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Phenotypic Characterization of Copper-Resistant Mutants of *Methylosinus trichosporium* OB3b

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Cultures of *Methylosinus trichosporium* OB3b grown in the presence of very low concentrations of copper synthesize a soluble methane monooxygenase (sMMO) that efficiently catalyzes the oxidation of trichloroethylene and other organic pollutants. Recently, we isolated five *M. trichosporium* OB3b mutants that express sMMO activity when grown in the presence of elevated copper concentrations (P. A. Phelps, S. K. Agarwal, G. E. Speitel, Jr., and G. Georgiou, Appl. Environ. Microbiol. 58:3701–3708, 1992). Here we show that, in contrast to the results for the wild-type cells, the addition of copper to mutant cultures grown on methane and nitrate as the nitrogen source has no noticeable effect on the growth rate and sMMO expression. In vitro experiments indicated that the copper-resistant phenotype does not arise from an increased stability of sMMO to copper deactivation. Furthermore, the mutant cultures exhibit altered speciation of copper in the extracellular fluid and have substantially decreased levels of cell-associated copper. On the basis of these results, we propose that the mutant phenotype arises from defects in copper uptake and metabolism rather than from changes in sMMO expression or enzyme stability.

Methanotrophs utilize methane as the sole carbon and energy source for growth. Certain methanotrophs have been shown to degrade trichloroethylene (TCE) and other halogenated aliphatic pollutants at rates significantly higher than those of any other microorganism (9). These bacteria are of considerable interest for the bioremediation of contaminated groundwater and air streams.

In methanotrophs, the first step in carbon assimilation is the oxidation of methane to methanol, a reaction catalyzed by methane monooxygenase (MMO). Some methanotrophs synthesize two forms of MMO: a particulate enzyme (pMMO), associated with the extensive internal membrane system of these organisms, and a soluble enzyme (sMMO) (1, 23). The sMMO exhibits much broader substrate specificity and greater catalytic activity than the particulate enzyme (7, 17, 22). Because of its broad specificity, sMMO can function as a detoxifying agent and is of great importance both ecologically and in bioremediation. sMMO-expressing cultures of *Methylosinus trichosporium* OB3b exhibit the highest known rates of biodegradation of TCE (9).

Copper has a central regulatory role in methanotrophs, affecting the synthesis of sMMO and pMMO, membrane organization, and the growth rate (13, 15, 16, 21). Cultures grown in the presence of copper produce the particulate enzyme exclusively and contain stacks of intracytoplasmic membranes which form parallel to the cell surface. In *M. trichosporium* OB3b, the presence of as little as 0.2 μM Cu(II) in the growth medium is sufficient to eliminate sMMO synthesis. Because such concentrations of copper are generally found in polluted waters (10), methanotrophs are unable to produce sMMO when grown under most conditions relevant to biotechnology and bioremediation.

The physiological and ecological significance of copper regulation is not fully understood. Green et al. (13) showed that submillimolar concentrations of Cu(I), Cu(II), Ag(I), or Cd(II) cause irreversible in vitro inactivation of sMMO from *Methylococcus capsulatus* (Bath). Four copper atoms appear to bind to the reductase (protein C) component (6) of sMMO, causing disruption of the protein structure and loss of the sulfur-iron center and of flavin adenine dinucleotide. Inactivation is very rapid, with nearly complete loss of sMMO activity occurring after 5 min of incubation with a 10-fold molar excess of copper.

We recently isolated a set of five *M. trichosporium* OB3b mutants that exhibit constitutive sMMO activity in the presence of copper (18). The mutants do not have pMMO activity and lack the characteristic stacked cytoplasmic membrane structures when grown in the presence of copper. In this report, we show that the mutations responsible for the constitutive expression of sMMO also give rise to complex phenotypes including alterations in copper speciation and uptake.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. trichosporium* OB3b was obtained from the American Type Culture Collection (ATCC 35070). A spontaneous rifamycin B-resistant colony, which exhibited good growth in liquid cultures and sMMO expression (as measured from the rate of TCE degradation) similar to that of ATCC 35070, was isolated by plating OB3b on plates containing 40 mg of rifamycin B per liter. This isolate was designated RTR and served as the wild-type control for these studies. The isolation of the copper-resistant mutants, PP311, PP319, PP323, PP333, and PP358 (ATCC 55314), has been described elsewhere (18).

Cultures were grown at ambient temperature (23°C) in triplicate either in 25 ml of medium in 250-ml screw-cap amber bottles sealed with Teflon-silicone rubber septa or in 5 ml of medium in 40-ml screw-cap vials similarly sealed. The composition of the nitrate salts medium (NSM) used

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was 5 mM Na_2HPO_4 and 5 mM KH_2PO_4 (both adjusted to pH 7.0), 10 mM $NaNO_3$, 1 mM $MgSO_4$, 100 μM $CaCl_2$, 10 μM $FeSO_4$, 10 μM MoO_3, 1 μM $MnCl_2$, 1 μM $CoCl_2$, 1 μM $ZnSO_4$, and 1 μM H_3BO_4. Methane and air (60 ml each) were injected into the culture in 250-ml bottles daily by a draw-and-fill method. Cultures in 40-ml vials received 10 ml of each gas by the same method. For cultures grown with ammonia as the nitrogen source, the medium used was NSM with 20 mM NH_4Cl substituted for the nitrate salt. Methanol-grown cells were supplemented with 1.0% (vol/vol) methanol, and only air was delivered in the draw-and-fill procedure.

Production of polyclonal antibodies. Polyclonal antibodies to the hydroxylase component (protein A) of sMMO from *M. trichosporium* OB3b were raised. The hydroxylase was purified as described by Fox et al. (11) except that the soluble cell fraction was subjected to ultrafiltration through an XM 300 membrane (Amicon) (300-kDa exclusion limit) prior to ion-exchange chromatography. The sMMO complex resided in the retentate of the ultrafiltration step. The presence of hydroxylase during purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified hydroxylase, mixed with Freund's incomplete adjuvant, was injected into a rabbit to produce antibodies. Sera were collected 1 to 2 weeks after a booster injection and were tested by Western blotting (immunoblotting) using purified hydroxylase protein and whole cells grown in the presence and absence of copper as controls.

Analytical techniques. A_{600} (measured as optical density at 600 nm [OD_{600}] centimeter^{-1}) was used to monitor growth in all experiments. The OD_{600} was related to cell dry weight as follows: an OD_{600} of 1.0 cm^{-1} was considered equal to 430 mg of cells (dry weight) per liter. Protein concentrations were assayed by the method of Bradford (3).

SDS-PAGE was performed as described by Laemmli (14). Polyacrylamide gels (12% acrylamide) were loaded with approximately 50 μg (dry weight) of whole cells, boiled in SDS-loading buffer for 5 min, and stained with Coomassie blue. Western blots were performed in the manner of Salinovich and Montelaro (19).

Copper analysis was performed with a Perkin-Elmer 303 atomic absorption spectrometer with an HGA-400 graphite furnace and a single-element copper lamp. Copper standards were prepared from copper wire dissolved in 1.0% HNO_3.

sMMO assays. MMO activity was assayed by propene oxidation (18). In some experiments, the presence of sMMO activity was determined qualitatively by the naphthalene colorimetric assay of Brusseau et al. (4) with modifications as described elsewhere (18). Quantitative determinations of sMMO activity were performed by measuring the rate of TCE degradation as reported previously (18, 20).

Copper addition to cultures expressing sMMO. To examine the effects of copper upon the wild-type and mutant OB3b strains, $CuSO_4$ was added at a final concentration of 5 μM to mid-exponential-phase cultures. Just prior to copper addition and at different times afterwards, samples were collected for naphthalene oxidation assays and gel electrophoresis.

Copper distribution. Cultures were grown as described above except for slight modifications in the composition of the NSM medium: 150 μM $MgSO_4$, 50 μM $CaCl_2$, 40 μM $FeSO_4$, and 1.5 μM $NaMoO_3$ were used instead of the concentrations indicated above. The growth medium also contained 5 μM copper, and the cells were grown at 30°C. All glassware was prewashed with 20 mM EDTA and rinsed at least three times with distilled deionized water to minimize copper contamination. Late-exponential-phase cells were harvested by centrifugation at 8,000 × *g* for 12 min, resuspended in 10 mM EDTA, and incubated for 10 min with mixing. The suspensions were centrifuged at 8,000 × *g* for 12 min, and the supernatants were saved. The cell pellets were resuspended in distilled deionized water, lysed, and separated into soluble and particulate cell fractions (38,000 × *g* for 90 min). The copper concentrations in all resultant fractions were determined.

TABLE 1. Specific growth rates at different copper levels[a]

Strain	Growth rate (h^{-1}) in Cu at:			
	0 μM	2 μM	5 μM	10 μM
RTR	0.032	0.080	0.077	0.023
PP311	0.048	0.047	0.043	ND[b]
PP319	0.053	0.059	0.049	0.025
PP323	0.063	0.047	0.046	0.021
PP333	0.056	0.044	0.049	ND
PP358	ND	ND	0.045	0.027

[a] Cells grown at 30°C.
[b] ND, not determined.

In vitro inactivation of sMMO. The susceptibility of sMMO to copper inactivation in vitro was determined as follows. Cultures were grown in NSM with or without 5 μM $CuSO_4$. Cells were harvested in mid-exponential phase, resuspended in 5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0) containing 5 mM $MgCl_2$, and lysed. Soluble fractions were prepared by centrifugation at 38,000 × *g* for 50 min and incubated with 15 and 75 μM copper at room temperature at a concentration of 2 to 3 mg of protein per ml. Samples were withdrawn at different times and diluted, and the rate of propene oxidation was measured to determine the remaining MMO activity.

RESULTS

Effects of copper on cell growth and sMMO expression. The growth of the mutants and the wild-type strain in the presence of various concentrations of copper was determined (Table 1). There were no appreciable growth rate differences among the five mutant strains under any of the conditions tested. However, a strain-dependent lag before the onset of exponential phase was evident in cultures grown in the presence of copper. This lag was a function of copper concentration and was greatest for strains PP323 and PP333. A lag was not observed with the wild-type strain (data not shown).

Cells grown in the absence of copper were used to inoculate copper-free medium. When the cultures reached mid-exponential phase (OD_{600}, ~0.4 cm^{-1}), copper was added to a final concentration of 5 μM. Wild-type cells quickly lost the ability to oxidize naphthalene (Fig. 1), a reaction catalyzed by sMMO (5). A significant decrease in the naphthalene oxidation rate was evident 24 h after the copper addition, and virtually no activity remained after 72 h. In contrast, all the mutants exhibited high rates of oxidation throughout the experiment. It should be noted that in our hands, naphthalene oxidation rates, though dependent on sMMO, do not correlate with TCE degradation rates.

Figure 2a shows the results of SDS-PAGE of the total proteins from wild-type cells harvested after the addition of 5 μM copper. Changes in the protein pattern characteristic

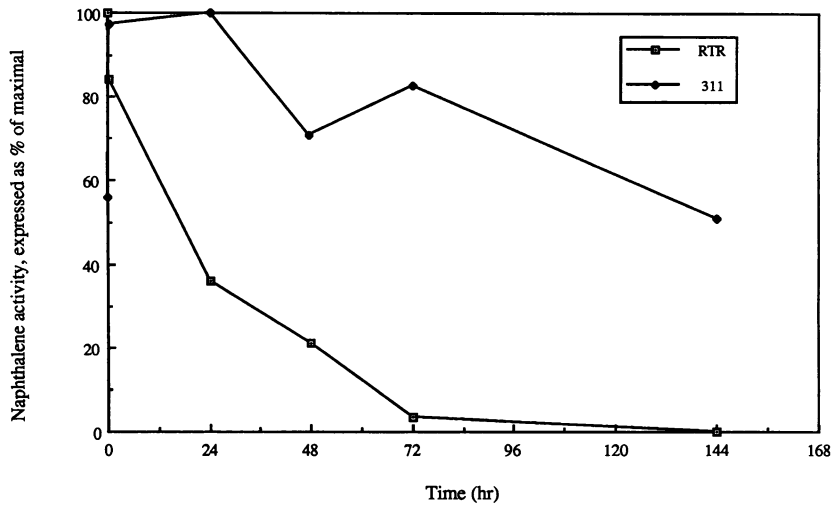

FIG. 1. Time course of sMMO activity (as shown by naphthalene oxidation, normalized by culture density) after copper addition to cultures. Data are normalized to the maximal naphthalene oxidation rate of each culture.

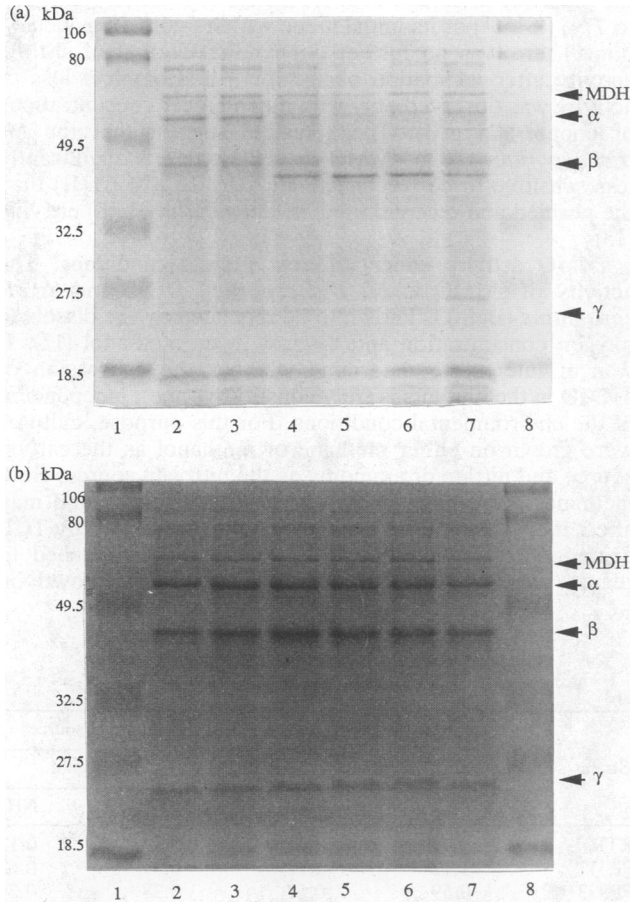

FIG. 2. SDS-PAGE of whole-cell lysates of the wild type (a) and PP311 (b), harvested following copper additions. Lanes 1 and 8, prestained molecular mass standards; lanes 2 through 7, 0, 0.5, 24, 48, 72, and 144 h after copper additions, respectively. Positions of the α, β, and γ subunits of sMMO are indicated. MDH, methanol dehydrogenase.

of the switch from the expression of sMMO to pMMO (5, 8), such as the disappearance of 57- and 45-kDa bands (corresponding to the α and β subunits of the sMMO hydroxylase component) and the appearance of an intense band at 43 kDa, were essentially complete within 24 h. In agreement with these observations, only a very weak signal was detected in Western blots of samples collected 24 h after copper addition and probed with antibodies raised against hydroxylase (Fig. 3a). The disappearance of the hydroxylase within a day after copper addition could not have been solely a result of repression of sMMO synthesis and subsequent dilution due to growth. The biomass increased by threefold by 72 h, and even if the sMMO that was initially present was diluted accordingly, it should still have been detectable in the Western blots. Thus, it appears that sMMO is subjected

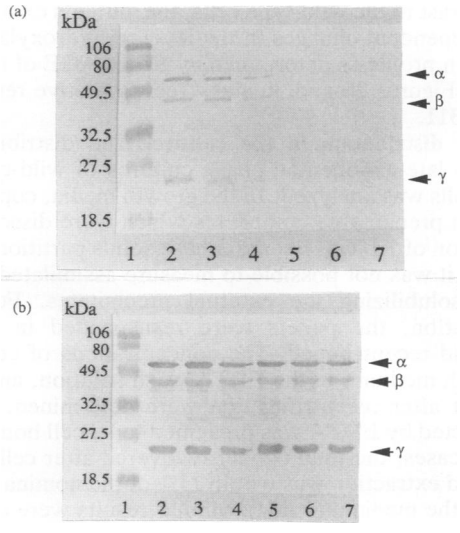

FIG. 3. Western blots of whole-cell lysates of the wild type (a) and PP311 (b), harvested during in vivo copper challenge. Lane 1, prestained molecular mass standards; lanes 2 through 7, 0, 0.5, 24, 48, 72, and 144 h after copper additions, respectively. Positions of the α, β, and γ subunits of sMMO are indicated.

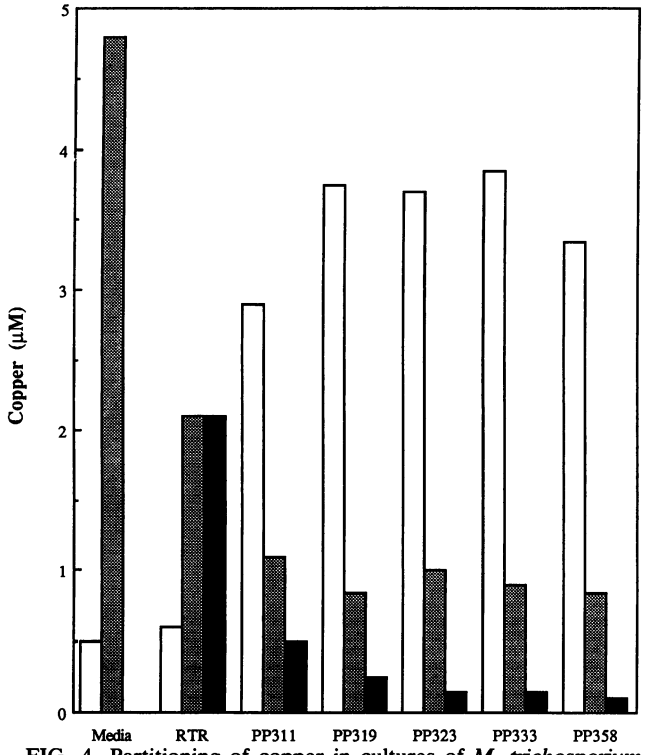

FIG. 4. Partitioning of copper in cultures of *M. trichosporium* OB3b grown to an OD_{600} of 0.8 cm^{-1} in NSM supplemented with 5.0 μM Cu. Copper in the medium (white bars), copper extracted from the pellet with EDTA (shaded bars), and residual particulate copper (black bars), which was assumed to be intracellular, are shown. Standard deviation of measurements, ±3%.

to proteolytic degradation after the addition of copper. Similar results were obtained in Western blots probed with antisera against the protein B and protein C subunits, indicating that all the subunits of the sMMO holoenzyme disappear in a coordinate fashion (data not shown).

In contrast to the wild-type cells, the mutants exhibited no copper-dependent changes in the level of hydroxylase or in the protein profile as determined by SDS-PAGE of total cell extracts. Figures 2b and 3b show representative results for strain PP311.

Copper distribution in the culture. The distribution of copper in late-exponential-phase cultures of wild-type and mutant cells was analyzed. In the growth media, copper was present in precipitable complexes which were dissolved by the addition of EDTA. Because these solids partitioned with the cells, it was not possible to measure assimilated copper without solubilizing the external precipitates. Following centrifugation, the pellets were resuspended in 10 mM EDTA and recentrifuged. The concentrations of copper in the growth medium, in the EDTA wash solution, and in the cell pellet after recentrifugation were determined. Copper not extracted by EDTA was presumed to be cell bound (Fig. 4). In all cases, the total copper recovered after cell precipitation and extraction was within 15% of the nominal copper added to the medium (5 μM). Similar results were obtained for cultures grown with 2.0 and 10.0 μM Cu(II), but at the lower concentration the difference between the wild-type and mutant strains was less pronounced (data not shown).

Following EDTA extraction, pelleted cells were lysed and separated into particulate (membrane) and supernatant fractions by ultracentrifugation. The concentrations of copper in the two fractions are shown in Table 2. Care was taken to minimize any cross-contamination during cell fractionation. An indication of the lack of cross-contamination is that no sMMO from the soluble fraction could be detected in the particulate fraction.

TABLE 2. Copper distribution within cells

Strain	Concn of copper present (μM/mg of cells [dry wt])		Ratio, particulate Cu/soluble Cu
	Soluble	Particulate	
RTR	0.228	2.02	8.6
PP311	0.306	0.698	3.3
PP319	0.166	0.21	1.3
PP323	0.084	0.242	2.9
PP333	0.144	0.264	1.8
PP358	0.094	0.31	3.3

Copper inactivation of sMMO in vitro. To examine the effect of copper on the sMMO from *M. trichosporium* OB3b, different concentrations of Cu(II) were added to the soluble fraction of cell lysates and the inactivation of the enzyme was monitored by propene oxidation. The results for all strains, both the mutants and the wild-type, were similar. In the presence of 15 μM copper, the sMMO activity dropped to 77% (±15%) of its initial value within 5 min after incubation. There was no further copper inactivation of sMMO activity after incubation on ice for 3 h. Complete loss of activity was observed only with much higher concentrations of copper (75 μM). These results suggest that the *M. trichosporium* OB3b sMMO in cell lysates is significantly less sensitive to copper inactivation by 10 μM Cu(II) than the purified and reconstituted *M. capsulatus* (Bath) enzyme (13).

sMMO activity under different growth conditions. The activity of sMMO in *M. trichosporium* OB3b and in *M. capsulatus* (Bath) is known to be regulated by the dissolved oxygen concentration and by growth on methanol (12). It was of interest to determine whether the expression of sMMO in the mutants is truly constitutive, i.e., independent of the environmental conditions. For this purpose, cultures were grown on either methane or methanol as the carbon source and nitrate or ammonia as the nitrogen source. Both methanol and ammonia are substrates of sMMO and may affect its expression. sMMO activity was measured by TCE degradation assays using cells that had been washed to remove residual ammonia or methanol (Table 3). Growth on

TABLE 3. sMMO activity in cultures grown on various substrates

Strain	sMMO activity with the indicated C and N source[a]			
	Methane		Methanol	
	NO_3	NH_4	NO_3	NH_4
RTR	0.45	0.26	0.14	0.13
PP311	1.12	1.12	0.39	0.62
PP323	0.59	0.51	0.38	0.39
PP333	1.12	1.18	0.44	0.49
PP358	1.54	1.52	0.66	0.41

[a] Activity is expressed for cultures grown with methane and nitrate as the TCE degradation first-order rate constant. All values are expressed as liters per milligram of cells (dry weight) per day, with standard deviations of less than ±0.1 liter per milligram of cells (dry weight) per day.

methane and ammonia did not affect the sMMO activity in any of the mutants but resulted in a statistically significant (n = 12) decrease in the activity found in wild-type cultures. In methanol-grown cultures, the sMMO specific activity was substantially reduced. However, strain PP358, when grown on methanol, had specific activities comparable to those of the wild-type cells grown on methane.

DISCUSSION

The five strains we isolated previously (18) have high levels of sMMO activity when grown in the presence of copper and are the only known stable *M. trichosporium* OB3b mutants exhibiting altered expression of MMOs. The experiments described in this paper reveal several aspects of the copper metabolism, sMMO expression, and sMMO activity in these strains. It was shown that the mutants maintain sMMO activity for long periods (at least 6 days) when challenged with copper. In contrast to the wild-type *M. trichosporium* OB3b, the growth rates of these strains did not increase upon copper addition and there were no apparent changes in the protein profile. Thus, the mutant strains failed to demonstrate any of the dramatic physiological changes that occur when cultures of OB3b are exposed to copper.

In the standard media used to grow *M. trichosporium* OB3b, most of the copper is present in complexes that can be solubilized by EDTA. However, growth of the mutant strains in these media resulted in a drastic increase in nonprecipitable-copper content. The solubilization of copper in extracellular media suggests that *M. trichosporium* OB3b excretes a Cu(II)-complexing agent(s), perhaps analogous to siderophores in Fe(III) metabolism (2). If this is the case, the increase in soluble copper in the mutant cultures may be due either to the overproduction of such agents or to a defect that prevents the cells from internalizing the complexes with copper.

The copper found in the cell pellet after extraction with EDTA (to solubilize inorganic complexes) is presumably tightly associated with the biomass. The levels of cell-associated copper in the mutant strains were substantially lower than in the wild-type. The maximum difference (almost 20-fold less copper) was observed with strain PP358. In the wild-type cells, most of the copper was found in the particulate fraction, where pMMO is found. In the mutants, which produce neither pMMO nor intracellular membranes (18), the cell-associated copper was more evenly distributed between the particulate and the soluble fractions of cell lysates.

The fact that an appreciable amount of copper is found in the soluble cell fraction raised the question of why sMMO is active in the mutants, since previous in vitro studies (13) had concluded that the enzyme loses its activity rapidly when exposed to copper concentrations comparable to those found in the soluble fraction (Table 2). However, we found that, contrary to the results of Green et al. (13), sMMO in cell lysate soluble fractions retained 77% (±15%) of activity when incubated with moderate copper concentrations. Thus, the sustained sMMO activity in the mutants is not inconsistent with the detection of copper in the soluble cell fraction.

The results of these studies suggest two hypotheses to explain the mutant phenotypes. First, there may be a defect in copper metabolism that affects copper assimilation. This hypothesis is supported to some extent by the solubilization and accumulation of external copper in mutant but not in wild-type cultures. However, it does not explain the markedly greater sMMO activity that was observed in the mutant strains (18). Also, the sMMO activity of the mutants was not affected by the growth with ammonia as the nitrogen source (Table 3), and this effect is not likely to be related to defects in copper uptake. A second possibility is that the mutation(s) may affect a more general, copper-dependent regulatory system which may be responsible for several cellular functions such as copper uptake, pMMO induction, sMMO repression, and production of intracellular membranes.

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REFERENCES

1. **Anthony, C.** 1986. Bacterial oxidation of methane and methanol. Adv. Microb. Physiol. **27**:113–209.
2. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51**:509–518.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brusseau, G. A., H. C. Tsien, R. S. Hanson, and L. P. Wackett.** 1990. Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation **1**:19–29.
5. **Burrows, K. J., A. Cornish, D. Scott, and I. J. Higgins.** 1984. Substrate specificities of the soluble and particulate methane monooxygenases of *Methylosinus trichosporium* OB3b. J. Gen. Microbiol. **130**:3327–3333.
6. **Colby, J., and H. Dalton.** 1979. Characterisation of the second prosthetic group of the flavoenzyme NADH-acceptor reductase (component C) of the methane mono-oxygenase from *Methylococcus capsulatus* (Bath). Biochem. J. **177**:903–908.
7. **Colby, J., D. I. Stirling, and H. Dalton.** 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate n-alkanes, n-alkenes, ethers and alicyclic, aromatic and heterocyclic compounds. Biochem. J. **165**: 395–402.
8. **Davis, K. J., A. Cornish, and I. J. Higgins.** 1987. Regulation of the intracellular location of methane mono-oxygenase during growth of *Methylosinus trichosporium* OB3b on methanol. J. Gen. Microbiol. **133**:291–297.
9. **Ensley, B. D.** 1991. Biochemical diversity of trichloroethylene metabolism. Annu. Rev. Microbiol. **45**:283–299.
10. **Forstner, U., and G. T. W. Wittman.** 1979. Metal pollution in the aquatic environment, p. 356. Springer-Verlag, New York.
11. **Fox, B. G., W. A. Froland, J. E. Dege, and J. D. Lipscomb.** 1989. Methane monooxygenase from *Methylosinus trichosporium* OB3b. J. Biol. Chem. **264**:10023–10033.
12. **Graham, D. W., J. A. Chaudhary, R. S. Hanson, and R. G. Arnold.** 1993. Factors affecting competition between Type I and Type II methanotrophs in two-organism, continuous-flow reactors. Microb. Ecol. **25**:1–17.
13. **Green, J., S. D. Prior, and H. Dalton.** 1985. Copper ions as inhibitors of protein C of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). Eur. J. Biochem. **153**:137–144.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Leak, D. J., and H. Dalton.** 1986. Growth yields of methanotrophs. 1. Effect of copper on the energetics of methane oxidation. Appl. Microbiol. Biotechnol. **23**:470–476.
16. **Park, S., M. L. Hanna, R. T. Taylor, and M. W. Droege.** 1991.

Batch cultivation of *Methylosinus trichosporium* OB3b. I. Production of soluble methane monooxygenase. Biotechnol. Bioeng. **38:**423–433.

17. **Patel, R. N., C. T. Hou, A. I. Laskin, and A. Felix.** 1982. Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium* sp. strain CRL-26. Appl. Environ. Microbiol. **44:**1130–1137.
18. **Phelps, P. A., S. K. Agarwal, G. E. Speitel, Jr., and G. Georgiou.** 1992. *Methylosinus trichosporium* OB3b mutants having constitutive expression of soluble methane monooxygenase in the presence of high levels of copper. Appl. Environ. Microbiol. **58:**3701–3708.
19. **Salinovich, O., and R. Montelaro.** 1986. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by SDS-PAGE. Anal. Biochem. **156:**341–347.
20. **Speitel, G. E., Jr., R. C. Thompson, and D. Weissman.** 1993. Biodegradation kinetics of *Methylosinus trichosporium* OB3b at low concentrations of chloroform in the presence and absence of enzyme competition by methane. Water Res. **27:**15–24.
21. **Stanley, S. H., S. D. Prior, D. J. Leak, and H. Dalton.** 1983. Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: studies in batch and continuous cultures. Biotechnol. Lett. **5:**487–492.
22. **Stirling, D. I., and H. Dalton.** 1979. Properties of the methane mono-oxygenase from extracts of *Methylosinus trichosporium* OB3b and evidence for its similarity to the enzyme from *Methylococcus capsulatus* (Bath). Eur. J. Biochem. **96:**205–212.
23. **Tsien, H.-C., and R. S. Hanson.** 1992. Soluble methane monooxygenase component B gene probe for identification of methanotrophs that rapidly degrade trichloroethylene. Appl. Environ. Microbiol. **58:**953–960.