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DEVELOPMENT OF COPPER-TOLERANT MUTANT OF SOLUBLE METHANE MONOOXYGENASE-PRODUCING METHANOTROPHS FOR APPLICATION IN BIOREMEDIATION

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The objective of this research was to create copper-tolerant soluble methane monooxygenase (sMMO)-producing methanotrophs capable of producing high sMMO activity for the degradation of trichloroethylene (TCE). Two methanotrophic strains were utilized for mutagenesis, *Methylosinus trichosporium* OB3b and *Methylosinus sporium* 2C10P. The work utilized three different mutagenesis systems that included dichloromethane mutagenesis; transposon mutagenesis; and UV mutagenesis. The data gathered were the measurement of chlorinated aliphatic transformation, growth kinetics, and calculation of sMMO activity. The dichloromethane mutagenesis method successfully produced copper-tolerant mutagenes. The mutants developed were able to stably express sMMO in the presence of copper levels up to 10 μM at approximately 50-80% of their maximal sMMO levels when cultivated in the absence of copper.

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PREFACE

This report summarizes research activities conducted by the Center for Environmental Biotechnology of the University of Tennessee under sub-contract through Batelle, Research Triangle Park, North Carolina for the Armstrong Laboratory, Environics Directorate (AL/EQ), Tyndall AFB, Florida "Development of Copper-Tolerant Mutants of Soluble Methane Monooxygenase Producing Methanotrophs for Application in Bioremediation" between 1 July 1993 and 31 October 1994.

The work activities summarized in this report have been conducted by the following researchers: John P. Bowman (Center for Environmental Biotechnology, University of Tennessee) and Gary S. Sayler (Department of Microbiology, Graduate Program in Ecology, and the Center for Environmental Biotechnology, University of Tennessee). The Project Officer was Alison Thomas (Armstrong Laboratory).

EXECUTIVE SUMMARY

A. OBJECTIVE

The overall goal of this work was to create copper-tolerant sMMO-producing methanotrophs able to produce high sMMO activity in the presence of copper ions. The mutants would be utilized in aboveground bioreactors and *in situ* systems for chlorinated aliphatic bioremediation of polluted aquifers.

B. BACKGROUND

Removal or mitigation of volatile organic compounds such as trichloroethylene (TCE) from groundwater using bioremediation technologies have been investigated over the last decade (Shannon and Unterman, 1993). One strategy involves utilization of methanotrophs (methane-utilizing bacteria) which are able to efficiently co-metabolize trichloroethylene to nontoxic metabolites. The enzyme responsible for the cometabolism of TCE by methanotrophs is soluble methane monooxygenase (sMMO). This enzyme converts methane to methanol, however due to its low level of substrate specificity, sMMO can also co-oxidize a wide range of compounds including aliphatic, alicyclic, heterocyclic, and aromatic compounds. The synthesis of sMMO is repressed in the presence of cupric or cuprous ions; instead a membrane-bound form of methane monooxygenase is formed, referred to as particulate methane monooxygenase (pMMO). This enzyme has a narrower substrate specificity and thus does not transform TCE efficiently with a catalytic rate only about 0.05% of sMMO. Due to the potential suppressive effects of copper ions on sMMO and thus on TCE transformation efficiency, strategies must be developed to counter problems that arise when copper containing groundwater requires treatment. This research follows the construction and demonstration of a bench-scale, two-stage bioreactor for TCE removal. In

this system a copper-tolerant sMMO-producing mutant of *Methylosinus trichosporium* designated PP358 was utilized. This strain was obtained under a materials transfer agreement from Dr. G. Georgiou, Department of Chemical Engineering, The University of Texas, Austin, Texas. In this study copper tolerant mutants will be developed for use by the U. S. Air Force in bioremediation.

C. SCOPE

This report encompasses work conducted at the Center for Environmental Biotechnology during the period from 1 July 1993 through to 31 October 1994. The work utilized three different mutagenesis systems. These included: (1) dichloromethane mutagenesis; (2) transposon mutagenesis; and (3) UV mutagenesis. Subsequent work involved characterizing the mutants.

D. METHODOLOGY AND TEST DESCRIPTION

Two methanotrophic strains were utilized for mutagenesis as they grew robustly and rapidly on methanol, the necessary carbon and energy source required to allow the mutagenesis to be effective. Three mutagenesis procedures were attempted to obtain mutants which were able to form sMMO in the presence of high copper ion levels. Mutagenesis systems included a dichloromethane-based system, another involved exposure to ultraviolet light, the final system utilized a molecular approach involving transposable element insertion. *In situ* plate screening for sMMO-producing colonies was use for mutant screening. Subsequent analyses were used to confirm mutant stability, growth characteristics, sMMO activity and ability to transform chlorinated aliphatic compounds.

E. RESULTS

Dichloromethane mutagenesis was used to obtain seven copper-tolerant sMMO-producing mutants of either *Methylosinus trichosporium* OB3b or *Methylosinus sporium* 2C10P (a strain

isolated from TCE contaminated aquifer sediments). Transposon and UV mutagenesis techniques did not succeed in obtaining mutants. Growth conditions were defined to allow maximal growth of the mutants and it was found for maximal growth that supplementation of the mineral salts medium was required. Supplements which were effective in boosting growth included yeast extract and certain vitamins. The mutants obtained had maximal sMMO activities 60-80% of their respective wild-type sMMO activities when grown in the absence of copper. In the presence of copper sMMO was also formed though it was partially repressed while the sMMO activities of wild-type strains in the same cultural conditions was completely repressed. Transformation rates of chlorinated aliphatics, including vinyl chloride, dichloromethane, cis-1,2-dichloroethylene, chloroform, trichloroethylene and 1,1,1-trichloroethane, were tested singly and as a mixture, and corresponded with the relative sMMO activity of the mutants.

F. CONCLUSIONS

1. Dichloromethane mutagenesis was effective in obtaining copper-tolerant sMMO-producing methanotroph mutants while other procedures including transposon mutagenesis using Tn5, Tn10, and Tn1721 delivery systems and using UV mutagenesis was not successful.
2. The copper-tolerant mutants were found to grow maximally in nitrate mineral salts media supplemented with yeast extract and a number of vitamins allowing growth equivalent to the parent wild-type strains.
3. The mutants developed were able to stably express sMMO in the presence of copper levels up to 10 μM at approximately 50-80% of their maximal sMMO levels when cultivated in the absence of copper.

4. The mutants were able to transform several chlorinated aliphatic compounds at a rate slightly less than the parent wild-type strains.

G. RECOMMENDATIONS

The copper-tolerant mutant strains developed in this investigation are suitable for research and application in TCE bioremediation. Further research will be required to obtain improved mutants for TCE bioremediation. A mutagenesis scheme needs to be developed which is able to increase the probability of obtaining stable mutants of specific phenotype, i.e. lacking pMMO activity. Most procedures available today, including DCM mutagenesis, are generally not adequate for selective mutagenesis of methanotrophs which have a molecular biology which still remains largely unknown. The mutants should not require medium supplementation for attaining good growth rates and should over-express sMMO, assuming requirements for reducing equivalents can be met.

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SECTION I

INTRODUCTION

A. OBJECTIVES

The overall goal of this work was to create copper-tolerant sMMO-producing methanotrophs able to produce high sMMO activity in the presence of copper ions. The mutants would be utilized in aboveground bioreactors and *in situ* systems for chlorinated aliphatic bioremediation of polluted aquifers.

B. BACKGROUND

Efficient and rapid treatment of TCE employing methanotrophic cometabolic strategies still face a number of problems. These problems include implementation of methane as the carbon and energy source; competitive inhibition of TCE transformation by methane; TCE-induced toxicity; and sMMO expression stability. In the latter case sMMO expression is dictated by several factors. The most critical of these is copper availability. Research has indicated that copper suppression of sMMO with simultaneous induction of pMMO activity depends on the relative availability of copper ions in relation to the biomass concentration. Eventually intracellular and extracellular compartmentalization of copper reaches an equilibrium at which the sMMO and pMMO levels have stabilized at certain points. For the purposes of TCE cometabolism, it is preferred that the transforming methanotrophic population utilized has a high sMMO level. Keeping sMMO stably expressed is problematic if TCE-contaminated groundwater has copper levels exceeding 0.1 μM (6.4 μg/L). By developing mutants that can maintain sMMO

activity in the presence of high copper ion concentrations is a useful strategy in avoiding this potential problem.

Copper-tolerant sMMO-producing mutants of *M. trichosporium* OB3b have been produced using dichloromethane mutagenesis (Nicolaidis & Sargent, 1985; McPheat et al. 1987) relatively recently (Phelps et al. 1992; Fitch et al. 1993). These mutants were found to partition copper from the medium into their cytosol unlike wild-type cells which tended to accumulate copper in their membrane fractions. Since pMMO is associated with methanotroph intra-cytoplasmic membrane (ICM) it was postulated by Fitch et al. (1993) that genes disrupted by the mutagenesis were associated with copper metabolism in cells. Recent research also has shown pMMO has an active site composed of a triplanar arrangement of copper molecules (Lidstrom, M. E. and colleagues, California Institute of Technology, Pasadena, Calif., unpublished). Additionally ICM formation seems to be directly dependent on copper availability suggesting additional genes are activated by copper ions leading to ICM formation. DCM mutagenic affects leading to nonrepression of sMMO by copper appears to involve inactivation of genes involved either in copper, transport, the copper activation of pMMO or in ICM formation.

Overall, the DCM mutagenesis procedure was utilized in this study to attempt to obtain additional pMMO-inactive mutants for utilization in TCE- cometabolic based bioremediation. Additionally transposon mutagenesis and UV mutagenesis were utilized in attempts at obtaining mutants. The rationale being that mutants formed by these procedures may produce different phenotypes which may have better growth characteristics, enzyme activity, stability or copper tolerance that contribute to more efficient use in bioremediation applications. It was further

attempted to obtain an improved form of mutants, more robust and less fastidious than mutants obtained previously developed by Phelps et al. (1992).

C. SCOPE

Research to develop copper-tolerant strains of sMMO-producing methanotrophs was conducted at the University of Tennessee, Center for Environmental Biotechnology during the period of 1 July 1993 through 31 October 1994. The work was conducted using two wild-type, Type II methanotroph strains, *Methylosinus tricosporium* OB3b and *Methylosinus sporium* 2C10P. These strains were subjected to chemical, ultra violet light and transposon mutagenesis procedures. Recovered mutants were characterized for copper tolerance, growth characteristics, MMO production and ability to degrade chlorinated aliphatics.

SECTION II

MATERIAL AND METHODS

A. STRAINS AND CULTIVATION

The strains which underwent mutagenization included the sMMO-producing group 11 methanotrophs, ***Methylosinus trichosporium OB3b*** (= ATCC 35070) and *Methylosinus sporium* strain 2C10P. Strain 2C10P was isolated from groundwater from the Savannah River Laboratory Integrated Demonstration Site (Bowman et al., unpublished). The strains were routinely cultivated in nitrate mineral salts (NMS) medium (Bowman & Sayler 1993) with either methane (provided as a 1:4 methane:air atmosphere) or 0.5% (v/v) methanol as sole carbon sources at 28°C. The NMS medium and its constituents and supplements are detailed in Table 1.

TABLE 1: NITRATE MINERAL SALTS MEDIUM USED FOR METHANOTROPH CULTIVATION AND MUTAGENISIS

Constituent	Concentration
$NaNO^3$	2 mM
Phosphate buffer, pH 6.8	2 mM
$MgCl_2.2H_2O$	150 μM
Ferric EDTA	50 μM
$CaCl_2.2H_2O$	50 μM
$MnSO_4.4H_2O$	2 μM
$ZnSO_4.7H_2O$	2 μM
H_3BO_3	2 μM
K_2SO_4	1 μM
Kl	1 μM
$CoCl_2.H_2O$	0.65 μM
$Na_2MoO_4.2H_2O$	0.4 μM
Yeast extract (optional)	0.02% (weight/volume)
Vitamin solution (optional)[a]	1 ml
Agar (optional)	1.5% (weight/volume)
Vitamin stock solution:	
d-biotin	5 mg/L
pyridoxine	2 mg/L
cyanocobalamine	0.1 mg/L

[a]The vitamin solution was sterilized using 0.2 μm filters and added to the medium cooled to 50°C following autoclave sterilization.

B. MUTAGENESIS EXPERIMENTS

1. Dichloromethane Mutagenesis

Strains were cultivated in NMS media with 0.5% (v/v) methanol for 3 days. Culture was serially diluted from 10^{-4} to 10^{-8} in NMS media and spread plated onto 0.5% (v/v) methanol NMS agar plates (1.5% weight/volume agar). Two sets of methanol-NMS plates were inoculated with one set supplemented with 0.02% (weight/volume) while the second set was un-supplemented. The plates were then incubated in 10 Liter desiccators at 28°C. Every 3 days, 0.6 mL of dichloromethane and 1.0 mL of methanol were allowed to volatilize in the desiccator. This was done by adding the solvents together in an open vial which was placed into the desiccator. Incubation then proceeded for 5-7 weeks with continual replenishment of the dichloromethane and methanol.

2. Transposon Mutagenesis

An attempt was made to create copper tolerant mutants by disrupting either pMMO or copper metabolism gene function using three different transposons. The transposons used included Tn5, Tn10 and Tn1721 systems (Table 2). The Tn5 and Tn10 plasmids carried on pUTHg and pLOFHg (Herrero et al. 1990), respectively, confer both mercury resistance and ampicillin resistance. Tn1721 was located on pUCD623 which confers tetracycline resistance and codes for *lux* genes (Shaw et al. 1988).

a. Transformation of E *coli* S17 (λ*pir*) with pUTHg and pLOFHg. *E. coli* S17 (λ*pir*) was suspended in transformation buffer to which was added 1 μg plasmid DNA. The

suspensions were initially incubated on ice for 30 minutes, then held at 42°C for 2 minutes and finally at 37°C for 10 min. LB broth at 50 μL was added to the suspension which was then incubated at 37°C. The suspension was then plated out onto LB supplemented with 10 μg/mL $HgCl_2$ and 50 μg/mL ampicillin and incubated at 37°C overnight. Transformants, checked with control plates lacking ampicillin, were restreaked onto LB supplemented with 10 μg/ml $HgCl_2$ and 50 μg/ml ampicillin.

b. Conjugation of pUTHg and pLOFHg to methanotrophs. Plasmids pUTHg and PUTLOF from *E.coli* S17*(λpir)* were transferred into the methanotroph test strains OB3b and 2C10P using triparental mating. The donor *E. coli* strain, a mobilization strain *E.coli* pRK2013 (grown in LB broth with 50 μg/ml kanamycin) (Figurski and Helsinki, 1979) and the methanotroph recipient strain were mixed in a 1:1:10 proportion and filtered onto sterile 0.45 μm polycarbonate filters. Filters were then placed onto Luria Broth agar medium (per 1-Liter distilled water: 10 grams tryptone, 5-gram yeast extract, 10 gram NaCl, 15 gram agar, pH 7.5) and incubated for 1 hour. The filters were then transferred to R2A agar plates under 1:1 methane:air atmosphere and incubated 24 hours. Finally, the filters were transferred to NMS agar with 0.02% (weight/volume) yeast extract and incubated for 4 days under a 1:4 methane:air atmosphere. Cell material on the filters was then removed using distilled water and washed via centrifugation. Cell material was serially diluted (10^0 - 10^{-3}) and spread plated onto media. For the mini-Tn5 and Tn10 mutagenesis the selective media used was NMS supplemented with 10 μg/mL $HgCl_2$ and 50 μg/mL ampicillin. For the Tnl721 mutagenesis, NMS was supplemented with 75 μg/mL tetracycline. All plates for these experiments were incubated at 28°C. Colonies appearing on these plates were screened as indicated below.

TABLE 2: STRAINS USED AND OBTAINED IN THIS STUDY

Strain	Feature	Reference
methylosinus trichosporium OB3b(= ATCC 35070)	sMMO producing type II methanotroph	Bowman et al. 1993
Methylosinus sporium 2C10P	sMMO producing type II methanotroph from TCE contaminated subsurface sediments	Bowman et al. unpubl.
E. coli SM10 (λpir)	pUTHg (Tn10 - HgR, ApR)	Herrero et al. 1990
E. Coli SM10 (λpir)	pLOFHg (Tn5 - HgR, ApR)	Herrero et al. 1990
E. Coli S17 (λpir)	Recipient for pUTHg & pLOFHg	C.A. Lajoie
E. coli DF1020	pRK2013 (for plasmid mobilization)	Figurski & Helsinki, 1979
E. Coli HB101	pUCD623 (Tn1721 - TcR, lux)	Show et al. 1988
M. Trichosporium JB22	DCM mutant from OB3b	This study
M. Trichosporium JB36	DCM mutant from OB3b	This study
M. Sporium JB69	DCM mutant from 2C10P	This study
M. Trichosporium JB208	DCM mutant from OB3b	This study
M. Sporium JB227	DCM mutant from 2C10P	This study
M. Sporium JB309	DCM mutant from 2C10P	This study

3. UV Mutagenesis

Short wavelength (245 nm) ultraviolet light induced mutagenesis was utilized to attempt to produce copper-tolerant sMMO-producing methanotroph mutants. Test strains were serially diluted with distilled water (10^0 - 10^{-5}) and spread plated onto 0.5% methanol NMS plates supplemented with 0.02% (weight/volume) yeast extract and exposed to different exposure levels of UV light using a 115 volt UV light chamber (Raytech Inc., Stafford Springs, Conn.). Following exposures plates were immediately transferred to black plastic bags to ensure photoactivated DNA repair mechanisms were not initiated. Varying exposure times was performed in order to create a lethality curve from which a LD_{50} could be obtained and thus utilized for optimal mutagenesis conditions. Following determination of the exposure required to kill 50% of the cells this exposure time was utilized in subsequent experiments to obtain mutants. Colonies arising on plates following exposure to UV were screened as indicated below.

C. SCREENING

Colonies obtained after the various different mutagenesis procedures were transferred to NMS agar supplemented with 0.02% (weight/volume) yeast extract via replicate plating and grown with methane as the sole carbon source. Replica plating was performed using sterile and disposable plate replicators available from FMC BioProducts (Rockland, Maine). The expression of sMMO by colonies arising on these plates were rapidly screened by an in-situ plate assay (Graham et al. 1992) and confirmed by assaying liquid NMS cultures with the naphthalene oxidation assay (Koh et al. 1993). The stability of the dichloromethane-resistant phenotype was

evaluated by plating the putative mutant onto methanol NMS agar and incubating under dichloromethane and methanol, as indicated above.

In situ plate screening for sMMO-producing colonies involved firstly covering colonies with a 5 mL overlay consisting of a 1% naphthalene solution in 20% Igepal CP-30 surfactant (Sigma, St. Louis, Miss.) diluted 1:50 into 1% SeaPlaque low melting point agarose (FMC Bioproducts, Rockland, Maine) held at 45°C. Plates were then incubated for 1-4 hour under a 30-50% O_2 atmosphere in a desiccator. Naphthol was then detected by adding a 0.2% (weight/volume) solution of tetraotized o-dianisidine. Colonies producing sMMO change to a pink-red color.

D. sMMO ACTIVITY ASSAY

Cell suspensions (2-5 ml) were diluted or concentrated via centrifugation to an absorbance of 0.2 (at 600 nm) and added to an equal volume of saturated naphthalene (30 mg/L) solution. The suspension was then incubated on **an orbital shaker for 1 hour. Approximately 100 µl of a 0.2%** (weight/volume) solution of tetraotized *o*-dianisidine was then added and the absorbance was measured at a wavelength of 540 nm on a DU-8 spectrophotometer. The sMMO activity was calculated from the naphthol formation rate and is given as nmol of naphthol formed/h/mg of cells (Brusseau et al. 1990-1 Koh et al. 1993).

E. CHLORINATED ALIPHATIC TRANSFORMATION ASSAYS

Cell suspensions (2-5 mL) were diluted or concentrated via centrifugation to an absorbance of 0.2 (at 600 nm) and placed into 32-mL glass vials with caps fitted with teflon-lined silicone rubber seals. Various concentrations of chlorinated aliphatics were added as saturated solutions or

as a mixed solution of different compounds (see below). Vials were then incubated at 28°C, with shaking before analysis. At the time of analysis vials were sacrificed with degradation terminated by the addition of 100 µl of 1 M NaOH. An internal standard, 5 mg/L 1,2-dibromoethane was then added.

Quantitative measurement of chlorinated aliphatic transformation was done by head-space analysis using a gas chromatograph equipped with an electron capture detector and a 60 m x 0.53 mm i.d. RTX Volatiles column (Restek Corp., Bellefonte, Penn.). GC conditions were as follows: column and detector temperatures were maintained at 120°C and 300°C, respectively while nitrogen was used as the carrier gas (flowrate 10 mL/min). Using a gas-tight syringe from 5-10 µl of vial head space was injected into the GC and the resultant peak was compared to a standard curve previously determined using killed cell biomass. The 1,2-dibromoethane internal standard was utilized to correct for injection variations and a killed control was always included to account for loss of compound due to adsorption to cell biomass.

F. GROWTH KINETIC MEASUREMENTS

The copper-tolerant mutants and comparison strains (OB3b and PP358) were cultivated on several different media to determine specific growth rates. The media used included those supplemented or unsupplemented with various factors that may affect sMMO activity including: copper sulfate, yeast extract, and vitamins. Media dispensed into 150 ml serum vials to a volume of 25 ml were inoculated with strains to an OD (600 nm) of 0.01. Samples of 0.5 mL were removed using a syringe at regular intervals. Vials were prepared in triplicate. Specific growth rates were determined from data plotted on logarithmic charts.

G. TCE AND MIXED CHLORINATED ALIPHATIC TRANSFORMATION EXPERIMENTS

The transformation efficiency of mutants was assessed by quantifying their ability to transform a mixture of chlorinated aliphatics over time compared to the wild-type strains. Cell suspensions were prepared as indicated for the single chlorinated aliphatic transformation experiments. The chlorinated aliphatic mixture was added such that the initial aqueous concentration were all at 2 mg/L and included: vinyl chloride, dichloromethane, cis-1,2-dichloroethylene, chloroform, trichloroethylene, and 1,1,1-trichloroethane. Tetrachloroethylene at 0.05 mg/L was added as an internal standard as it is not degraded by either OB3b and 2C10P and thus would be useful for accounting for losses due to adsorption, leakage and account for injection variation for the GC analysis. The GC conditions utilized were the same as used for the TCE analysis except the oven temperature was reduced to 800C.

H. COPPER EXPERIMENTS

The bathocuproine procedure (Franson, 1992) was used to assay soluble copper ion concentrations in growth media and solutions of yeast extract. The effect of different copper concentrations on sMMO activity was determined by the addition of different concentrations of $CuS0_4.5H_20$ to the NMS medium (supplemented with yeast extract and vitamins), including 0, 1, 5, and 10 μM.

SECTION III

RESULTS

A. MUTAGENESIS EFFICIENCY

The plating efficiency of both OB3b and 2C10P on NMS media was quite low even when supplemented with yeast extract and having methanol as the sole carbon source. The recovery ranged from only 1 to 10%. Colonies usually appeared within 5-7 days when grown on either methane or methanol.

1. Dichloromethane mutagenesis

Colonies appearing on NMS methanol plates (with or without yeast extract) were exposed for several weeks to a dichloromethane. After about 6 weeks the colonies were transferred using nylon transfer membranes and regrown under a methane atmosphere. About 90% of the colonies were capable of growing on methane following the DCM exposure. Colonies producing sMMO were found to occur approximately at an efficiency of 2×10^{-6}. As a result from a 1 mL culture with 10^8 cells/mL approximately 20 colonies able to form sMMO in the presence of 5 μM $CuSO_4$ were derived using dichloromethane-induced mutagenesis. Of these, only about 2-3 mutants remained stable with the majority losing detectable activity (when grown on copper) after 1 to 3 subcultures presumably reverting back to their wild-type form. NMS plates lacking yeast extract completely failed to form any stable mutants while the mutagenesis efficiencies of OB3b and 2C10P were practically the same. Mutants which appeared to be stable were stored in NMS

supplemented with 0.02% yeast extract and 30% (v/v) glycerol at -20°C. Overall, 8 mutants (Table 2) were obtained using chemical mutagenesis, all of which were analyzed as indicated below.

2. Transposon Mutagenesis

High efficiency transformation of *E. coli* SV17λ was achieved using the plasmids pUTHg and pLOFHg. The transformants were checked for stability by repeated transfers on LB supplemented with 5 μg/ml $HgCl_2$ and 50 μg/ml ampicillin. Transformants of *E.coli* SV17 were then used in triparental mating with OB3b or 2C10P along with a mobilizer strain (HB1 Ol:pRK2013). Conjugants of both OB3b and 2C10P were obtained on NMS containing both mercury and ampicillin. These were then directly transferred to NMS containing mercury only in order to cause loss of the plasmid and induce transposition of Tn5 and Tn10 and thus cause mutagenesis. Though colonies were obtained readily on the NMS with 10 mg/L HgCl none of these proved able to form sMMO. In any case, the stability of these transconjugants appeared poor as following only 1-3 transfers over 90% of the colonies had lost the ability to grow in the presence of HgCl. In the case of the Tnl721 mutagenesis both OB3b and 2C10P had the disadvantage of having high natural resistance to tetracycline (approx. 60 μg/mL). This made determination of actual transconjugants very difficult and this procedure was not pursued further.

3. UV Mutagenesis

UV lethal dosage curves were derived for both strains OB3b and 2C10P in order to determine the level of exposure most likely to have a high level of mutagenicity. The UV lethal dosage curves of OB3b and 2C10P were very similar and a UV dosage of 5 J M^{-2} was utilized in subsequent attempts to obtain copper-tolerant mutants of OB3b and 2C10P. All colonies were

able to grow on NMS plates under methane following transfer indicating methane oxidation genes were still intact. However screening failed to detect any sMMO producing colonies on NMS supplemented with or without yeast extract though several thousand colonies were screened.

B. GROWTH KINETICS OF COPPER-TOLERANT MUTANTS

Analysis of the growth rates of the copper-tolerant mutants (obtained by DCM mutagenesis) and their wild-types (Table 3) indicated mutant specific growth rates were on average 77% of those of the wild type under optimal growth conditions. In the absence of yeast extract and vitamin supplementation this proportion declined to 54%. With the addition of these supplements the average generation time of the mutants was reduced considerably from 18 ± 6 hours to only 5.8 ± 0.5 hour. The absence of copper did not appear to affect the growth rates of the mutants significantly, an observation also reported by Phelps et al. (1992). The absence of copper however reduced the growth rates of the wild-types with strain 2C10P much more severely affected than OB3b. Though vitamin supplementation had no apparent affect on wild-type growth rates as seen previously (Bowman & Sayler 1993) there seemed to be some indication that mutant growth rates were slightly stimulated from 6.5 ± 0.4 hour to 5.8 ± 0.5 hour. Variance analysis indicated this stimulation was statistically significant ($p < 0.05$). The addition of individual amino acids at either 0.1 or 0.01 mM failed to yield the same stimulatory effect of yeast extract but were actually inhibitory to varying extent.

TABLE 3: GROWTH KINETICS OF WILD TYPE AND COPPER-TOLERANT MUTANTS IN DIFFERENT MEDIA

	OB3b	**2C10P**	**PP358**	**JB22**	**JB36**	**JB69**	**JB147**	**JB208**	**JB227**	**JB309**
Medium:	Specific Growth Rate (μ h^{-1})									
NMS	0.075	0.081	0.035	0.044	0.042	0.053	0.050	0.021	0.038	0.048
NMS+Cu	0.112	0.108	0.043	0.041	0.048	0.058	0.055	0.047	0.051	0.058
NMS+YE	0.135	0.17	0.129	0.099	0.108	0.119	0.117	0.086	0.104	0.096
NMS+Cu+Ye	0.147	0.167	0.126	0.098	0.110	0.117	0.119	0.092	0.110	0.091
NMS+Cu+Ye+vit	0.158	0.153	0.140	0.106	0.117	0.123	0.130	0.116	0.113	0.110

TABLE 4: MAXIMAL SMMO ACTIVITY OF WILD-TYPE AND COPPER-TOLERANT MUTANTS IN DIFFERENT MEDIA

	OB3b	2C10P	PP358	JB22	JB36	JB69	JB147	JB208	JB227	JB309
Medium:	**sMMO activity (nmol naphthol formed h^{-1} mg cells $^{-1}$)**									
NMS	302	258	278	232	180	201	207	201	177	267
NMS+Cu[b]	n.d.[a]	14	189	154	121	106	133	97	148	135
NMS+YE[c]	247	221	210	166	123	116	128	103	135	125
NMS+Cu+Ye	n.d.	n.d.	194	160	96	110	118	105	115	93
NMS+vitamins[d]	390	341	301	240	176	227	199	231	221	258
NMS+Cu+Ye+vit	n.d.	n.d.	204	177	122	124	122	145	132	112

[a]n.d., sMMO not detectable
[b]Copper concentration was 5 μM.
[c]Yeast extract concentration was 0.02% (weight/volume).
[d]Vitamin solution in detailed in Table 1.

C. sMMO ACTIVITY OF COPPER TOLERANT MUTANTS

Different media were utilized to determine optimal conditions for sMMO activity in the copper-tolerant mutants (Table 3). The presence of copper only suppressed the sMMO activity of the mutants from 16-52% compared to the wild-types in which sMMO activity was almost completely suppressed. A saturation response was observable with copper suppression of sMMO as shown in Figure 1 with copper ions at 5 and 10 μM showing a similar level of suppression compared to a much lower degree of suppression for 1 μM copper ion concentration. The moderately suppressive effect of yeast extract on sMMO activity appears simply due to the presence of copper in the yeast extract product. There is approximately 5 mg of copper in 1 gram of yeast extract as shown by copper analysis by the bathocuproine procedure. Certain vitamins, including biotin, pyridoxine, and cyanocobalamine have been previously shown to be stimulatory for sMMO activity. Though the highest sMMO activity was recorded in NMS medium supplemented with vitamins only the relative growth rates of the mutants was relatively poor in this medium. The addition of yeast extract stimulated the growth dramatically but only reduced activity slightly. The addition of copper did not improve growth and caused further suppression of the sMMO activity.

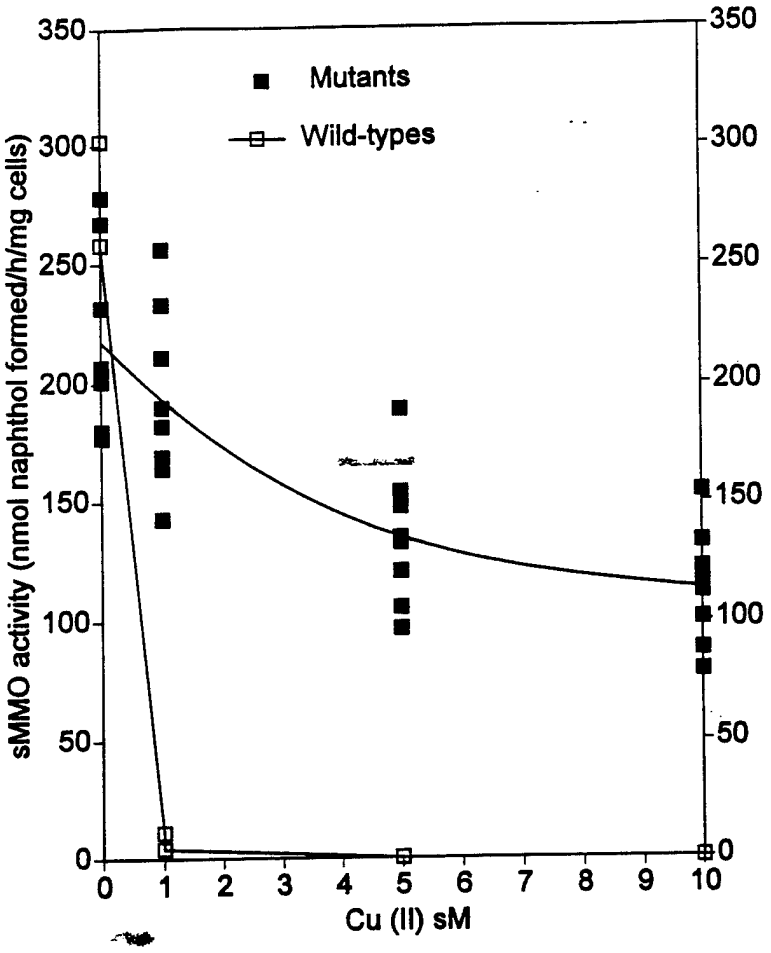

Figure 1: The Effect of Medium Copper Concentration on the sMMO Activity of Copper-Tolerant Mutants and their Parent Wild-Type Strains

D. TRANSFORMATION OF CHLORINATED ALIPHATICS BY COPPER-TOLERANT MUTANTS

Mutants were examined for their ability to transform chlorinated aliphatics in the presence and absence of copper ions (Table 5). The transformation rate of the six compounds tested appeared to correspond quite closely to the inherent sMMO activity of the various strains. The rates of vinyl chloride and dichloromethane transformation by the wild-type and mutant strains were quite similar since the transformation kinetics of these compounds is similar for both sMMO and pMMO. The copper tolerant mutants however transformed these compounds somewhat slower than the wild-type cultures. This apparently corresponds with the lower maximal sMMO activities found in the mutants (Table 4). In the presence of 5 μM copper ions the transformation of TCE and 1,1,1 -trichloroethane by OB3b and 2C10P was almost completely suppressed while the transformation of *cis*-1,2-dichloroethylene and chloroform was reduced considerably (Table 5). The mutants instead transformed these compounds much more efficiently when grown with copper though the rates were still lower compared to resting cells grown in the absence of copper.

TABLE 5: WILD -TYPE AND COPPER-TOLERANT MUTANT MIXED CHLORINATED ALIPHATIC TRANSFORMATION

Compound[a]:	Cu^{2+}	OB3b	2C10P	PP358	JB22	JB36	JB69	JB147	JB208	JB227	JB309
		Average transformation rate (µmol/h/mg cells)									
VC	+	140	>161	79	104	97	80	90	111	92	73
	-	161	>161	106	121	113	110	106	126	98	100
DCM	+	108	>118	71	81	76	69	82	92	85	58
	-	118	>118	111	107	99	101	103	>118	100	88
cis-1,2-DCE	+	11.5	7.3	13.7	10.7	10.8	9.8	11.3	9.6	12.7	7.5
	-	>20	>20	18.0	16.2	15.4	15.6	15.1	14.1	14.8	12.3
CF	+	2.0	<1	9.3	8.9	11.1	9.0	9.2	6.3	9.9	6.1
	-	16.7	15.4	13.7	12.7	14.8	15.5	13.1	10.8	11.9	9.8
TCE	+	<1	<1	7.9	8.7	10.1	7.3	8.1	5.7	9.3	5.8
	-	>15	>15	13.2	10.5	13.7	13.8	12.5	11.9	10.6	9.6
TCA	+	<1	<1	1.6	0.9	1.1	1.2	1.5	0.6	1.9	1.0
	-	3.4	4.6	2.8	1.7	2.1	1.8	2.2	1.0	2.5	1.4

[a]Abbreviations of chlorinated aliphatics (initial concentration): VC, vinyl chloride (50 mg/L); DCM, dichloromethane (50 mg/L); cis-1,2-DCE, cis-1-2dichloroethylene (10mg/L); CF, chloroform (10 mg/L); TCE, trichloroethylene (10mg/L); and TCA, 1,1,1-trichloroethane (10 mg/L); <1, no significant transformation detected.

TABLE 6: REMOVAL OF COMPONENTS OF A CHLORINATED ALIPHATIC MIXTURE BY WILD-TYPE AND COPPER-TOLERANT MUTANTS

	OB3b	**2C10P**	**PP358**	**JB22**	**JB36**	**JB69**	**JB147**	**Jb208**	**Jb227**	**JB309**
Component:[a]	Removal of VOC component (%) (minus/plus copper)									
VC	>99	>99	>99	>99	>99	>99	>99	92	>99	89
	>99	>99	>99	>99	>99	>99	>99	>99	>99	>99
DCM	>99	>99	>99	>99	>99	>99	>99	>99	>99	>99
	>99	>99	>99	>99	>99	>99	>99	>99	>99	>99
cis-1,2-DCE	52	58	42	40	31	38	27	29	33	30
	74	79	62	51	47	52	39	46	47	49
CF	28	45	25	39	25	29	20	21	28	22
	67	81	41	56	39	43	36	38	44	33
TCE	8	<3	32	38	17	32	21	33	20	18
	>99	>99	43	48	32	41	39	40	36	29
TCA	<3	<3	11	10	<3	9	6	8	12	<3
	27	49	19	18	11	16	14	19	21	13
Total mass removed	47	57	51	54	45	51	45	47	49	43
	73	85	61	62	55	58	54	57	58	54

[a]Chlorinated aliphatic mixture components were all at 2 mg/L and included: VC, vinyl chloride; DCM, dichloromethane; 1,2-DCE,cis-1,2-dichloroethylene; CF, chloroform; TCE, trichloroethylene; and TCA, 1,1,1-trichloroethane.

E. TRANSFORMATION OF CHLORINATED ALIPHATIC MIXTURE

Resting cell suspensions of mutants and their wild-types grown with or without copper were incubated with a chlorinated aliphatic mixture for 4 hours and then analyzed by GC. Transformation of the combined chlorinated aliphatics were relatively similar for both wild-types and mutants (Table 6). In the absence of copper the wild-types appeared better able to transform the mixture compared to the mutants. Vinyl chloride and dichloromethane were almost completely transformed under both copper-free and copper-rich conditions in the presence of other chlorinated aliphatics. Approximately 20-40% and 40-50% of the *cis*-1,2-dichloroethylene, chloroform, and TCE were transformed in copper-free and copper-rich conditions, respectively. Transformation of these compounds and 1,1,1 -trichloroethane by wild-type cultures was significantly greater than the copper-tolerant mutants in copper-free conditions but comparable or less in copper-rich conditions.

F. DISCUSSION

Several forms of chemical mutagenesis have been found to be relatively ineffective in obtaining methanotrophic mutants. The following mutagens were found to be ineffective for forming mutants: UV and gamma irradiation, methane methyl sulfonate, ethyl methane sulfonate, and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Higgins et al. 1981). It was proposed this may be due to low permeability of the cell wall or due to the lack of a error-prone SOS repair process typical in *Escherichia coli* (Murrell 1992) though none of these speculations have been followed up.

Dichloromethane mutagenesis on the other hand have been found to be an effective means of obtaining mutants of methanotrophs. Dichloromethane appears to act as a "suicide-substrate"

which is co-oxidized by sMMO or pMMO to carbon monoxide which is cytotoxic. Nicolaidis and Sargent (1985) used this procedure to obtain pMMO-deficient mutants of methanol-adapted *Methylomicrobium album* (previously *"Methylomonas albus")* and *Methylosinus trichosporium* cells. Phelps et al. (1992) further refined this procedure to obtain mutants of *Methylosinus trichosporium* OB3b which were unable to functionally form pMMO thus allowing sMMO activity to exist in the presence of high copper levels. Fitch et al. (1993) found the pMMO-deficient mutants actually had a disabled copper uptake or metabolism thus disrupting both pMMO formation which requires copper for its active site and for ICM formation.

The UV and transposon mutagenesis procedures used in this study unfortunately failed to create any stable mutants. It was confirmed that successful conjugation of pLOFHg and pUTHg into both OB3b and 2C10P since mercury and ampicillin resistant colonies were formed. The transfer frequency to *Methylosinus* strains (10^{-2} to 10^{-1}) was similar to those reported in other studies (Al-Taho & Warner 1987; Murrell 1992). However removal of the ampicillin selection in order to promote transposition failed to yield stable colonies on the mercury containing NMS medium. This seems to suggest that the Tn5 and Tn10 transposons are either not stably inserted into either the OB3b or 2C10P genome. Conjugal transfer of the Tnl721 containing plasmid pUCD623 carrying a tetracycline resistance marker gene was inconclusive since both OB3b and 2C10P both have high natural tetracycline resistance. No other transposon systems were utilized for mutagenesis due to time constraints.

DCM mutagenesis proved to be effective for generating mutants with sMMO copper-tolerant phenotypes. In this study a total of 8 mutants were obtained. Optimal growth was only obtained when the NMS medium was supplemented with yeast extract and vitamins. A similar response

was found for mutant PP358 which was one of the more active and better growing mutants obtained by Phelps et al. (1992). This moderate degree of fastidiousness seems related to the genetic lesions produced by the DCM mutagenesis. Supplementation of the NMS medium with single amino acids failed to pin point a specific auxotrophic response. This suggests that some other aspect of anabolism in the mutants has been affected by the mutagenesis. All the mutants including PP358 still showed some sMMO activity repression in the presence of 5 μM copper ions (Table 4; Figure 1). This would seem to indicate DCM mutagenesis had not completely disable copper metabolism/ uptake mechanisms in the mutants. In general the mutant's maximal sMMO activity was distinctly lower than the wild-types possibly related to an overall reduction in the individual mutant's biosynthetic capability. Again sMMO activity was stimulated by vitamin supplementation however it is not as pronounced as what is observed for wild-type sMMO-producing methanotrophs (Bowman & Sayler 1993). The ability to transform individual or mixtures chlorinated aliphatics (Table 5 and 6) essentially corresponded to the relative sMMO activity and production. The mutants have in general only a slightly lower rate of transformation efficiency compared to the wild-types when grown in copper-free conditions. The major advantage the mutants share is that their sMMO is active in a wide range of copper concentrations.

Recent studies have demonstrated that new bioreactor technology and reactor operating regimes can be applied for maximizing methanotrophic TCE removal from contaminated groundwaters (Tschantz et al. 1995). These results were based on investigations employing the copper-tolerant strain *Methylosinus tricosporium* PP358. Utilization of this strain was deemed necessary to overcome copper inhibition experienced in a laboratory reactor system and to

investigate performance of copper-tolerant methanotrophs that may be applied in environmental bioremediation applications.

In these studies a novel operating design was used to produce an sMMO active biomass in continuous stirred tank reactor (CSTR) with TCE degradation accomplished by co-metabolic oxidation compartmentalized in a series of methane limited plug flow reactors. This reactor was designed as a recirculating system with dewatering and return of methanotrophic biomass to the CSTR following TCE contacting and biodegradation. These studies demonstrated that the copper-tolerant methanotroph could maintain significant TCE removal (78.7% to 93.5%) in a waste stream containing 20 mg^{-l} TCE within a 4 hour retention time over an operating run of 90 hours.

A dominant variable contributing to efficiency of the process was found to be methanotrophic biomass and sMMO specific activity. In future application of such bioreactor strategies for TCE bioremediation, sMMO active biomass must be maintained for extended treatment times and most likely maintained in a background of indigenous and competing organisms within the treatment system.

The results of the current research permit evaluating other copper-tolerant strains for improved performance in bioreactors designed for co-metabolic TCE biodegradation. The current availability of additional copper-tolerant mutants generated in this study, permits investigations to evaluate the competitive maintenance of strains and system performance in order to ultimately define parameters controlling performance at the field treatment scale.

G. CONCLUSION

1. Dichloromethane mutagenesis was effective in obtaining copper-tolerant sMMO-producing methanotroph mutants while other procedures including transposon mutagenesis using Tn5, Tn10, and Tn1721 delivery systems and using UV mutagenesis was not successful.
2. The copper-tolerant mutants were found to grow maximally in nitrate mineral salts media supplemented with yeast extract and a number of vitamins allowing growth equivalent to the parent wild-type strains.
3. The mutants developed were able to stably express sMMO in the presence of copper levels up to 10 μM at approximately 50-80% of their maximal sMMO levels when cultivated in the absence of copper.
4. The mutants were able to transform several chlorinated aliphatic compounds at a rate slightly less than the parent wild-type strains.

H. RECOMMENDATIONS

The mutants obtained in this study are proficient in TCE degradation and should be examined at the level of bench scale pilot processes. The organisms should be tested in a comparative sense, to strain PP358 and nonmutant wild types, for advantageous characteristics that may lead to maintenance of higher levels of active biomass in mixed culture reactor systems for TCE bioremediation.

Further research will be required to obtain additional improved mutants for TCE bioremediation. A mutagenesis scheme needs to be developed which is able to increase the probability of obtaining stable mutants of specific phenotype,i.e. lacking pMMO activity. Most

procedures available today, including DCM mutagenesis, are generally not adequate for selective mutagenesis of methanotrophs which have a molecular biology which still remains largely unknown. The mutants should ideally not require medium supplementation for attaining good growth rates and should over express sMMO assuming requirements for reducing equivalents can be met.

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