biphosphate (FBP). These molecules are then cleaved by aldolases to glyceraldehyde 3-phosphate plus either pyruvate (KDPG) or dihydroxyacetone (FBP). In the rearrangement part of the pathway, the glyceraldehyde phosphate and F6P undergo a series of reactions which lead to regeneration of ribulose 5-phosphate. There are two variants in this step; the sedoheptulosebiphosphate variant and the transalddolase variant. The most common variant is the KDPG aldolase/transaldolase (TA) and less common variant is the FBP aldolase/SBP phosphatase. The energetics are shown in the Table 1.2.

Table 1.2. Energetics of the RuMP pathway of formaldehyde fixation. Pyruvate is considered the end-product (Dijkhuizen *et. al.*,1992)

	Energy change (3HCHO $\longrightarrow$ 1 pyruvate)	
Variant	$NAD(P)H_2$	ATP
KDPGA/TA	+1	0
FBPA/SBPase	+1	0
KDPGA/SBPase	+1	-1
FBPA/TA	+1	+1

Serine cycle

Studies with short term incubations of *Pseudomonas* AM1, which was renamed*Methylobacterium extorquens* AM1, with ^{14}C-labeled methanol resulted in sequential labeling of serine followed by glycine, malate and aspartate leading to the hypothesis that the serine cycle was a C_1 assimilatory pathway (Large et. al., 1961). Unlike the RuMP pathway in which carbohydrates serve as intermediates, the serine cycle utilizes carboxylic acids and amino acids. Carbon is assimilated via formaldehyde and carbon dioxide. The serine cycle genes in *M. extorquens* (AM1) are transcribed from three separate units that are positively regulated by a LysR-Type transcriptional regulator that is homologous to CbbR (Kalyuzhnaya andLidstrom. 2003). Glycine is the starting compound in the serine cycle, which is condensed with formaldehyde by serine trans-hydroxymethylase to yield serine. Serine-glycine aminotransferase converts serine to hydroxypyruvate which is then reduced to glycerate at the expense of NADH. Phosphoglycerate is one of the compounds from which carbon can be drawn for cell material and it is produced from glycerate at the expense of 1 ATP, catalyzed by glycerate kinase. Phosphoenolpyruvate (PEP) is the compound that condenses with the carbon dioxide to form oxaloacetate and then malate. Malate then condenses with CoA giving rise to malyl-CoA. Malyl-CoA is then split into glyoxylate and acetyl-CoA by malyl-CoA lyase. Glyoxylate is finally converted to glycine by serine-glycine aminotransferase which completes the cycle. In isocitratelyase positive (*icl*$^{+}$) organisms, the acetyl-CoA is oxidized to glyoxylate with a set of reactions that involves isocitratelyase and this pathway also occurs in bacteria growing on ethanol or acetate. However, most of the methylotrophic microorganisms are known to be *icl*$^{-}$.

Regeneration of glyoxylate from acetyl-CoA in *icl*$^{-}$ microorganisms remained an enigma until a new pathway was recently proposed. Erb and coworkers have shown the transformation of isotopically labeled acetate and bicarbonate to ethylmalonyl-CoA by cell extracts from cultures grown in acetate by the isocitratelyase negative bacterium *Rhodobactersphaeroides* (Erb et. al., 2007). They identified crotonyl-CoA carboxylase/reductase as a key enzyme in the ethylmalonyl-CoA pathway which catalyzes the condensation of carbon dioxide with crotonyl-CoA to yield ethylmalonyl-CoA. In short (Figure 1.2), two acetyl-CoA molecules and 1 molecule of carbon dioxide condense yielding a C5 compound methylmalyl-CoA which is then split into propionyl-CoA (C3) and glyoxylate (C2). Glyoxylate enters into the serine cycle while propionyl-CoA is carboxylated to a C4 compound eventually yielding succinate which then enters the serine cycle as malate.

Putative genes encoding for these enzymes were also identified in the *M.extorquens* AM1 genome (Vuilleumier et. al., 2009), but not in the Type X methylotroph *M. capsulatus* (Bath) genome (Ward et. al., 2004).

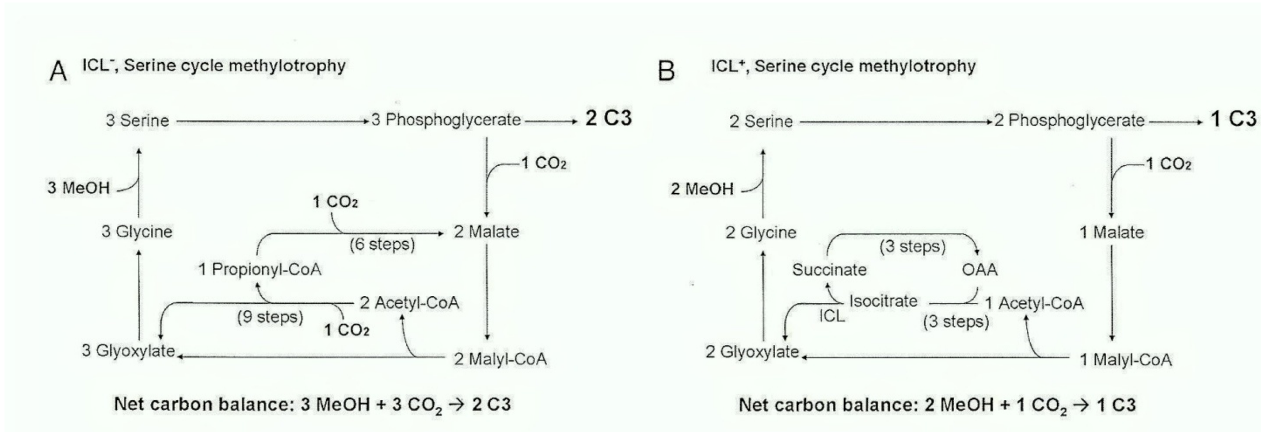


Figure 1.2. Comparison of C1 assimilation pathways by isocitratelyase-negative (A) and positive (B) methylotrophs (Peyraud et. al., 2009).

Dissimilation of carbon in methylotrophs

Methanol dehydrogenase

The first step in the methylotrophy is the oxidation of methanol into formaldehyde. This conversion is carried out by methanol dehydrogenase in bacteria and by methanol oxidase in yeast. Only the methanol dehydrogenase will be summarized here. Cell lysis achieved by using the French press or by sonication provides sufficient enzyme for purification, but when assayed, some MDH is also found associated with the membranes. MDH has been found in membranes or in membrane vesicles in a wide variety of bacteria including *Methylococcus capsulatus* (Wadzinski and Ribbons, 1975), *Hyphomicrobium* X (Duine et al., 1978), and*Methylophilusmethylotrophus* (Cross and Anthony, 1980).

Pyrroloquinoline quinone (PQQ) is ubiquitous as a cofactor of alcohol dehydrogenases. Gram-negative microorganisms employ PQQ-containing methanol dehydrogenases like MDH of *Methylobacteriumextorquens* and *Methylophilus* sp. (Anthony and Williams, 2003). It has a $\alpha_2\beta_2$tetrameric structure and each α subunit contains one PQQ molecule and one Ca^{2+} ion. The αsubunit is around 66 kDA while the β subunit is 8.5 kDA (Anthony and Williams, 2003). The first step is the transfer of an electron to cyctochrome c_L through PQQ. The PQQ of MDH is reduced by methanol, and formaldehyde is released. Then, two electrons are transferred to cyctochrome c_L from $PQQH_2$ which oxidizes $PQQH_2$ back to quinone by the way of a free radical semiquinone (Anthony and Williams, 2003). Different mechanisms have been proposed as to how methanol is oxidized by MDH (Leopoldini et. al., 2007), but the overall reaction is thought to be exothermic (Zhang et. al., 2007).

Most emphasis has been put on the MDHs of Gram-negative bacteria since most of the research has been done on them, and there is less data regarding MDHs of the Gram-positive methylotrophs. Gram-positive bacteria lack a clear periplasmic space, and they do not posses PQQ (Hektor et. al., 2000). The methanol dehydrogenases of Gram-positive bacteria are different than those of Gram-negative in that they are NAD-dependent. They may constitute up to 22% of total protein when grown on methanol (Arfman et.al., 1992). The MDH of thermotolerant *Bacillus methanolicus* C1 is a decameric protein with an estimated size of 43 kDA (Vonck et. al., 1991). Each subunit contains one zinc and one or two magnesium ions. Each subunit also contains one tightly bound NAD(H) molecule as a cofactor. This NAD molecule is oxidized by formaldehyde and reduced by methanol while remaining bound to the protein (Arfman et al., 1997). The *B. methanolicus* C1 MDH also requires exogenous NAD that re-oxidizes the cofactor NADH. A protein called activator protein (ACT) increases the affinity of MDH for methanol and may enhance the methanol turnover rate up to 40 fold (Arfman et. al., 1991). The exogenous NAD and Mg^{2+} are strictly required for the function of the activator enzyme (ACT).

Acetobacterpasteurianus SKU 1108 is found to possess both PQQ-containing alcohol dehydrogenase (ADH) and NAD-dependent ADH. A mutant strain deficient in PQQ-containing ADH has elevated levels of NAD-dependent ADH and grows better on ethanol which leads to the conclusion that PQQ-dependent ADHs function in catabolic acetate production while NAD-dependent ADHs function in assimilation of ethanol (Chinnawirotpisanet.al., 2003).

Formaldehyde

Formaldehyde is the simplest of the aldehydes and ranks as one of the largest volume commodity chemicals with production of 29 million tons/yr in 2010 which is expected to grow 5% per year from 2011-2015 (Merchant Research & Consulting ltd, 2012). It is one of the most important basic chemicals required for manufacture of industrial and consumer products. It is industrially produced from methanol by different synthesis routes with up to 97-98 % reported yield (Reuss et. al., 2005). The largest amount of formaldehyde is used in the manufacture of resins for the production of adhesives and impregnating resins which are employed for manufacturing particle boards, plywood and furniture. These resins are also used as auxiliaries in the textile, leather, rubber and cement industries. In agriculture, formaldehyde is used as a fumigant and as a preservative in animal feed (O'Brien et. al., 2005). The second largest use of formaldehyde is as an intermediate for synthesizing other chemical compounds. This constitutes about 40% of total formaldehyde consumption while direct use only constitutes a small fraction of total consumption, 1.5%. As a C_1 compound, formaldehyde is irreplaceable with any other compound (Reuss, 2005).

A trace amount of formaldehyde is present in the air as a result of photochemical oxidation of hydrocarbons. It is also emitted to air from plastics and resin glues (WHO, 2005). In rural air, terpenes and isopropene emitted by foliage also react with hydroxyl radicals to form formaldehyde (Atkinson 1990). Formaldehyde concentrations of up to 30µg/L have been found in ozonated drinking water (Krasner et. al., 1976). Formaldehyde is also present in rain water (1.38 mg/L) (Kitchens et. al., 1989) and fog water (6.8 mg/L) (Muir, 1991). It is even found in the Antarctic troposphere up to 0.7 ppbv, fluctuating seasonally (Riedel, 1999). The general route of exposure to formaldehyde is through inhalation (US NRC, 1980). The concentration of formaldehyde in food is found to be in the range of 3-23 mg/kg (NTP, 2010). Trezl et. al., (1997) found the level of formaldehyde to range from 3.6 mg/kg to 35 mg/kg in different plants most of which are used as human food.

Endogenous resources of formaldehyde include lipid peroxidation, carbohydrate auto-oxidation, carbohydrate metabolism, amine oxidase-catalyzed metabolic activation and cytochrome P-450 catalyzed metabolic activation (O'Brien et. al., 2005). One other important source of formaldehyde is methanol, generated by methanol dehydrogenases in methylotrophic microorganisms. It is a key metabolite in methylotrophy and its concentration should be controlled since it is also very reactive and can be toxic to microorganisms.

Formaldehyde toxicity

Formaldehyde is the most reactive aliphatic aldehyde molecule and takes part in many well-known chemical reactions (Walker, 1975). This reactivity is the major reason for its toxicity. It also has mutagenic effects. With the use of xanthine guanine phosphoribosyltransferase gene as a genetic target, 4mM formaldehyde was found to induce 41% large insertions, 18% large deletions and 41% point mutations in *E. coli*(Crosby et. al., 1988). Formaldehyde forms monohydroxymethyl adducts with four major ribonucleosides which occur at the exocyclic amino groups of deoxyguanosine, deoxycytidine, deoxyadenosine and at the endocyclicimino group of deoxtymidine. (McGhee and von Hippel, 1975a and 1975b). Incubation of single-stranded polyadenine with formaldehyde at 23 °C results in the bonding of the formaldehyde at a ratio of 1.00±0.05 per base pair. The initial denaturation of DNA by formaldehyde occurs in AT-rich regions in the interior of the DNA molecule. The denaturation occurs as follows: (i) a small sequence of DNA base pairs opens as a result of local thermal fluctuations or for transcription, (ii) the exocyclic amino group of an adenine residue is exposed, (iii) the adenine reacts with formaldehyde which either reforms in a hydroxymethylated AT base pair or remains as unbound to the complementary strand depending on the temperature and other environmental factors (McGhee and von Hippel, 1977a). Once bound to the DNA, formaldehyde lowers the T_m of the DNA sequence and the change in T_m increases with the AT content of the DNA (McGhee and von Hippel, 1977b).

Reactions of formaldehyde with amino acids, peptides and proteins were investigated using ^{13}C and ^{15}N NMR. The reactions of formaldehyde with proteins start with the formation of methylol adducts on amino groups. The major derivatives formed with single amino acids are monohydroxymethyl adducts, dihydroxymethyl adducts and cyclic products (Heck et. al., 1990). The methylol adducts of primary amino groups are partially dehydrated, yielding Schiff-bases, which can form cross-links with several amino acid residues and produce irreversible covalently cross-linked complexes between proteins and single stranded DNA (Bolt, 1987). Thus, organisms should keep metabolic formaldehyde levels low to avoid these undesired reactions.

Formaldehyde oxidation

Studies of methylotrophic microorganisms growing on methanol have shown the need for keeping the methanol concentration limited since specific growth rates declined after methanol concentration in the culture medium of *B. methanolicus* reached a critical level of 12g/L (Jakobsen et. al., 2006). In the same study, it was shown that formaldehyde is responsible for the decrease of the specific growth rate by adding extracellular formaldehyde (Pluschkell, 1998) but there are no published studies reporting that the formaldehyde produced as a metabolic intermediate inhibits microbial growth. An increase in the level of intracellular formaldehyde exerts stress on the microorganisms which may lead to induction of multiple stress response pathways including general stress response mechanisms (Nguyen et. al., 2009). The level of formaldehyde can be lowered by either assimilation by the RuMP or the serine cycle or dissimilation by oxidation to carbon dioxide. The same study has shown the specific induction of the ribulose monophosphate (RuMP) pathway in response to formaldehyde among other carbonyl electrophiles in non-methylotrophic *B. subtilis* where it is thought to serve as a detoxification pathway (Nguyen et. al., 2009). Accordingly, bacteria have capabilities to dissimilate excess formaldehyde as carbon dioxide. Dissimilatory pathways also generate energy required for growth on C_1 compounds in methylotrophic organisms although the reactions may differ as described below.

Linear formaldehyde oxidation pathways by aerobic methylotrophic organisms are summarized in Figure 1.3. The simplest pathway that converts formaldehyde to formate is catalyzed by the dye-linked formaldehyde dehydrogenase (DL-FALD) enzyme. This enzyme in *M. capsulatus* (Bath) is associated with membranes, and it has been shown to be the major formaldehyde oxidizing enzyme when *M. capsulatus* (Bath) cultures proliferate in medium supplemented with high copper concentration (Zahn et. al., 2001). The redox cofactor of the DL-FALD has been identified as the pyrroloquinoline quinone (PQQ) (Zahn et. al., 2001). However, another compound that can act as cofactor is mycothiol. In Gram-positive methylotrophs, NAD- and mycothiol-linked FALD have been identified as the major formaldehyde oxidation enzymes consisting of a single type of apo-protein (van Ophem et. al., 1992). The presence of bacillithiol as a thiol containing antioxidant in *Bacillus* species was demonstrated recently (Newton et. al., 2009). However, to date, no FALD was shown to be bacillithiol-dependent.

The third pathway to oxidize formaldehyde to formate in Gram-negative methylotrophs such as *Paracoccusdenitrificans* takes three steps, two enzymes and glutathione (GSH) as a cofactor of the FALD (Ras et. al., 1995 and Harms et. al., 1996). In this case, it requires two enzymes to oxidize formaldehyde to formate. Formaldehyde spontaneously condenses with GSH to form S-hydroxylmethyl GSH which is then converted to S-formyl GSH by NAD-GSH dependent FALD. The second enzyme, S-formyl GSH hydrolase catalyzes the conversion into formate while releasing GSH.

There are two routes for folate-linked formaldehyde oxidation; THF- or THMPT-linked pathways. In the former route, THF spontaneously condenses with formaldehyde to yield methenyl-THF. This compound is oxidized to formate and then carbon dioxide by the following enzymes: methylene THF dehydrogenase, methenyl THF cyclohydrolase, formyl THF synthetase and finally formate dehydrogenase. The THMPT-linked pathway was thought to be specific for archaea, and its presence in bacteria was considered unlikely. However, Chistoserdova et al., showed the presence of these enzymes in *M. extorquens* AM1 (Chistoserdov et. al., 1994). Indeed, it was shown to be more prevalent in bacteria than previously thought (Vorholt et al., 1999). It is also present in *M. capsulatus* (Bath), in which DL-FALD is thought to be the major dissimilatory enzyme (Ward et. al., 2004 and Zahn et. al., 2001). Unlike condensation of formaldehyde with THF, which occurs spontaneously, an enzyme was suggested to help condense formaldehyde with THMPT, named formaldehyde activating enzyme (Fae) (Vorholt et. al., 2000). An analog of Fae was also suggested that helps condense formaldehyde with GSH which was termed glutathione-dependent formaldehyde-activating enzyme (Gfa) (Goenrich et. al., 2002)

THF-linked enzymes formate-THF ligase, methenyl-THF cyclohydrolase and methylene-THF ligase also function in the anaerobic Wood-Ljungdahl pathway where carbon dioxide and carbon monoxide are converted into acetyl-CoA. Acetogenic bacteria assimilate carbon dioxide into biomass via this route (Ragsdale, 2008).

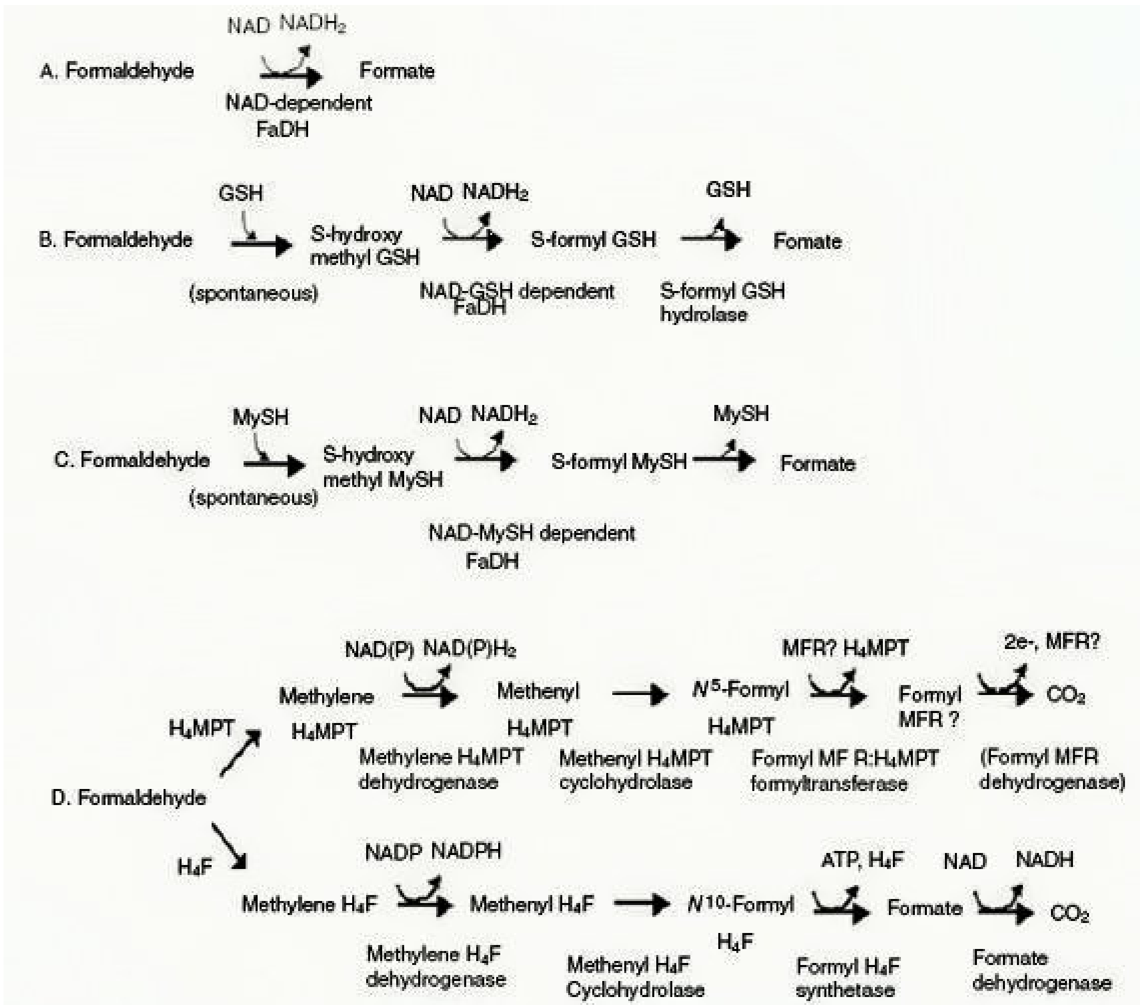


Figure 1.3. Formaldehyde oxidation pathways by aerobic methylotrophic bacteria.A. NAD-linked formaldehyde dehydrogenase (FALD), B. Glutathione (GSH)-linked FALD, C. Mycothiol (MySH)-linked FALD, D. Folate-linked pathways, (upper) involving THMPT and (lower) involving THF(Lidstrom, 2006).

Formate Dehydrogenase

Formate is the next oxidation step of methanol after formaldehyde. As can be seen from Figure 1.1, no methylotrophic organism assimilates methanol carbon at the level of formate, but formate represents a valuable source for recycling cofactors ($NAD(P)^+$ to NAD(P)H) and also for energy generation. Under anoxic conditions formate, produced from pyruvate by the enzyme pyruvate formate-lyase (PFL), serves as a major electron donor for a variety of inducible respiratory pathways that use terminal acceptors other than molecular oxygen in *E. coli* (Unden and Bongaerts, 1997). Formate is also an important branch point in the anaerobic Wood-Ljungdahl pathway. Carbon dioxide is reduced to formate by FDH before the enzymes mentioned above (see Formaldehyde oxidation section) can react with formate.

Formate dehydrogenase (FDH) is one of the most extensively studied microbial enzymes. It catalyzes the two electron oxidation of formate to carbon dioxide. It also reduces $NAD(P)^+$ to NAD(P)H during this reduction. Because of this reduction, it is one of the most frequently used enzymes in dehydrogenase-based synthesis of optically active compounds (Tishkov and Popov, 2006). The availability of formate as a cheap substrate also makes FDH an enzyme of choice. There have been extensive efforts to engineer FDHs from different microorganisms to have a higher catalytic activity and increased stability (Tishkov and Popov, 2006). Most microorganisms have been shown to carry multiple copies of formate dehydrogenases. *E. coli* has three distinct FDH isoenzymes (Sawers, 1994). *M. extorquens*AM1 has four functional FDHs (Chistoserdova et. al., 2004 and 2007) although only one has been shown to be indispensable for growth on methanol (2007); mutations in *fdh4A* resulted in a phenotype that cannot grow on methanol. *Methylobacillusflagellatus* is another microorganism that possesses more than one formate dehydrogenase. Like *M. extorquens* AM1, only one of the genes was crucial for growth of *M. flagellatus* (Hendrickson et. al., 2010).

Also, if nitrate is present in the medium when *E. coli* is grown anaerobically, a formate-nitrate respiratory chain may be formed by FDHs with nitrate reductase as a terminal electron acceptor (Enoch and Lester, 1974, 1975). Fdh-N of *E. coli* is part of nitrate reduction where under anaerobic conditions formate oxidation is coupled to nitrate reduction via a lipid-soluble quinone (Jormakka*et. al*., 2003). The capability of FDHs functioning in the formate-nitrate respiratory chain may bestow methylotrophic bacteria with the ability to grow in anaerobic environments.

Molybdenum and tungsten are the most abundant metals found in FDHs, although selenium may also present in some FDHs. The FDH from the sulfate reducing bacterium*Desulfovibrio alaskensis* NCIMB 13491 was shown to contain either molybdenum or tungsten (Brondino et. al., 2004). In *Methylobacterium*sp. RXM, the addition of molybdenum (0.6 µM) and tungsten (0.6 to 0.9 µM) to shake flask cultures yielded the highest formate dehydrogenase activity but these two metals were found to have antagonistic effects on the formate dehydrogenase enzyme that contains the other metal (Girio et. al., 1998). The FDH-H of *E. coli*was shown to have selenium in the active site of the enzyme, and to carry out oxidation of formate into CO_2 without an oxygen transfer (Khangulov et al., 1998).

Anaerobic methylotrophic microorganisms

The long held belief that some microorganisms such as *Bacillus subtilis* are strictly aerobic has been changed recently (Nakano and Zuber, 1998). *B. subtilis* has been shown to grow under an anaerobic atmosphere where nitrate or nitrite is present as an electron acceptor. Similarly, another bacillus, *B. macerans* was shown to respire anaerobically in the presence of nitrate or nitrite with glycerol as an electron donor (Glaser et. al., 1995). Although the presence and functionality of nitrogenase genes in methane oxidizing microorganisms may have suggested the possibility of anaerobic growth (Oakley and Murrel, 1988), methylotrophic microorganisms were not demonstrated to grow anaerobically until recently (Raghoebarsing et. al., 2006; Ettwig et. al., 2008).

Researchers have isolated methylotrophic microorganisms from environments where oxygen is thought to be scarce or absent. Strains of *Methylobacterium* were isolated from legume nodules and shown to fix nitrogen where the strict absence of oxygen is required (Sy et. al., 2001). Oxygen is thought to be a strict requirement for the oxidation of methanol or methyl amines as the terminal electron acceptor. A terminal electron sink is required in both oxidation cases, either for the removal of electrons from methanol through the electron transport chain in the membrane or by the oxidation of NAD^+ to NADH. Methylotrophs are thought to be strict aerobes because of this requirement. The necessity for oxygen is more profound for oxidation of methane to methanol where molecular oxygen is added to methane. However, recent studies demonstrated that sulfate and nitrate can serve as a source of molecular oxygen under anaerobic conditions (Knittel and Boetius, 2009). This proof of concept study has shown that anaerobic oxidation of methane can be coupled to denitrification (Raghoebarsing et. al., 2006). Although this oxidation was achieved by a consortium of microorganisms that include both Archaea and bacteria, researchers were able to separate the bacteria from the Archaea and demonstrate the anaerobic oxidation of methane by bacteria (Ettwig et. al., 2008).

Genetic manipulations of methylotrophs

The development of genetic and molecular biology techniques available for use in methylotrophs traditionally lagged behind those of other microorganisms. The nature of obligate methylotrophy and the limited number of antibiotic markers that can be employed in methylotrophs accounted for this lag. The published reports of the genome sequences of model methylotrophic organisms like *M. capsulatus* (Bath) and *M. extorquens* AM1 lagged behind reported sequences of other microorganisms and only recently became available (Chistoserdova et. al., 2009 and Ward et. al., 2004). Triparental or biparental conjugative mating techniques have been preferred as a method of genetic transformation rather than techniques like electroporation. The *E.coli* strain S17-1 was successfully used as donor in mating experiments with Gram-negative bacteria (Simon et. al., 1983). Matings are usually carried out on unselective solid media (Ali et. al., 2006, Smith et. al., 2002 and Warner et. al., 1980). Stolyar and coworkers (Stolyar et. al., 1995) proved the possibility of transfer and expression of the Inc-P incompatibility group in cells of obligate methane-oxidizing bacteria *Methylomonasrubra* 15sh and *Methylococcus thermophilus* 111p. They also showed the intraspecific gene transfer in methane-oxidizing bacteria for the first time. The cloning and usage of the methanol-regulated promoter sequence from the methanol oxidation gene *moxF* found in facultative methylotrophic bacterium *Methylobacteriumorganophilum* XX has made the homologous and heterologous expression in methylotrophic bacteria possible (Choi et. al., 2006 and Xu et. al., 1993). However, expression of methane monooxygenase from *M. capsulatus* (Bath) in *E.coli* did not yield a fully functional enzyme (West et. al., 1992). Promoter-probe vectors with reporter genes encoding green fluorescent protein (GFP), catechol 2,3-dioxygenase (XylE) or β-galactosidase (LacZ) were also designed and used in the methylotroph *M. capsulatus* (Bath) (Ali and Murrel, 2009). Methane monooxygenase mutants of *Methylosinustrichosporium* have been successfully constructed by marker-exchange mutagenesis (Martin and Murrel, 1995).

Bacillus methanolicus

B. methanolicus is a Gram-positive aerobic methylotroph growing at temperatures between 35 and 60 °C. Various wild type strains that are able to grow on methanol as a sole carbon source were isolated by different researchers (Arfman et. al., 1992). Wild-type strains of *B. methanolicus*isolated at the University of Minnesota have been reported to secrete 58 g/l of L-glutamate in fed-batch cultures (Schendel et. al., 2000) while classical mutants can secrete 37 g/l of L-lysine (Hanson et. al., 1996), at 50 °C. The ribulose monophosphate (RuMP) pathway is used to assimilate the carbon derived from methanol. Methanol is converted to formaldehyde by a methanol dehydrogenase (MDH) which is encoded by a gene carried on a 19 kb endogenous plasmid (Brautaset et. al., 2004). The MDH in *B. methanolicus* is shown to be an NAD-dependent, decameric protein with 43,000-Mr subunits. Each subunit contains a tightly bound NAD(H) molecule, one zinc and one or two Mg ions

(Arfman et. al., 1997). The only gene described encoding MDH was reported to be on the pBM19 plasmid (Brautaset et al., 2004).

This plasmid pBM19 also has additional genes, encoding enzymes of the RuMP pathway, *glpX*, *fba*, *tkt*, *pfk* and *rpe*, which encode the enzymes fructose-1,6-biphosphatase (FBPase), fructose-1,6-biphospate aldolase (FBPA), transketolase, phosphofructokinase, and Ru-5-P-3 epimerase, respectively. Five of the corresponding genes of RuMP pathway enzymes are carried on the pBM19 along with *mdh*. However, the genes *hps* and *phi*, encoding the first two enzymes of the RuMP pathway, hexulose phosphate synthase and phosphohexuloisomerase, are located on the chromosome. The overexpression of these genes resulted in reported increased tolerance to higher formaldehyde concentrations in shake flask studies compared to wild type strains (Jakobsen et. al., 2006). There had been attempts to increase the amino acid production by overexpression of the RuMP pathway or TCA cycle enzymes. The overexpression of aspartokinase increased the L-lysine production while overexpression of pyruvate carboxylase did not have a positive effect on the rate of L-lysine or L-glutamate production (Brautaset et. al., 2010 and Jakobsen et. al., 2009).

Genetic manipulations in *B. methanolicus* were made possible by the pioneering work of Cue *et. al.,*1997: *B. methanolicus* was successfully transformed by protoplast formation for the first time. *E.coli-B.methanolicus* shuttle vectors, pDQ507 and pDQ508 were successfully constructed and shown to be stable in *B. methanolicus* up to 60 generations even in nonselective conditions. The stability of *E.coli-B.subtilis* shuttle vector pHP13 was also assessed. All three plasmids were shown to replicate by a rolling-circle mechanism which contributed to their stability in *B. methanolicus*. Methylation of the restriction site *Bme*TI, isoschizomer of *Bcl*I, also added stability to the transformed plasmids, since *B. methanolicus* was shown to have the *Bme*TI restriction-modification system (Cue et. al., 1996).

The dissimilation of carbon from formaldehyde via conversion into formate and then carbon dioxide, which leads to loss of carbon, has been shown for *B. methanolicus* MGA3 by ^{13}C NMR and isotope-ratio mass spectrometry (Pluschkell and Flickinger, 2002). However, the difference in carbon dissimilation rates of strains MGA and PB1, as shown in Figure 1.4, suggests that there should be significant difference in how these two strains dissimilate the carbon (Gutmacher, 2009).

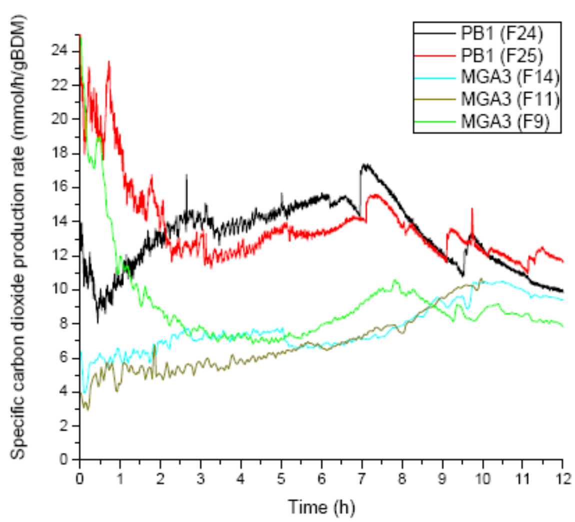


Figure 1.4. Specific CO_2 production rates of *B. methanolicus* MGA3 and PB1 in a 1-L fed-batch fermentation (Gutmacher, 2009).

The presence of formaldehyde and formate dehydrogenase enzymes was shown by enzyme assays of the crude cell extracts in Minnesota (Smith, 2007), but were never shown on the genetic level. Candidate genes were cloned from *B. methanolicus* PB1 by a research group in Bath, U.K. using degenerate primers but the expression of these clones did not confer any formaldehyde or formate dehydrogenase activity either in*E. coli*or in *B. subtilis*(Mozzanega, 2010). Furthermore, the expression of plasmid-borne *mdh* in *E. coli* or *B. subtilis*carried out by this group did not yield an active enzyme in the same study (Mozzanega, 2010). The lack of activity from the *mdh* enzyme raises the question about the validity of the enzyme assays Mozzanega conducted since the enzyme was characterized before and the activity was shown clearly (Arfman et. al., 1997). Identification of a thermo-stable formaldehyde dehydrogenase and formate dehydrogenase from *B. methanolicus* may represent a significantly improved enzyme for use as biocatalysts by the chemical industry (Schrader et. al. 2009).

Recently, the genome sequences of both *B. methanolicus* MGA3 and PB1 were published ahead of us by our former collaborators in Norway (Heggeset *et. al.,* 2012). The difference of *B. methanolicus* strains MGA3 and PB1 in terms of carbon dissimilation rate is confirmed in the same report. This report summarizes the methanol assimilation and dissimilation pathways of *B. methanolicus* as depicted in Figure 1.5 along with key findings including possible growth on glucose. Some of these findings were already written in Chapter 3 before the report was published. The Norwegian report is presented as Appendix C.

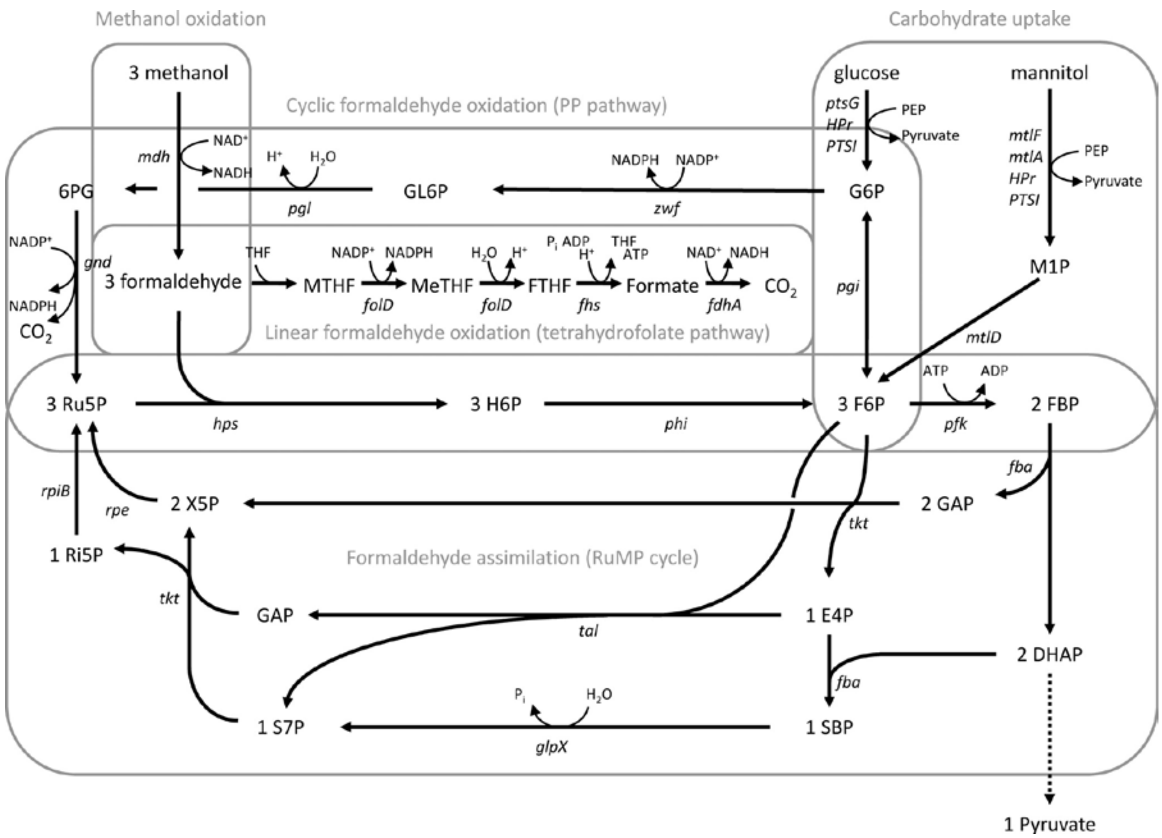


Figure 1.5. Proposed methylotrophic and important carbohydrate assimilation pathways in *B. methanolicus* (Heggeset *et. al.*, 2012)

Thesis Goals

The goals of this thesis are to:

- Investigate the tolerance of *B. methanolicus* for methanol, formaldehyde, and formate
- Sequence the *B. methanolicus* MGA3 genome, annotate the draft sequence.
- Depict the methanol dissimilation pathway from the annotated draft genome sequence

- Elucidate the methanol and formaldehyde assimilation and dissimilation pathways of *B. methanolicus* strains MGA3 and PB1
- Investigate the regulation of formaldehyde dissimilation pathway genes
- Characterize the enzymes involved in formaldehyde assimilation and dissimilation
- Compare the carbon dioxide evolution of different *B. methanolicus* strains in a small methanol-fed bioreactor
- Clone, characterize and compare methanol, formaldehyde and formate dehydrogenases of different bacilli with those of *B. methanolicus*
- Investigate the possible anaerobic growth of *B. methanolicus* on different media and gases

CHAPTER 2

GROWTH CHARACTERISTICS OF *B. methanolicus*

Introduction

Various wild type strains of *B. methanolicus* have been described by different research groups from various environments (Dijhuizen et. al., 1988, Arfman et. al., 1992, Schendel et. al., 1990). Most of these strains are restricted facultative methylotrophs in that they can also grow on a limited number of sugars like maltose or mannitol (Arfman et. al., 1992). The growth energetics of methylotrophs differs according to the pathway they employ to fix the formaldehyde produced from methanol. Extensive theoretical modeling studies have been conducted to estimate the growth yield from methanol and its metabolites (Anthony, 1978 and Van Dien and Lidstrom, 2002). The work of Anthony (1978) modeled the growth yield of different variants of RuMP and serine pathway methylotrophs based on the Y_{ATP} while Dijkhuizen et. al. (1992) summarized (see Introduction) the energetics of formation of pyruvate from formaldehyde by different variants of the RuMP pathway. In addition, Van Dien and Lidstrom modeled the growth of *M. extorquens* AM1 (Serine pathway) and *Methylobacillus falgellatus* KT (RuMP pathway). Anthony (1978) found that 1.5 moles of NADH, 29 moles of ATP and 3 moles of NH_3 are required to synthesize 306 g of biomass from 12 moles of formaldehyde molecules by the FBPA variant of the RuMP pathway, while 17.5 moles of NADH, 41 moles of ATP, 4 moles of CO_2, 3 moles of NH_3, and 4 moles of O_2 are needed to synthesize the same amount of biomass from 8 moles of formaldehyde via the *icl*$^-$ variant of serine cycle. These models have important implications for the biotechnological use of methylotrophs.

The biotechnological aspects of methylotrophs have been reviewed by Schrader et. al. (2009), Trotsenko et. al. (2005), and Brautaset et. al. (2007). Methylotrophs are promising candidates for the production of forage proteins for livestock, production of Poly-β-hydroxybutyrate/valerate, exopolysaccharides, ectoine, phytohormones and vitamins (Trotsenko et. al., 2005). They also contain high levels of various dehydrogenase enzymes that may be used in generation of reduced compounds like NADH. The RuMP pathway methylotrophs are superior to serine pathway microorganisms in that they produce almost two fold higher cell yield per gram of methanol carbon (Schrader et al., 2009). Another advantage of the RuMP pathway organisms is the higher biomass productivity; RuMP pathway methylotrophs have an average productivity of 28.4 g_{CDM}/l/h, while productivity of serine cycle microorganisms ranges between 1.2-3.6 g_{CDM}/l/h. A portion of methanol is dissimilated as carbon dioxide and lost. In RuMP pathway methylotrophs, approximately 38% of methanol carbon is dissimilated (Schrader et. al., 2009). In this regard, understanding of the formaldehyde dissimilation pathway is equally important as the assimilation pathway since it determines the portion of substrate carbon that is lost to carbon dioxide.

An important limitation of the methylotrophs is the toxicity of the methanol or its metabolites. While this toxicity can lower the contamination risk during the fermentation process, it can also suppress the growth at higher than the optimum concentrations. Thus, any fermentation process is required to control the methanol level. This chapter aims to characterize the toxicity of methanol and its metabolites on the growth of *B. methanolicus* strains MBA3 and PB1. Toxicity of hydrogen peroxide and acetaldehyde were also assessed as oxidative stress inducing compounds.

Materials and Methods

Growth Media: A modified version of the minimal media (MTYM) developed by Dijkhuizen et al. (1988) was used as growth media. It contains 10.9 mM $NaH_2PO_4 \cdot H_2O$ (1.5g/l), 23.5 mM K_2HPO_4 (4.1 g/l), 15.9 mM $(NH_4)_2SO_4$(2.1 g/l), and 0.25g/l of yeast extract, pH 7.0. The trace metal stock solution (Table 2.1) and vitamin stock (Table 2.2.) solution were added to the final concentration of 1X. The trace metal solution was sterilized by autoclaving for 20 min and vitamin stock solution was filter sterilized with a 0.22µ filter. The $MTYM_{MeOH}$ medium contained 150 mM of methanol added to the medium while $MTYM_{MAN}$ medium contained 55 mM mannitol (10.02 gr/L), unless otherwise stated. Chloramphenicol is added to the media when required at the concentration of 5µg/l.

Table 2.1. Composition of trace metal solution (1000X)

Ingredient	**F.W. g/mol**	**Stock 1000x mM**	**Stock 1000x g/L**
Citrate·H_2O	210.14	124.4	26.14
$MgCl_2 \cdot 6\ H_2O$	203.29	1000	203.3
$MnCl_2 \cdot 4\ H_2O$	197.91	100	19.8
$CaCl_2 \cdot 2\ H_2O$	147.02	100	14.7
$FeSO_4 \cdot 7\ H_2O$	278.02	40	11.12
$ZnCl_2$	126.29	2	0.27
H_3BO_3	61.84	1	0.06
$Na_2MoO_4 \cdot 2\ H_2O$	241.98	0.4	0.1
$CoCl_2 \cdot 6\ H_2O$	237.9	0.34	0.08
$CuCl_2 \cdot 2\ H_2O$	170.49	0.32	0.05

Bacterial stratins: *B. methanolicus* MGA described by Schendel et al. (1990) was germinated from spores as follows; 50 µl of spore stock was incubated at 80 °C for 15 min and then transferred into a pre-warmed $MTYM_{MeOH}$ media and incubated at 50 °C, 270 rpm in a shaker. *B. methanolicus* PB1 (ATCC 51375), first isolated and described by Arfman et. al. (1992), was purchased from the ATCC. The cryoculture stock was prepared from the exponentially growing *B. methanolicus* culture with 10% final glycerol concentration and by flash freeze in liquid nitrogen.

Table 2.2. Composition of vitamin stock solution (1000X)

Vitamin	**F.W. g/mol**	**Stock 1000x mM**	**Stock 1000x mg/L**
d-biotin (B_7)	244.31	0.409	100
thiamine·HCl (B_1)	337.27	0.296	100
riboflavin (B_2)	376.37	0.266	100
pyridoxine·HCl (B_6)	169.18	0.591	100
pantothenic acid (B_5)	219.24	0.456	100
nicotinic acid (B_3)	123.11	0.812	100
p-aminobenzoic acid (B_x)	137.14	0.146	20
folic acid (B_9)	441.41	0.023	10
cyanocobalamin (B_{12})	1355.39	0.007	10
lipoic acid	206.33	0.048	10

Methanol and *B. methanolicus* growth:*B. methanolicus* was grown in 50 ml $MTYM_{MeOH}$ medium in 250 ml baffled shaker flasks at 50 °C and 270 rpm in a shaker (HT-INFORS) for 10-11 h and inoculated into pre-warmed MTYM media with methanol concentrations ranging between 6 to 2000 mM. The specific growth (μ) rate is calculated from the exponential phase of the growth. The growth of *B. methanolicus* in $MTYM_{MeOH}$ with 25 mM nitrate, 2X YE and without YE were also evaluated.

***B. methanolicus* growth on mannitol:** Two hundred fifty microliters of *B. methanolicus* was inoculated into $MTYM_{MAN}$ medium supplied with 1X vitamin and trace metal stock solution and grown for 10-11 hours. Then enough volume was transferred into 50 ml pre-warmed $MTYM_{MAN}$ medium in 250 ml baffled shaker flask to have an OD_{600} ~0.05, with mannitol concentrations of 5, 10, 25 and 55 mM. The specific growth rate (μ) was calculated from exponential phase of the growth.

***B. methanolicus* growth on glucose:** A tube of cryostock was thawed and inoculated into MTYM as media as described above and mannitol was replaced with 50 mM of glucose. The culture was incubated at 50 °C overnight and enough volume was transferred into 50 ml pre-warmed MTYM medium in 250 ml baffled shakers flask to have an OD_{600} ~0.05, with glucose concentrations of 5, 10, and 20 mM. The specific growth rate (μ) was calculated from exponential phase of the growth.

Growth response to different methanol metabolites: Three hundred microliter of frozen *B. methanolicus* is inoculated into 50 ml of $MTYM_{MeOH}$ or $MTYM_{MAN}$ medium in 250 ml baffled shaker flasks and grown 10-11 hours. Then, enough volume was transferred into a 50 ml pre-warmed $MTYM_{MeOH}$ or $MTYM_{MAN}$ to have an OD_{600} ~0.05. The culture was sampled every hour for OD_{600} measurements and spiked with formaldehyde and formate at the concentrations of 0.5, 1, and 2 mM when the OD_{600} of culture is 0.250-0.350. Formaldehyde was prepared fresh from paraformaldehyde as 2M stock in a serum bottle and autoclaved. Formate was prepared from sodium formate in distilled water as 2M stock. For the methanol spike in mannitol-grown cells, the culture was spiked with 100 mM methanol when the OD_{600} was ~0.25.

Growth response to hydrogen peroxide and acetaldehyde: As described above, *B. methanolicus* was grown either on $MTYM_{MeOH}$ or $MTYM_{MAN}$ and spiked with 0.5, 1 and 2 mM hydrogen peroxide and acetaldehyde. The growth was monitored by measuring OD_{600} every hour.

Detection of formaldehyde in the media: A colorimetric method originally developed by Chrastil and Wilson (1975) and modified by Topp and Knowles (1984) was used to measure the formaldehyde concentration in the culture. Briefly; 1 ml of sample was added into 1 ml of tryptophan-ethanol reagent and then, 1 ml of concentrated (90%) H_2SO_4 was added immediately. The mixture was agitated with a vortex mixer and 200 µl of 0.2% $FeCl_3$ (wt/v) was added for color development. Then, mixture was incubated for 1 hour at 70 °C in an oven. The color development was measured at 575 nm after the samples cooled. A standard curve was obtained by diluting formaldehyde stock solution (2M) in MTYM to have a concentration range of 10 to 700 µM in MTYM. No attempt was made to detect formate in the culture.

Detoxification of formaldehyde by methanol and mannitol grown *B. methanolicus* MGA3: *B. methanolicus* MGA3 was grown to OD_{600} of 0.8 in 50 ml of either $MTYM_{MeOH}$ or $MTYM_{MAN}$ in a 250 ml shaker flask and then formaldehyde was added to final concentration of 2 mM. The formaldehyde concentration in the culture media was measured via the method described above by using 1 ml of culture at the time points of 5, 10, 20 and 60 minutes after formaldehyde addition.

Results

Methanol and *B. methanolicus* growth: The growth rate of *B. methanolicus* MGA3 grown on different methanol concentrations is shown in Figure 2.1. The maximum specific growth rate (μ) was achieved (0.65±0.01 h^{-1}) when *B. methanolicus* MGA3 is grown in the MTYM with 10 mM methanol. The growth rate decreased when the methanol concentration is higher than 25 mM, from 0.65±0.007 h^{-1}at 25 mM to 0.23±0.004 h^{-1} at 2 M. It is also noteworthy that *B. methanolicus* MGA3 culture growing in 2 M methanol achieved a final OD_{600} of 3.76±0.09. Figure 2.2 displays *B. methanolicus* MGA3 culture grown in $MTYM_{MeOH}$ and spiked with methanol concentrations of 10, 50 and 100 mM. As seen from the figure, there is no change in the growth of *B. methanolicus* MGA3 growing on the methanol when challenged with the mentioned methanol concentrations. However, a significant growth disturbance is seen with the same methanol concentrations when $MTYM_{MAN}$-grown *B. methanolicus* MGA3 is challenged (figure 2.3).

Mannitol and *B. methanolicus* growth: The growth rate of *B. methanolicus* MGA3 in different mannitol concentrations is shown in Figure 2.4. The specific growth rate (μ) is relatively constant for different mannitol concentrations, 0.53±0.002. As shown in the figure 2.4, there is no decrease or increase in the growth rate depending on the mannitol concentration.

Glucose and *B. methanolicus* growth: Although it was suggested by Heggeset et. al. (2012), growth of *B. methanolicus*on glucose is demonstrated for the first time in this study.

The growth rate of *B. methanolicus* MGA3 in different glucose concentrations is shown in Figure 2.5. The specific growth rate does not change with the glucose concentrations tested, 0.336±0.003 h^{-1} which is considerably lower than growth rate of *B. methanolicus* on mannitol.

Growth response of *B. methanolicus* to formaldehyde and formate: The exponentially growing culture of *B. methanolicus* was spiked with different concentrations of formaldehyde and formate to elucidate the effect of these methanol metabolites on the growth of the microorganism. Figure 2.6 demonstrates the *B. methanolicus* MGA3 grown on methanol and spiked with different concentrations of formaldehyde. As seen from the figure, *B. methanolicus*MGA3 is able to cope with 0.5 mM formaldehyde, but 1 mM formaldehyde almost completely halts the growth of the microorganism, and 2 mM formaldehyde is enough to kill the culture. However, mannitol grown *B. methnaoliucus* MGA3 is more tolerant to formaldehyde. As shown in Figure 2.7, *B. methanolicus* MGA3 is able tolerate even 2 mM formaldehyde.

Figures 2.8 and 2.9 demonstrate the response of *B. methanolicus* MGA3 grown in $MTYM_{MeOH}$ and $MTYM_{MAN}$ respectively, and spiked with the same concentrations of formate as was formaldehyde. As seen from the figures, formate at the spiked concentrations does not interfere with*B. methanolicus* MGA3 growth.

Growth response of *B. methanolicus* to acetaldehyde and hydrogen peroxide: The growth response of *B. methanolicus* MGA3 to acetaldehyde, to compare with the stress response of formaldehyde and hydrogen peroxide as general oxidative stress agents, was tested. As can be seen from the figures 2.12 and 2.13, acetaldehyde with the tested concentrations does not interfere with growth of *B. methanolicus* MGA3, grown in either $MTYM_{MeOH}$ or $MTYM_{MAN}$. However, figures 2.10 and 2.11 demonstrate that even 0.5 mM hydrogen peroxide was enough to halt the growth of *B. methanolicus* MGA3 completely, whether the cells are grown on methanol or mannitol.

Detoxification of formaldehyde by methanol and mannitol grown *B. methanolicus* MGA3: The standard curve obtained with known formaldehyde concentrations in MTYM medium is shown in figure 2.14. A linear curve with the R-square value of 0.997 was obtained with the formaldehyde range of 10 to 700 µM.

Detoxification (removal) of formaldehyde from the media is shown in figure 2.15. *B. methanolicus* MGA3 detoxifies formaldehyde faster when grown in $MTYM_{MAN}$. The concentration of formaldehyde falls below detection limit (10 µM in sample) in 60 minutes when *B. methanolicus* MGA3 is grown in $MTYM_{MAN}$ whereas in $MTYM_{MeOH}$ culture, it is still at the level of 457 µM. The concentrations of formaldehyde in both $MTYM_{MeOH}$ and $MTYM_{MAN}$ grown cultures were below the detection limit prior to the addition.

Conclusion

Toxicity of methanol and its metabolite formaldehyde is one of the major concerns for the biotechnological uses of methylotrophs. *B. methanolicus* gains the highest specific growth rate with methanol concentrations of 10 and 25 mM. The specific growth rate decreases in methanol concentrations higher than 25 mM. The response of $MTYM_{MAN}$ grown cells to methanol spikes suggests that methanol or its metabolites are toxic to *B. methanolicus* MGA3 even at 50 mM which is lower than $MTYM_{MeOH}$. Considering this with the specific growth rate in $MTYM_{MeOH}$ being lower than maximum, it would not be misleading to state that growth of *B. methanolicus* MGA3 in $MTYM_{MeOH}$ is stressful and *B. methanolicus* MGA3 successfully adapted to this stress albeit with lower specific growth rate. The addition of 100 mM methanol into the culture growing in $MTYM_{MeOH}$ did not produce repression of the growth due most probably to adaptation to stress environment. Taking the methanol evaporation rate (Nilasari et. al., 2012) and growth of *B. methanolicus* MGA3 into account, the maximum methanol concentration in $MTYM_{MeOH}$ does not exceed 250 mM. The growth rates at 150 mM and 300 mM methanol concentrations are comparable, 0.55±0.007 and 0.52±0.01, respectively.

Formaldehyde is an important branch point of methanol metabolism in that carbon of formaldehyde is either assimilated into biomass or lost into the air as carbon dioxide. Because of its toxicity, the intercellular concentration of formaldehyde needs to be controlled to avoid any toxic effects. As seen from figures 2.6, formaldehyde is toxic to *B. methanolicus* even at the 1 mM concentration in the culture supernatant when *B. methanolicus* MGA3 is grown in $MTYM_{MeOH}$.However, when grown in $MTYM_{MAN}$ (figure 2.7), even 2 mM formaldehyde can be tolerated by *B. methanolicus* MGA3. This is most probably due to saturation of formaldehyde detoxification pathways in methanol grown cells, since mannitol grown cells are also able to remove formaldehyde from the media faster (figure 2.15). One route of formaldehyde removal is the assimilation into biomass via the RuMP pathway. Jakobsenet. al., (2006) demonstrated the upregulation of RuMP pathway genes on both chromosome and plasmid. Another possible route of formaldehyde removal is the dissimilation into carbon dioxide through oxidation to first formate and then carbon dioxide. This pathway is also important since it determines the oxidative capacity of the microorganism. Microorganisms with higher oxidative capacity require more effective cooling due to increased heat output (Schrader et. al., 2009) beside the loss of substrate carbon into air.

Formate is the next compound after formaldehyde in the dissimilation pathway. Addition of formate into $MTYM_{MeOH}$ or $MTYM_{MAN}$ grown cells did not produce any growth inhibition as can be seen from the figures 2.8 and 2.9. This is most probably due to low concentrations of formate being added since Pluschkell (1998) showed the inhibition with 25 mM formate. The accumulation of formaldehyde and formate in continuous *B. methanolicus* MGA3 culture when spiked with methanol was also shown by Pluschkell and Flickinger (2002), and formaldehyde and formate dehydrogenase activity in the crude cell extract was shown by Smith (2007). However, no formaldehyde or formate dehydrogenase genes have been identified and characterized from *B. methanolicus* MGA3. Identification of these genes and elucidation of their regulation are important to understand formaldehyde dissimilation in *B. methanolicus*.

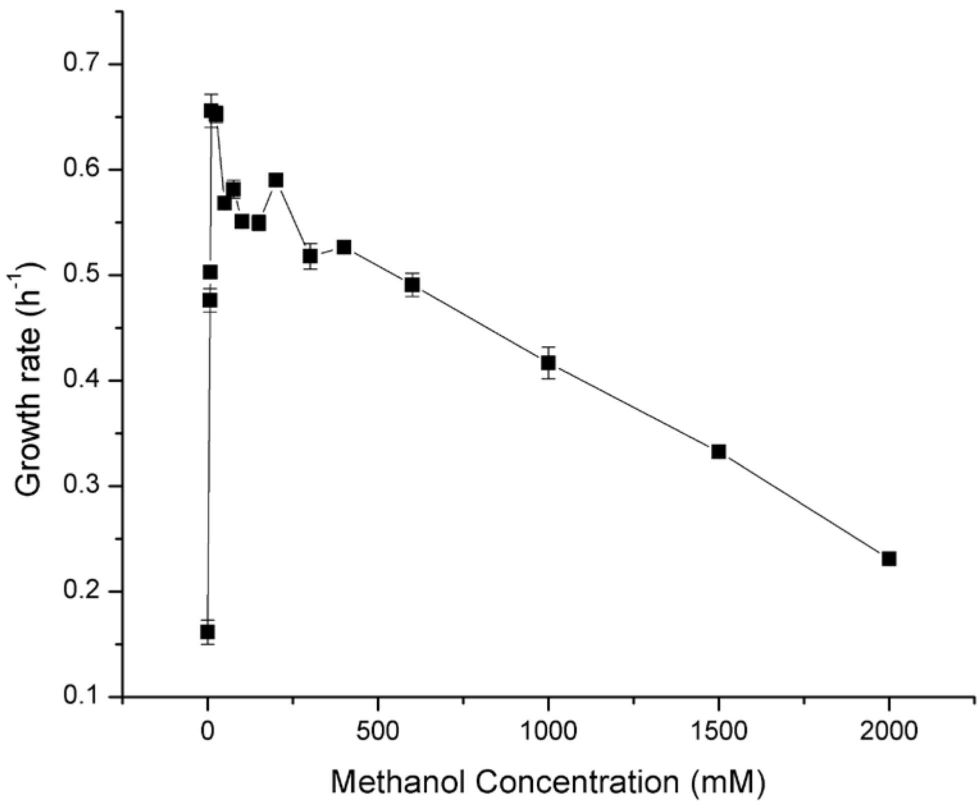


Figure 2.1. The maximum specific growth rate of *B. methanolicus* MGA3 in different methanol concentrations (n=3). *B. methanolicus* MGA3 was grown in 50 ml MTYM medium in 250 ml shaker flasks with different methanol concentrations. The µ was calculated from the exponential phase of the growth.

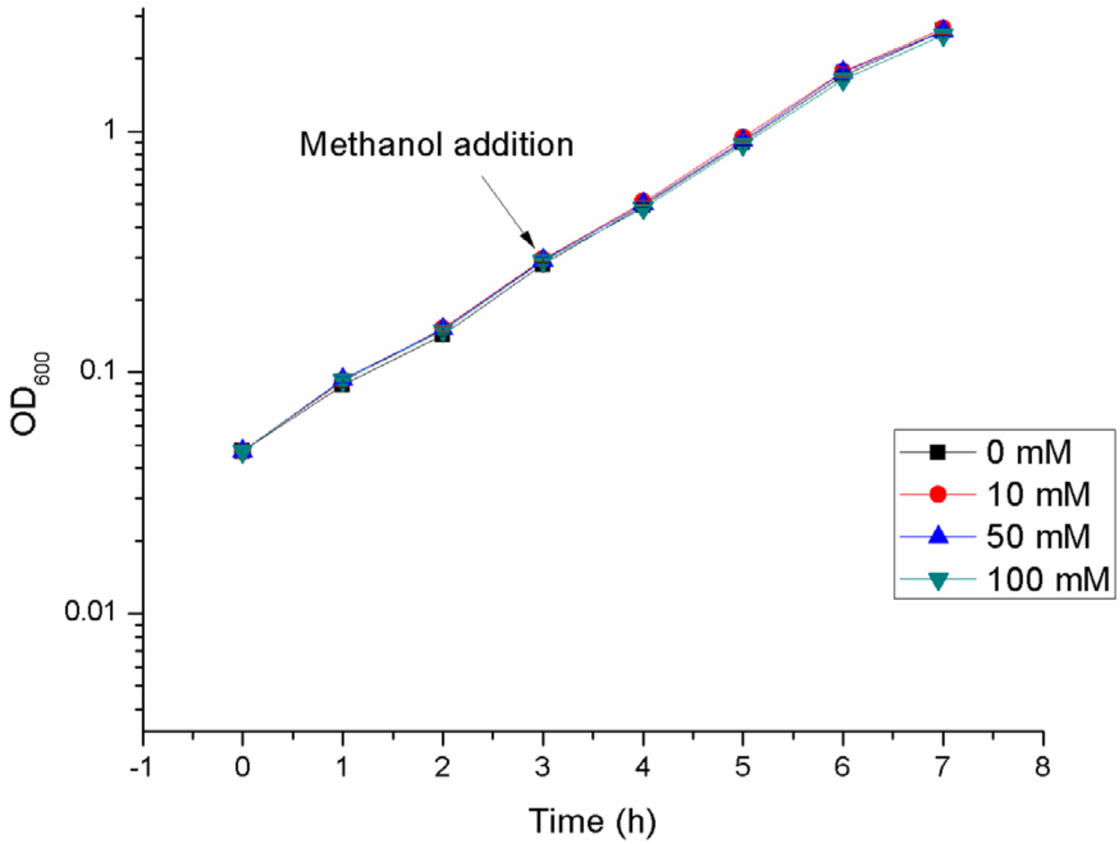


Figure 2.2. Response of $MTYM_{MeOH}$ grown *B. methanolicus* MGA3 to methanol spikes.*B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MeOH}$ in 250 ml baffled shaker flasks and challenged with different concentrations of methanol (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

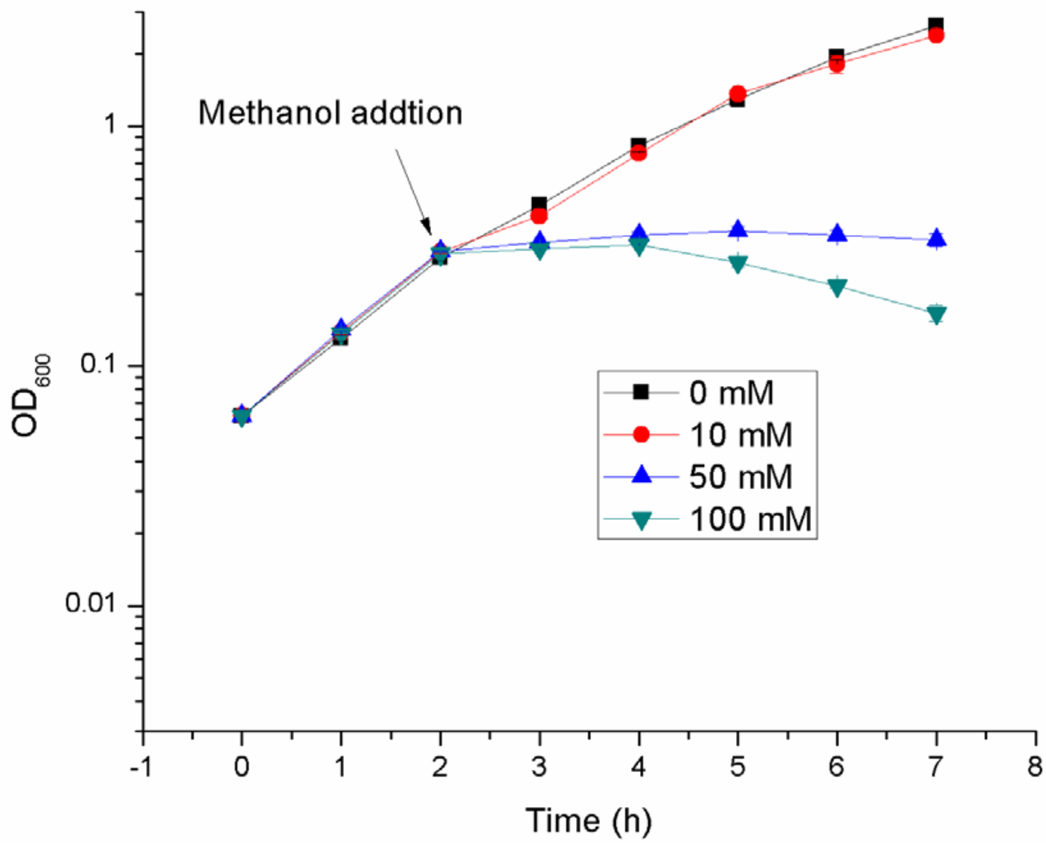


Figure 2.3. Response of $MTYM_{MAN}$ grown *B. methanolicus* MGA3 to methanol spikes.*B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MAN}$ in 250 ml baffled shaker flasks and challenged with different concentrations of methanol (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

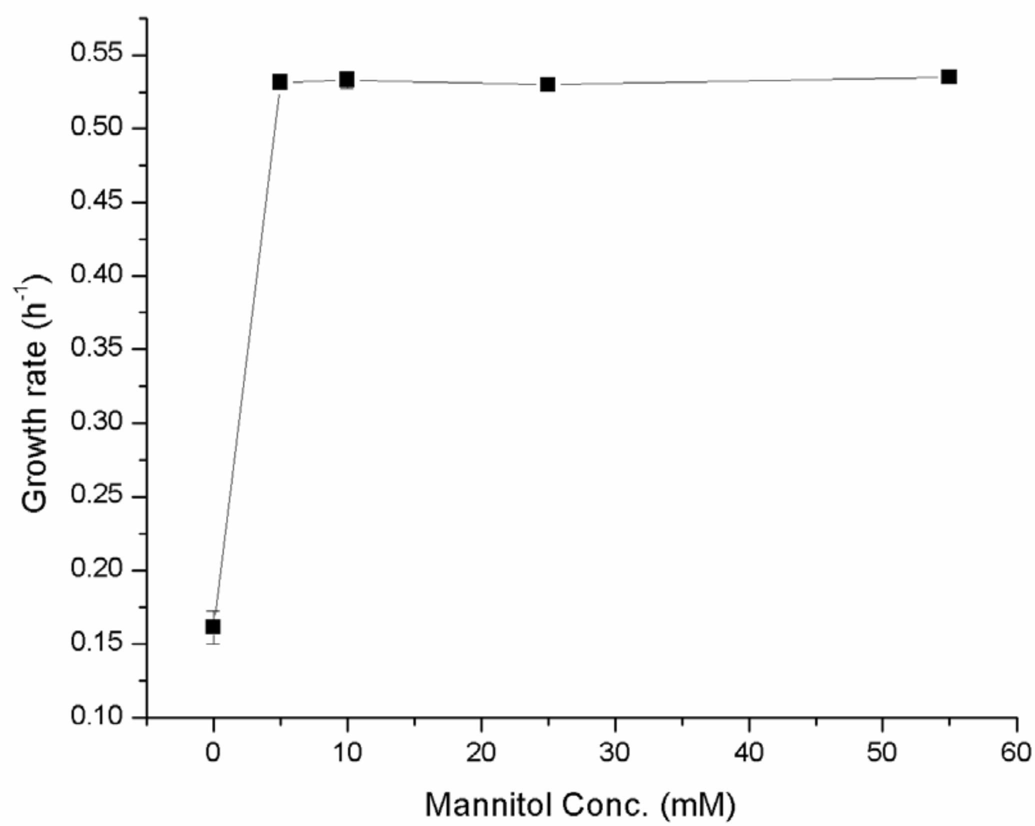


Figure 2.4. The maximum specific growth rates of *B. methanolicus* MGA3 on different mannitol concentrations (n=3). *B. methanolicus* MGA3 was grown in MTYM medium with different mannitol concentrations and μ was calculated from exponential phase of the growth.

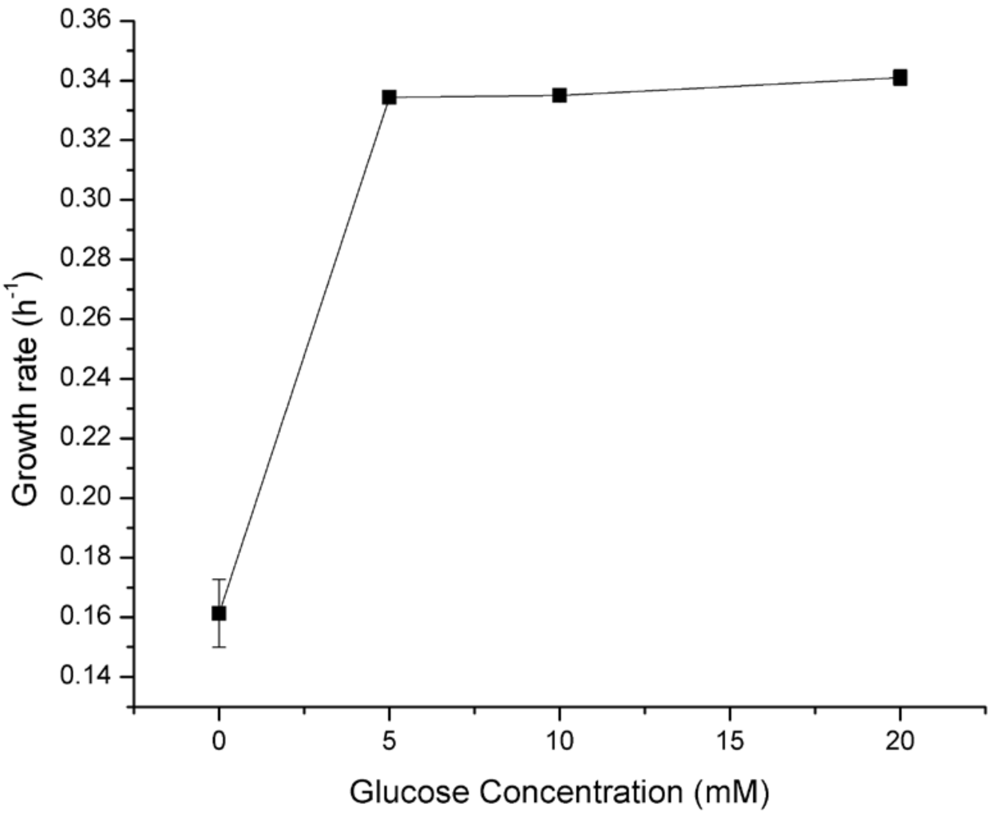


Figure 2.5. The maximum specific growth rates of *B. methanolicus* MGA3 on different glucose concentrations (n=3). *B. methanolicus* MGA3 was grown in MTYM medium with different glucose concentrations and μ was calculated from the exponential phase of the growth.

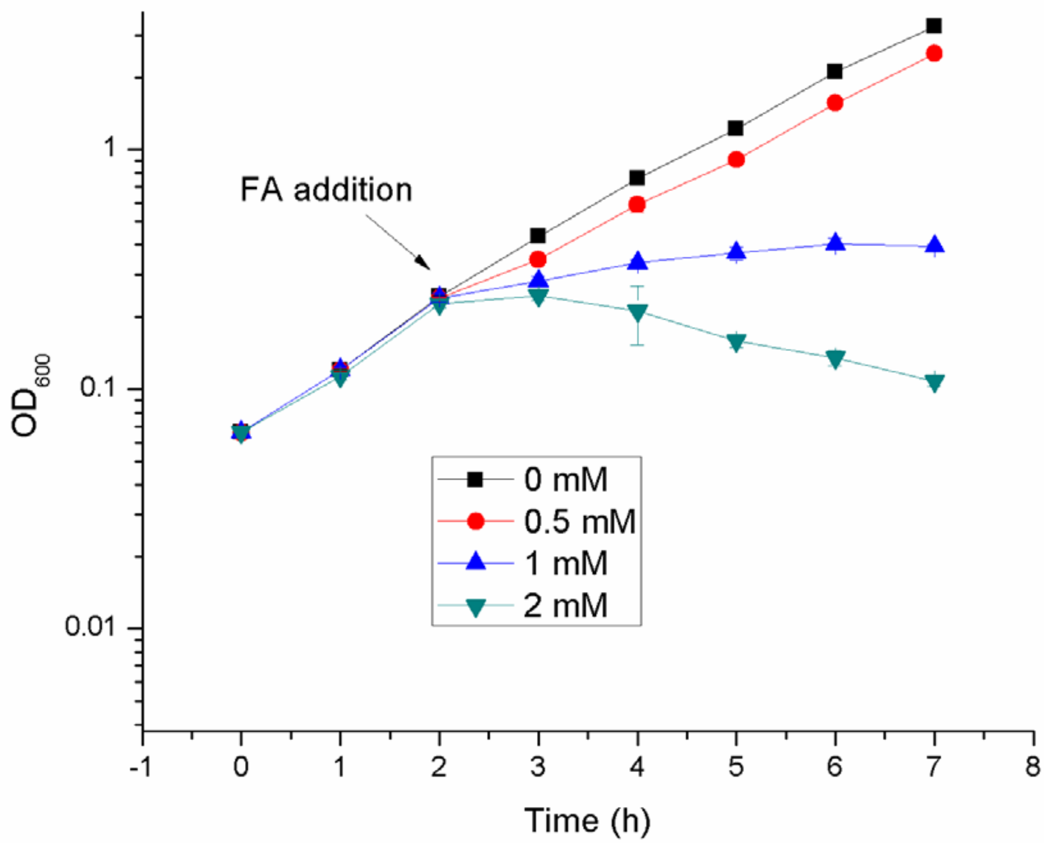


Figure 2.6. Response of $MTYM_{MeOH}$ grown *B. methanolicus* MGA3 to formaldehyde spikes. Formaldehyde was added to the exponentially growing *B. methanolicus* MGA3 culture (OD_{600} ~0.25) and growth was monitored by optical density at 600 nm (n=3).

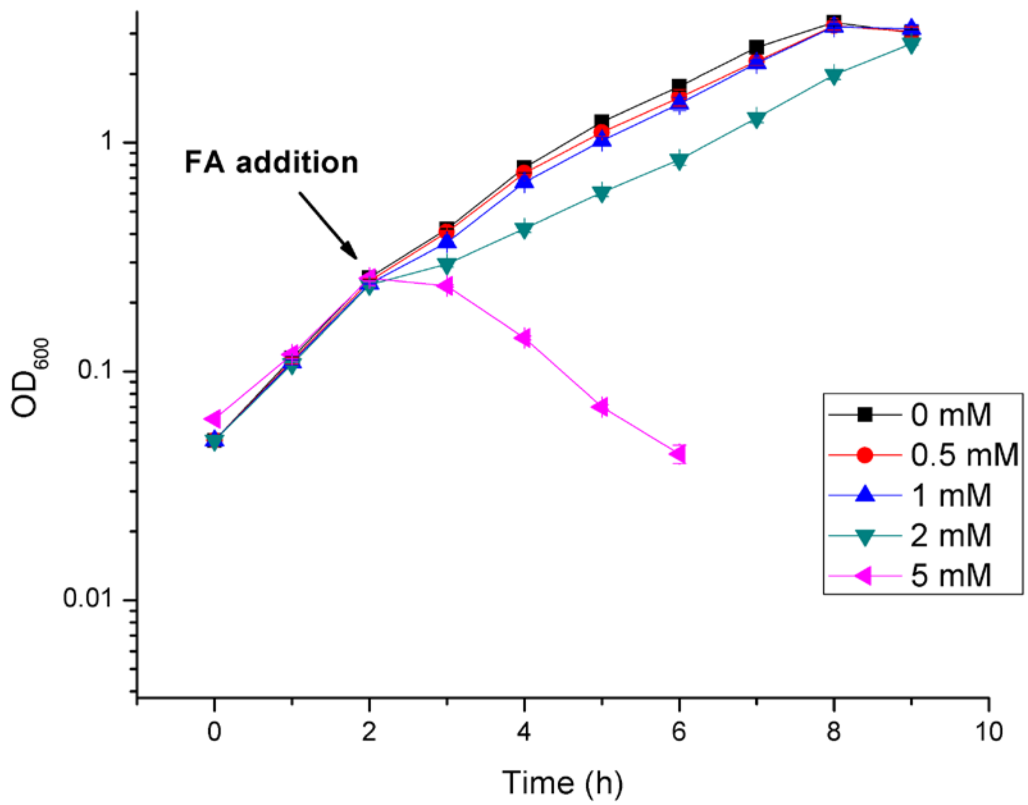


Figure 2.7. Response of $MTYM_{MAN}$ grown *B. methanolicus* MGA3 to formaldehyde spikes. Formaldehyde was added to the exponentially growing *B. methanolicus* MGA3 culture (OD_{600} ~0.25) and growth was monitored by optical density at 600 nm (n=3).

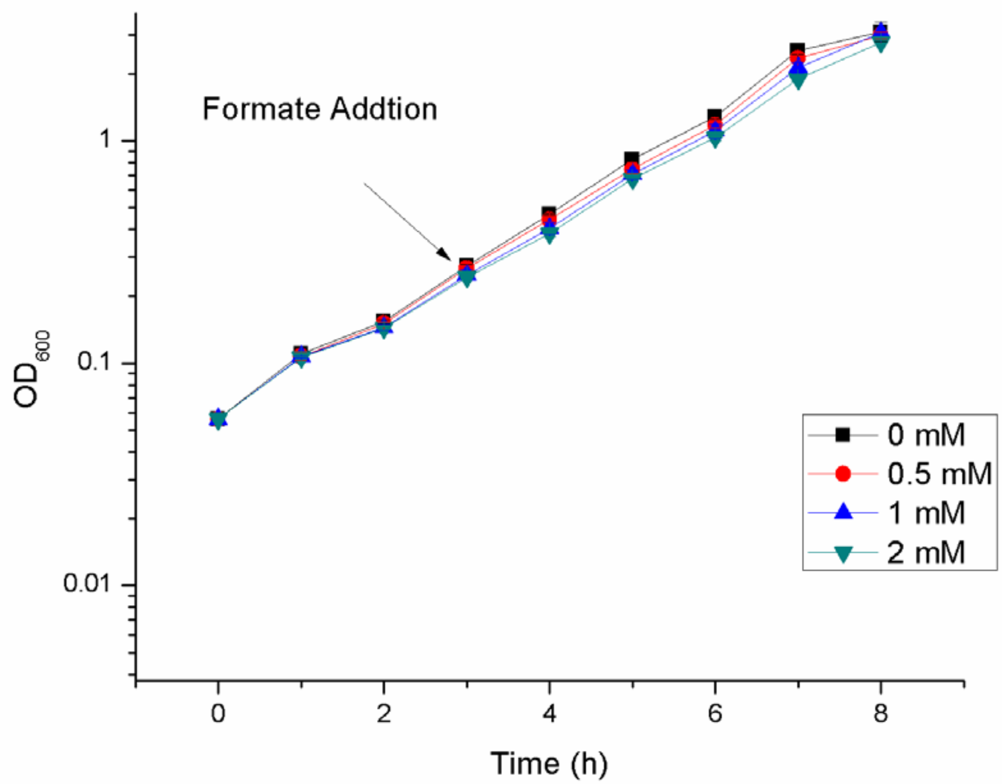


Figure 2.8. Response of $MTYM_{MeOH}$ grown *B. methanolicus* MGA3 to formate spikes. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MeOH}$ in 250 ml baffled shaker flasks and challenged with different concentrations of formate (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

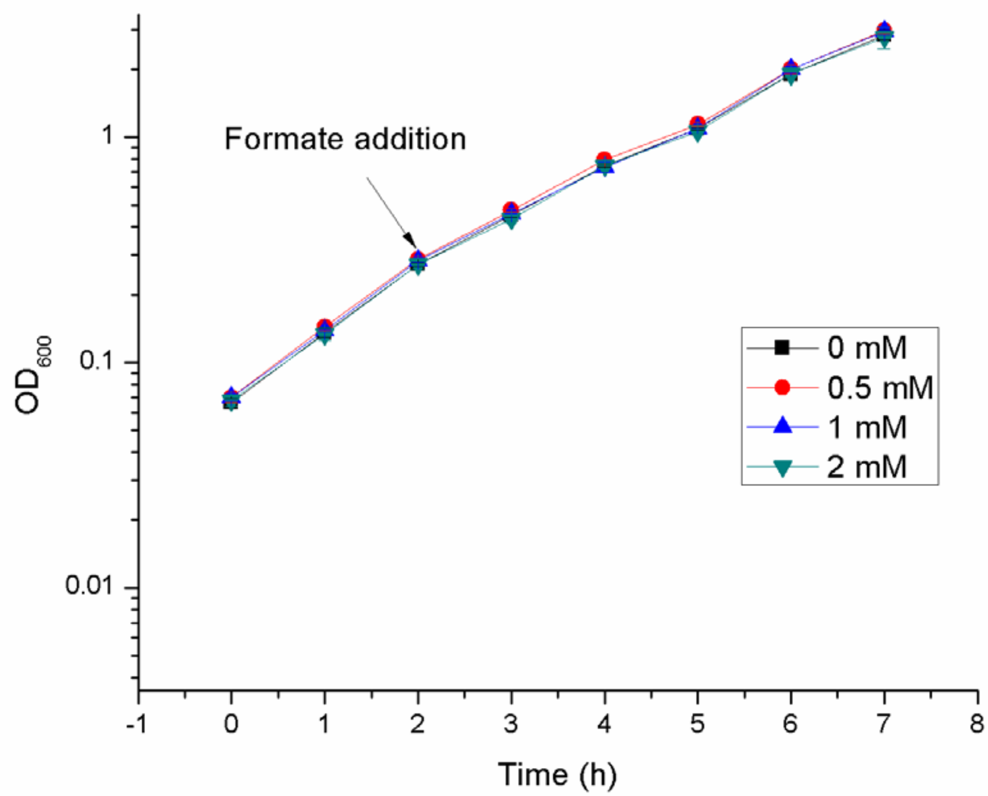


Figure 2.9. Response of $MTYM_{MAN}$ grown *B. methanolicus* MGA3 to formate spikes. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MAN}$ in 250 ml baffled shaker flasks and challenged with different concentrations of formate (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

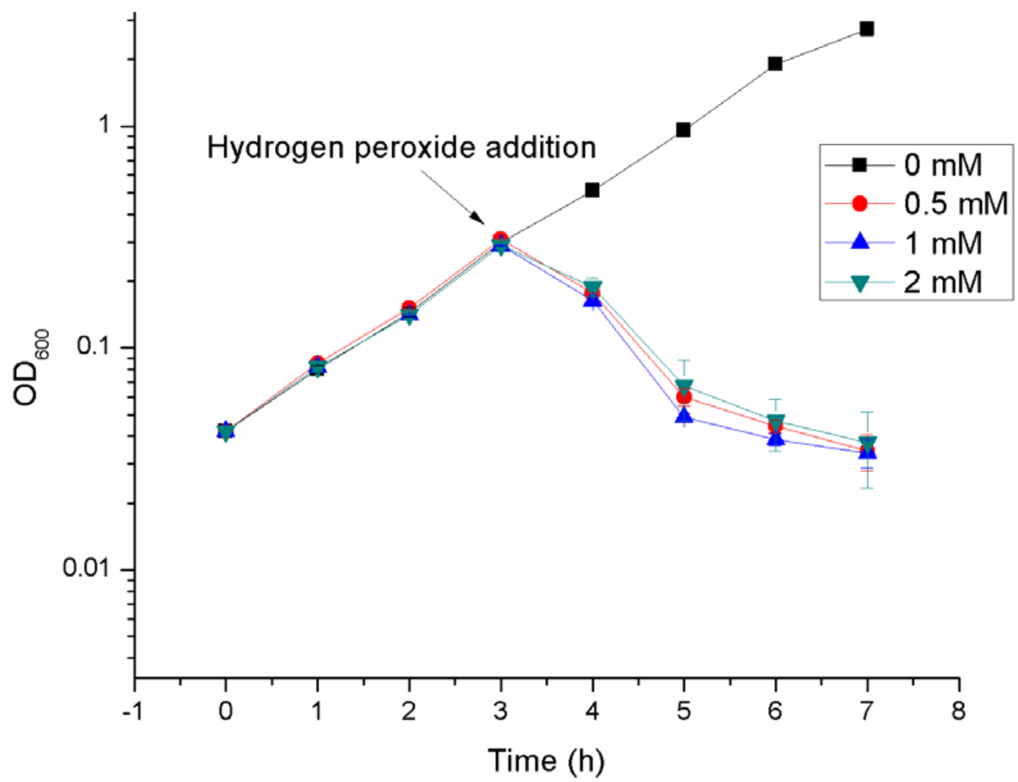


Figure 2.10. Response of $MTYM_{MeOH}$ grown *B. methanolicus* MGA3 to hydrogen peroxide spikes. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MeOH}$ in 250 ml baffled shaker flask and challenged with different concentrations of hydrogen peroxide (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

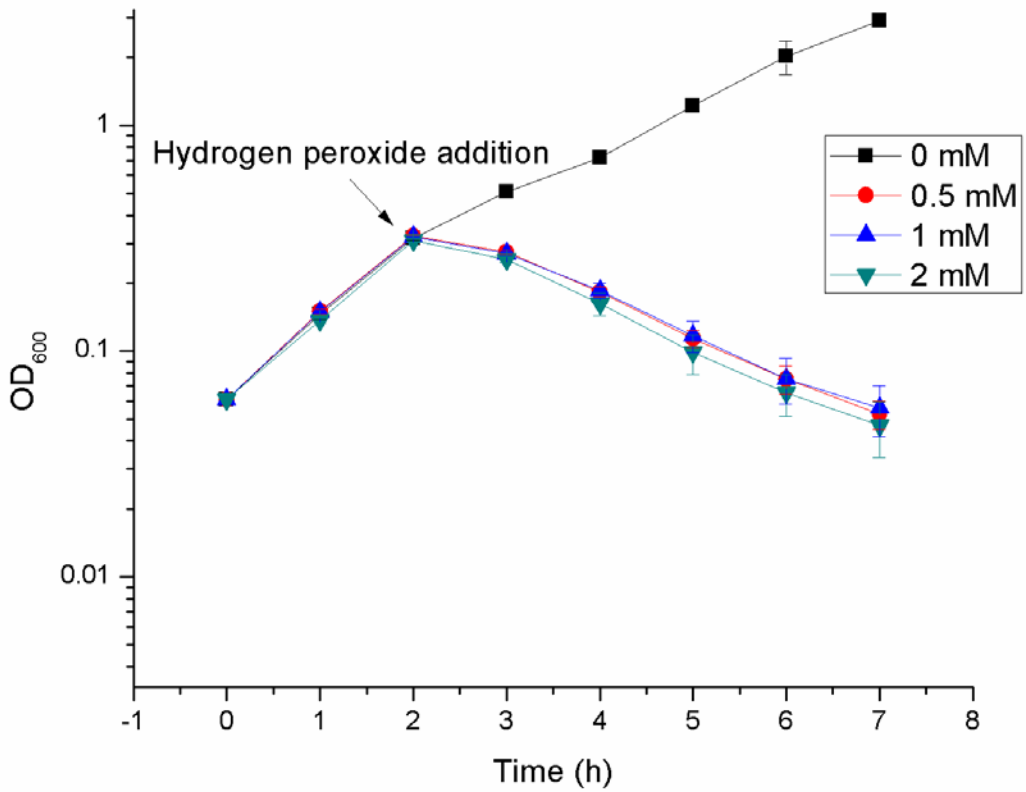


Figure 2.11. Response of $MTYM_{MAN}$ grown *B. methanolicus* MGA3 to hydrogen peroxide spikes. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MAN}$ in 250 ml baffled shaker flask and challenged with different concentrations of hydrogen peroxide (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

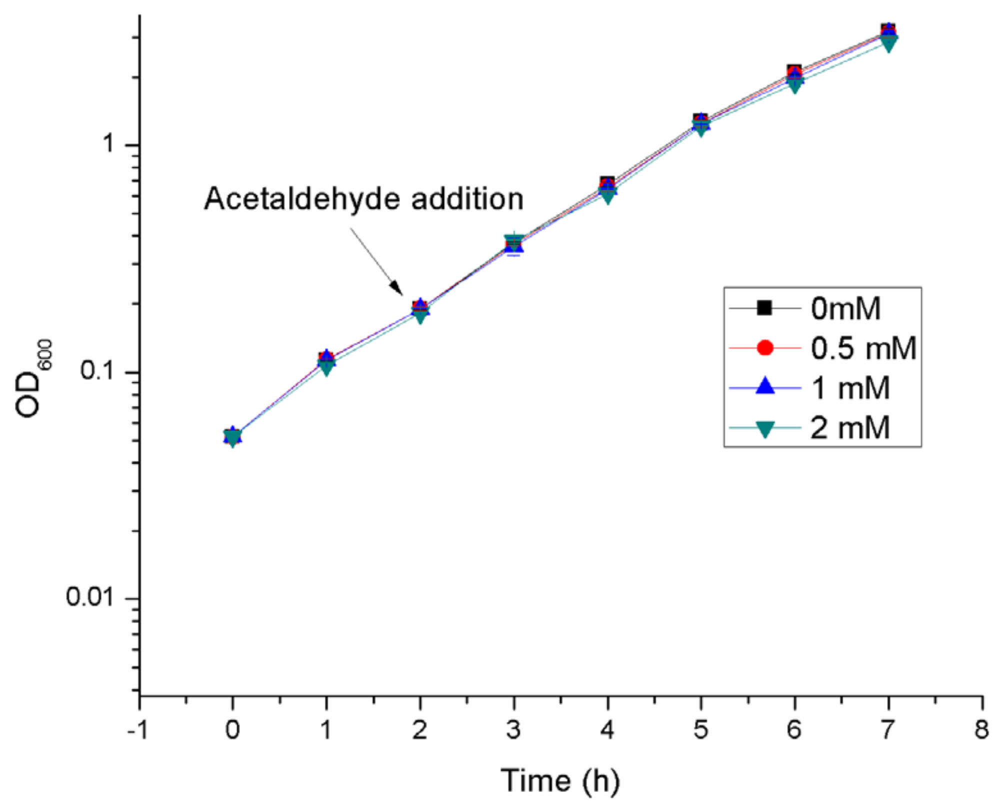


Figure 2.12. Response of $MTYM_{MeOH}$ grown *B. methanolicus* MGA3 to acetaldehyde spikes. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MeOH}$ in 250 ml baffled shaker flasks and challenged with different concentrations of acetaldehyde (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

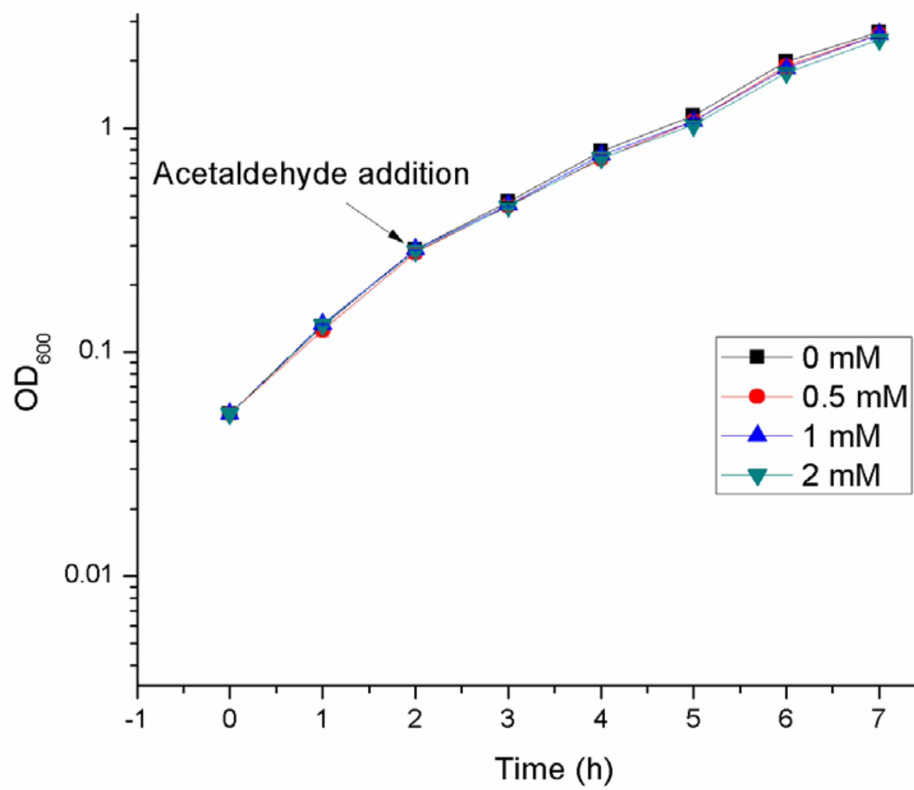


Figure 2.13. Response of $MTYM_{MAN}$ grown *B. methanolicus* MGA3 to acetaldehyde spikes. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MAN}$ in 250 ml baffled shaker flasks and challenged with different concentrations of acetaldehyde (n=3) when the cells were growing exponentially (OD_{600}~0.25) .

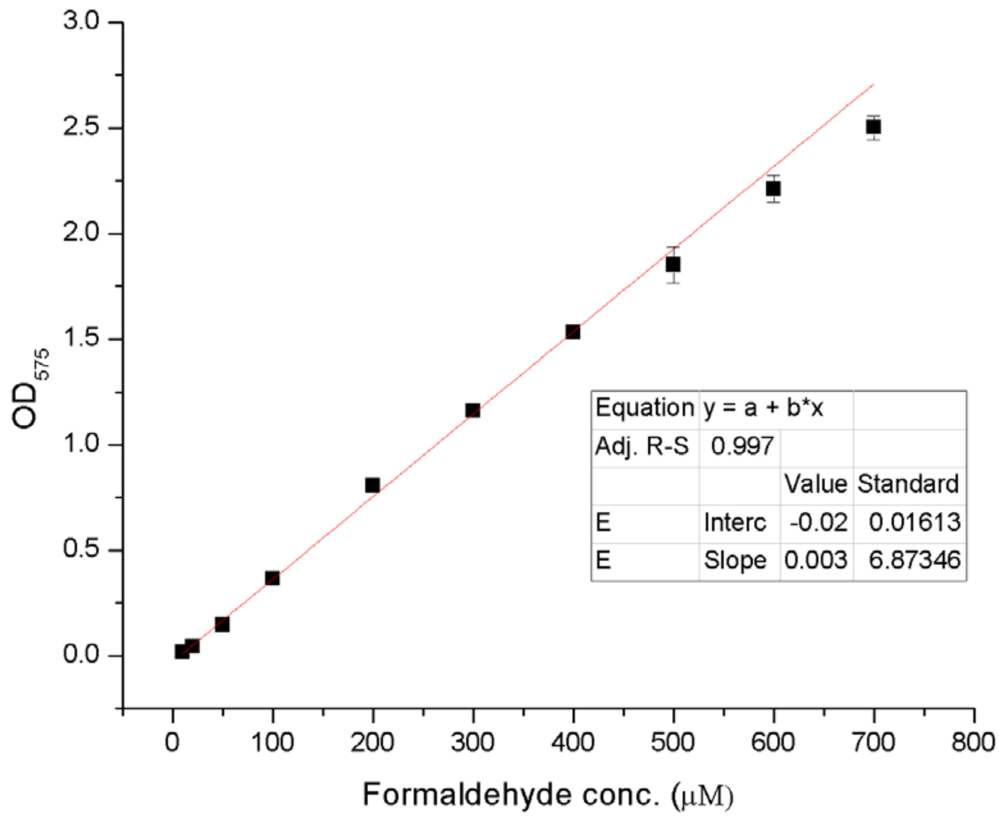


Figure 2.14. Standard curve obtained by the method of Topp and Knowles (1984) that was used to calculate the formaldehyde concentrations in the bacterial culture. Formaldehdye was diluted in MTYM medium to have a range of 10 to 700 µM.

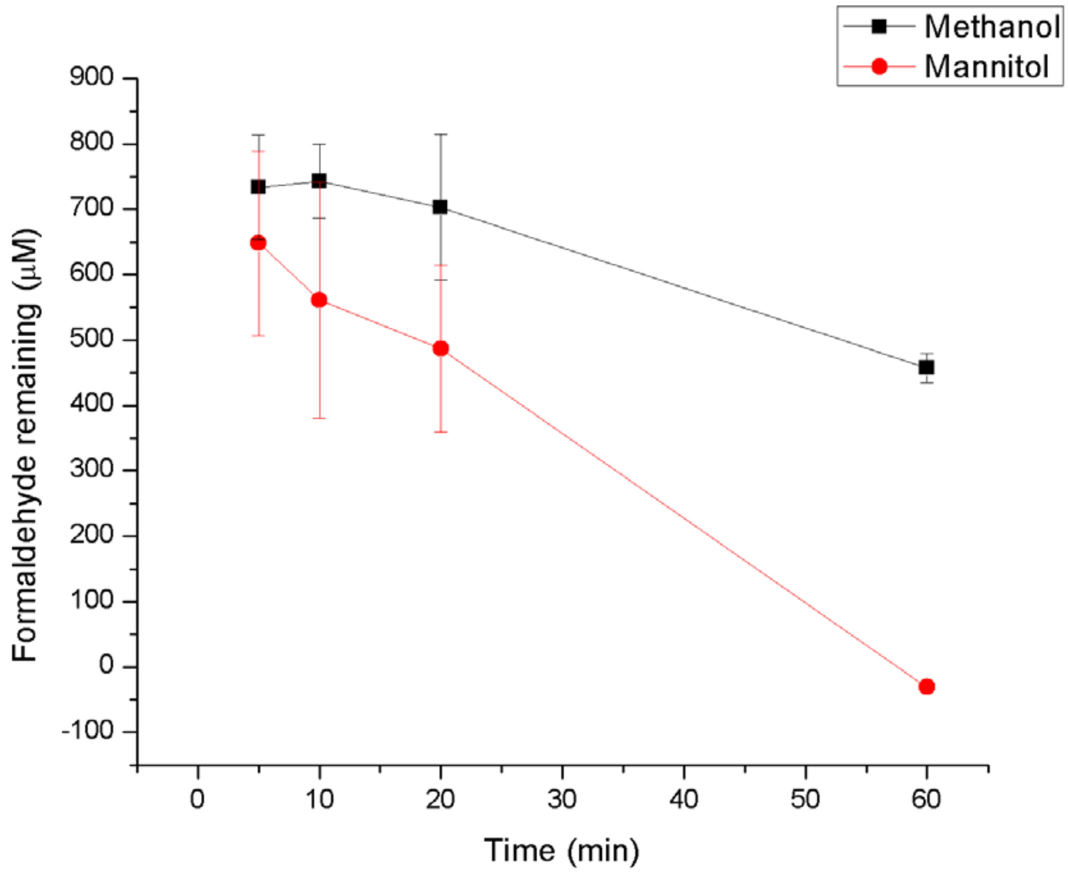


Figure 2.15. Remaining formaldehyde in the *B. methanolicus* MGA3 culture media. Formaldehyde (2 mM final) was added to *B. methanolicus* MGA3 growing in $MTYM_{MeOH}$ (Methanol) or $MTYM_{MAN}$ (Mannitol) at the OD_{600} of 0.8. One milliliter sample was used to measure the formaldehyde concentration in the culture media at 5, 10, 20 and 60 minutes post addition. (n=3)

CHAPTER 3

GENOME SEQUENCE of *Bacillus methanolicus* MGA3 and ELUCIDATION OF CARBON DISSIMILATION PATHWAY

Introduction

Advances in genome sequencing technologies greatly reduced the cost of whole genome sequencing. The time required to sequence a whole bacterial genome has also been reduced significantly since current sequencing technologies do not require cloning the DNA fragments into a bacterial host which is usually *E. coli*. These advances resulted in a geometric increase in the number of sequenced microorganisms (Binnewies et. al., 2006). The number of bacteria that have complete genome sequence has increased from 792 as of February 2009 (Tettelin and Feldblyum, 2009) to 2824 by February 2012 (GOLD, www.genomesonline.org). There are also 7931 ongoing bacterial genome sequence projects that are not complete yet. The presence of a vast amount of DNA sequences led to the birth of the genomics discipline.

The advances in sequencing also substantially widened the scope of metagenomic analysis of environmentally derived samples. In the pre-genomic era, it was required to isolate and culture a bacterium in the laboratory and run biochemical assays in order to obtain useful information about the metabolism of the bacterium. Having less than a half of 1% of 2-3 billion microbial species being identified (Fraser et. al., 2002), and only a very small proportion of these bacterial species being cultured (Handelsman, 2004), this limited our understanding of the microbial world and the metabolic diversity in the environment. However, advances in sequencing led researchers to overcome this difficulty. Qin et. al.

(2010) catalogued all microbial genes in the human gut, and constructed and compared the gut microbial flora between healthy and diseased individuals without isolating and culturing the individual bacteria. Similarly, Tyson et. al. (2004) revealed the metabolism and structure of a microbial community in the environment through reconstruction of microbial genomes from the environmental samples. Environmental genomes of SaragossaSea organisms were shotgun sequenced (Venter et. al., 2004) and revealed 1800 genomic species, 148 of which were unknown previously. This study also identified 1.2 million previously unknown genes. These studies show the power of sequencing; it would not be possible to isolate and culture all the 1800 microbial species and identify their unique genes.

Genome sequencing of obligate methanotrophic bacterium *Methylococcuscapsulatus* (Bath) (Ward et. al., 2004) led to the discovery of serine pathway genes in RuMP pathway methylotrophs which might have significant implications to the C1 metabolism of the bacterium. *M. capsulatus* (Bath) has a 3.3-Mb genome which encodes genes suggesting the ability to grow on sugars, to oxidize the hydrogen and sulfur and to grow under reduced oxygen tension. Another model microorganism that has been sequenced is the facultative methylotrophic bacterium *Methylobacteriumextorquens* AM1 (Vuilleumier et. al., 2009). It has a genome size of 6.88-Mb. Given both of these microorganisms are Gram-negative bacteria with high G+C content (63.6% and 68.7% for *M. capsulatus*(Bath) *and M. extorquens*AM1, respectively), it is necessary to sequence the genome of Gram-positive, thermotolerant, restrictive methylotroph*B. methanolicus* MGA3. Sequence of *B. methanolicus* MGA3 genome will serve as a reference to Gram-positive RuMP pathway methylotrophs.

Materials and Methods

Isolation of a plasmid from *B. methanolicus* PB1: The plasmid from *B. methanolicus* PB1 is isolated using Qiagen® Plasmid Midi Kit with the following modifications: 20 mg/ml lysozyme was added to Buffer P1 and incubated overnight at 37 °C and the mixture was centrifuged for 90 min at 14780g for the precipitation of plasmid with isopropanol at the precipitation step. The rest of the procedure is as written in the Qiagen manual.

Sequencing of the *B. methanolicus* PB1 plasmid: The plasmid from *B. methanolicus* PB1 was cloned into MCS of pUC18 after digestion with proper restriction enzymes. The clones were sequenced using M13 forward and reverse primers at DNA sequencing facility of University of Chicago. Primers were designed to sequence long inserts when needed.

Preparation of total DNA:*B. methanolicus*MGA3 was grown in MTYM media as described in Chapter 2 and 20 mL of cells were harvested by centrifugation (5 min x 10,000g) at the mid-exponential phase (OD_{600} of 1-1.5). The pellet was resuspended in 540 µl of lysis buffer (20 mMTris.Cl, 2 mM EDTA, 1.2% Triton X-100) with 20 mg/ml lysozyme and incubated at 37 °C for two hours. Total DNA was extracted with Qiagen® DNeasy® Blood and Tissue kit according to the manufacturer's instruction, albeit reagent volumes used were multiplied by 3 until filter binding. DNA was eluted in the buffer AE after extended incubation (up to 4 hours) of filter-bound DNA at room temperature. Eluted DNA was run on agarose gel (0.8% w/v) and quantified with a NanoDrop 1000.

Sequencing of *B. methanolicus*MGA3 total DNA and annotation: Sample preparation of *B. methanolicus* MGA3 total DNA was carried out at the North Carolina State University Genomics Sciences Laboratory (GSL). Total DNA of *B. methanolicus* MGA3 was shot-gun sequenced on the IlluminaGAIIx platform and raw sequences assembled *de novo* at GSL. Draft sequence of 117 contigs were analyzed and annotated using sequence visualization and annotation software Artemis (Trust Sanger Institute). Identified open reading frames (ORFs) were translated *in silico* and blasted against the protein database at NCBI.

Results

Sequence of plasmid from *B. methanolicus* PB1:Figure 3.1 presents the map of the plasmid isolated from *B. methanolicus* PB1. The sequences from the clones totaled to 20,187 bps. In compliance with the naming of pBM19 from *B. methanolicus* MGA3 (Figure 3.2), this plasmid is called pBM20. Like pBM19, pBM20 carries the RuMP pathway genes, *glpX, fba, tkt, pfk, rpe*along with *mdh.* The transposon *tnpI* is also present on the pBM20. The sequence of pBM20 presented in Appendix A.

Sequencing and assembly of *B. methanolicus* MGA3 genome: Theresults of the draft genome sequencing are summarized in Table 3.1 below. A total of 27,934,202 readings with 72 bp-long sequences yielded 2,011,262,544 bp total read length. There were 28,002 reads in 2,676,600 total sequence lengthin non-specific matches that were not mapped. *De novo* assembly of mapped reads resulted in a ~3.3 Mb genome from 117 contigs with an average coverage of 309 to 9091X. Based on the draft genome sequence obtained, *B. methanolicus* MGA3 genome has a GC content of 38.35%.

Table 3.1. Summary of *B. methanolicus* MGA3 genome sequencing

Contig count	117
Total read count	27,934,202
Mean read length	72.00
Total read length	2,011,262,544
Mean contig length	28,460
Total contig length	3,329,851
GC contents in %	38.35

Annotation: Annotation of the ORFs in *B. methanolicus* MGA3 draft genome is presented in Appendix B. The *B. methanolicus* MGA3 draft genome contains 2773 ORFs longer than 100 bp. Endogenous plasmid pBM19 sequence was assembled into Contig1. Previously reported methylotrophy genes (Brautaset et. al., 2004) were identified in the genome. Highlights from the genome sequence of *B. methanolicus* MGA3 are presented below.

Methanol dehydrogenase (*mdh*): Methanol dehydrogenase (MDH) is the first enzyme in the methanol utilization pathways of methylotrophs. Methanol is oxidized to formaldehyde by MDH. Methylotrophy in *B. methanolicus* was shown to be plasmid dependent since most of the RuMP genes and *mdh* were carried on the endogenous plasmid pBM19 (Brautaset et. al., 2004). Exceptions to these genes are hexulose phosphate synthase (*hps*) and phosphohexuloseisomerase (*phi)* which are located on the chromosome. Plasmid-borne *mdh* was shown to be the only gene responsible for the conversion of methanol to formaldehyde. Southern blots with plasmid born *mdh* as probe did not yield any hybridization on the chromosome. In addition, pBM19 cured *B. methanolicus* MGA3 was shown to lose the ability to grow on methanol. Genome sequence reveals two additional putative *mdh*genes in *B. methanolicus* MGA3; on contig27 and contig93 (ORFs 01267 and 02737). Both of these *mdh* genes are clearly different from the plasmid-borne *mdh*. The *mdh* on contig27 aligns with *Lysinibacillussphaericus*NAD-dependent methanol dehydrogenase. On the protein level, the *mdh* on contig27 has 70% identity with the *mdh* of *L. sphaericus*. The *mdh* on contig93 is similar to the *mdh* on contig27 with 96% identity on the protein level, and it aligns better with *L. fusiformis* ZC1. Both of these *mdh* genes are different from the previously reported methanol dehydrogenase (*mdh*) gene of *B. methanolicus* C1 (de Vries et. al., 1992). We also report the sequence of *mdh* from pBM20 of *B. methanolicus* PB1 for the first time. Comparison of *mdh* sequences is presented in Figure 3.3 and the relationship of the sequences are depicted in Figure 3.4.

Formaldehyde dehydrogenase (*faldh*): The next reaction in the dissimilation of methanol is the oxidation of formaldehyde to formate. Methanol was shown to be dissimilated through formaldehyde and formate in *B. methanolicus* MGA3 by ^{13}C NMR mass and isotope-ratio mass spectrometry (Pluschkell and Flickinger, 2002). Continuous cultures of *B. methanolicus* MGA3 were shown to accumulate formaldehyde and formate when spiked with [^{13}C] methanol at 50 °C (Pluschkell and Flickinger, 2002). Although this accumulation clearly demonstrates the conversion of methanol into formaldehyde and then formate, no *faldh* gene was reported from *B. methanolicus*. Attempts to demonstrate the presence of formaldehyde dehydrogenase by Southern blot using *faldh* from three *Bacilli* failed to provide the identity of the long sought genes (Smith, 2007 and Chapter 4).However, three ORFs in the genome of *B. methanolicus* MGA3 on contig19 (BMETHMGA300554c), contig20 (BMETHMGA300623) and contig36 (BMETMGA301733c) align with aldehyde dehydrogenases of *Geobacillus* sp. WCH70, *Geobacillusstearothermophilus,* and Bacillus sp. NRRL B-14911 with identities of 95%, 64%, and 91%, respectively.These genes are annotated as putative aldehyde dehydrogenases which might be the long soughtgenes. The FADH activities of the proteins encoded by these putative genes needs to be demonstrated biochemically.

THF- and THMPT-linkded enzymes: There is more than one possible route for conversion of formaldehyde into formate as mentioned in Chapter 1. *M. extorquens*AM1 oxidizes formaldehyde into formate mainly through THF- and THMPT-linked enzymes, although it also possesses a*faldh* gene (Crowther et. al., 2008). Putative genes encoding for the enzymes Methenyl-THF dehydrogenase and cyclohydrolase and Formate-THF ligase were found to be present in the genome of *B. methanolicus* MGA3. The presence of these enzymes suggests a possible alternative route for oxidation of C_1 compounds.

Formate dehydrogenase (*fdh)*: The last oxidation step in the dissimilation of methanol is the oxidation of formate into carbon dioxide. Oxidation of methanol to formate was known, as formate was the other intermediate shown to accumulate when *B. methanolicus* MGA3 was spiked with ^{13}C methanol (Pluschkell and Flickinger, 2002). Formate is thought to be oxidized to carbon dioxide by FDH since *B. methanolicus*MGA3 does not grow on formate. Although FDH activity was shown with crude cell extracts of *B. methanolicus* MGA3 (Smith, 2007), no *fdh* gene was reported. Two ORFs in the genome of *B. methanolicus*MGA3 encodes for putative FDHs. ORFs on contig32

(BMETHMGA301578c and BMETHMGA301579) encode two peptides; accessory protein and alpha chain of FDH, respectively. The alpha chain shares 81% identity with the *fdh* of *Bacillus halodurans* C-125. The accessory protein is on the complementary strand and transcribed in the opposite direction of the alpha chain. It shares 72% identity with formate dehydrogenase accessory protein of Bacillus sp. 2_A_57_CT2. The ORF on contig48 (BMETHMGA301931) encodes a putative *fdh* which is 82% identical to the *fdh* of *Geobacillus thermodenitrificans* NG80-2. As for putative formaldehyde dehydrogenases, the function of these *fdh* genes also needs to be verified biochemically.

Carbon monoxide dehydrogenase *(codh)*: Carbon monoxide can be used as a sole source of carbon and energy by microorganisms that employ the Wood-Ljungdahl pathway (Ragsdale, 2004). Carbon monoxide dehydrogenase (*codh*) plays a significant role in reduction of CO_2 and it has been extensively studied in anaerobic microorganisms (Ferry, 1999, Seravalli and Ragsdale, 2000, Ragsdale 2008). In *Moorella acetica* (formerly *Clostridium thermoaceticum*), it is found as a Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase (CODH/ACS) complex that reduces CO_2 to Acetyl-CoA by the Wood-Ljungdahl pathway (Seravalli and Ragsdale, 2000). The *codh* is widely distributed in Desulfuricants, Hydrogenogens, Acetogens and Methanogens (Oelgeschlager and Rother, 2008). The genome of *B. methanolicus* MGA3 also contains a cluster of ORFs (BMETHMGA301736-01742) that encodes for CODH protein subunits. The ORF BMETHMGA301738 encodes the 773 aa. large subunit of CODH that has 75% identity with the CODH of *Thermaerobacter subterraneus* DSM 13965. The ORFs BMETHMGA301736-01742 encode for CODH medium subunit, CODH small subunit, CODH large subunit, CODH subunit F, CODH subunit G, CODH subunit D and CODH subunit E, respectively. Considering the presence of THF- and THMPT linked enzymes together with CODH, possibility of anaerobic growth on CO_2 through Wood-Ljungdahl pathway deserves further attention and experimentation.

Conclusion

The sequencing of the genome of the *B. methanolicus* MGA3 is an important step in understanding the metabolism of this bacterium as a promising industrial candidate. Genome sequence analysis has revealed the presence of two more *mdh*s in the chromosome in addition to the plasmid borne gene. Putative genes of *aldh*s and *fdh*s were identified in the genome. In addition to the candidate genes with possible roles in dissimilation of methanol, *B. methanolicus* MGA3 genome sequence has also revealed the presence of putative THF- and THMPT linked enzymes that may function in dissimilation of methanol. Another important outcome of the genome sequence is the presence of putative *codh* in the *B. methanolicus* MGA3 genome which may offer versatility broader than previously thought (Brautaset et. al., 2007).

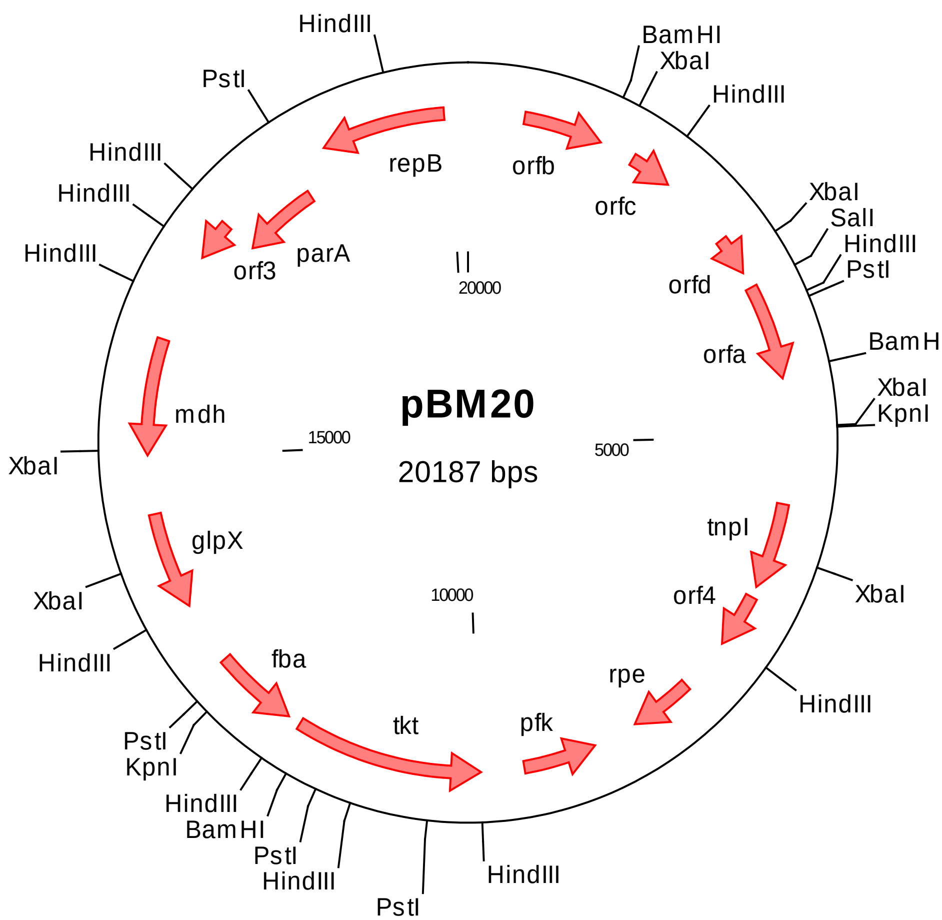


Figure 3.1. Plasmid from *B. methanolicus* PB1, pBM20.

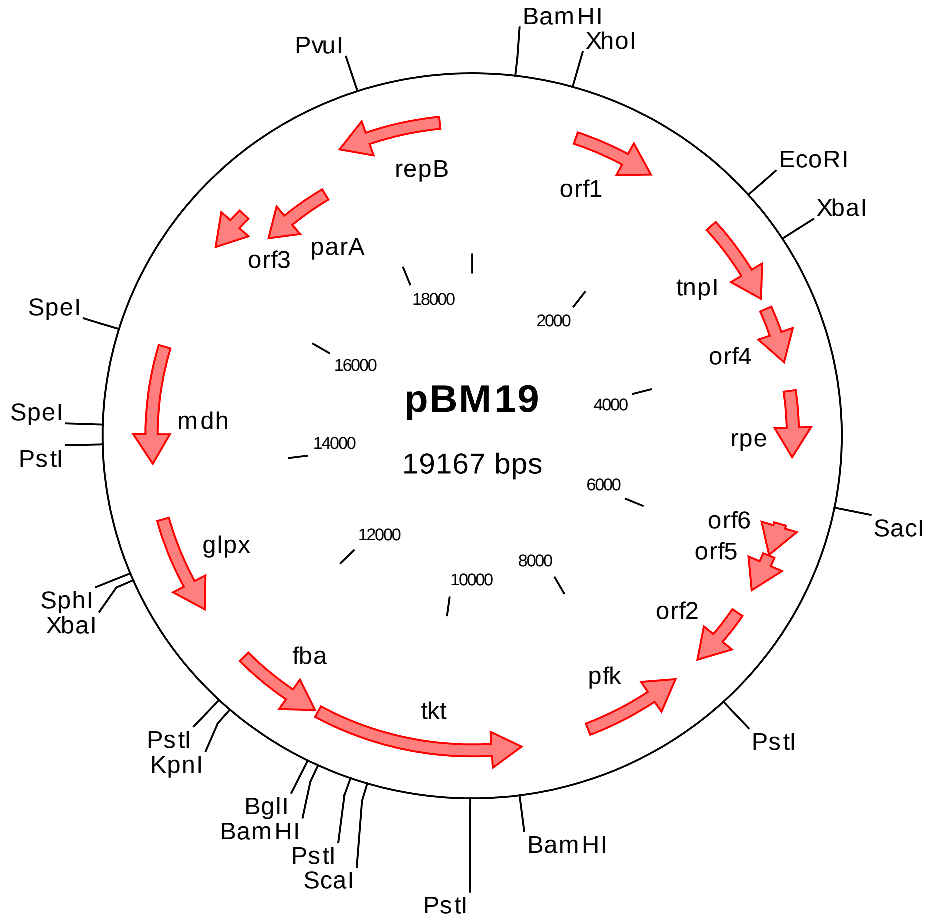


Figure 3. 2. Plasmid from *B. methanolicus* MGA3, pBM19 (Brautaset et. al., 2004).

```
B.methC1        ----MTNFFIPPASVIG--AVKEVGTRLKQIG--KALIVTDAFLHSTG--EEVAKNIREA 50
B.methPB1       --MTQRNFFIPPASVIGRGAVKEVGTRLKQIGATKALIVTDAFLHGTGLSEEVAKNIREA 58
B.methMGA3      ---MTTNFFIPPASVIGRGAVKEVGTRLKQIGAKKALIVTDAFLHSTGLSEEVAKNIREA 57
B.meth27        MKNTQSAFYMPSVNLFGAGSVNEVGTRLAGLGVKKALLVTDAGLHSLGLSEKIAGIIREA 60
B.meth93        MTNTQSAFFMPSVNLFGAGSVNEVGTRLADLGVKKALLVTDAGLHGLGLSEKISSIIRAA 60
                       *::*...::*  :*:******  :*  ***:**** **. *  *:::  ** *

B.methC1        GLD--VFPKAQPDPADTQVH--VDVFKQENCDALVS--GGSSHDTAKAIGLV--NGGRIN 102
B.methPB1       GLDAVIFPKAQPDPADTQVHEGVDIFKQEKCDALVSIGGGSSHDTAKAIGLVAANGGRIN 118
B.methMGA3      GVDVAIFPKAQPDPADTQVHEGVDVFKQENCDSLVSIGGGSSHDTAKAIGLVAANGGRIN 117
B.meth27        GVEVAIFPKAEPNPTDKNVAEGLEAYNAENCDSIVTLGGGSSHDAGKAIALVAANGGTIH 120
B.meth93        GVEVSIFPKAEPNPTDKNVAEGLEAYNAENCDSIVTLGGGSSHDAGKAIALVAANGGKIH 120
                *::  :****:*:*:*.:*   :: :: *:**::*:  ******:.***.**  *** *:

B.methC1        DYQGVNSV--PVVPVVAITTTAGT--ETTSLAVITDSAR--KMPVIDEKITPTVAI--PE 154
B.methPB1       DYQGVNSVEKPVVPVVAITTTAGTGSETTSLAVITDSARKVKMPVIDEKITPTVAIVDPE 178
B.methMGA3      DYQGVNSVEKPVVPVVAITTTAGTGSETTSLAVITDSARKVKMPVIDEKITPTVAIVDPE 177
B.meth27        DYEGVDVSKKPMVPLIAINTTAGTGSELTKFTIITDTERKVKMAIVDKHVTPTLSINDPE 180
B.meth93        DYEGVDVSKEPMVPLIAINTTAGTGSELTKFTIITDTERKVKMAIVDKHVTPTLSINDPE 180
                **:**:    *:**::**.*****  * *.:::***: *  **.::*:::***::*  **

B.methC1        LMVKKPAGLTIA--MDALSHAIEAYVAK--TPVTDAFAIQAMKL--EYLPKAVANGEDIE 208
B.methPB1       LMVKKPAGLTIATGMDALSHAIEAYVAKRATPVTDAFAIQAMKLINEYLPRAVANGEDIE 238
B.methMGA3      LMVKKPAGLTIATGMDALSHAIEAYVAKGATPVTDAFAIQAMKLINEYLPKAVANGEDIE 237
B.meth27        LMVGMPPSLTAATGLDALTHAIEAYVSTGATPITDALAIQAIKIISKYLPRAVANGKDIE 240
B.meth93        LMVGMPPSLTAATGLDALTHAIEAYVSTGATPITDALAIQAIKIISKYLPRAVANGKDIE 240
                ***  *..** *  :***:*******:.  **:***:****:*:  :***:*****:***

B.methC1        --EAMAYAQYMAGVAF--GGLGLVHSISHQVG--YKLQHGICNSVNMP--CAFNLIAKTE 260
B.methPB1       AREAMAYAQYMAGVAFNNGGLGLVHSISHQVGGVYKLQHGICNSVNMPHVCQFNLIARTE 298
B.methMGA3      AREKMAYAQYMAGVAFNNGGLGLVHSISHQVGGVYKLQHGICNSVNMPHVCAFNLIAKTE 297
B.meth27        AREQMAFAQSLAGMAFNNAGLGYVHAIAHQLGGFYNFPHGVCNAILLPHVCRFNLISKVE 300
B.meth93        AREQMAFAQSLAGMAFNNAGLGYVHAIAHQLGGFYNFPHGVCNAVLLPYVCRFNLISKVE 300
                  * **:** :**:**  .*** **:*:**:*  *:: **:**:: :*  * ****::.*

B.methC1        RFAH--ELLGENVSGLSTA--AERAIVALERYNKNF--PSGYAEMGVKEE--DILAKNAF 312
B.methPB1       RFAHIAELLGENVSGLSTASAAERAIVALQRYNKNFGIPSGYAEMGVKEEDIELLANNAY 358
B.methMGA3      RFAHIAELLGENVAGLSTAAAAERAIVALERINKSFGIPSGYAEMGVKEEDIELLAKNAY 357
B.meth27        RYAEIAAFLGENVDGLSTYEAAEKAIKAIERMARDLNIPKGFKELGAKEEDIETLAKNAM 360
B.meth93        RYAEIAAFLGENVDGLSTYDAAEKAIKAIERMAKDLNIPKGFKELGAKEEDIETLAKNAM 360
                *:*.   :***** ****   **:** *::*  :.:  *.*: *:*.***  : **:**

B.methC1        EDVCTQSN--VATVQDIAQIIKNAGALAEIAEGVVTGIANGHIAGEPLRKSIGGAKSKDG 370
B.methPB1       QDVCTLDNPRVPTVQDIAQIIKNAL----------------------------------- 383
B.methMGA3      EDVCTQSNPRVPTVQDIAQIIKNAM----------------------------------- 382
B.meth27        NDACALTNPRKPKLEEVIQIIKNAM----------------------------------- 385
B.meth93        KDACALTNPRKPKLEEVIQIIKNAM----------------------------------- 385
                :*.*:  *   ..:::: ******

B.methC1        ANRNVVAAILR 381
B.methPB1       -----------
B.methMGA3      -----------
B.meth27        -----------
B.meth93        -----------
```

Figure 3.3. Alignment of methanol dehydrogenase sequences from *B. methanolicus* strains. B.methC1; *B. methanolicus* C1, B.methPB1; *B. methanolicus* PB1, B.methMGA3; *B. methanolicus* MGA3, B.meth27; *B. methanolicus* MGA3 ORF01267, B.meth93; *B. methanolicus* MGA3 ORF02737. "*"; identical in all sequences, ":"; conserved substitutions are observed, "."; semi-conserved substitutions are observed.

Figure 3.4. Relatedness of methanol dehydrogenase sequences from *B. methanolicus* strains by Neighbour-Joining method (Thompson et. al., 1994). B.methC1; *B. methanolicus* C1, B.methPB1; *B. methanolicus* PB1, B.methMGA3; *B. methanolicus* MGA3, B.meth27; *B. methanolicus* MGA3 ORF 01267, B.meth93; *B. methanolicus* MGA3 (ORF 02737)

CHAPTER 4

ELUCIDATION OF C_1 ASSIMILATION AND DISSIMILATION PATHWAYS IN *B. methanolicus*

Introduction

Methanol assimilation pathway: The RuMP pathway was briefly described in Chapter 1. *B. methanolicus* employs the FBPA/SBPase variant of the RuMP pathway. There are two enzymes that function in the fixation part. The first enzyme of the RuMP pathway is the hexulose phosphate synthase (HPS). This enzyme catalyzes the synthesis of hexulose-6-phosphate from ribulose-5-phosphate and formaldehyde produced by the oxidation of methanol by methanol dehydrogenase. Three molecules of formaldehyde are condensed with ribulose-5-phosphate to yield three molecules of hexulose-6-phosphate. The second reaction in the fixation part is the isomerisation of hexulose-6-phosphate to fructose 6-phosphate (F6P) by 6-phospho-3-hexuloisomerase (PHI). The ORFs BMETHMGA300426 and BMETHMGA300427 located on the chromosome annotated as *hps* and *phi* encode for the enzymes HPS and PHI respectively. The first reaction in the cleavage part is the conversion of the F6P to fructose-1,6-bisphosphate (FBP) by phosphofructokinase (PFK). FBP is then cleaved to glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by fructose-1,6-bisphosphate aldolase. GAP enters into the regeneration part along with two FBPs where three ribulose 5-phosphate molecules are regenerated while three-carbon DHAP is the net gain of the RuMP pathway. *B. methanolicus* MGA3 is known to carry both genes encoding for PFK and FBPA on pBM19 (Brautaset et al., 2004). There are three more RuMP pathway genes that are located on pBM19, namely transketolase (*tkt*), SBPase (*glpX*), and ribulose 5-phosphate 3-epimerase (*rpe*) (Brautaset et. al., 2004).

Methanol dissimilation pathway: The methanol dissimilation pathway, oxidation of methanol to carbon dioxide through formaldehyde and formate, is not as well characterized in *B. methanolicus* as the RuMP pathway. Although the formation and accumulation of formaldehyde and formate were demonstrated with ^{13}C NMR studies by Pluschkell and Flickinger (2002) attempts to identify possible formaldehyde and formate dehydrogenases from *B. methanolicus*by PCR failed (Smith, 2007).

This chapter reports the identification of formaldehyde and formate dehydrogenases in *B. methanolicus* strain PB1 by PCR and Southern Blot, and also proposes the possibility of an alternative dissimilation pathway through the presence of THF- and THMPT-linked enzymes. This chapter also reports the presence of additional copies of RuMP pathway genes on the chromosome.

Materials and Methods

Cloning *B. methanolicusfaldh*:Total DNA was isolated from *B. methanolicus* and *B. subtilis*, *Geobacillus kaustophilus* and *Bacillus licheniformis* as described in Chapter 3. Primers with indicated restriction sites at the end (Table 4.1) were designed specific to the putative aldehyde dehydrogenases (*aldh*) that have high homology to known formaldehyde dehydrogenases (*faldh)*. The *yycR* gene of *B. subtilis*, *ywdH* gene of *B. licheniformis* and *G. kaustophilus* ORF between 2797314-2798810 bases were selected as candidate *faldh* genes. These primers were used to amplify candidate *faldh* genes by PCR using the total DNA of *B. methanolicus* strains MGA3 and PB1 with the following parameters: 5 min initial denaturation at 94 °C then 25 cycles of 1 min annealing at 52 °C, 1 min extension at 72 °C, and 30 sec denaturation at 94 °C. A final extension at 72 °C was added at the end of the cycle. The PCR reactions contained 500 nM forward and reverse primers, 1.5 mM $MgCl_2$, 500 µM of dNTP mix, and 50-200 ng of template DNA. The PCR mixture was then run on a 0.8 % agarose gel and visualized with Kodak Gel Documentation. The PCR fragment then was digested with respective restriction enzymes and ligated into pET16-b or pET17-b. *E. coli* DH5α was transformed with the plasmid as described by Maniatis et al., (1982), and the colonies were screened for the insert using T7 forward and reverse primers with the PCR parameters as described above.

Cloning of *B. methanolicus fdh*: The same approach was used for the cloning of *fdh* genes of *B. methanolicus* using the primers designed for putative *fdh* (*yrhE*) genes of *B. subtilis*, *B. licheniformis* and *G. kausotphilus* ORF located in between bases 498622-501585 using the primers listed in Table 4.1.

Southern Blot: Southern blotting was employed to demonstrate the presence of putative *faldh* and *fdh* genes in *B. methanolicus* strains. The primers in Table 4.1 were used for the synthesis of Digoxigenin (DIG)-labeled DNA probes by Roche's PCR DIG Probe Synthesis Kit (Cat# 11 636 090 910) as described in the manufacturer's manual using the total DNA from the respective *Bacilli* species as template. A DIG-labeled DNA probe was also synthesized for the citrate synthase (*citY*) gene of *B. methanolicus* MGA3 as a control using Bm_CitY_Fw and Bm_CitY_Rv primers in Table 4.1. Incorporation of DIG-labeled nucleotides into the PCR amplicon was checked with gel electrophoresis as DIG-labeled DNA moves slower than its non-labeled counterpart. The total DNA from *B. methanolicus* strains were digested completely using restriction enzyme NdeI. Different amounts of DNA were blotted to find the required amount for the hybridization and 12 µg was found to give good hybridization. Twelve µg of digested DNA was loaded into an agarose gel and run until a good separation was achieved. Then the DNA was transferred onto a nylon membrane by capillary transfer as described in the Roche's application manual. Prehybridization, hybridization, washing and detection were carried out according to the instructions in the manual. Hybridization was carried out at 55 °C in 50 ml falcon tubes in HYBAID incubator. The membrane was washed and blocked using the Roche's DigWash and Block Buffer Set (Cat# 11 585 762 001). Anti-Digoxigenin-AP, Fab fragments (Cat# 11 093 274 910) along with the NBT/BCIP tablets, were used for the detection and visualization of the hybridization.

Results

RuMP pathway genes: *B. methanolicus* is known to employ the RuMP pathway for the assimilation of C_1-carbon into biomass, and it was shown to have a plasmid-dependent methylotrophy (Brautaset et al., 2004) since the entire RuMP pathway genes except the *hps* and *phi* were carried on a native plasmid pBM19. Here we describe additional copies of RuMP pathway genes on the chromosome: The *B. methanolicus* MGA3 ORF BMETHMGA301123 encodes a ribulose phosphate epimerase enzyme which has 80% identity to *Bacillus* sp. 2_A_57_CT2. It also shares a 74% identity with the plasmid borne *rpe*, the ORF BMETHMGA300014c. The *pfk* gene also has an additional copy on the chromosome; BMETHMGA300592. It is 81% identical to *pfk* of *Bacillus* sp. NRRL B-14911and 61% identical to plasmid-borne *pfk*. The chromosomal copy of the *tkt* gene is located on the ORF BMETHMGA300446c. The chromosomal *tkt* has 84% identity to *Bacillus* sp. 2_A_57_CT2 and shares 76% identity with *tkt* carried on the pBM19. *B. methanolicus* MGA3 has a chromosomal copy of *fba* on ORF BMETHMGA302111 which has 88% identity to *fba* of *B. licheniformis* ATCC 14580. The ORF BMETHMGA302111 shares 74% identity with the previously reported *fba* on pBM19 (Brautaset et al., 2004). The chromosomal copy of SBPase (*glpX*) is located down stream of the *fba*, on ORF BMETHMGA302113. It has 87% identity to the *glpX* gene of *B. licheniformis* ATCC14580 and a comparatively lower identity to the plasmid borne *glpX*, 54%. In summary, all of the RuMP genes carried on the pBM19 have chromosomal counterparts although they are not identical.

Cloning *faldh* of *B. methanolicus* PB1: The primers in Table 4.1 were used to target *faldh* genes of *B. methanolicus* strains MGA3 and PB1 by PCR. The annealing temperature of the PCR was varied to optimize the amplification and the best temperature was found to be 52 °C. As can be seen in the picture of the gel electrophoresis (Figure 4.1), only primers designed for *B. licheniformis ywdH* produced amplification. This fragment was digested with NdeI and BamHI sites incorporated into the primers and cloned into the respective site of pET16-b. The insert was sequenced with T7 forward, T7 reverse. The partial sequence obtained shares 97% identity with the *ywdH* gene of *B. licheniformis* ATCC14580.

Southern Blot: Attempts to clone prospective *faldh* and *fdh* genes from *B. methanolicus* were unsuccessful with *B. licheniformis yydH* primers except for the putative *faldh* gene from strain PB1. Therefore, I aimed to show the presence of these genes in *B. methanolicus* by Southern blotting. The restriction enzyme NdeI produces a good smear as can be seen in Figure 4.2. The *B. methanolicus citY* gene was used as a control. Figure 4.3 clearly shows the efficiency of hybridization with different amounts of DNA as template. It can be seen from the Figure 4.4 that there is similarity of DNA between strains MGA3 and PB1, and *B. methanolicus* PB1 contains three copies of the citrate synthase gene. Figures 4.5-4.7 displays the hybridization of probes from genes of three *Bacilli* with *B. methanolicus* PB1 DNA. As can be seen, *B. methanolicus* PB1 DNA hybridizes with the *B. licheniformisywdH* gene but the *B. subtilis* or *G. kaustophilus* probes did not produce any hybridization with either of the strains. As for the *fdh* (Figures 4.8-4.10), the probes from *B. subtilis*, *B. licheniformis* and *G. kaustophilus* all hybridized with the *B. methanolicus* PB1 DNA although the hybridization of *B. subtilis* was weaker than the others. The absence of hybridization with the *B. methanolicus* MGA3 DNA is most probably due to DNA structure of the strain, since putative *fdh* of strains MGA3 and PB1 are 90% identical. Similarly, putative *fdh* of *G. kaustophilus* is 53% identical to *fdh* of PB1, and 54% to the *fdh* of strain MGA3. However, it only hybridizes with strain PB1 DNA, which suggests difference in the DNA structure rather than DNA sequence between the strains MGA3 and PB1.The relative places of the hybridization bands on the membrane suggest that *B. methanolicus* PB1 carries more than one copy of the *fdh* gene.

None of the tested probes produced hybridization with *B. methanolicus* MGA3 while they hybridized with strain PB1 DNA. This might be either because of the structure of the DNA or the difference in the base composition of the target genes. To test this, probes of *B. subtilis citZ* gene were hybridized against both strains. Although *B. methanolicus* MGA3 has a citrate synthase II gene, *citY* (Brautaset et. al., 2003),the *B. subtilis citZ* probe hybridized only with PB1 DNA as it can be seen in Figure 4.11. It is also noteworthy that putative *fdh* genes and *aldh* genes, possibly *faldh* were identified in the genome of *B. methanolicus* MGA3 (below). This suggests that the base composition of the genes, *citY* gene in this case, in strain MGA3 is different than strain PB1.

Methanol dissimilation pathways of *B. methanolicus*: The presence of putative *faldh* and *fdh* genes of *B. methanolicus* in addition to chromosomal *mdh* genes were explained in Chapter 3. However, these three genes are not the only possible route for the dissimilation of methanol. The presence of THF- and THMPT-linked pathway genes also suggests alternative routes for carbon dissimilation. Figure 4.12 outlines the possible routes methanol carbon may follow to carbon dioxide. The first reaction of the THF-linked pathway, the condensation of formaldehyde with THF, occurs non-enzymatically. The *B. methanolicus* MGA3 genome possesses a putative methenyl-THF cyclohydrolase & dehydrogenase gene (ORF 00712) that is 76 % identical to the *Bacillus sp.* 1NLA3E. This is a bifunctional enzyme that can catalyze the conversion of methylene-THF to methenyl-THF first and then to formyl-THF. It is possible for the THF- and THMPT-linked enzymes to cross-catalyze the substrates as in the example of the MtdA enzyme of *M. extorquens* AM1 (Hagemeier et. al. 2000), and these enzymes may function in detoxification of formaldehyde (Marx et. al., 2003). The next reaction of the pathway is the conversion of the formyl-THF into formate. This reaction is catalyzed by formyl-THF cyclo-ligase (ORF BEMTHMGA300665). This putative enzyme shares 58% identity with the formyl-THF cyclo-ligase of *Bacillus* sp. 2_A_57_CT2. The last step in the dissimilation is the oxidation of formate into carbon dioxide by formate dehydrogenase. As discussed in Chapter 3, *B. methanolicus* MGA3 possesses two putative formate dehydrogenases.

Table 4.1. List of primers used for cloning of respective genes and synthesis of DIG-labeled probes. Respective restriction sites are underlined.

Microorganism	Gene	Primer	Sequence
B. subtilis	*yycR*	Bs_faldh_Fw	5'-ATG GTA CTC GAG ATG GGA GGA ATG GCG TTG ACA G-3'(XhoI)
B. subtilis	yycR	Bs_faldh_Rv	5'-ATG GTA GGA TCC TTA CTT CAA TGT CCC ATG-3' (BamHI)
B. subtilis	yrhE	Bs_fdh_Fw	5'-ATG GTA CTC GAG ATG AGA TCT TTT GCC ACA CAA C- 3'(XhoI)
B. subtilis	yrhE	Bs_fdh_Rv	5'-ATG GTA GGA TCC TCA ATC TGT ACA CGG CCT G-3' (BamHI)
B. licheniformis	ywdH	Bl_faldh_Fw	5'- ATG GTA CAT ATG GCA GAA CGA ACA ACC TCC-3' (NdeI)
B. licheniformis	ywdH	Bl_faldh_Rv	5'-ATG GTA GGA TCC CTA CCT GAG AAG TTT TTT G-3' (BamHI)
B. licheniformis	yrhE	Bl_fdh_Fw	5'- ATG GTA CAT ATG GAC GGA AAG CCA ATC AG-3' (NdeI)
B. licheniformis	yrhE	Bl_fdh_Rv	5'- ATG GTA GGA TCC TTA GTC TGT TAA ATG AAC G-3' (BamHI)
G. kaustophilus	2797314-	Gk_faldh_Fw	5'- ATG GTA CAT ATG ACA ATG ATT TCG GCG-3' (NdeI)
G. kaustophilus	2797314-	Gk_faldh_Rv	5'- ATG GTA GGA TCC TTA AAA CAA CGT CAT CGG-3' (BamHI)
G. kaustophilus	498622-	Gk_fdh_Fw	5'-ATG GTA CAT ATG GAA GCG CAA ATG GTG-3'(NdeI)
G. kaustophilus	498622-	Gk_fdh_Rv	5'-ATG GTA GGA TCC TTA GCC ACC GAT CGT CAC-3' (BamHI)
B. subtilis	citZ	Bs_CitZ-Fw	5'-ATG ACA GCG ACA CGC GG-3'
B. subtilis	citZ	Bs_CitZ-Rv	5'-TTA GGC TCT TTC TTC AAT CG-3'
B. methanolicus		Bm_citY_Fw	5'-ATG ACA GTT ACA CGT GGT C-3'
B. methanolicus		Bm_citY_Rv	5'-TTA TCC TCT TTG CTC AAT TGG-3'

Wood-Ljungdahl pathway and Carbon monoxide dehydrogenase: The anaerobic bacterium *Clostridium ljungdahlii* uses the Wood-Ljungdahl pathway to fix carbon dioxide. The enzymology of the pathway is discussed in detail by Ragsdale (2008). The THF-linked enzymes mentioned above also take part in the fixation of carbon from carbon dioxide (Ragsdale, 2008). With the addition of methylene tetrahydrofolate reductase and carbon monoxide dehydrogenase enzymes, all enzymes of the eastern branch of the Wood-

Ljungdahl pathway can be located in the genome of *B. methanolicus* MGA3 (Figure 4.13). Briefly, carbon dioxide is reduced to formate by formate dehydrogenase. Then, it is condensed with THF to form formyl-THF by formate-THF ligase (ORF BMETHMGA300091). The putative *B. methanolicus* MGA3 formate-THF ligase is a 562 aa protein with 82% identity to the gene of *Bacillus* sp. 2_A_57_CT2. The next two reactions, conversion of the formyl-THF to 5,10-methenyl-THF and then to 5,10-methylene-THF are catalyzed by the methenyl-THF cyclohydrolase&dehydrogenase enzyme described above. Then, methylene-THF reductase (*metF*) converts 5,10-methylene-THF into 5-methyl-THF. *B. methanolicus* MGA3 possesses two copies of *metF*, ORFs BMETHMGA300824c and BMETHMGA301597c. The former 310 aa-long *metF* has 97% identity to the *metF* gene of *Geobacillus thermoglucosidans* while the latter 621 aa-long *metF* shares 77% identity with the *metF* gene of *Bacillus* sp. 2_A_57_CT2. Then, the methyl group of methyl-THF is condensed with carbon dioxide by carbon monoxide dehydrogenase (CODH) to produce acetyl-CoA. CODH enzymes may be bifunctional and also catalyze the reaction of Acetyl-CoA synthase enzyme (ACS). *B. methanolicus* MGA3 has a multimeric carbon monoxide dehydrogenase. The *codh* operon consists of seven ORFs, BMETHMGA301736-42. The large subunit of the *B. methanolicus* MGA3 *codh* is 75% identical to *Thermaerobacter subterraneus codh* large subunit. It should also be noted here that *T. subterraneuscodh* encodes an aerobic-type CODH which catalyzes the oxidation of carbon monoxide to carbon dioxide.

Conclusion

The genomic data revealed the presence of additional chromosomal copies of all RuMP pathway genes except the *hps* and *phi*. Being the first two reactions of the RuMP pathway, it is clear that the rate of down stream reactions will depend on the rates of these two enzymes and upregulation of these enzymes will result in an increased rate of the overall RuMP pathway (Brautaset 2004). The southern blot results demonstrate that the DNA composition of *B. methanolicus* strain MGA3 is different from the strain PB1. Combined with the southern blot results, genomic data suggest the presence of the *faldh* and *fdh* genes in both MGA3 and PB1 strains. For the first time, we propose putative *faldh* and *fdh* genes in *B. methanolicus* MGA3. In addition to *faldh* and *fdh* genes, the presence of the THF-linked enzymes implies an alternative route for oxidation of the formaldehyde to formate as depicted in Figure 4.12

Bacillithiol is a newly described antioxidant compound in *Bacillus*species (Newton et. al., 2009) that functions similar to mycothiol. *B. methanolicus* carries some of the bacillithiol synthesis genes, like*Bsh*A(ORF 2041) which suggests the synthesis of bacillithiol. However, no *faldh* enzyme was described to be bacillithiol dependent.The proposed formaldehyde dissimilation pathway does not change in the presence of bacillithiol in *B. methanolicus*.

Figure 4.13 displays a possible Wood-Ljungdahl pathway in *B. methanolicus* MGA3. With the inclusion of methenyl-THF reductase (BMETHMGA300824c and 01597c), the Wood-Ljungdahl pathway is completed. The presence of this pathway is rather surprising for an aerobic methylotrophic microorganism. As stated by Cordwell (1999), the presence of a gene sequence does not directly imply that the enzyme is functional. The gene might be a remnant nonactive gene through the evolutionary history. The functionality of these genes needs to be confirmed biochemically.

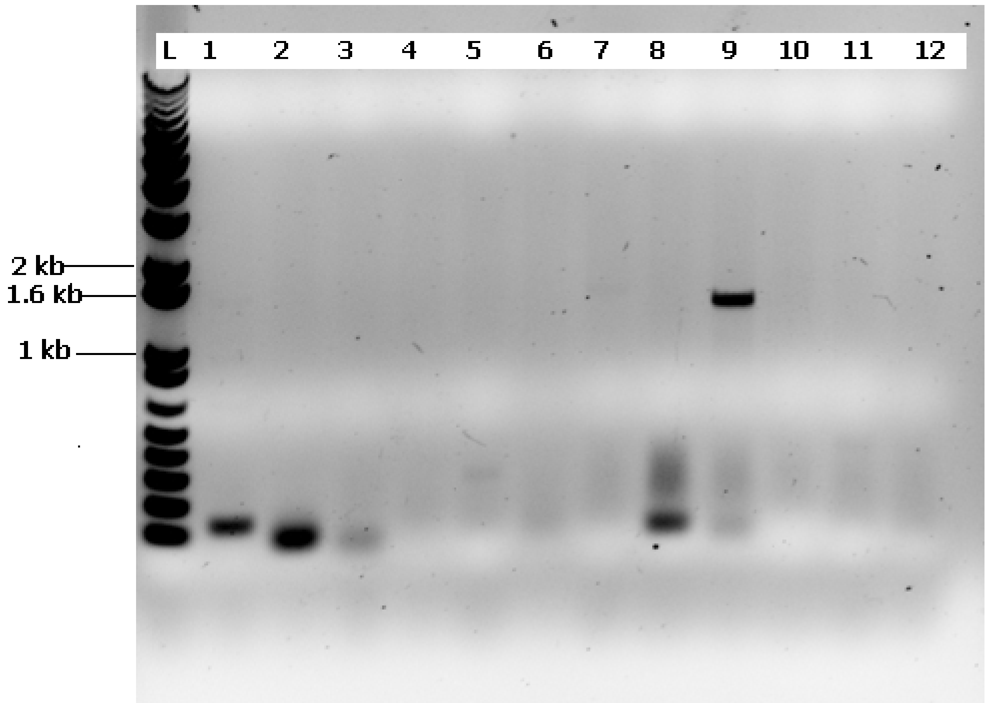


Figure 4.1. PCR amplification of *faldh*from*B. methanolicus* MGA (wells 1-6) and PB1 strains (wells 7-12) with *G. kaustophilus* putative *faldh* (1 and 7) and *fdh* (2 and 8), *B. licheniformisywdH* (3 and 9) and *yrhE* (4 and 10), *B. subtilis yycR* (5 and 11) and *yrhE* (6 and 12) primers. *B. licheniformisywdH* primers produced a similar length (1500 bp) amplicon from strain PB1.

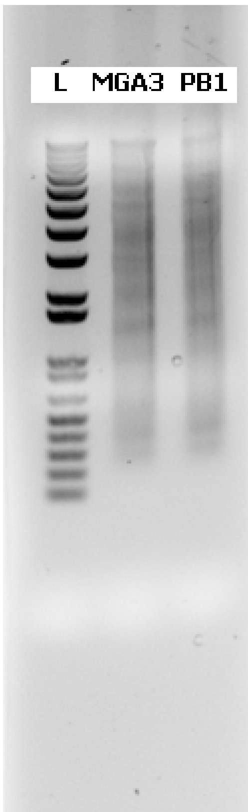


Figure 4.2. Digest of *B. methanolicus* strains MGA3 and PB1 with restriction enzyme NdeI. L; DNA ladder, MGA3: total DNA from strain MGA3, PB1; total DNA from strain PB1.

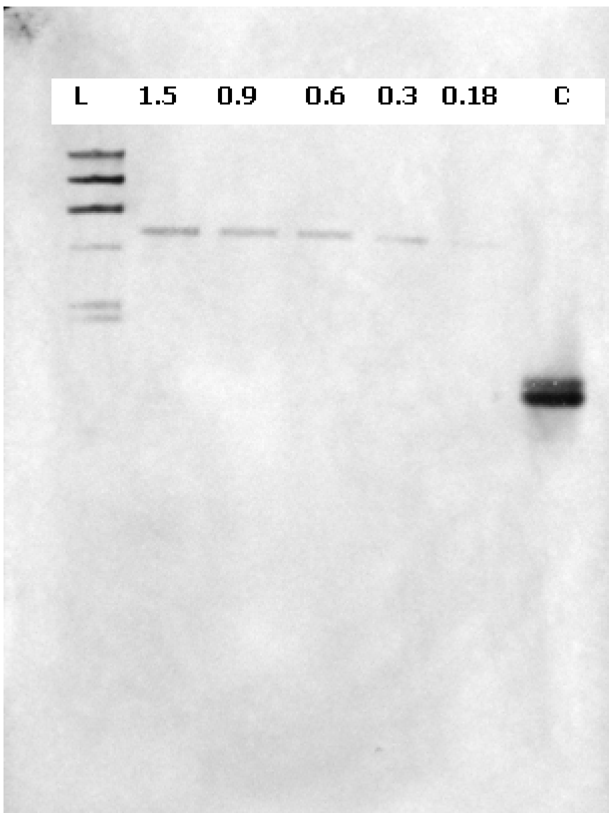


Figure 4.3. Southern blot control experiment with *B. methanolicus* MGA3 *citY* gene as probe and *B. methanolicus* MGA3 DNA digested with NdeI as target. L; Dig-labeled ladder, 1.5, 0.9, 0.6, 0.3 and 0.18; amount of DNA loaded to the gel before blotting to the membrane. C; control with *B. methanolicus* MGA3 *citY* gene, amplified by PCR.

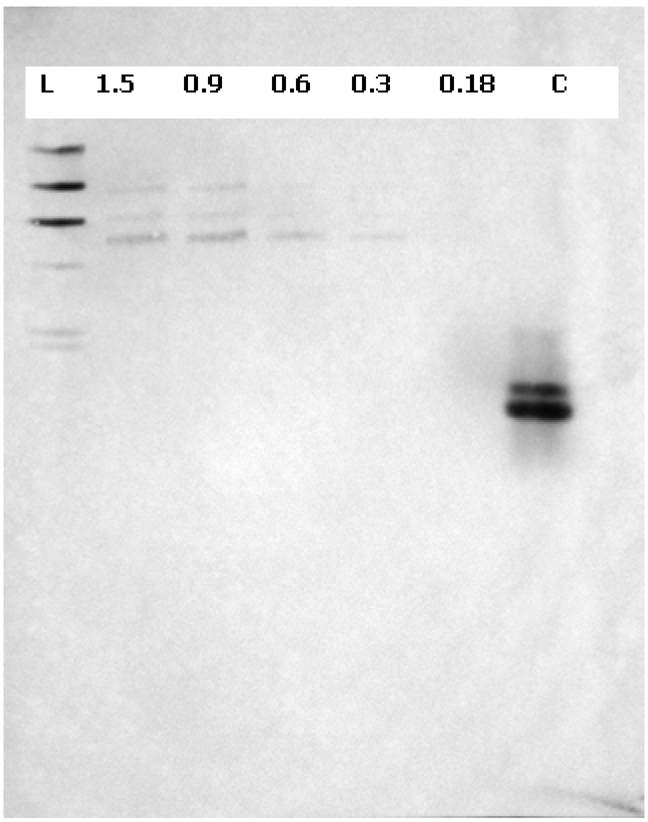


Figure 4.4. Hybridization of *B. methanolicus* MGA3 *citY* gene as probe with *B. methanolicus* PB1 DNA digested with NdeI as target. L; Dig-labeled ladder, 1.5, 0.9, 0.6, 0.3 and 0.18; amount of DNA loaded to the gel before blotting to the membrane (µg). C; control with *B. methanolicus* MGA3 *citY* gene, amplified by PCR.

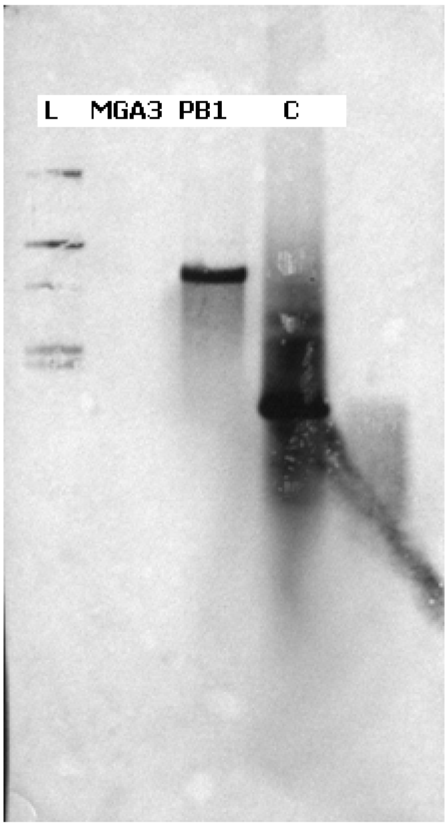


Figure 4.5. Hybridization of *B. licheniformi sywdH* gene as probe against*B. Methanolicus* MGA3 and PB1. L; Dig-labeled DNA ladder, MGA3; *B. methanolicus* MGA3, PB1; *B. methanolicus* PB1, C; control (PCR amplicon of *B. licheniformisywdH* gene).

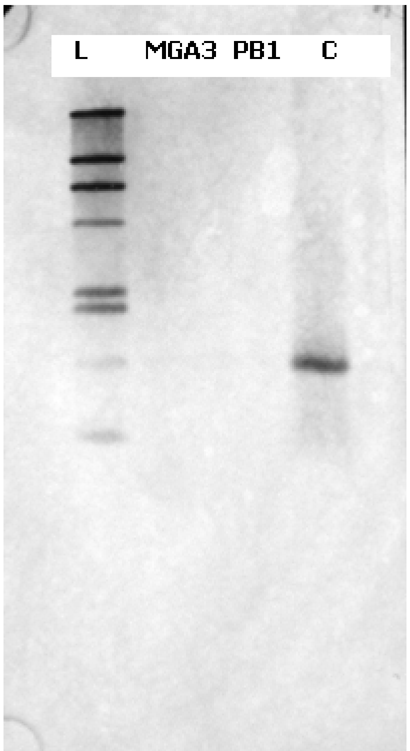


Figure 4.6. Hybridization of *B. subtilis ywdH* gene as probe against *B. methanolicus* MGA3 and PB1. L; Dig-labeled DNA ladder, MGA3; *B. methanolicus* MGA3, PB1; *B. methanolicus* PB1, C; control (PCR amplicon of *B. subtilis ywdH* gene).

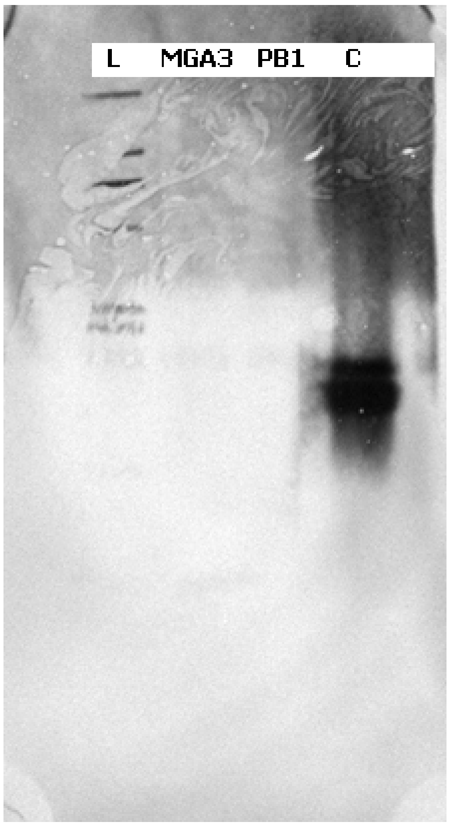


Figure 4.7. Hybridization of *G. kaustophilus* putative *faldh* gene as probe against *B. methanolicus* MGA3 and PB1. L; Dig-labeled DNA ladder, MGA3; *B. methanolicus* MGA3, PB1; *B. methanolicus* PB1, C; control (PCR amplicon of *G. kaustophilus*putative *faldh* gene).

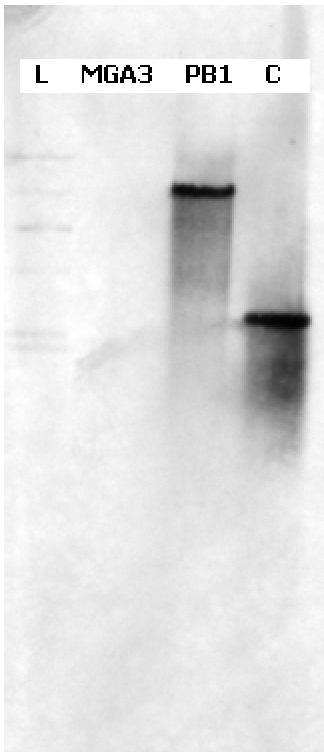


Figure 4.8. Hybridization of *B. licheniformis yrhE* gene as probe against *B. methanolicus* MGA3 and PB1. L; Dig-labeled DNA ladder, MGA3; *B. methanolicus* MGA3, PB1; *B. methanolicus* PB1, C; control (PCR amplicon of *B. licheniformisyrhE* gene).

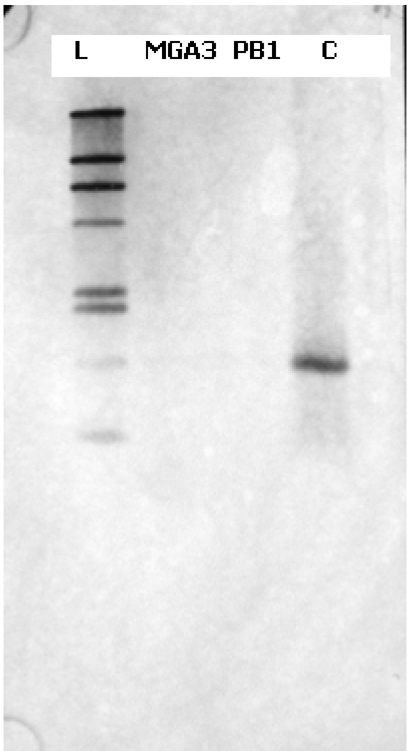


Figure 4.9. Hybridization of *B. subtilis yrhE* gene as probe against *B. methanolicus* MGA3 and PB1. L; Dig-labeled DNA ladder, MGA3; *B. methanolicus* MGA3, PB1; *B. methanolicus* PB1, C; control (PCR amplicon of *B. subtilisyrhE* gene).

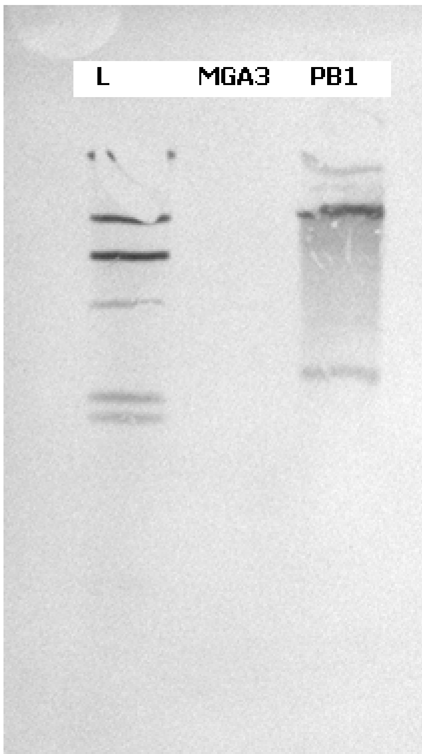


Figure 4.10. Hybridization of *G. kaustophilus* putative *fdh* gene as probe against *B. methanolicus* MGA3 and PB1. L; Dig-labeled DNA ladder, MGA3; *B. methanolicus* MGA3, PB1; *B. methanolicus* PB1.

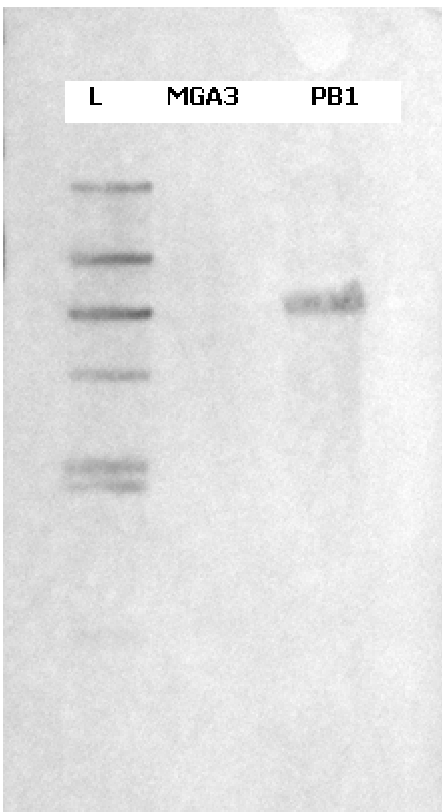


Figure 4.11. Hybridization of *B. subtilis citZ* gene against total DNA from *B. methanolicus* strains MGA3 and PB1. L; Dig-labeled DNA ladder, MGA3; total DNA from strain MGA3, PB1; total DNA from strain PB1.

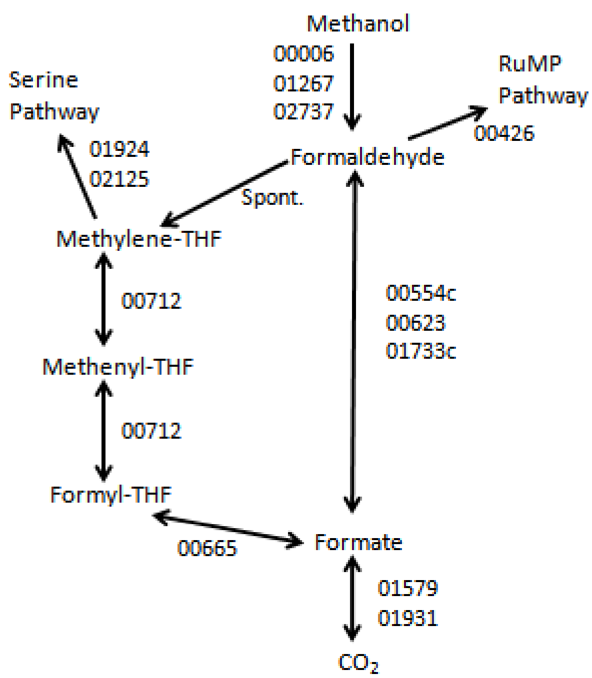


Figure 4.12. Proposed routes for the fate of methanol carbon based on the *B. methanolicus* MGA3 (NCSU) genomic data. ORFs encoding for the putative enzymes are indicated with their respective numbers.

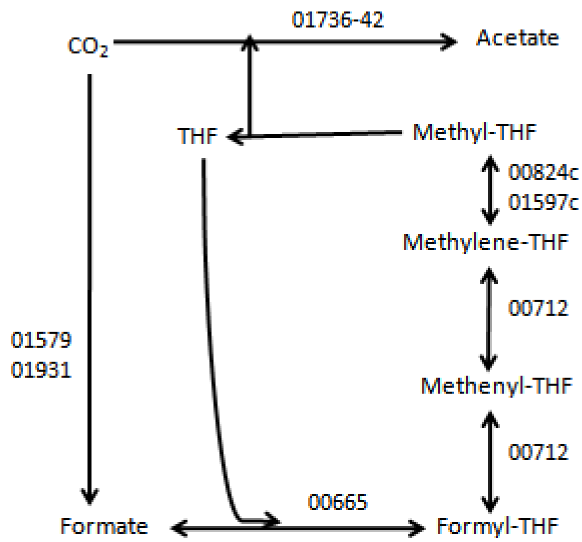


Figure 4.13. Construction of Wood-Ljungdahl pathway from the genomic data. ORFs encoding for the putative enzymes are indicated with their respective numbers.

CHAPTER 5

REGULATION OF METHANOL AND FORMALDEHYDE METABOLISM BY MeOH, FORMALDEHYDE AND FORMATE

Introduction

B. methanolicus is a restrictive methylotroph that is able to grow on a few sugar compounds other than methanol. Arfman *et al*., (1992) summarized the compounds that *B. methanolicus* species can utilize. All *B. methanolicus* species can grow on mannitol at comparable levels with methanol. Although they report data regarding growth on sucrose (Arfman *et al*., 1992), there are no published data regarding growth of *B. methanolicus* on glucose to date. Recently, Heggeset *et al*., suggested (2012) that *B. methanolicus* may also be able to grow on glucose and this was shown in Chapter 2. However, mannitol was the choice of sugar when *B. methanolicus* was intended to grow non-methylotrophically. Mannitol was also the choice of sugar as control when the gene expression of methylotrophic pathways were analyzed and compared to non-methylotrophic gene expression (Jacobsen et. al., 2006).

The regulation of C_1 assimilation in the serine cycle methylotroph *Methylobacterium extorquens* AM1 is achieved via a LysR-type transcriptional regulator that is a homolog to CbbR (Kalyuzhnaya and Lidstrom, 2003). Kalyuzhnaya and Lidstrom (2003) have shown that the *qscR* mutant was not able to grow on C_1 compounds; the activity of the key enzyme of the serine cycle, serine-glyoxylate aminotransferase, was lacking in the qscR mutants. The binding of QscR upstream of most serine cycle enzymes was shown via gel retardation assays in the same study.

There is no study to date to report the regulation of methylotrophy in *B. methanolicus*. The existing data on the differential expression of genes in *B. methanolicus* is based on the growth of the microorganism on methanol and mannitol (Jacobsen *et al*., 2006, Heggeset *et al.*, 2012). Jacobsen *et al*., (2006) reported expression levels of RuMP pathway genes using Real Time-qPCR. They compared the expression levels of *B. methanolicus* MGA3 genes grown on methanol or mannitol.As mentioned, two of these genes, *hps* and *phi*, are known to be on the chromosome while the rest are plasmid-bound. The pBM19 plasmid was shown to be lost after prolonged incubation of *B. methanolicus* MGA3 on mannitol (Brautaset *et. al.*, 2004).

There is a need for a regulated promoter to be used in *B. methanolicus* as a candidate industrial microorganism for the production of amino acids and fine chemicals from methanol. The reported expressed genes, either homologous (Jacobsen et al. 2006 and 2009, Brautaset et al. 2010) or heterologous (Nilasari et al. 2012), used the promoter P_{mdh}. Jacobsen et al. (2006) expressed genes of the carbon assimilation pathway under the P_{mdh} on the shuttle vector pHP13 in order to achieve higher carbon flux through the end product of L-lysine. However, Nilasari *et al.* used another vector pNW33N to successfully transform *B. methanolicus* and express heterogeneous GFP. These studies were constitutive, i.e unregulated. The GFP protein was expressed regardless of the substrate the microorganism was grown on (Smith, 2007).

This chapter investigates the regulation of methanol dissimilation pathway genes that are mentioned in Chapter 4 in response to a spike of methanol, formaldehyde and formate by

RT-qPCR. The strengths of possible promoters to be used in *B. methanolicus* are also assessed.

Materials and Methods

Growth conditions of *B. methanolicus*:*B. methanolicus* MGA3 was grown as described in Chapter 2 in either $MTYM_{MeOH}$ or $MTYM_{MAN.}$The OD_{600} of culture to be spiked was determined experimentally to have sufficient cells (exponentially growing) for RNA extraction. The formaldehyde concentration was also experimentally determined to have sufficient amount to arrest the cell growth for desired time (at least 1 hour) but not to kill the culture. In this regard, combination of OD_{600} of 0.8 and 2 mM formaldehyde was chosen (Figure 5.1). A lower cell density would not be sufficient for desired RNA amount and higher cell density would remove formaldehyde from the media faster. Similarly, a lower formaldehyde concentrarion would be removed from the media faster and higher concentration would kill the cells. The OD_{600} of the culture remained the same during sampling for RNA extraction (Figure 5.2).Thus, exponentially growing cultures were spiked with methanol (100 mM), formaldehyde (2 mM) or formate (2 mM) at $O.D_{600}$ ~0.8. The total RNA was extracted 20 minutes after the spike unless otherwise stated.

Determination of cell lysis and sporulation: DNA concentration in the spent media was measured as an indication of cell lysis for a period of 1 hour after spiking with formaldehyde at above mentioned conditions. Standards of DNA was prepared from Lambda DNA by diluting in MTYM media. A standard curve was constructed using the dilutions of 5ng/ml to 250 ng/ml (Figure 5.3). One milliliter of culture was centrifuged for 1 min at 16,000 g. DNA in the supernatant was measured using NanoDrop-1000. No significant cell lysis was detected during the time frame the RNA extractions were carried out (Figure 5.4)Malachite Green was used to detect sporulation of the cells upon formaldehyde addition. No spore formation was detected for the period of 1 hour past formaldehyde addition.

Extraction of total RNA: Three milliliters of culture was directly transferred into a falcon tube containing 6 ml of Qiagen RNAprotect® Bacteria Reagent (Cat. 76506) and incubated for 5 minutes at room temperature. Then, cells were centrifuged at 10,000g for 5 minutes and the pellet was re-suspended in 0.2 ml TE containing 20 mg/ml lysozyme (Sigma cat. L6876). The sample was then processed according to the Qiagen RNeasy Mini Kit manual. Extracted RNA was quantified using a NanoDrop-1000 and aliquoted into tubes for single use.

Primers and Standards: Primers used for qPCR (listed in Table 1) were designed using software Primer Express 3.0 of Applied Biosystems to be 120-150 bp long and have similar annealing temperatures (~60 °C). For absolute quantification, standards were synthesized by PCR using the Expand High Fidelity PCR System. The reaction mixture contained 400 nmol of primers, 400 µmol of dNTP mix, 0.5 µl of enzyme mix, 1.5 mM $MgCl_2$ and 50-200 ng of total DNA of *B. methanolicus* MGA3. The reaction conditions were: 5 min initial denaturation at 94 °C then, 25 cycles of 1 min annealing at 52 °C, 1 min extension at 72 °C, and 30 sec denaturation at 94 °C. A final extension at 72 °C was added at the end of the cycle. The PCR product was run on an 0.8 % agarose gel and purified by using the Wizard® SV Gel and PCR Clean-Up System (Promega cat. A9281). Purified PCR product was quantified using NanoDrop-1000 and the concentration was calculated based on the molecular mass of the amplicon. A calculated amount of sample was then serially diluted for the standards with the range of 10^{10} to 10^5. Five of these serial dilutions were used to obtain a standard curve depending on the sample to be analyzed.

Table 5.1. List of primers used for RT-qPCR. Underlined sequences are sites for respective restriction sites.

Gene	**Sequence**	**Reference**
Formate THF-ligase(ORF 000 91)	Fw:ATGCTCTGGCAGCTTTAATTGATA Rev: ATGAACAGGTCCGCCTAAACC	This study
Aldehyde dehydrogenase (ORF 00554)	Fw: TGCCTCTACTGCGCGATCTA Rv: GAAGCAGGAAAGCGTGTAGGA	This study
Aldehyde dehydrogenase ORFO 00623)	Fw: AAATGTCCGCCAGTGAAAGAA Rv: TTTGTTTCCCTAATGGGTTTTCC	This study
Methylene tetrahydrofolate dehydrogenase/ cyclohydrolase (ORF 00712)	Fw:GAAGGAGCCGTTGTAATTGATGTA Rv: CGTAATCGTCATCGGTCCAA	This study
Formate dehydrogenase alpha chain (ORF 01579)	Fw: TACGAACGGCTTTGAAGAACTGA Rv: GCATCGGCAAGCAGTTTTG	This study
Methylene tetrahydrofolate reductase (ORF 0 1597)	Fw: TGGCGTTTGGATAAGCTGATAA Rv: TAGAGGGCCTGGGAGCAGAT	This study
CO dehydrogenase large subunit (ORF 01738)	Fw: AGAATTGATACGTCGGCTGCTT Rv: CACCATCTGTTTGTCGCCATT	This study
Formate dehydrogenase (ORF 01931)	Fw: TGTTGCTCCTGAAGCCAATAAAG Rv: AAAGGATGATGTTGCAGGAAGTACA	This study
Pyruvate formate lyase (ORF 02694c)	Fw: CCCTGGTCCATGTGAGGTAATC Rv: TCCGACTGAAGCTACAACAAAATT	This study
Methanol dehydrogenase (ORFs 01267 and 02737)	Fw: TGAAGGTGTCGATGTATCAAAAGAA Rv: TTCACTTTGCGTTCAGTATCTGTGA	This study
Superoxide dismutase (ORF 00650)	Fw: AAACAATGAACATCCATCACACAAA Rv: GCATCAAGGTTGGAAATGACTTCT	This study
mdh (plasmid borne)	Fw: CAAACGTGTGGCATATTAACTGAGT Rv: GGCAGGAGTGGCATTTAACAAC	This study
mdh (chromosomal)	Fw: TGAAGGTGTCGATGTATCAAAAGAA Rv:TTCACTTTGCGTTCAGTATCTGTGA	This study
Pfk	Fw: AAGTGCCATCTCCACCAATC Rv: CCAGGAATGAACGCTGCTAT	Jacobsen et al.
P_{pfk} promoter	Fw: ATGGTACGATCGCAAAGCAAACGG TAC(PvuI) Rv:ATGGTAGAATTCTCTTGTTTTCCTCCATATC(EcoRI)	This study
Alu156	Fw:ATGGTAGAATTCAAGATTATTATGCCACAC(EcoRI) Rv:ATGGTACGATCGCCCGGATCCGTCCTTCC (PvuI)	This study
repB	Fw: GTCTGTTCGTCCTTGGACTT Rv: AATGCTTGGAGCCGAGGATA	Jacobsen et.al.,
Gfpuv (qPCR)	Fw2: TGTTCAATGCTTTTCCCGTTATC Rv2: TTGACTTCAGCACGCGTCTT	This study

Table 5.1 Continued

GFPuv (Cloning)	Fw:ATGGTAGAATTCATGAGTAAAGGAGAAG(EcoRI) Rv:ATGGTAAAGCTTTTATTTGTAGAGCTCATC(HindIII)	This study
hps	Fw: CCTTGTTGACATGATCGCAGTT Rv: AATGGGTTTTTACCTACTGCTTGAA	This study
G. kaustophilus 2797314-2798810	Fw:ATGGTAGAATTCATGACAATGATTTCGGCG(EcoRI) Rv:ATGGTAGGATCCTTAAAACAACGTCATCGG(BamHI)	This study
P_{mdh} promoter	Fw:GTAGAATTCTCTTACTACCTCCTATTTATG(EcoRI) Rv: ATGGTACGATCGAATCGTTCATTAAAGAGC(PvuI)	This study
Hps+phi	Fw:ATGGTAGAATTCATGGAACTTCAATTAG(EcoRI) Rv:ATGGTAAAGCTTCTACTCAAGATTAGC(HindIII)	This study

RT-qPCR: The reactions were conducted with an Applied Biosystems 7300 Real-Time PCR system and a Quantitect SYBR Green PCR Kit was used for qPCR experiments. For absolute quantification, both reverse transcription and qPCR were conducted in the same tube. The plate contained the samples and the standards with 5-log range for the standard curve. The reaction mixture contained 25 µl of PCR mix with SYBR Green, 200 nmol of forward and reverse primers, 50 ng of total RNA as template, and 0.5 µl of Reverse Transcriptase (RT) mix in a total of 50 µl. The RT mix was omitted from the standards. The reaction conditions were as follows; 30 minutes at 50 °C for RT reaction, 15 min at 95 °C for activation of Taq polymerase and then 30 cycles of 30 sec. denaturation at 95 °C, 30 sec. annealing at 55 °C and 30 sec. extension at 72 °C and then dissociation curve from 60 °C to 95 °C with 1 °C increment at every 15 sec. while recording fluorescence. The results were normalized using 16S rRNA.

One-step RT-qPCR, the reverse transcription and qPCR reactions in the same tube, was used for relative quantification. The conditions of the one-step RT-qPCR are the same as the absolute quantification. Relative expression of genes in methanol grown cells was assessed with respect to mannitol grown cells as the calibrator. Both *repB* and 16S rRNA genes were used as endogenous controls mainly to compare the results with previous reports. The results are presented as relative to counterparts of genes from mannitol grown cells.

Construction of GFPvectors: The *E.coli*-*B.subtilis* pHP13 shuttle vector was used as the backbone for cloning genes into *B. methanolicus* MGA3. The propagation and transformation of the vector was conducted in *E. coli* as described by Maniatis et. al.(1982). The transformants were screened via PCR for positive colonies. Upstream (500 bp) sequences of *mdh,* and *pfk* were amplified by PCR using the primers shown in Table 5.1 and cloned into pHP13 via respective restriction sites. The Alu156 promoter was synthesized by Integrated DNA Technologies (Coralville, IA, US) as single strand and then was amplified using the primers Alu156Fw and Alu156Rv. The GFPuv gene was amplified using PCR and cloned downstream of promoters of P_{mdh},$P_{pfk,}$ and $P_{Alu156.}$ The sequences of the clones were confirmed by sequencing the vectors with custom designed and commercially available primers. The pNW33N (Nilasari et al., 2012) vector was kindly provided by Dr. Claire Komives and it is labeled as P_{mdhK} for distinction from the P_{mdh}. It contains 11 amino acids of methanol dehydrogenase before the GFPuv.

Construction of pTS123 and pHPS12: The P_{mdh} promoter was amplified by PCR and cloned into pHP13 using respective restriction enzyme sites. The putative *faldh* of *G. kaustophilus* (ORF 2797314-2798810) was also PCR amplified using primers shown in Table 5.1 and cloned downstream of P_{mdh}. This vector is called pTS123. Similarly, the P_{pfk} was also cloned into pHP13. Genes of *hps*and *phi* were amplified by PCR monocistronically using the primers in Table 5.1 and cloned into downstream of P_{pfk}. This vector is called pHPS12.

Electroporation of *B. methanolicus*: The electroporation of *B. methanolicus* was conducted with the following modifications to published protocols (Jacobsen et al. 2006, Nilasari et al. 2012). The cells were grown in 5 ml of SOBsuc after electroporation for 2 hours in a 50 ml falcon tube and then 10 ml SOBsuc with 10 μg/ml final concentration of choloramphenical was added to the tube and incubated 6 more hours before plating. A colony from the plate was inoculated into 5 ml of MTYM with 60 mM methanol and incubated for 6 hours. The entire volume was then transferred into 50 ml MTYM media in a 250 ml shake flask with 150 mM methanol and 5 μg/ml chloramphenicol. The culture was incubated overnight and frozen stocks were prepared by flash freeze in liquid nitrogen.

Results

Methanol dissimilation pathway genes are down regulated by methanol, formaldehyde and formate: The proposed methanol oxidation pathway derived from the genome sequence of *B. methanolicus* MGA3 is presented in Figure 4.12. To summarize briefly, methanol is oxidized to formaldehyde by methanol dehydrogenases (ORFs 00006, 01267, 02737)in the first step. Formaldehyde is then oxidized to formate either by putative formaldehyde dehydrogenases (ORFs 00554c, 00623c, 01733c) or THF-linked methylene tetrahydrofolate dehydrogenase/cyclohydrolase (ORF 00712) and formyl-THF ligase (ORFs 00665).

Regulation of methanol oxidation: Figures 5.5-5.9 depict the expression of *mdh* genes in response to methanol addition. As can be seen from the figure 5.5, the expression level of pBM19 *mdh* stays stable over the course of 60 minutes after methanol addition when *B. methanolicus* MGA3 was grown in MTYM methanol media. However, the expression is significantly down-regulated when mannitol grown *B. methanolicus* MGA3 was exposed to a 100 mM methanol spike, as can be seen in Figure 5.6. The expression level of *mdh* was decreased almost one-log in 20 minutes. The expression of chromosomal *mdh* genes in response to methanol addition is different from pBM19 *mdh*in methanol-grown *B. methanolicus* MGA3 in that it was down regulated immediately (until 10 minutes post-spiking) and then starts to recover back although, the expression level after 60 minutes is still significantly lower than pre-spiking level. The expression of pBM19 *mdh* is also down-regulated when methanol or mannitol grown *B. methanolicus* MGA3 was subjected to formaldehyde (2 mM) and formate spike (2 mM). As seen in Figures 5.9 and 5.10, pBM19 *mdh* was down-regulated in response to a formaldehyde and formate spike although formaldehyde was more potent to down-regulate the gene.

An important observation from the expression levels of *mdh* is that it is expressed constitutively both in mannitol and methanol-grown *B. methanolicus* MGA3. This result is consistent with the expression of GFP under P_{mdh} in both methanol and mannitol grown cells (Nilasari et. al., 2012). The expression levels of pBM19 *mdh* and chromosomal *mdh* genes are comparable between methanol and mannitol grown cells. However, there are clear differences in expression levels of pBM19 and chromosomal *mdh* genes. The expression level of pBM19 *mdh* is one-log higher than the combined expression of *mdh* genes from ORFs 01267, 02737 (the qPCR primers for chromosomal *mdh* genes amplify both genes). This might be because of the copy number of pBM19, ~10-16 copies per cell (Brautaset et. al., 2004).

Regulation of formaldehyde oxidation: The second step in methanol dissimilation is the conversion of formaldehyde to formate which can be either through THF-linked enzymes or formaldehyde dehydrogenases. The expression levels of putative genes for these pathways are assessed via RT-qPCR.

Figures 5.11 and 5.12 depict the regulation of putative aldehyde dehydrogenase (possibly formaldehyde) in response to formaldehyde, formate and methanol spiking. Both putative aldehyde dehydrogenases are down regulated in response to spikes by methanol and its metabolites. However the down-regulation is more profound in methanol-grown cells.

Figures 5.13 and 5.14 summarize the regulation of putative THF-linked enzymes as a second possible pathway for the oxidation of formaldehyde to formate. The putative methylene tetrahydrofolate dehydrogenase/cyclohydrolase (ORF 00712) is down regulated by methanol, formaldehyde and formate in both methanol and mannitol grown cells. The putative formyl-THF ligase enzyme (ORF 00665) is also down regulated by these compounds. However, the effect of formate is more profound on Formyl-THF ligase than formaldehyde while it is reversed for methylene tetrahydrofolate dehydrogenase/cyclohydrolase.

Regulation of formate dehydrogenase: The third step in the dissimilation of methanol is the oxidation of formate to CO_2. This reaction is catalyzed by formate dehydrogenase. Two putative formate dehydrogenases are ORFs 01579 and 01931. Figures 5.15 and 5.16 summarize the regulation of these genes in response to methanol and its metabolites. Both of the formate dehydrogenases are down-regulated in response to spiking of methanol and its metabolites. Although expression levels of ORF 01931 are comparable between methanol and mannitol grown cells, the ORF 01579 in mannitol-grown cells is expressed as twice the level that it is expressed in methanol-grown cells.

Regulation of methanol assimilation (RuMP) pathway gene *hps*: Figures 5.17 and 5.18 summarize the regulation of *hps* in response to methanol and its metabolites. The *hps* gene encodes for the first enzyme of the RuMP pathway that draws the formaldehyde to assimilative pathways. It is surprising that the expression level of *hps*(7.42×10^{-1}) which is located in the chromosome, is significantly higher than the expression levels of pBM19 *mdh* (Met-5 sample 2.23×10^{-1}, see Figures 5.17 and 5.5). The *hps* gene in methanol grown cells is up-regulated approximately 3-times compared to mannitol grown cells (7.42×10^{-1} vs 2.15×10^{-1}). Although not at the same level, this result confirms the upregulation of *hps* reported by Jacobsen et. al., (2006). However, the *hps* is also down regulated when methanol-grown cells are subjected to formaldehyde and formate spikes. Despite this down-regulation in methanol-grown cells, it is clearly upregulated when mannitol-grown cells are subjected to methanol, formaldehyde or formate. Almost 2-fold (2.15×10^{-1} vs 4.17×10^{-1}**)** up-regulation is evident in Figure 5.18.

Regulation of pfk: Another RuMP pathway gene investigated was the phosphofructokinase (*pfk*), which is located on the pBM19 plasmid. It catalyzes the next reaction after *hps* and *phi* in the RuMP pathway; conversion of fructose-6 phosphate to fructose-1,6-biphosphate. Figure 5.19 depicts its regulation in response to formaldehyde. The upregulation of *pfk* in methanol grown cells is apparent in Figure 5.19 which confirms the upregulation reported before (Jacobsen et. al., 2006) although at a significantly lower level. Despite being encoded on the pBM19 plasmid, the expression level of *pfk* is significantly lower than *hps* when figures 5.17 and 5.19 are compared. The *pfk* gene is upregulated in methanol-grown cells compared to mannitol grown cells and spiking mannitol-grown cells with formaldehyde caused about 2-fold upregulation. Although small, spiking methanol-grown cells with formaldehyde also caused an upregulation of *pfk*.

Regulation of Wood-Ljungdahl pathway enzymes: The Wood-Ljungdahl pathway was described in Chapter 4. The THF-linked formaldehyde oxidation enzymes methylene tetrahydrofolate dehydrogenase/cyclohydrolase (ORF 00712) and formyl-tetrahydrofolate ligase (ORF 00665) along with formate dehydrogenase (ORFs 01579, 01931) also function in Wood-Ljungdahl pathway as depicted in Figure 4.13. The regulation of these genes in response to methanol and its metabolites was discussed above. The expression of CO dehydrogenase large subunit (ORF 01738) and methylene tetrahydrofolate reductase (ORF 01597) are also investigated. Figure 5.20 shows that the expression levels of putative methylene tetrahydrofolate reductase in methanol and mannitol-grown *B. methanolicus* are comparable and is down regulated in both samples. However, the expression level of CO dehydrogenase (ORF 01738) in mannitol-grown cells is higher than in methanol grown cells while the gene is also down-regulated by methanol and its metabolites (Figure 5.21).

Superoxide dismutase: The methanol metabolite formaldehyde is a reactive oxygen species, and it reacts nonspecifically with bio-molecules. Accumulation of formaldehyde alone may cause an oxidative stress which can result in down regulation of metabolism as a whole except for the stress response genes. Therefore, the expression level of putative superoxide dismutase (ORF 00650) was investigated. Figure 5.22 shows the response of putative superoxide dismutase in response to formaldehyde and formate spikes. As seen in the figure, the expression level of superoxide dismutase is comparable between the methanol-grown and mannitol grown cells. A spike of formaldehyde did not cause any down or upregulation of superoxide dismutase in methanol-grown cells, although there is a slight upregulation in mannitol-grown cells. However, a spike of formate caused down regulation only in methanol-grown cells, not in the mannitol grown cells. This result suggests that the down regulation of genes discussed above was not due to a global oxidative stress in response to formaldehyde.

Evaluation of promoter strength for regulated protein expression in *B. methanolicus*: Regulation of recombinant protein expression is an important aspect of recombinant protein production and pathway engineering. In this regard, well established systems are available especially for *E. coli* like pET expression systems from Clontech labs (Mountain View, CA) which utilizes bacteriophage T7 RNAP and the T7 promoter for induction of transcription. The T7 expression system was also engineered for expression in *B. subtilis* (Chen et. al., 2010). A methanol induced promoter is desirable for a possible *B. methanolicus* expression system. The P_{mdh} of *B. methanolicus* has been used for both homologous and heterologous gene expression as discussed above. To elucidate the behavior of P_{mdh} and compare with $P_{pfk}$and P_{Alu156}, the transcription level of the reporter GFPuv was assessed. Figure 5.23 summarizes the expression of GFPuv by these promoters. As seen in the figure, the expression level of GFP under P_{mdhK} is comparable to the expression level of pBM19 *mdh*, and it is also down regulated by a spike of formaldehyde. The expression level underthe control of P_{mdh}, P_{pfk} and P_{Alu156} are significantly lower than the expression level under P_{mdhK}. However, there is a slight upregulation in P_{pfk} regulated expression with a formaldehyde spike which is again consistent with upregulation of *pfk* with formaldehyde spike in mannitol grown cells. The difference in expression levels of P_{mdhK} and P_{mdh} is most probably due to introduction of 6-bp between the promoter region and start codon during vector construction.

Formaldehyde tolerance of *B. methanolicus* MGA3 carrying pTS123 and pHPS12:The vectors pTS123 and pHPS12 carry genes for enzymes responsible for removal of formaldehyde via oxidation to formate and assimilation into RuMP pathway, respectively. Growth inhibition experiments were conducted to test the formaldehyde tolerance of recombinant *B. methanolicus* MGA3, as described in Chapter 2. Figure 5.25 demonstrates the formaldehyde tolerance of *B. methanolicus* MGA3 carrying putative *G. kaustophilus faldh* under P_{mdh}. As can be seen from the figure, recombinant *B. methanolicus* MGA3 is able to tolerate formaldehyde spikes up to 1 mM. Similarly, recombinant *B. methanolicus* MGA3 carrying extra copies of *hps* and *phi*genes under P_{pfk} is also able to tolerate 1 mM formaldehyde as can be seen on figure 5.26 (See figure 2.6 for comparison).

Conclusions

B. methanolicus is a restrictive methylotrophic microorganism that is also capable of utilizing mannitol. As a candidate industrial microorganism, understanding the regulation of methanol metabolism, especially methanol dissimilation, is crucial for possible manipulations since the substrate carbon is lost to the atmosphere as CO_2. There exist reports comparing the expression profiles of methanol and mannitol-grown cells (Jacobsen et. al., 2006, Heggeset et.al., 2012), but these do not answer the question of how methanol and formaldehyde metabolism are affected by methanol, formaldehyde and formate.

I employed RT-qPCR to elucidate the regulation of methanol and formaldehyde metabolism, especially the methanol dissimilation routes, in response to methanol, formaldehyde and formate, but also included some of the methanol assimilation genes for reasons of comparison with the published reports which gave important insights into the regulation of the metabolism. First of all, my results agree with the report of Jacobsen et. al, (2006) in that the *hps* and *pfk* genes are expressed at higher levels in methanol-grown cells. However, expression of pBM19 *mdh* does not agree with their report that they are comparable in methanol and mannitol-grown cells. This is most probably due to the difference of quantification; absolute vs relative. They used pBM19-borne *repB* to normalize the expression levels which may cause miscalculation since it is known that pBM19 is lost from *B. methanolicus* MGA3 after prolonged incubation on mannitol (Brautaset et. al., 2004). However, in the experimental settings used in this study, the *repB* expression, thus copy number of pBM19, is comparable between methanol and mannitol grown *B. methanolicus* MGA3 (Figure 5.24).Considering the regeneration time of *B. methanolicus* MGA3 (1 hour), it is safe to assume that copy number of pBM19 will stay the same for 1-hour period. Additionally, the absolute expression level of *repB* changes when the methanol or mannitol grown *B. methanolicus* MGA3 is spiked with methanol (Figure 5.24).

The presence of two more *mdh* genes on the chromosome was described in Chapter 3. The qPCR primers for chromosomal *mdh* genes were designed to amplify both of these genes. Thus, Figures 5.7 and 5.8 depict combined expression of chromosomal *mdh* genes. The expression of pBM19 *mdh* is one-log higher than the combined expression of ORFs 01267 and 02737. From this we can infer that pBM19 *mdh* is responsible for most if not all of the methanol dehydrogenase activity. Another striking result is the down regulation of the *mdh* genes in response to methanol spike when *B. methanolicus* MGA3 was grown on mannitol. Contrary to expectations, spiking with methanol did cause the down-regulation of *mdh* genes.

The same pattern of down regulation is also true for the putative methanol dissimilation pathway genes. The putative genes for formaldehyde and formate oxidation are down regulated in response to not only methanol but also formaldehyde and formate spikes. Down regulation with formate spike is also interesting since formate does not have any inhibitory effect on the growth of *B. methanolicus* at a concentration of 25 mM (Pluschkell, 1998) as discussed in Chapter 2.

Contrary to the dissimilation pathway genes, the formaldehyde assimilation pathway genes *hps* and *pfk* are upregulated in response to methanol, formaldehyde and formate (Figures 5.17 and 5.18) in mannitol grown cells. The upregulation by methanol and its metabolites are clearly seen in Figures 5.17 and 5.18 after spiking. But, in methanol-grown cells, the *hps* is down regulated from its already high levels. The expression of *hps* in methanol-grown *B. methanolicus* MGA3 is almost twice that compared to mannitol-grown cells. As mentioned above, the *mdh* is expressed constitutively which will result in conversion of methanol into formaldehyde and the formaldehyde assimilation pathway is saturated, while in mannitol grown cells it is idle. Spiking of formaldehyde or formate will cause the already saturated assimilation pathway to overflow in methanol-grown cells which will cause accumulation and toxicity. However, any additional formaldehyde or formate will not cause any accumulation in mannitol-grown cells as the cells can tolerate it. This also explains the interesting result in Chapter 2, Figures 2.6 and 2.7 where mannitol-grown *B. methanolicus* MGA3 is more tolerant to formaldehyde spike than methanol-grown cells.

In the search for suitable promoters for protein expression and pathway engineering in *B. methanolicus*, I assessed the expression level of GFPuv from different promoters. The low expression levels from P_{mdh}, P_{pfk} and P_{Alu156} are most probably due to the 6-bp introduced between the start codon and the promoter regions since the promoter sequence of P_{mdhK} and P_{mdh} are otherwise identical. From this low level of expression it will be misleading to conclude if P_{Pfk} and P_{Alu156} are better than P_{mdhK}. However, as seen from Figure 5.23, P_{mdhK} is capable of transcribing high levels of protein comparable to its native downstream *mdh* although the expression from P_{mdhK} is also down-regulated by formaldehyde. From the expression of *hps*, it would be proper to recommend usage of P_{hps} since it has high expression levels comparable to plasmid encoded genes, and is upregulated in response to formaldehyde.

The overexpression of genes responsible for formaldehyde removal (either by dissimilatory or assimilatory pathways) confers *B. methanolicus* an ability to tolerate higher formaldehyde concentrations. As can be seen from the Figure 5.25, overexpression of putative *G. kaustophilusfaldh* results in higher formaldehyde tolerance when compared to wild type *B. methanolicus* MGA3. Moreover, the growth rate of *B. methanolicus* MGA3 (pTS123) in 150 mM methanol is also comparable to the growth rate of wild type *B. methanolicus* MGA3, 0.547±0.006 h^{-1} and 0.549±0.007 h^{-1}, respectively. However, growth rate of *B. methanolicus* MGA3 (pHPS12) is higher than growth rate of wild type, 0.599±0.004 $h^{-1}$and 0.549±0.007 h^{-1}, respectively, in addition to the higher tolerance of formaldehyde (Figure 5.26). This suggests that more formaldehyde is being assimilated by *B.*

methanolicus MGA3 (pHPS12). Dissimilation of carbon by both recombinant and wild type strains need to be compared to have better picture of formaldehyde assimilation by *B. methanolicus* MGA3.

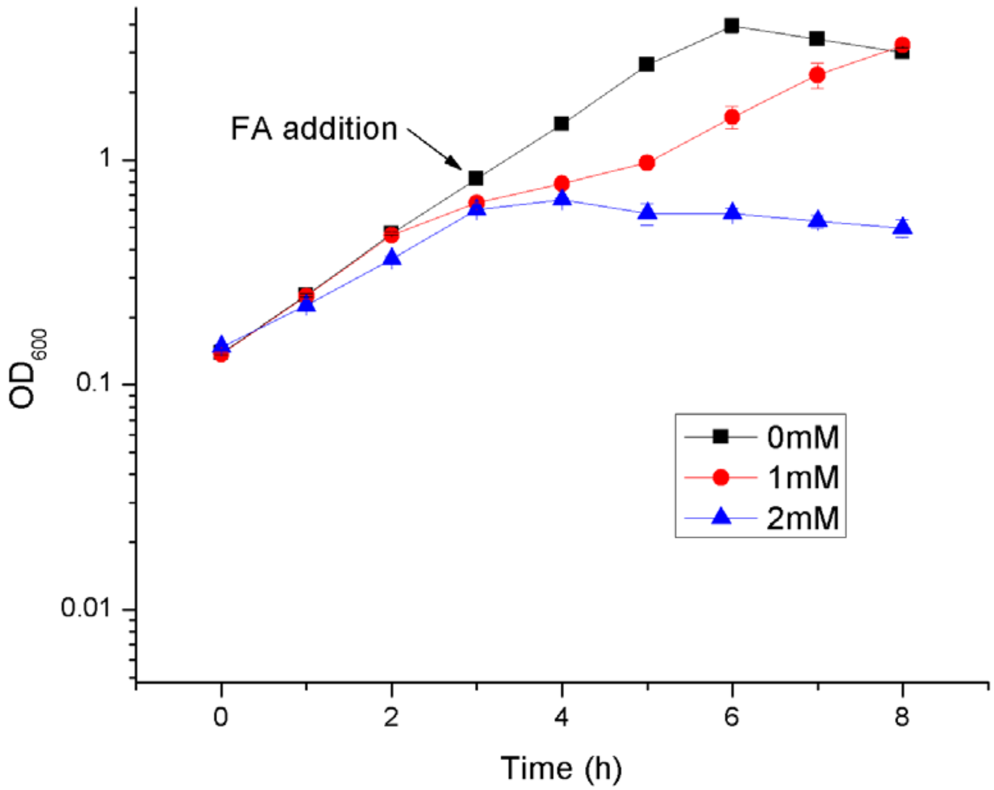


Figure 5.1. Response of $MTYM_{MeOH}$ grown *B. methanolicus* MGA3 to formaldehyde spikes. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MeOH}$ in 250 ml baffled flask and challenged with different concentrations of formaldehyde when the cells were growing exponentially (OD_{600} ~0.8). (n=3)

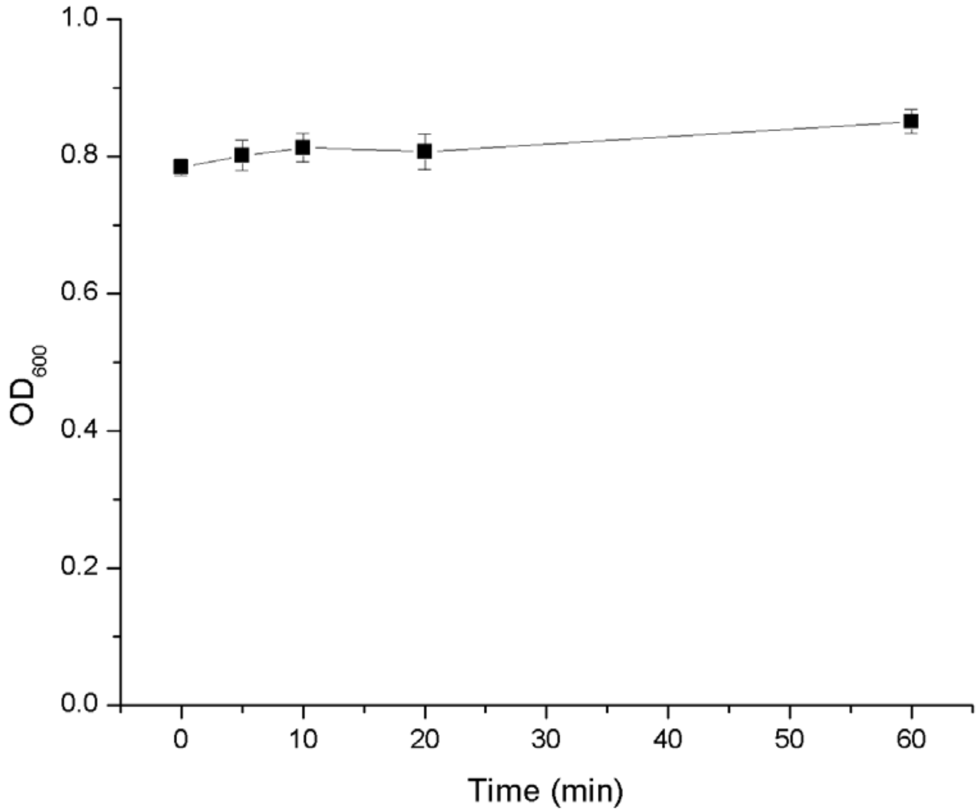


Figure 5.2. Change in OD_{600} of *B. methanolicus* MGA3 after spiking with 2 mM formaldehyde. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MeOH}$ in 250 ml baffled flask and challenged with 2 mM of formaldehyde when the cells were growing exponentially (OD_{600} ~0.8). (n=3)

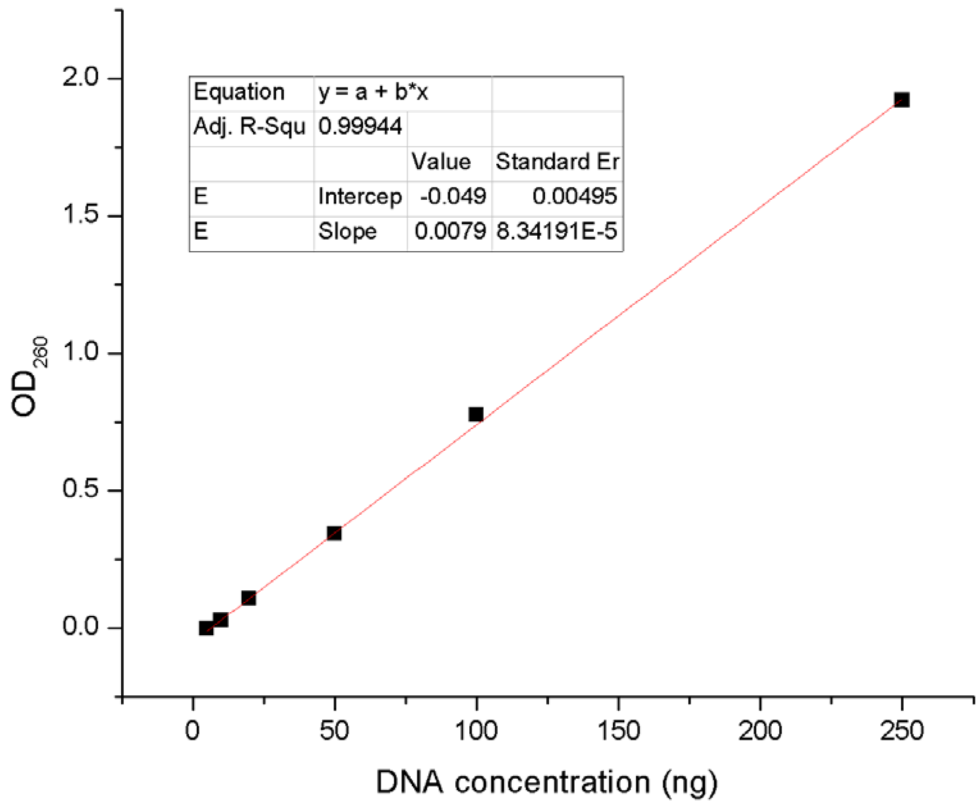


Figure 5.3. Standard curve for calculation of DNA concentration in the culture supernatant.Standards were prepard using Lambda DNA by diluting in MTYM to have a range of 5 ng/ml to 250 ng/ml. The DNA concentration was measured using NanoDrop-1000.

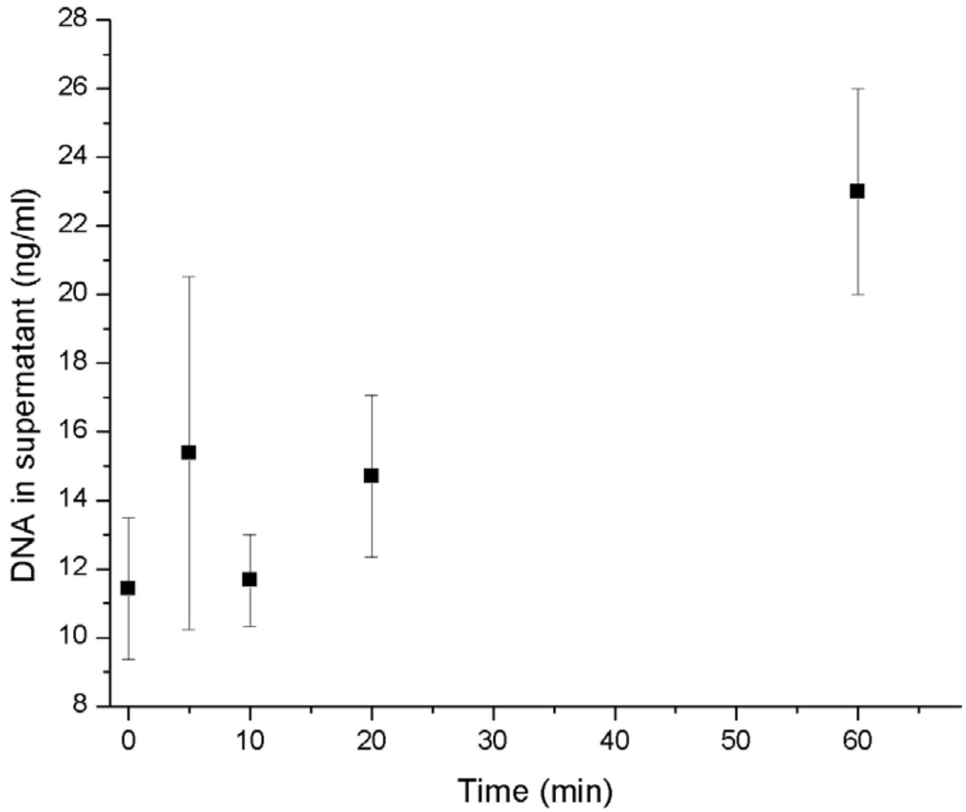
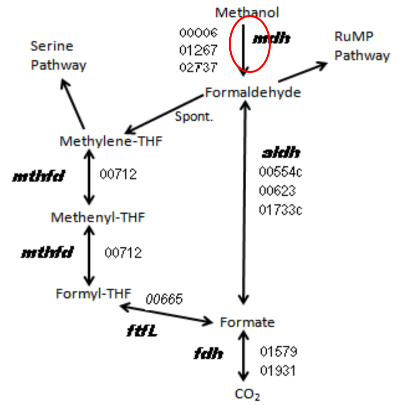


Figure 5.4. DNA concentration in the supernatant of *B. methanolicus* MGA3 after spiking with formaldehyde. The DNA in the supernatant was measured using NanoDrop-1000.



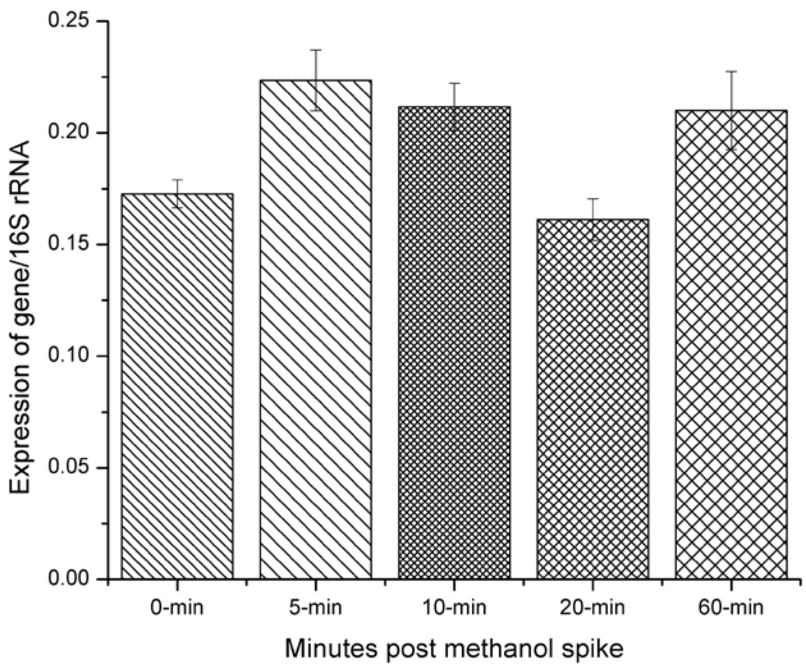
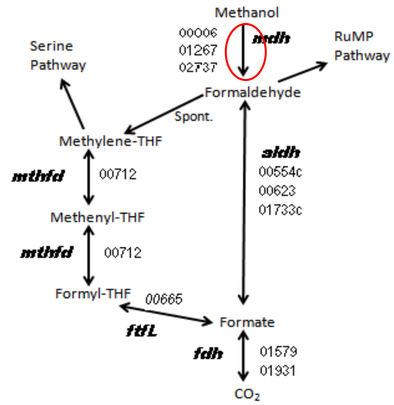


Figure 5.5. Regulation of pBM19 *mdh* in response to 100 mM methanol addition to methanol grown *B. methanolicus*.*B. methanolicus* MGA3 was grown in $MTYM_{MeOH}$ and spiked with methanol at $O.D_{600}$ of ~0.8. Then, the total RNA was extracted as described after 5, 10, 20, and 60 min. The sample “0-min” was taken immediately before the spike. (n=3)



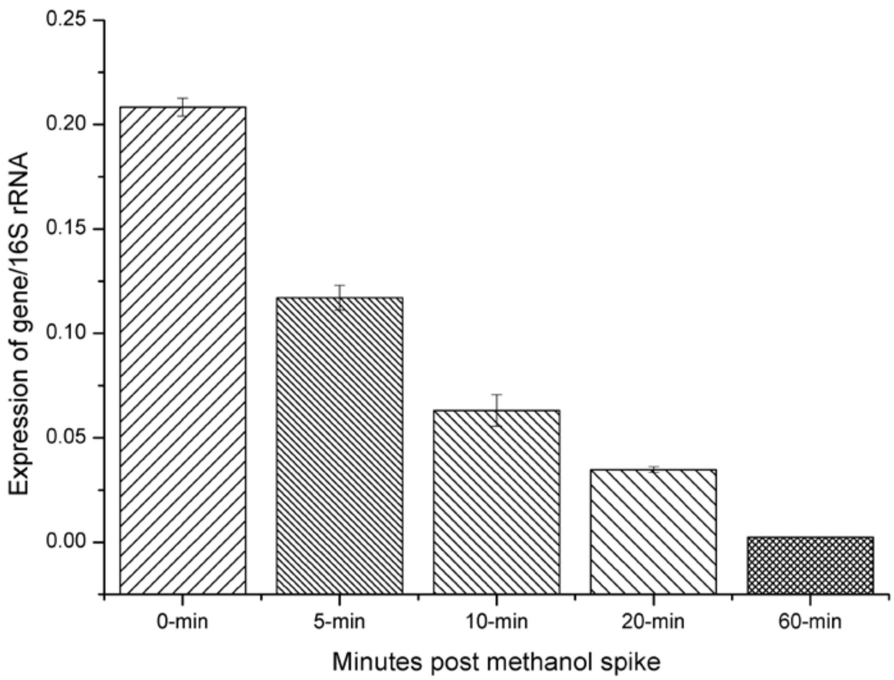
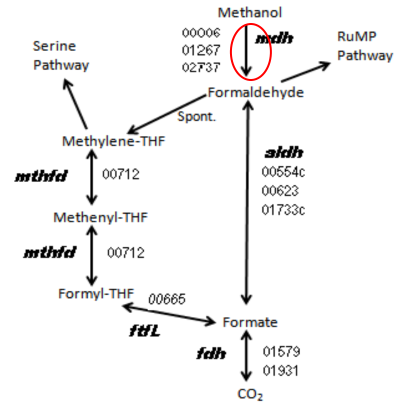


Figure 5.6. Regulation of pBM19 *mdh* in response to 100 mM methanol addition in mannitol grown *B. methanolicus*.*B. methanolicus* MGA3 was grown in $MTYM_{MAN}$ and spiked with methanol at $O.D_{600}$ of ~0.8. Then, the total RNA was extracted as described after 5, 10, 20 and 60 min post spike. The sample "0-min" was taken immediately before the spike. (n=3)



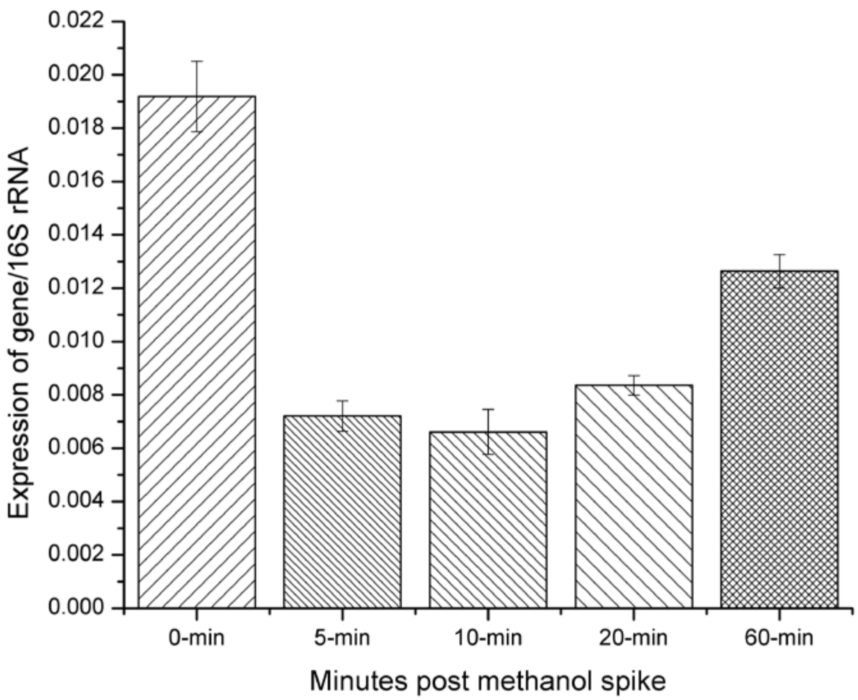
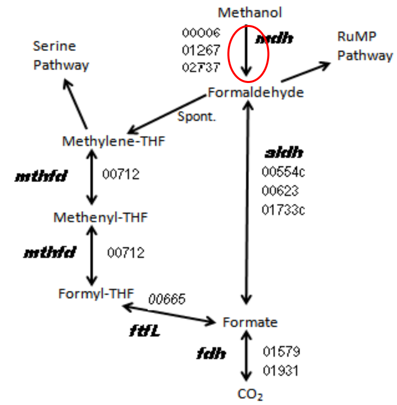


Figure. 5.7. Regulation of chromosomal *mdh* (ORFs 01267 and 02737) genes in response to 100 mM methanol addition. *B. methanolicus* MGA3 was grown in $MTYM_{150}$ and spiked with methanol at $O.D_{600}$ of ~0.8. Then, the total RNA was extracted as described after 5, 10, 20, and 60 min. The sample "0-min" was taken immediately before the spike. (n=3)



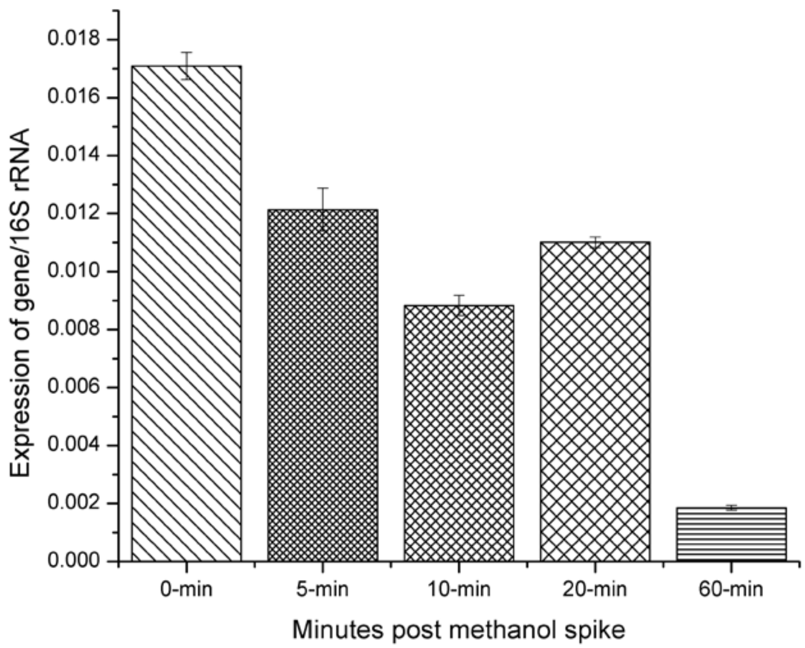
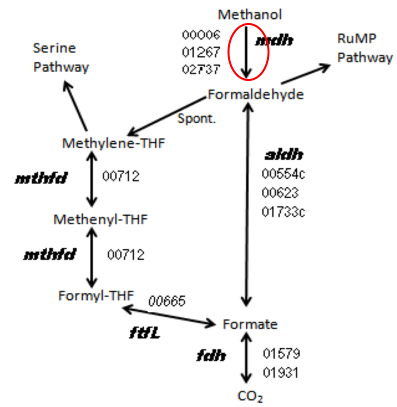


Figure 5.8. Regulation of chromosomal *mdh* genes (ORFs 01267 and 02737) in response to 100 mM methanol addition.*B. methanolicus* MGA3 was grown in $MTYM_{MAN}$ and spiked with methanol at $O.D_{600}$ of ~0.8. Then, the total RNA was extracted as described after 5, 10, 20, and 60 min. The sample "0-min" was taken immediately before the spike. (n=3)



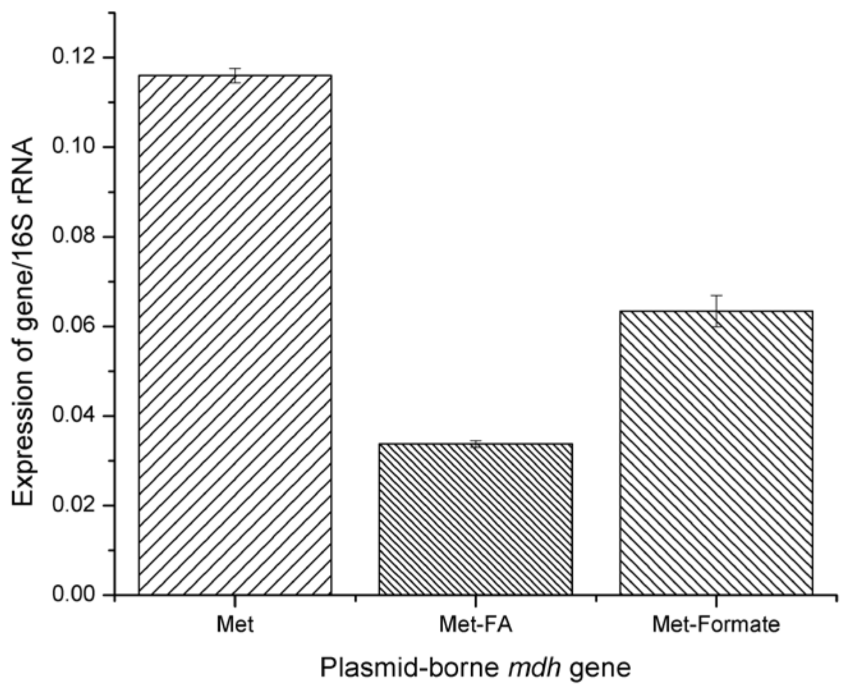
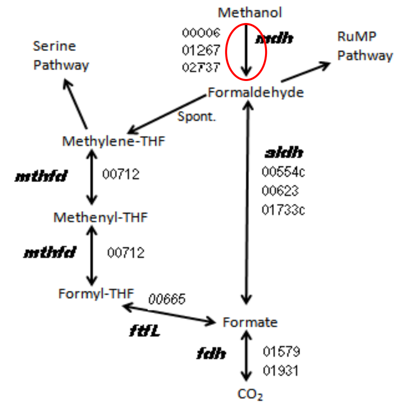


Figure 5.9. Regulation of pBM19 *mdh* in response to formaldehyde (2 mM) and formate (2 mM) addition.*B. methanolicus* MGA3 was grown in $MTYM_{150}$ and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking. Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate. (n=3)



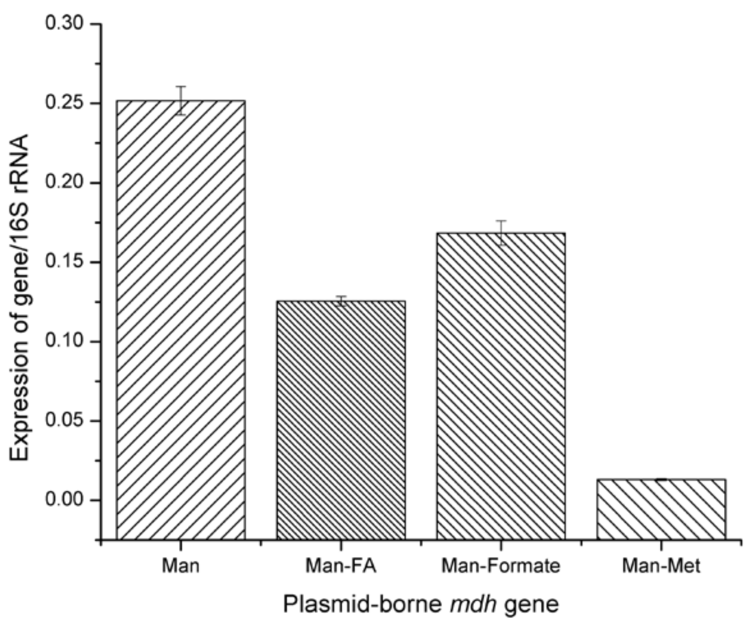
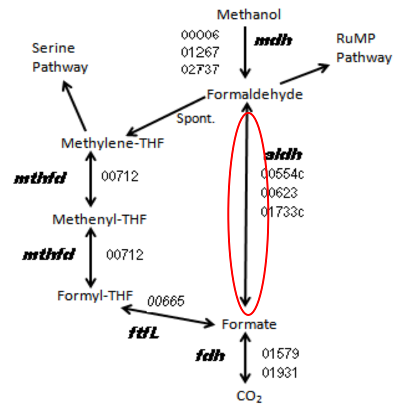


Figure 5.10. Regulation of pBM19 *mdh* in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM) addition. *B. methanolicus* MGA3 was grown in $MTYM_{MAN}$ and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking. Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)



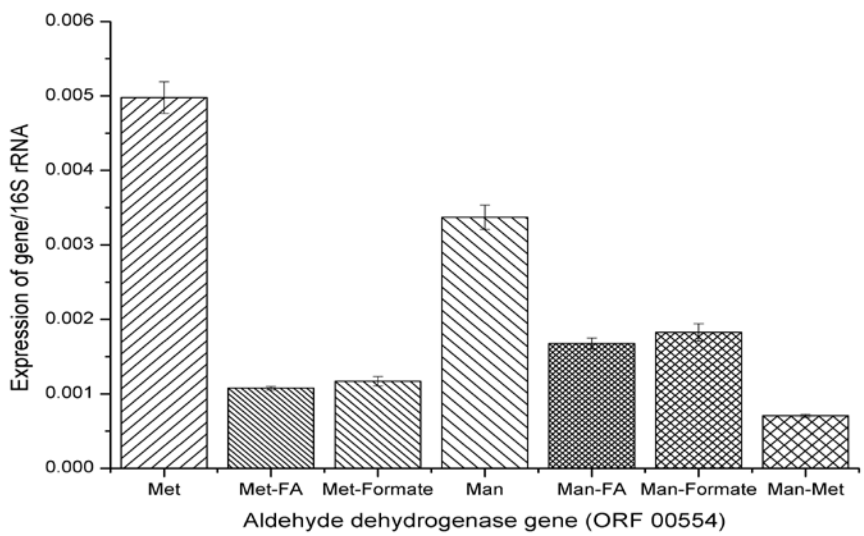
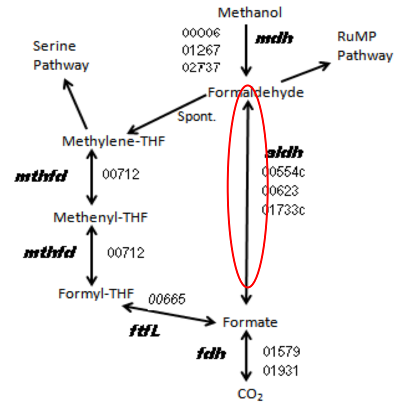


Figure 5.11. Regulation of putative aldehyde dehydrogenase (ORF 00554c) in response to formaldehyde (2 mM), formate (2 mM) and methanol (for mannitol-grown cells, 100 mM). *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol.(n=3)



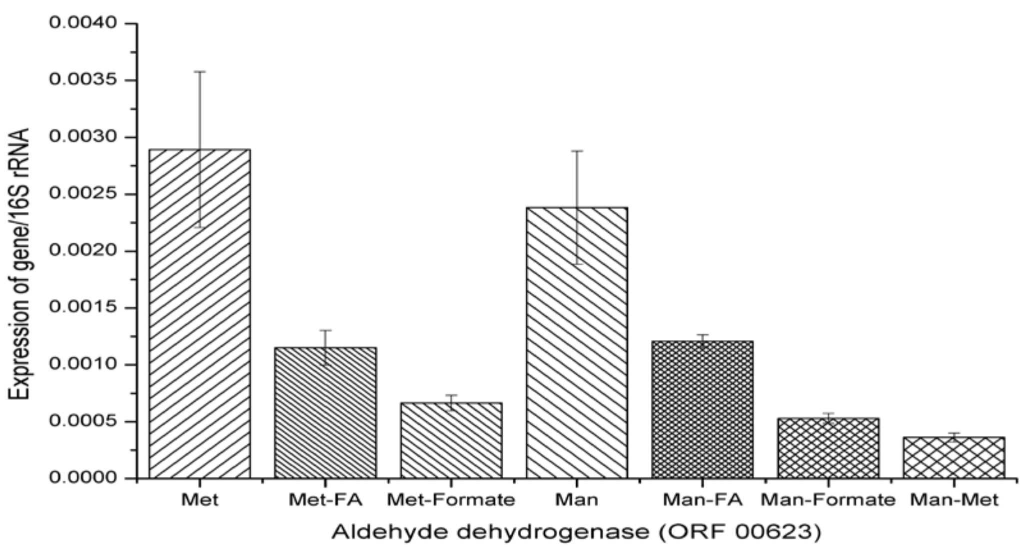
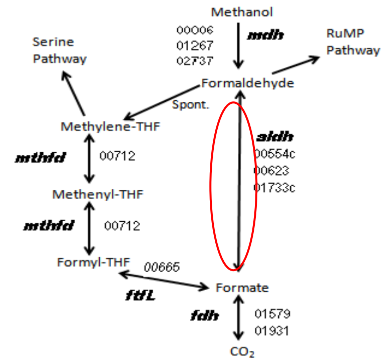


Figure 5.12. Regulation of putative aldehyde dehydrogenase (ORF 00623) in response to formaldehyde (2 mM), formate (2 mM) and methanol (for mannitol-grown cells, 100 mM). *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the O.D_{600} is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)



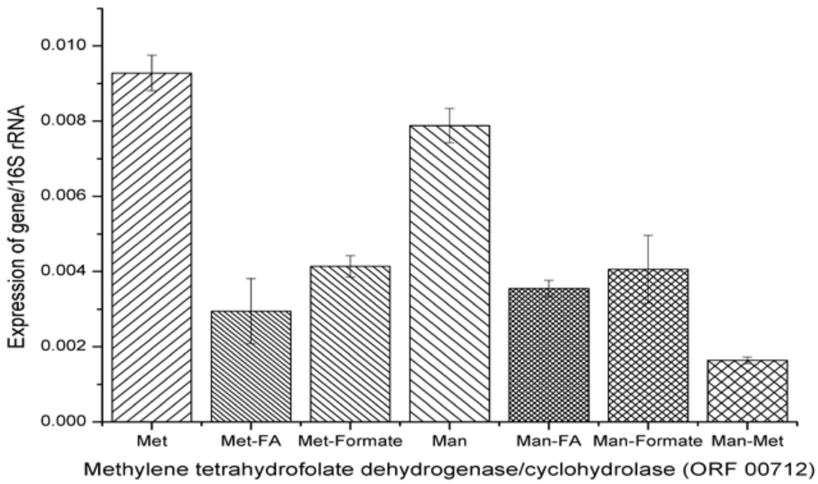
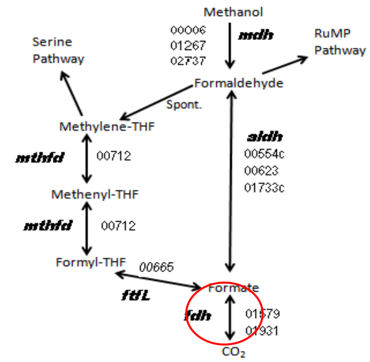


Figure 5.13. Regulation of putative methylene tetrahydrofolate dehydrogenase/cyclohydrolase (ORF 00712) in response to formaldehyde (2 mM), formate (2 mM), and methanol (100 mM) spikes. *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)



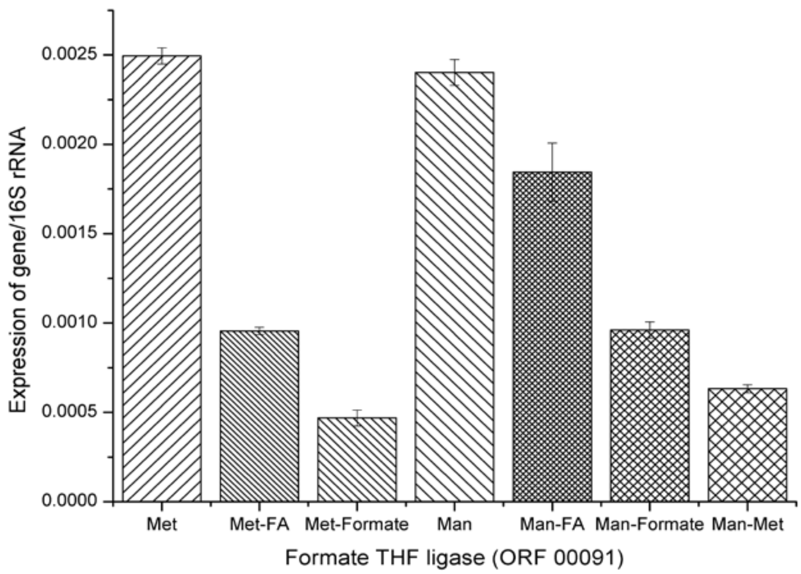
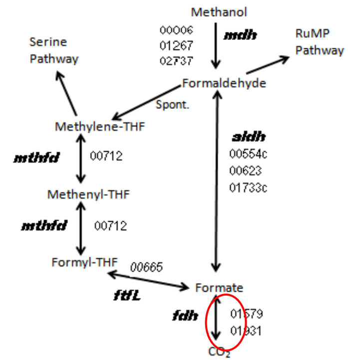


Figure 5.14. Regulation of putative formate THF ligase gene (ORF 00665) in response to formaldehyde (2 mM), formate (2 mM), and methanol (100 mM) spikes.*B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the O.D$_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)



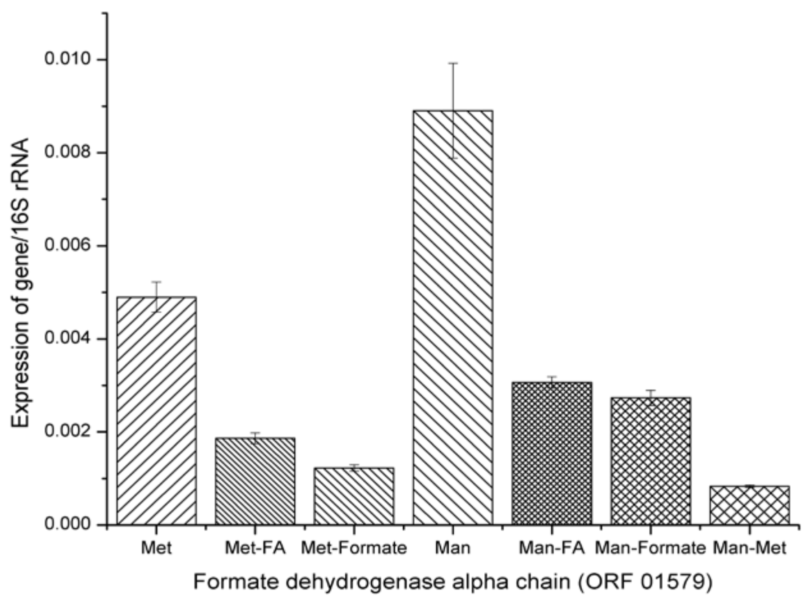
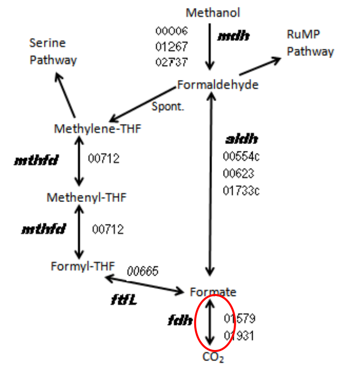


Figure 5.15. Regulation of putative formate dehydrogenase gene (ORF 01579) in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM). *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)



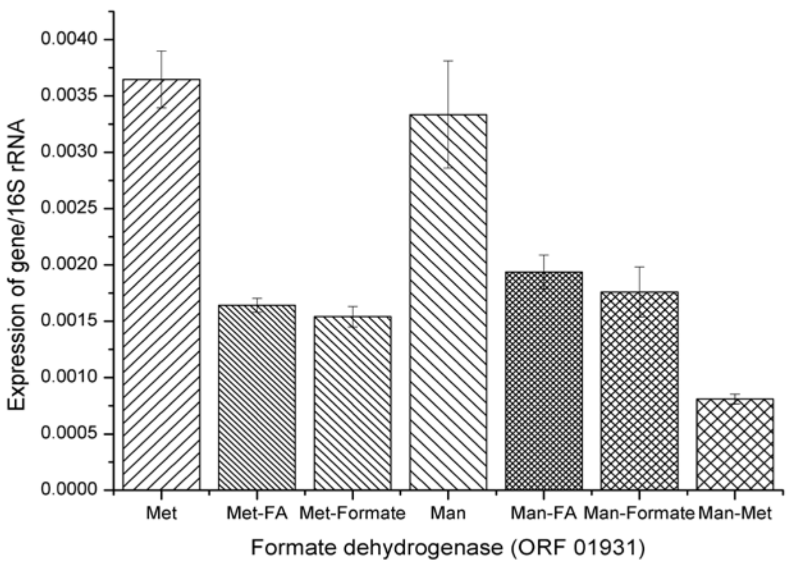
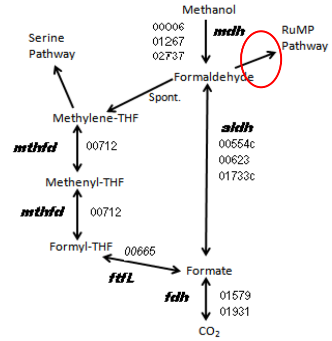


Figure 5.16. Regulation of putative formate dehydrogenase gene (ORF 01931) in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM). *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)



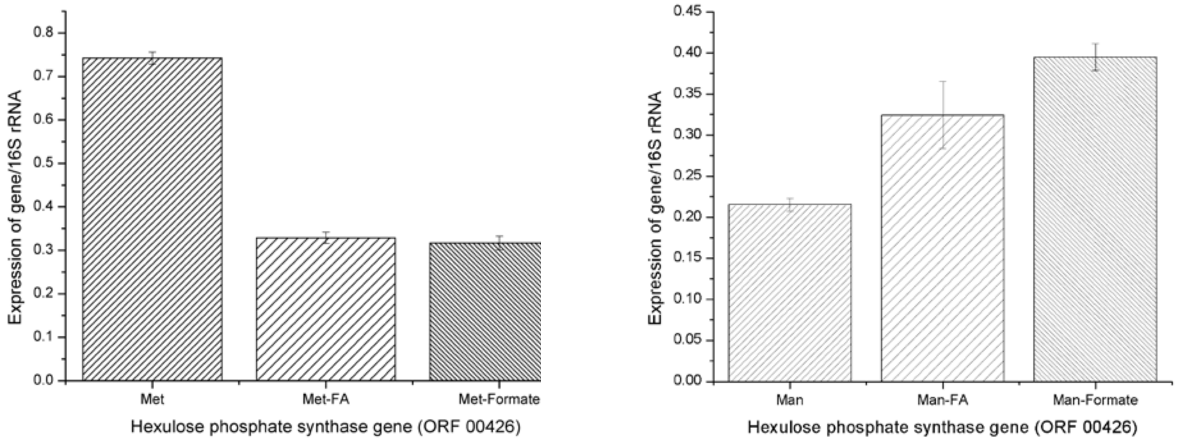
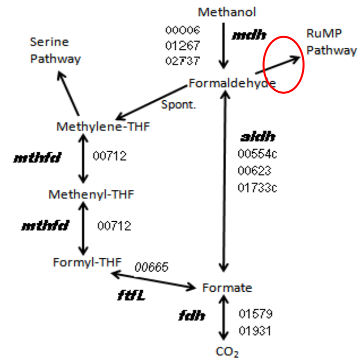


Figure 5.17. Regulation of hexulose phosphate synthase (*hps*) in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM) spikes in methanol and mannitol-grown cells. *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate. (n=3)



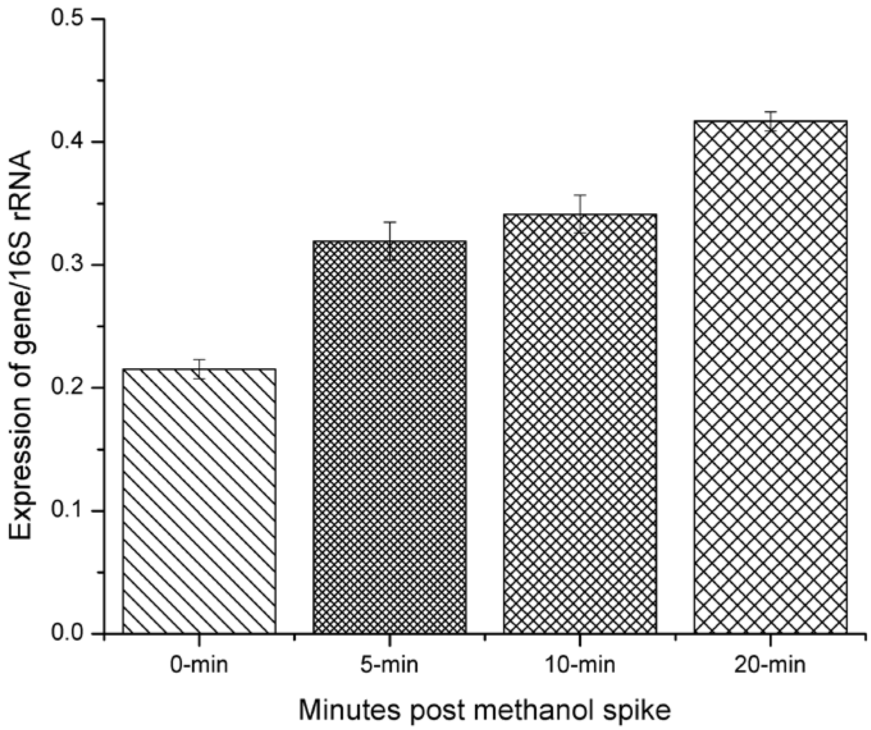


Figure 5.18. Regulation of hexulose phosphate synthase (*hps*) in response to methanol (100 mM) spike.*B. methanolicus* MGA3 was grown in $MTYM_{MAN}$ and spiked with methanol at $O.D_{600}$ of ~0.8. Then, the total RNA was extracted as described after 5, 10, and 20 min. The sample "0-min" was taken immediately before the spike. (n=3)

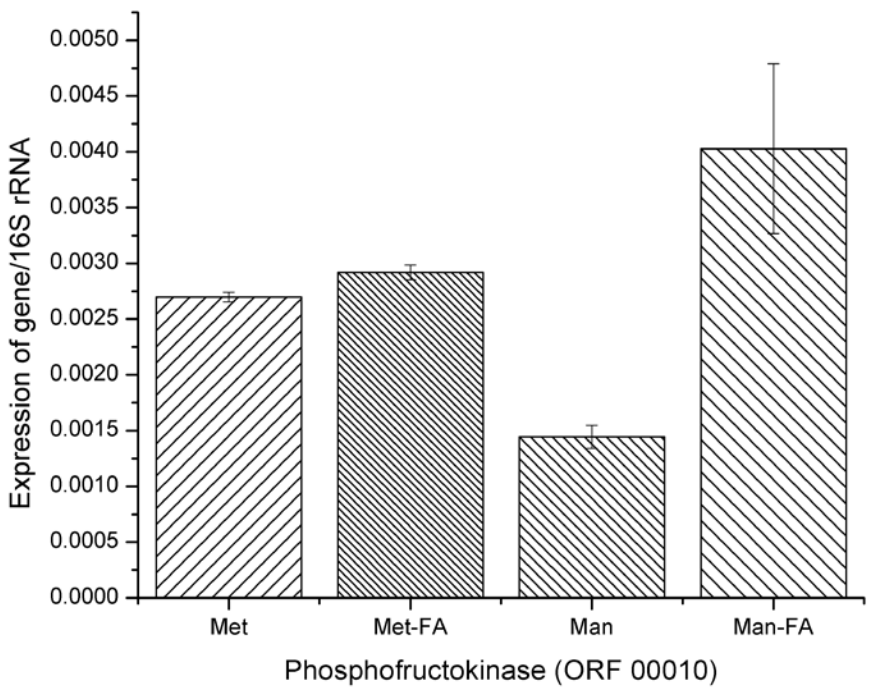


Figure 5.19. Regulation of phosphofructokinase (*pfk*) in response to formaldehyde (2 mM) spike.*B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Man; immediately before spike, Man-FA; spiked with formaldehyde. (n=3)

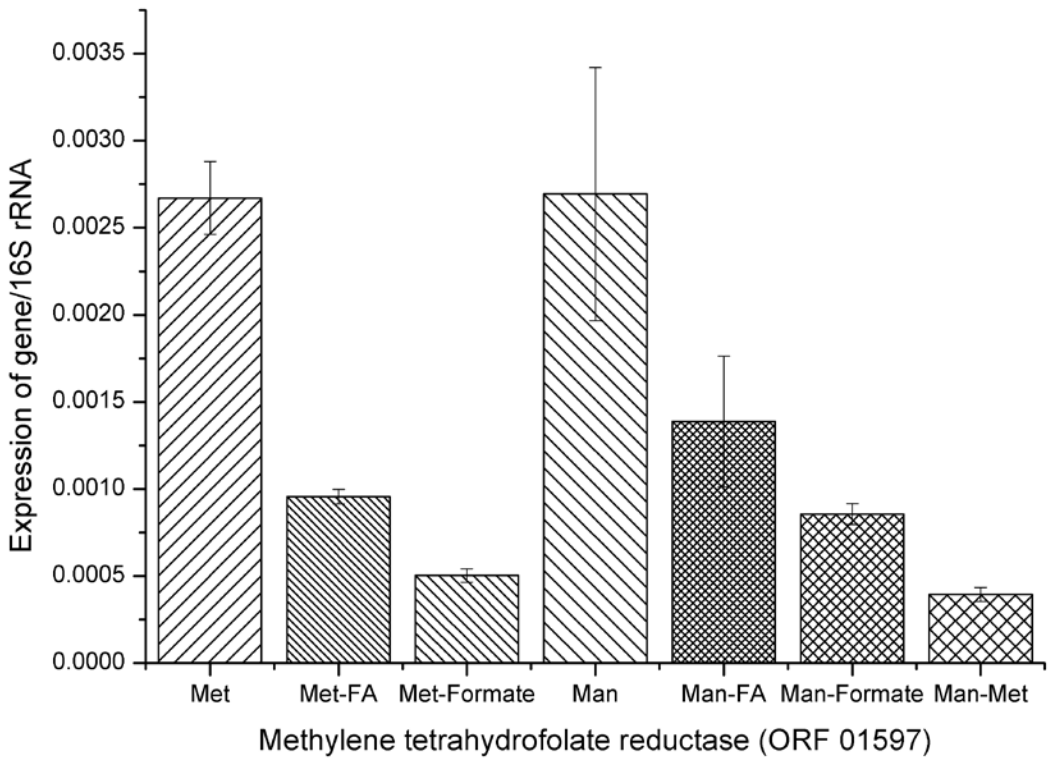


Figure 5.20. Regulation of methylene tetrahydrofolate reductase in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM).*B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)

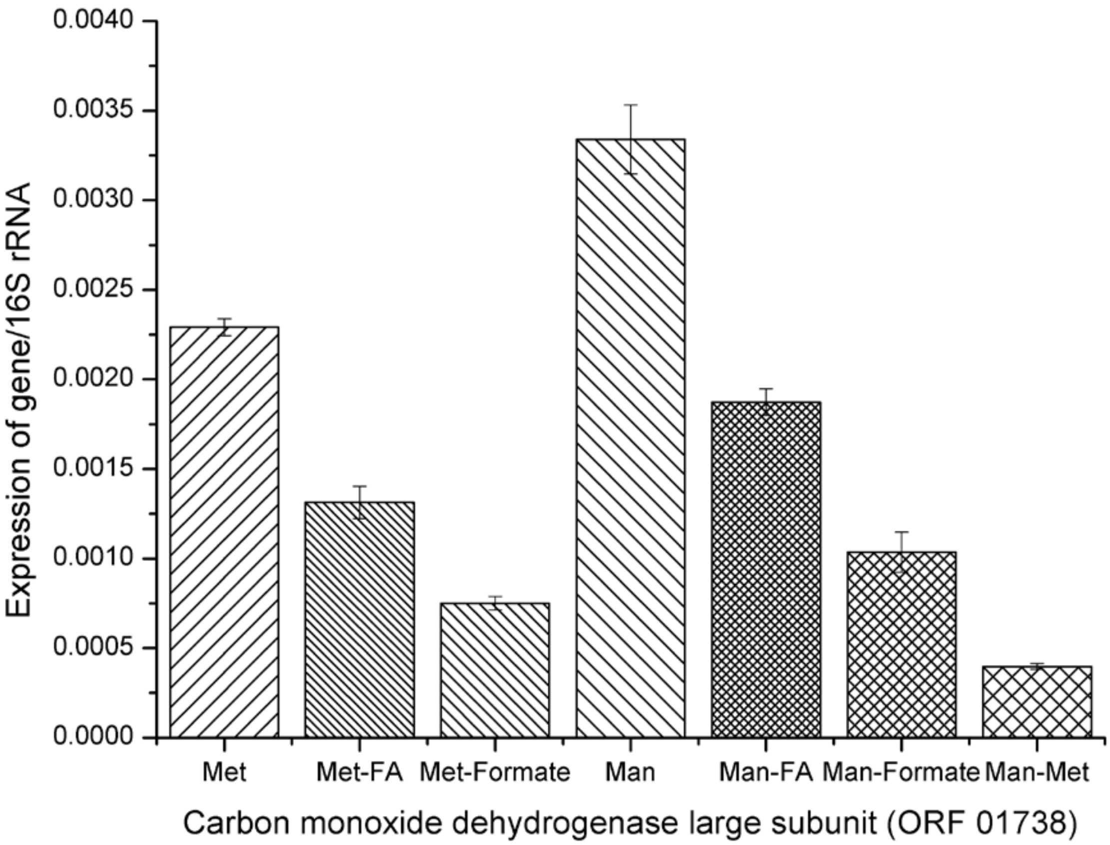


Figure 5.21. Regulation of CO dehydrogenase in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM).*B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the O.D_{600} is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)

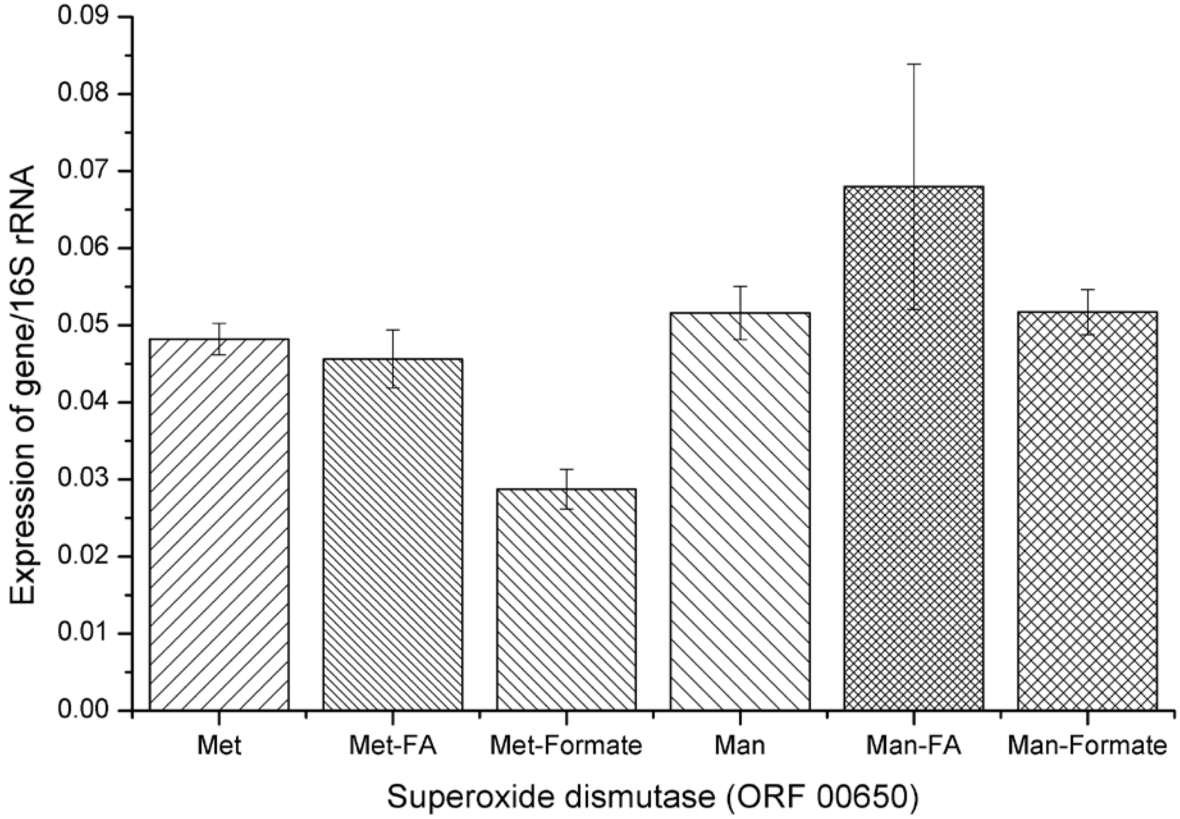


Figure 5.22. Regulation of superoxide dismutase in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM).*B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the $O.D_{600}$ is ~0.8. The total RNA was extracted 20 minutes post-spiking.Met; immediately before spike, Met-FA; spiked with formaldehyde, Met-Formate; spiked with formate, Man; immediately before spike, Man-FA; spiked with formaldehyde, Man-Formate; spiked with formate, Man-Met: spiked with methanol. (n=3)

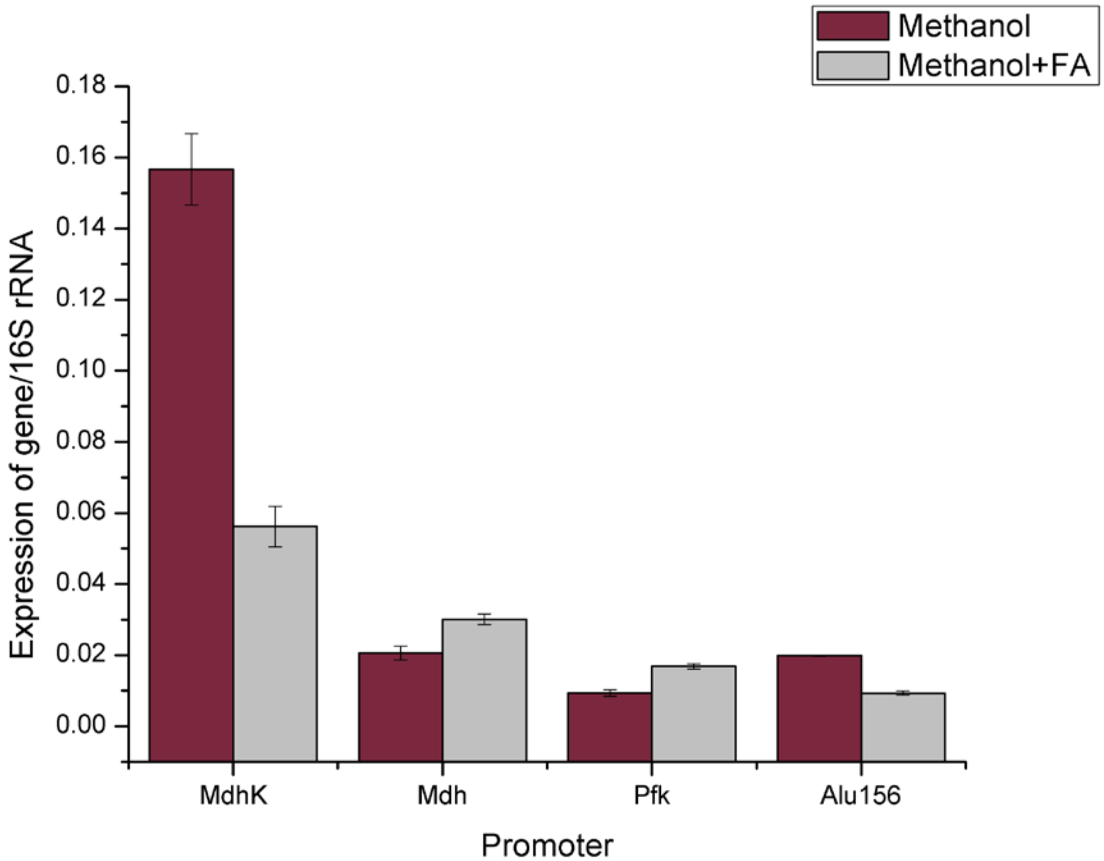


Figure 5.23. Expression GFPuv under different promoters.*B. methanolicus* MGA3 was grown in $MTYM_{150}$ (sample Methanol) and spiked with formaldehyde (sample Methanol+FA) at OD_{600} of ~0.8. The total RNA was extracted 20 min post-spiking. MdhK; GFPuv expressed from pNW33N as described in Nilasari et. al., (20012), Mdh; GFPuv expressed under P_{mdh} from pHP13, Pfk:GFPuv expressed under P_{pfk} from pHP13,Alu156: GFPuv expressed under P_{Alu156} from pHP13. (n=3)

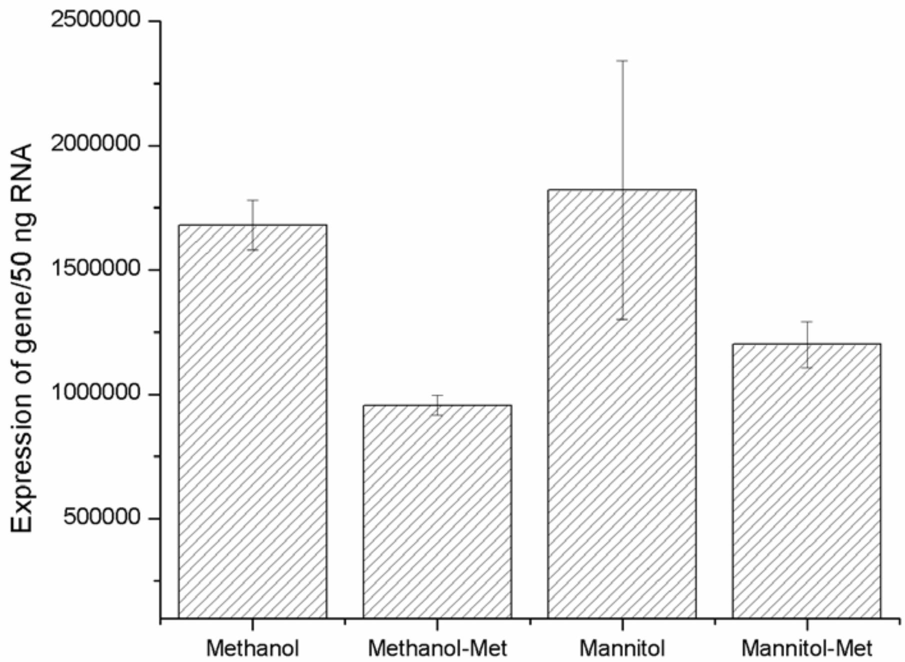


Figure 5.24. The expression level of *repB* in *B. methanolicus* MGA3. Met: Methanol grown cells, Met-Met: Methanol grown cells spiked with methanol, Man: Mannitol grown cells, and Man-Met: Mannitol grown cells spiked with methanol. Fifty ng of total rRNA was used for the assay. (n=3)

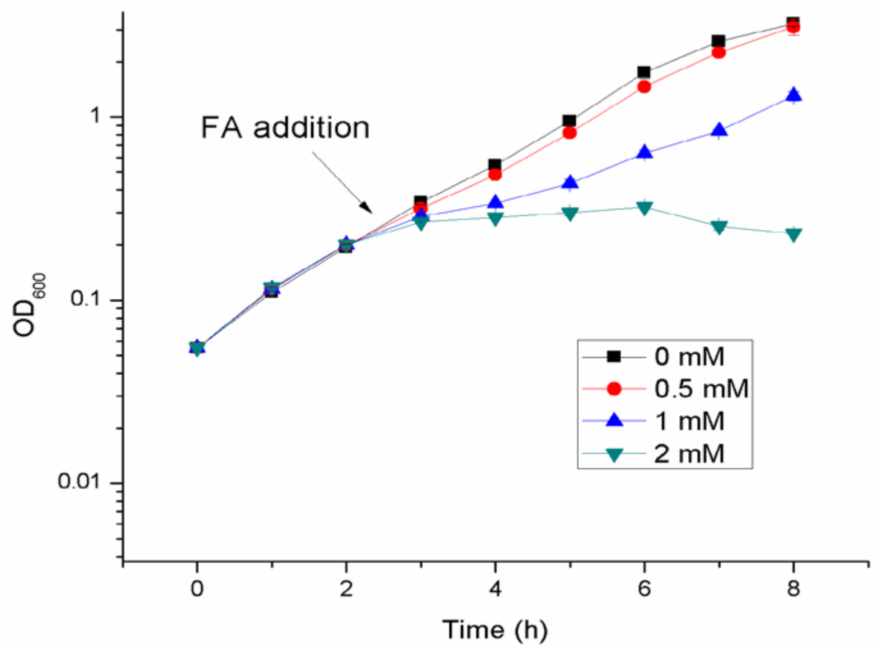


Figure 5.25. Formaldehyde tolerance of recombinant *B. methanolicus*MGA3(pTS123). *B. methanolicus* MGA3 (pTS123) was grown in MTYM with 150 mM methanol and spiked with formaldehyde at OD_{600} of ~0.250. (n=3)

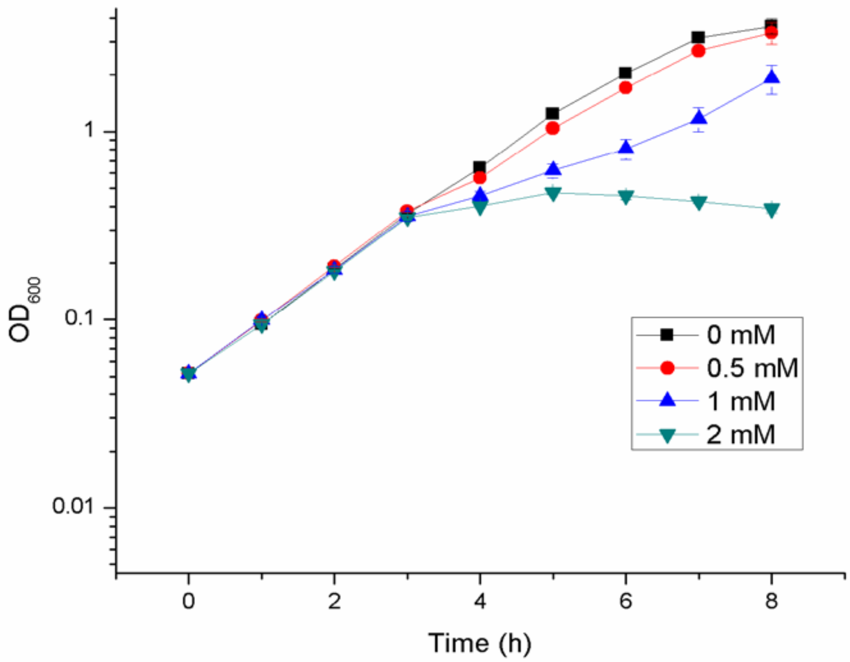


Figure 5.26. Formaldehyde tolerance of recombinant *B. methanolicus*MGA3(pHPS12). *B. methanolicus* MGA3 (pHPS12) was grown in MTYM with 150 mM methanol and spiked with formaldehyde at OD_{600} of ~0.250. (n=3)

CHAPTER 6

FORMALDEHYDE AND FORMATE DEHYDROGENASES OF *Bacilli*

Introduction

The concentration of CO_2in the atmosphere has been increasing since the start of the Industrial age, mostly due to the use of fossil fuels. Since the start of the Industrial Revolution, the carbon concentration has risen 30% (Vitousek et. al., 1997). This accumulation of CO_2 causes irreversible effects on the climate that last for 1000 years (Solomon et. al, 2009).

There are constant efforts for new technologies to limit the release of CO_2 into the atmosphere and fixation of atmospheric carbon to use as fuel, and as a substrate for production of intermediate products. One way of limiting the CO_2 emission is trapping it at the end of a pipeline. This is usually achieved by dissolving CO_2 in aqueous solution and absorbing it to an absorber like NaOH or seawater (Lin et.al. 2003). The second method of sequestering CO_2 is achieved through biology, which is conducted by nature; fixing it into biomass. For this, $CO_2$from the atmosphere is assimilatedinto the biomass, and biomass is then used as a substrate for production of biofuel and other metabolic intermediates. In this regards, the anaerobic bacterium *Clostridium ljungdahlii* was used to produce bioethanol using synthesis gas as substrate (Tirado-Acevedo, 2010).

Yet, another possibility is the enzymatic reduction of CO_2 to methanol, instead of ethanol. This pathway employs aerobic methylotrophic enzymes to synthesize methanol from CO_2. Practically, this is the reverse of the pathway that methylotrophic microorganisms use to dissimilate methanol to CO_2as discussed in Chapter 1. The simplest model of this pathway includes methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase. Theoretically, formate dehydrogenase reduces CO_2 to formate which is then reduced to formaldehyde by formaldehyde dehydrogenase. Finally, formaldehyde is reduced to methanol by methanol dehydrogenase. Baskaya et. al. (2010), reported the thermodynamic feasibility of this route. The reverse of this biological pathway is feasible at low pH, low ionic strength and elevated temperatures. The biggest pitfall in this pathway is the source of reducing equivalentsin the form of NADH.

The enzymes of this route were discussed in Chapter 1. As a thermotolerant methylotrophic microorganism, *B. methanolicus* might serve as a source for these enzymes. The growth of *B. methanolicus* PB1 in sea-water based media at 50 °C was shown by Komives et. al. (2005).*B. methanolicus* carries methanol dehydrogenase genes both on a plasmid and on the chromosome. The presence of putative aldehyde (formaldehyde) and formate dehydrogenases was presented in Chapter 3. The methanol dehydrogenase enzyme from*B. methanolicus* PB1 was characterized before, although it is not identical to methanol dehydrogenase genes of strain MGA3 carried both on the pBM19 plasmid and chromosome (See Figure 3.4).

In this chapter, I report cloning, expression and characterization of putative formaldehyde and formate dehydrogenase genes of *B. methanolicus* MGA3, along with the putative respective genes of *Bacillus subtilis* BD170, *Bacillus licheniformis* and *Geobacillus kaustophilus*.

Materials and Methods:

Growth of microorganisms: *Bacillus subtilis*, *Bacillus licheniformis*, and *Geobacillus kaustophilus* HTA426 were obtained from ATCC. They were grown in 250 ml baffled shake flasks fitted with milk filter discs containing 50 ml of Luria-Bertani (LB) with 270 rpm shaking. The growth temperatures for microorganisms were 37 °C for *B. subtilis*, and *B. licheniformis*, and 60 °C for *Geobacillus kaustophilus* HTA426. *B. methanolicus* MGA3 was grown as described in Chapter 2.

Cloning of genes: The *B. subtilis* genes *yycR* and *yrhE*, *B. licheniformis* genes *ywdH* and *ywrH*, *G. kaustophilus* ORFs between the nucleotides of 2797314-2798810 and 498622-501585 were amplified using the Expand High Fidelity PCR kit from Roche, and primers listed in Table 6.1. Correct band size was verified using agarose gel electrophoresis. The PCR mixture was purified using Wizard® SV Gel and PCR Clean-Up System (Promega cat. A9281) and ligated into cloning vector pGEM-T using T_4 ligase (New England Biolabs, Ipswich MA). The ligated pGEM-T was transformed into *E. coli* DH5α using $CaCl_2$ competency as described in Maniatis et.al. (1982). Colonies were screened for the insert using PCR, and pGEM-T was isolated using Qiagen's QIAprep Spin Miniprep Kit. The insert was then subcloned into pET16-b or pET17-b using the respective restriction sites as listed in Table 6.1. The ligation, transformation and screening were carried out again as above and presence of correct size insert was verified using restriction enzyme digestion. For *B. methanolicus* MGA3+pTS123, the putative formaldehyde dehydrogenase gene of *B. kaustophilus* (2797314-2798810) was cloned into pHP13 under the control of P_{mdh} promoter. The manipulations were carried out using *E. coli* and then transformed into *B. methanolicus*

MGA3 as described in Chapter 5. The expression vectors were then submitted for sequencing for verification of nucleotide sequence. The same pattern was followed for the cloning of ORFs from *B. methanolicus* MGA3 except for subcloning into pGEM-T. The PCR amplified ORFs were directly cloned into either pET16-b or pET17-b, and nucleotide composition was verified again via sequencing.

Expression of recombinant genes: The expression of genes was carried out in *E. coli* BL21 (DE3). The host *E. coli* was grown in the presence of ampicillin at 37 °C, in 25 ml LB media in 250 ml shake-flasks to OD_{600} of ~0.8 and induced by 1 mM final IPTG. The temperature was lowered to 30 °C and culture was incubated for four hours. Cells were harvested by centrifugation for 5 min at 10,000xg and washed with 10 ml phosphate buffer saline (PBS). The pellet was resuspended in 2 ml PBS.

Lysis of cells: Twenty-microliter of Halt Protease Inhibitor Cocktail was added to resuspended cells in PBS and the suspension was transferred to 15 ml falcon tubes. Cell lysis was achieved using a Qsonica sonicator with micro tip in ice. The sonication parameters were; 50% probe density, 10 sec on, 20 sec off for one and half minute for *E.coli* and 3 minutes for *B. methanolicus*. The lysis of *B. methanolicus* differed from *E. coli* in that exponentially growing cells (OD_{600} ~1.5-2.0) were harvested and resuspended in 5 ml PBS with 20 mg/ml lysozyme after washing in 10 ml PBS. The cells were then incubated at 37 °C for an hour to digest the cell wall, washed again with 10 ml PBS to remove remnants of lysozyme and resuspended in 2 ml PBS toa final OD_{600} of ~20. Twenty microliters of protease inhibitor cocktail were added before sonication. The lysate was transferred back to a 50 ml falcon tube and centrifuged for 10 min at 10,000xg to remove the cell debris. The supernatant was used as crude cell extract. The protein concentration was measured using a Bradford assay.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGEwas performed using a Mini-Protean Tetra System (BioRad Laboratories). Fifteen microliters of sample were mixed with 15 µl 2x sample buffer containing 5% β-mercaptoethanol and denatured for 10 min at 90 °C before loading into a 12% nUView Tris-HEPES gel (NuSep, Bogart GA). The gel was run at 100 V for 45-60 minutes and stained in 25 ml Instant Blue (Kampenhout, Belgium). The gel was visualized using Kodak Gel Logic 200 imaging system after destaining with a solution containing 5% methanol and 7% acetic acid.

Purification of His-tagged proteins: Thermo Scientific HisPur Ni-NTA resin was used to purify the His-tagged proteins from the crude cell extracts according to the manufacturer's manual.

Formaldehyde dehydrogenase assay: The formaldehyde dehydrogenase of *Pseudomonas putida* (Sigma F1879, 3U/mg solid) was used as standard enzyme to construct a standard curve for the formaldehyde dehydrogenase activity. The entire sample in the tube was dissolved in 100 µl of PBS and protein content was measured using a Bradford assay. Then, it was diluted to the range of 0.01 to 0.0005 units, in PBS. Formaldehyde activity was assayed by measuring formation of diformazan. The reaction mixture, (1 ml) containing 26 mM potassium phosphate buffer (pH 8.0), 0.2% Triton X, 5 mM formaldehyde, 0.005 % NBT, 0.0005% PMS and 0.5 mM NAD^+, was prewarmed for 5 minutes in a water bath and the reaction was started by adding the enzyme in a 1 ml disposable cuvette. The change in OD_{570} was measured at exactly 3 minutes. The standard curve obtained is shown in Figure

6.1. One unit of enzyme is defined as amount of enzyme that will oxidize 1 µmol of formaldehyde into formic acid per minute.

Formate dehydrogenase assay: The assay for formate dehydrogenase was the same as with formaldehyde dehydrogenase except that formaldehyde was replaced with formate. The standard curve obtained is shown in Figure 6.2. A standard enzyme isolated from*Candida boidinii* was purchased from Sigma. One unit of enzyme is defined as amount of enzyme required to oxidize 1 µM of formate into CO_2 per min.

Results:

Expression of clones: The formaldehyde dehydrogenase genes of *B. subtilis* and *B. kaustophilus* were successfully expressed from pET16-b vector (Figure 6.3). However, the expression of formate dehydrogenase from *B. subtilis* (yrhE), *B. licheniformis* (yrhE) and *G. kaustophilus*(498622-501585) was not assessed. *B. methanolicus* MGA ORFs 01733 (putative formaldehyde dehydrogenase) and ORF 01931 (putative formate dehydrogenase) were successfully overexpressed in *E. coli* BL21 as seen in Figure 6.4. There was no distinct overexpression seen on the SDS-PAGE for clones of ORFs 00554, 00623 and 01579.

Formaldehyde dehydrogenase activity of *yycR*: The putative formaldehyde dehydrogenase of *B. subtilis* was successfully overexpressed and purified by metal affinity chromatography (Figure 6.4). Figure 6.5 describes the enzymatic assay of purified FADH of *B. subtilis* as a function of pH.

Formaldehyde dehydrogenase activities of *E. coli, B. subtilis, B. methanolicus* MGA3, *B. methanolicus* PB1 and *B. methanolicus* MGA3 (pTS123): The formaldehyde and formate dehydrogenase activities of samples from crude cell extracts were assayed using the calibration curves constructed via standard enzymes as described above. One unit of enzyme activity is defined as the amount of enzyme activity that oxidizes 1 µmol of substrate (formaldehyde or formate) into product per min. As seen from Table 6.2, *B. methanolicus* strains MGA3 and PB1 have significantly higher formaldehyde dehydrogenase activities than that of *B. subtilis* and *E. coli* BL21. Also, the formaldehyde dehydrogenase activity of *B. methanolicus* PB1 is two-fold higher than that of strain MGA3. This is consistent with Figure 1.4 which demonstrates a higher CO_2 dissimilation rate from the strain PB1 and also with the Sothern Blot data, on which the probes were able to hybridize with putative formaldehyde gene(s) of strain PB1 but not with that of MGA3. Although there was no evident expression on the gel (Figure 6.6), the difference between formaldehyde dehydrogenase activities of *E. coli* BL21 and *E. coli* BL21+ORF00554 and ORF00623 (Table 6.2) suggests possible expression of the respective genes, albeit very weakly. The specific formaldehyde dehydrogenase activity of *B. methanolicus* is comparable to that of *M. capsulatus* (Bath), (Adeosun et. al., 2004). This difference deserves further investigation. Additionally, no formaldehyde dehydrogenase was detected from*B. methanolicus* MGA3 (pTS123). This requires further investigation as*B. methanolicus* MGA3 (pTS123) is more tolerant to formaldehyde spike than the parent strain (Figure 5.25)

Formate dehydrogenase activities of *B. subtilis, E. coli, B. methanolicus* strains MGA3 and PB1 and ORF01931: Table 6.3 summarizes the formate dehydrogenase activity from crude cell extracts of the samples. The enzyme activities were measured as described above and calculated using the calibration curve in Figure 6.2. One unit of enzyme activity is defined as the activity that oxidizes 1 µmol of formate into CO_2 per minute. As seen in Table 6.3, the formate dehydrogenase activity of *B. methanolicus* strain PB1 was significantly higher than that of strain MGA3. The formate dehydrogenase activities of *B. subtilis* and *E. coli* are comparable to each other. The possible overexpression of ORFs 01579 and 01931 (see figure 6.4 for overexpression of ORF01931) in *E. coli* caused two fold increase in specific activity of crude cell extract. In addition, the purified FDH (ORF1931) (Figure 6.7) had ~3-fold higher formate dehydrogenase activity than that of crude cell extract. However, this activity was too small for a purified enzyme. This might be either due to very low affinity of the expressed protein, the His-tag that it contains may alter its activity or the reaction conditions may require further optimization.

Conclusion:

The putative formaldehyde and formate dehydrogenases of *B. subtilis, B. licheniformis, G. kaustophilus* HTA 426 and *B. methanolicus* MGA3 were investigated in this chapter. The crude cell extract of *B. subtilis*, and *B. methanolicus* strains MGA3 and PB1 were tested for formaldehyde dehydrogenase activity. Among them the crude cell extract of *B. methanolicus* PB1 had shown the highest enzymatic activity. Up to 7 mU/mg protein of activity was detected in *B. methanolicus* strain PB1 whereas the MGA3 strain had only half of it, 3.057 mU/mg protein. *B. subtilis* had even less formaldehyde dehydrogenase activity, 1.192 mU/mg protein. This activity of formaldehyde dehydrogenase from crude cell extracts of *B. methanolicus* is comparable to that of *M. capsulatus* Bath which had activity of 5 mU/mg protein (Adeosun et. al., 2004).

The difference in enzymatic activity was more profound between the formate dehydrogenases of strains MGA3 and PB1, 3.053 mU/mg protein vs. 8.574 mU/mg protein. The activities of *B. subtilis* and *E. coli* BL21 are 1.473 and 1.873 mU/mg protein respectively. The *E. coli* strains that harbor putative *fdh* genes in pET system hada 2-fold increased formate dehydrogenase activity compared to the parent strain.

The simplest route for the dissimilation of methanol into CO_2 is oxidation through the enzymes of formaldehyde and formate dehydrogenases, which requires molecular O_2. Gutmacher (2009) has shown that the strain PB1 has higher O_2 demand during fed-batch fermentations (See figure 1.5), and incorporates less carbon into biomass per consumed methanol. The differences in carbon dissimilation pathway of strains MGA3 and PB1 were also emphasized in Chapter 4 with Southern Blot. Since the genome sequence revealed putative formate dehydrogenases in both strains (Chapter 2 and Heggeset et al., 2012), the difference in CO_2 evolution rate was most probably due to the efficiency of these enzymes.

The purified FDH of *B. methanolicus* MGA3 had activity of 13.116 mU/mg protein. This is a significant increase from the crude cell extract, but further investigation is required to optimize the purification and enzymatic assay for FDH. It is possible that the reaction conditions or expression parameters require optimization for maximum activity. Also, the FDH of strain PB1 may have a better activity, given the crude cell extract of *B. methanolicus* PB1 is 3-fold higher than the strain MGA3.

Among the FADHs of *B. subtilis, B. licheniformis* and *G. kaustophilus,* the *B. subtilis* and *G. kaustophilus* enzymes were overexpressed in *E. coli* as seen in Figure 6.3. The *B. subtilis* FADH was further characterized and compared to standard enzyme from *P. putida* (Degenkolbe, 2010). The activity of *B. subtilis* FADH with varying temperatures is summarized in Figure 6.5. Despite the initial demonstration of FADH activity of *G. kaustophilus* at 65 °C (Figure 6.9), no activity was detected when the crude cell extract was tested at 37 °C. The optimal growth temperature of *G.kaustophilus* is 65 °C and this might be the reason for lacking activity at 37 °C. This clone also needs further investigation.

Table 6.1. Primers used to amplify the respective gene for cloning and target cloning vectors. The restriction sites for respective enzymes are underlined.

Microorganism	Gene	Primer	Exp. Vector	Sequence
B. subtilis	*yycR*	Bs_faldh_Fw	pET16-b	5'-ATG GTA CTC GAG ATG GGA GGA ATG GCG TTG ACA G-3'(XhoI)
B. subtilis	yycR	Bs_faldh_Rv	pET16-b	5'-ATG GTA GGA TCC TTA CTT CAA TGT CCC ATG-3' (BamHI)
B. subtilis	yrhE	Bs_fdh_Fw	pET16-b	5'-ATG GTA CTC GAG ATG AGA TCT TTT GCC ACA CAA C- 3'(XhoI)
B. subtilis	yrhE	Bs_fdh_Rv	pET16-b	5'-ATG GTA GGA TCC TCA ATC TGT ACA CGG CCT G-3' (BamHI)
B. licheniformis	ywdH	Bl_faldh_Fw	pET16-b	5'- ATG GTA CAT ATG GCA GAA CGA ACA ACC TCC-3' (NdeI)
B. licheniformis	ywdH	Bl_faldh_Rv	pET16-b	5'-ATG GTA GGA TCC CTA CCT GAG AAG TTT TTT G-3' (BamHI)
B. licheniformis	yrhE	Bl_fdh_Fw	pET16-b	5'- ATG GTA CAT ATG GAC GGA AAG CCA ATC AG-3' (NdeI)
B. licheniformis	yrhE	Bl_fdh_Rv	pET16-b	5'- ATG GTA GGA TCC TTA GTC TGT TAA ATG AAC G-3' (BamHI)
G. kaustophilus	2797314-	Gk_faldh_Fw	pET16-b	5'- ATG GTA CAT ATG ACA ATG ATT TCG GCG-3' (NdeI)
G. kaustophilus	2797314-	Gk_faldh_Rv	pET16-b	5'- ATG GTA GGA TCC TTA AAA CAA CGT CAT CGG-3' (BamHI)
G. kaustophilus	498622-	Gk_fdh_Fw	pET16-b	5'-ATG GTA CAT ATG GAA GCG CAA ATG GTG-3'(NdeI)
G. kaustophilus	498622-	Gk_fdh_Rv	pET16-b	5'-ATG GTA GGA TCC TTA GCC ACC GAT CGT CAC-3' (BamHI)
B. methanolicus	ORF00554	19-Alde Fw	pET17-b	5'-ATG GTA AAG CTTATGGAATATCAAGTTACG-3'
B. methanolicus	ORF00554	19-Alde Rv	pET17-b	5'-ATG GTA CTC GAGTTAAAACGGAAATTTGCG-3' (XhoI)
B. methanolicus	ORF00623	20-Alde Fw	pET16-b	5'-ATG GTA CAT ATGGTTGTTTTGAGTGAAACC-3' (NdeI)
B. methanolicus	ORF00623	20-Alde Rv	pET16-b	5'-ATG GTA GGA TCCTCAATGTAAGTTTACCC-3' (BamHI)
B. methanolicus	ORF01733	36 Alde-Fw	pET16-b	5'-ATG GTA CAT ATGAGTCATTTAACCGTCG-3'(NdeI)
B. methanolicus	ORF01733	36 Alde-Rv	pET16-b	5'-ATG GTA GGA TCCTTAGTTTAAAGAAATCC-3'(BamHI)
B. methanolicus	ORF01579	32-Form-Fw	pET17-b	ATG GTA AAG CTTATGGGAAAAACACAACATAC-3'
B. methanolicus	ORF01579	32-Form-Rv	pET17-b	ATG GTA CTC GAGTCGAGACGTAACATAAAAATGC-3'
B. methanolicus	ORF01931	48-Forma-	pET16-b	ATG GTA CTC GAGATGCACTCTTATGTGGATCAG-3'
B. methanolicus	ORF01931	48-Forma-	pET16-b	5'-ATG GTA GGA TCCTTACAGTTTCTCCACATC-3'

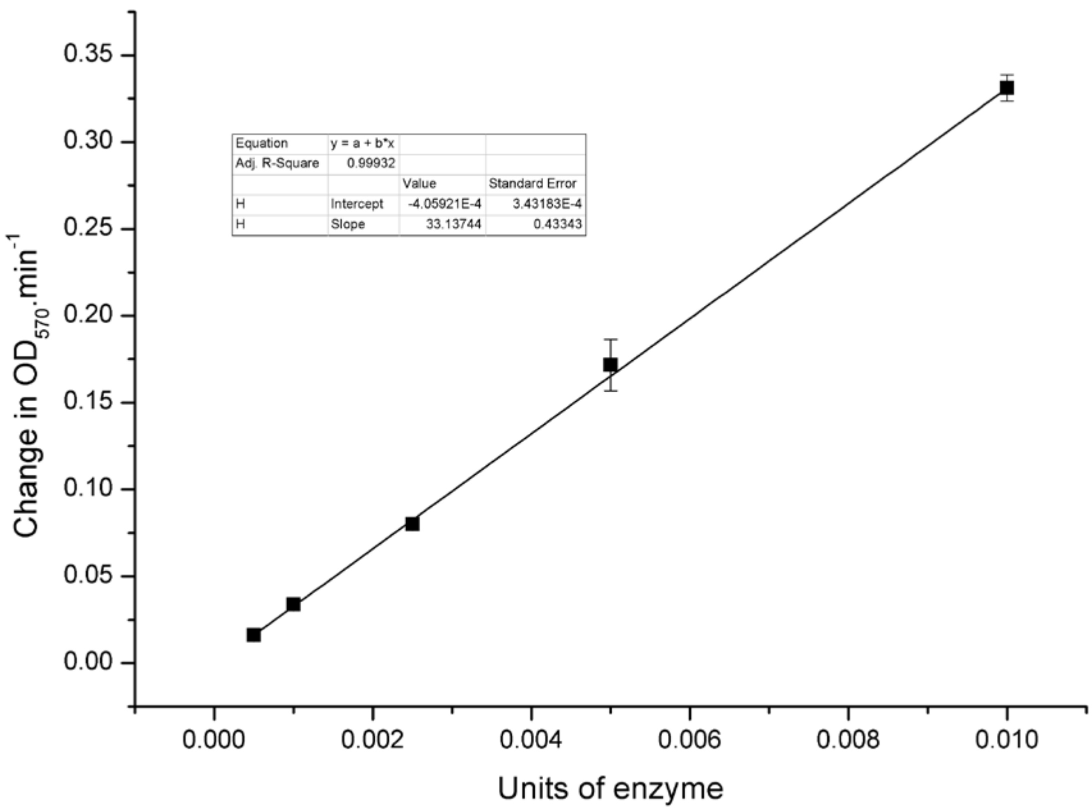


Figure 6.1. The calibration curve for measurement of formaldehyde dehydrogenase activity. *P. putida* formaldehyde dehydrogenase was used as the standard enzyme for the construction of standard curve.

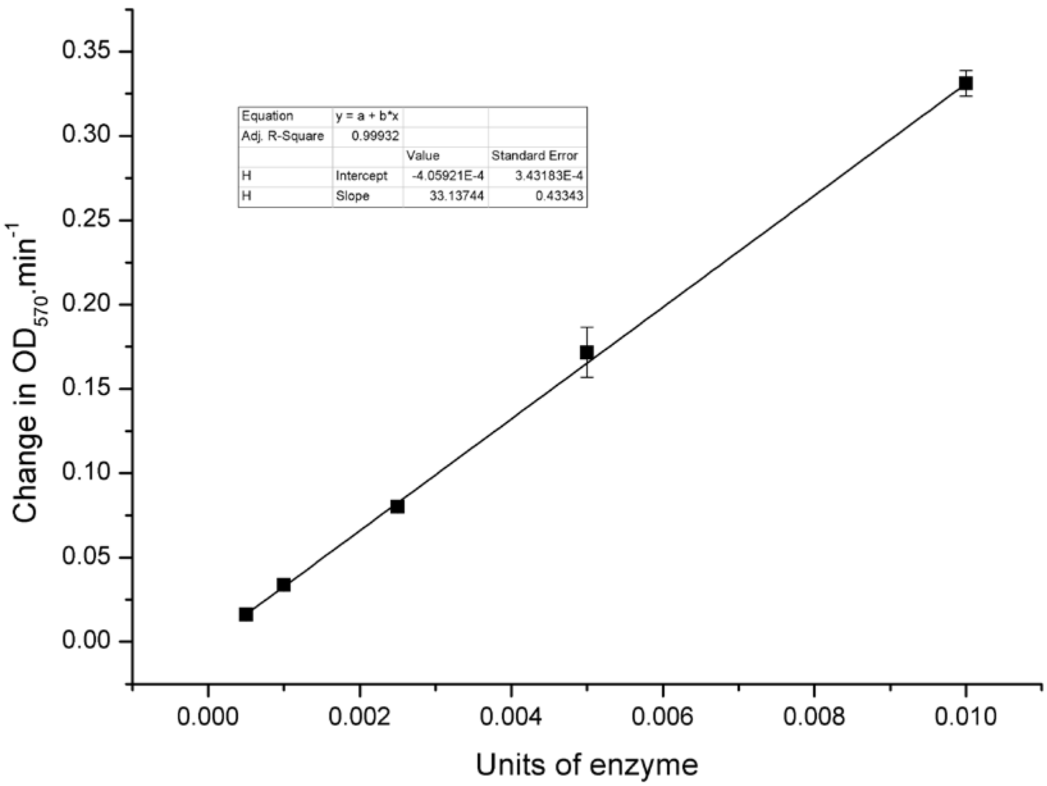


Figure 6.2. The calibration curve for measurement formate dehydrogenase activity. *Candida boidinii* formate dehydrogenase was used as standard enzyme for construction of standard curve.

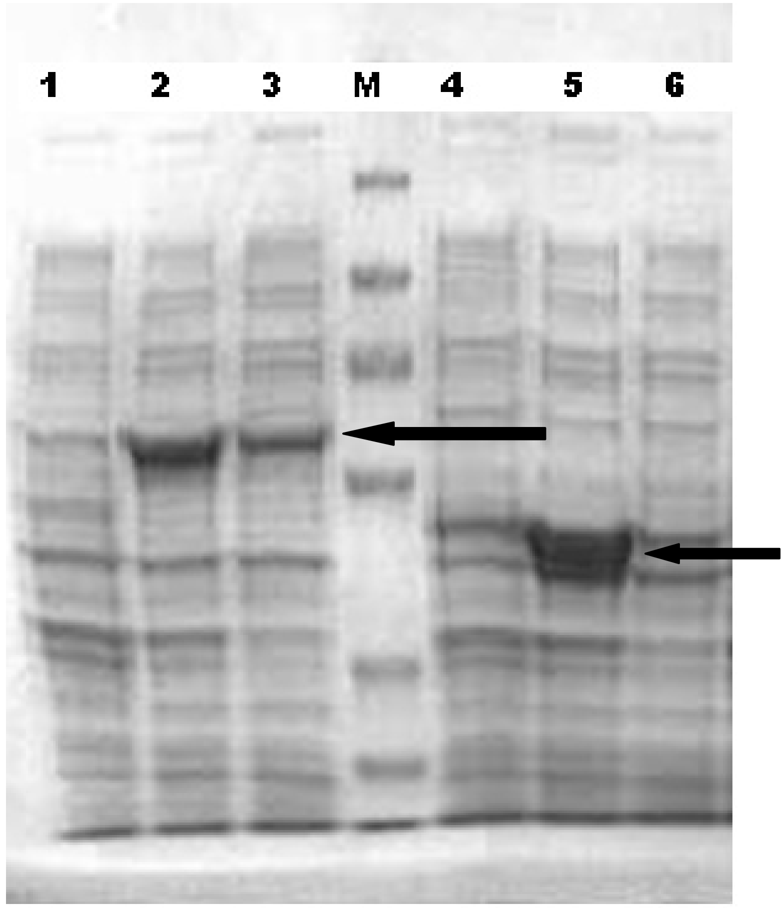


Figure 6.3. Overexpression of *B.kaustophilus* (53.49 kDa) and *B. subtilis faldh* (42.95 kDa) genes. Lines 1: *B. kaustophilus* uninduced, 2: *B. kaustophilus* induced insoluble, 3: *B. kaustophilus* induced soluble. M: Protein molecular marker, 4: *B. subtilis* uninduced, 5: *B. subtilis* induced insoluble, 6: *B. subtilis* soluble. (Image courtesy of Degenkolbe, 2010).

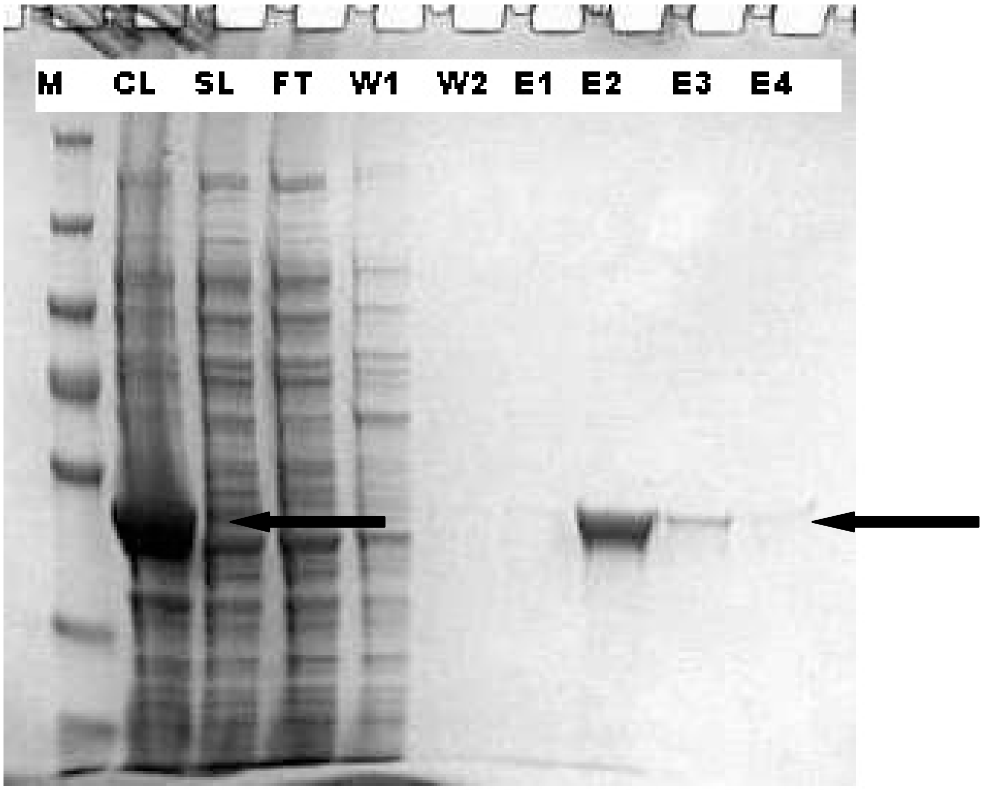


Figure 6.4. Purification of overexpressed *B. subtilis faldh* (42.95 kDa) via IMAC. M: Molecular marker, CL; crude cell lysate, SL; soluble fraction from the lysate, FT: flow through from IMAC column, W1 and W2: washes, E1-4: Elutions (Courtesy of Degenkolbe, 2010).

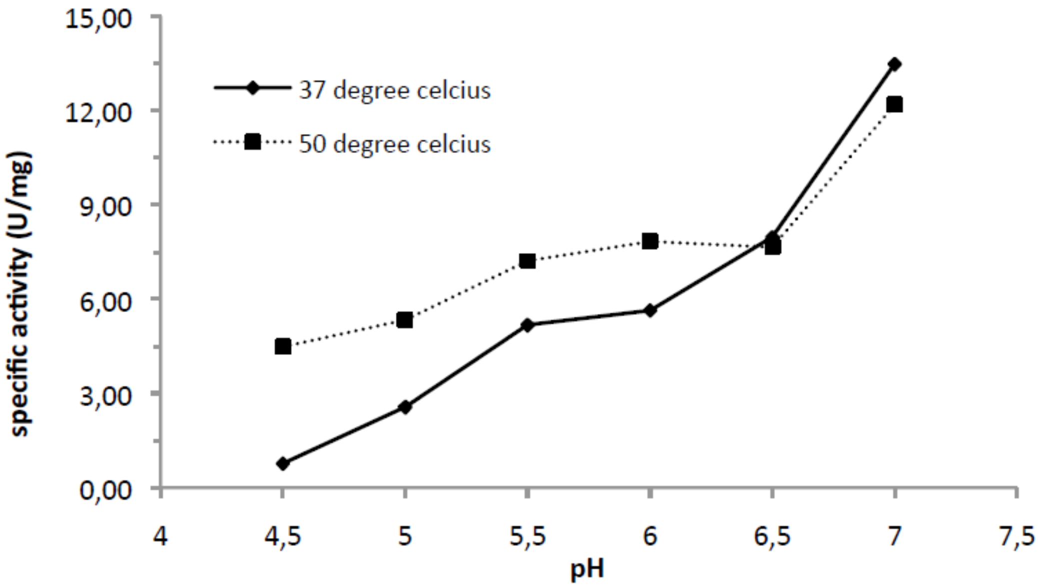


Figure 6.5. Specific activity of *B. subtilis* FADH at varying pH. (Degenkolbe, 2010)

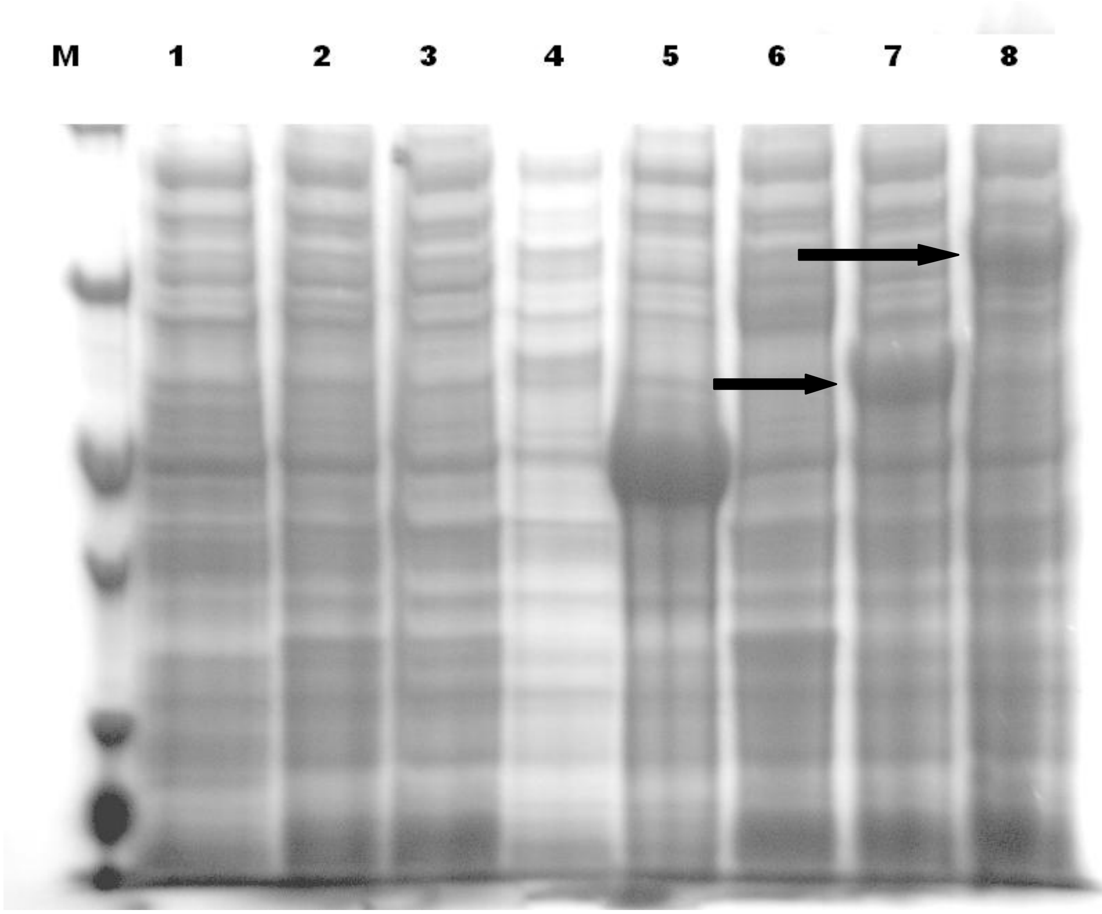


Figure 6.6. Analysis of crude cell extract of *E. coli* BL21 cells transformed with respective genes. M: marker, 1: *E. coli* BL21, 3: *E. coli* with ORF 554, 4: *E. coli* with ORF 623, 6: *E.coli* with ORF 1532, 7: *E. coli* with ORF 1733 (53.89 kDa) and 8: *E. coli* with ORF 1931 (75.69 kDa). The samples in lanes 2 and 5 are not directly related with this topic.

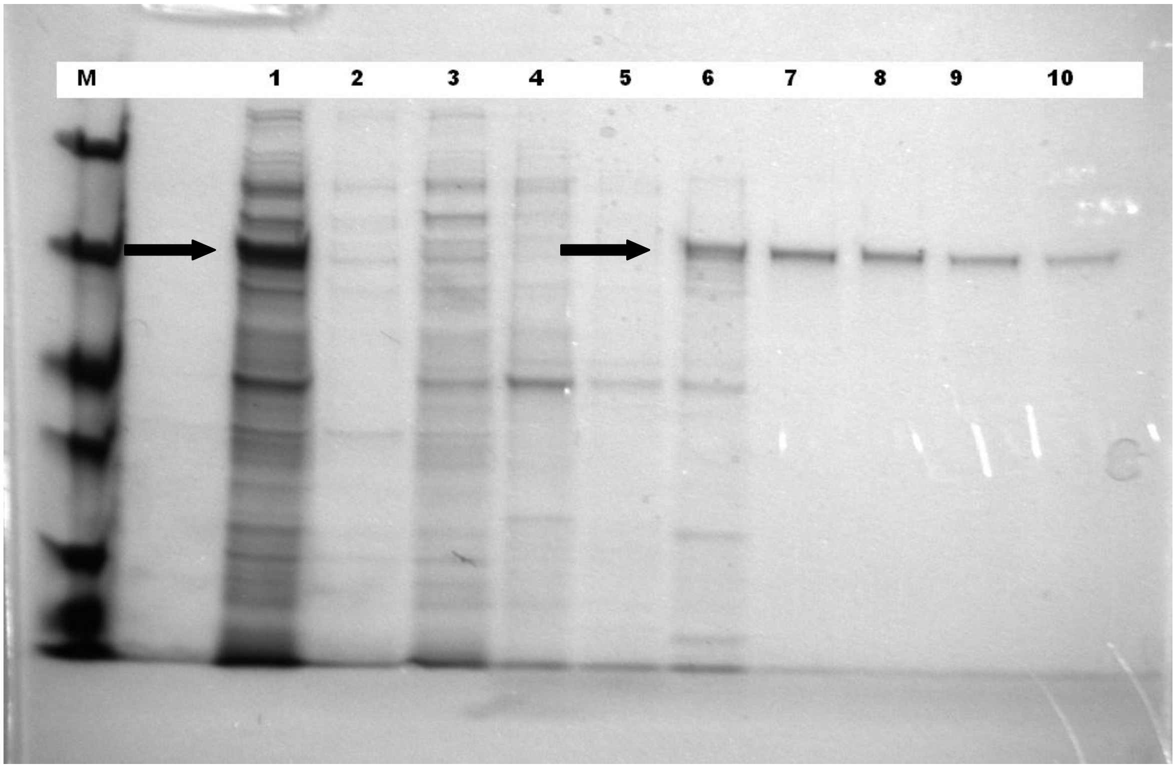


Figure 6.7. Purification of putative formate dehydrogenase (ORF01931-75.69 kDa) using IMAC. M: Molecular marker, 1: Crude cell extract, 2: Flow through, 3:Wash 1, 4: Wash 2, 5-10: Elution 1-6.

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Table 6.2. Formaldehyde dehydrogenase activities from crude cell extract. One unit of enzyme oxidizes on µmol of formaldehyde into formate per min (n=3). ND: Not detected.

Sample	FADH activity mU/mg protein	STD
B. subtilis	1.19	±0.08
B. methanolicus MGA3	3.08	±0.12
B. methanolicus PB1	6.97	±1.94
B. methanolicus MGA3 pTS123	ND	ND
E. coli BL21	0.45	±0.02
E. coli BL21+ORF00554	3.76	±0.04
E. coli BL21+ORF00623	1.98	±0.03
E.coli BL21+ORF01733	0.86	±0.03

Table 6.3. Formate dehydrogenase activities of crude cell extract. One unit of enzyme oxidizes one µmol of formate into CO_2 per min.

Sample	FDH activity mU/mg protein	STD
B. subtilis	1.44	±0.06
B. methanolicus MGA3	3.05	±0.46
B. methanolicus PB1	8.57	±2.39
E. coli BL21	1.84	±0.05
E. coli BL21+ORF1579	3.84	±0.07
E. coli BL21+ORF1931	3.86	±0.17
Purified FDH (ORF 1931)	13.12	±1.46

CHAPTER 7

INVESTIGATION OF ANAEROBIC GROWTH OF *B. methanolicus* MGA3

Introduction

The anaerobic growth of a strict aerobe,*B. subtilis*, was discussed in Chapter 1. Glaser et. al., have grown *B. subtilis* anaerobically in rich medium (2XYT) supplemented with 0.2 % KNO_3 as terminal electron acceptor (Nakano and Zuber, 1998). Another bacillus, *Bacillus infernus* can grow on formate or lactate with Fe(III), MnO_2 or nitrate as an electron acceptor (Boone et. al., 1995). Interestingly, methylotrophic bacteria were also isolated from legume nodules, which are known for the requirement of strict anaerobic conditions (Sy et. al., 2001).The growth of methylotrophic microorganisms presents a challenge since they require molecular oxygen in the dissimilation of methanol, especially for the oxidation of methane to methanol. Yet, oxidation of methane under anaerobic conditions has been reported (Knittel and Boetius, 2009). Although it was a consortium of bacteria and *Archaea* that oxidized the methanol without molecular oxygen, the bacterial members of the consortia were also shown to oxidize methane in the absence of *Archaea*. Nakano and Zuber (1998) discussed the importance of nitrate reductase genes for the anaerobiosis of *B. subtilis*.

The examples mentioned above were grown on rich media using oxygenated compounds like nitrate as the terminal electron acceptor. However, the anaerobiosis of Wood-Ljungdahl pathway organisms are different from above mentioned microorganisms in that they fix CO_2 into biomass. The Wood-Ljungdahl pathway was briefly discussed in Chapter 1.

The *B. methanolicus* MGA3 genome was shown to harbor genes of the Wood-Ljungdahl pathway (4.13). Especially, the presence of carbon monoxide dehydrogenase (CODH) necessitated an investigation of possible anaerobiosis of *B. methanolicus*.

Materials and Methods

Growth of *B. methanolicus*: *B. methanolicus* MGA3 was grown overnight on mannitol in the presence of 25 mM sodium nitrate to acclimatize *B. methanolicus* to nitrate as an electron acceptor. Anaerobic serum bottles containing 50 ml of either $MTYM_{MeOH}$ or $MTYM_{MAN}$ media were prepared by flushing the head space with nitrogen gas for 5 minutes. The culture grown in the presence of 25 mM nitrate was inoculated (with varying initial concentrations) into the bottles using a syringe and then incubated at 50 °C. For comparison, bottles with aerobic head spaces were also inoculated with the *B. methanolicus* MGA3. For possible acclimatization, *B. methanolicus* MGA3 was grown with aerobic (air) headspace and allowed to deplete the oxygen in the headspace. The growth was monitored by measuring OD_{600} by taking samples from the bottle using syringes. The change in gas composition was measured via gas chromatography.

Results and conclusion

As seen from the Figure 7.1, death of *B. methanolicus* MGA3 starts immediately on inoculation of the seed culture into the anaerobic bottle. However the culture in the bottle with aerobic headspace has limited growth (OD_{600} of 1.336) until the oxygen was depleted. No further growth was observed after this point.

The head space gas compositions of aerobic bottles with either $MTYM_{MeOH}$(Figure 7.2) or $MTYM_{MAN}$(Figure 7.3) were monitored for any change over 72 hours after inoculation of *B. methanolicus* to OD_{600} of 1. In both bottles, the oxygen in the headspace was depleted in 6 hours and the CO_2 concentration peaked at the same time. The bottles were monitored for 72 h without any change in the composition of headspace gas, which indicates no growth of *B. methanolicus*. The bottles were incubated for an extended period of time (30 days). The culture during this period stayed dormant as demonstrated by the fact that one half a milliliter of culture from the bottle was able to start aerobic growth when transferred into 50 ml $MTYM_{MeOH}$ in shake flasks (data not shown). This might be the spores *B. methanolicus* formed during oxygen depletion since it is known to form spores. With the aim of acclimatizing *B. methanolicus* MGA to anaerobiosis, 5 ml of liquid from the anaerobic bottles were transferred to fresh anaerobic bottles every other day. Limited growth (OD_{600} of 0.350) was observed after several passages. The total DNA from this culture was isolated as described in Chapter 3. The primers designed to amplify the entire 16S rRNA of *B. methanolicus* MGA3 failed to amplify the target. Another primer set, which amplifies a shorter (~500 bp) fragment of 16S rRNA, amplified an expected size fragment but sequencing of the amplicon identified the microorganism as *Bacillus sonorensis*. The sequence is 99% identical to *B. sonorensis* while it matched 97% to *B. methanolicus* MGA3 16S rRNA. This is most probably a contamination introduced during passages. The trials of growing *B. methanolicus* MGA3 with different media (SOBsuc, 2XYT) and different gas composition in the head space (N_2, Ar, Syngas) were also unsuccessful.

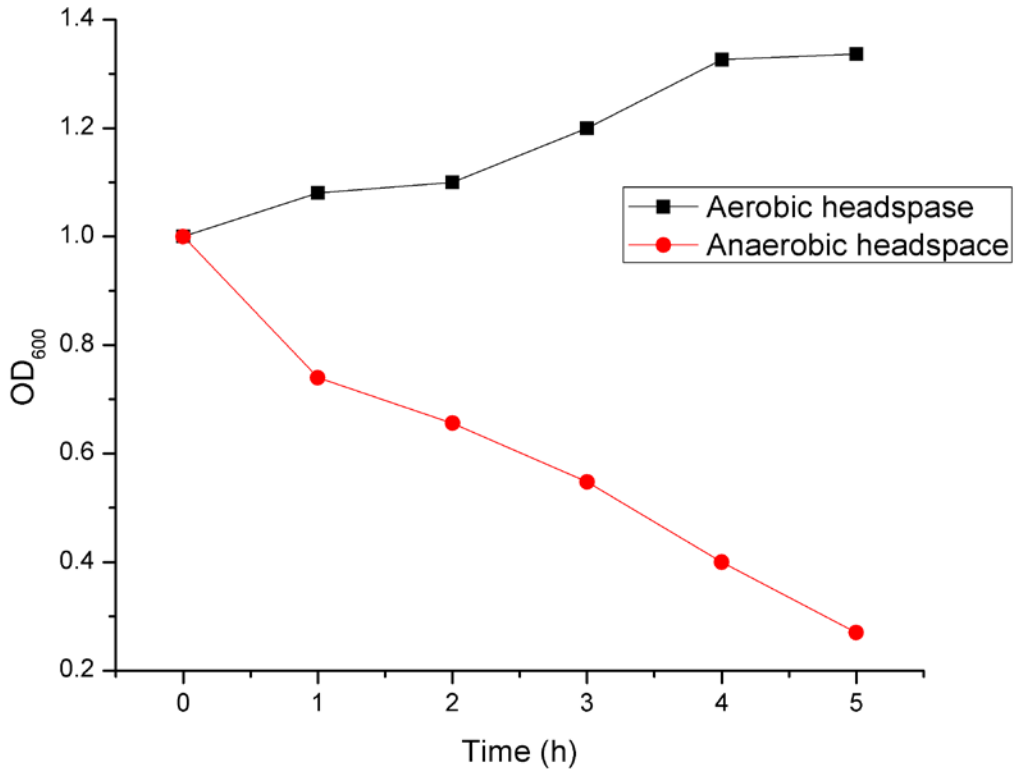

Figure 7.1. The growth of *B. methanolicus* MGA3 in serum bottles ($MTYM_{MAN}$) under aerobic (air) and anaerobic (N_2) head space. The bottles were inoculated to final OD_{600} of 1 and the growth was monitored for 5 hours. (n=1)

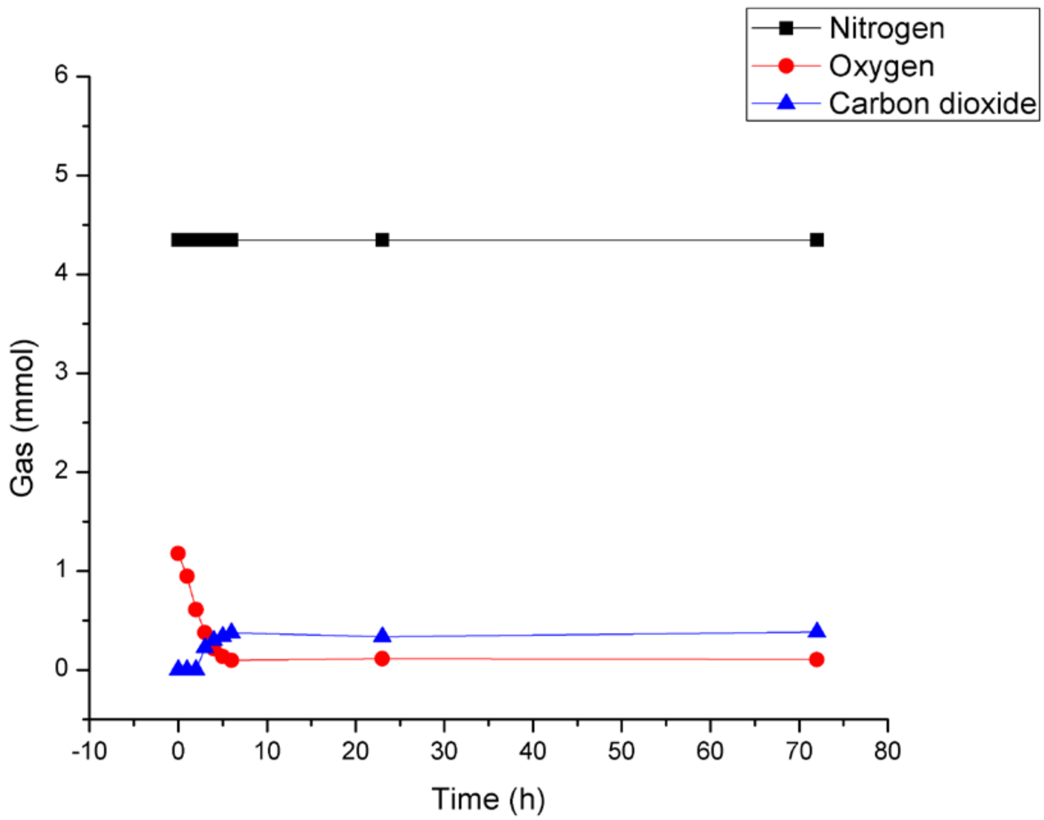

Figure 7.2. Analysis of gas composition of *B. methanolicus* MGA3 grown in $MTYM_{MeOH}$. *B. methanolicus* MGA3 was grown in $MTYM_{MeOH}$ in shake flasks aerobically as described in Chapter 2, in the presence of 25 mM sodium nitrate and inoculated to a final OD_{600} of 1.

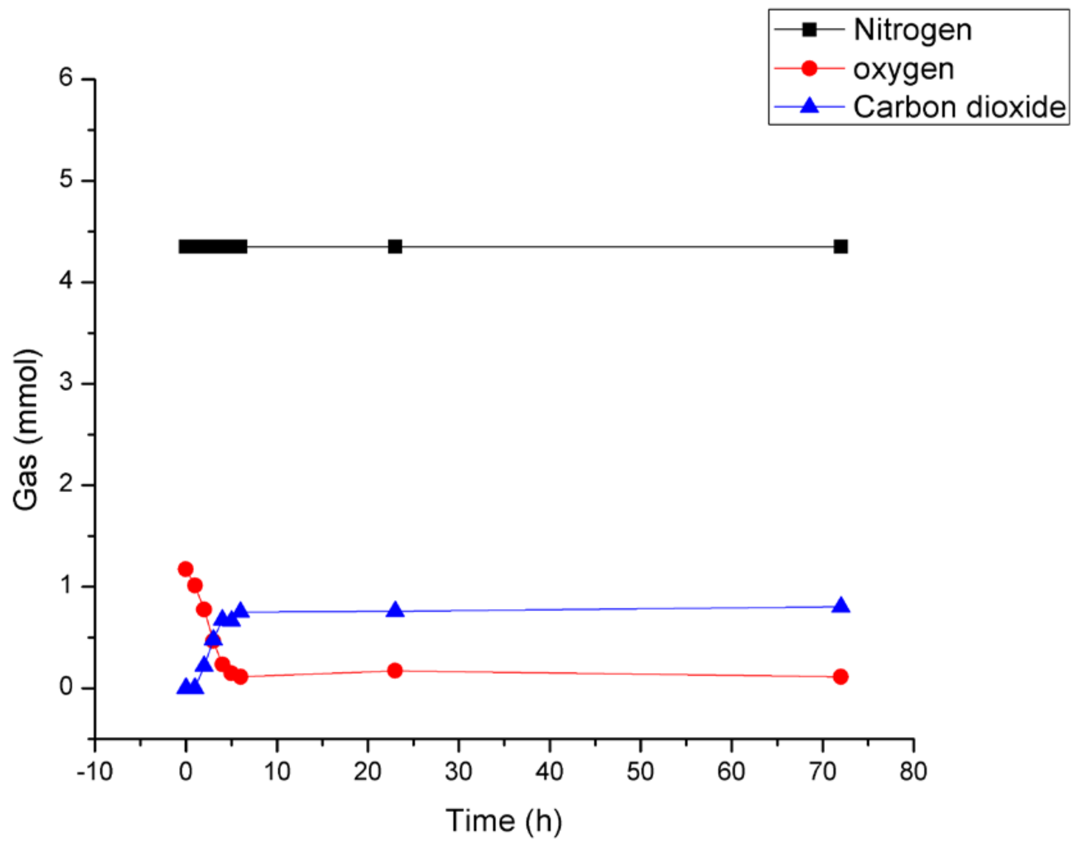

Figure 7.3. Analysis of gas composition of *B. methanolicus* MGA3 grown in $MTYM_{MAN}$. *B. methanolicus* MGA3 was grown in $MTYM_{MAN}$ in shake flasks aerobically as described in Chapter 2 in the presence of 25 mM sodium nitrate and inoculated into the bottle to final OD_{600} of 1.

CHAPTER 8

STUDY CONCLUSIONS

Bacillus methanolicus is a gram-positive, thermotolerant, restrictive methylotroph capable of using few substrates other than methanol like mannitol and glucose. The methylotrophy in *B. methanolicus* is known to be encoded by a plasmid. *B. methanolicus* employs the RuMP pathway for assimilation of carbon from formaldehyde. The formaldehyde is either assimilated into biomass via the RuMP pathway or dissimilated to CO_2. The rate of CO_2dissimilation increases when fed-batch cultures of *B. methanolicus* are subjected to a methanol or formaldehyde spike.

In this study we demonstrated that growth on methanol is challenging due to the possible accumulation of the metabolically important compound formaldehyde, despite the higher growth rate of *B. methanolicus*when grown on methanol than on mannitol or glucose. The growth rate on methanol is highest at methanol concentrations of 10 mM and 25 mM and then decreases as the methanol concentration is elevated (0.65±007 h^{-1} at 25 mM to 0.231±0.004 h^{-1} at 2 M). We have demonstrated that *B. methanolicus* growing on methanol can tolerate higher concentrations of methanol spike when compared to mannitol grown cells. However, it is interesting to note the growth inhibition when mannitol grown *B. methanolicus*cells were subjected to a methanol spike.*B. methanolicus* can utilize both substrates, and it can grow on 2 M methanol with a growth rate of 0.231±0.004 h^{-1}, yet 50 mM methanol was enough to perturb the growth of *B. methanolicus* when grown on mannitol. In this study we have also shown that methanol grown *B. methanolicus* is more susceptible to formaldehyde toxicity than mannitol grown cells probably due to the already high intracellular levels of formaldehyde in methanol grown cells. The detoxification pathways of formaldehyde assimilation and dissimilation, are saturated in methanol grown cells, and further addition of formaldehyde increases the level to toxic levels. The methanol metabolite formate was shown not to have any visible effect on the growth of either methanol or mannitol grown *B. methanolicus*.

B. methanolicus has a genome of ~3.3 Mb with the GC content of 38.35%. Its 2773 ORFs which are longer than 100 bp were annotated. Interestingly, duplicates of RuMP pathway genes were found on the chromosome. Also, putative genes of formaldehyde dissimilation were identified on the genome. It carries two additional *mdh* genes besides the gene on the pBM19; however, the plasmid-borne *mdh* is responsible for most ofthe methanol dehydrogenase activity as the RT-qPCR results demonstrate. No gene was annotated as a formaldehyde dehydrogenase while a number of putative aldehyde dehydrogenases were identified. The genome carries two putative formate dehydrogenases and the THF-linked pathway for the oxidation of formaldehyde. Surprisingly, it also harbors genes of the Wood-Ljungdahl pathway, including CO dehydrogenase and methylene-THF reductase.

In this study we demonstrate that the formaldehyde dissimilation metabolisms of *B. methanolicus* MGA3 and PB1 are different. The Southern Blots using formaldehyde and formate dehydrogenases of other bacilli demonstrated the difference between the metabolism of *B. methanolicus* MGA3 and PB1. The formaldehyde dissimilation pathway of strain PB1 is more efficient than that of MGA3, as it has higher formaldehyde and formate dehydrogenase activity in the crude cell extract.

We have shown the down regulation of methanol dissimilation pathway genes by methanol, formaldehyde and formate. In this regard, formate is more potent as a regulator despite the fact that it does not have an inhibitory effect on the growth of *B. methanolicus*. The expression of methanol dehydrogenase, formaldehyde and formate dehydrogenases, and THF-linked enzymes are all down regulated by methanol and its metabolites. Interestingly, the *mdh* in mannitol-grown *B. methanolicus* is also down regulated in response to methanol. But, the RuMP pathway genes, i.e *hps* and *pfk*, are upregulated in response to methanol and its metabolites in mannitol grown *B. methanolicus*. The expression level of *hps* is comparable to that of *mdh*, even though the later is encoded by a plasmid with 10-16 copies per cell. However, the *hps* is down regulated in methanol-grown *B. methanolicus* from its already high levels in response to formaldehyde and formate.

Methanol and formaldehyde metabolisms and their regulation were investigated in this study. This is the first study to report the effects of methanol, formaldehyde and formate when spiked in growing *B. methanolicus*cultures rather than comparison of methanol and mannitol grown cells.

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APPENDICES

Appendix A

Sequence of pBM20

AACGTCAAAAACCCTTATTTTTACTGGTTTTTTAGACGTCGAAAACAATAAAATA
TTTAAATATTTAAAATGTATTATTGTAAAACGACAAAACAAAGATGTATAAAAA
ATTTTTTTCATGTTATTTACTCAACTTTCGCAGAGTAATTTCTATATGCAACTCTT
GATACAACTGCTTTGCTTCGTCTTTCATTATTTCTCTTGTTTATAATGGACCCATA
AAACTAGACACGAAATGAGGAGATGTTAAAGTAGGTTTATGAAAAGAAAACGA
TATACACCAGAGTTTAAATCCCAGATCGTGCTAGAGGTCCTAAAAGAAGAAAAA
ACTATGAGTGAAATTGCTTCCTCTCATGGAATCCATGTGAATCAGATTCGGCAAT
GGAGGAATGCGTTTCTTGAACAAATGCCCCAGTTGTTTGCGAAAGAAAATAAGA
AAGTAGATCAAATGAAGGCTGACTATGAACAACAAATTGAAAACCTTTATGCCG
AAGTCGGTCGTTTAACCACGCAATTGTCGTGGCTTAAAAAAAAATCTGGCCTTAA
GGAGTAGGGCAGAACGTATTGACATGATTGATTGGGACGATGCAGAACTCCCTA
TTGCCACCCAGGCTGAATTGCTCGGACTCAATCGGTCCAGCTTGTATTATAAGCC
TGTGGAGCCTTCTCCTGAAGAAGTGTTCATCAAGCACCGAATTGATGAAATTTAC
ACCAAATATCCGTTTTTTGGCTCCCGGCGGATCACGGCCGTTCTGAATGAAAACG
GGTTACACATTAACCGAAAGGCCGTTCAGCGCCATATGAGGGAGATGGGGATTG
CCGGGATTTCACCTGGCCCCAACCTTAGTAAGCGCAACCTGGAACACCGGATTT
ATCCCTATCTTCTTAGAAATCTCGACATCACTCACCCCAACCAGGTATGGGGGAT
TGATATCACTTACATCCGGCTTCAACGCGGTTGGATGTATCTCGTCGCCATTATT
GACTGGTATTCCCGTTACGTCGTTAGTTGGGAGCTGGATCAGACGCTGGAAATCG
ATTTTGTCCTTGAAGCCGTCAAACGGGCGCTGTCGCACGCTCAACCTGAGATTAT
AAACAGTGATCAGGGTAGCCACTTCACCAGCCCGAAATATACCGAGCTGGTACT
GGATAAGAAAGTTCAAATCAGTATGGACGGCAAAGGTCGGGCTCTTGACAATAT
CATTACCGAGCGATTGTGGCGGACGATTAAATATGAAGAGGTCTACTTGAAGGA
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TAACCATGAACGCCCTCATCAATCATTAGGCTATAAACCACCTGCCTCTGTTTAT
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APPENDIX B
Annotation of *B. methanoliucus* MGA3 genome

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CDS             complement(2677961..2678206)
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CDS             2682096..2683577
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CDS             2687587..2688570
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CDS             2695931..2698048
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       alpha"
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CDS             2698110..2699156
                /note="ribonucleotide-diphoshpate reductase subunit
       beta"
                /gene="BMETHMGA302342"
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                     /note="monogalactosyldiacylglycerol synthase"
                     /gene="BMETHMGA302356"
     CDS             2715729..2716277
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                /note="ADP-glucose pyrophosphorylase"
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                /note="glycogen synthase"
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CDS             complement(2733647..2734579)
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                /note="isochorismate synthase"
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CDS             2736508..2738151
                /note="2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-
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CDS             2740050..2741528
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                /gene="BMETHMGA302378"
CDS             2742217..2742666
                /note="carbonate dehydratase"
                /gene="BMETHMGA302379"
CDS             2743036..2743509
                /note="S-ribosylhomocysteinase"
                /gene="BMETHMGA302380"
CDS             complement(2743803..2744372)
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                /gene="BMETHMGA302381c"
CDS             2745272..2745703
                /note="transcriptional regulator, ArsR family"
                /gene="BMETHMGA302382"
CDS             2746025..2747017
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CDS             2747092..2747532
                /note="DNA-binding ferritin-like protein"
                /gene="BMETHMGA302384"
CDS             2747652..2748083
                /note="hypothetical protein"
                /gene="BMETHMGA302385"
CDS             2748912..2749271
                /note="hypothetical protein"
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                /note="nucleoside triphosphatase YtkD"
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CDS             complement(2749960..2750859)
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                /gene="BMETHMGA302388c"
CDS             complement(2750750..2751526)
                /note="nitrate/sulfonate/taurine/bicarbonate ABC
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CDS             complement(2751565..2752557)
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                transporter"
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CDS              complement(2756177..2756725)
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CDS              complement(2758046..2758948)
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                 /gene="BMETHMGA302396c"
CDS              complement(2759076..2760275)
                 /note="3-phosphoshikimate 1-carboxyvinyltransferase"
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CDS              complement(2760393..2761367)
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CDS              complement(2761515..2762609)
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                 /gene="BMETHMGA302399c"
CDS              complement(2762765..2763130)
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                 /gene="BMETHMGA302400c"
CDS              complement(2763131..2764219)
                 /note="3-dehydroquinate synthase"
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                 /gene="BMETHMGA302402c"
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CDS              complement(2766571..2767017)
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CDS             complement(2770185..2770751)
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CDS             complement(2771896..2773263)
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                /gene="BMETHMGA302410c"
CDS             complement(2773621..2774319)
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CDS             complement(2775006..2775989)
                /note="glycerol-3-phosphate dehydrogenase"
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CDS             complement(2776073..2777392)
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                /gene="BMETHMGA302416c"
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CDS             complement(2783323..2784222)
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                /gene="BMETHMGA302426"
CDS             2793287..2793958
                /note="Transcriptional activator tenA"
                /gene="BMETHMGA302427"
CDS             2793966..2794781
                /note="ABC-type nitrate/sulfonate/bicarbonate
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CDS             2797627..2798616
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CDS             complement(2799860..2800495)
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CDS             complement(2800492..2801154)
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CDS             2804036..2804818
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CDS             complement(2821516..2822136)
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                /gene="BMETHMGA302455c"
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CDS             complement(2823595..2826231)
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CDS             complement(3103024..3103677)
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CDS             complement(3104383..3106002)
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CDS             3106828..3107538
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                /gene="BMETHMGA302700"
CDS             3107548..3109368
                /note="PAS sensor protein"
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CDS             3111485..3112279
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CDS             3112819..3113961
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                /gene="BMETHMGA302705"
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     CDS             3129036..3129920
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                     /note="putative ABC-2 type transport system permease
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     CDS             3139528..3139827
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                     /gene="BMETHMGA302732c"
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CDS                  3162654..3164387
                     /note="methyl-accepting chemotaxis protein"
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CDS                  3169746..3171080
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CDS                  3171173..3171835
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CDS               3222340..3222666
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                  /gene="BMETHMGA302795"
CDS               complement(3223526..3224122)
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CDS               complement(3224206..3224772)
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CDS               complement(3227210..3227551)
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CDS               3228423..3228998
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CDS             complement(3239749..3241224)
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CDS             3246475..3247686
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CDS                  3277090..3278532
                     /note="putative activator of 2-hydroxyglutaryl-CoA
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CDS                  complement(3279375..3280094)
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CDS                  complement(3280448..3280870)
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APPENDIX C

Applied and Environmental Microbiology

Genome Sequence of Thermotolerant Bacillus methanolicus: Features and Regulation Related to Methylotrophy and Production of l-Lysine and l-Glutamate from Methanol

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