A Study on an Interaction of Copper-Binding Compounds Between Methanotrophs

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1. Introduction

Methanotrophs have been known as gram-negative and aerobic bacteria and they use only methane for their carbon and energy source [3]. The initial oxidation of methane to methanol is catalyzed by methane monooxygenase (MMO) that can be expressed differently depending on the environmental factors. The most well known factor is copper concentration by which two different MMOs can be expressed: a soluble cytoplasmic MMO (sMMO) and a membrane-associated, or particulate, MMO (pMMO) [1,3,4,6]. Under low ratio of copper to biomass (≤ 0.9 nmol of Cu/mg of cell protein), the sMMO is expressed; at higher value, the pMMO is [6]. That is mainly because pMMO is a copper-based enzyme [4].

While copper plays an important role in the physiology of methanotrophs, the mechanism of copper uptake system by methanotrophs is still unclear [2]. However, sMMO's mutant of *Methylosinus trichosporium* OB3b [5, 7] which can express either sMMO or pMMO depending on copper concentration, suggested the presence of an extracellular copper-binding compound (CBC). The CBC was further isolated from *M. capsulatus* Bath and was shown to be small polypeptides with a molecular mass of 1,232 Da [5, 8, 9]. The two strains, *M. capsulatus* Bath and *M. trichosporium* OB3b, have been known to make CBC, but other methanotrophs have never been reported to make CBC. Since methanotrophs such as *Methylomicrobium album* BG8 and *Methylocystis parvus* OBBP need copper to express pMMO for the oxidation of methane and appear not to possess CBC, they either have a different copper uptake mechanism or have to utilize the CBC made by other methanotrophs.

This independent study was performed to achieve two goals. The first one was to determine if there was an interaction among methanotrophs for copper uptake; i.e. CBC, the sole copper uptake mechanism. To do so, three methanotroph strains were chosen, *M. trichosporium* OB3b, *M. album* BG8, and *M. parvus* OBBP to compare their growth trends in response to the presence of CBC in the growth media. The second goal of this study was to acquire the basic knowledge and technology of molecular analysis. Hence,

the characteristics of the bacterial strains were investigated using naphthalene assay for a verification of sMMO expression, trichloroethylene (TCE) degradation, polymerase chain reaction (PCR), gel electrophoresis (GE), and capillary electrophoresis (CE).

2. Materials and Methods

2-1 Characterization of two methanotrophs

Organisms and growth conditions

M. trichosporium OB3b, *M. album* BG8, and *M. parvus* OBBP were grown at 30℃ on the agar plates of nitrate mineral salts (NMS) medium with the presence of 10 μM copper as $Cu(NO_3)_2 \cdot 5H_2O$ under a methane-air mixture (1:2 ratio) [10] and cells were transferred to a fresh NMS liquid medium. The liquid culture medium of NMS did not exceed 15 % of the total flask volume to prevent mass transfer limitations of methane and oxygen from the headspace to liquid medium [10].

Naphthalene assay

Whole-cell sMMO activity of *M. trichosporium* OB3b was examined using the colorimetric naphthalene assay of Brusseau et al. [11]. Since only sMMO can oxidize naphthalene to 1- or 2- naphthol, it could be determined whether or not *M. trichosporium* OB3b expressed sMMO rather than pMMO depending on copper concentration by adding tetrazotized *o*-dianisidine to form a purple naphthol diazo complex [12].

NMS media with different copper concentrations, either 0 μM or 20 μM, were prepared to evaluate sMMO expression. As *M. trichosporium* OB3b can express either sMMO or pMMO, all flasks were acid-washed in 2 N HNO_3 for 2 days and 20 μM copper was added aseptically as $Cu(NO_3)_2 \cdot 5H_2O$. Cells were grown until they were in an exponential growth phase (optical density (OD) between 0.2 and 0.5) and then 2 ml of the liquid culture was aseptically transferred to 20 ml vials. Naphthalene was then added to the cell-transferred vials, sealed and incubated at 30℃, 270 rpm for 1 hour.

35 µl of 5 N NaOH was added to the samples to disrupt cell activities. 1.5 ml of the samples were taken and centrifuged at 12,000×g for 5 min. Lastly 130 µl of 4.21mM tetrazotized *o*-dianisidine was added to 1.3 ml of the supernatant for the absorbance measurement at 528 nm using Milton Roy Company Spectronic 20. Duplicate samples were measured.

TCE degradation measurement

Trichloroethylene (TCE) degradation assay was performed with *M. trichosporium* OB3b given with either 0 µM or 20 µM copper as $Cu(NO_3)_2 \cdot 5H_2O$ to evaluate the ability of sMMO and pMMO-expressing cell to degrade chlorinated solvents. Stock liquid culture was prepared as described earlier and methane was removed from the stock culture flask by evacuating the flask and reequilibrating with air performing seven cycles [13]. 3 ml of the stock culture was aseptically transferred to 20 ml vials with Teflon-coated rubber butyl stoppers and aluminum crimp caps, and sealed [13]. TCE concentrations for standard calibration curve varied from 0 to 33 µM in aqueous phase. 9.8 µM of TCE in the aqueous phase of 20 ml vials was added to evaluate the ability of *M. trichosporium* OB3b to degrade TCE. In addition, the role of formate was investigated adding 20 mM of formate in the form of sodium formate to the samples. By using a dimensionless Henry's constant of TCE as 0.42, the partitioning amount of TCE between the liquid space and the headspace was calculated [13]. The 20 ml vials containing cells and certain amount of TCE were incubated at 30℃, 270 rpm for 6 hours. Control samples for monitoring any abiotic losses were treated with 50 µl of 5 N NaOH to lyse the cells [13]. TCE analysis was performed using an Hewlett Packard 5890 Series II gas chromatograph with an FID detector and the temperature of the injector, oven, and detector were 250, 120, and 250℃, respectively. Triplicate samples were prepared and analyzed in the experiment. All gas phase concentrations were calculated by the standard curve equation.

***M. album* BG8 purification**

Since *M. album* BG8 has been contaminated, it had to be purified before the

experiment. The purification was verified using nutrient agar evaluation, and polymerase chain reaction (PCR) and gel electrophoresis. The contaminated cells were diluted 10, 100, and 1,000 times and then spread on NMS agar medium plates to pick up some colonies of *M. album* BG8-like cells, all of which were aseptically transferred to other NMS agar medium plates and nutrient agar medium plates at the same time and the contamination was observed for more than a week. By performing the procedure repeatedly, cells that could grow on a NMS agar medium but not on a nutrient agar medium were finally selected as potential *M. album* BG8.

Microscopy observation

A microscope was used to verify the rod shape of *M. album* BG8. A simple staining was conducted using methylene blue. A small amount of cells was placed in a drop of Milli Q water on a glass slide and fixed by heating the glass slide. The heat fixed smear was covered by 1 % of methylene blue for approximately 1 min, and the excess stain was washed off. The stained cells were observed using a microscope (Olympus Tokyo Model E 324059).

PCR and gel electrophoresis

PCR and gel electrophoresis were conducted to verify if the purified *M. album* BG8-supposed cells possessed *pmoA* gene but *mmoX* gene. For positive controls and a negative control, both *M. trichosporium* OB3b and *M. capsulatus* Bath, and *Escherichia coli* were used, respectively. DNA samples of the bacteria were prepared using a freezing-thawing method and a beadbeater-using method, both of which were developed earlier [14] were performed and the effectiveness of the methods were compared each other.

In the freezing-thawing method, approximately ten loopful-amount of cells in 1 ml of TE buffer that played a role in repressing the activity of DNA degrading enzyme were frozen at -70℃ for 30 min, boiled for 10 min, and mixed strongly for 3 min using a vortexing device. This cycle was repeated 3 times and the samples were finally centrifuged at 13,000 rpm for 5 min. The supernatants were taken as whole-cell

DNA samples. In the other method using a beadbeater, 0.1 mm glass beads were filled half of 2 ml Ependoff tube and TE buffer was also filled two-third of the tube. The same amount of cells used in the freezing-thawing method was placed in the tube, shaken at 5,000 rpm for 30 sec, and cooled in ice water for 1 min. Total 6 cycles of the procedure were conducted and the samples were finally centrifuged at 13,000 rpm for 5 min. The supernatants were taken as whole-cell DNA samples. The recipe of PCR is shown in Table 1 and the condition of temperature control is indicated in Table 2. Thermocycle device made by a company automatically performed the temperature cycles and it kept the samples at 4 °C after finishing the thermocycles.

After completing the PCR, 5 µl of sample loading buffer made of 20 % of glycerol and bromophenol blue was added to each sample and DNA molecular weight marker (DNA molecular weight marker VIII, 19~1114 bp, Roche) which was prepared with 20 µl of Milli Q water and 5 µl of the marker. All samples were briefly centrifuged to be mixed and loaded into 1.8 % of agarose gel with TAE electrophoresis buffer (Tris-acetate-EDTA made of 0.04 M Tris-acetate and 2 mM EDTA, pH 8) and 0.5 ppm of ethidium bromide which could bind very tightly to DNA molecules and form a strong fluorescent complex resulting in being visualized by exposure to UV light. Current was applied as 125 volts for 45 min. The separated PCR products in the agarose gel were exposed to a strong UV light using UV device.

Capillary electrophoresis (CE)

Gel electrophoresis is able to show clear bands of PCR products under UV light but it usually requires a relatively long time to see the results of PCR. However, much more precise and rapid results of PCR products can be obtained using capillary electrophoresis (CE).

P/ACE^{TM} MDQ capillary electrophoresis system from Beckman Coulter was used under the condition of the reverse polarity mode with either 4 kV or 6 kV applied voltage. The CE separation buffer was prepared as described earlier in Han and Semrau [10]; 50 mM HEPES sodium salt (N-2-hydroxylethylpiperazine-N'-2-ethanesulfonic acid), 65 mM boric acid, 0.5 % HPMC

(hydroxypropylmethylcellulose), 6 % mannitol, and 1 μg/ml ethidium bromid. The final volume of the buffer was adjusted to 100 ml. Since 6 % mannitol made the buffer so sticky and bubbling that the buffer had to be degassed overnight by a stirring device using a low stirring speed. The buffer was then sonicated for 30 min to completely remove remained bubbles and stored in a refrigerator before CE analysis. The capillary used was an uncoated silica capillary whose total length, effective length, and inner diameter were 31 cm, 21 cm, and 75 μm, respectively [10].

Both 0.2 N NaOH and 0.2 N HCl as well as the separation buffer were used to rinse the CE capillary before analyzing samples. Each rinsing solution rinsed the capillary for 5 min in order of 0.2 N NaOH, 0.2 N HCl, and the separation buffer under a pressure of 25, 25, and 30 psi, respectively. A sample was then injected at 1 psi for 50 sec and measured at 254 nm of UV detector. To be sure of the CE result quality, all CE analysis was performed after confirming that the current was maintained consistently during CE analysis.

Copper analysis

Copper analysis was performed using an atomic absorption spectrophotometer (Perkin-Elmer, model Z5100) with a furnace mode. The amount of sample injection was 20 μl with 5 μl of dilution Milli Q water. All samples were properly diluted to a final concentration within 100 ppb because the detection range of the AA instrument was from 0 ppb to approximately 100 ppb. Energy lamp strength was always maintained over 50 %.

Copper samples were analyzed under the presence of nitric acid that could dissolve copper bound to cell materials. Since heavy metals tend to be precipitated with ligands depending on pH conditions, copper might also be bound to cells or precipitated in a sample, which might cause error in a copper analysis due to the unbalanced distribution of copper concentration in a sample vial. Thus, by making the sample be acidic, copper could be present in a dissolved form in the liquid sample. The proper concentration of nitric acid was investigated analyzing a known copper concentration solution with different concentration of nitric acid ranging from 2 % to

10 %.

2.2 CBC interaction between methanotrophs

Copper analysis of methanobactin (MB)

MB was isolated from the spent medium of *M. trichosporium* OB3b and treated with either copper or EDTA: copper-bound MB and EDTA-treated MB. The copper concentrations of the treated MB and the original MB were analyzed. In addition, the copper concentration of a fraction of MB eluted from HP20 column after regeneration was measured as well. Stock solutions of each sample were prepared dissolving 10 mg of each sample into 1 ml of Milli Q water. The samples were diluted 1,000 to 3,300 times depending on the copper concentrations before injecting to the AA instrument.

M. parvus OBBP growth under the presence of MB

M. parvus OBBP was grown under the presence of CBC to evaluate the effect of CBC on the growth of *M. parvus* OBBP. A stock liquid culture was prepared as described earlier including 10 μM of copper as $Cu(NO_3)_2 \cdot 5H_2O$. After harvesting cells in the exponential growth phase, cells were washed with pre-warmed fresh NMS medium to remove loosely cell-bound copper, and resuspended on 50 ml of NMS medium samples with different MB and copper concentration.

Two kinds of MB were added to the *M. parvus* OBBP cultures: copper-bound MB and EDTA-treated MB. Since MB could be destroyed by heating, it was added to the culture after autoclaving the medium. The copper concentrations for the samples were either 0 or 10 μM. In addition, a negative control was prepared adding neither copper nor MB to the *M. parvus* OBBP culture and a positive control adding just 10 μM copper to the culture. An optical density of each sample was measured at 600 nm. After completing the growth monitoring, copper concentrations of the spent media were analyzed.

3. Results

3.1 sMMO and pMMO activity measurement

Naphthalene assay

Table 3 indicates the results of optical density at 528 nm depending on different copper concentration. The naphthalene assay is based on the fact that only sMMO can oxidize naphthalene to 1- or 2- naphthol. While the color of the 20 μM-copper sample was not changed, the naphthol diazo complex samples of 0 μM copper were in bright purple color. Based on the results, 0 μM copper condition made *M. trichosporium* OB3b to express sMMO and oxidize naphthalene to 1- or 2- naphthol; on the other hand, 20 μM copper condition to express pMMO that could not oxidize naphthalene. These results were well matched with the known fact that *M. trichosporium* OB3b can express either sMMO or pMMO depending on copper concentration [3].

TCE degradation depending on copper concentration in NMS medium

The standard concentrations for the calibration varied from 0 to 33 μM in the gas phase. Figure 1 shows a standard curve between TCE concentration in gas phase and peak area. Figure 2 indicates TCE concentrations in the gas phase of the 20 ml tube samples after 6 hour incubation with the initial TCE concentration of 4.12 μM in the gas phase. Although all gas phase concentrations were calculated by the standard curve equation, two data of GC peak area, 0 μM and F 0 μM samples in Figure 2 (b), were converted to the concentration using just two points of the standard concentration because the peak areas were too small to be calculated by the standard curve.

Since *M. trichosporium* OB3b has been known to express either pMMO or sMMO depending on copper availability [3], *M. trichosporium* OB3b was expected to express pMMO under 20 μM copper concentration (Figure 2 (a)) and sMMO under 0 μM copper concentration (Figure 2 (b)). As shown in the two figures, sMMO showed approximately 10 times higher rate in TCE degradation than pMMO and TCE degradation efficiency by *M. trichosporium* OB3b grown with 20 mM formate was

7 % higher than that with no formate. Table 4 shows the TCE degradation rate of the samples. These results were also well matched with those of the naphthalene assay.

3-2 *M. album* BG 8 purification and verification

M. album BG 8 purification and microscopy observation

Two kinds of potential *M. album* BG 8 could be picked up from contaminated plates; one was from a very small colony in light yellow, and the other from a streak. The two potential samples were spread on NMS and nutrient agar media at the same time. After 5 days of the incubation, the colony samples showed much higher cells on NMS media and much less cells on nutrient agar media than the streak sample, suggesting that the colony sample had contained much more methanotrophs than the streak sample. The colony samples were then diluted and transferred to fresh NMS media repeatedly. Finally cleaned cells from the contaminated cells showed no growth on a nutrient agar medium but on a NMS medium.

The purified *M. album* BG 8-like cells and *M. album* BG 8 that had been stored in a refrigerator for 6 years were observed using a microscope. Simple staining with 1 % of methlylene blue showed the shape of the cells. Both of the cells seemed to be a round shape and seemed alike much. Also there were no other shapes of cells except for the round type, so they might not to be contaminated by other microorganisms.

PCR and gel electrophoresis for *M. album* BG 8 verification

The PCR product bands shown in Figure 3 were obtained using the freezing-thawing method. While *pmoA* gene indicated strong bands of both *M. trichosporium* OB3b and *M. album* BG8, *mmoX* gene rarely showed any bands except for one weak band (marked with a circle) in Figure 3 (b). After some consecutive results of no PCR products in *mmoX* gene such as Figure 3 (a), the quality of the primer set of *mmoX* gene were suspected, so they were replaced with another one which was also not a new one. With the replaced *mmoX* primer set, a weak PCR product of *mmoX* gene of *M. trichosporium* OB3b could be obtained (Figure 3 (b)). However, no PCR product of *mmoX* gene of *M. album* BG8 was shown in all the PCR experiments. The

unexpected results of *mmoX* gene of *M. trichosporium* OB3b might result from either the poor quality of the primer set or not proper method of DNA sample preparation, or both of them.

With the same DNA preparation method, a freezing-thawing method, the following PCR products shown in Figure 4 and 5 were obtained using three different methanotrophs: *M. trichosporium* OB3b, *M. capsulatus* Bath and *M. album* BG8. Both *M. trichosporium* OB3b and *M. capsulatus* Bath were used as positive controls in producing the products of *pmoA* and *mmoX* genes at the same time. As shown in Figure 4, *M. capsulatus* Bath showed two clear bands of *pmoA* and *mmoX* as expected, but no bands of *M. trichosporium* OB3b was indicated. However, the reverse results of those in Figure 4 were obtained in Figure 5; *M. trichosporium* OB3b showed two distinguish bands of *pmoA* and *mmoX,* but *M. capsulatus* Bath did not.

Although one of two positive controls for the detection of *pmoA* and *mmoX* genes did not work properly at the same time in the two PCR experiments and nothing was surely confirmed, a clear PCR product of only *pmoA* gene of *M. album* BG 8-like cells was shown in all PCR experiments and also the negative control of E. coli showed no products of *pmoA* and *mmoX* genes. Therefore, the cleaned *M. album* BG 8-like cells obtained another positive evidence for real *M. album* BG 8.

Capillary electrophoresis (CE) for DNA molecular weight marker

CE performance for DNA molecular marker VIII from Roche (0.25 μg/μl, molecular size 1,114 ~ 19 bp) was conducted to make sure of the separation quality of the marker prior to an analysis of PCR products of *pmoA* and *mmoX* genes. Han [10] applied 1.0 psi for 50 sec and 4 kV for 25 min for sample injection pressure and separation voltage, respectively. With the same conditions as Han [10], the separation of DNA molecular marker VIII was performed and Figure 6 showed the results and a semi-log graph between molecular size (bp) and retention time (RT). According to the composition datum of the DNA marker offered by Roche (Figure 7), the separation quality using Han [10]'s method was quite good except for the baseline at the end of the analysis. So, better separation quality and faster method was investigated changing

the sample injection pressure and separation voltage based on the Han's method [10].

Figure 8 indicated the results of the modified method of Han [10]. With 0.5 psi for 30 sec and 6 kV for 15 min for sample injection pressure and separation voltage, respectively, the separation quality was good enough to distinguish each peak, it was faster to separate all peaks, and the baseline during the analysis was fairly good. The current was maintained as -20 μA during the CE analysis. In addition, the DNA marker separation was conducted with the same method as the modified method except for the applied voltage 8 kV instead of 6 kV to make the analysis much faster. As shown in Figure 9, the separation of the peaks performed at 8 kV was good enough and the analyzing time was shorter, but the base line was not as good as that of 6 kV.

Capillary electrophoresis (CE) for PCR products

PCR products of *pmoA* and *mmoX* genes of *M. capsulatus* Bath were detected using CE analysis. The growth condition of *M. capsulatus* Bath was the same as shown in Materials and method. DNA samples were prepared using the beadbeater-using method. Although the same method as used in the previous PCR experiments was applied, PCR products did not clearly shown on both gel and capillary electrophoresis (data not shown). So, each PCR chemical and DNA samples were analyzed using CE to figure what shape of peaks could be indicated on CE. Figure 10 shows each peak of the chemicals and DNA samples.

One suspected thing from Figure 10 was that the peaks of the primer sets were much smaller than those of dNTPs. Actually the primer sets were not fresh ones and also they had been stored even in a refrigerator with no power for about 2 days due to the US-nationwide power failure in 2003. Hence, the concentration of the primer sets was adjusted to 0.4 μM (proper concentration range of a primer set is 0.1 ~ 0.6 μM) and the amount of each primer set was recalculated according to the information shown on the labels of the primer sets. The information on each label was as follows: *pmo*f 334 (MW=6,885.5); 2.3 OD_{260} = 10.8 nmol = 0.07 mg. That information was not enough to calculate the required amount of the primer sets, so the total volume of each

primer was assumed 1.8 ml which was the volume of the primer-contained vial. Based on the information, the required amount of each primer set was calculated as 0.4 μM in 25 μl of a sample. The amount of each primer of *pmo*f 334, *pmo*r 640, *mmox*f 882, and *mmox*r 1403 were 1.77, 0.64, 1.11, and 0.49 μl, respectively. Figure 11 shows the results of CE analysis of *pmoA* and *mmoX* genes using either the pre-existed amount of each primer (Figure 11 (a)) or the recalculated amount of each primer (Figure 11 (b)).

With the same PCR products as shown in Figure 11 (b), CE analysis was conducted and the peaks of the PCR products are indicated in Figure 12. As shown in Figure 11 (b) and Figure 12 (a and b), clear bands of *pmo*A and *mmo*X genes appeared on both GE and CE results. The sizes of *pmo*A and *mmo*X genes were 327 bp and 547 bp according to the DNA marker. The two obvious results were the clearest results after changing the DNA sample preparation method and adjusting the concentration of the primer sets. In addition, both gel and capillary electrophoresis results were well matched each other.

3-3 CBC interactions between methanotrophs

Copper measurement of methanobactin (MB)

5 % of nitric acid showed a proper concentration of copper, so all copper analysis were performed under the presence of 5 % of nitric acid. Copper concentrations of four different MB are shown in Table 5 and Figure 13. According to the results, MB was proved to have a high affinity to copper binding approximately 260 time-higher copper amount of original MB. Also, MB could still retain about 11 % of the total copper amount even after the ethylenediaminetetraacetic acid (EDTA) treatment.

M. parvus OBBP growth in methanobactin (MB) with different copper concentrations

M. parvus OBBP growth was monitored under the presence of 20 mg/L of MB. Figure 14 and 15 shows the growth curve of the optical density and the relative optical density, respectively.

As shown on the graphs, *M. parvus* OBBP grown with MB-Cu did not show any growth in both 0 μM and 10 μM copper concentrations of NMS media; however, *M. parvus* OBBP grown with MB-EDTA was grown well similar to the positive control, *M. parvus* OBBP grown in 10 μM copper-NMS, except for longer lag phase than that of the positive control. In addition, the growth rates of the three samples were so similar one another even though the growth rate of the positive control was a little higher than those of the others (Table 6). After completing the monitoring of growth rates, whole-cell copper concentrations of the samples were analyzed to compare copper concentrations between initially intended and actual concentrations. Table 7 and Figure 16 indicate the actual copper concentrations of *M. parvus* OBBP-containing samples.

Figure 16. Whole-cell copper concentrations of *M. parvus* OBBP-containing samples shown in Figure 14

4. Discussion

4.1 Characterization of methanotrophs

Basic molecular analysis on methanotrophs was performed to understand the characteristics of the bacterial strains and acquire the analysis techniques. To examine MMO expressions in *M. trichosporium* OB3b depending on copper availability, naphthalene assay and TCE degradation assay were conducted using non-copper NMS medium and 20 μM copper-containing NMS medium. Since *M. trichosporium* OB3b expresses either sMMO or pMMO under low copper and high copper concentrations, respectively (the critical concentration was reported as 0.89 μmol copper per gram cell dry weight [10, 13]), *M. trichosporium* OB3b was expected to express sMMO at non-copper NMS medium and pMMO at 20 μM copper-containing NMS medium. In addition, while the substrate specificity of sMMO is lower than that of pMMO,

halogenated hydrocarbon degradation rate of sMMO is higher than that of pMMO [3, 10, 15]; in other words, sMMO can oxidize much more kinds of halogenated hydrocarbons at faster degradation rate than pMMO. In both of the assays, sMMO-expressing cells showed much more degradation efficiency of naphthalene and TCE than pMMO-expressing cells (Table 3 and 4, Figure 2). Also, 20 mM formate played a role in an outside source of reducing equivalent that facilitated the TCE degradation rate. Thus, making methanotrophs express sMMO and adding a proper reducing equivalent in a chlorinated compound-contaminated site would help *in situ* bioremediation strategy be optimized. During the experiments, it was important for better activities of cells to transfer a part of a liquid stock culture to sample vials when cells were in the initial exponential phase rather than other phases.

PCR and gel electrophoresis were performed to verify a purified *M. album* BG8. Since both *M. trichosporium* OB3b and *M. capsulatus* Bath possess two types of MMO genes, they were used as positive controls to show the PCR products of *pmo*A and *mmo*X genes on agarose gel. However, initial continuative experiments could not show clear bands of PCR products of either *pmo*A or *mmo*X gene but indicated a thick and blunt band on agarose gel. Three explainable reasons were investigated changing the components of PCE experiment. The first one was the method of DNA sample preparation; using a beadbeater with 0.1 mm glass beads rather than a freezing-thawing method to disrupt cell membrane showed better PCR results. Although the freezing-thawing method could also indicate quite good PCR products, it sometimes failed to show proper products (Figure 3, 4, and 5). The reason why the freezing-thawing method sometimes failed to show good results might be either that the method was too strong to remain a proper gene size or that it was too weak to disrupt the cell membrane. In Figure 3 and 4 using the freezing-thawing method, both *pmo*A and *mmo*X genes did not appear at the same time but one of the gene products was shown, suggesting that the preparation method was not too weak to disrupt cell membrane. Thus, the former reason

might be a better explanation. The second reason could be the quality of the primer sets that were exposed to room temperature for about 48 hours, because oligoneucletide primers were vulnerable to freezing-thawing cycles and room temperature. After changing another primer set of *mmox*f 882 and *mmox*r1403, a *mmo*X gene product could be obtained (Figure 3). The last possible explanation would be not proper amount of the primers used in PCR experiment. Although PCR recipe in Jong-In's lab note [14] suggested taking 0.5 μl of each primer whose concentration should be 20 μM, the concentration of the primers used in this study was so doubtful that the concentrations of the primers were recalculated based on the label information and the required amount of each primer was then calculated. As shown in Figure 11, PCR containing recalculated amount of each primer showed very clear product bands on 1.0 % agarose gel (Figure 11 (b)) even though the DNA sample was the same as used in Figure 11 (a).

According to the PCR results and nutrient agar assay for the verification of the purified *M. album* BG8, it not only indicated a *pmo*A gene product in all experiment (Figure 3 (lane 3), Figure 4 (lane 2), and Figure 5 (lane 2)), but also showed no growth on nutrient agar plates. As *M. album* BG8 can express only pMMO, the *M. album* BG8-like cells might be purified based on the two assay results. However, the purified *M. album* BG8 did not grow as well as other methanotrophs in a liquid NMS medium (data not shown). It took more than 8 days to start to grow in the liquid medium, suggesting that the purified *M. album* BG8-like cells might not be *M. album* BG8. Thus, it is not clear whether the purified *M. album* BG8-like cells were completely purified or not. Since the *M. album* BG8-like cells have been contaminated on agar plates for a long time, it might be possible that they could not be easily accustomed to the liquid medium. Based on the assumption, the *M. album* BG8-like cells will be continuously tried to grow in the liquid medium and 16S rRNA analysis will be performed if necessary in the future study.

As a more convenient and faster analysis of PCR products, capillary

electrophoresis (CE) was used to separate the PCR products of *pmo*A and *mmo*X genes. CE method was modified and developed from Han's method [10]. Faster and better peaks could be obtained using 6 kV separation voltages with 0.5 psi sample injection pressure for 30 sec; especially the peaks of 900 and 1114 bp of the DNA molecular weight marker were sharper than those of the previous study [10]. Also, 8 kV separation voltages were applied to achieve much faster results, but as shown in Figure 9, the baseline was not as stable as that of 6 kV voltages. With the modified method, the PCR products of *pmo*A and *mmo*X genes were separated and the size of the genes were estimated. Two distinguishable peaks could be observed in Figure 12 (a and b). A *pmo*A gene product appeared at 10.34 min of the retention time and a *mmo*X gene product at 12.03 min; the gene sizes were 327 bp and 547 bp for *pmo*A and *mmo*X, respectively. These results of CE analysis were well matched with those of gel electrophoresis analysis but showed much faster and sharper peaks.

4.2 Role of CBC in the growth of methanotrophs

Methanobactin (MB) is a copper-binding compound (CBC) which was isolated from the spent medium of *M. trichosporium* OB3b [9]. Although the role of CBC is not known, it is assumed that it might play a role in up-taking copper outside cells because CBC has high affinity for binding copper. Table 5 showed the high affinity of MB to copper. One unusual and interesting thing in Table 5 was that the copper concentration of EDTA-treated MB was higher than that of the original MB. Since EDTA is a strong chelating agent [16] resulting in binding copper much more strongly than MB, the copper concentration of MB-EDTA was expected to be lower than that of the original MB; however, the reverse was true, suggesting that MB could retain the bound copper against the strong chelating agent, EDTA. In addition, *M. parvus* OBBP could utilize the copper bound to MB-EDTA and showed almost the same growth pattern as that grown under the presence of 10 μM copper. The results

indicated that the EDTA-treated MB did not badly affect on the growth of *M. parvus* OBBP but assisted the growth supplying copper to *M. parvus* OBBP for the expression of pMMO. However, the lag phase of *M. parvus* OBBP with MB-EDTA was longer than that of the normal growth of *M. parvus* OBBP, probably because MB still affected on the growth to some extent, so it took much time for *M. parvus* OBBP to be accustomed to the MB environment. Also the two growth of 0 μM-ED and 10 μM-ED in Figure 15 showed exactly the same pattern each other. It might be explained that *M. parvus* OBBP could be grown normally once copper concentration in NMS media met the minimum of the required amount of copper to express pMMO. On the other hand, *M. parvus* OBBP could not survive under the presence of copper-bound MB (0 μM-Cu and 10 μM-Cu in Figure 14), suggesting that copper-bound MB seriously badly affected on the growth or killed the cells. The cause(s) might be either the effect of MB or the toxicity of high copper concentration, or both of them. According to the copper analysis of the whole-cell and NMS medium (Table 7), the copper concentration of 0 μM-Cu and 10 μM-Cu were 22.0 and 22.9 μM, respectively. 22 μM of copper was usually not a high concentration to methanotrophs, so it could be hypothesized that MB might play a serious role in impeding the cell growth.

5. Future Study

Two methanotrophs, *M. parvus* OBBP and *M. album* BG8, were purified from contaminated plates but it will be necessary to verify them using PCR and CE with the recalculated amounts of primer sets (Figure 11 (b) and Figure 12 (a and b)). Also, based on the preliminary data of the growth of *M. parvus* OBBP under the presence of different-copper concentrations of MB, it will be required to determine if *M. parvus* OBBP is able to show the same growth pattern with the same copper concentrations under no presence of MB. It will be able to suggest which of the factors caused no growth of *M. parvus* OBBP

under the presence of copper-bound MB in Figure 14. In addtion, the growth monitoring of other methanotrophs that can express pMMO but not produce CBC under the presence of the treated MB will make the role of CBC better clear. With the results, it will be possible to give an answer to a hypothesis if there are some important groups that provide copper-needed methanotrophs with CBC among methanotrophs. Ultimately, the way that methanotrophs interact one another based on the necessity of copper will be figured out to understand the copper uptake mechanism of methanotrophs.

6. Summary

Methanotrophs oxidize methane to methanol using either soluble cytoplasmic MMO (sMMO) or a membrane-associated, or particulate, MMO (pMMO). pMMO expression requires high ratio of copper to biomass (> 0.9 nmol of Cu / mg of cell protein), but the copper uptake mechanism remains vague. One of the assumptions of the copper uptake system lies in copper-binding compounds (CBC) that are small extracelluar polypeptides and have high affinity for binding copper. In this independent study, preliminary data suggesting the effect of CBC on the growth of *Methylocystis parvus* OBBP were obtained and the characterizations of some methanotrophs were performed as well.

Methylosinus trichosporium OB3b showed the effect of copper concentration on the expression of either sMMO or pMMO using naphthalene and trichloroethylene (TCE) assays. Non-copper environment caused *M. trichosporium* OB3b to express sMMO and the cells then not only showed to oxidize naphthalene to naphthol changing the color of the NMS medium to bright purple in the naphthalene assay, but also indicated approximately 10 times higher TCE degradation rate than those grown with 20 μM copper. Also, 20 mM formate played a role in an outside source of reducing equivalent that facilitated the TCE degradation rate.

Polymerase chain reaction (PCR), gel electrophoresis (GE), and capillary electrophoresis (CE) were performed to verify purified *Methylomicrobium album* BG8. The purified cells showed a distinct PCR product of the *pmo*A gene on GE, but no product of the *mmo*X gene, suggesting that the purified cells might be *M. album* BG8; however, the cells were not grown as well as other methanotrophs in a liquid nitrate mineral salts (NMS) medium. Thus, the cells need to be monitored continuously in the liquid medium and further analysis such as 16S rRNA analysis will be required to verify them. In addition, the method of CE analysis could be modified from Han's [10] using 0.5mpsi sample injection pressure for 30 seconds and applying 6 kV separation voltages; it showed as better peaks as Han's [10] and faster analysis.

Methanobactin (MB) could, to some extent, hold bound copper against a strong chelating agent, ethylenediaminetetraacetic acid (EDTA). EDTA-treated MB assisted the growth of *M. parvus* OBBP supplying copper to the cells even though the lag phase under the presence of EDTA-treated MB was approximately 20 hours longer than that of a normal one. However, copper-bound MB killed the cells; it might be caused by either MB toxicity or high copper concentration, or both of them. With the results, it is assumed that MB might be able to play a different role according to a certain environment in an interaction among methanotrophs based on the copper nessecity.

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