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Effect of Copper on *Methylomonas albus* BG8†

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Addition of copper to the medium for *Methylomonas albus* BG8 increased cell yield and methane monooxygenase activity. Intracytoplasmic membrane was formed only in cells grown with copper supplementation. Additionally, the abundances of two major membrane proteins were affected by copper in the growth medium. These findings indicate that effects of copper on the physiology of methanotrophic bacteria are not limited to those on types II and X.

Methanotrophs are gram-negative bacteria that use methane as a sole source of carbon. These organisms oxidize methane to carbon dioxide and thus play an important role in the global carbon cycle. Methanotrophs are classified (17) as type I, II, or X on the basis of the pathways used for carbon assimilation and the structure of the intracytoplasmic membrane (ICM).

Methanotrophs respond to environmental conditions by physiological adaptations. It is important to understand these physiological responses both for optimization of applied uses of methanotrophs and for interpretation of field measurements and estimation of in situ rates of methane oxidation. This information is required for calculation of methane fluxes, which is needed to develop models of global climate change.

In some type II and X methanotrophs, the presence of ICM has been correlated with the type of methane monooxygenase (MMO) present (11, 13). The occurrence of either a particulate (pMMO) or a soluble (sMMO) MMO is affected by copper in the growth medium (4, 11, 15). Such an effect of copper on the ICM has not previously been reported in any type I methanotroph. In the present communication, we report that copper affects ICM presence and other aspects of the physiology of the type I methanotroph *Methylomonas albus* BG8. These findings are remarkable in that *Methylomonas albus* BG8 does not switch from pMMO to sMMO under conditions of copper limitation. On the basis of biochemical (4, 15) and molecular analyses (14), it has been suggested that this organism lacks an sMMO.

(A preliminary report of this work has been presented [2].)

Growth of bacteria. *Methylomonas albus* BG8 was grown in batch culture in nitrate minimal salts medium (18) at 30°C with shaking at 150 rpm under an atmosphere that was 50% methane–50% air. Precultures for inocula were grown without copper supplementation. Copper was excluded from the medium. To prevent contamination by copper, the water and vessels used for cell culture and analytical procedures were treated. Water was purified by treatment with a Barnstead (Boston, Mass.) water purification system. Glassware was washed in 30% HNO_3 and in 0.3% EDTA, followed by rinses in treated water. The chemicals were of the highest quality available and were handled so as to avoid any contamination. For cultures receiving copper supplementation, $CuSO_4$ was added from a stock solution to a nominal concentration of 5 or 10 μM.

Electron microscopy. In preparation for electron microscopy, cells were harvested in 1.5-ml Eppendorf tubes, washed twice in 10 mM potassium phosphate buffer (pH 7.0), fixed for approximately 1 h in 0.5% glutaraldehyde prepared in buffer, postfixed for approximately 2 h in 1% OsO_4 prepared in buffer, dehydrated in ethanol, and embedded in LR White resin (Polysciences, Inc. Warrington, Pa.), which was polymerized by 24 h of incubation at 60°C. Ultrathin sections were poststained with 2% uranyl acetate and Reynolds (12) lead citrate, as described previously (3), and examined on a Hitachi H600CR/CR.

Cell fractionation and analytical procedures. Particulate and soluble fractions were obtained essentially as described previously (9), except that 10 μM potassium phosphate buffer (pH 7.0) was used. Protein was estimated by the method of Lowry et al. (8) modified as described previously (9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was run by the method of Laemmli (7) modified as described previously (10). The copper content of the medium was determined on an Instrumentation Laboratory Video 12 aa/ae spectrophotometer (Thermo Jarrell Ash Corp., Franklin, Mass.); samples were atomized with either an air-acetylene flame or a graphite furnace. The latter was used to obtain accurate measurements on samples with copper concentrations of less than 1.3 μM. Standard curves ($r = 0.997$) demonstrated that copper concentrations of ≥0.02 μM could be accurately determined. MMO activity of whole cells was measured by monitoring the oxidation of an alternative substrate, propylene, to propylene oxide essentially as described by others (1). Briefly, 1-ml reaction mixtures containing 4 to 9.5 mg (dry weight) of cells were incubated in the presence of 2 mM formate in 10-ml serum vials at 30°C with shaking at 150 rpm. Two milliliters of 100% propylene was added to initiate the reaction. Samples were withdrawn at intervals, and propylene oxide was monitored by gas chromatography on a Hach Carle flame ionization detector chromatograph equipped with an LKB 2200 recording integrator. The rate of propylene oxidation was linearly related to the mass of cells used in the assay. All values are based on three to six replicate samples.

Effect of copper on growth. The presence of copper in the medium was shown to have a substantial effect on the growth of *Methylomonas albus* BG8. The results of typical experiments are shown in Table 1. For each experiment, identical inocula grown on medium without copper were used. In the experiments whose results are shown, the

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TABLE 1. Effect of copper supplementation on growth of *Methylomonas albus* BG8

Expt	Without Cu^{2+}		With Cu^{2+}	
	$[Cu^{2+}]^a$, μM	A_{650}	$[Cu^{2+}]^a$, μM	A_{650}
1	≤0.350	0.175[b]	5.14	0.915[b]
2	0[c]	0.140[b]	5[c]	1.05[b]
3	≤0.02	0.101[b]	11.8	0.891[b]
4	0.074	0.075[b]	11.1	0.650[b]
5	0.056	0.109[d]	10.6	0.630[b]
6	≤0.02	0.034[d]	10.8	0.479[b]
7	0.069	0.378[d]	9.22	1.055[b]

[a] Copper concentrations were determined by atomic absorption spectroscopy.
[b] Determined on a Perkin-Elmer Lambda 3B spectrophotometer at harvest after 36 to 48 h of incubation.
[c] Nominal concentration; atomic absorption measurement was not made.
[d] Determined at harvest after 96 to 112 h of incubation.

nominal copper concentration was 0 (i.e., unsupplemented), 5, or 10 μM; the concentrations measured by atomic absorption spectroscopy were tabulated. In all experiments, the mass of cells measured by absorbance obtained in the unsupplemented cultures was limited by the amount of copper in the medium. Cultures grown for 48 h without added copper achieved absorbances of 0.075 to 0.175 (experiments 1 to 4), while Cu-supplemented cultures achieved absorbances of 0.650 to 1.05 (experiments 1 to 4 and 7). While growth was limited by lack of copper in unsupplemented cultures, some growth did occur, as evidenced by the increase in absorbance, e.g., cultures in experiment 5 (Table 1) increased from an absorbance of 0.001 to 0.034 ± 0.007 in 96 h, representing approximately five divisions. The copper requirement of *Methylomonas albus* BG8 is satisfied with 5 to 10 μM Cu; 40 μM copper was inhibitory (data not shown). Differences between experiments are probably attributable to differences in the inocula used, as well as differences in incubation time (as noted in Table 1), and in the case of the unsupplemented cultures, differences in copper concentration.

Effect of copper on ICM formation and membrane protein composition. Cells grown with copper supplementation formed abundant ICM. The morphology of this ICM was typical of type I methanotrophs. ICM was not present in cells grown without copper supplementation. This phenomenon was confirmed by four independent experiments (Table 1, experiments 1, 2, 4, and 7). Typical examples are shown in Fig. 1. While cells grown without added copper lacked ICM, these cells had the typical peripheral membrane structure of *Methylomonas albus* BG8. The cytoplasmic and outer membranes, as well as the surface structures that we have previously described (6), were apparent. Additionally, electron-dense granules were present in the cytoplasm of cells grown under both conditions. Similar granules have been observed in *Methylococcus capsulatus* (11).

The overall membrane protein composition of cells grown with and without copper supplementation was evaluated. Crude membrane fractions were obtained from extracts of cells broken by French pressure cell treatment. The particulate fractions obtained at 223,000 × *g* should contain cell envelope material as well as ICM if present. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the membrane fractions of cells grown with and without copper supplementation revealed 21.8 ± 0.6- and 40.7 ± 1.0-kDa polypeptides present at high levels only in membrane fractions from cells grown with added copper (Fig. 2). This was found to be reproducible in three independent experiments (Table 1, experiments 3, 4, and 7). No major differences in the soluble fractions (Fig. 2) composed of the periplasm and cytoplasm were noted.

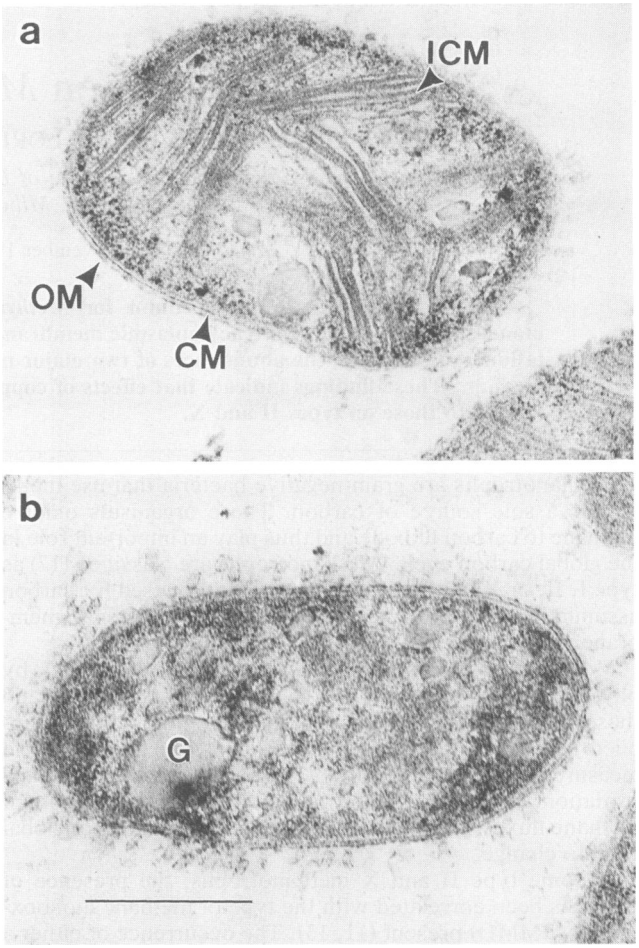

FIG. 1. Electron micrographs of ultrathin sections of *Methylomonas albus* BG8 grown with (a) and without (b) copper supplementation. ICM formed only in cells grown with copper. Outer membrane (OM), cytoplasmic membrane (CM), and granules (G) are indicated. Bar, 0.5 μM.

Effect of copper on MMO. The level of MMO activity in cells grown with and without copper supplementation was evaluated (Table 2). While we were unable to observe MMO activity in cell extracts, measurements of whole-cell MMO activity revealed that cells grown with added copper had 6.5- to 13-fold greater MMO activity. This was attributable to the presence of copper during growth. Addition of copper to the assay medium to a final concentration of 10 μM did not affect the activity of cells grown under either condition.

Bacteria respond to environmental conditions by physiological adaptations. ICM formation in the type II methanotrophs *Methylosinus trichosporium* and *Methylomonas margaritae* is affected by growth conditions (13, 16). Scott et al. (13) demonstrated that when growth was limited by oxygen, abundant ICM was formed by *Methylosinus trichosporium*. Experiments with the type X methanotroph *Methylococcus capsulatus* Bath showed that ICM presence,

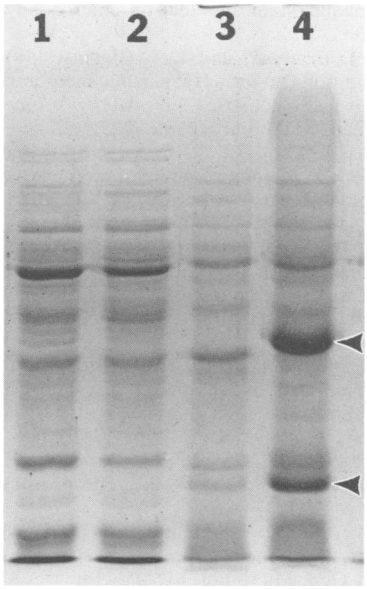

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of soluble (lanes 1 and 2) and particulate (lanes 3 and 4) fractions of cell extracts of *Methylomonas albus* BG8 grown with (lanes 2 and 4) and without (lanes 1 and 3) copper supplementation. The upper and lower arrowheads, respectively, indicate bands of 41 and 22 kDa detected only in particulate fractions from cells grown with copper.

which was correlated with the type of MMO present, was affected by copper in the growth medium (11, 15). *Methylococcus capsulatus* cells grown with sufficient copper produced a pMMO and abundant ICM; conversely, *Methylococcus capsulatus* grown with insufficient copper produced an sMMO and no ICM (11, 15). These studies with *Methylococcus capsulatus* led to reevaluation of the factors controlling the physiological responses of *Methylosinus trichosporium*. Dalton and coworkers (15) suggested that when the biomass-to-copper ratio was limited by the atmosphere used for growth, a pMMO was formed. The effect of copper on the MMO type formed by *Methylosinus trichosporium* was confirmed by studies with chemostat cultures (4). Higgins and coworkers correlated the change to a pMMO type with the appearance of a 42-kDa protein in the membranes of *Methylosinus trichosporium* OB3b (1, 5). Similarly, Prior and Dalton showed an increase in proteins of 46, 35, and 25 kDa when copper was increased in the growth medium for *Methylococcus capsulatus* (11). These findings have significance for commercial applications of methanotrophs because the pMMO and sMMO of *Methylosinus trichosporium* and *Methylococcus capsulatus* have different substrate specificities (1, 4).

TABLE 2. Effect of copper in the growth medium on MMO activity of *Methylomonas albus* BG8 cells

Trial[a]	Mean MMO activity (nmol/min/mg [dry wt]) ± SA	
	Without Cu^{2+}[b]	With Cu^{2+}[b]
1	7.73 ± 0.72	49.8 ± 3.7
2	3.16 ± 1.10	41.5 ± 9.3

[a] Trials 1 and 2 refer to experiments 5 and 6 of Table 1.
[b] Copper concentrations of growth medium determined by atomic absorption spectroscopy are reported in Table 1.

Methylomonas albus BG8 is a type I methanotroph. While copper has been shown to effect physiological changes in type II and X methanotrophs, this has not been previously reported for any type I methanotroph. In the present communication, we show that the level of copper in the growth medium affects cell growth, ICM presence, MMO activity, and two major membrane polypeptides. It is possible that these effects are manifestations of a general stress response. We demonstrate that ICM formation is directly or indirectly affected by the presence of copper in the growth medium. These studies also demonstrate that ICM is not required for growth of *Methylomonas albus* BG8 on methane. We were unable to determine any effect on the localization of the MMO because we could not detect MMO activity in vitro. On the basis of the characteristics of the enzyme measured in in vivo assays and on the basis of molecular hybridization analysis, it has been suggested that *Methylomonas albus* BG8 is unable to make an sMMO (4, 14, 15). While it appears that *Methylomonas albus* BG8 does not adapt to low-copper conditions by making an sMMO similar to that of *Methylosinus trichosporium* or *Methylococcus capsulatus*, the MMO activity of *Methylomonas albus* BG8 is affected by the level of copper present in the growth medium. This may reflect either a different form of MMO or differences in the abundance or activity of a single form of MMO. It is possible that the 21- and 40-kDa membrane proteins are subunits of MMO.

This study demonstrates that physiological responses to copper concentration are not limited to type II and X methanotrophs and do occur in a type I methanotroph, *Methylomonas albus* BG8. Moreover, since the levels of copper that cause this effect are characteristic of various environments where methanotrophs may be present, these effects should be considered when estimating rates of methane oxidation in natural environments.

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