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Chloromethane stimulates growth of *Methylomicrobium album* BG8 on methanol

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Abstract

Studies were performed to determine if the growth of *Methylomicrobium album* BG8 on methanol could be enhanced through the provision of chloromethane. *M. album* BG8 was found to be able to oxidize chloromethane via the particulate methane monooxygenase with an apparent K_s of 11 ± 3 μM and V_{max} of 15 ± 0.6 nmol (min mg protein)$^{-1}$. When up to 2.6 mM chloromethane was provided with 5 mM methanol, methanotrophic growth was significantly enhanced in comparison to the absence of chloromethane, indicating that methanotrophs can utilize chloromethane to support growth, although it could not serve as a sole growth substrate. [^{14}C]chloromethane was found to be oxidized to [^{14}C]CO_2 as well as incorporated into biomass. These results indicate that reactions previously thought to be cometabolic may actually provide some benefit to methanotrophs and that these cells can use multiple compounds to enhance growth.

Keywords: Methanotroph; Cometabolism; Chloromethane; Particulate methane monooxygenase

1. Introduction

Methanotrophs, bacteria that utilize methane as their source of carbon and energy, are commonly found in marine, terrestrial and freshwater environments [1]. Through a series of two-electron transfers these cells convert methane first to methanol, then to formaldehyde where it can either be assimilated into biomass or further oxidized to formate and ultimately to carbon dioxide. The enzyme catalyzing the transformation of methane to methanol, the methane monooxygenase (MMO), has been found in two forms, one associated with the cytoplasm or soluble methane monooxygenase (sMMO) and the other associated with the membranes or particulate methane monooxygenase (pMMO). Both forms of the MMO can oxidize numerous other alkanes and alkenes due to their broad substrate range [2]. These transformations, however, are of little benefit to the cells as they typically derive neither reducing equivalents nor biomass from these transformations. Such cometabolic reactions can only be maintained in the obligate presence of a second substrate that can support growth (usually methane in the case of methanotrophs). Although these cometabolic reactions often consume reducing equivalents and as such are a burden to the cell, the phenomenon of cometabolism has been successfully used for the degradation of hazardous wastes in situ [3].

Recently it has been speculated that some of the compounds degraded cometabolically by methane monooxygenase may actually serve as either a carbon or energy source for methanotrophs [4]. To be of any value to the cells, formaldehyde must be made in the oxidation of organic compounds such that the carbon can be converted either into biomass or further oxidized to carbon dioxide to generate more reducing equivalents than that consumed by the methane monooxygenase. Cell-free extracts of the methylotroph *Methylobacterium* sp. strain CRL-26 showed that formaldehyde was generated from the oxidation of chloromethane [5]. Furthermore, *Methylococcus capsulatus* (Bath) was observed to degrade chloromethane, and formaldehyde did not enhance the oxidation rate, suggesting that such oxidation was not limited by reducing equivalents [6]. Therefore it is possible that methanotrophs, when given chloromethane, may be able to utilize the products of oxidation to enhance growth. As it is estimated that at least 5×10^6 tons of chloromethane are produced each year from natural sources [7], it is possible that in situ methanotrophic growth can be aided from the

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oxidation of chloromethane. In this paper, we present results showing that *Methylomicrobium album* BG8, a methanotroph that can only express the particulate form of the methane monooxygenase (pMMO), does indeed derive some benefit from oxidizing chloromethane in the presence of methanol, although chloromethane can not serve as a sole growth substrate.

2. Materials and methods

2.1. Kinetics of chloromethane oxidation

All chemicals in the preparation of media were of reagent grade or better. The kinetic parameters of apparent maximal degradation rate, V_{max}, and apparent affinity, K_s, of chloromethane degradation were determined using nonlinear regression to fit the Michaelis–Menten formula to initial rates of chloromethane degradation measured over a 1 h time frame as described previously [8].

2.2. Growth on chloromethane

To determine if chloromethane could enhance growth of *M. album* BG8, the cells were grown in the presence of 5 mM methanol and varying concentrations of chloromethane in 20 ml vials capped with teflon-coated silicone septa. Duplicate vials were prepared for both the positive control (no chloromethane) and samples with chloromethane. Methanotrophic growth was monitored by measuring OD_{600} over time which was converted to protein concentrations using a pre-determined linear correlation ($r^2 = 0.997$). Upon entering the stationary phase, 200 μl headspace samples were removed to determine the amount of methanol and chloromethane consumed using gas chromatography.

2.3. Products of chloromethane oxidation

To determine the fate of chloromethane oxidation, *M. album* BG8 was grown in 70 ml vials with methanol and [^{14}C]chloromethane obtained from American Radiolabeled Chemicals, Inc. St. Louis, MO, USA. Methanol was added at an aqueous concentration of 5 mM while chloromethane was added at either 1.3 or 2.6 mM for 28.1 and 58.0 μCi activity, respectively. After the cells had grown to the mid-exponential phase ($OD_{600} = 0.2$), CO_2 was collected from a NaOH trap and analyzed using a Rackbeta 1219 scintillation counter (LKB Wallac). The vials were then unsealed and unreacted [^{14}C]chloromethane removed. Cells were collected by centrifugation at 12 000 rpm for 30 min, washed three times in 25 mM phosphate buffer (pH 7.0) and analyzed using the scintillation counter.

To determine if proteins were labeled from [^{14}C]chloromethane utilization, *M. album* BG8 grown in the presence of 5 mM methanol and 2.6 mM chloromethane was collected as described above and resuspended in SDS loading buffer. Cells were disrupted by bead beating (Bead Beater; Biospec Products, Bartlesville, OK, USA) and then boiled for 10 min. The broken cells were then loaded on 10% NuPAGE SDS–PAGE with MES (2-[*N*-morpholino]ethanesulfonic acid) running buffer (Novex, San Diego, CA, USA). The gel was stained with Coomassie blue R-250, destained with methanol–acetic acid–water (10:10:80, vol/vol/vol) and dehydrated by 10 min incubations with 25, 50, and 100% acetic acid. The gel was then soaked for 2 h in 20% (wt/vol) 2,5-diphenyloxazole (PPO) scintillant in 100% acetic acid. After washing with water for 2 h, the gels were dried and placed in an X-ray cassette with Kodak X-ray film at −80°C for 3 h.

3. Results and discussion

Although methanotrophs have been shown by others to degrade chloromethane [5,6], the kinetics of chloromethane oxidation have not been reported. When *M. album* BG8 was incubated with up to 400 μM chloromethane, the kinetics of chloromethane degradation followed Michaelis–Menten kinetics with apparent values of V_{max} and K_s 15 ± 0.6 nmol (min mg protein)$^{-1}$ and 11 ± 3 μM respectively. If *M. album* BG8 was inactivated with acetylene, a specific inhibitor of pMMO activity [9], no chloromethane degradation was observed, indicating that pMMO was responsible for chloromethane degradation.

Table 1
Growth characteristics of *M. album* BG8 in the presence of 5 mM methanol and varying concentrations of chloromethane

Chloromethane (mM)	Specific growth rate (h^{-1})[a]	ΔMethanol[b] (mM)	ΔChloromethane[b] (mM)	pH_{final}[c]
0	0.094 (0.002)	3.5 (0.35)	–	6.6 (0.10)
1.3	0.14 (0.010)	4.1 (0.07)	1.0 (0.015)	4.7 (0.25)
2.6	0.18 (0.006)	4.1 (0.11)	0.95 (0.13)	4.1 (0.05)
5.2	0.13 (0.006)	3.9 (0.40)	0.67 (0.21)	4.4 (0)
7.8	0.085 (0.002)	1.0 (0)	0.009 (0.09)	6.5 (0.05)

[a]Number in parentheses indicates 95% confidence interval of measured growth rates.
[b]Based on amount of methanol or chloromethane consumed upon entering stationary phase. Number in parentheses indicates range of duplicate samples.
[c]pH upon entering stationary phase. Number in parentheses indicates range of duplicate vials.

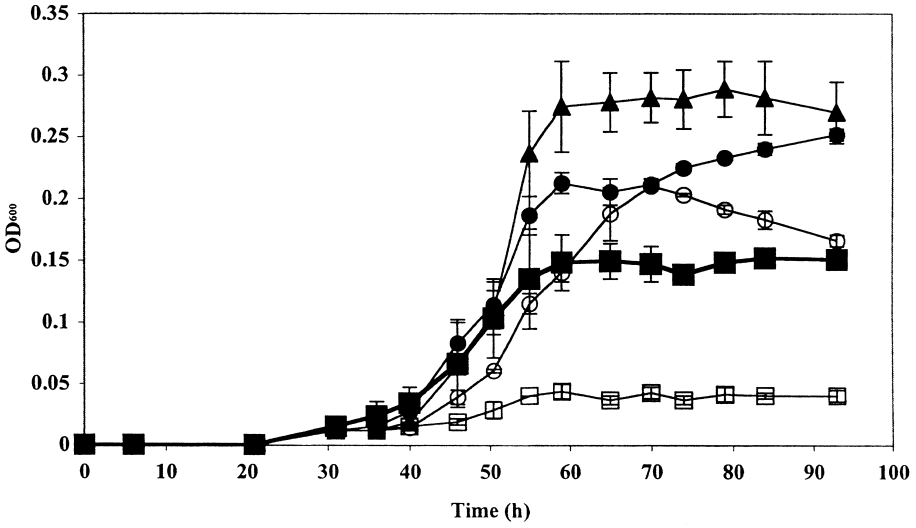

Fig. 1. Growth of *M. album* BG8 in the presence of 5 mM methanol and varying concentrations of chloromethane. ■, 0 mM; ●, 1.3 mM; ▲, 2.6 mM; ○, 5.2 mM; and □, 7.8 mM chloromethane, respectively. Error bars indicate range of duplicate samples.

To determine if chloromethane could stimulate methanotrophic growth, methanol rather than methane was used as the primary carbon source to avoid any competition for binding to pMMO that could complicate data analysis. As shown in Fig. 1 and Table 1, the growth rate, μ, and maximum cell of *M. album* BG8 grown with 5 mM methanol doubled if 2.6 mM chloromethane was also provided. Methanol consumption only increased by 17% in the presence of 2.6 mM chloromethane, suggesting that chloromethane served as a carbon and/or energy source for *M. album* BG8.

To determine the role of chloromethane in enhancing methanotrophic growth, experiments were performed with [^{14}C]chloromethane. Of the [^{14}C]chloromethane consumed when provided at an initial concentration of 1.3 or 2.6 mM in the presence of 5 mM methanol, approximately half was converted to CO_2 while 35 to 38% was associated with the biomass as shown in Fig. 2. To further verify that the cell-associated [^{14}C] was not due to reactive intermediates binding to pMMO or other nearby macromolecules, SDS–PAGE analysis was performed using whole cells grown to the mid-exponential phase with 2.6 mM chloromethane (58 μCi). As can be seen in Fig. 3, all polypeptides were labeled, indicating that chloromethane was converted to an intermediate of the ribulose monophosphate pathway of carbon assimilation, i.e. formaldehyde. From

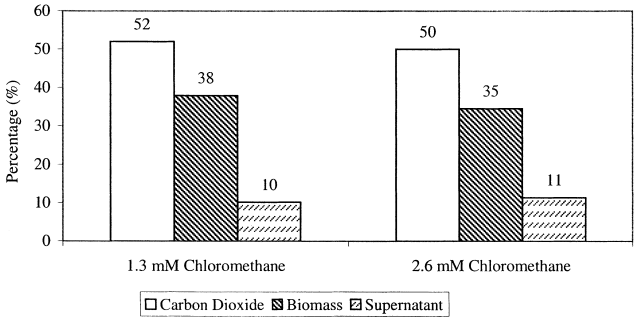

Fig. 2. Product distribution of [^{14}C]chloromethane degradation by *M. album* BG8 grown with 5 mM methanol and either 1.3 or 2.6 mM chloromethane.

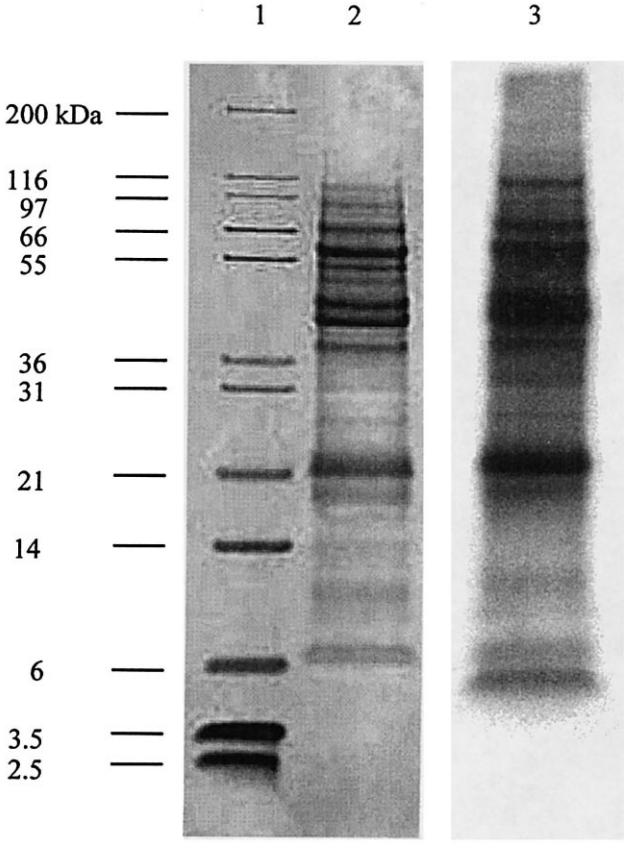

Fig. 3. SDS–PAGE analysis of whole cells of *M. album* BG8 incubated with 2.6 mM [^{14}C]chloromethane and 5 mM methanol. Lanes: 1, molecular mass standards; 2, Coomassie-stained proteins from whole cells; 3, fluorograph of proteins in lane 2.

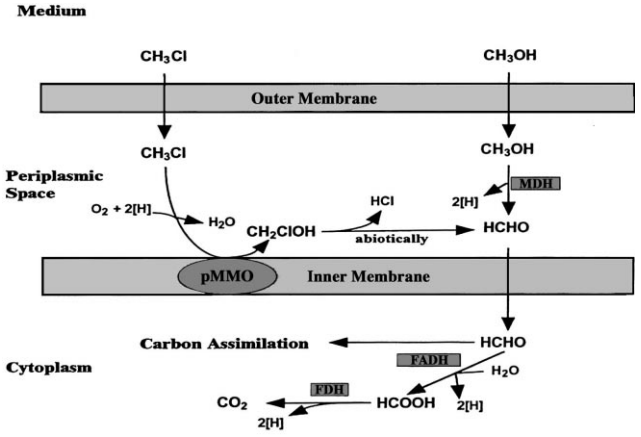

Fig. 4. Proposed mechanism of chloromethane oxidation by *M. album* BG8. pMMO, particulate methane monooxygenase; MDH, methanol dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; 2[H], two reducing equivalents.

these results, it is clear that chloromethane served to provide both energy and carbon to *M. album* BG8.

Although oxidizing chloromethane was beneficial to the cells, two points should be discussed. First, at chloromethane concentrations of 5.2 and 7.8 mM, cell growth was inhibited as indicated by reduced specific growth rates and lower cell densities as noted in Table 1 and Fig. 1. The amount of chloromethane at these concentrations consumed was less than that at lower concentrations, indicating that substrate inhibition was occurring, i.e. chloromethane itself was toxic to the cells at high concentrations. Second, the growth medium was acidified upon the oxidation of chloromethane. The initial pH of the growth medium was 6.8 for all experiments, but as can be seen in Table 1, the pH of the medium dropped over 2 pH units when chloromethane was substantially degraded. Such a pH drop can also inhibit growth, suggesting that product toxicity also occurred during the oxidation of chloromethane. To explain this pH drop, we believe that formaldehyde is not the initial product of chloromethane oxidation, rather it appears that chloromethanol is produced with the concomitant production of formaldehyde through the elimination of hydrochloric acid. Such an oxidative dehydrohalogenation scheme is not unprecedented as it has been imputed for oxidation of halogenated alkanes by cytochrome P450 [10]. Based on these observations, we propose chloromethane is oxidized via the pathway outlined in Fig. 4.

As both substrate and product inhibition occurred during the exposure of *M. album* BG8 to chloromethane, it was not possible to construct an analytical model that could predict the growth rate in the presence of chloromethane and methanol. It should be noted, however, that as discovered earlier [6] it was found here that chloromethane could not serve as a sole source of carbon and energy (data not shown). This was not due solely to the acidification of the medium from the oxidation of chloromethane. When the buffer strength was increased 10-fold to prevent the pH from dropping, no cell growth was observed in the presence of chloromethane alone at a concentration of 1.3 mM. This is interesting as the cells did get some benefit from chloromethane oxidation in the presence of methanol. Although chloromethane can act as a carbon source, *M. album* BG8 may not be able to produce enough reducing equivalents to support growth. As indicated on Fig. 4, during complete oxidation of chloromethane, the net production of reducing equivalents is a third of that produced from complete oxidation of methanol. Apparently the reduced energy yield prevents these cells from utilizing chloromethane as a sole carbon and energy source. If an alternative substrate is provided, however, chloromethane can be used to enhance growth.

The finding that chloromethane stimulated growth on methanol is exciting as it suggests that methanotrophs can use a variety of carbon sources to stimulate growth, provided they are transformed to formaldehyde. This could be particularly important when these cells are exposed to low concentrations of any one growth substrate that may poorly support active growth. By utilizing multiple substrates, these cells may have a selective advantage over other cells in some environments and thus be able to survive and grow under sub-optimal conditions, e.g. with low concentrations of the primary substrate, methane. Further work should be done to determine if other mono-halogenated methanes, particularly bromomethane, can also enhance methanotrophic growth. As bromomethane has been extensively used as a soil fumigant and methanotrophs have been shown to oxidize it to carbon dioxide [11], it is possible that methanotrophs can supplement their growth by utilizing this compound as a carbon or energy source. Finally, as methanol has been shown to promote methane oxidation [12], it may also stimulate oxidation of other substrates by pMMO.

Acknowledgements

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