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Study on the production and species-specificity of methanobactin

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ABSTRACT

Methanobactin is a small peptide that appears to function as an agent for copper sequestration and uptake in methanotrophs. It isn't known whether the ability to produce methanobactin is wide-spread in methanotrophs and whether the methanobactin characteristics are identical or they vary with strain. In this work, methanobactin production was determined by split mineral salts/Cu–CAS plates. The effect of copper concentration on the methanobactin production was investigated. To examine the species-specificity of methanobactin, four kinds of methanotrophic cells grown in copper-free mineral salts medium were washed and resuspended in high copper concentrations medium with or without Methylosinus trichosporium 3011 Mb addition. Methanobactin from Methylosinus trichosporium 3011 can shorten the copper-shock lag phase of all tested methanotrophs upon sudden exposure to elevated copper concentration and can stimulate Methane Monooxygenase activity of all tested methanotrophs in the presence of copper. The results suggested that the production of methanobactin may be a widespread characteristic of methanotrophs and methanotrophs may take delivery of copper from methanobactin released by other species. This result would be beneficial to insight into the diversity of methanobactin structure and what role it may play in interspecies competition or cooperation for copper ion.

Key words: copper; lag phase; methane monooxygenase; methanobactin; methanotrophs

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INTRODUCTION

Methanotrophs are a group of gram-negative eubacteria that utilize methane as the sole energy and carbon source [1]. In the first step of carbon assimilation by methanotrophs, methane is oxidized to methanol by Methane Monooxygenase (MMO). MMO exists in both the iron-containing soluble form (soluble methane monooxygenase, sMMO) and the copper-containing membrane bound particulate form (particulate methane monooxygenase, pMMO) [2].

Methanotrophs require substantial amounts of copper as it is an integral part of the pMMO and stimulates the formation of extensive intracytoplasmic membranes that house pMMO. In addition, in methanotrophs that have both forms of MMO, copper represses the transcription of the sMMO genes while leading to the expression of pMMO [2].

The copper requirement for methanotrophs is higher than that observed in other aerobic bacteria [3]. Taking into consideration the low bioavailability of copper in natural environment and the central role of copper in metabolism of methanotrophs, it has been speculated that methanotrophs must have an effective copper acquisition strategy to collect copper.

A small, copper-binding, siderophore-like molecule called Methanobactin (Mb) appears to fulfill the roles of copper-trafficking molecule [4]. Mb has been identified in the extracellular fractions of both *Methylosinus trichosporium* OB3b [5] and *Methylococcus capsulatus* Bath [6]. In the growth medium with low copper concentration, high concentrations of Mb have been detected. After copper was added to this previous copper-limited culture, the Mb and copper concentrations in the medium decreased and membrane bound copper-Mb complexes were found [7]. These results suggested that Mb should be the extracellular component of a copper uptake system. Mb may not only function in copper uptake, but also as a defense system in case of high copper concentration. A marked effect was observed upon addition of Mb to *Methylosinus trichosporium* OB3b growth medium concomitant with a switch from copper-starved conditions to 10 μM copper. The growth lag was shortened significantly and the cells grew faster [8]. Furthermore, Mb was possibly in direct association with the pMMO and plays a more direct role in pMMO activity [9].

If Mb is indeed a copper-acquisition compound, whether the production of Mb is a universal or wide-spread characteristic of methanotrophs and whether the Mb characteristics are identical or they vary with strain must be determined.

Yoon et al. developed a split nitrate mineral salts/Cu–chrome azurol S (CAS) plate to screen for Mb production. Assay results indicated that *Methylosinus trichosporium* OB3b, *Methylococcus capsulatus* Bath and *Methylomicrobium album* BG8 have the ability to export Mb [10].

Recently, we have described a methanotrophic strain *Methylosinus trichosporium* 3011. Like *Methylosinus trichosporium* OB3b, *M. trichosporium* 3011 is a Type 2 bacterium and can express both pMMO and sMMO. However, unlike *M. trichosporium* OB3b, *M. trichosporium* 3011 has shown to be capable of growing on methanol and also of constitutively expressing MMO, in a manner independent of the growth substrate [11].

To determine how widespread the expression of Mb may be, in this paper, the Mb production of *M. trichosporium* 3011 and other three tested methanotrophs (*M. trichosporium* OB3b, *Methylococcus capsulatus* 3021 and *Methylomonas* sp. GYJ3) were assayed by split mineral salts/Cu–CAS plate. Because *M. trichosporium* 3011 can growing on methanol and has not absolute requirement of copper for pMMO activity, the effect of copper concentration on the Mb production of *M. trichosporium* 3011 was investigated using methane and methanol as Carbon sources.

Progress in the area of detailed structural determination was difficult, primarily due to the difficulty in obtaining Mb preparations that were pure, homogeneous, and stable. To resolve this problem, we seek to understand whether or not Mb produced by one species of methanotroph is able to be utilized by other species as a source of and buffer for copper ions. The results of the specificity experiment would be helpful to assessing the comparability or diversity of Mb from different methanotrophs, especially since it is not known whether the Mb molecules from different methanotrophs have the same chemical composition.

In this paper, to examine the species-specificity of Mb, *M. trichosporium* 3011, *M. trichosporium* OB3b, *M. capsulatus* 3021 and *M.* sp. GYJ3 cells grown in copper-free mineral salts medium were washed and resuspended in high copper concentrations medium with or without *M. trichosporium* 3011 Mb addition. The curve-fitting software in conjunction with a logistic growth model has been used to analyze growth data exactly and reliably.

Previous studies showed that addition of purified Mb to *M. capsulatus* Bath cells could increase the activity of pMMO at whole cell level [6]. Here the effect of addition Mb from *M. trichosporium* 3011 on other methanotrophic pMMO activity has been studied. The results would be beneficial to insight into the diversity of Mb structure and what role it may play in interspecies competition or cooperation for copper ion.

EXPERIMENTAL SECTION

Culture of methanotrophs

Methylosinus trichosporium 3011 and *Methylococcus capsulatus* 3021 were obtained from the Institute of Microbiology and Virology (Kiev. Ukraine) [12]. *Methylomonas sp.* GYJ3 was isolated from soil samples of Yumen oil fields, Gansu Province, China. *Methylomonas* sp. GYJ3 was classified as type I methanotrophs and cultivated as described by Shen *et al.*[13]. *Methylosinus trichosporium* OB3b was kindly provided by Prof. Xinhui Xing of the Tsinghua University. Cultivation was carried out in a 250 ml closed vial with 50 ml minimal salt medium on a shaker at 150 rpm and 28-30 ℃. The minimal salt medium was modified according to Xin *et al.*, [11]. $CuSO_4$•$5H_2O$ was omitted from the medium. Methane was added periodically by establishing a partial vacuum in the flask and backfilling with methane and air (1:10). The gas phase methane level was maintained at about 10%

(V/V) by exchanging the headspace volume three times per day.

Determining of Mb production on split mineral salt/Cu–CAS plates

To determine if methanotrophic cells produce Mb, a split mineral salt/Cu–CAS plate assay developed by Yoon [10] was modified and used. The concentration of phosphate salt in mineral salt medium was reduced by factors of 10 for the Cu–CAS blue agar preparation to avoid the interference of phosphate salt.

To prepare 500ml of 50μM Cu–CAS blue agar, 50 ml of 2mM CAS solution was mixed with 10 ml of a 5mM $CuSO_4$ solution. Under stirring this solution was slowly added to 40 ml of 5mM HDTMA (hexadecyltrimethylammonium). This gives a final copper concentration of 0.5 mM, a CAS concentration of 1mM and a HDTMA concentration of 2mM. The resultant dark blue liquid was autoclaved. Also autoclaved was 450 ml of mineral salt agar. Concentrations of salts were adjusted for 500 ml mineral salt medium, considering later addition of the Cu–CAS solution. After cooling to ~50°C, 50 ml of Cu–CAS solution was carefully added into mineral salt agar medium along the glass wall, with enough agitation to achieve mixing. After the agar plates cooled and solidified, half of the agar gel was carefully excised with a sterilized razor. The empty space was then filled with sterilized mineral salt agar with 1 μM copper. Methanotrophs were streaked onto mineral salt agar with 1 μM copper immediately adjacent to 50 μM Cu–CAS agar; a color change in modified CAS plate was observed as copper ion weakly bound to CAS is taken up by Mb.

Methanotrophs were inoculated on split mineral salt/Cu–CAS plates in a desiccator jar under methane or methanol vapor atmosphere at 28-30 ℃. Methane was added periodically by establishing a partial vacuum in the desiccator and backfilling with methane and air (1:10). The methanol vapor was supplied by adding liquid methanol at the bottom of desiccator jar.

Culture conditions for Mb production

M trichosporium 3011 was cultured in a 5 l Baoxing bioreactor containing 3 l of copper deficient mineral salt medium. Methanol was added to 0.2% (v/v) and supplied every 24h to keep the same concentration. Cells were grown at 28-30℃ and an agitation rate of 250-300 rpm. Ambient air was bubbled through the fermentor continuously at 0.5-0.8 l/min. The cultures were grown to stationary phase for Mb production.

Isolation of Mb

Mb from the spent medium of *M. trichosporium* 3011 was isolated as previously described for *M. capsulatus* Bath by Choi *et al.* [6]. The cells were removed by centrifugation at 10000 g for 30 min. The supernate was loaded onto a 2.5×20 cm Diaion HP-20 column (Mitsubishi Chemical Holdings, Japan). The bound Mb was washed with two column volumes of H_2O and eluted with 30% methanol:60% H_2O. The eluant was lyophilized for concentration and storage. The freeze-dried samples following chromatography on Diaion HP-20 columns were the source of Mb used in this study. Typically, 5 to 20 mg of Mb is isolated per liter of spent medium with copper-limited cultures. Freeze-dried samples were stored under moisture-free conditions at -20℃ until enough Mb was collected for the entire experimental program.

Purity of Mb samples was checked at this stage by HPLC equipped with multiwavelength UV-vis detector by using a XDB C-18 (15cm×4.6mm) reverse-phase column at a flow rate of 1.0 mL/min, with 10 mM sodium phosphate buffer, pH 6.6 (solvent A) and acetonitrile (solvent B) as the mobile phase. A linear gradient consisting of an initial solvent B concentration of 20% following injection to 40% solvent B at 20 min and 100% at 55 min was used in this analysis step. The sample injection volume was 10μl.

Quantitative measurements of Mb production by colorimetric assays

CAS assay solution was prepared as following procedure: A 4ml volume of 0.02mM HDTMA solution was placed in a 10 ml volumetric flask and diluted with dH_2O. A mixture of 1ml Cu solution (0.02mM $CuSO_4$•$5H_2O$) and 2 ml 0.02mM aqueous CAS solution was slowly added under stirring. The volumetric flask was then filled with dH_2O to afford 10 ml of CAS assay solution. The solutions were stored in the dark.

Phosphate in the growth media interfere determination of Mb and have to be removed. A 25-ml volume of supernate containing Mb was loaded onto a 0.2×5 cm Diaion HP-20 column (Mitsubishi Chemical Holdings, Japan). The bound Mb was washed with two column volumes of H_2O and eluted with 30% methanol:60% H_2O. The eluant was placed in a 25 ml volumetric flask and then filled with d H_2O to afford 25 ml. A 1.0-ml Mb eluant was mixed with 9.0 ml-CAS assay solution. A reference control was prepared exactly using the uninoculated medium by the same procedure. After reaching equilibrium the absorbance was measured at 605 nm. All samples were measured in triplicate.

sMMO and pMMO enzyme activity assays

The activity of sMMO was evaluated by naphthalene assays described by Brusseau *et al*. [14] and Chu *et al*. [15]. sMMO can oxidize naphthalene while pMMO cannot. When no sMMO activity in methanotrophs was detected by naphthalene oxidation, pMMO activity can be determined by the epoxidation of propylene.

Bacteria were harvested by centrifugation at 9,000×g for 10 min at 4 °C. The cell pellets were washed twice with cold 20 mM phosphate buffer (pH 7.0). Cells were resuspended in the same buffer containing 5 mM $MgCl_2$ (at a cell concentration of 2–3 mg dry weight of cell [dwc]/ml) and used for the experiment. The sample solution (1.0 ml) was added to 10mM sodium phosphate buffer containing 1mM $MgCl_2$. The flask (10 ml) was sealed with a Teflon-sealed septum and then incubated for 5 min at 30℃. The reaction was initiated by injecting 2.5 ml propene into the flask using a gas-tight syringe. Epoxypropane formation from propene was determined chromatographically using a gas chromatograph equipped with a capillary GC column (0.23mm×30 m; stationary phase, SE-54) and a flame ionization detector (FID).

UV–visible absorption spectroscopy UV–visible absorption spectroscopy was performed by using a Shimadzu UV-2550 spectrophotometer. All samples were protected from ambient light to prevent possible photo-oxidation.

Effect of Mb on the Growth of Methanotrophs All strains were subcultured three times at copper-free conditions to OD_{600} of 0.5 to 1.0. After the third subculturing, the cultures were washed with mineral salt medium containing 30μM Copper and then transferred to fresh mineral salt medium containing 30μM Copper at a starting OD of ~0.1 to avoid the decline of copper concentration of the medium by inoculum. To assess the influence of Mb on the growth and toxicity reduction in methanotrophic bacteria, 10mg of purified Mb were provided to a series of 100 ml cultures before incubation and growth. The OD was measured periodically with spectrophotometer until the stationary phase. All measurements were performed in biological triplicates, and the resulting standard deviations are computed and reported.

RESULTS

Detection of Mb Production by split mineral salt/Cu–CAS plates Detection of Mb production by streaking methanotrophs directly onto 50 μM Cu–CAS mineral salt plate was unsuccessful duo to the toxicity of HDTMA to methanotrophs. So Mb production was determined by the methodology developed by Yoon *et al.*[10]. Methanotrophs were streaked onto mineral salt agar with 1 μM copper immediately adjacent to 50 μM Cu–CAS agar as previously described. It has been found that phosphates may act as interfering chelates and the interference of phosphates in Cu–CAS mineral salt agar solution was obvious. For phosphates with lower copper affinity, this interference is observed only at higher concentrations. Thus, the concentration of phosphates in mineral salt medium was reduced by factors of 10 for the Cu–CAS blue agar preparation. This lower concentration added as phosphorus source did not interfere.

As shown in Figure 1, all tested strains showed significant change in the coloration

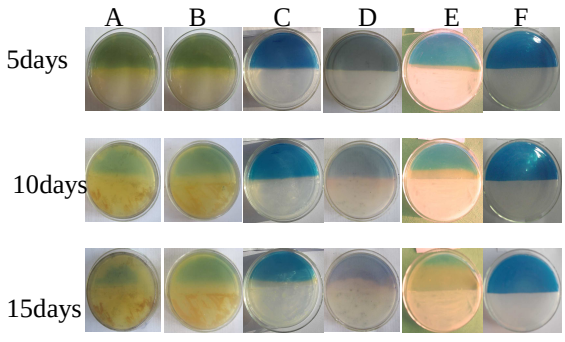

Fig.1: Split mineral salt/Cu–CAS plates for detection of Mb production over time by methanotrophs

A. *M. trichosporium* 3011 grown on methane; B. *M. capsulatus* 3021 grown on methane; C. *M. trichosporium* OB3b grown on methane; D. *Methylomonas* sp. GYJ3 grown on methane; E. *M. trichosporium* 3011 grown on methanol; F. Control of the Cu–CAS agar within 15 days (from blue to light yellow). For all tested strains, the color change in Cu–CAS agar increased over time, which revealed that all the tested strains secreted Mb. It is implied that the ability to produce Mb may be wide-spread in methanotrophs.

Because *M. trichosporium* 3011 was capable of growing on methanol and of constitutively expressing pMMO, the production of Mb during growth of *M. trichosporium* 3011 on methanol was also determined by split mineral salts/Cu–CAS plate. Interestingly, the results showed that *M. trichosporium* 3011 grown on methanol reacted in different manners to the split mineral salts/Cu–CAS plate assay and the change in the coloration of the Cu–CAS agar occurred earlier and faster. *M. trichosporium* 3011 grown on methane showed significant change in the coloration of the Cu–CAS agar within 15 days, while plates incubated with *M. trichosporium* 3011 grown on methanol showed a similar color change within 10 days.

UV-Visible Absorption Spectra of Mb from *Methylosinus trichosporium* 3011

The UV-visible absorption spectroscopy of Mb from *M. trichosporium* 3011 was shown in Figure 2. UV-visible absorption spectra in the 200-600 nm range of Mb showed absorption peak at 403 nm. The addition of copper ion caused the change of UV-visible spectrum at 394 nm and 282 nm. which were consistent with previous studies using Mb from *M. trichosporium* OB3b [8]. On the basis of the revised structure of Mb [16], Cu binding is associated with

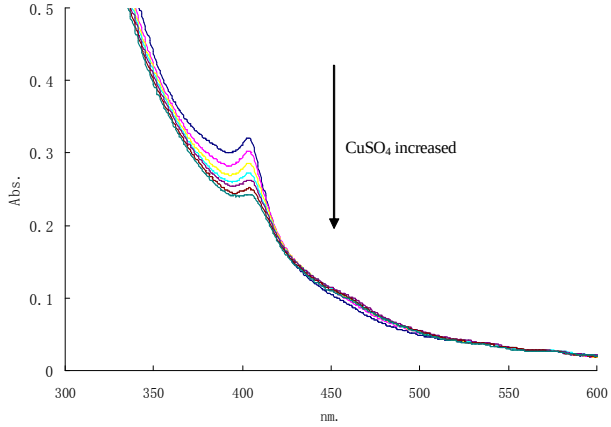

Fig.2: UV-vis absorption spectra of Mb and Cu-Mb

Cu absorption spectra were measured after 5 mg of preparation Mb was dissolved into 50 ml of 0~30μM $CuSO_4$ solution

two alkylidene oxazolone rings, each associated with an enethiol group, that together create a site for copper ion binding. The absorption peak at 403 nm can be assigned to the alkylidene oxazolone ring which has a longer conjugated system. It has been reported that the two mercapto-oxozolone groups of Mb are involved in copper-binding [17]. Consistent with this report, the selective decreased absorption at 403 nm have been observed with copper binding. These results suggested that Mb from *M. trichosporium* 3011 have affinity for copper ion.

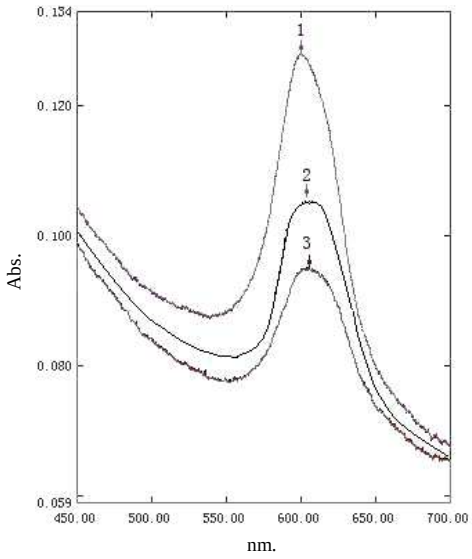

Fig 3: The absorbance spectra of the CAS reagent

1. Cu:0.2μM; CAS: 0.4 μM; HDTMA:0.8μM; 2. Cu:0.2μM; CAS: 0.4 μM; HDTMA:0.8μM; EDTA: 0.05 μM; 3. Cu:0.2μM; CAS: 0.4 μM; HDTMA:0.8μM Mb sample: 10μg/ml

Quantitative measurements of Mb by calorimetric assays

The quantitative measurement of Mb in supernatant solutions by calorimetric assays is very useful. The agar method developed by Yoon *et al*.[10] can be easily used for screening purposes because the dye is incorporated into the agar medium and strains producing Mb can be detected by color-change in the Cu-CAS blue agar section of split mineral salt/Cu–CAS plates. However, quantitative measurements of Mb production on the agar test are not possible. It has been reported that CAS was used to determine serum iron by calorimetric assays and the method has an extremely high sensitivity [18]. Here we developed a method to determine Mb quantitatively by using its high affinity for copper.

As shown in Figure 3, the absorbance spectra of the CAS reagent clearly showed an absorbance peaks at 605 nm. When a strong chelator (Mb or EDTA) is added to a highly colored Cu-CAS-HDTMA complex, the release of the copper is accompanied by a 605 nm absorbance peak decrease.

Using the ternary complex Cu-CAS-HDTMA as an indicator, both Mb and EDTA exhibit a linear dependence of the absorbance at 605 nm versus concentration of the chelator (data not show). So the absorbance wavelength of 605 nm was chosen as the measurement point for detection of Mb in solution.

Effect of Copper concentration on the Mb production of *M. trichosporium* 3011

In solutions probably the most important application is the qualitative check for the concentration of Mb by calorimetric assays. This quantitative method allowed us to study the dependence of Mb excretion on the concentration of copper. It is postulated that Mb is excreted by the cells and then reinternalized again after combination with copper. Copper concentrations in medium solutions influence the pMMO activity expression and the extracellular Mb concentration.

Previous studies on *Methylosinus trichosporium* OB3b demonstrated that a limitation of copper may trigger extracellular accumulation of Mb by this bacterium. High concentrations of Mb could be detected only in growth conditions with low copper concentration in the nutrient solution [8, 19]. Laboratory experiments also shown that a significant dependence of the growth of methanotrophic microorganisms on copper.

M. trichosporium 3011 can growing on methanol and has not absolute requirement of copper for pMMO activity. In order to investigate the effect of copper concentration on the Mb production, *M. trichosporium* 3011 was cultivated in mineral salt medium with methane or methanol as carbon sources. It was of interest to measure the extracellular Mb concentrations of batchcultured cells in medium containing varying copper concentration.

The relationship between the ratio of extracellular Mb concentration to the cell density and the copper concentrations for methane-growth *M. trichosporium* 3011 was examined firstly. The concentration of Mb was monitored as absorbance change at 605 nm ($\Delta Abs._{605nm}$) result from 1.0-ml Mb eluant. Both cell density and extracellular copper concentration may influence accumulation of Mb. The first step in the conversion of methane into biomass is catalyzed by methane monooxygenase (MMO). The form and catalytic activity of MMO can be controlled by copper (Balasubramanian and Rosenzweig 2008). The copper demanding enzyme pMMO is not expressed if copper is absent and this decreases the growth rate of methanotroph itself. As shown in Figure 4, a corresponding decrease in the ratio of extracellular Mb concentration to the cell density was found. The reason for this may be an excess of copper accelerate the uptake of Cu-Mb and prevent the accumulation of extracellular Mb. These data are in agreement with previous reports that a copper limitation triggers the accumulation of Mb in methane-grown bacteria [8]. Consistent with methane-growth *M. trichosporium* 3011, the highest ratio of extracellular Mb to the cell density in methanol-growth *M. trichosporium* 3011 was also observed in copper-free growth media. Higher copper concentrations depress the extracellular Mb accumulation. It is noteworthy that the Mb production ability of methanol-grown cells was obviously higher than that of methane-grown cells. For methanotrophic bacteria, methanol can produce more reductive energy (NADH) than that of methane [11]. It has been speculated that methanol might act as the electron-donating substrate to regenerate the NADH and drive Mb synthesis.

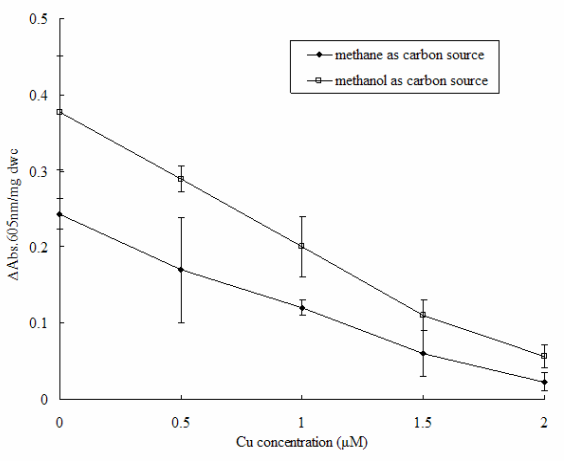

Fig 4: Effect of copper concentration on the Mb production of *M. trichosporium* 3011

Effect of Mb on the Growth of Methanotrophs

The lag phase duration and the maximum growth rate can be used to characterize the bacterial growth curve. Previous research has shown that Mb from *M. trichosporium* OB3b suppressed the "copper-shock" lag phase in *M. trichosporium* OB3b upon sudden exposure to elevated copper [8]. Therefore, the species-specificity of Mb can be assessed by providing one strain's Mb to other methanotroph cultures grown under copper limited conditions that were rapidly shifted to high copper concentrations. Here, *M. trichosporium* 3011, *M.s trichosporium* OB3b, *M. capsulatus* 3021 and *M.* sp. GYJ3 cells grown in copper-free mineral salt medium were washed and resuspended in high copper concentrations (30μM) medium with or without Mb from *M. trichosporium* 3011 addition. Some main growth parameters such as length of lag phase, maximal growth rate and maximal OD_{600} were analyzed to assess whether Mb suppressed the copper-shock lag and increased the growth rate upon sudden exposure to elevated copper. In order to assess growth parameters exactly and reliably, the growth data were fitted to the Logistic models using MATLAB 7.1. The parameters fitted were lag phase and maximal growth rate. Lag phase was quantified as the time obtained by extrapolating the tangent at the exponential part of the growth curve, back to the inoculum level. This definition is nowadays most widespread. As shown in Figure 5, *M. trichosporium* 3011 cultures that were switched from copper-free conditions to 30.0 μM copper without Mb exhibited much extended growth lag; however, the growth lag was reduced from 31 to 2 h as 10 mg Mb was provided. Mb addition also stimulated growth as indicated by increased growth rates and OD levels.

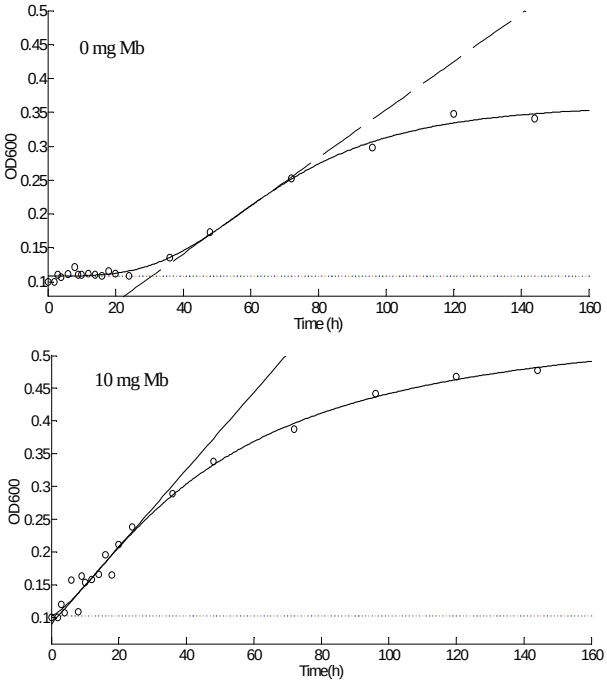

Fig5: Effects of Mb on the growth of *M. trichosporium* 3011 in mineral salts medium containing 30 μM copper

Table 1 showed that Mb from *M. trichosporium* 3011 also enhanced growth and reduced lags in other methanotrophic cultures grown under elevated copper conditions. Mb produced by strain *M. trichosporium* 3011 was readily utilized by other growing methanotrophic culture and support copper uptake by other methanotrophs. The results presented here suggested that Mb may be functionally same or similar in different methanotroph species. Methanotrophs may take delivery of copper from Mb released by other species without species-specific recognition.

Tab. 1 Effects of methanobactin on various growth parameters on methanotrophs in mineral salt medium containing 30 μM copper

Strain	3011 Mb addition amount (mg/100 mL)	Lag phase (h)	Maximal specific growth rate $\times 10^{-3}$ (h^{-1})	Doubling time (h)	OD_{600} max
3011	0	31.0 ± 1.2	16.2 ± 0.9	42.8 ± 2.5	0.34 ± 0.02
	10	2.3 ± 0.8	41.5 ± 2.9	16.7 ± 1.2	0.48 ± 0.03
3021	0	51.1 ± 1.5	17.8 ± 0.4	38.9 ± 0.8	0.28 ± 0.03
	10	3.0 ± 0.4	32.9 ± 2.8	21.1 ± 1.8	0.50 ± 0.01
OB3b	0	41.7 ± 0.2	20.9 ± 1.0	33.1 ± 1.5	0.27 ± 0.01
	10	4.9 ± 0.5	28.4 ± 1.2	24.4 ± 1.0	0.52 ± 0.02
GYJ3	0	34.8 ± 0.4	13.0 ± 0.1	52.1 ± 0.4	0.30 ± 0.01
	10	3.6 ± 0.7	14.5 ± 0.5	47.9 ± 1.5	0.44 ± 0.05

Effects of Mb on pMMO activity

Previous studies have shown that Mb usually stimulates pMMO activity in whole-cell by the form of Cu-Mb and suggested that the stimulation is due to an increased rate of electron flow to the Cu(II) centers of the pMMO [6]. In this study, by using Mb from *M. trichosporium* 3011, the effects of Mb on pMMO activity were examined in the whole-cell samples from *M. trichosporium* 3011, *M. trichosporium* OB3b, *M. capsulatus* 3021 and *Methylomonas.* sp. GYJ3, respectively. The cells used were cultured in medium with a copper concentration of 16μM for higher expression levels of pMMO. Whole-cell sMMO enzyme activity was measured by monitoring the production of naphthol from naphthalene. No sMMO activity was observed. The initial propylene-oxidation activities were in the range of 3.8-10.3 nmol/min·mg dwc, before copper or Cu-Mb additions. By using *M. trichosporium* 3011 cell with propylene-oxidation activities in the 7.6 nmol/min·mg dwc range, activities approaching 8.9 nmol/min·mg dwc were observed at optimal copper addition. The stimulation of pMMO activity by copper can also be found in other methanotrophic whole-cell samples. An optimal copper concentration was always observed, followed by a decrease in pMMO activity as the copper concentration was increased further. The stimulation of pMMO activity by Mb alone can not be found in whole-cell samples. Mb samples were mixed with copper and incubated for a minimum of 5 min before addition to the whole-cell samples. The stimulation of pMMO activity by copper in the presence of Mb was greater than that observed in the absence of Mb. The effects of Mb under different copper concentration on pMMO activity in *M. trichosporium* 3011, *M. trichosporium* OB3b, 3021 and *Methylomonas.* sp. GYJ3 whole cells were studied. Addition of Mb from 3011 to other methanotrophic cells increased pMMO activity by 46-55% as compared with 17-28 % by adding copper alone. The increase in pMMO activity following Mb addition indicated that Mb produced by *M. trichosporium* 3011 may be functionally indistinguishable to *M. trichosporium* OB3b, *M. capsulatus* 3021 and *Methylomonas.* sp.GYJ3 cell.

DISCUSSION

Mb may function in copper acquisition, protection against copper toxicity, and pMMO activity. Although the existence of Mb has been approved in laboratory experiments and the structure of Mb isolated from *M. trichorsporium* OB3b has been published by Kim *et al.* [5], relatively little is known about whether the production of Mb is a universal characteristic of methanotrophs and whether the Mb characteristics are identical or they vary with strain. In this study, Split mineral salts/ Cu–CAS plates were used for detection of Mb production. The discovery of Mb in all of the four tested methanotrophs suggests that this may be a widespread if not universal mechanism used by methanotrophs for copper sequestration. We recommend that Mb production should be characterized using a broader range of methanotrophs. The methodology developed by Yoon *et al.*[10] can only be carried out in solid medium. However, but only in liquid solution it can be used for estimating the quantity of Mb present. Here the quantitative determination of Mb from culture supernatant by the colorimetric assays method has been used for studying the effect of copper concentration on the Mb production of *M. trichosporium* 3011. Phosphate in the supernatant interfere determination of Mb and Mb must be separated from phosphates-containing supernatant by Diaion HP-20. Thus, the colorimetric assays method is still arduous and expensive and should be further improved. Mb from *M. trichosporium* 3011 stimulated growth of all the culture of methanotrophs at a sudden elevation of copper concentration. Moreover, Mb from *M. trichosporium* 3011 stimulated all four methanotrophs pMMO activity in the presence of copper. These data suggested that methanotrophic cell may take delivery of copper from Mb released by other species and Mb may be structurally same or similar in different methanotroph species. The results would be helpful to estimate the diversity of Mb structure, since it is not known whether the Mb

molecules from the different methanotrophs have the same chemical composition. The results could also help determine the role Mb may play in interspecies competition or cooperation for copper uptake.

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