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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Growth of *Bacillus methanolicus* in 2 M methanol at 50 °C: the effect of high methanol concentration on gene regulation of enzymes involved in formaldehyde detoxification by the ribulose monophosphate pathway

Ahmet Bozdag[1,5] · Claire Komives[4] · Michael C. Flickinger[2,3]

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Abstract *Bacillus methanolicus* MGA3 is a Gram-positive aerobic methylotroph growing optimally at 50–53 °C. Methylotrophy in *B. methanolicus* is encoded on pBM19 and by two chromosomal copies of the methanol dehydrogenase (*mdh*), hexulose phosphate synthase (*hps*) and phosphohexuloisomerase (*phi*) genes. However, there are no published studies on the regulation of methylotrophy or the dominant mechanism of detoxification of intracellular formaldehyde in response to high methanol concentration. The μ_{max} of *B. methanolicus* MGA3 was assessed on methanol, mannitol and glucose. *B. methanolicus* achieved a μ_{max} at 25 mM initial methanol of 0.65 ± 0.007 h^{-1}, which decreased to 0.231 ± 0.004 h^{-1} at 2 M initial methanol. Slow growth was also observed with initial methanol concentrations of >2 M. The μ_{max} on mannitol and glucose are 0.532 ± 0.002 and 0.336 ± 0.003 h^{-1}, respectively. Spiking cultures with additional methanol (100 mM) did not disturb the growth rate of methanol-grown cells, whereas, a 50 mM methanol spike halted the growth in mannitol. Surprisingly, growth in methanol was inhibited by 1 mM formaldehyde, while mannitol-grown cells tolerated 2 mM. Moreover, mannitol-grown cells removed formaldehyde faster than methanol-grown cells. Further, we show that methanol oxidation in *B. methanolicus* MGA3 is mainly carried out by the pBM19-encoded *mdh*. Formaldehyde and formate addition down-regulate the *mdh* and *hps* genes in methanol-grown cells. Similarly, they down-regulate *mdh* genes in mannitol-grown cells, but up-regulate *hps*. Phosphofructokinase (*pfk*) is up-regulated in both methanol and mannitol-grown cells, which suggests that *pfk* may be a possible synthetic methylotrophy target to reduce formaldehyde growth toxicity at high methanol concentrations.

Keywords Methylotrophy · *Bacillus methanolicus* · Formaldehyde tolerance · Formaldehyde toxicity · RUMP pathway

Introduction

Methylotrophs are microorganisms able to grow at the expense of reduced carbon compounds containing one or more carbon atoms, but no carbon–carbon bonds [1]. Obligate methylotrophs utilize C1 compounds as a sole carbon and energy source, while facultative methylotrophs can utilize multi-carbon compounds as well. The presence of methylotrophic bacteria in nature may be attributed to the abundance of methane in the environment generated by methanogens. Methanol produced as the end product of pectin metabolism also offers a suitable niche for methylotrophs to survive in [26]. Another compound that may serve as a source of methylotrophy is lignin. Formaldehyde (FA), but not methanol, is the by-product of lignin biodegradation [14].

✉ Michael C. Flickinger
michael_flickinger@ncsu.edu

1 Department of Plant and Microbial Biology, North Carolina State University, Raleigh, NC, USA

2 Golden LEAF Biomanufacturing Training and Education Center, North Carolina State University, Campus Box 7928, Raleigh, NC 27695-7928, USA

3 Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC, USA

4 Department of Chemical Engineering, San Jose State University, San Jose, CA, USA

5 Present Address: Department of Biomedical Engineering, Duke University, Durham, NC, USA

Methylotrophic bacteria have not been engineered by industry as biocatalysts to efficiently convert C1 carbon into low molecular weight commodity chemicals in high yield due to (1) methanol toxicity which requires complicated methanol sensing and feeding to achieve high cell density and high product concentration, (2) loss of methanol carbon by metabolism as carbon dioxide, (3) poorly developed genetic systems and (4) the inability to grow at high methanol concentration which may occur in very large methanol-fed bioreactors that are not homogenous. These limitations have recently stimulated interest in engineering surrogate easily manipulated model host organisms, such as *E. coli* for methanol assimilation—known as synthetic methylotrophy [20, 33]. However, *E. coli* as a surrogate host has not been reported to be capable of growth in moderate to high methanol concentrations as a sole carbon source, a fundamental characteristic required for any methylotroph for efficient fuel or chemical production from methanol [33].

Formaldehyde is the most reactive aliphatic aldehyde molecule and takes part in many well-known chemical reactions [20, 32, 33]. This reactivity is the major reason for its toxicity. Thus, organisms have evolved multiple mechanisms to bind intracellular formaldehyde to avoid these undesired reactions [16, 20, 33].

Studies of methylotrophic microorganisms have shown methanol toxicity (substrate toxicity) and methods have been developed for sensing and controlling the dissolved methanol concentration or sensing exhaust gas methanol concentration in bioreactors to minimize the inhibitory effect on the specific growth rate [9]. The growth rate has been reported to decline after the methanol concentration in the culture medium of *Bacillus methanolicus* exceeded 12 g/l, 375 mM [12]. It was also shown that formaldehyde was responsible for the decrease of the specific growth rate by adding extracellular FA [23], but there have been no published studies reporting that the formaldehyde produced in vivo 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 well studied in bacilli [21]. The intracellular level of formaldehyde can be controlled via either assimilation by the ribulose monophosphate (RuMP) pathway or the serine cycle, or dissimilation by oxidation to carbon dioxide. The same study reported the specific induction of the RuMP pathway in response to formaldehyde among other carbonyl electrophiles in non-methylotrophic *Bacillus subtilis* where it may serve as a detoxification pathway [21].

The biotechnological importance of methylotrophs has been reviewed by Schrader et al. [27], Trotsenko et al. [31] and Brautaset et al. [6], and all of these earlier studies have been recently reviewed as the foundation for current work toward synthetic methylotrophy [20, 33]. Methylotrophs are promising candidates for the production of microbial protein for livestock or aquaculture, production of poly-β-hydroxybutyrate/valerate, exopolysaccharides, ectoine, phytohormones and vitamins [31]. A critical limitation for industrial application is how they can be grown at high methanol concentrations with minimal methanol toxicity and minimal loss of carbon as carbon dioxide [20, 33]. Methylotrophs also contain high levels of dehydrogenase enzymes that may be used in the generation of reduced compounds like NADH. The RuMP pathway methylotrophs are energetically superior to serine pathway microorganisms, in that they produce almost twofold higher cell yield per gram of methanol carbon [27]. Another advantage of the RuMP pathway organisms is the higher biomass productivity; RuMP pathway methylotrophs have an average productivity of 28.4 g cell dry mass (g_{CDM})/l/h, while the productivity of serine cycle microorganisms ranges between 1.2 and 3.6 g_{CDM}/l/h. A portion of the assimilated methanol is dissimilated as carbon dioxide and lost. In RuMP pathway methylotrophs, ~38 % of methanol carbon is dissimilated [27]. Understanding the regulation of the formaldehyde dissimilation pathway is equally important as the assimilation pathway since it determines the portion of substrate carbon lost as carbon dioxide [20, 33].

Unfortunately, little is known about the cellular mechanisms of methanol growth toxicity in natural methylotrophs which is required to engineer synthetic methanotrophs to be less sensitive to high methanol concentration, so that they can be developed into biocatalysts for large-scale conversion of C1 compounds into commodity chemicals and fuels.

Bacillus methanolicus

B. methanolicus is a Gram-positive aerobic halotolerant restrictive methylotroph growing at temperatures between 35 and 60 °C [2]. It is able to grow on a few sugars in addition to methanol [2]. All *B. methanolicus* strains can grow on mannitol at comparable levels with methanol. Although they report data for growth in sucrose [2], there are no published data regarding the growth rate of *B. methanolicus* on glucose to date. Recently, Heggeset et al. [11] suggested that *B. methanolicus* may also be able to grow on glucose. However, mannitol was the choice of sugar when *B. methanolicus* was intended to grow non-methylotrophically. Mannitol was also the choice of sugar as the control when the gene expression of methylotrophic pathways was analyzed and compared to non-methylotrophic gene expression [12] along with a recent genomic and proteomic comparison analysis [19, 20, 33].

Wild-type strains of *B. methanolicus* strains isolated at the University of Minnesota have been reported to secrete 58 g/l of L-glutamate in fed-batch cultures [25], while classical mutants (non-GMO) can secrete 37 g/l of L-lysine [10] at 50 °C. Several strains have also been reported to be adapted to rapid growth in seawater, making them valuable for very large-scale processes for conversion of C1 carbon to chemicals without using freshwater [13]. The 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 [5]. The MDH in *B. methanolicus* is an NAD-dependent decamer protein with 43,000-Mr subunits. Each subunit contains a bound NAD(H), one zinc and one or two Mg ions [3]. It was previously reported that the only gene encoding MDH was on the pBM19 plasmid in strain MGA3 and on pBM20 in strain PB1 [4, 5].

The plasmid, pBM19, also carries genes encoding enzymes of the RuMP pathway (Fig. 1), *glpX, fba, tkt, pfk* and *rpe*, which encode the enzymes fructose-1,6-bisphosphatase (FBPase), fructose-1,6-bisphospate aldolase (FBPA), transketolase, phosphofructokinase and Ru-5-P-3 epimerase, respectively. Although five of the corresponding genes of the RuMP pathway enzymes are carried on the pBM19 along with *mdh,* two additional *mdh* genes and 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 the *hps* and *phi* genes resulted in increased tolerance to higher formaldehyde concentrations in shake flask studies compared to wild-type strains [12].

The dissimilation of carbon from formaldehyde via conversion into formate and then carbon dioxide, which leads to loss of carbon, has been shown by ^{13}C NMR and isotope-ratio mass spectrometry [23, 24]. The presence of formaldehyde and formate dehydrogenase enzymes was shown by enzyme assays of the crude cell extracts [28], but was never shown on the genetic level. Candidate genes were cloned from *B. methanolicus* PB1 using degenerate primers, but the expression of these clones did not confer any formaldehyde or formate dehydrogenase activity *in E. coli* or *B. subtilis* [18]. Furthermore, the expression of plasmid-borne *mdh* in *E. coli* or *B. subtilis* did not yield an active enzyme in the same study [18]. The lack of activity from the expressed MDH raises questions about the validity of the enzyme assays conducted since the enzyme was characterized by others and the activity was shown clearly [3].

There is no study to date that reports the regulation of methylotrophy in *B. methanolicus*, particularly at high initial methanol concentrations. The existing data on the differential expression of genes in *B. methanolicus* is based on the growth of the microorganism on methanol and mannitol [11, 12]. Jacobsen et al. [12] 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. Moreover, the

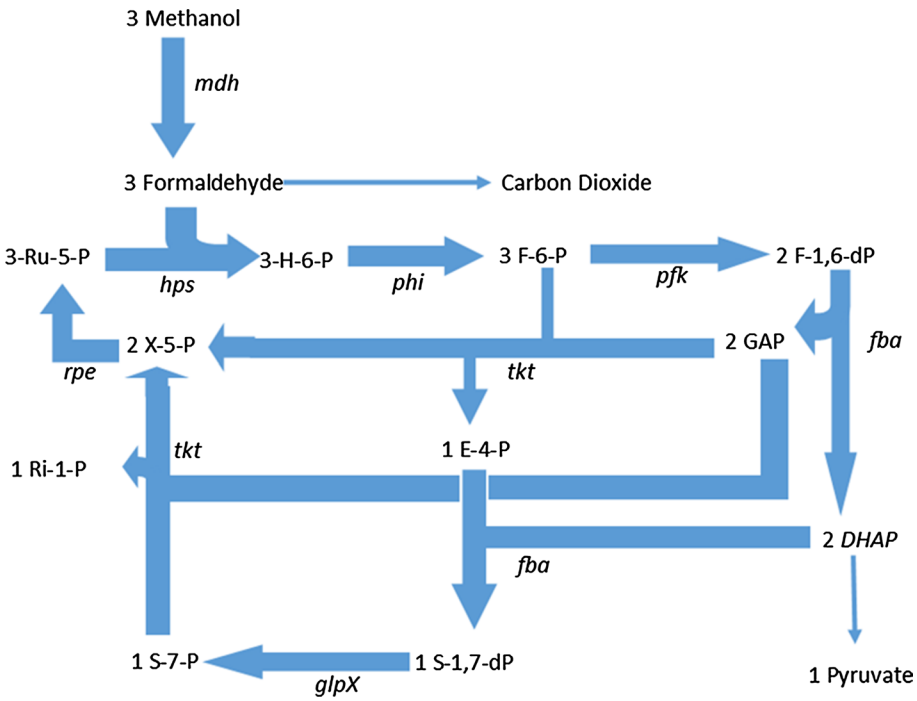

Fig. 1 Genes and metabolites of the RuMP pathway: *hps* hexulose phosphate synthase, *phi* phosphohexuloisomerase, *mdh* methanol dehydrogenase, *pfk* phosphofructokinase, *fba* fructose bisphosphate aldolase, *tkt* transketolase, *glpX* fructose/sedoheptulose bisphosphatase, *rpe* ribulose phosphate epimerase. Metabolites: *H-6-P* hexulose-6-phosphate, *F-6-P* fructose-6-phosphate, *F-1,6-dP* fructose-1,6-bisphosphate, *DHAP* dihydroxy acetone phosphate, *GAP* glyceraldehyde-3-phosphate, *E-4-P* erythrose-4-phosphate, *S-7-P* sedoheptulose-7-phosphate, *S-1,7-dP* sedoheptulose-1,7-bisphosphate, *X-5-P* xylulose-5-phosphate, *Ri-5-P* ribose-5-phosphate, *Ru-5-P* ribulose-5-phosphate. Adapted from [12]

pBM19 plasmid was shown to be lost after prolonged incubation of *B. methanolicus* MGA3 on mannitol [5].

In this study, we report for the first time the maximum specific growth rate (μ_{max}) of *B. methanolicus* MGA3 at previously unreported high initial methanol concentrations along with the growth in glucose. We also show that mannitol-grown *B. methanolicus* MGA3 is more tolerant to formaldehyde, contrary to expectations. Additionally, we show that the pBM19-encoded *mdh* is the gene mainly responsible for the methanol oxidation, although there are two more *mdh* genes on the chromosome. We also report the down-regulation of the methanol dehydrogenase gene of *B. methanolicus* MGA3 in response to the methanol metabolites, formaldehyde and formate. However, the RuMP pathway gene *hps* is also down-regulated in response to formaldehyde and formate in methanol-grown cells, but up-regulated in mannitol-grown cells. The *pfk* is the only gene tested that was up-regulated by formaldehyde and formate in both methanol and mannitol-grown cells.

Materials and methods

Bacterial strain and cultivation

A modified version of the minimal media (MTYM) developed by Dijkhuizen et al. [8] was used as growth media. It contains 10.9 mM $NaH_2PO_4{\cdot}H_2O$ (1.5 g/l), 23.5 mM K_2HPO_4 (4.1 g/l), 15.9 mM $(NH_4)_2SO_4$ (2.1 g/l) and 0.25 g/l of yeast extract (Fisher Scientific), pH 7.0. The trace metal stock solution and vitamin stock solution [8] were added to the final concentration of 1X. The $MTYM_{MeOH}$ medium contained 150 mM of methanol added to the medium, while the $MTYM_{MAN}$ medium contained 55 mM mannitol (10.02 g/l) unless otherwise stated.

B. methanolicus MGA3 growth in methanol, mannitol and glucose

B. methanolicus MGA3 (obtained from the University of Minnesota Biotechnology Institute, St. Paul, MN, USA) was grown in 50 ml of the respective MTYM medium (with methanol, mannitol or glucose) in 250 ml baffled shaker flasks at 50 °C and 270 rpm in a shaker (HT-INFORS) for 10–11 h. Then, enough volume was transferred into pre-warmed respective MTYM media to have an OD_{600}~0.05. The concentrations of methanol in MTYM media ranged between 6 and 2000 mM, while mannitol was tested at 5, 10, 25 and 55 mM concentrations. Glucose was tested at 5, 10 and 20 mM concentrations. The growth was monitored by sampling and measuring the OD_{600} every hour. The specific growth (μ) rate was calculated from the exponential phase of the growth curve by determination of the slope of a semi-log plot of OD_{600} versus time.

Growth response to different methanol metabolites

Three hundred microliters of frozen *B. methanolicus* was inoculated into 50 ml of $MTYM_{MeOH}$ or $MTYM_{MAN}$ medium in 250 ml baffled shaker flasks and grown for 10–11 h. 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 different concentrations when the OD_{600} of the culture was 0.250–0.350. Formaldehyde was prepared fresh from paraformaldehyde as 2 M stock in a serum bottle and autoclaved. Formate was prepared from sodium formate in distilled water as 2 M stock. For the methanol spike in mannitol-grown cells, the culture was spiked with 100 mM methanol when the OD_{600} was ~0.25.

Detection of formaldehyde in the culture

A colorimetric method originally developed by Chrastil and Wilson [7] and modified by Topp and Knowles [30] was used to measure the formaldehyde concentration in the culture. Briefly; 1 ml of sample (culture) 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 and the mixture incubated for 1 h 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 (2 M) in MTYM to have a concentration range of 10–700 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 a final concentration of 2 mM. The formaldehyde concentration in the culture media was measured via the method described above using 1 ml of culture at the time points of 5, 10, 20 and 60 min after formaldehyde addition.

Extraction of total RNA

B. methanolicus MGA3 was grown in either $MTYM_{MeOH}$ or $MTYM_{MAN}$. The OD_{600} of the culture to be spiked was determined experimentally to have sufficient exponentially

Table 1 List of primers used for RT-qPCR experiments

Target gene	Primer	Reference
pBM19 *mdh*	Fw: CAAACGTGTGGCATATTAACTGAGT	This study
	Rv: GGCAGGAGTGGCATTTAACAAC	
Chromosomal *mdh* genes	Fw: TGAAGGTGTCGATGTATCAAAAGAA	This study
	Rv: TTCACTTTGCGTTCAGTATCTGTGA	
pfk	Fw: AAGTGCCATCTCCACCAATC	[12]
	Rv: CCAGGAATGAACGCTGCTAT	
hps	Fw: CCTTGTTGACATGATCGCAGTT	This study
	Rv: AATGGGTTTTTACCTACTGCTTGAA	

growing cells for RNA extraction. The formaldehyde concentration was experimentally determined to have sufficient amount to arrest cell growth for at least 1 h, but not to kill the culture. Cell lysis was monitored by measuring the DNA concentration in the medium. In this regard, a combination of OD_{600} of 0.8 and 2 mM formaldehyde was chosen. A lower cell density would not be sufficient for recovery of a sufficient amount of RNA and a higher cell density would remove formaldehyde from the media faster. Similarly, a lower formaldehyde concentration 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. 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 min after the spike unless otherwise stated. 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 min at room temperature. Then, cells were centrifuged at 10,000*g* for 5 min and the pellet was re-suspended in 0.2 ml TE (10 mM Tris, 1 mM EDTA, pH 8.0) 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.

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 h after spiking with formaldehyde at the above-mentioned conditions. Standards were prepared from Lambda DNA by diluting in MTYM media using dilutions of 5–250 ng/ml. 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. Malachite Green was used to detect sporulation of the cells upon formaldehyde addition. No spore formation was detected for the period of 1 h past formaldehyde addition.

RT-qPCR Standards and primers

Primers used for qPCR (listed in Table 1) were designed using Primer Express 3.0 software 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 s denaturation at 94 °C. A final extension at 72 °C was added at the end of the cycle. The PCR product was run on a 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}–10^5. Five of these serial dilutions were used to obtain a standard curve depending on the sample to be analyzed.

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 min at 50 °C for RT reaction, 15 min at 95 °C for activation of Taq polymerase and then 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C and then dissociation curve from

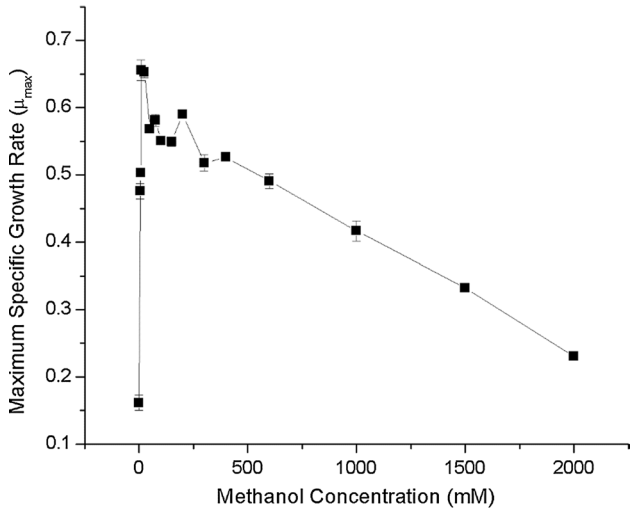

Fig. 2 The maximum specific growth rates of *B. methanolicus* MGA3 in methanol. *B. methanolicus* MGA3 was grown in 50 ml MTYM medium in 250 ml shaker flasks with different substrate concentrations. The μ_{max} was calculated from the exponential phase of growth ($n = 3$)

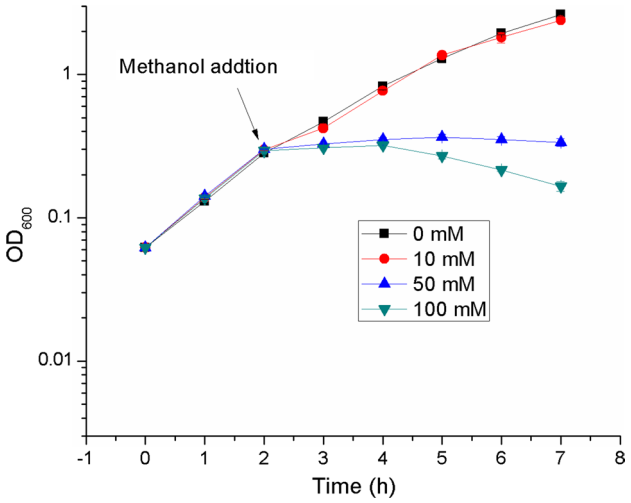

Fig. 3 Response of *B. methanolicus* MGA3 to methanol spikes grown in $MTYM_{MAN}$. *B. methanolicus* MGA3 was grown in $MTYM_{MAN}$ in 250 ml baffled shaker flask and challenged with different concentrations of methanol ($n = 3$) when the cells were growing exponentially (OD_{600}~0.25)

60 °C to 95 °C with 1 °C increment at every 15 s while recording fluorescence. The results were normalized using 16S rRNA.

Determination of specific growth rate

Maximum specific growth rate (μ_{max}) was used to evaluate the effect of methanol, sugars and methanol-derived metabolites on *B. methanolicus* physiology instead of growth extent (maximum cell density achieved, X_{max}), because dissolved methanol is continuously stripped from the culture liquid in proportion to the aeration rate, temperature and mole fraction. The maximum specific growth rate was determined from the exponential phase of triplicate shake flask cultures with different initial substrate concentration by calculating the slope of the curve using a semi-log plot. Then, the maximum specific growth rates were plotted against the substrate concentration (Fig. 2).

Results

Methanol and *B. methanolicus* growth

The maximum specific growth rate of *B. methanolicus* MGA3 grown on different methanol concentrations is shown in Fig. 2. The highest maximum specific growth rate (μ_{max}) was achieved (0.65 ± 0.01 h^{-1}) when *B. methanolicus* MGA3 is grown in the MTYM with 10 mM initial methanol. The maximum specific growth rate decreased when the initial methanol concentration was higher than 25 mM, from 0.65 ± 0.007 h^{-1} at 25 mM to 0.23 ± 0.004 h^{-1} at 2 M. The MTYM medium without any carbon source other than the low level of yeast extract supports growth to OD_{600} of 0.15 ± 0.009 with μ_{max} of 0.16 ± 0.01. It is also noteworthy that *B. methanolicus* MGA3 cultures growing in 2 M methanol (64 g/l) achieved a final OD_{600} of 3.76 ± 0.09.

B. methanolicus growth in glucose and mannitol

Although it was suggested but not reported by Heggeset et al. [11], the maximum specific growth rate of *B. methanolicus* on glucose was determined in this study. The maximum specific growth rate does not change with the glucose concentrations tested, 0.336 ± 0.003 h^{-1}, which is considerably lower than maximum specific growth rate of *B. methanolicus* on methanol and mannitol. The maximum specific growth (μ_{max}) rate of *B. methanolicus* MGA3 in different mannitol concentrations is relatively constant at 0.53 ± 0.002. There is no decrease or increase in the maximum specific growth rate depending on the mannitol concentration in the range tested.

Growth response of *B. methanolicus* MGA3 to methanol additions

Exponentially growing *B. methanolicus* cultures were spiked with different methanol concentrations. *B. methanolicus* MGA3 culture grown in $MTYM_{MeOH}$ and spiked with methanol concentrations of 10, 50 and 100 mM. There is no change in the growth of *B. methanolicus* MGA3

when challenged with the above methanol concentrations. However, a significant growth disturbance was seen with the same methanol concentrations (data not shown) when $MTYM_{MAN}$-grown *B. methanolicus* MGA3 is challenged (Fig. 3).

Growth response of *B. methanolicus* to formaldehyde additions

The exponentially growing culture of *B. methanolicus* was spiked with different concentrations of formaldehyde to investigate the effect of this methanol metabolite on growth. Figure 4a 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, which was apparent by the cell lysis which decreased the OD_{600} of the culture and the release of DNA. However, mannitol-grown *B. methanolicus* MGA3 is more tolerant to formaldehyde. As shown in Fig. 4b, *B. methanolicus* MGA3 is able to tolerate even 2 mM formaldehyde.

Detoxification of formaldehyde by methanol- and mannitol-grown *B. methanolicus* MGA3

A linear curve with the R^2 value of 0.997 was obtained with the formaldehyde range of 10–700 µM. Detoxification (removal) of formaldehyde from the media is shown in Fig. 4. *B. methanolicus* MGA3 detoxifies external formaldehyde faster when grown in $MTYM_{MAN}$. The concentration of formaldehyde falls below detection limit (10 µM in sample) in 60 min 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.

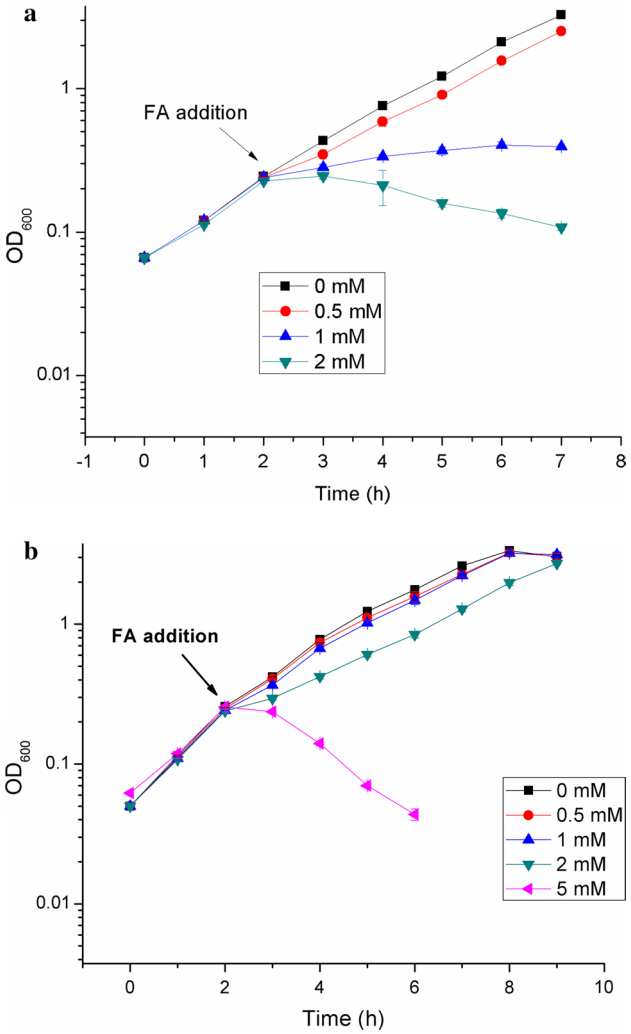

Fig. 4 Response of *B. methanolicus* MGA3 to formaldehyde spikes grown in **a** $MTYM_{MeOH}$ and **b** $MTYM_{MAN}$. *B. methanolicus* MGA3 was grown in a 50 ml $MTYM_{MeOH}$ (**a**) or $MTYM_{MAN}$ (**b**) in 250 ml baffled shaker flask and challenged with different concentrations of formaldehyde ($n = 3$) when the cells were growing exponentially (OD_{600} ~ 0.25)

Methanol dissimilation pathway genes are down-regulated by methanol, formaldehyde and formate

Regulation of methanol oxidation

Figures 6 and 7 show the expression of *mdh* genes in response to methanol addition. As can be seen from Fig. 6a, the expression level of pBM19 *mdh* stayed stable over the course of 60 min after methanol addition when *B. methanolicus* MGA3 was grown in $MTYM_{MeOH}$ media. However, the expression was significantly down-regulated when mannitol-grown *B. methanolicus* MGA3 was exposed to a 100 mM methanol spike, as can be seen in Fig. 6b. The expression level of *mdh* was decreased almost one-log in 20 min. The expression of chromosomal *mdh* genes in response to methanol addition was different from pBM19 *mdh* in methanol-grown *B. methanolicus* MGA3, in that it was down-regulated immediately (until 10 min post-spiking) and then started to recover, although, the expression level after 60 min was still significantly lower than the pre-spiking level (Fig. 7a). The expression of pBM19 *mdh* was 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 Figs. 8 and 9, pBM19 *mdh* was down-regulated in response to a formaldehyde and formate spike, although formaldehyde was more potent in down-regulating the gene. Based on this, we

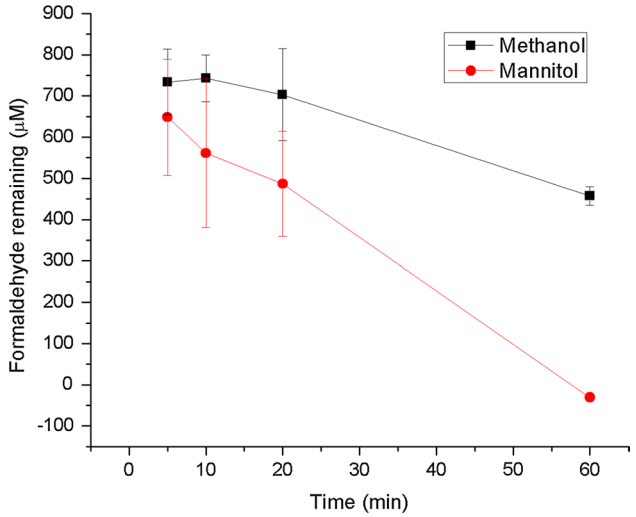

Fig. 5 Remaining formaldehyde in the *B. methanolicus* MGA3 culture media. Formaldehyde (2 mM final) was added to *B. methanolicus* MGA3 growing in $MTYM_{MeOH}$ (methanol) or $MTYM_{MAN}$ (mannitol) at the OD_{600} of 0.8. One milliliter sample was used to measure the formaldehyde concentration in the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

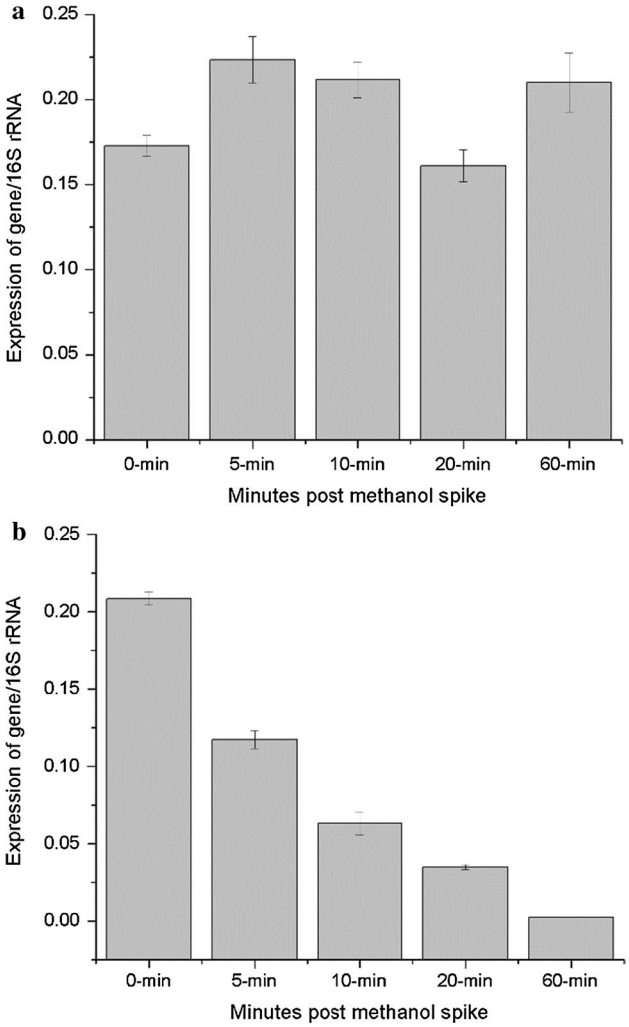

Fig. 6 Regulation of pBM19 *mdh* in response to 100 mM methanol addition to **a** methanol-grown and **b** mannitol-grown *B. methanolicus*. *B. methanolicus* MGA3 was grown in $MTYM_{MeOH}$ or $MTYM_{MAN}$ and spiked with methanol at OD_{600} of ~0.8. Then, the total RNA was extracted as described after 5, 10, 20 and 60 min. The sample "0-min" was taken immediately before the spike ($n = 3$)

suggest that down-regulation of *mdh* in response to methanol spike is due to accumulation of intercellular formaldehyde, since *mdh* is constitutively expressed and RuMP pathway genes are not up-regulated [12] to encounter accumulated formaldehyde.

An important observation from the expression levels of both chromosomal and pBM19 *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 [22]. The expression levels of *mdh* genes (both pBM19 *mdh* and chromosomal *mdh*) are comparable between methanol- and mannitol-grown cells. However, there are clear differences in the 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 and 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 [5].

Regulation of methanol assimilation (RuMP) pathway gene hps

Figure 10a, b summarizes the regulation of *hps* in response to methanol and its metabolites when added to the culture media. 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* (see Fig. 6). The *hps* gene in methanol-grown cells is up-regulated approximately three 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 up-regulation of *hps* reported by Jacobsen et al. [12]. 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 up-regulated when mannitol-grown cells are subjected to methanol, formaldehyde or formate. An almost twofold (2.15×10^{-1} vs 4.17×10^{-1}) up-regulation is evident in Fig. 10b.

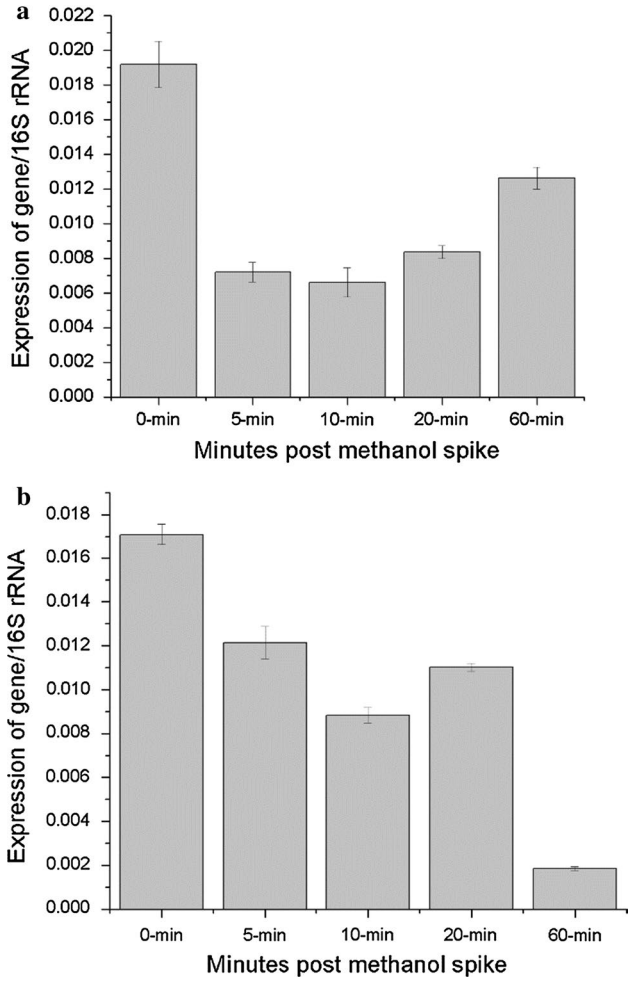

Fig. 7 Regulation of chromosomal *mdh* (ORFs 01267 and 02737) genes in response to 100 mM methanol addition to **a** methanol-grown and **b** mannitol-grown *B. methanolicus*. *B. methanolicus* MGA3 was grown in $MTYM_{MeOH}$ or $MTYM_{MAN}$ and spiked with methanol at OD_{600} of ~0.8. Then, the total RNA was extracted as described after 5, 10, 20 and 60 min. The sample "0-min" was taken immediately before the spike ($n = 3$)

Regulation of pfk

Another investigated RuMP pathway gene 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 11 depicts its regulation in response to formaldehyde. The up-regulation of *pfk* in methanol-grown cells is apparent in Fig. 11 which confirms the up-regulation reported earlier [12], although at a significantly lower level. Despite being encoded on the pBM19 plasmid, the expression level of *pfk* is significantly lower than *hps* when Figs. 10a and 11 are compared. The *pfk* gene is up-regulated in methanol-grown cells compared to mannitol-grown cells, and spiking mannitol-grown cells with formaldehyde resulted in a twofold up-regulation.

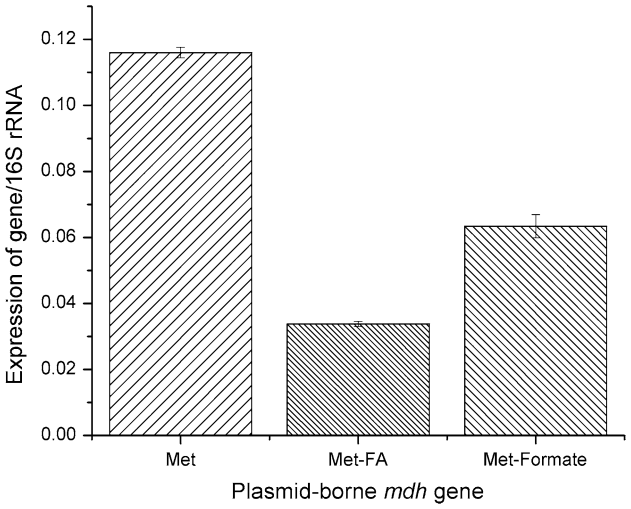

Fig. 8 Regulation of pBM19 *mdh* in response to formaldehyde (2 mM) and formate (2 mM) addition.*B. methanolicus* MGA3 was grown in $MTYM_{MeOH}$ and spiked when the OD_{600} was ~0.8. The total RNA was extracted 20 min post-spiking. *Met* immediately before spike, *Met-FA* spiked with formaldehyde, *Met-Formate* spiked with formate ($n = 3$)

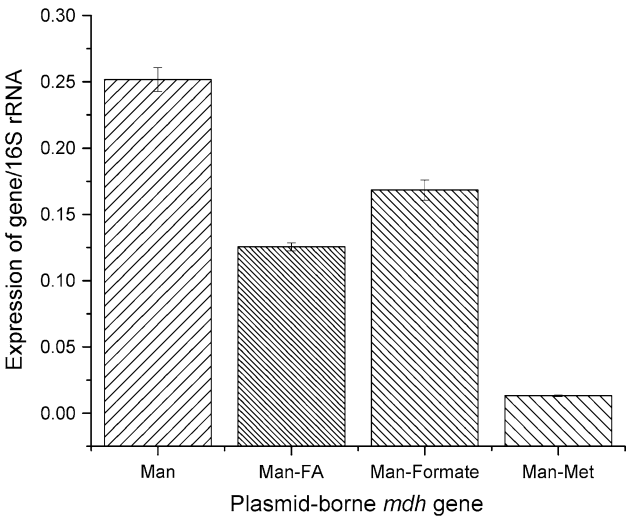

Fig. 9 Regulation of pBM19 *mdh* in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM) addition. *B. methanolicus* MGA3 was grown in $MTYM_{MAN}$ and spiked when the OD_{600} was ~0.8. The total RNA was extracted 20 min post-spiking. *Man* immediately before spike, *Man-FA* spiked with formaldehyde, *Man-Formate* spiked with formate, *Man-Met* spiked with methanol ($n = 3$)

Although small, spiking methanol-grown cells with formaldehyde also caused an up-regulation of *pfk*.

Discussion

The growth of *B. methanolicus* MGA3 on carbohydrates such as glucose and mannitol, in addition to methanol,

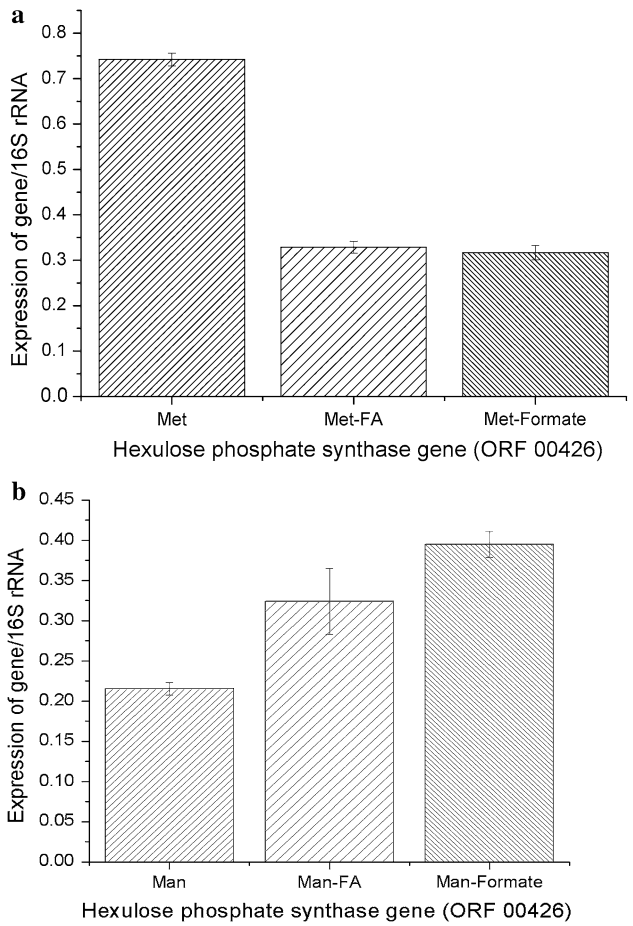

Fig. 10 Regulation of hexulose phosphate synthase (*hps*) in response to formaldehyde (2 mM), formate (2 mM) and methanol (100 mM) spikes in **a** methanol- and **b** mannitol-grown cells. *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the OD_{600} was ~0.8. The total RNA was extracted 20 min 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 ($n = 3$)

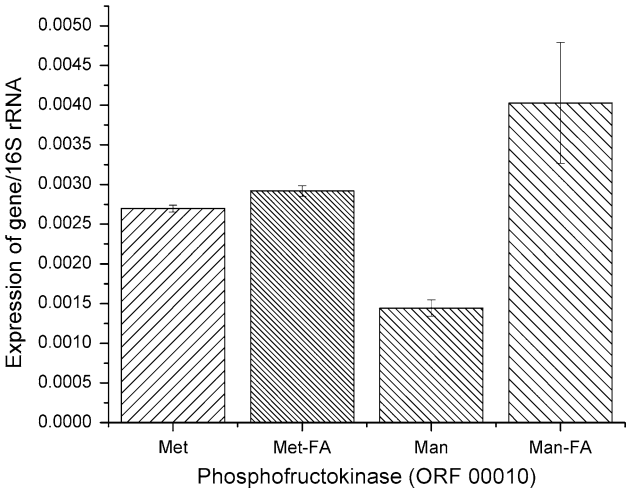

Fig. 11 Regulation of phosphofructokinase (*pfk*) in response to formaldehyde (2 mM) spike. *B. methanolicus* MGA3 was grown in either methanol (Met) or mannitol (Man) and spiked when the OD_{600} was ~0.8. The total RNA was extracted 20 min post-spiking. *Met* immediately before spike, *Met-FA* spiked with formaldehyde, *Man* immediately before spike, *Man-FA* spiked with formaldehyde ($n = 3$)

gives it an important advantage as an industrial platform microorganism for conversion of inexpensive carbon compounds into fuels and chemicals. *B. methanolicus* can utilize C1 or C6 substrates depending on the availability and price. Its ability to grow on 2 M methanol or higher levels also provides it a unique advantage in biomanufacturing commodity fuels and chemicals using C1 carbon sources as few microorganisms are able to grow at high methanol levels. Fed-batch growth of *B. methanolicus* to a dry cell mass of 20 g/l (OD_{600} > 55) using a controlled dissolved methanol level of 100 mM and oxygen-enriched aeration has been achieved in 2- and 14-l bioreactors by our group in both stirred tank and airlift bioreactors [15, 17].

Further investigation of the physiology of growth at methanol concentrations of >2 M and the physiological response to different levels of dissolved methanol during fed-batch culture is warranted. The ability to grow at high methanol concentrations may be due in part to the abundant S-layer proteins found in the cell wall of *B. methanolicus* strains and other thermotolerant bacilli such as *Geobacillus stearothermophilus* [29]. The disadvantage of these S-layers is that they complicate transformation (require very high lysozyme levels to remove), complicate regeneration of protoplasts and therefore frustrate development of genetic tools and pathway engineering these potentially industrially useful hosts.

In this study, we demonstrated that control of growth in methanol without substrate inhibition was challenging due to the possible intracellular accumulation of formaldehyde (FA), 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 ($0.65 \pm 007\ h^{-1}$ at 25 mM to $0.231 \pm 0.004\ h^{-1}$ at 2 M). We have demonstrated that *B. methanolicus* growing on methanol can tolerate higher concentrations of methanol addition when compared with 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 as shown in Fig. 1 it can grow on 2 M methanol with a growth rate of $0.231 \pm 0.004\ h^{-1}$, yet 50 mM methanol was enough to disturb the growth of *B. methanolicus* when grown on mannitol. This is most probably due to the formaldehyde that is transiently accumulated in mannitol-grown cells beyond its

tolerable concentration since (a) the *mdh* genes are constitutively expressed in both methanol- and mannitol-grown cells [22] and (b) RuMP pathway genes that remove the formaldehyde by assimilating are not up-regulated [12]. The methanol dehydrogenases of methanol-grown cells are most probably saturated and addition of methanol to the media does not increase the formaldehyde concentration in the cells.

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, i.e., assimilation and dissimilation pathways are saturated in methanol-grown cells, and further addition of formaldehyde increases the level to toxic levels. However, these metabolic pathways are idle in mannitol-grown cells and can overcome a 2 mM formaldehyde addition to the medium as evidenced by the faster removal of added formaldehyde (Fig. 5).

We have shown the down-regulation of methanol dehydrogenase genes by methanol, formaldehyde and formate using quantitative RT-qPCR. It is interesting to note that all three methanol dehydrogenase genes in mannitol-grown cells are down-regulated by methanol along with formaldehyde and formate. This down-regulation might be a general stress response to formaldehyde; however, down-regulation by formate might be due to feedback inhibition as formate does not inhibit *B. methanolicus* growth [23]. In this regard, formate is more potent as a regulator despite the fact that it does not have an inhibitory effect on the growth rate of *B. methanolicus*. But the RuMP pathway genes, i.e., *hps* and *pfk*, are up-regulated 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 latter is encoded by a plasmid with 10–16 copies per cell. However, *hps* is down-regulated in methanol-grown *B. methanolicus* from its already high levels in response to formaldehyde and formate.

As a candidate industrial platform microorganism, it is important to understand what effects methanol growth toxicity and its metabolites, formaldehyde and formate as well as loss of carbon as carbon dioxide. This is critical for industrial applications of methylotroph bacteria, so that high concentrations of methanol can be fed to achieve high cell density, efficient product production with minimal substrate inhibition of growth rate and simple dissolved methanol control. Dissolved methanol control is needed to avoid localized growth toxicity which occurs in very large bioreactors depending on how and where the methanol is added in fed-batch processes. In this sense, understanding the regulation of intracellular formaldehyde detoxification is especially important, as it poses both a threat and an opportunity for engineering enhanced formaldehyde detoxification pathways without loss of carbon to carbon dioxide. From our study, it is apparent that removal of formaldehyde by rapid assimilation into the RuMP pathway is an efficient way of detoxification. Furthermore, since the expression levels of *mdh* and *hps* are comparable, the bottleneck reaction that might be enhanced by future pathway engineering is most probably *pfk* levels and activity, as its transcription level is very low compared to *mdh* and *hps* genes. Thus, any attempt to increase the yield of products produced by natural aerobic methylotrophs such as *B. methanolicus* should consider the overexpression of *pfk*, after assessment of phosphofructokinase enzymatic activity rate, to avoid methanol toxicity using the RuMP pathway and achieve rapid growth in high methanol concentrations.

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