To the Graduate Council:

I am submitting herewith a dissertation written by Sung-Cheol Koh entitled "Trichloroethylene Degradation by a Type I Methanotroph, *Methylomonas methanica* 68-1, and Molecular Analysis of the Population Dynamics in the Two Methanotrophic Species Competition." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology.

Gary S. Sayler, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

TRICHLOROETHYLENE DEGRADATION BY A TYPE I METHANOTROPH, *METHYLOMONAS METHANICA* 68-1, AND MOLECULAR ANALYSIS OF THE POPULATION DYNAMICS IN THE TWO METHANOTROPHIC SPECIES COMPETITION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Sung-Cheol Koh

May 1994

DEDICATION

This dissertation is dedicated to my parents, Mr. Young-Suck Koh and Mrs. Jeom-Duk Yang, my parents-in-law, Mr. In-Sang Im and Mrs. Eul-Boon Yoo, and my wife, Hea-Ok Koh. I enjoyed their unwavering support, guidance, and patience during this sometimes difficult, always intense period. They have encouraged me to complete this dissertation.

ACKNOWLEDGMENTS

First of all I would like to thank Drs. Gary S. Sayler, Gary Stacey, Barbara T. Walton, and David C. White for service on my committee, helpful comments on this research, and detailed review of this dissertation.

My sincere thanks go to my comrades in the laboratories in the Center for Environmental Biotechnology, University of Tennessee, Knoxville , both former and present for discussion and assistance: John Bowman, Michael Tschantz, Luis Jimenez, Igrid Gregory, David Ringelberg, Bruce Applegate, and Udayakumar Matrubutham.

I am also grateful to Jong-Yoon Chun for his assistance in DNA sequencing at Dr. Gary Stacey's laboratory and to Mr. Dick Williams for his help in electron microscopic procedures in the electronmicroscopy laboratory at the University of Tennessee, Knoxville.

I thank to both former and present staff in the Environmental Sciences Division at Oak Ridge National Laboratory for their providing strains and useful comments: Dr. Tony Palumbo, Dr. Dean Little, and William Eng.

In addition, I have to thank Drs. Richard S. Hanson and Bernard Tsien at Gray Freshwater Biological Institute, University of Minnesota for their cooperation and support by performing experiments and providing molecular probes during the course of this research.

I thank Dr. Terry Hazen of the Savannah River Laboratory (SRL) for providing soil samples used for microbial community analysis.

I am grateful to the Waste Management Research and Education Institute, University of Tennessee, Knoxville for financial support through research assistantships.

Finally I am especially indebted to my academic and research advisor, and mentor, Dr. Gary S. Sayler, Center for Environmental Biotechnology, University of Tennessee, Knoxville, for his ideas, guidance support, advice, and patience, without which this research would have been impossible. I will remember him as a creative scientist setting a high standard of research.

This research was sponsored by the Waste Management Research and Education Institute, University of Tennessee, Knoxville, and by the Savannah River Laboratory of the U. S. Department of Energy and U. S. Air Force subcontracts DE-AC05-840R21400 and F49620-92-J-0147 through Oak Ridge National Laboratory, respectively.

ABSTRACT

The goal of this study was to determine whether type I and II methanotroph specific DNA probes could specifically detect and monitor the relevant populations in a methanotrophic mixed culture that degrades TCE. A model mixed culture was composed of a type I methanotroph, strain 68-1, isolated from a TCE-contaminated well and an extensively characterized type II methanotroph, *Methylosinus trichosporium* OB3b.

The strain 68-1 was identified as *Methylomonas methanica* by pattern of intracytoplasmic membrane ultrastructure, 16S rRNA signature probe hybridizations, and PLFA and LPS-OHFA profiles. 68-1 could produce sMMO under copper-limiting condition, and oxidize naphthalene and degrade TCE more rapidly than *M. trichosporium* OB3b at room temperature despite its higher K_m values for these substrates. There was little genetic homology between the sMMO genes of 68-1 and OB3b, indicating the diversity of sMMO genes in methanotrophs. However, substrate specificity indicated a potential functional similarity between of the sMMOs of the type I and II methanotrophs. The putative sMMO gene fragment (4 kb) of 68-1 was cloned and partially identified by Southern and slot blots, and DNA sequencing. The DNA fragment cross-hybridized to the genomes of a few pink-pigment type I methanotrophs (not producing sMMO) and a type X methanotroph (producing sMMO), indicating the conservation of the putative gene at least within the RuMP pathway methanotrophs. The putative sMMO DNA probe was successfully used to monitor the 68-1 population in the mixed culture containing OB3b. The monitoring data were supported by plate count data and hybridization results

using 16S rDNA-targeting oligodeoxynucleotide probes while AODC data generally complemented the sMMO gene probe results with certain levels of error. The 68-1 population was out-competed by OB3b population in both flask and continuous cultures under copper-limiting condition. The 68-1 population was also out-competed by OB3b under lower concentration of methane while higher methane concentration favored 68-1. Moreover, the OB3b population was dominant over 68-1 regardless of copper and nitrate concentrations. The putative sMMO gene probe was successfully used to detect the potential homologous methanotrophic genes and predict the population density in TCE-contaminated subsurface environments.

Therefore, the putative sMMO gene from 68-1, if confirmed, could be used to monitor the populations of the potential type I sMMO producers growing or present in the various environments.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Trichloroethylene (TCE) is an organic chemical that has been used primarily as an industrial solvent for the last several decades. The deliberate discharge of TCE into the environment has resulted in a persistent contamination problem in subsurface soils, sediments, and groundwaters. TCE contamination has been of heightened concern due to its toxicity and potential carcinogenicity. Since the first discovery of TCE mineralization by a methane-oxidizing consortium (Wilson and Wilson, 1985) a decade ago, various pure cultures of heterotrophic and methanotrophic organisms have been reported to cometabolically degrade and/or mineralize TCE under aerobic conditions without generating toxic metabolites. The methanotrophic bacteria able to produce methane monooxygenase (MMO), have received considerable attention for *in situ* TCE biodegradation. In *in situ* bioremediation, whether it is accomplished through natural conditions without human intervention (intrinsic bioremediation) or by a human-controlled optimizing process requiring engineering infrastructures (engineered bioremediation), the central role of the remediation is played by microorganisms (especially bacteria) (National Research Council, 1993). Therefore detection of the specific degradative organisms, and monitoring and prediction of degradation activity of the organisms will be a required procedure in evaluating *in situ* bioremediation.

There are three types of methanotrophs, I, II, and X. Both type II and X have been known to produce both soluble methane monooxygenase (sMMO) and particulate MMO (pMMO; membrane-bound MMO), which has a narrower

substrate range than the sMMO. Type I methanotrophs have been reported to produce only pMMO, which degrades TCE at rates in several orders of magnitude lower than those of sMMO. Therefore, those organisms with pMMO are less likely to be important for *in situ* bioremediation. The physiological and biochemical characterization of sMMOs of *Methylosinus trichosporium* OB3b (type II) and *Methylococcus capsulatus* Bath (type X) is extensive while no information is available on sMMO of type I methanotrophs. This led to the recent cloning and sequencing of sMMO gene clusters of the type X and type II methanotrophs, thus making available gene probes useful for the detection of the homologous genes and organisms.

All the three types of methanotrophs have been isolated from a variety of pristine or contaminated environments. Their distribution and population density in the environment appears to be significantly influenced by the availability of nutrients and other growth conditions (e.g., methane, oxygen, nitrate, copper, pH, and temperature).

The process analysis of methanotrophic TCE bioremediation in the environment should include information about the genetic capabilities of microorganisms to degrade TCE. Two types of genetic information important for analyzing the biological process of TCE degradation are: the total pool of genes within the population and the individual genes within the individual cells in the population (Rittman et al., 1990). In this context nucleic acid (DNA or RNA) probes should be useful in detecting and monitoring genes, specific methanotrophic populations, and for predicting potential TCE degradation activities of the populations. Information of this nature will eventually contribute

to the elucidation of the role and dynamics of methanotrophs in environments contaminated with TCE and their relationship to TCE degradation.

The goal of this study was to determine whether type I and II methanotroph specific DNA probes could specifically detect and monitor the relevant populations in a methanotrophic mixed culture that degrades TCE. A model mixed culture was composed of a type I methanotroph, strain 68-1, isolated from a TCE-contaminated well and a well-characterized type II methanotroph, *Methylosinus trichosporium* OB3b. Three research objectives were set up to accomplish the research goal: (i) to determine whether the strain 68-1 can degrade TCE via expression of sMMO or pMMO; (ii) to develop a type I methanotroph-specific DNA probe for the strain 68-1 and examine feasibility of the DNA probe in detection and monitoring of the relevant population in mixed cultures; and (iii) to measure the dynamics of a mixed methanotrophic bacterial population consisting of 68-1 and *M. trichosporium* OB3b by using DNA probes and then to determine the relationships between the dynamics and their activities in TCE degradation.

CHAPTER II

LITERATURE REVIEW

A. TCE as an environmental pollutant

Trichloroethylene is an organic compound that is a colorless volatile, nonflammable liquid at ambient temperatures. It is primarily used as an excellent solvent in metal and fabric degreasing processes. The compound is also used in textile treatment, polyvinyl chloride production, food processing, and as a medicinal anesthesia (U. S. E. P. A., 1979). TCE has been discharged to the nation's surface waters and groundwaters during the last several decades without an awareness of TCE becoming a persistent contamination problem (Schaumburg, 1990). Therefore, TCE has been listed as one of the most frequently reported contaminants at hazardous waste sites (U. S. E. P. A., 1985a) and is found in significant quantities in contaminated groundwaters (Westrick et al., 1984). It is a persistent problem due to its long half-life (300 days) in aquifers (Roberts et al., 1982). The presence of this organo-chlorine pollution in water threatens ecological and human health because of its toxicity and suspected carcinogenicity (U. S. E. P. A., 1985b; Craun, 1991).

B. TCE biodegradation

Using mixed cultures grown under anaerobic conditions, TCE is transformed to dichloroethylene and vinyl chloride by sequential reductive dehalogenation (Vogel and McCarty, 1985; Barrio-Lage et al., 1986). Vinyl chloride is known to be recalcitrant and a more potent carcinogen than TCE (Infante and Tsongas, 1987). The anaerobic studies of TCE degradation by pure

cultures are few. The methanogens *Methanobacterium tiedjieii* DCB-1 and *Methanosarcina* spp. DSM was observed to perform the reductive dechlorination of PCE and TCE utilizing an electron donating substrate such as acetate and pyruvate (Fathepure et al., 1987). Suspected methanogenic mixed cultures enriched from contaminated groundwaters could completely dechlorinate PCE and TCE to ethylene, but the rate-limiting step in the pathway appeared to be the reduction of vinyl chloride to ethylene (Freedman and Gossett, 1989). Evidence for the involvement of sulfate-reducing cultures in TCE dechlorination was reported but the predominant biotransformation product was *cis*-1,3-dichloroethylene (cDCE) (Pavlostathis and Zhuang, 1991) (Table II-1).

However, under aerobic conditions, TCE is degraded and/or mineralized by heterotrophic and methanotrophic bacteria (methanotrophs) without producing carcinogenic toxic metabolic intermediates. Heterotrophic bacteria have been tested for their ability to degrade TCE, including toluene degraders (Zylstra and Gibson, 1989), phenol oxidizers (Nelson et al., 1987), ammonia oxidizers (Arciero et al., 1989), and propane oxidizers (Wackett et al., 1989). Their cometabolic TCE degradation rates appear to be significantly less than sMMO-producing methanotrophs (Tsien et al., 1989; Fox et al., 1990) (Table II-1).

Several studies (Wilson and Wilson, 1985; Fogel et al., 1986; Fliermans et al., 1988; Henson et al., 1988; Henson et al., 1989) have shown that TCE could be degraded aerobically by microbial consortia under methane-induced conditions. Some recent investigations have revealed that pure cultures of methanotrophs can degrade TCE (Little et al., 1988; Oldenhuis et al., 1989; Tsien et al., 1989; Uchiyama et al., 1989) (Table II-1). The methane-oxidizing

Table II-1. Comparison of TCE degradations by various microorganisms in pure or mixed culture.

Organism or consortium	Cosubstrate as a carbon source; enzyme system[A]	TCE degradation rate (nmol/min/mg protein)	Reference
Aerobes			
Methylosinus trichosporium OB3b	methane; sMMO	290-456	Oldenhuis et al., 1989; Tsien et al., 1989
Pseudomonas cepacia G4	phenol, toluene, *o*, *m*-cresols; *o*-TMO	8	Folsom et al., 1990
Pseudomonas putida F1	toluene; TDO	1.8	Wackett et al., 1988
Pseudomonas mendocina KR-1	toluene; *p*-TMO	1.5	Richardson et al., 1984
Nitrosomonas europaea	ammonia; AMO	1	Arciero et al., 1989
Strain M (type II methanotroph)	methane; pMMO	0.69	Uchiyama et al., 1989
Methylomonas spp.	methane; pMMO	0.67	DiSpirito et al., 1992
Mycobacterium vaccae JOB5	propane; PMO	~ 0.5	Wackett et al., 1989
Strain 46-1 (type I methnaotroph)	methane, methanol; pMMO	< 0.1	Little et al., 1988
Anaerobes			
Methanobacterium tiedjieii DCB-1;*Methanosarcina* sp.	acetate, methanol, pyruvate	Not showm	Fathepure et al., 1987
Sulfate-reducing consortium	sodium dithionite	Not shown	Pavlostathis et al., 1991

[A] sMMO soluble methane monooxygenase; pMMO particulate MMO; TMO toluene monooxygenase; TDO toluene dioxygenase; AMO ammonium monooxygenase; PMO propane monooxygenase.

bacteria are capable of this co-metabolic TCE degradation using methane as sole carbon and energy source for growth. The mineralization of TCE by cometabolism does not produce any toxic metabolic intermediates. TCE treatment by methanotrophs, when economics, availability, and suitability of electron donors and acceptors are considered, has become a favorable choice for the bioremediation of groundwater contaminated with halogenated alkenes, including TCE (Semprini et al., 1990). Recently, there has been increasing interest in methanotrophic bacteria as biodegradative agents (Chaudhry and Chapalamadugu, 1991).

C. Biochemical mechanisms of TCE degradation

TCE cannot be used as sole carbon and energy source by methanotrophs but can be cometabolized in the presence of methane. It appears that TCE mineralization (direct transformation to CO_2) solely through the action of sMMO is unlikely. The mineralization may occur through other enzyme systems in the methanotrophs or in methanotrophic mixed cultures involving heterotrophic bacteria. The TCE biodegradation pathway is shown in Figure II-1. The degradation reaction is initiated when an activated oxygen species bound to an iron atom of sMMO hydroxylase component attacks the double bond of TCE and preferentially inserts an oxygen atom into the most substituted carbon atom to generate a free radical form. This intermediate rapidly forms TCE epoxide, which is also a highly unstable chemical species. During this reaction the iron atom is lost causing the enzyme inactivation of sMMO. The TCE epoxide chemically breaks down to yield formate, carbon monoxide, HCl, and glyoxylate in alkaline aqueous condition. Under acidic conditions, the dominant breakdown products

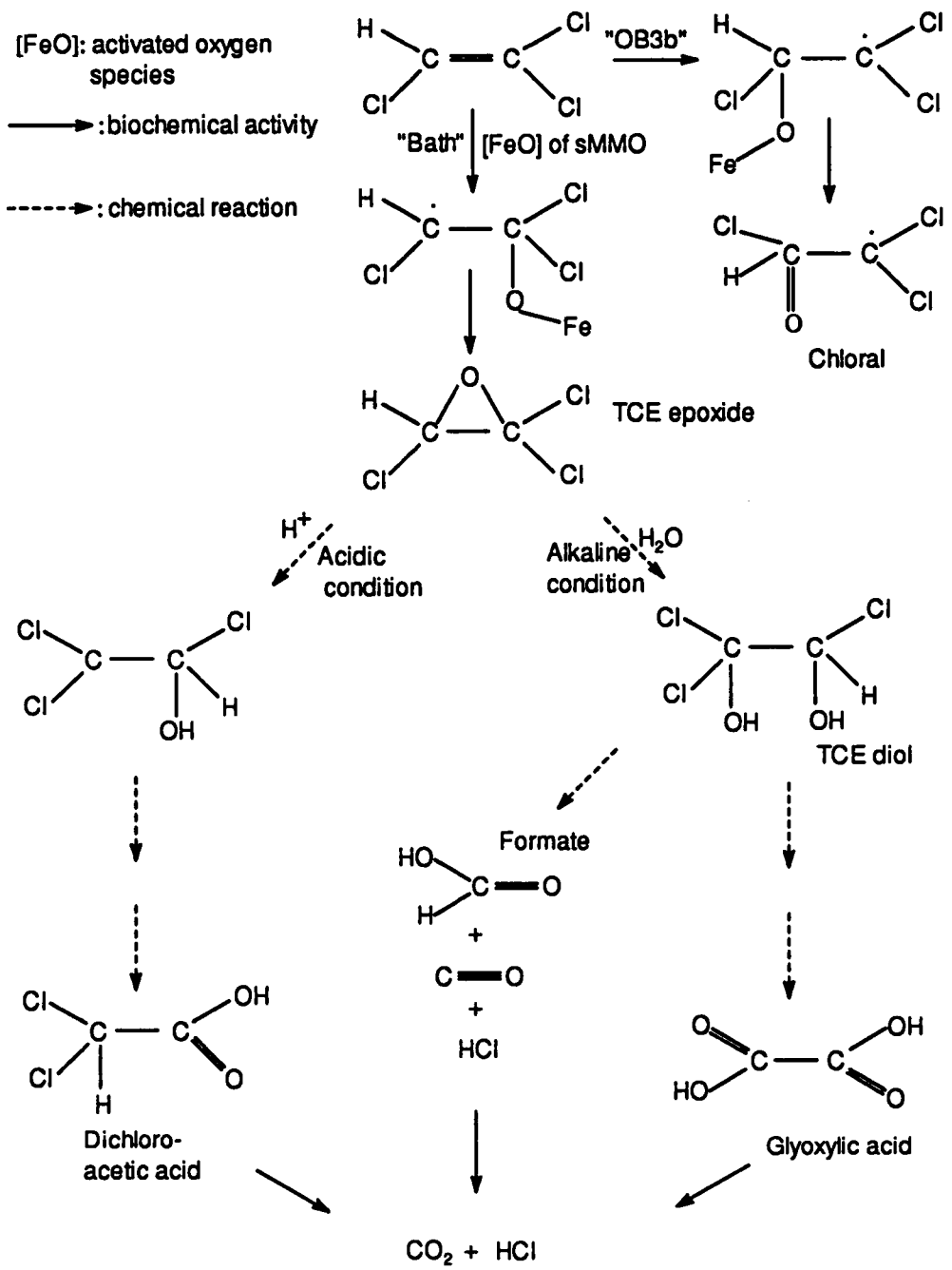


Figure II-1. The proposed pathway of TCE degradation by sMMO of *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b (Fox et al., 1989; Green and Dalton, 1989; Little et al., 1988)

would be dichloroacetic acid. The chemical reaction occurs spontaneouly in aqueous systems and no trichlorinated C_1 or C_2 molecules are detected. Finally, formate and carbon monoxide can be oxidized (mineralized) to carbon dioxide by other oxidation systems in the methanotrophic cells. Dichloroacetic acid and glyoxylic acid can accumulate in the aqueous phase of the pure medium but can be mineralized in cooperation with heterotrophic bacteria in a mixed culture

D. Biology and ecology of methanotrophs

It has been estimated that 355-870 trillion grams of atmospheric methane are produced annually by biological processes (Tyler, 1991). The amount available from non-biological sources has been estimated as between 20% and 100% of that of biological sources (Gold, 1979). Methane is, therefore, one of the more abundant and widely distributed organic compounds on the earth. This is reflected by the wide distribution and abundance of methanotrophs in the environment including freshwater, marine, and terrestrial ecosystems (Whittenbury et al., 1970; Whittenbury et al., 1976; Colby et al., 1979). They are known to play a significant role in the carbon cycle of aquatic ecosystems (Hanson, 1980). Recently methanotrophs are being recognized as an important sink for methane that is the most abundant organic gas in the atmosphere and is generally accepted to be one of the so-called "green house" gases (Cicerone and Oremland, 1988; Gal'chenko et al., 1989).

The dominant methanotrophs in any environment have not been identified. The methanotrophs isolated as colony forming units from pure cultures have been a small fraction of the viable cells recovered from environmental samples (Hanson, 1980). Methanotrophs examined from

freshwater and marine environments appear to exist under microaerophilic conditions and require concentrations of methane well below that required for saturation of aqueous solutions (Abramochinka, et al., 1987; Lidstrom, 1988). The growth dependence of methanotrophs on low oxygen tensions indicates that their populations vary spatially and seasonally. For example, methane oxidation occurred in the metalimnion of a low-dissolved-nitrogen lake in Canada during summer stratification (Rudd and Hamilton, 1978) where the oxygen concentration was the growth limiting factor. It was observed (Reed and Dugan, 1978) that the maximum populations of both *Methylomonas methanica* (type I) and *M. trichosporium* (type II) were observed at the oxic and anoxic interface at the sediment surface.

It appears that habitats can determine the types of methanotrophs and their abundance. Oligotrophic and acidic environments seem to select type II methanotrophs over type I, while the reverse is true for the eutrophic environments. Type I strains appear to be present in almost all methane-enriched locations when other nutrients, such as dissolved nitrogen source, are available. The type I methanotrophs *Methylomonas methanica* and *Methylomonas rubrum* were dominant over type II methanotrophs in the metalimnion of a eutrophic lake (Saralov et al., 1985). *Methylomonas methanica* was the dominant species over type II methanotrophs, *Methylosinus trichosporium* and *Methylocystis methanolicus,* in the bottom sediments in a eutorphic lake (Abramochinka et al., 1987). Putzer et al. (1991) found predominantly type I organisms in high-methane and low oxygen river water. However, type II methanotrophs such as *Methylosinus* sp. and *Methylocystis* sp. were dominant over type I methanotrophs in acidic peat bog lakes (pH 3.8-5.3)

(Heyer and Suchow, 1985), and polyhumic acidic lakes and oligotrophic lakes (Saralov et al., 1985). The domination of type II methanotrophs in the nitrogen-limiting oligotrophic lakes can be explained, in part, by the fact that type II methanotrophs can fix dinitrogen but not type I (Whittenbury and Krieg, 1984). Samples collected from the liquid-sediment interface in a nitrogen-limited treatment pond for pulp mill wastes generated predominantly type II strains (Graham et al. 1993). It is generally believed that type II methanotrophs appear to be selected in nitrogen-limited and perhaps low-pH zones. Both type I and type II methanotrophs were isolated from seawater. The type I organisms (*Methylobacter* sp. and *Methylomonas* sp.) were more abundant in bottom sediments potentially high in methane concentration while both type I (*Methylobacter* sp.) and type II (*Methylocystis* sp.) were found in the water above the sediments (Gal'chenko et al., 1988). Graham et al. (1993) examined competition between type I and type II methanotrophs in continuous-flow systems in order to further define factors leading to type selection. This report showed that the primary determinants of species selection (independent variables) were concentrations of methane, copper, and nitrogen.

Traditional classification has long been a problem with methylotrophs because there is insufficient information available for the design of a useful taxonomic system (Whittenbury et al., 1984). Methylotrophs can be divided into functional groups depending on whether or not they can utilize methane (methanotrophs) and on which carbon assimilation pathway is used. A new alternative method that is just beginning to emerge is classification based on 16S or 5S ribosomal RNA sequence analyses (Ando et al., 1989; Tsuji et al., 1990). This new classification system also reflects the classical taxonomic

system based on the physiological and phenotypic characteristics of methylotrophs. Tsien et al. (1990) developed RuMP pathway and serine pathway methylotroph specific oligodeoxynucleotide signature probes complementary to 16S rRNA. Recently Brusseau et al. (1994) reported type I and type II methanotroph specific signature probes which were used to detect methanotrophs in soil samples and differentiate their types by total RNA isolation and hybridization.

E. Methane oxidation and biochemistry of methane monooxygenase

Methanotrophs are a highly specialized class of aerobic bacteria that obligately utilize methane as a sole source of carbon and energy and are capable of completely oxidizing methane to carbon dioxide. The biochemical pathway of methane oxidation is shown in Figure II-2. The crucial step in biological oxidation of methane is performed by methane monooxygenase (MMO), which catalyzes the NAD(P)H-dependent cleavage of molecular oxygen and insertion of one oxygen atom into methane to produce methanol. The second oxygen atom is reduced to water. An intermediate in the oxidation pathway is formaldehyde which can either be assimilated into the cell to provide carbon for growth or further oxidized in dissimilatory fashion to CO_2 providing energy for the assimilatory functions (Dalton and Leak, 1985; Anthony, 1986). The carbon is assimilated through the ribulose monophosphate pathway (RuMP)(type I) or the serine pathway (type II), although both pathways may occur in the same organism (Whittenbury et al., 1976) which are named type X methanotrophs (Whittenbury and Dalton, 1984).

Biochemical studies of methane oxidation have been extensively

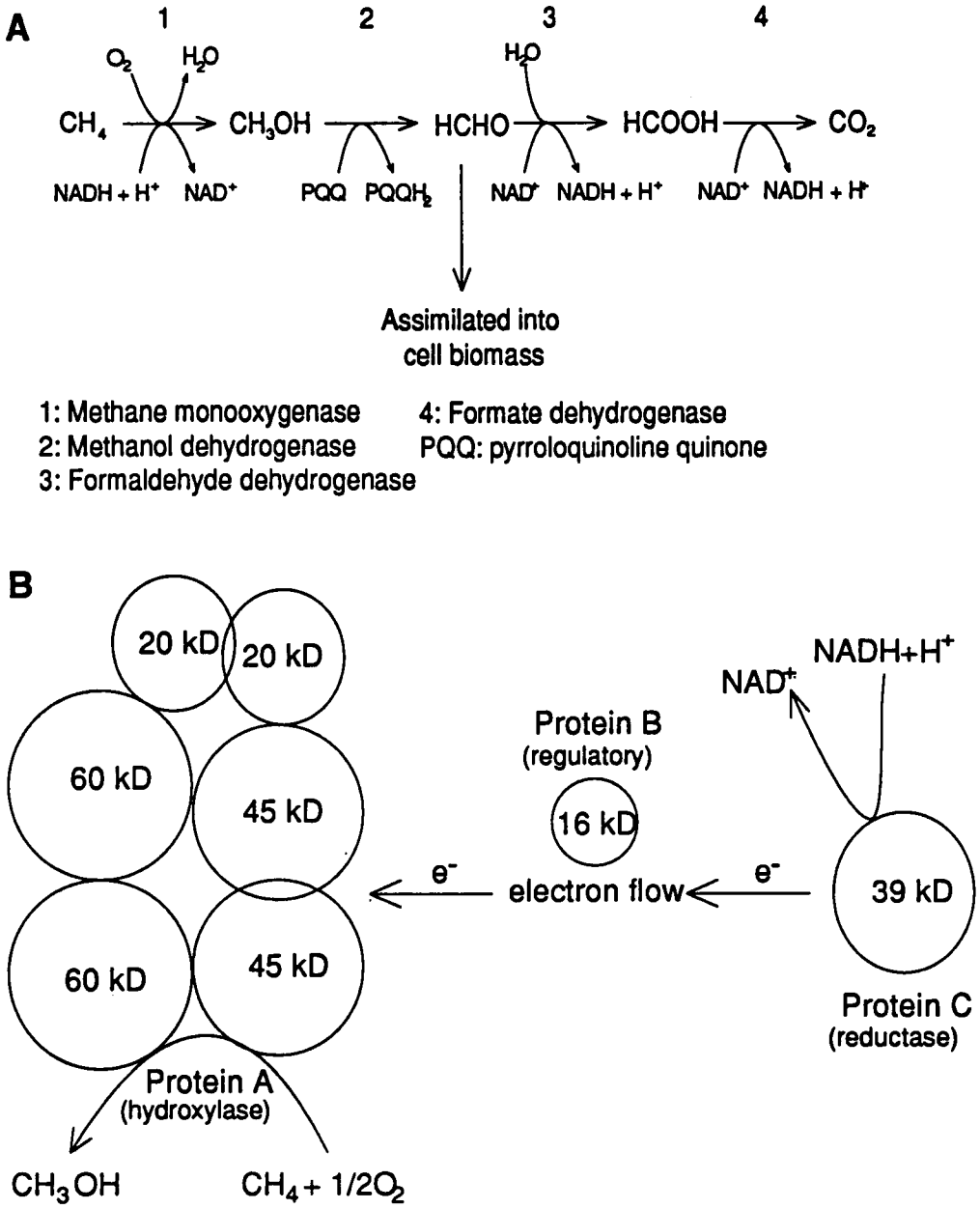


Figure II-2. The methane oxidation pathway (A) and soluble methane monooxygenase enzyme complex from *Methylococcus capsulatus* Bath (B) (Colby et al., 1979; Murrell, 1992).

performed and reviewed recently (Dalton and Leak, 1985; Anthony, 1986; Dalton et al., 1992). The first step of methane oxidation is catalyzed by methane monooxygenase (MMO) which has been extensively investigated in the two species of obligate methanotrophs, *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b. These two organisms can produce a membrane-bound (particulate) MMO (pMMO) and a cytoplasmic (soluble) MMO (sMMO). However, it is not entirely clear whether soluble and particulate forms are distinct or whether they share components (Dalton et al., 1984; Fox and Lipscomb, 1988). In addition, at least two type II methanotrophs, *Methylobacterium* sp. strain CRL-26 and *Methylosinus sporium* 5, can express both a soluble and a particulate enzyme (Patel et al., 1979; Patel et al., 1982; Pilkington and Dalton, 1990). *Methylomonas alba* BG8 (type I) and *Methylocystis parvus* (type II) may possess only a particulate form (Dalton and Leak, 1985).

The sMMO of *M. capsulatus* Bath has been purified and extensively characterized (Colby and Dalton, 1978; Colby and Dalton, 1979; Woodland and Dalton, 1984; Green and Dalton, 1985; Green and Dalton, 1986). The sMMO consists of three components: A, B, and C. The schematic diagram of the sMMO components and their interaction in methane oxidation is shown in Figure II-2. Component A (M_r. 226,000 Da) is a nonheme iron protein which binds methane. This component apparently utilizes reducing equivalents obtained from component C to activate O_2. Component B (M_r. 17,000 Da) is a low molecular weight protein lacking any prosthetic groups. It converts the MMO from an oxidase to an oxygenase. Component C (M_r. 44,600 Da) is an iron-sulfur flavoprotein. It is a reductase component of the MMO, transporting electrons

from NADH to component A. Recently the three components of *M. trichosporium* OB3b have been purified and characterized (Fox and Lipscomb, 1988; Fox et al., 1989) and shown to possess similar physical properties to those of *M. capsulatus* Bath (Table II-2). A sMMO from *Methylobacterium* sp. strain CRL-26 has been partially purified and characterized (Patel et al., 1982; Patel, 1984). It also consists of three components. Immunological studies have shown that there are similarities among different groups of methylotrophs and have suggested that type I methanotrophs can produce sMMOs (Table II-2).

In contrast, much less information is available on pMMO. A particulate MMO has been purified from cell extracts of *M. trichosporium* OB3b (Tonge et al., 1975; Tonge et al., 1977) and has three components: a soluble CO-binding cytochrome (M_r. 13,000), a copper protein (M_r. 47,000), and a small protein (M_r. 9400). Their functions are not yet clearly elucidated mainly because of difficulty in solubilization in active form *in vitro* (Stanley et al., 1983; Burrows et al., 1984; Prior and Dalton, 1985; Drummond and Dalton, 1989). However, the pMMO activity was stabilized and enhanced in high copper concentrations *in vivo* and *in vitro* (Burrows et al., 1984; Prior and Dalton, 1985). Three polypeptides (46 kDa, 35 kDa, and 26 kDa) were newly observed on SDS-polyacrylamide gels when cells of *Methylococcus capsulatus* Bath expressing sMMO were switched to copper-enriched media (Stanley et al., 1983; Burrows et al., 1984; Prior and Dalton, 1985). This polypeptide pattern was confirmed and the pMMO-associated polylpeptide pattern of *M. trichosporium* OB3b (25.5 kDa and 42 kDa) was shown by DiSpiritio et al. (1992). This pattern of OB3b peptides is in contrast to that reported by Tonge et al. (1975; 1977). Recently extensive EPR and nuclear magnetic studies on the membrane-bound proteins of *M.*

Table II -2. Characteristics of purified or partially purified soluble methane monooxygenases from several representatives of methanotrophs and methylotrophs.[A]

Characteristics	*Methylococcus capsulatus* Bath	*Methylosinus trichosporium* OB3b	*Methylobacterium* CRL-26	*Methylomonas* sp. 761M
Classification	Type I, obligate methanotroph	Type II, obligate methanotroph	Type II , facultative methylotroph	Type I, obligate methanotroph
Components	A, Hydroxylase B, Regulatory C, Acceptor reductase	A, Hydroxylase B, Regulatory C, Acceptor reductase	A, Hydroxylase B, ? C, acceptor reductase	
Component A Mr. (Da)	226 000	245 000	220 000	
α	54 000	54 400	55 000	
β	42 000	43 000	42 000	
γ	17 000	22 700	20 000	
Metal content (mol/mol protein)	Fe 2.3	Fe 4.3	Fe 2.8	
Component B Mr. (Da)	17 000	15 000	ND	
Metal content (mol/mol protein)	None	None	ND	

Table II-2 cont'd.

Characteristics	*Methylococcus capsulatus* Bath	*Methylosinus trichosporium* OB3b	*Methylobac-terium* CRL-26	*Methylomonas* sp. 761M
Component C Mr. (Da)	44 600	38 300	40 000	
Prosthetic group	1 FAD + 1 Fe_2S_2	1 FAD + 1 Fe_2S_2	1 FAD + 1 Fe_2S_2	
Catalytic activity	NADH:acceptor reductase	NADH:acceptor reductase	NADH:acceptor reductase	
Other characteristics	Components cross-reacts with those of strain OB3b		The antibody probes ofcomponents A & C react with the corresponding components of M. organophilum XX and Methylococcus sp. CRL-25 (obligate type I methanotroph	The crude extract react with antibody probe of component A of SB-I (obligate type II methanotroph)

A From Allen et al., 1984; Dalton, 1992; Patel, 1984; Patel and Savas, 1987; Pilkington and Dalton, 1991.
ND Not determined.

capsulatus Bath suggested that the trinuclear copper cluster could be the site of monooxygenase activity of the pMMO system (Chan et al., 1993).

F. Molecular genetics of MMO

In contrast to the extensive studies on the biochemistry of MMO systems, genetic studies on MMO have lagged. This is mainly due to the difficulty in producing useful mutants in methanotrophs (Williams and Bainbridge, 1971; Williams et al., 1977).

A useful approach to identify MMO genes has been the employment of oligodeoxynucleotide probes specific to the N-terminal amino acid sequence of individual polypeptides of the soluble MMO. This method has paved the way for cloning and sequencing the sMMO gene cluster of *M. capsulatus* Bath (Mullens and Dalton, 1987; Stainthorpe et al., 1989; Pilkington et al., 1990; Stainthorpe et al., 1990). Recently Stainthorpe et al. (1990a) have shown that a cloned DNA probe containing *M. capsulatus* Bath soluble MMO genes specifically hybridized to the DNAs of type II methanotrophs (*Methylosinus* sp. strains including *Methylosinus trichosporium* OB3b) but not to type I (*Methylomonas albus* BG8 and *Methylomonas methanica* S1). This indicates that the sMMO genes appear to be conserved in limited groups of type II methanotrophs.

As shown in Table II-3, the sMMO genes from *M. capsulatus* Bath and *M. trichosporium* OB3b have very similar organizations. The soluble MMO gene probe (5.8 kb *Bam* HI fragment of pCH4) cloned from *M. capsulatus* has homology to *Methylosinus* sp. strains producing similar sMMOs. In contrast to the genetic distinctiveness of sMMO of *M. capsulatus* Bath, other data show that the sMMO is not confined to only type X and some limited numbers of type II

Table II-3. Comparative molecular analysis of the genes and the deduced protein sequences of soluble methane monooxygenases of *Methylococcus capsulatus* Bath (Type I) and *Methylosinus trichosporium* OB3b (Type II).[A]

		Methylococcus capsulatus Bath			*Methylosinus trichosporium* OB3b		
Compo-nent		ORF size (bp)	No. of amino acids	% G + C	ORF size (bp)	No. of amino acids	% homology (a.a.) to 'Bath'
A subunit	α	1584 (*mmoX*)	527	60	1578 (*mmoX*)	525	94
	β	1164 (*mmoY*)	387	61	1185 (*mmoY*)	394	84
	γ	513 (*mmoZ*)	170	60	510 (*mmoZ*)	169	85
B		426 (*mmoB*)	141	54	417 (*mmoB*)	138	89
C		1047 (*mmoC*)	348	63	1023	340	78
Plasmid carrying sMMO gene cluster		pCH4			pDVC210		

Table II-3 cont'd.

Compo-nent	*Methylococcus capsulatus* Bath ORF size (bp)	No. of amino acids	% G + C	*Methylosinus trichosporium* OB3b ORF size (bp)	No. of amino acids	% homology (a.a.) to 'Bath'
Organisms carrying homologous gene cluster	*Methylosinus sporium* 5 and12 *Methylosinus trichosporium* OB3b, OB4, and OB5b			ND		

[A] From Cardy et al., 1991; and Stainthorpe et al.,1989.

methanotrophs. To date, no sMMO genes of type I methanotrophs have been cloned and characterized.

G. Nucleic acid probes for identifying and detecting methanotrophs and other techniques monitoring the methanotrophic populations

It may be possible to use the sMMO ORFs (*mmoX, mmoY, mmoZ, and mmoB*) of *M. capsulatus* Bath as specific sMMO probes. The sMMO gene fragment carried on plasmid pCH4 has been used to detect similar sMMO systems of new and uncharacterized environmental methanotrophs by colony hybridization under conditions of low stringency (Stainthorpe et al., 1990b). A sMMO gene probe of *M. trichosporium* OB3b has also been used to identify homologous genes of a few type II methanotrophs enriched from a groundwater sample contaminated with TCE and isolated from environmental samples (Tsien et al., 1992). However, this probe failed to hybridize to the homologous region of the sMMO gene cluster of *M. capsulatus* Bath and to detect the potential sMMO genes of type I methanotrophs. Thus it seems that sMMO genes are not universally conserved in methanotrophs. Hanson (1992) suggested that the methanol dehydrogenase (MDH) gene could be used to measure the total population of gram-negative methylotrophs due to the universal conservation of the genes in the methylotrophs. In this case it is assumed that all strains have one copy of the target gene per genome, and an average genome molecular weight and the number of genomes per cell are known.

A 16S rRNA sequence analysis made it possible to classify methylotrophs (Tsuji et al., 1990), and unique sequences within 16S rRNA were used to construct group-specific oligodeoxynucleotide probes. The 16S rRNA probe for

RuMP methylotrophs (10-γ) and the 16S rRNA probe for serine pathway methylotrophs (9-α) were developed. However, these probes cannot exclusively detect methanotrophs (Tsien et al., 1990). Recently five additional 16S rRNA-targeting oligodeoxynucleotide probes have been developed allowing the differentiating the methanotrophs and methylotrophs within the two different pathways for carbon assimilation (Brusseau et al., 1994).

One of the typical methods to enumerate methanotrophs in environmental samples was to plate serially diluted samples onto agar media and count the number of colonies formed following incubation under an atmosphere of methane and air (Hanson, 1980; Abramochinka et al., 1987; Gal'chenko et al., 1988; Hanson et al., 1991a). The population density typically found in soils, sediments, and waters ranged from 10^3 to 10^6 CFU/g (Heyer et al., 1985). The plate count procedure, however, tends to underestimate the population size and diversity of methanotrophs due to the poor plating efficiency of the organisms (Hanson, 1980; Bone and Balkwill, 1986). The plating efficiency may be dependent on the various incubation conditions such as substrate concentrations, nutrient supplements, oxygen concentrations, pH, and temperatures (Hanson et al., 1991). Methanotrophs are frequently out-grown by colonies of oligocarbophilic bacteria growing on the same agar media (Hanson, 1980).

Direct identification and enumeration of methanotrophs without culturing may be possible by using fluorescent antibody techniques (Reed and Dugan, 1978; Saralov et al., 1985; Abramochinka et al., 1987; Gal'chenko et al., 1988). However, the insufficient information on the number of serotypes of

methanotrophs hampers the wide application of these techniques to enumerate the populations (Boxrukova et al., 1983).

Direct counting and enumeration of methanotrophic mixed populations was performed in the two species competition studies (Graham et al., 1993). In this case acridine orange direct count (AODC) technique was used as a complementary method to the fluorescently labeled oligo probe method to differentiate the two taxonomically different methanotrophs (*M. albus* BG8 and *M. trichosporium* OB3b) where BG8 and OB3b cells fluoresced orange and bright green after staining by acridine orange, respectively. The monitoring data by the two methods were in agreement within 20 % variation. It is generally agreed in acridine orange staining that the single-stranded nucleic acids emit orange while the double-stranded ones fluoresce green *in vivo* (Daily, 1979; McFeters et al., 1991). However, variations in the staining procedure (Jones, 1974; Van Es and Meyer-Reil, 1982) and the status of the physiological activity of cells (Moyer and Morita, 1989; McFeters et al., 1991) can significantly affect the results of the staining.

H. TCE degradation by different types of methanotrophs

The ability of MMOs from a number of methanotrophs to oxidize a wide range of substrates is well documented (Colby et al., 1977; Burrows et al, 1984). These substrates include n-alkanes, n-alkenes and ethers, and aromatic, alicyclic, and heterocyclic compounds. Both sMMO and pMMO can oxidize alkanes and alkenes up to pentane (Burrows et al, 1984; Dalton et al., 1984). However, pMMO cannot oxidize aromatic and alicyclic compounds or higher ($n>5$) alkanes while sMMO can (Burrows et al, 1984; Stirling et al., 1979).

Ethylene, similar in structure to TCE, is oxidized to epoxyethane by sMMO of *M. capsulatus* Bath (Colby et al., 1977), by sMMO and pMMO of *Methylobacterium* sp. CRL-26, and by resting cell suspensions of *M. trichosporium* OB3b (Higgins et al., 1979; Hou et al., 1979; Patel et al., 1982).

A methanotrophic mixed culture responsible for TCE degradation was first isolated from an unsaturated surface soil (Wilson and Wilson, 1985). Subsequently type II methanotrophs were identified by phospholipid fatty acid analysis of the same soil samples enriched with natural gas (Nichols et al., 1986). Freshwater lake sediments and aquifer samples were also sources of mixed methanotrophic cultures mineralizing TCE (Fogel et al., 1986; Broholm et al., 1990; Lanzarone and McCarty, 1990). Recently pure cultures of type I methanotrophs and a mixed culture containing a type II methanotroph were isolated from TCE contaminated wells and groundwater aquifers and found to degrade TCE (Henry and Grbic-Galic, 1990; Henry and Grbic-Galic, 1991a). A pure type II methanotroph was also isolated from soil and could degrade TCE (Uchiyama et al., 1989). Methane oxidation can occur even within a pristine, well-oxygenated region of an aquifer (Mancinelli and White, 1991; Smith et al., 1991). Generally, in ecosystems where nitrogen is in excess, and in those environments where methane limits growth and copper is in excess, type I methanotrophs are dominant. Type II methanotrophs dominate in environments in which methane is non-limiting and nitrogen, copper or oxygen are growth-limiting substrates (personal communication, R. S. Hanson, University of Minnesota). Hence, it might be possible that type I and type II methanotrophs are under selection pressure depending on the carbon source and the mineral nutrient availability in certain environments.

I. Growth regimes affecting TCE degradation

Environmental factors may limit or preclude the biodegradation of a subsurface organic pollutant, even in the presence of adapted organisms (Lee et al., 1988). Even though most pristine drinking water aquifers do contain a certain amount of O_2 it may be rapidly consumed by an active population in highly organically polluted environments and O_2 (Ghiorse et al., 1988) supply may become the key limitation of the biodegradation rate (Morgan and Watkinson, 1989). O_2 concentrations of uncontaminated groundwater range from 0.02 to 8.26 mg/L (Fliermans and Balkwill, 1989). Another recent report shows that chemical oxygen demand in groundwaters heavily contaminated with organics is 200 - 2000 times as high as uncontaminated ones (Kastner, 1991). This indicates that there is a great gap between O_2 demand and supply in the contaminated groundwaters. Indeed, methanotrophs, that are indigenous to a subsurface aquifer environment, can be successfully stimulated to promote the TCE degradation by injecting O_2 and CH_4 (Semprini et al., 1990). However, due to the low solubilities of these gases, the delivery of dissolved O_2 and CH_4 to methanotrophs may significantly affect the growth and maintenance of the bacterial population in a saturated aquifer.

Copper may significantly influence the expression of MMO and cell morphology (Burrows et al., 1984; Dalton and Leak, 1985; Prior and Dalton, 1985; Collins et al., 1991). In the case of *M. capsulatus* Bath and *M. trichosporium* OB3b grown in copper limiting conditions, methane is oxidized mostly by the sMMO. The pMMO becomes dominant when copper is present in non-limiting quantities (Burrows et al., 1984; Dalton et al., 1984). Cu(I) and Cu(II) can inactivate the soluble enzyme of *M. capsulatus* Bath (Green and

Dalton 1985). Apparently copper is required for pMMO activity in both these species (Prior and Dalton, 1985), and *Methylobacterium* sp. strain CRL-26 (Patel et al., 1982). The pMMO content of intracytoplasmic membranes in the type X and type II methanotrophs increases as the Cu (II) availability increases (Scott et al., 1981; Stanley et al., 1983; Prior and Dalton, 1985). *Methylomonas albus* BG8 (type I), known to produce only pMMO, increases intracytoplasmic membranes and pMMO activity at enhanced copper availability (Collins et al., 1991). sMMO expression of a pure continuous culture of *M. trichosporium* OB3b is completely inhibited by copper ion (4.8 μM) but pMMO activity is unaffected and contributes to approximately 50 % of total TCE degradation (Oldenhuis et al., 1989). Here, the optimal level of copper ion and biomass were not examined to get maximum TCE degradation by pMMO. sMMO expression and TCE degradation of OB3b occurred in the presence of less than 0.25 μM copper (Tsien et al., 1989). At this copper concentration, cell densities should be more than 1.0 g per liter to reach a significant TCE oxidation rate. Again in this study, the role of pMMO activity in TCE degradation under copper limitation conditions was not addressed. The TCE oxidation rate of *Methylomonas* sp. MM2 (type I) appears to be inhibited by copper ion (1.6 μM) but methane oxidation rate and growth yield do not (Henry and Grbic-Galic, 1990). Although minimum copper ion concentration inhibiting TCE oxidation was not determined in this study, the oxidation rate under copper limitation is comparable to that of *M. trichosporium* OB3b considering the lower optimum growth temperature of the strain MM2.

Taking into account the contribution of pMMO of type II methanotrophs to TCE oxidation (Oldenhuis et al., 1989; Tsien et al., 1989), the pMMOs of type I methanotrophs could play a role in TCE degradation. This is supported by the

recent evidence showing that pMMOs of various type I methanotrophs isolated from marine, freshwater, and groundwater are capable of TCE oxidation (DiSpirito et al., 1991). However, their degradation rates appear to be significantly lower than sMMO.

This study had three specific aims:

A. To determine whether strain 68-1 can degrade TCE as a type I methanotroph

A pure culture of the TCE-degrading methanotroph, strain 46-1 (type I), was first isolated from TCE-contaminated wells (Little et al., 1988). Later several other type I (Henry and Grbic-Galic, 1990; DiSpirito et al., 1992) and type II (Uchiyama et al., 1989; Alvarez-Cohen et al., 1992; Henry and Grbic-Galic, 1990) methanotrophs were isolated from aquifers, soil, and other environmental samples and found to degrade TCE. Recently, a type I methanotroph, *Methylomonas* sp. MM2 was isolated from a TCE-contaminated aquifer and shown to degrade TCE efficiently enough to have the potential for bioremediation applications (Henry and Grbic-Galic, 1990). In our laboratory we have been working on strain 68-1 which is tentatively identified as a type I methanotroph based only on stacked intracytoplasmic membrane structures and hexulose phosphate synthase activity (Little et al., 1988). In this study, we have further characterized the strain 68-1 using more sensitive and specific molecular biological and biochemical techniques, and confirmed that this strain is a type I methanotroph with the ability to degrade TCE via sMMO.

B. To clone type I specific DNA probes and use them for quantitative measurement of the genes and populations in methanotrophic pure and defined mixed cultures

Currently the sMMO gene clusters from *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b have been cloned and sequenced (Mullens and Dalton, 1987; Stainthorpe et al., 1989; Stainthorpe et al., 1990; Cardy et al., 1991). The sMMO structural genes are clustered in a 7 kb chromosomal DNA region and closely linked to one small open reading frame of unknown function. However, no sMMO genes have been cloned from type I methanotrophs. Therefore, in this study an attempt was made to clone the sMMO genes from a type I methanotroph and to develop a type I methanotroph-specific DNA probe. It was also examined whether a known sMMO gene clone carrying a sMMO B component gene of *Methylosinus trichosporium* OB3b (Tsien et al., 1992) could be used as a type II methanotroph-specific gene probe. In this study these probes were used to monitor populations of the strain 68-1 and *M. trichosporium* OB3b in pure and defined mixed cultures for TCE biodegradation study.

C. To measure the dynamics of mixed methanotrophic bacterial populations consisting of 68-1 and *M. trichosporium* OB3b by using DNA probes and to determine the quantitative relationships between the population dynamics and their activities in TCE degradation

Gene probe technology has been shown to be useful for the efficient detection of specific populations (Sayler et al., 1985; Steffan et al., 1989) and specific genes (Ogram and Sayler, 1988; Barkay et al., 1991) and for relating

gene frequency with catabolic activity (Blackburn et al., 1987) in given microbial communities. So far, two DNA probes (pCH4 and pDVC201) coding for sMMOs of type X and II methanotrophs, respectively, have been successfully used to detect similar sMMO genes in pure methanotrophic cultures and environmental samples (Stainthorpe et al., 1990b; Alvarez-Cohen et al., 1992; Tsien et al., 1992).

Among growth regimes affecting TCE degradation by methanotrophs, methane (Oldenhuis et al., 1989; Broholm et al., 1990; Lanzarone and McCarty, 1990; Semprini et al., 1990; Graham et al., 1993), nitrogen source (nitrate; Graham et al., 1993), copper ion concentrations (Oldenhuis et al., 1989; Tsien et al., 1989; Henry and Grbic-Galic, 1990; Graham et al., 1993), and oxygen (Mayer et al., 1988; Lanzarone and McCarty, 1990; Semprini et al., 1990; Alvarez-Cohen and McCarty, 1991a; Graham et al., 1993) appear to be important. To date, it has been difficult to quantitatively relate methanotrophic gene frequencies with their population densities (and then densities with activities) in TCE degradation studies. This study addressed this issue under different growth regimes, such as different methane, nitrate, copper, and oxygen concentrations.

CHAPTER III

MATERIALS AND METHODS

The overall experimental design was set up to accomplish three objectives of this study: (i) to characterize the strain 68-1 taxonomically, physiologically, and genetically; (ii) to develop a DNA probe allowing specific detection and monitoring of the 68-1 population in mixed cultures; (iii) to perform molecular analysis of the species competition of the strain 68-1 and *Methylosinus trichosporium* OB3b in batch (flask) and continuous bioreactor cultures under varying growth regimes.

A. Identification of strain 68-1

The strain 68-1, originally isolated from TCE-contaminated wells (Little et al., 1988), was characterized and identified by electron microscopy, phospholipid fatty acid analysis, and 16S rRNA signature-probing.

Bacterial strains and cultural conditions

Bacterial strains used in this study are listed in Table III-1. Methanotrophic bacteria were grown on a nitrate mineral salts (NMS) medium (Cornish et al., 1984) under an atmosphere of 4:1 air to methane. Most methanotrophic and heterotrophic bacteria were grown at 26-28°C except *Methylococcus capsulatus* Bath and *Methylococcus capsulatus* ATCC 19069^T which were grown at 37°C. The RuMP pathway methylotrophs were grown in NMS containing 0.1 % (v/v) methanol and the serine pathway methylotrophs in

Table III-1. Bacterial strains used in this study.

Type and species of bacterial strain	Source[A] or reference
Type I methanotrophs:	
Methylomonas agile	ATCC 35008
Methylomonas alba	ATCC 33003 (=BG8)
Methylomonas methanica	ATCC 35067 [T]
Methylomonas methanica [*"Methylomonas rubra"* [B]]	NCIMB 11913
Strain 68-1	Little et al., 1988
Strain 46-1	Little et al., 1988
Type X methanotrophs:	
Methylococcus capsulatus	ATCC 19069 [T] (=Texas)
Methylococcus capsulatus	ATCC 33009 (=Bath)
Type II methanotrophs:	
Methylocystis parvus	ATCC 35066 (= OBBP)
Methylosinus sporium	ATCC 35069 (=5)
Methylosinus trichosporium	ATCC 35070
RuMP pathway methylotrophs[C]:	
Methylophilus methylotrophus ["*Methylomonas clara*"]	ATCC 31226 (Jenkins and Jones, 1987)
Methylophilus methylotrophus	ATCC 53528 [T]
Serine pathway methylotrophs[C]:	
Hyphomicrobium sp.	ATCC 43129
Methylobacterium extorquens	ATCC 14718 [T] (=AM1)

Table III-1 cont'd.

Type and species of bacterial strain	Source[A] or reference
Methylobacterium organophilum	ATCC 27886[T] (=XX)
"Methanomonas methylovora"	ATCC 21852 [T]
Unidentifled methane or methanol utilizers:	
IAM-012A (methane)	C. E. B., UTK[D]
IAM012B (methane)	C. E. B., UTK
IAM-026 (methanol)	C. E. B., UTK
IAM-030 (methanol)	C. E. B., UTK
Heterotrophs:	
Agrobacterium tumefaciens	ATCC 23308
Bacillus subtilis	ATCC 6051 [T]
Escherichia coli	ATCC 23716 (=K12)
Escherichia coli	HB101
Escherichia coli XL1-Blue	Stratagene Cloning System, Inc.
Rhizobium leguminosarum	strain 248 (Hirsch, 1979)
Pseudomonas putida	strain F1 (Wackett and Gibson, 1988)

[A] ATCC, American Type Culture Collection, Rockville, MD, U. S. A.; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. [B] Inverted commas indicate the species has a taxonomically invalid status (Holt et al., 1992). [C] RuMP, ribulose monophosphate; Serine, serine transhydroxymethylase. [D] Center for Environmental Biotechnology, University of Tennessee, Knoxville.

NMS containing methylamine-HCl (6.75 g/L) except *Methylobacterium rhodesianum* ATCC 43882^T which was grown in nutrient broth. All heterotrophs were grown in nutrient broth unless otherwise mentioned. Copper-free NMS media were prepared with double glass-distilled water in glassware washed according to the procedure of Tsien et al. (1989). Growth of cultures and density of cell suspension were monitored at 600 nm using a Spectrophotometer DU-70 (Beckman, Palo Alto, CA).

Electron microscopy

The strain 68-1 was grown to late log phase in NMS medium in the presence or absence of 1 μM $CuSO_4$. Pellets of bacteria were fixed in 3 % glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.0) for 1 h at room temperature. After being washed three times in 0.1 M phosphate buffer for 1 h, the cells were then post-fixed in 2 % (w/v) OsO_4 for 1 h. The cells were dehydrated in a graded series of alcohol : water mixtures and then embedded in Epon 812 (Electronmicroscopy Sciences, Fort Washington, PA) as described (Luft, 1961). The embedded cells were cut with a diamond knife and stained with uranyl acetate (Watson, 1958) and basic lead citrate (Reynolds, 1963) prior to observation using Hitachi H-600 Electron Microscope (Hitachi Co., Tokyo, Japan) in the electronmicroscopy laboratory of the University of Tennessee, Knoxville.

Phospholipid fatty acid (PLFA) and lipopolysccharide hydroxy fatty acid (LPS-OHFA) analyses

The total lipid of lyophilized 68-1 cell material was extracted and fractionated with activated silicic acid (Nichols et al., 1985; White et al., 1979). The phospholipid fraction was trans-esterified to their corresponding methyl esters using mild alkaline methanolysis and analyzed by gas chromatography (Nichols et al., 1985). Lipid isomers and double-bond position was determined by dimethyl-disulfide derivitization (Dunkleblum et al., 1985; Nichols et al., 1986).

Specific 16S rRNA signature probes for methanotrophs and methylotrophs and their hybridization to total RNA or DNA

Oligodeoxynucleotide probes complementary to 16S rRNA specific to ribulose-monophosphate pathway-containing (10-γ or 1035-RuMP) and serine pathway-containing (9-α or 1034-Ser) methylotrophs were used. Characteristics and sequence of the probes are shown in Table III-2. Total RNA's were extracted from strain 68-1 and 9 other strains including methanotrophs, methylotrophs, and one heterotroph by the hot phenol extraction method (Oelmuller et al., 1990). The method was modified as follows:1.5 ml of cell culture (10^{7-8} cells/ml) was harvested by 3 min centrifugation at 14000 rpm in a microfuge. The cells was then washed once with 1 ml ice-cold AE buffer (20 mM sodium acetate and 1 mM disodium EDTA; pH 5.5) and resuspended in 0.5 ml ice-cold AE buffer. The cell suspensions were immediately transferred to a 50 ml polypropylene tube containing 6 ml of a hot (60°C) phenol-chloroform mixture [one volume of phenol (equilibrated with 20 mM sodium acetate, pH 5.5, 1 mM

Table III-2. Nucleotide sequence of oligodeoxynucleotide signature probes complementary to 16S rRNA of methantrophs and methylotrophs used in this study.

Probe	Description of specificity	Nucleotide sequence	Reference
10-γ	RuMP pathway methylotrophs	5'-GGTCCGAAGATCCCCCGCTT-3'	Tsien et al., 1990
9-α	Serine pathway methylotrophs	5'-CCCTGAGTTATTCCGAAC-3'	Tsien et al., 1990
Eubacterial probe	Eubacteria	5'-ACCGCTTGTGCGGGCCC-3'	Tsien et al., 1990
1035-RuMP	RuMP pathway methanotrophs	5'-GATTCTCTGGATGTCAAGGG-3'	Brusseau et al., 1994
1034-Ser	Serine pathway methanotrophs	5'-CCATACCGGACATGTCAAAAGC-3'	Brusseau et al., 1994

disodium EDTA, and 0.1 % (w/v) SDS) and one volume of chloroform-isoamyl alcohol (24:1, v/v)], 150 µl of 10 % (w/v) SDS and 2.5 ml of AE buffer. The tubes were incubated at 60°C for 10-15 min with intermittent shaking. The tubes were then chilled immediately on ice. The organic and aqueous phases were separated at 4°C by 15 min centrifugation at 13000× g and the aqueous phase was taken. After addition of sodium acetate solution (2 M; pH 5.2) to the aqueous phase to give a final concentration of 0.25 M, the phase was repeatedly extracted (2-3 times) with phenol-chloroform mixture until no protein was detected in the interface. Total RNA was precipitated after adding 2.5 volume of 96% ethanol and incubating at -80°C for 15 min, and pelleted by centrifugation for 15 min at 13000× g. The pellet was washed once with 96 % ethanol and dried at 37°C for 15 min or at room temperature. The pellet was resuspended in 100 µl of TM buffer (40 mM Tris-HCl, pH 7.5 and 6 mM $MgCl_2$). The approximate concentration of RNA was determined by running the sample on non-denaturing agarose mini-gels. Residual DNA was removed by addition of 100 U of DNase free of RNase (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and incubation at 37°C for 30 min, if necessary.

The deoxyoligonucleotide probes were labeled with [γ-^{32}P]-dATP (ICN Biomedicals, Inc., Irvine, CA; 4500 Ci/mmol) by following the method described by Ausubel et al. (1987). Total RNA (100-500 ng) was immobilized on a nylon membrane (ICN Biomedicals, Inc., Cleveland, OH) for slot blot hybridization (Sambrook et al., 1989) and hybridized to each target 16S rRNA probe according to the procedure of Tsien et al. (1990).

B. Measurement of growth of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b

Maximum specific growth rate

Cultures were grown in shaking flasks containing NMS medium (Cornish et al., 1984) with or without copper (1 μM $CuSO_4$) at room temperature (25-26°C). The culture volume did not exceed 20 % of the total volume of flask in order to maintain a good mass transfer of air (oxygen) and methane gas mixture (4:1 v/v ratio). Growth of cultures was determined by measuring optical density at 600 nm with a spectrophotometer DU-70 (Beckman, Palo Alto, CA). The measurements were made over all the growth period covering lag phase, log phase, and stationary phase. More frequent measurements were performed during the log phase to obtain reproducible specific growth rate data. The optical density values were converted into log values and plotted over time (h). Linear regression equations were determined by fitting at least 3 consecutive time points over the growth period and the slope of the equation was calculated to obtain the specific growth rate according to Monod growth kinetics equation in batch culture (Pirt, 1975). The maximum specific growth rate could be determined from measurements at log phase.

Determination of total biomass density and growth yield

To compare the growth state among cultures, total biomass density and growth yield of each culture was determined. For total biomass density determination, duplicates of cell suspension (5 ml) at early stationary phase were taken into individual preweighed aluminum boats and dried at 105°C at least several hours until the total weight reached equilibrium or overnight. The

biomass density was expressed as mg dry cell per liter culture. For growth yield determination both the dry cell weight of each culture and methane consumption observed in headspace of flask were measured and expressed as g cell dry weight per g methane consumed.

C. Measurement of sMMO activity by naphthalene oxidation

The sMMO activity was measured for both whole cells and cell fractions. The cell fractions were prepared as soluble fraction containing sMMO and membrane fraction (or particulate fraction) containing particulate MMO (pMMO). The measurement of the sMMO activity is based on the fact that sMMO can oxidize naphthalene while pMMO cannot.

Quantitative measurement of soluble MMO activity of 68-1 whole cells

A slightly modified version of the naphthalene oxidation assay of Brusseau et al. (1990) was used to measure soluble MMO activity. Ten ml of each culture was degassed in 58 ml serum bottles sealed with rubber stoppers by evacuating the head space and backfilling with air to remove residual methane. Cultures were then diluted to an absorbance of 0.2 (600 nm; 0.08 to 0.09 mg dry cell weight per ml) with NMS media. The diluted cultures were transferred in 1 ml aliquots to 10 ml screw cap test tubes and 1 ml prefiltered saturated naphthalene solution [234 μM at 25°C; (Verschueren,1977)] was added to each aliquot. The samples were prepared in triplicate. The reaction mixtures were incubated at 200 rpm on a rotary shaker at 25°C for 1- 3 h. Heat-killed control and sterile medium control as blanks were also prepared and processed as tests. After incubation, 100 ml of freshly prepared tetrazotized-*o*-

dianisidine solution (4.21 μM) was added to each tube and the formation of colored diazo-dye complexes was immediately monitored by recording the absorbance at 525 nm using spectrophotometry. The intensity of diazo-dye formation is proportional to the naphthol concentration (1-naphthol and 2-naphthol). Wackett and Gibson (1983) determined the molar extinction coefficient (e) of the diazo-dye to be 38000 M^{-1} cm^{-1}. The protein content of the cell suspension was determined by the micro-Biuret procedure (Munkres and Richard, 1965). The specific activity of soluble MMO was expressed as nmoles naphthol formed per mg cell protein per h.

Quantitative measurement of soluble MMO activity of 68-1 cell fractions

Preparation of cell-free extracts were performed according to the modified method of Nakajima et al. (1992). Cells, grown at shaking flasks up to early stationary phase, were harvested by centrifugation at 13,200× g for 10 min at 4°C and washed in 100 mM PIPES (piperazine-N, N' bis [2-ethane sulfonic acid]) buffer (pH 7.0) containing 5 mM sodium thioglycolate as a reducing agent. The washed cells were resuspended in the buffer and sonicated with 20 × 30 s pulses and 1.5 min cooling on ice between each pulse at an output of 0.4 - 0.6 using a Virsonic 300 sonicator (The Viritis Co., Gardiner, NY). Cell debris was removed by centrifugation at 20,000× g for 15 min at 4°C and then the pellets were discarded and the supernatants centrifuged at 150,000× g for 1.5 h. The supernatants from this preparation were used as the soluble fractions and the pellets were resuspended in the PIPES buffer (pH 7.0) containing 5 mM sodium thioglycolate and used as the particulate fractions. For the assay of naphthalene oxidation activity by sMMO, 1 ml of the soluble fraction was dispensed into each

cap tube and then NADH (3.5 mM) and $FeCl_3$ (200 μM) were added while the particulate fraction containing NADH (3.5 mM) and $CuSO_4$ (20 μM) was used for the assay of naphthalene oxidation activity by pMMO. Triplicate vials were prepared for each fraction and the subsequent naphthalene oxidation assay was performed as in the assay of sMMO activity of whole cells described above.

D. Determination of TCE degradation activity

Quantitative measurement of TCE degradation by whole cells

Diluted cell suspensions (0.2 absorbance at 600 nm) were prepared as described in the above procedure and transferred in 1 ml aliquots to screw cap septum vials (14 ml; Pierce, Rockford, IL). Sodium formate was then added to each vial to a final concentration of 20 mM. Heat-killed control samples were prepared for all experiments. TCE degradation was initiated by adding 5 μl of a TCE aqueous stock solution (1100 μg/mL at 25°C; [Verschueren, 1977]) to each vial to a final concentration of 5.5 μg/mL (42 μM). The vials were then sealed with Teflon-lined silicone septa, inverted, and incubated on a rotary shaker (200 rpm) at room temperature (25°C) for various time periods. Triplicate samples and their corresponding heat-killed controls were sacrificed at designated time intervals by adding 2 ml of *n*-hexane containing 1,2-dibromoethane (10 ppm) as an internal control. The undegraded TCE was extracted into the organic phase by shaking and centrifugation (2000 x *g* for 20 min). The degradation analyses were performed on a Shimadzu GC 9AM gas chromatograph (Shimadzu Analytical Instruments Co., Kyoto, Japan) equipped with a a split injection port (1:1) run at 220°C, a 60 m x 0.53 mm i.d. RTX volatiles capillary column (Restek Corp., Bellefonte, PA) run isothermically at 80°C, and an electron capture

detector run at 220°C. Nitrogen was used as the carrier gas (flow rate, 10 ml/min). The peak areas were calculated by using a Shimadzu intergrator, C-R6A Chromatopac. Five or 10 µl of the organic phase was sampled by using a gas tight syringe (Hamilton Co., Reno, Nev.) and injected into the sample injection port. Under these conditions, TCE and 1,2-dibromoethane exhibited retention times of 4.3 and 1.7 min respectively.

Quantitative measurement of TCE degradation by cell fractions

Preparation of cell fractions were performed according to the method as in the sMMO activity assay of cell fractions described above. One ml of the soluble fraction was dispensed into each vial and then NADH (3.5 mM) and $FeCl_3$ (200 µM) were added while the particulate fraction containing NADH (3.5 mM) and $CuSO_4$ (20 µM) was used for the assay of TCE degradation activity by pMMO. Triplicate vials were prepared for each fraction and the subsequent TCE degradation assay was performed as described above.

E. Determination of kinetic parameters of naphthalene oxidation and TCE degradation

The strain 68-1 and *Methylosinus trichosporium* OB3b were grown to early stationary phase in 500 ml of copper-free NMS (in 2 L flasks). The cell suspensions were diluted to an absorbance of 0.2 (600 nm) and 1 ml of each of the suspensions was added to screw cap septum vials (14 ml; Pierce, Rockford, IL). Different concentrations of naphthalene (7.3 to 234.0 µM) and TCE (5.0 to 500.0 µM) were added to the various vials. In addition, 20 mM sodium formate was added to ensure that NADH pools were saturated (Brusseau et al., 1990).

The vials were incubated for 1 h at 25°C. Two independent experiments using triplicate vials for each concentration were performed to determine K_m and V_{max} values. It was found that the Lineweaver-Burke plots were not statistically adequate because inversion distorted the error span in lower substrate concentrations. Thus rectangular hyperbolic curve fits were performed using the computer program DeltaGraph (Microsoft Corp., Redmond, WA). The maximum dissolved substrate concentrations for naphthalene and TCE were assumed to be 234 μM and 8.365 mM, respectively. These concentrations are the points at which these compounds are saturated in water at 25°C (Verschueren, 1977).

F. DNA slot blotting and Southern blot hybridization for genetic homology study

Plasmid DNA isolation

The plasmids, pBluescript II SK and pUC119, were used and carried on Epicurean Coli (Stratagene Cloning Systems, Inc., La Jolla, CA) and *E. coli* DH1 cells respectively. For the muti-copy plasmid isolation at small scale preparation, the method described by Sambrook et al. (1989) was followed. By this procedure, several micrograms of plasmid DNA was routinely obtained from 3 ml culture of the host cells grown in LB containing ampicillin (100 μg/ml) at 37°C overnight and subsequently used for making of the DNA probe, slot-blotting, and Southern blotting.

Large scale preparations of multi-copy plasmids were done by following the alkaline lysis method (Ausubel et al. 1987). The host cells were grown in 1 liter LB broth containing ampicillin (100 μg/ml) at 37°C overnight. Cells were pelleted by centrifugation at 4600× g for 10 min in 250 ml centrifuge tubes and resuspended in 10 ml glucose (50 mM)/Tris (25 mM; pH 8) /EDTA (10 mM)

solution. Cells were then lysed by adding 20 ml of NaOH (0.2 M) /SDS (1 %) solution, gently mixed , and incubated at room temperature for 5 to 10 min. Fifteen ml of 3 M potassium acetate solution was added, mixed gently, and left at room temperature for 5 min. The tubes were centrifuged at 4600× g for 15 min and the supernatants were filtered through cheese cloth into 250 ml centrifuge tubes. At this point the supernatants were precipitated by adding 100 ml isopropanol for CsCl-EtBR gradient purification by ultracentrifuge described above or were subjected to extraction by an equal volume phenol/chloroform/isoamyl alcohol for further plasmid purification using the pZ523 Spin Column (5 Prime -> 3 Prime, Inc., Boulder, Co.). After ultracentrifugation the CsCl-EtBr gradient purification clearly showed plasmid and chromosomal DNA bands under UV light. The spin column also allowed a good plasmid purification by trapping linearized chromosomal DNA within a Sephadex G-50 column. The plasmid DNA was identified by agarose gel electrophoresis.

Chromosomal DNA isolation

For chromosomal DNA isolation at small scale, cultures were grown in appropriate media up to 10^8 cells/ml and the cells (3 ml) of each culture were harvested by centrifugation. The cell pellet was then washed with 1-1.5 ml of 0.5 % N-lauroyl sarcosine in TSE buffer (TE buffer containing 100 mM NaCl). The washed pellet was resuspended in 450 µl of TE buffer, and 75 µl each of 10 % SDS and proteinase K (2.5 mg/ml) were added and the cell lysis was performed at 37°C for 1 h. The reaction mixture was extracted with equal volumes of phenol/chloroform (2-3 times) and chloroform (once) in order. The DNA in the

aqueous phase was precipitated by ethanol, dried, and resuspended in 50-100 µl of TE buffer. This procedure routinely yielded several micrograms of DNA and was used for slot-blotting and Southern blotting.

For large scale chromosomal DNA isolation from strain 68-1 and *Methylosinus trichosporium* OB3b, cultures were grown in 500 ml of NSM up to 10^8 cells/ml and the total DNA isolation protocol (Russell et al., 1985) was followed. The cells were spun down at 4600x g for 10 min, and resuspended in 10 ml of TSE buffer containing 100 mg lysozyme and incubated at 37°C for 1h. 2.5 ml of 10 % SDS and 0.5 ml of Proteinase K [5 mg/ml; prepared in Tris-Ca buffer (10 mM Tris-HCl pH 8.0 and 5 mM $CaCl_2$)] were then added and incubated at 65°C for 1 h. The lysate was put into a 50 ml sterile tube and brought up to 30 ml with TE buffer, and 28.54 g of CsCl was added to adjust buoyant density to 1.55-1.59. The mixture was put in Ti80 seal top centrifuge tube (Beckman Polyallomer Quick Seal Centrifuge tube 25x89 mm) and mineral oil was added on top of the mixture, and the tubes balanced. The tubes were subjected to centrifugation at 300,000× g for 16-18 h. The viscous chromosomal DNA fraction was collected in a 10 ml tube by inserting needles (16G) at the top and near the bottom of the centrifuge tube. The DNA was precipitated by adding 2.5 volume of ethanol.

DNA slot blotting and Southern blot hybridization

For DNA slot blot hybridization, 2 µg of each DNA sample dissolved in 25 µl of water was added to 25 µl of 1 M NaOH and denatured at 95°C water bath for 10 min. The denatured DNA solution was then cooled rapidly on ice, and 50 µl of 0.5 M NaOH and 100 µl of water were then added. A nucleic acid slot blot

system (BioDot SF Microfiltration Apparatus, BioRad, Richmond, CA) was assembled and mounted with a 6× SSC (0.9 M sodium chloride and 0.09 M trisodium citrate, pH 7.0) prewetted Biotrans nylon membrane (ICN Biomedicals, Cleveland, OH). The denatured DNA samples were loaded into wells of the slot blot apparatus and transferred to the filter by applying a vacuum. The filter was then air-dried and baked at 80°C for 1-2 h to immobilize the DNA. For DNA Southern blot hybridization, 10 µg of each genomic DNA was digested with *Eco* RI enzyme and separated by electrophoresis on 0.8 % agarose gels in 1× TBE buffer (50 mM Tris base, 50 mM boric acid, and 1 mM EDTA; pH 8.3). The electrophoresed DNA was transferred to Biotrans nylon membranes by using the Vacuum-Blotting System (Pharmacia Biotechnology AB, Sweden) following the manufacturer's instruction. A DNA probe complementary to 2.2 kb *Eco* RI fragment of the *Methylosinus trichosporium* OB3b sMMO gene cluster [carrying the *mmoB, mmoZ, orfY,* and part of the *mmoC* genes; (Stainthorpe et al., 1990)] was labeled by using the Genius Nonradioactive Labeling and Detection Kit (Boehringer Mannheim Corp., Indianapolis, IN). All the subsequent prehybridization, hybridization, and detection of the labeled DNA probe were performed by following the Genius System User's Guide for Filter Hybridization (Boehringer Mannheim Corp., Indianapolis, IN). Detection in this experiment was carried out by using a chemiluminescent detection system using Lumi-Phos 530 provided by the manufacturer. In some cases (e.g., population monitoring by total DNA extraction and hybridization) DNA probes were labeled by using the Prime-It Random Primer Labeling Kit (Stratagene Cloning Systems Inc., La Jolla, CA) and hybridizations were performed by essentially following the procedure of

Church and Gilbert (1984) in which 7 % of SDS was used as a blocking agent for the non-specific binding of DNA probe.

G. Degradation of various chlorinated aliphatic hydrocarbons

Cell suspensions (absorbance at 600 nm) were prepared as described in the above procedure and transferred in 5 ml aliquots to Mininert-valve cap septum vials (32 ml; triplicates). Sodium formate was then added to each vial to a final concentration of 20 mM. Heat-killed control samples were prepared for all experiments. The degradation assay was initiated by adding each aqueous stock solution to each vial to a final concentration of 10 μM. The vials were then closed with Teflon-lined valve and incubated on a rotary shaker (200 rpm) at room temperature (25°C) for 1 h, 4 h, 24h, and 48 h. After incubation, 10 μl of head space gas of samples and their corresponding heat-killed controls were taken and subjected to GC analysis. The analyses were performed using s Shimadzu GC 9AM GC equipped with a 60 m × 0.53 mm i.d. RTX volatile capillary column: column temperature was raised from 50°C to 150°C (5°C/min) according to a program while the injector and ECD detector temperatures were maintained at 250°C and 300°C, respectively. Nitrogen gas was used as the carrier gas (flow rate, 10 ml/min). Under these conditions retention times for the chlorinated aliphatic hydrocarbons were observed as follows: dichloromethane 2.62 min; chloroform 4.01 min; 1,2-dichloroethane 3.2 min; 1,1-dichloroethylene 2.34 min; *cis*-1,2-dichloroethylene 3.81 min; *trans*-1,2-dichloroethylene 2.87 min; 1,1,1-trichloroethane 4.59 min; 1,1,2-trichloroethane 10.1 min; 1,1,1,2-tetrachloroethane 13.6 min; 1,1,2,2-tetrachloroethane 16.6 min. carbon tetrachloride 5.1 min; tetrachloroethylene 11.1 min.

H. Measurement of TCE transformation capacity of resting cells

Preparation of reaction mixture and degradation analyses were similar to those of TCE degradation assay mentioned above. The duplicate reaction mixtures were composed of 1 ml cell suspension (0.25-0.5 mg cell dry weight/ml) and 20 mM of sodium formate. TCE (1 μl of TCE stock solution) was repeatedly injected at time intervals 0 h, 2 h, 4.3 h, and 16.3 h. The TCE load at each addition was approximately 0.064 mg/mg dry weight cells. Duplicate heat-killed controls were also prepared similarly. Samples for analysis were taken by stopping the reaction by adding 2 ml of hexane containing 1,2-dibromoethane as an internal standard at both the TCE addition time points and appropriate time points between the TCE addition. Five ml of the organic phase after extraction was analyzed on GC as in the TCE degradation assay described above.

I. Construction of genomic DNA library for cloning a sMMO gene of *Methylomonas methanica* 68-1

Bacterial strains, plasmids, and oligodeoxynucleotides

The bacterial strains are listed in Table III-1. The cloning vectors were plasmids pBluescript II SK(+) (Stratagene Cloning Systems, Inc., La Jolla, CA) and pUC18 (Bethesda Research Labs; Gaithersburg, MD). The former plasmid has multiple cloning sites located between the *lacZ* and *lacI* genes, phage f1 origin, and ampicillin resistant genes so that it was useful for cloning and DNA sequencing. pUC18 also has a *lacZ-I* gene carrying multiple cloning sites and ampicillin resistant gene. Degenerate oligodeoxynucleotide probes were used for the screening of the genomic DNA library constructed. The probes were designed from amino-terminal of hydroxylase γ-subunit of sMMO of *Methylosinus*

trichosporium OB3b and was a gift of R. S. Hanson, University of Minnesota, Minneapolis, MN.

Chromosomal and plasmid DNA isolation

Both large scale plasmid DNA and chromosomal DNA isolations were performed by essentially following methods described by Ausubel et al. (1987). For isolation and purification of chromosomal DNA from strain 68-1, cells were grown to early stationary phase in 500 ml nitrate mineral salts medium (Little et al., 1988) each contained in two 2-liter flasks. Cells (10^8/ml) were then harvested by centrifugation at 4600× g in 250 ml tubes. Cells were resuspended in 23.8 ml TE buffer (pH 8.0), 1.3 ml SDS (10 %), and 100 µl lysozyme (20 mg/ml) and lysed by incubating at 37°C for 1 h. Four and half ml of 5M NaCl was added and mixed thoroughly, followed by the addition of 3.8 ml CTAB (hexadecyltrimethyl ammonium bromide)/NaCl solution with incubating at 65°C for 20 min. The reaction mixture was extracted with equal volumes of chloroform/isoamyl alcohol and phenol/chloroform/isoamyl alcohol once each respectively. The aqueous phase was precipitated with a 0.6 volume of isopropanol, and the pellet was washed with 70 % ethanol and resuspended in 10 ml of TE buffer. The DNA solution was then subjected to CsCl / EtBr gradient ultracentrifugation. The centrifugation was performed at 55000 rpm for 16 -18 h at 20°C. One chromosomal DNA band was clearly visible under UV light after the centrifugation. The DNA solution was then subjected to EtBr removal by water saturated butanol and alcohol precipitated and the DNA pellet was resuspended in TE buffer. The DNA concentration was determined by spectrophotometry and agarose gel electrophoresis.

Construction and screening of genomic DNA library of *Methylomonas methanica* 68-1

DNA manipulations were carried out by following protocols described by Ausubel et al. (1987). Chromosomal DNA of a type I methanotroph, 68-1, was completely digested with restriction endonuclease *Eco* RI, and was ligated into *Eco* RI- restricted and dephosphorylated pBluescript II SK(+) vector (Stratagene Cloning Systems, Inc., La Jolla, CA). Ligated DNA was used to transform *E. coli*, and the resulting transformants were selected on Luria agar plates containing ampicillin (100 μg/ml) and tetracycline (20 μg/ml).

Eighteen-mer degenerate oligodeoxynucleotide probes coding for the N-terminal amino acid of γ-subunit of sMMO of *Methylosinus trichosporium* OB3b were 5'-GAA-CCC-ATC-CAC-GAC-AAC-3' and 5'-GAG-CCG-ATC-CAT-GAT-AAC-3'. The equal mixture of each of the two probes were used in colony and Southern hybridizations. The expected degeneracy of the 18-mer probe was 16 [$2^4 = 16$ from 5'-GAA(G)-CCC(G)-ATC-CAC(T)-GAC(T)-AAC-3']. The oligodeoxynucleotides were end-labelled by 5' addition of ^{32}P using [γ-^{32}P]dATP (ICN Biomedicals, Inc., Irvine, CA; 4500Ci/mmol) and T_4 polynucleotide kinase (Bethesda Research Labs; Gaithersburg, MD) following methods described by Ausubel et al. (1987). Biotrans nylon membranes (ICN Biomedicals, Inc., Irvine, CA) carrying the 68-1 genomic clones were washed to remove cell debris in 0.1× SSPE [1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.7], 1 mM EDTA) and 0.1% (w/v) SDS at 65°C overnight. The membranes were prehybridized in a buffer consisting of 6× SSC, 0.4% SDS, 20 mM NaH_2PO_4, and denatured herring testes DNA(200 μg/ml). The prehybridization solution was then replaced by fresh buffer, and the labelled oligodeoxynucleotide probe (2×10^6 cpm/ml) was

added. After overnight incubation the membranes were washed initially at room temperature for 10 min in 6× SSC containing 0.1% SDS at room temperature (repeated three times), and then at 42°C for 10 min. The final wash was done for 10 min in 6× SSC containing 0.2% SDS at 42°C. The filters were air-dried and subjected to autoradiography at -85°C for 1 to 4 days.

DNA Southern blot and slot blot

Genomic DNA's from methanotrophs and plasmid DNA from *E. coli* were digested to completion with the desired restriction enzymes and separated by electrophoresis on 0.8 - 1.0% agarose gels in 1× TBE buffer. DNA from the gel was transferred to Biotrans nylon membranes (0.2 mm) by the Vacuum-Blotting System (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The DNA slot blot was prepared by using the Biodot Blot SF apparatus (Bio-Rad Laboratories, Richmond, CA). Double-stranded DNA probes were prepared using a DNA nick translation kit (Bethesda Research Labs; Gaithersburg, MD) and [α-^{32}P]dCTP (ICN Biomedicals, 3000 Ci/mmol, Irvine, CA). Blotted filters were prehybridized at 65°C for 1 hr in a buffer consisting of 5× SSPE, 5× Denhardt solution (Denhardt, 1966), 0.2% SDS, and denatured herring testes DNA (200 µg/ml), and were then hybridized in the fresh buffer containing the labelled probe (2×10^6 cpm/ml) overnight at 65°C. An initial wash was done in 2× SSC containing 0.2% SDS for 15 min at room temperature (two times) and 1× SSC containing 0.1% SDS for 15 min at room temperature. Finally the filters were washed in 1× SSC containing 0.1% SDS for 30 min at 65°C (repeated two times). The filters were air-dried and subjected to autoradiography at -85°C for 1 to 5 days.

Amplification of putative sMMO gene fragment by PCR

A putative MMO insert cloned into pBluescript II SK(+) vector was amplified by using the 18-mer synthetic oligodeoxynucleotide and M13 reverse sequencing primer (New England Biolabs, Inc. Beverly, MA) as primers and Gene AMP DNA Amplification Kit (Perkin Elmer Cetus, Corp., Norwalk, CT) including DNA *Taq* polymerase. The amplification was done by the following thermal process: melting at 95°C for 1 min, annealing at 42°C for 2 min, and polymerization at 72°C for 3 min (repeated 38 cycles) (Figure IV-11, Chapter IV). The DNA thermal cycling was carried out using the Automatic DNA Thermal Cycler (Perkin Elmer Cetus, Corp., Norwalk, CT).

J. DNA sequencing of putative sMMO gene

Sequencing strategy

From restriction mapping of pSK5 (Figure IV-14, Chapter IV) it was assumed that the putative sMMO gene (*mmoZ*) of strain 68-1 was located on the upstream of *Sph* I fragment of the 4 kb insert of the recombinant plasmid. It was therefore decided that sequencing of the 18-mer probe binding region would be necessary to pick up a possible homologous region between the two strains. This strategy allowed subcloning of a 1.8 kb *Sph* I fragment of the 4 kb insert into pUC18 carrying a binding site of M13 forward sequencing primer (#1211, New England Biolabs, Inc., Beverly, MA) located next to *Hind* III site of the vector. The putative sMMO probe binding site was located 200 bp downstream of a *Sph* I site near a *Hind* III site. Hence sequencing of 230-240 bp downstream from the *Sph* I site will make it possible to read through the target the 18 mer-probe binding site (Figure IV-15, Chapter IV).

Sequencing procedure and analysis

The DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) following the manual of Sequenase Version 2.0 or Taquenase DNA polymerase (United States Biochemical, Cleveland, OH). For single-stranded template DNA preparation the procedure for obtaining and purifying single-stranded DNA was generally followed from Stratagene Cloning Systems, Inc. (La Jolla, CA). A single colony from a ampicillin plate was grown overnight in 3 ml of LB medium containing ampicillin. The culture (100 µl) was inoculated into 10 ml of Superbroth (gram per liter: Bacto-tryptone 10; Bacto-yest extract 20; NaCl 5 ; pH adjusted to 7.5; no antibiotics added) and grown up to 0.3 OD_{600} by vigorous shaking (225 rpm). Helper phage R408 (Stratagene, La Jolla, CA) was then added to the culture at a mutiplicity of infection 20:1 (phage to cell). The growth was continued for an additional 8 h. Cells were pelleted at 800 rpm with a JA14 rotar (Beckman, Palo Alto, CA) for 15 min and the supernatant was taken into a 30 ml Oak Ridge tube. The phage particles were then precipitated out by adding 0.25 volume of phage precipitation buffer (20 % polyethyleneglycol 8000 in 3.75 M ammonium acetate, pH 7.5) and incubating the mixture at room temperature for 15 min, and pelleting at 11000 rpm for 15 min. The pellet was resuspended in 700 µl of TE buffer (pH 8.0). The solution was extracted with phenol/chloroform 3-5 times followed by 2 extractions with chloroform. The DNA was precipitated by addition of an equal volume of 7.5 ammonium acetate and 2 volumes of ethanol. For double-stranded DNA template preparation an alkaline denaturation method from Sequenase Version 2.0 manual or TaqTrack sequencing Systems manual (Promega Corp., Madison,

WI) was utilized. For preparation and running of sequencing gel the protocol of Lang and Burger (1990) was essentially followed.

Sequence analysis was performed by using programs (PFASTA) of Genetics Computer Group (GCG), Biotechnology Center of the University of Wisconsin (Madison, WI) and DNA and protein sequences database analysis programs (Blastn, Blastp, and Blastx) of National Center for Biotechnology Information, Bethesda, MD.

K. Microbial community analysis of environmental samples using gene probes

In this section methods will be described to examine community diversity of a mixed culture sample enriched in a bioreactor and environmental samples from the sites heavily contaminated with TCE using the putative sMMO gene probe developed from *Methylomonas methanica* 68-1 and other characterized oxidative and degradative genes.

DNA extraction from bioreactor samples and DNA dot blotting

A biofilm sample was from a fixed-film packed-bed bioreactor enriching a methanotrophic consortium isolated from TCE-contaminated groundwater monitoring wells (Strandberg et al., 1989) from which strain 68-1 and 46-1 were isolated (Little et al., 1988). The sample was enriched again in 100 ml of mineral salts medium (Little et al., 1988) under 30-40 % methane in head space of the 500 ml shaking flask. Total genomic DNA was isolated and purified by following the genomic DNA isolation procedure for a large scale described in the Section I of this documentation. The DNA dot blot hybridization was then carried out using the 4 kb insert as a DNA probe to detect the possible specific gene in the DNA

isolated from the enrichment culture. The genomic DNA's of 68-1 and *Pseudomonas fluorescens* LOS1 were used as positive and negative controls respectively. DNA ranging from 10 ng to 10 µg was denatured by 0.5 M NaOH and immobilized in each well of Minifold Dot Blot system of Schleicher and Schuell Inc. (Keene, NH) according to the manufacturer's procedure. Preparation of labeled DNA probe (pSK5) and hybridization procedure were as described in the Section I of this documentation.

Direct DNA extraction from Savannah River Site samples and DNA slot blot hybridization

The map of sampling sites (MHT1C; MHT6C; MHT9C) and characteristics of their corresponding groundwater has been reported previously (Bowman et al., 1993). The procedure for the direct DNA extraction from the contaminated soils was a modification of the method of Ogram et al. (1987). Modifications included 3-5 times phenol extraction after PEG extraction and elimination of DNA purification by hydroxyapatite column. The extracted DNA solution (6.3 to 200 µl; 1 ng/µl) was adjusted to 200 µl and then denatured with an equal volume of 1 M NaOH by incubating at 90-95°C for 10 min and cooling rapidly each solution on ice for 5 min. The denatured DNA solution was loaded into the well of BioDot Blot System (Bio-Rad, Richmond, CA) using Biotrans nylon membranes (ICN Biomedicals, Costa Mesa, CA). The subsequent DNA hybridization was carried out by following the procedure described in the Section F of this documentation. The DNA probes used were: the 4 kb putative sMMO gene of 68-1 (type I and X specific), 2.1 kb fragment carried on pBluescript II (coding sMMO B gene of *M. trichosporium* OB3b), 2.5 kb *Sma* I fragment in

pUS325 (a structural gene coding methanol dehydrogenase of *Methylobacterium organophilum* XX; *moxF*), and 2.5 kb *Sma* I -*Not* I in pDTG601 [a chromosomal gene (*todC2C1BA*) coding toluene dioxygenase of *Pseudomonas putida* F1].

L. Procedure for determination of the relationship between DNA amount and hybridization signal

Determination of a standard curve showing the relationship between DNA amount loaded and the corresponding hybridization signal was tried for each probe (4 kb putative sMMO gene fragment for *Methylomonas methanica* 68-1 and 2.1 kb fragment carrying sMMO B gene of *Methylosinus trichosporium* OB3b). The target DNA for each probe was isolated by the chromosomal DNA large scale preparation method as previously described (refer Section F). Each target genomic DNA (ng; 1000, 500, 250, 125, 63, 32, 16, 4, 1) was denatured by 0.5 M NaOH and immobilized on a nylon membrane. DNA probes were labeled with [^{32}P]dCTP by using Prime It Random Primer Labeling Kit (Stratagene Cloning Systems Inc., La Jolla, CA). DNA slot blot hybridization was performed as described in Section F. Hybridization and washing were performed under stringent conditions allowing 30 % mismatch. The hybridization signals developed on X-ray film were quantified with the BioImage Analysis System (Eastman Kodak Co., Rochester, NY). The optical density (OD) values from the analysis were plotted against the amount of DNA loaded on the membrane and the plot fitting was done to find standard curve equation using the graphics computer program DeltaGraph Professional Version 2.0 (Microsoft Corp., Redmond, WA). The results are shown in Figures III-1 and 2. There was a linear relationship between the logarithmic DNA amount and the optical density for

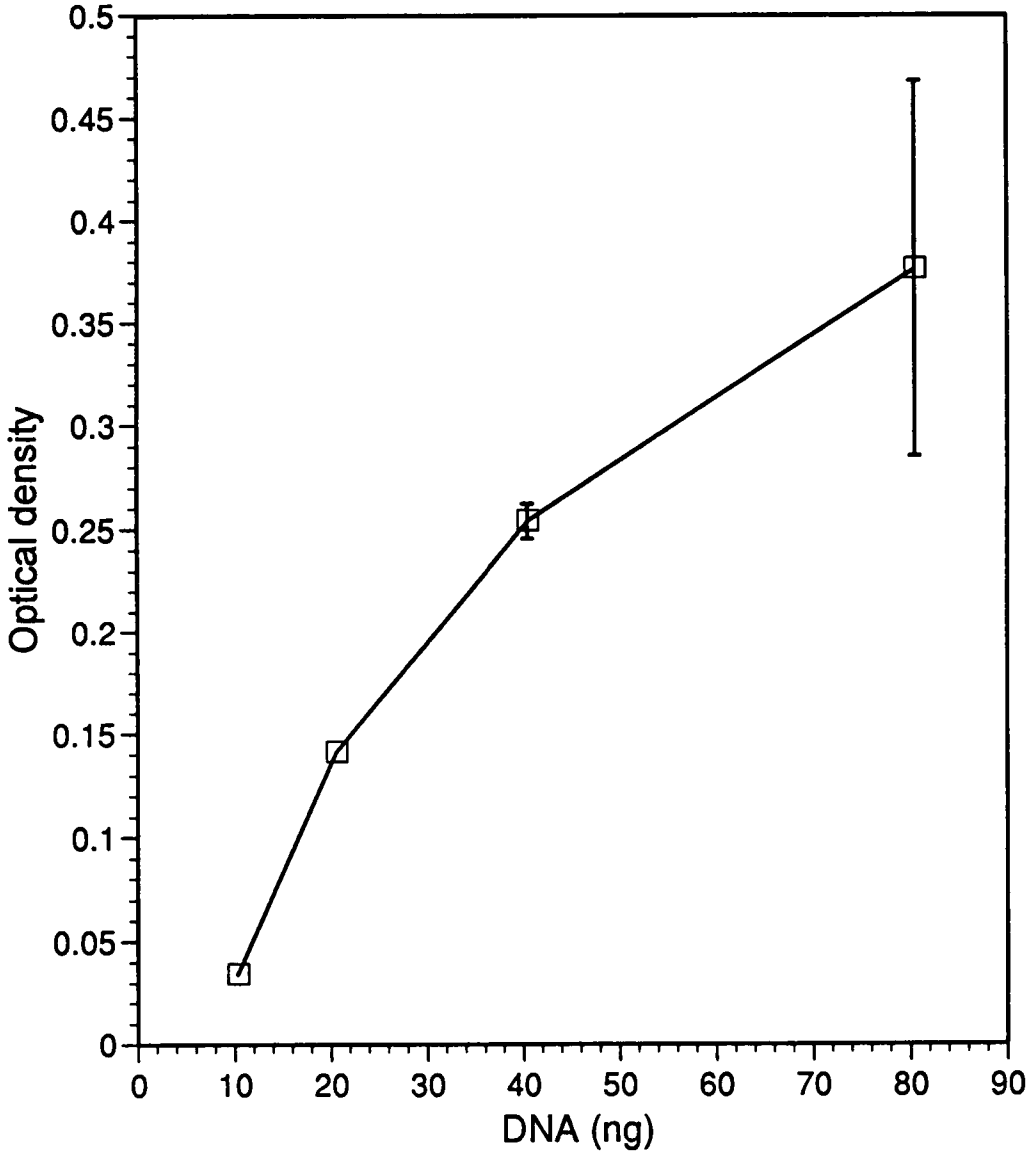


Figure III-1. The standard curve for the relationship between total DNA amount of *Methylomonas methanica* 68-1 and optical density from image analysis after hybridization to the DNA probe (4 kb insert of pSK5). The regression equation is $y = 0.166 \ln(x) - 0.3588$ ($r^2 = 0.998$).

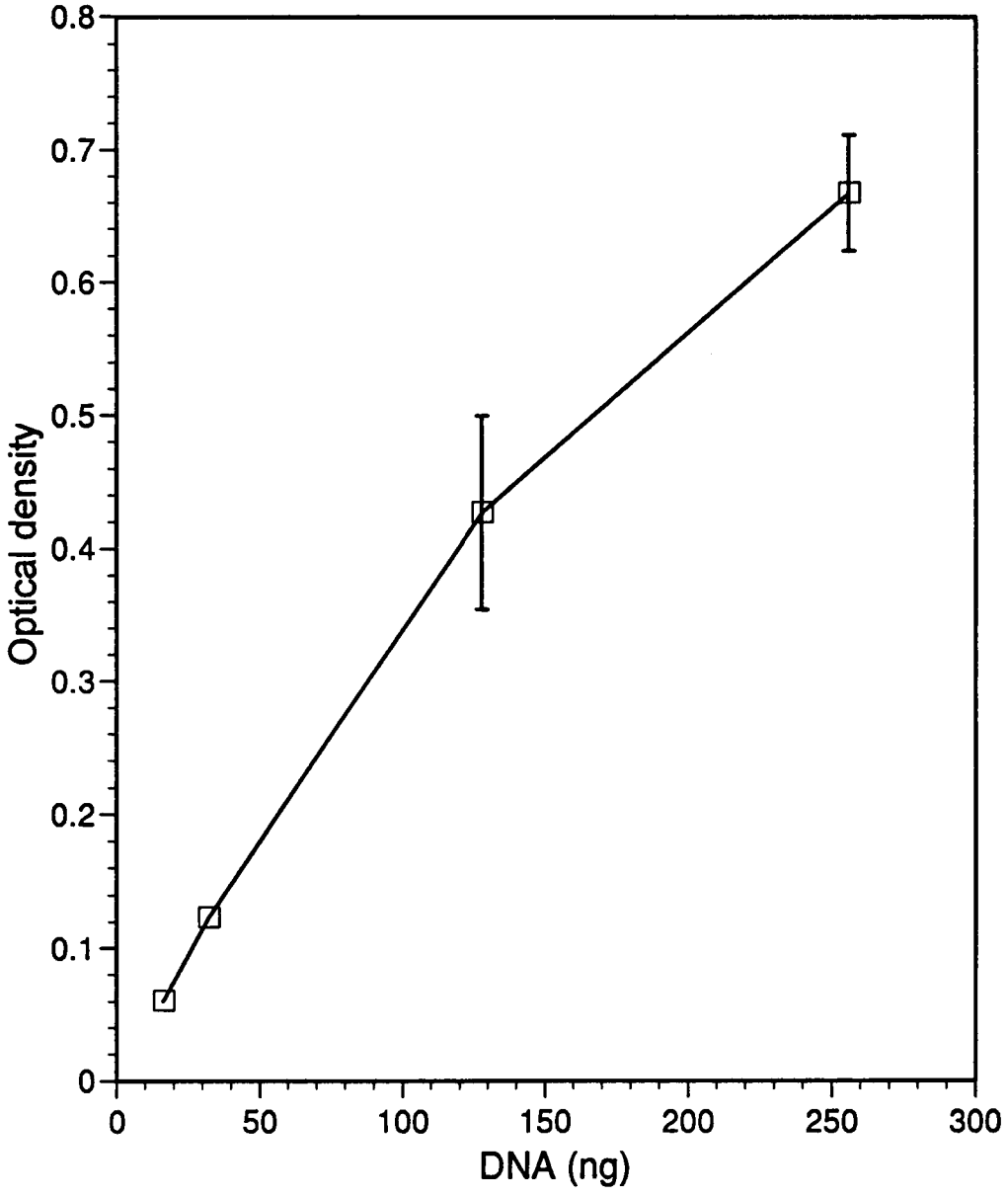


Figure III-2. The standard curve for the relationship between DNA amount of *Methylosinus trichosporium* OB3b and optical density from image analysis after hybridization to the 2.1 kb DNA probe carrying MMO B gene. The regression equation is $y = 0.22 \ln(x) - 0.599$ ($r^2 = 0.968$).

both 68-1 and OB3b. This relationship was prominent for optical density values from strong hybridization signals. For relatively weak hybridizational signals there seemed to be a direct linear relationship between DNA amount and optical density value. However, the regression relationships need to be determined each time when a target sample DNA is hybridized to a concerning DNA probe and quantified. This is because the probe specific activity, and target DNA immobilization and hybridization efficiency may be changed each time.

M. Procedure for determination of DNA extraction efficiency

Measurement of DNA extraction efficiency is necessary to predict the amount of DNA extracted from which the population density is subsequently monitored. Determination of the genomic DNA extraction efficiency of 68-1, OB3b, and their mixed culture was based on an extraction efficiency of added to the DNA extraction procedure. Phage λ DNA (cut with *Hind* III; 640 ng) was added to cell lysis mixture of each culture (3×10^8 cells) and the procedure ofgenomic DNA extraction at small scale was followed. Total DNA (each genomic DNA plus phage) from the extraction was dissolved in 100 µl of TE buffer. The dissolved DNA solutions (14 µl and 1 µl) were immobilized on a nylon membrane using the DNA slot blot system described before and subjected to DNA hybridization. During the procedure, various amounts of phage λ DNA as standard (ng; 128, 63, 32, 16, 4, 1, 0.5, 0.05) immobilized on a nylon membrane were also hybridized to the phage λ DNA (cut with *Hind* III) itself as a probe. All the hybridization signals were quantified as described above. Amounts of the extracted λ DNA were determined based on the standard curve equation generated. The extraction efficiency is a % ratio of the extracted λ DNA to the

added λ DNA (640 ng). Two important factors involved in determination of a population density by DNA probing are lysis efficiency of the target cells and extraction efficiency of the DNA carrying the target gene concerned. In this study the cell lysis efficiency was not determined because pure cultures and the mix of the pure cultures were used for the DNA isolation. Under the conditions of the chromosomal small scale isolation procedure (Section F of Chapter III), the cells of 68-1 and OB3b appeared to be completely lysed when observed using a microscope. Hence the cell lysis efficiency was considered to be 100 %. For determination of the DNA extraction efficiency, the standard curve for the relationship between phage λ DNA quantity and its relevant optical density is shown in Figure III-3. There was also a linear relationship observed between logarithmic DNA quantity and optical density. The DNA extraction efficiency for each culture (68-1, OB3b, and their mixed culture) is shown in Table III-3. The extraction efficiencies of 68-1 and OB3b were similar (60 % and 56 % respectively) while the efficiency for the mixed cell sample was higher (80 %). The reason for higher DNA extraction efficiency at the mixed cells was not clear but appeared to be due to less cell loss during the cell washing in 0.5 % N-lauroyl sarcosine in TSE buffer. The 80 % DNA extraction efficiency was used for all the following DNA quantitation of mixed culture samples by DNA:DNA hybridization.

N. Competition experiment using flask culture

Effect of NMS medium strength on growth of *Methylomonas methanica* 68-1

Park et al. (1991) reported that the two fold strength of NMS medium

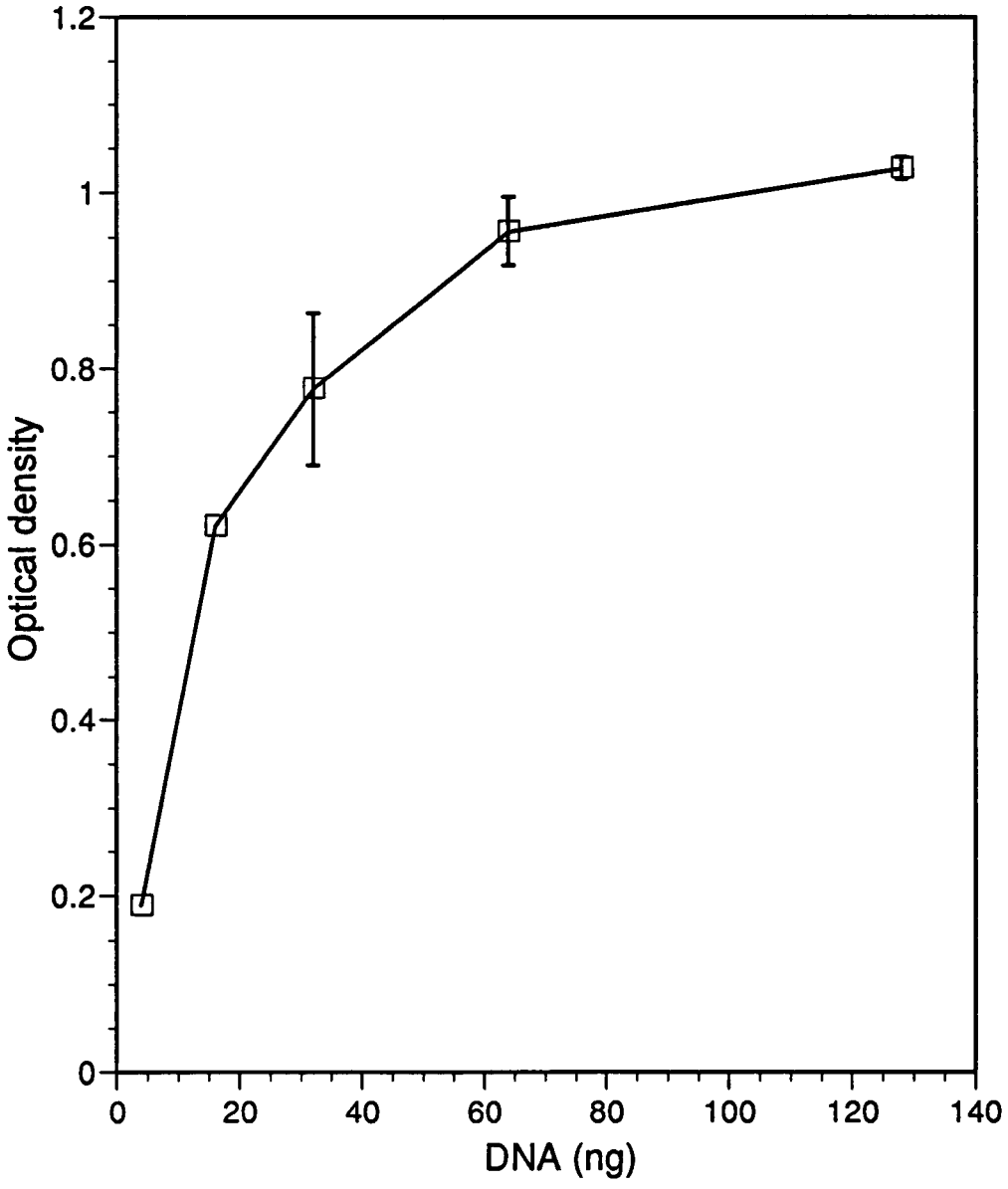


Figure III-3. The standard curve for the relationship between lambda DNA amount and optical density from image analysis after hybridization to the lambda DNA itself (cut with *Hind*III) used as a DNA probe. The regression equation is $y = 0.247 \ln(x) - 0.109$ ($r^2 = 0.977$).

Table III-3. Determination of DNA extraction efficiency during genomic DNA isolation from *Methylomonas methanica* 68-1, *Methylosinus trichosporium* OB3b, and their mixed culture. The data are obtained from duplicate or triplicate experiments and shown as average ± standard deviation.

Culture for DNA extraction[A]	Sample volume used for hybridization (ml)[B]	O. D. from image analysis[C]	Total λ DNA extracted (ng)	DNA Extraction efficiency (%)
68-1	14	0.862 ± 0.008	363.6 ± 10.3	56.8 ± 1.6
	1	0.235 ± 0.011	404.0 ± 19.8	63.1 ± 3.1
OB3b	14	0.900 ± 0.009	423.9 ± 14.9	66.2 ± 2.4
	1	0.159 ± 0.022	296.5 ± 27.6	46.4 ± 4.3
Mixed	14	0.956 ± 0.011	531.7 ± 22.5	83.1 ± 3.5
	1	0.288 ± 0.006	497.5 ± 10.6	77.6 ± 1.6

[A] Phage λ DNA (cut with *Hind*III; 640 ng) was added to the lysis mixture of each cells (3×10^8) during the genomic DNA mini-prep extraction procedure. [B] Total DNA from the extraction and purification was dissolved in 100 µl of TE buffer. [C] The curve fitting equation of the relationship between the standard λ DNA amount and optical density from image analysis after the hybridization with the λ DNA (cut with *Hind*III) as a DNA probe: $y = 0.247 \ln(x) - 0.109$ ($r^2 = 0.977$) where x is the amount of standard λ DNA (cut with *Hind*III) loaded on the filter and y is O. D. from image analysis.

increased the biomass of cells of *Methylosinus trichosporium* OB3b. It was of interest to examine the effect of the medium strength on the growth of the strain 68-1. In this experiment two concentrations of NMS (1.5× and 2×) were tried and compared with single strength NMS. As shown in the Figure III-4, the 50% increase of NMS strength slightly decreased the specific growth rate of 68-1 while the 100% increase caused a significant reduction of the growth rate (40 % decrease). Thus all the species competition experiments in this study were performed in the single strength NMS. All NMS media used for inoculum preparation and competition studies contained 10 vitamins (reagent grade; Sigma Chemical Co., St Louis, MO) in the following concentrations (mg/L): biotin 2, folic acid 2, pyridoxine-HCl 10, thiamine-HCl 5, riboflavin 5, niacin 5, pantothenic acid 5, cyanocobalamin 0.1, *p*-aminobenzoic acid 5, and lipoic acid 5.

Relationship between OD_{600} and cell density

For rapid population density monitoring in pure cultures to be used for mixed culture experiments, the regression relationship between OD_{600} and cell number of each culture from AODC was determined (Figures III-5 and 6). This relationship was quite useful in preparing inocula for the subsequent mixed culture experiment.

Procedure for acridine orange direct count (AODC)

It was reported by Graham et al. (1993) that the populations of *Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3b in a mixed culture could be differentiated and monitored by acridine orange direct count

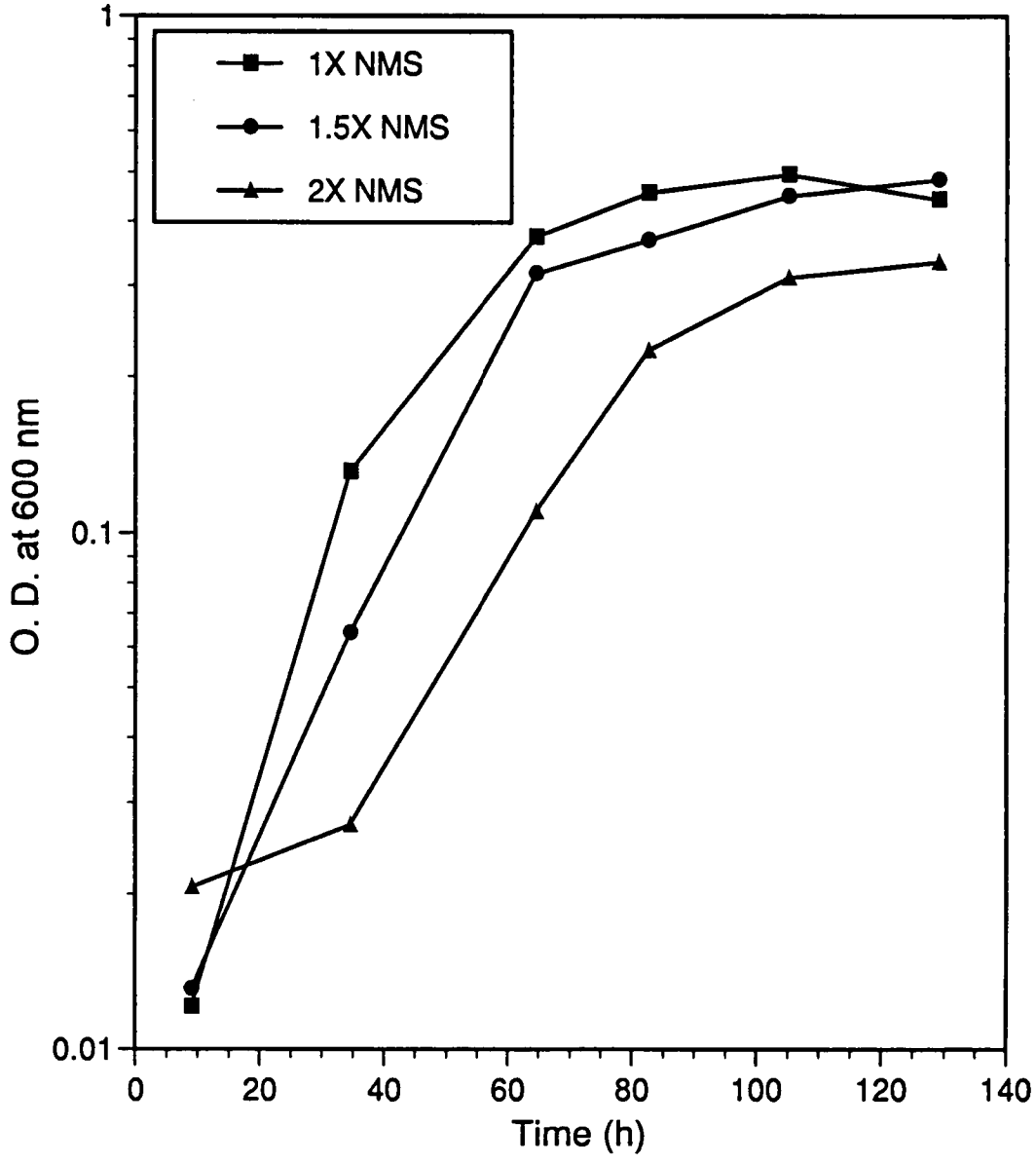


Figure III-4. Effect of NMS medium strength on the growth of *Methylomonas methanica* 68-1 in flask culture. The specific growth rate in each strength (1X, 1.5X, and 2X) of the NMS was determined to be 0.015, 0.012, and 0.009 h^{-1} repectively.

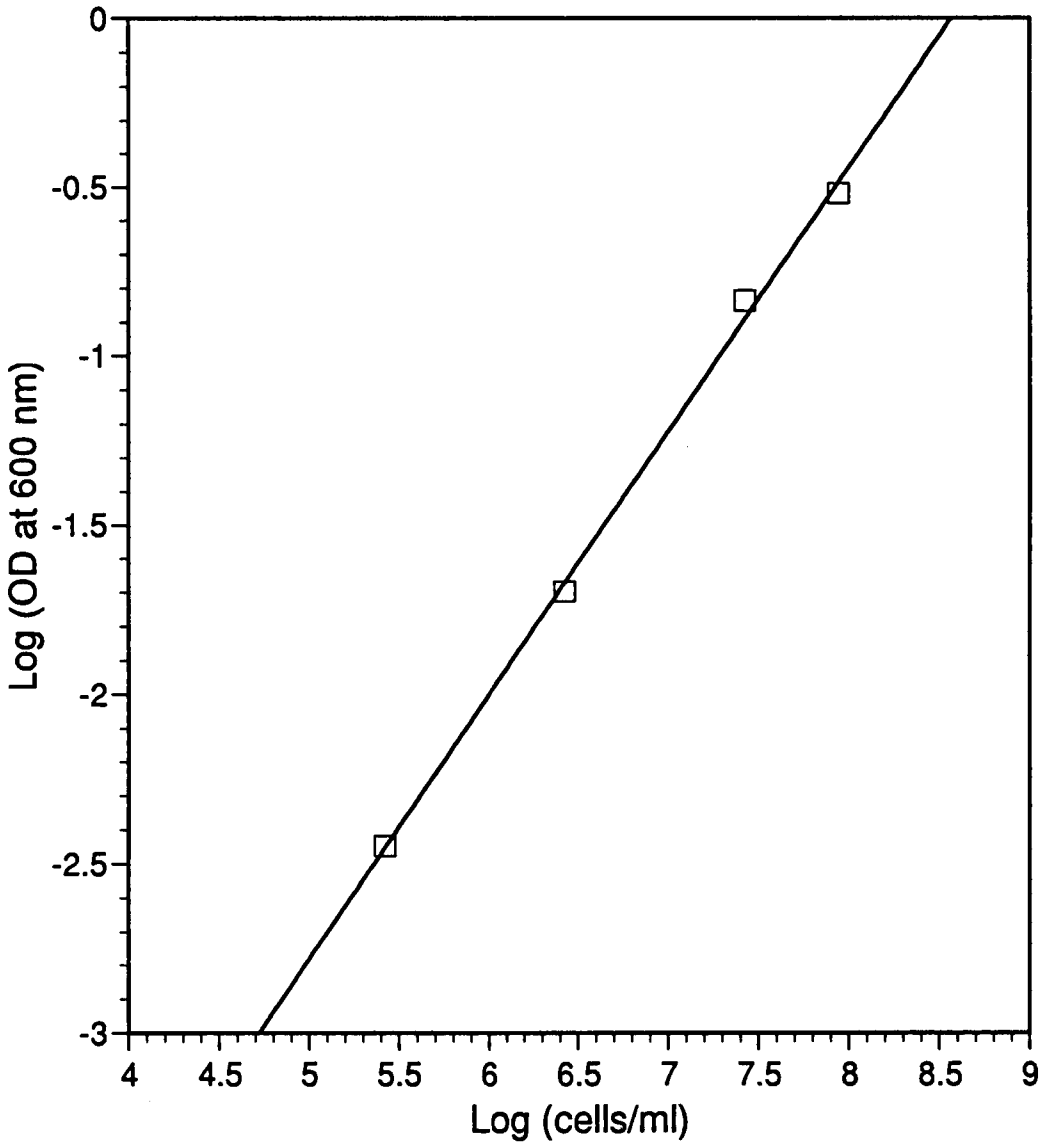


Figure III-5. The relationship between OD and cell density in *Methylomonas methanica* 68-1. The cell density was determined by acridine orange direct count (AODC). The regression equation is $y = 0.780x - 6.685$ ($r^2 = 0.998$).

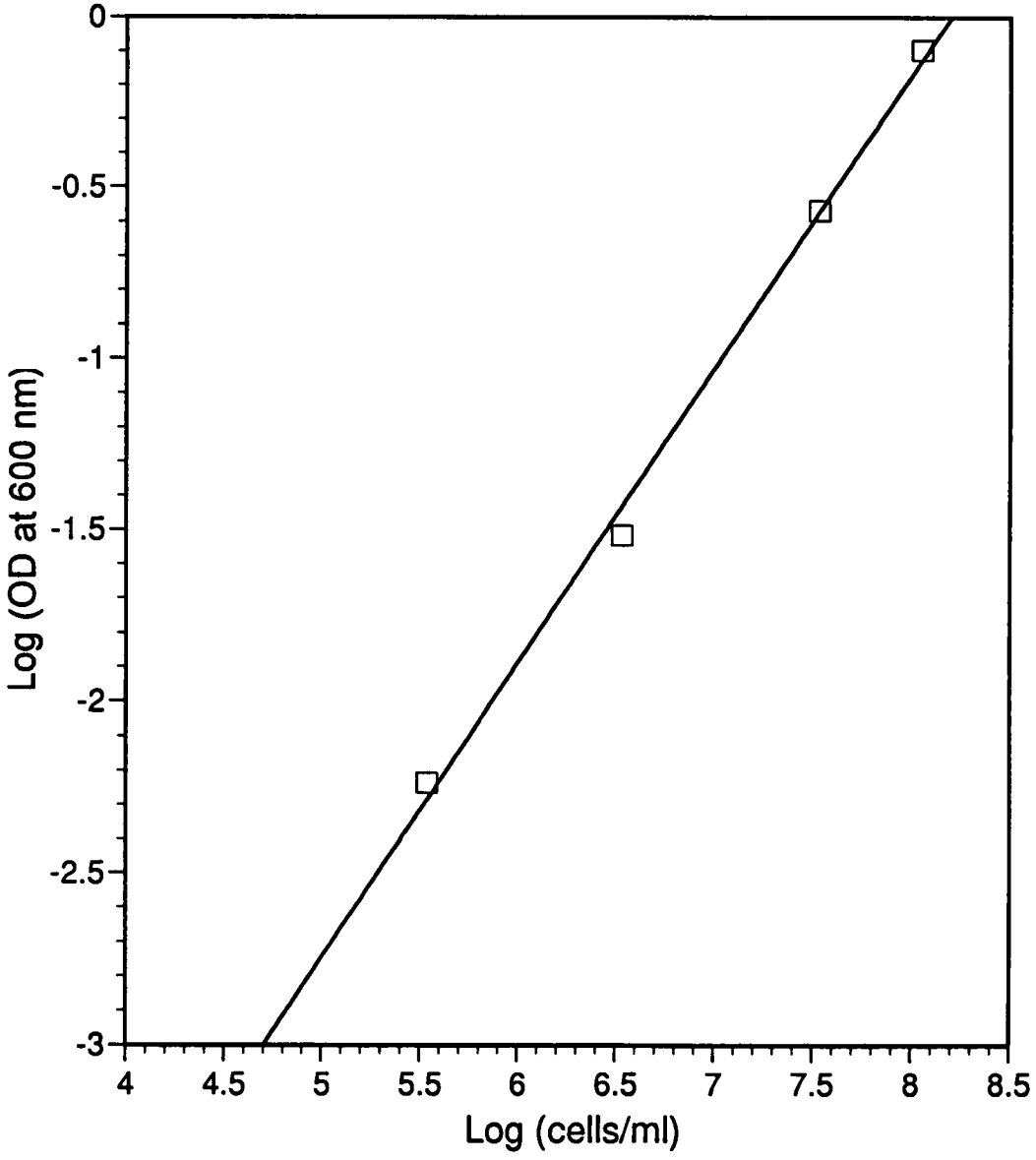


Figure III-6. The relationship between OD and cell density in *Methylosinus trichosporium* OB3b. The cell density was determined by acridine orange direct count (AODC). The regression equation is $y = 0.857x - 7.034$ ($r^2 = 0.996$).

(AODC) for the following reasons: OB3b fluoresced bright green while BG8 fluoresced orange under epifluorescence and morphological differences of the two organisms. The AODC was performed by modifying the ASTM method (ASTM, 1985). Cell suspensions were diluted to appropriate density (e.g., cells per field: 100 grids; 0.01 $mm^{2)}$. A 15 ml Deltaware glass filter unit (Kimble Glass, Vineland, NJ) was set up carrying a polycarbonate membrane black filter (Costar Mesa, CA) was placed underneath the membrane filter. One ml of the diluted cell suspension was taken into the filter unit and then cells were stained by adding 2ml of acridine orange solution (0.01 % w/v) for 3-5 min. The staining mixture was then filtered under a gentle vacuum. The membrane filter was removed from the filter system and dried in the air, and mounted on top of a drop of immersion oil (Fisher Scientific Co., Pittsburgh, PA) carried on slide glass. After loading one drop of the immersion oil on the top of filter, a cover glass was placed. The cells were then counted under oil immersion with fluorescence microscopy (1000×) using Nikon Optiphot microscope equipped with a Mercury-100 Lamp (Nikon Inc., Garden City, NY). Three to five fields (0.01 mm^2 per field) were counted per the sample prepared in duplicates. Fluorescence microscopy was carried out with a camera mounted on the microscope using Kodak Ektachrome color slide film (ASA 400) (Eastman Kodak Co., Rochester, NY), if necessary.

The relationship between OD at 600 nm and cell density of each culture (68-1 or OB3b) was determined for a rapid population monitoring purpose. Each culture (10^8 cells/ml; measured by AODC) was serially diluted ranging from 10^8 to 10^5 cells/ml and OD at 600 nm was measured for each diluent. Logarithm of OD values were plotted against logarithm of cells/ml values and curve fitting was

performed for each culture by using the graphics program mentioned Section N of this documentation.

Procedure for plate count

Cultures were diluted to give 50-300 colonies per plate. Fifty or 100 µl of diluted cell suspensions was plated onto NMS medium (Cornish et al., 1984) and the plates were placed in Oxoid anaerobic jars (Unipath Limited, Hampshire, United Kingdom) containing 30 % methane in the air. The jars were then kept at room temperature for at least two weeks. The 68-1 and OB3b cells grown from mixed culture could be differentiated by colony type and color.

Determination of cell density by DNA:DNA hybridization and image analysis

The general scheme for determination of cell density by DNA:DNA hybridization and image analysis was shown in Figure III-7. The chromosomal DNA preparation at small scale (refer Section F) was used for the isolation of DNA from pure or mixed culture. A cell density of culture was determined from the amount of DNA quantified by DNA extraction and hybridization using a DNA probe. An example of the procedure is shown as follow:

Example:

Assume that 1 ng of specific target genomic DNA each of *M. methanica* 68-1 and *M. trichosporium* OB3b was detected by DNA slot blot by the DNA probes, 4 kb of pSK5 and MMO B gene (2.1 kb) fragment respectively. What are the predicted cell numbers for each slot?

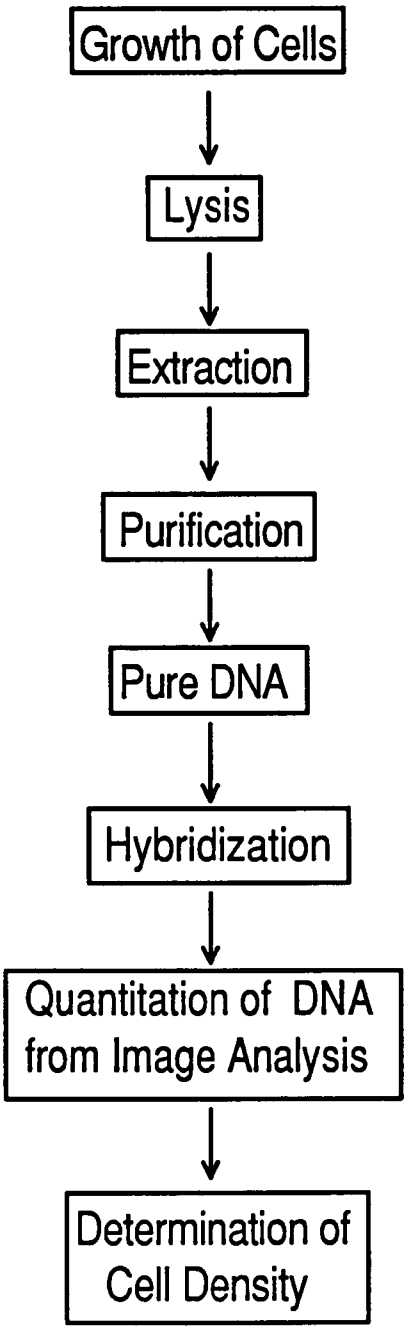


Figure III-7. Procedure of quantifying cell density of culture by DNA:DNA hybridization and image analysis techniques.

Solution:

1. Conversion factors

- Genome size of *M. methanica*: 3,060 kb
- Genome size of *M. trichosporium* OB3b: 2,015 kb
- Under the standard temperature and pressure (STP):
  1 pmol of 1 kb DNA = 0.66 μg

2. Hybridization using the 4 kb putative sMMO gene probe for detection of *M. methanica* 68-1

Under STP, 1 mole of the gene molecule = 6.02×10^{23} molecules
Therefore, 1 molecule = $(1/6.02) \times 10^{-23}$ moles
and 1 pmol of 4 kb DNA = 2.64 μg

10^{-12} moles	--->	2.64 μg
$(1/6.02) \times 10^{-23}$ moles (= 1 molecule)	--->	X μg

$X = (1/6.02) \times (10^{-23} \text{ moles}) \times (2.64 \text{ μg}) \times (10^{-12} \text{ moles}^{-1})$
$= 4.38 \times 10^{-12}$ μg per gene molecule

The genome size of OB3b is 2,015 kb so that the fraction of the 2.1 kb as to the genome size is: 4 kb/ 3,060 kb = 1.3×10^{-3}.

In this case 1 ng of genomic DNA was detected by the 4 kb probe. Hence the actual amount of gene in the 1 ng genomic DNA loaded will be:

(1 ng) × (1.3×10^{-3}) × $(10^{-3}$ μg/ng) = 1.3×10^{-6} μg of the pure gene detected*.

As shown above, a gene molecule weighs 4.38×10^{-12} μg. Therefore, the number of gene molecule contained within the 1 ng genomic DNA loaded will be:

$(1.3 \times 10^{-6}$ μg of the pure gene detected)/$(4.38 \times 10^{-12}$ μg per gene molecule)
= 2.96×10^5 genes (or cells) per slot (only if one cell has a single copy of the target gene)

*For actual application, this value can be divided by the cell lysis efficiency and genomic DNA extraction efficiency.

3. Hybridization using the sMMO B gene probe (2.1 kb) for detection of *M. trichosporium* OB3b

Under STP, 1 mole of the gene molecule = 6.02×10^{23} molecules
Therefore, 1 molecule = $(1/6.02) \times 10^{-23}$ moles

and 1 pmol of 2.1 kb DNA = 1.38 μg

10^{-12} moles ---> 1.38 μg

(1/6.02) x10^{-23} moles (= 1 molecule) ---> X μg

$X = (1/6.02) \times (10^{-23} \text{ moles}) \times (1.38 \text{ μg}) \times (10^{-12} \text{ moles}^{-1})$

$= 2.29 \times 10^{-12}$ μg per gene molecule

The genome size of OB3b is 2,015 kb so that the fraction of the 2.1 kb as to the genome size is: 2.1 kb/ 2,015 kb = 1.04×10^{-3}.

In this case 1 ng of genomic DNA was detected by the 2.1 kb probe. Hence the actual amount of gene in the 1 ng genomic DNA loaded will be:

$(1 \text{ ng}) \times (1.04 \times 10^{-3}) \times (10^{-3} \text{ μg/ng}) = 1.04 \times 10^{-6}$ μg of the pure gene detected*.

As shown above, a gene molecule weighs 2.29×10^{-12} μg. Therefore, the number of gene molecule contained within the 1 ng genomic DNA loaded will be:

$(1.04 \times 10^{-6}$ μg of the pure gene detected)/$(2.29 \times 10^{-12}$ μg per gene molecule)

$= 4.54 \times 10^{5}$ genes (or cells) per slot (only if one cell has a single copy of the target gene)

*For actual application, this value can be divided by the cell lysis efficiency and genomic DNA extraction efficiency.

Comparative analysis of population monitoring by 16S rDNA-targeting signature oligodeoxynucleotide probes

Relative population density was also monitored by using 16S rDNA-targeting signature oligodeoxynucleotide probes (1035-RuMP for type I methanotroph, 1034-Ser for type II methanotroph, and a eubacterial probe for total eubacteria) (refer Table III-2). These data were comparatively analyzed with those from AODC, viable cell count, and cell count predicted by DNA extraction and hybridization. Genomic DNA extraction (small scale) and image analysis was similar to those of Section F and N in order. Labeling of 16S rDNA-targeting oligodeoxynucleotide probes and their hybridization were performed according to the protocols from Ausubel et al. (1987) and Stratagene Cloning

Systems, Inc. respectively. Hybridization was carried out at 42°C and washings were at room temperature (three times) and at 47°C (once) in 6× SSC containing 0.1 % SDS. Autoradiography with intensifying screens was performed at -80°C for 3-5 days.

Determination of TCE degradation rate

The rates of TCE degradation by a mixed culture grown in flask (2 liter) at various time points were determined by the protocol described in Section D. The degradation rates were shown in pmoles per mg cell dry weight per h.

O. Growth of *Methylomonas methanica* 68-1 in batch culture bioreactor

Batch culture of 68-1 in bioreactor was performed to find optimal growth conditions such as methane and oxygen flow rates, gas pressure within bioreactor, and agitation speed. The growth conditions allowing the maximum specific growth rate were determined under fixed concentrations of phosphate buffer (10 mM), sodium nitrate (10 mM), ferric chloride (80 μM), and no added copper.

P. Competition experiment in continuous bioreactor system

Description of bioreactor system

The schematic diagram of bioreactor system used in this study was shown in Figure III-8. The system included continuous stirred tank bioreactor (Figure III-9), medium pump system, oxygen probe, and back-pressure regulator as major

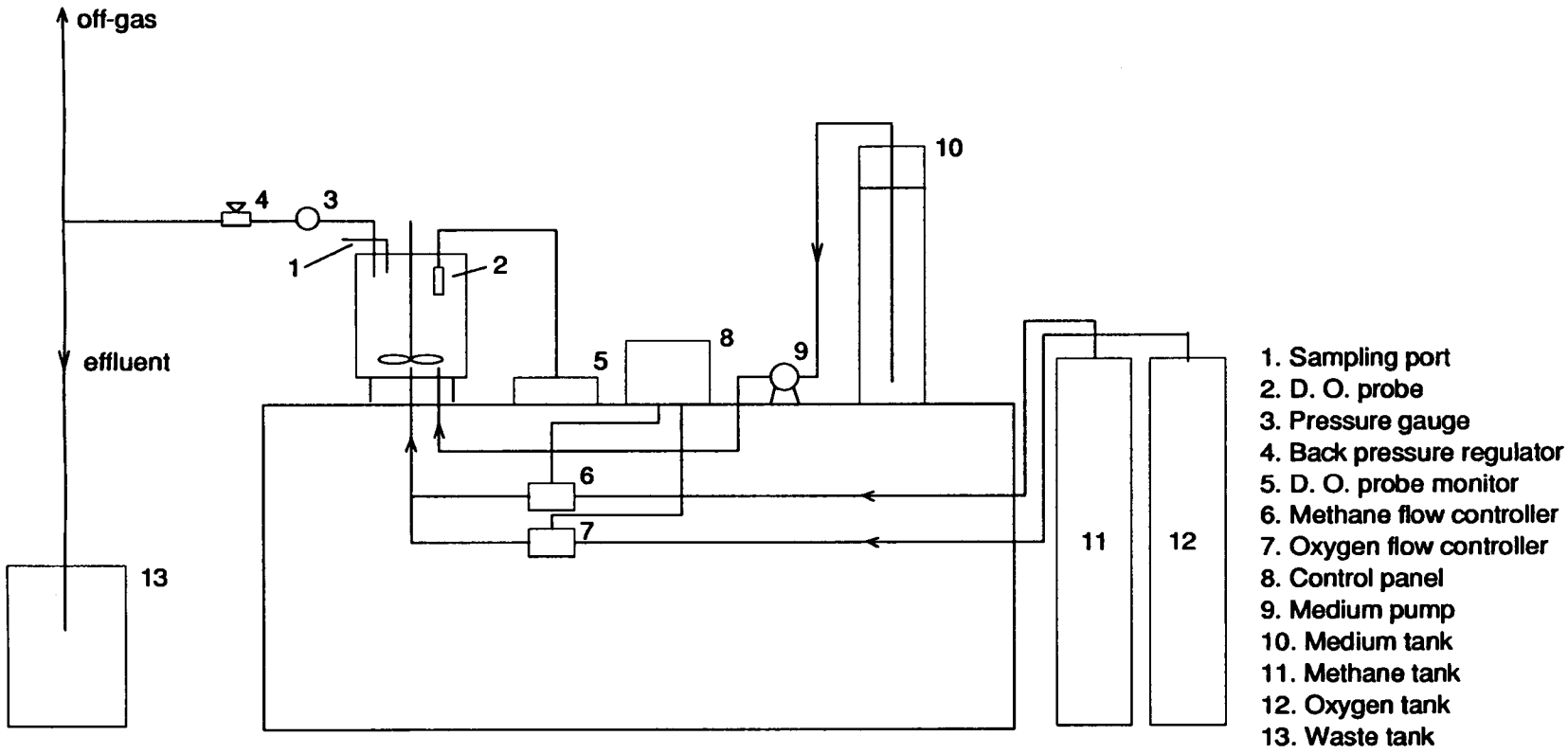


Figure III-8. The schematic diagram of continuous stirred tank reactor system.

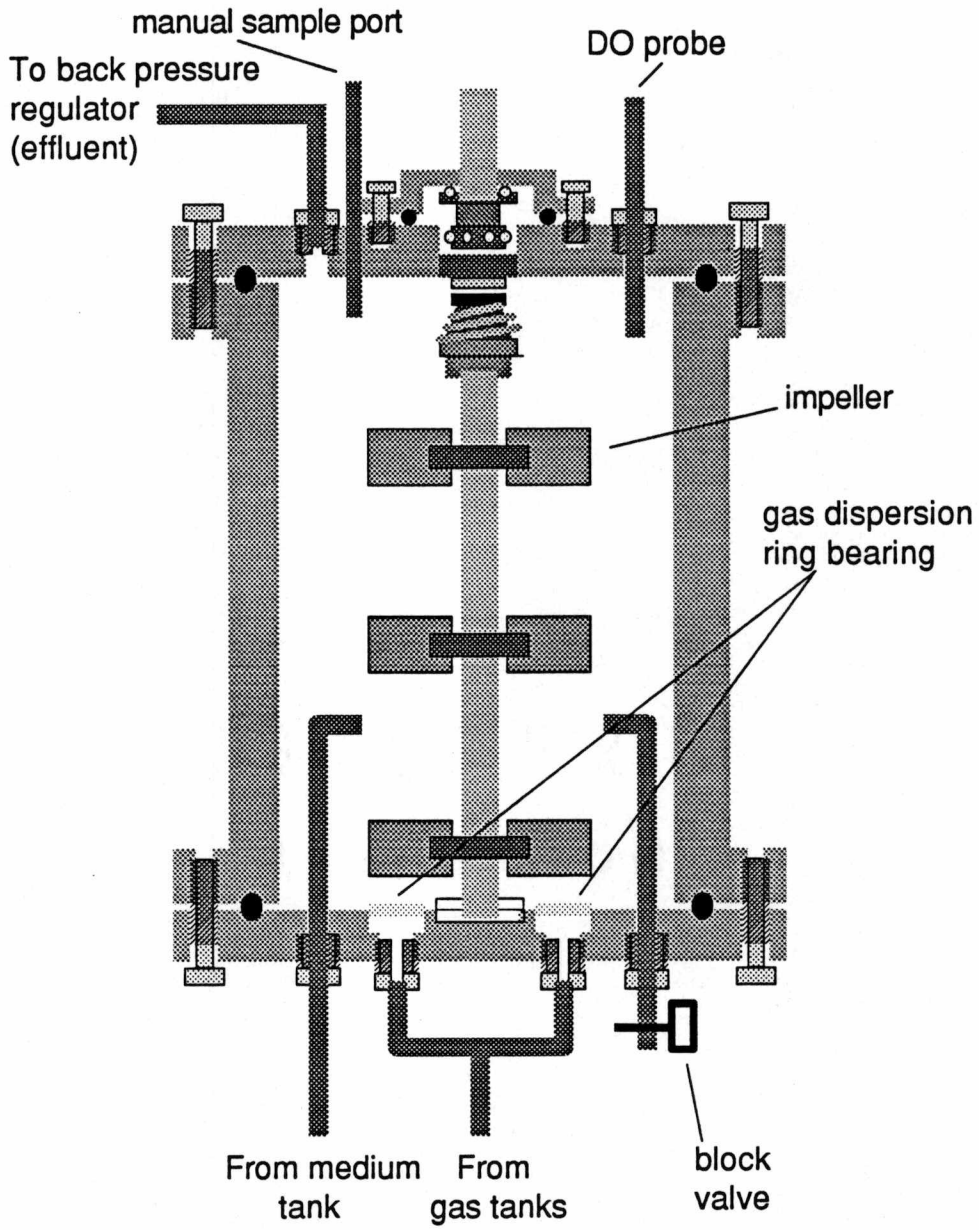


Figure III-9. The scheme of continuous stirred tank reactor.

components. The reactor was primarily designed by Dr. P. Bienkowski and custom-made by the chemical engineering shop located at the University of Tennessee, Knoxville campus.

The medium was pumped in continuous mode by a FMI low speed drive pump Model QG50-0-SSY (FMI Pump Inc., Oyster Bay, NY). The maximum pumping speed and pressure allowed was 4 ml/min and 100 psi. The flow of methane and oxygen gases were controlled by Matheson mass flow controller Model 8102-1412-FC (Matheson Instruments, Horsham, PA) and control panel Model Multiple Dyna-Blender 8219 (Matheson Instruments, Horsham, PA). Dissolved oxygen was measured using an Ingold Sterilizable Dissolved Oxygen Probe (Ingold Electrodes, Inc., Wilmington, MA) and the probe was controlled by Cole-Palmer Panel-Mount Dissolved Oxygen Meter/Controller Model 5513 (Cole-Parmer Intr, Co., Chicago, IL). The probe was calibrated using oxygen and helium gases (0 -100 % of oxygen saturation) and maintained according to the manufacturer's manual. The back-pressure regulator used was a back-pressure regulator Model 26-2320-24 (Tescom Corp., Elk River, MN), and assembled and maintained according to manufacturer's manual.

The use of bioreactor was primarily intended to study population dynamics (e.g., competition) of mixed cultures under various growth regimes.

Population and TCE degradation activity monitoring

The population(s) in pure or mixed culture and TCE degradation activity were monitored by AODC and specific DNA probing as described in the Section N.

Q. Effect of growth regimes on the species competition in flask cultures

The two species competition between *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b was done under various growth regimes such as, methane gradient (5-50 %), copper gradient (0-10 μM), and nitrate gradient (0.1-10 mM) using NMS (Cornish et al., 1984) without nitrate and copper as a basal salts medium.

CHAPTER IV

RESULTS

A. Identification of strain 68-1

The strain 68-1, a methanotroph originally isolated by Little et al. (1988) from a TCE-contaminated aquifer, when grown under copper limitation, was found to produce sMMO as indicated by the naphthalene oxidation assay of Brusseau et al. (1990). It was suspected that this strain was a *Methylomonas methanica* by virtue of its prominent pink carotenoid pigmentation (Whittenbury and Krieg, 1984). This was confirmed through the use of 16S rRNA signature oligodeoxynucleotide probing, electron microscopy, and by phospholipid fatty acid analysis.

The total RNA of strain 68-1 was found to hybridize to the 10-γ 16S rRNA oligodeoxynucleotide probe which is specific for ribulose monophosphate pathway-containing methylotrophs (Tsien et al., 1990) which include both type I and type X methanotrophs (Green, 1992). No hybridization occurred with the 9-α 16S rRNA probe which is specific for serine pathway-containing methylotrophs. Other methanotrophs whose RNA's hybridized to the 10-γ were *Methylomonas methanica, Methylomonas rubra, Methylomonas alba* BG8, and *Methylococcus capsulatus* Bath while the probe 9-α hybridized to *Methylosinus trichosporium, Methylosinus sporium, Methylobacterium organophilum*, and *Methylobacterium extorquens*. Neither probe hybridized to *Agrobacterium tumefaciens* which was used as a negative control (Table IV-1).

The strain 68-1 exhibits intracytoplasmic membrane (ICM) ultrastructures that are typical of type I and X methanotrophs not type II (Figure IV-1). (Jensen

Table IV-1. Identification of strain 68-1 by hybridization of their total RNA with 16S rRNA signature probes, 10-γ and 9-α. Other strains were used as positive and negative controls.

Type and species of bacterial strain	Source or reference	16S rRNA probes	
		10-γ	9-α
Type I methanotrophs:			
Methylomonas alba	ATCC 33003 (=BG8)	+	–
Methylomonas methanica	ATCC 35067	+	–
Methylomonas rubra	NCIMB 11913	+	–
Strain 68-1	Little et al., 1988	+	–
Type X methanotrophs:			
Methylococcus capsulatus	ATCC 33009 (=Bath)	+	–
Type II methanotrophs:			
Methylocystis parvus	ATCC 35066 (= OBBP)	ND	ND
Methylosinus sporium	ATCC 35069 (=5)	–	+
Methylosinus trichosporium	ATCC 35070	–	+
RuMP pathway methylotrophs:			
Methylophilus methylotrophus	ATCC 53528	ND	ND
Serine pathway methylotrophs:			
Methylobacterium extorquens	ATCC 14718 (=AM1)	–	+
Methylobacterium organophilum	ATCC 27886 (=XX)	–	+
Heterotrophs:			
Agrobacterium tumefaciens	ATCC 23308	–	–

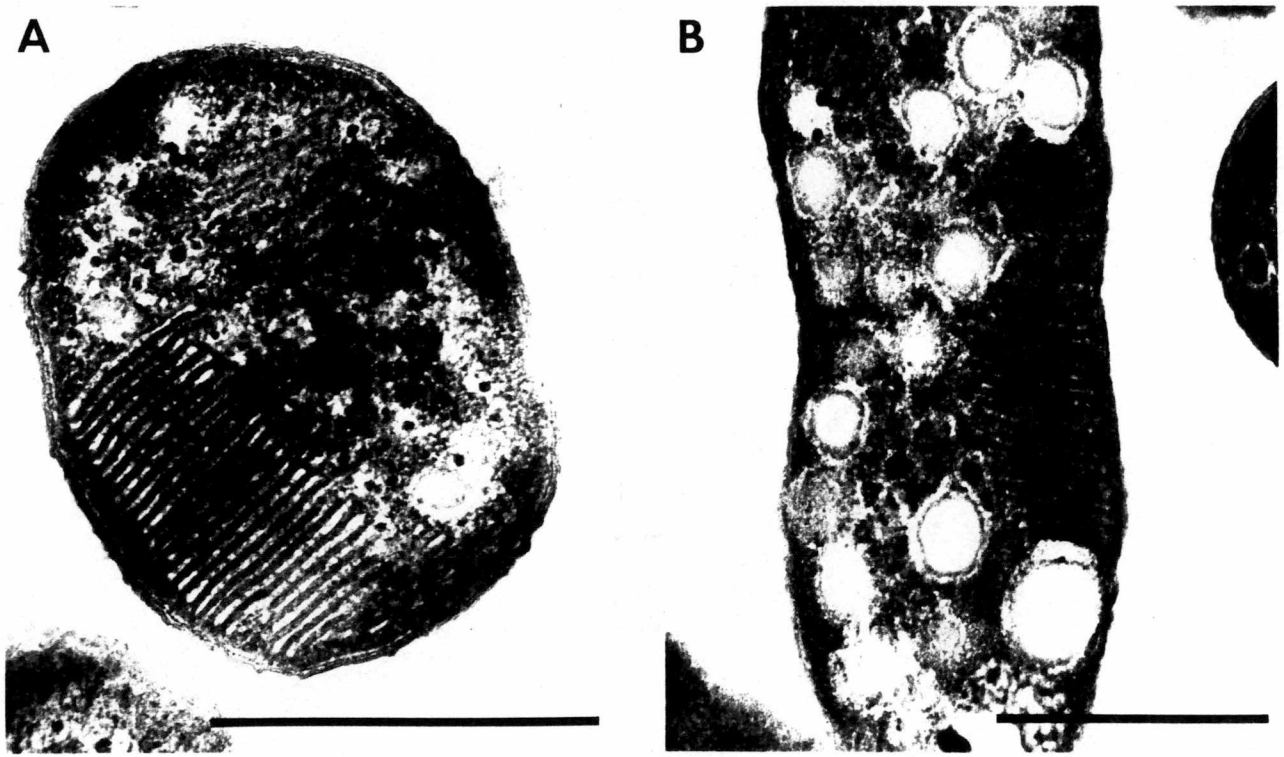


Figure IV-1. Electronmicrographs of thin sections of strain 68-1 grown in the presence (A) and absence (B) of copper (1.0 μM of $CuSO_4.5H_2O$). Bars are equal to 1 μm.

and Corpe, 1991). The structures were present as bundles of vesicles (Figure IV-1) and were produced in the absence of copper.

Strain 68-1 was found to contain 14:0 (30 %), 16:1w8c (18 %), 16:1w7c (15 %), 16:1w6c (13 %), and 16:1w5t (13 %) as its major phospholipid fatty acids (Table IV-2). Of these fatty acids 16:1w8c and 16:1w6c were unique monounsaturates. A previous report (Bowman et al., 1990) showed that the type strain of this genus, *Methylomonas methanica* ATCC 35067^T, had a similar profile of PFLA except that the contents of 16:1w8c and 16:1w6c were significantly different from each other. The presence or absence of copper did not affect significantly the profiles of PLFA. This result is supported by the previous report (Guckert et al., 1991) showing that low or high copper concentration did not cause a significant change in membrane lipid content of *Methylococcus capsulatus* Bath. The lipopolysaccharide hydroxy fatty acid (LPS-OHFA) profiles of 68-1 also complemented those of the type I methanotrophs presented in the previous work (Guckert et al., 1991). Mid-chain hydroxy branched 16 carbon fatty acids were found in 68-1 as in the type strain (ATCC 35067) of *Methylomonas methanica* (Guckert et al., 1991). Therefore, we conclude that 68-1 belongs within the group of type I pink pigment methanotrophs (Figure IV-2).

B. Effect of copper absence on strain 68-1

When grown in a state of copper absence the biomass level and specific growth rate of 68-1 declined significantly. *Methylosinus trichosporium* OB3b, however, showed no significant differences in the parameters (Table IV-3). A

Table IV-2. The profiles of membrane phospholipid fatty acids (PLFA) of strain 68-1 and other representatives of type I, II, and X methanotrophs.

PLFA	68-1 (+Cu)[A]	68-1 (-Cu)[B]	*Methylomonas methanica*[C]	*Methylomonas* sp.[D]	*Methylosinus trichosporium*[C]	*Methylococcus capsulatus*[C]
i14:0				0-0.1		
14:1ω7c				0-0.2		
14:0	29.76	24.57	20.76	18.9-25.1	0.01	2.35
i15:0		0.19		0-2.5		
a15:0		0.15		0-2.4		
15:0	0.45	0.45	0.30	0-1.2		1.22
i16:0						
16:1ω8c	17.94	18.69	40.90	25.7-41.3		
16:1ω7c	14.68	15.29	10.86	7.7-14.0	10.71	18.66
16:1ω6c	12.90	13.26	3.35	4.5-8.5		10.26
16:1ω7t			1.92		0.37	8.54
16:1ω6t			0.57			
16:1ω5c	3.84	5.81	0.83	1.9-6.3		12.36
16:1ω5t	12.75	16.63	15.57	7.9-15.3		0.38
16:0	5.04	4.32	4.29	5.0-8.7	1.17	32.53
i17:0				0-0.1	0.06	
a17:0					0.10	
17:1ω7c				0-0.7		
17:1ω7t				0-0.3		
cy17:0(ω7,8)	0.91	0.26		0-2.1		7.37
cy17:0b						5.17

Table IV-2 cont'd.

PLFA	68-1 (+Cu)[A]	68-1 (-Cu)[B]	*Methylomonas methanica*[C]	*Methylomonas* sp[D]	*Methylosinus trichosporium*[C]	*Methylococcus capsulatus*[C]
17:0					0.03	
18:1ω9c	0.24			0-0.2		
18:1ω8c					62.02	
18:1ω7c	0.89	0.18	0.25	1.0-2.5	17.35	0.81
18:1ω8t					7.50	
18:1ω7t						0.14
18:1ω5c				0-0.2		
18:0				0-0.1	0.66	0.21
br19:0				0-0.5		
cy19:(ω7,8)	0.61	0.20		0.2-0.4		
Total	**100.00**	**100.00**	**100.00**	**100.0**	**100.00**	**100.00**

[A] 1.0 μM of copper sulfate added to NMS liquid medium. [B] 0.0 μM of copper sulfate added to NMS liquid medium. These data were courtesy of Ringerberg.[C] Bowman et al., 1990; abbreviation for *Methylomonas methanica* ATCC 35067, *Methylosinus trichosporium* OB3b, and *Methylococcus capsulatus* Bath in order. [D] Adapted from the data of Bowman et al. (1990): *Methylomonas methanica* ATCC 35067, *Methylomonas fodinarum* ACM 3268, and *Methylomonas aurantiaca* NCIB 11915.

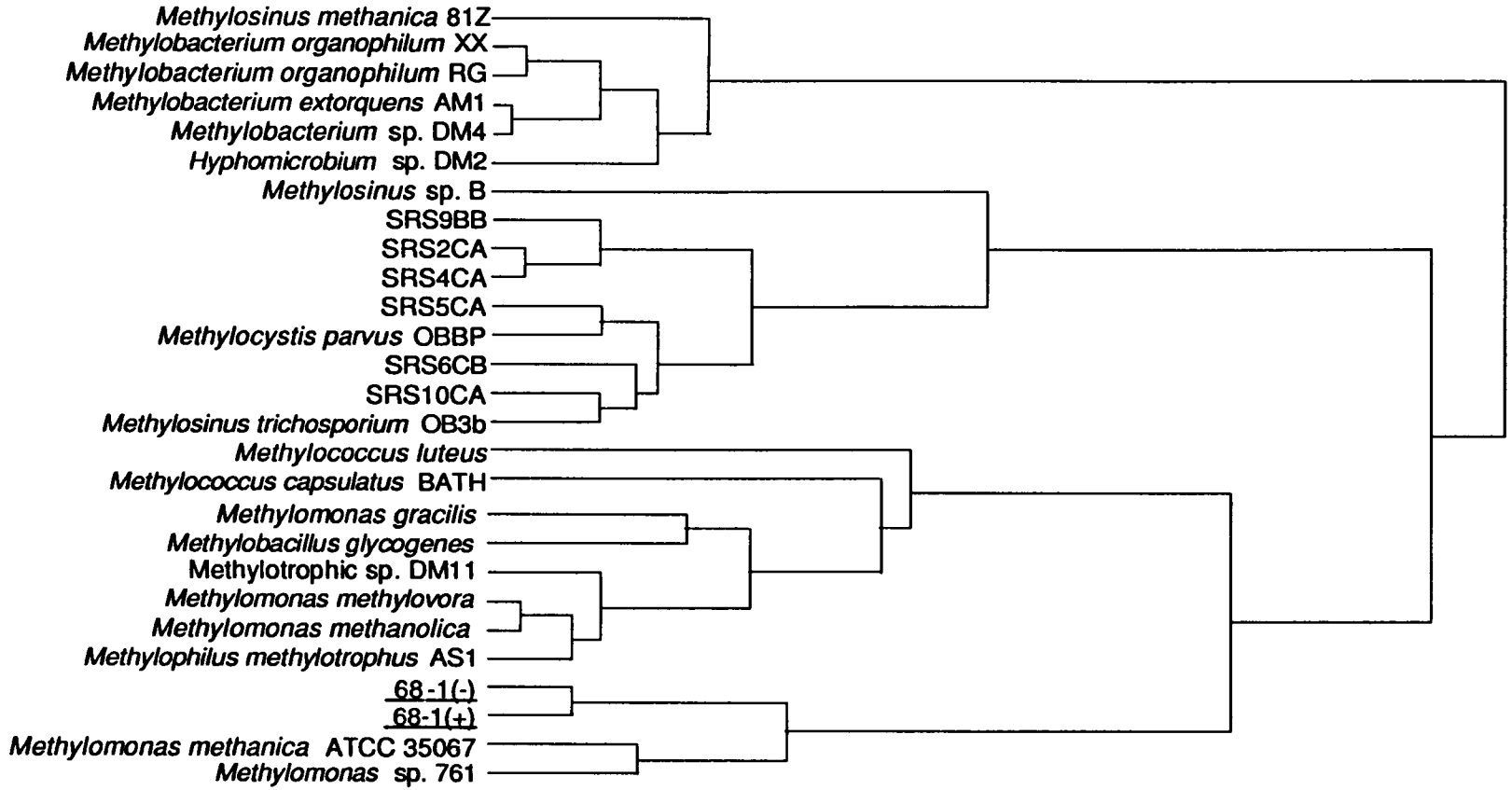


Figure IV-2. The taxonomic status of strain 68-1grown in presence (+) or absence (-) of copper. The strain belongs to the typical pink-pigmented type I methanotrophic group based on its PLFA analysis in comparison with other methanotrophic and methylotrophic eubacteria shown by Guckert et al. (1991) and Bowman et al, 1993.

Table IV-3. Growth parameters of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b grown during copper-free conditions.

Parameters	*Methylomonas methanica* 68-1	*Methylosinus trichosporium* OB3b
NMS[A] + 1 μM $CuSO_4$:		
$\mu_{max}(h^{-1})$[B]	0.074	0.060
Biomass (mg/L)	202.9 ± 8.1	160.1 ± 8.0
Y_s (g cells/g CH_4)	0.80	0.67
NMS + 0 μM $CuSO_4$		
$\mu_{max}(h^{-1})$[B]	0.060	0.053
Biomass (mg/L)	93.0 ± 10.1	178.8 ± 10.1
Y_s (g cells/g CH_4)	0.73	0.63

[A] NMS, nitrate mineral salts medium. [B] Cultures were grown in 500 ml of NMS medium in 2 L flasks under 4:1 air to methane. Flasks were shaken at 200 rpm at 25°C.

decline in ICM was observed in 68-1, however, many cells still showed obvious ICM formation (Figure IV-1B). In this study some cells of 68-1 seemed to lack ICM when they were grown in the absence of copper. The increase in poly-β-hydroxybutyrate storage granules was striking in most cells (Figure IV-1B). This has also been observed in *Methylomonas alba* (Collins et al., 1991) and *Methylococcus capsulatus* (Prior and Dalton, 1985). In contrast to 68-1, the maximum specific growth rate and growth yield of *M. trichosporium* OB3b were not significantly affected by copper absence.

C. Soluble methane monooxygenase activity measured by naphthalene oxidation

The sMMO activity of *M. methanica* 68-1 was measured for both whole cells and cell fractions to locate the sMMO in the cells and comparatively analyzed with *M. trichosporium* OB3b.

Naphthalene oxidation by whole cells

Naphthalene oxidation has been used to indicate the presence of sMMO activity (Brusseau et al., 1990). When grown in copper-free conditions strain 68-1 readily oxidized naphthalene to 1- or 2- naphthol (Figure IV-3). Naphthalene oxidation in 68-1 and *Methylosinus trichosporium* OB3b was found to be highest in early stationary growth phase. The naphthalene oxidation rate of 68-1 was at least two times as high as that of OB3b. In a state of methane limitation enzyme activity declined. This may be due to reduced electron transfer through sMMO as a NADH conservation mechanism in these organisms (Dalton and Leak, 1985). The strain 68-1 did not express sMMO and did not oxidize naphthalene when

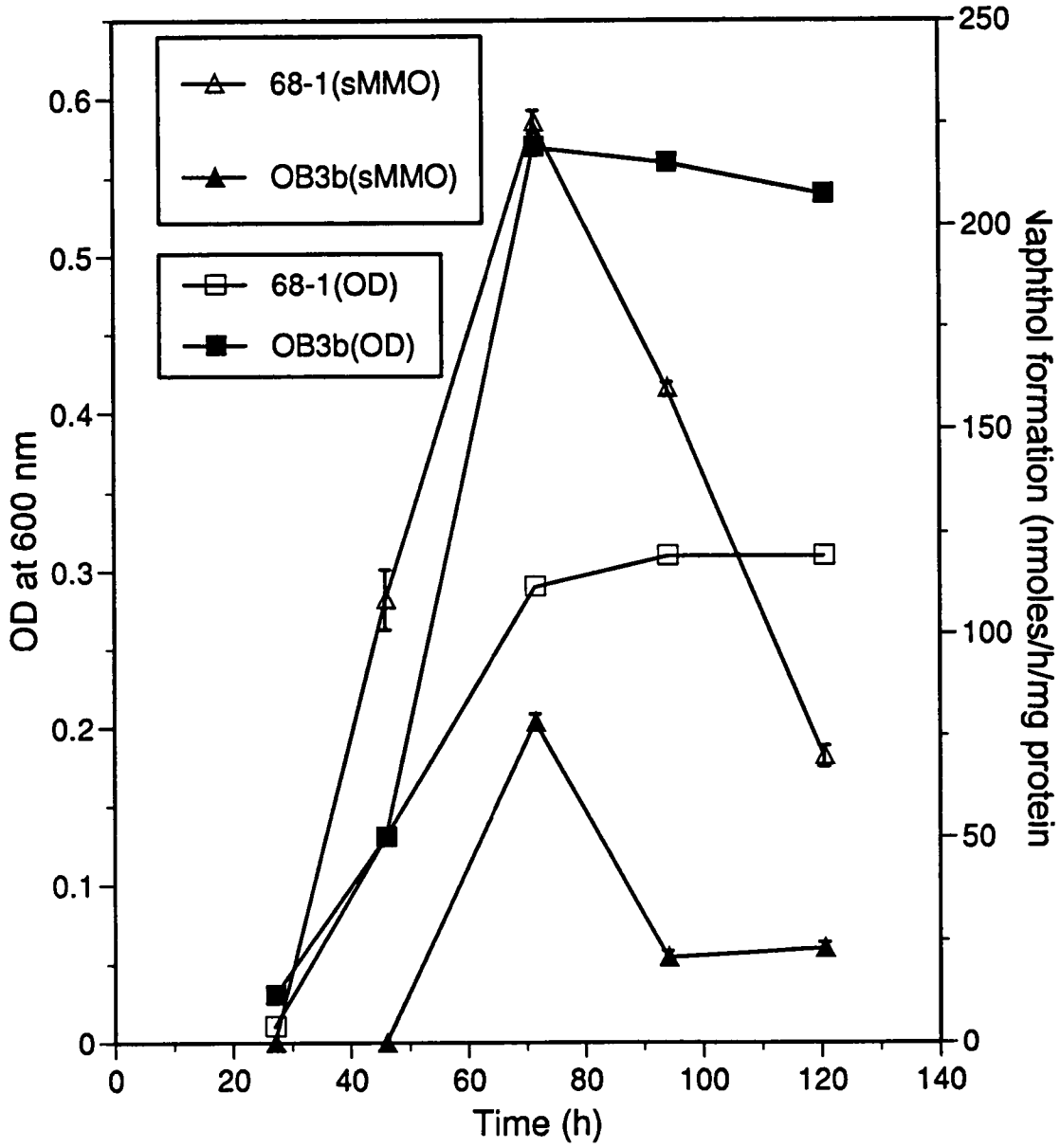


Figure IV-3. Growth curves of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b grown under copper-free conditions and sMMO activity measured by naphthalene oxidation. Naphthalene oxidation to naphthol by soluble methane monooxygenase of these strains peak in early stationary growth phase.

grown in the presence of 1 μM of $CuSO_4$ as previously shown in *M. trichosporium* OB3b (Brusseau, 1990). Other *Methylomonas* spp. strains investigated including *M. methanica* ATCC 35067^T, *M. alba* BG8, and *M. rubra* NCIMB 11913 did not oxidize naphthalene and grew poorly in copper-free NMS medium. This suggests sMMO activity is a strain-specific feature at least in 68-1.

Naphthalene oxidation by cell fractions of strain 68-1

The sMMO activity assay in cell fractions (soluble and particulate fractions) was not possible by naphthalene oxidation method. It seemed that the dithiothreitol (DTT) (20 mM) contained in PIPES buffer used in the preparation of cell fractions caused the yellow precipitation with tetrazotized-*o*-dianisidine. If DTT were replaced by sodium thioglycolate (5 mM), the precipitation was not observed. However, this modification still did not give the typical purple color seen when 1-naphthol or 2-naphthol (product of naphthalene oxidation) reacted with the azo dye. This reason would be the decrease of enzyme activity for each cell fraction which was caused during the fraction preparation procedures. It turned out that the TCE degradation activity of these cell fractions was low by several orders of magnitude (refer the Section C of the Chapter IV).

D. TCE degradation by soluble methane monooxygenase activity of strain 68-1

TCE degradation by whole cells

Strain 68-1 was able to degrade TCE more rapidly than OB3b (Figures IV-4 and IV-5) in the conditions employed. The time course of TCE degradation was shown in Figure IV-4. The remaining TCE concentration in the 68-1 reaction mixture decreased more rapidly than that of OB3b.

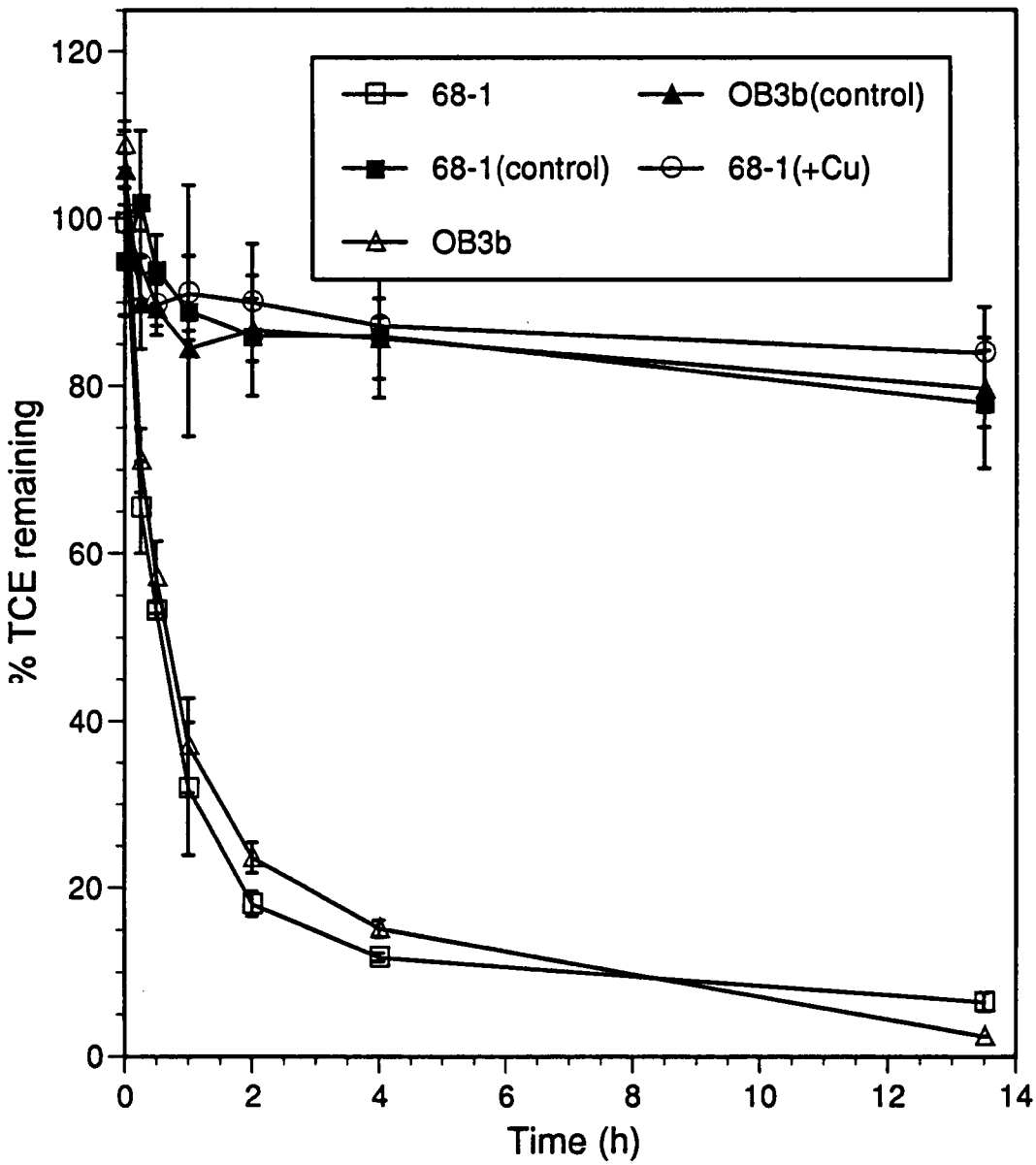


Figure IV-4. TCE degradation by viable cell suspensions of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b. Each diluted cell suspension (0.08 to 0.09 mg dry weight of cells /ml) in the early staionary phase was incubated with 42 μM of TCE and 20 μM of sodium formate and the reamining TCE concentrations were determined at the given time points within 24 h. Heat-killed cells of both strains were used as controls.

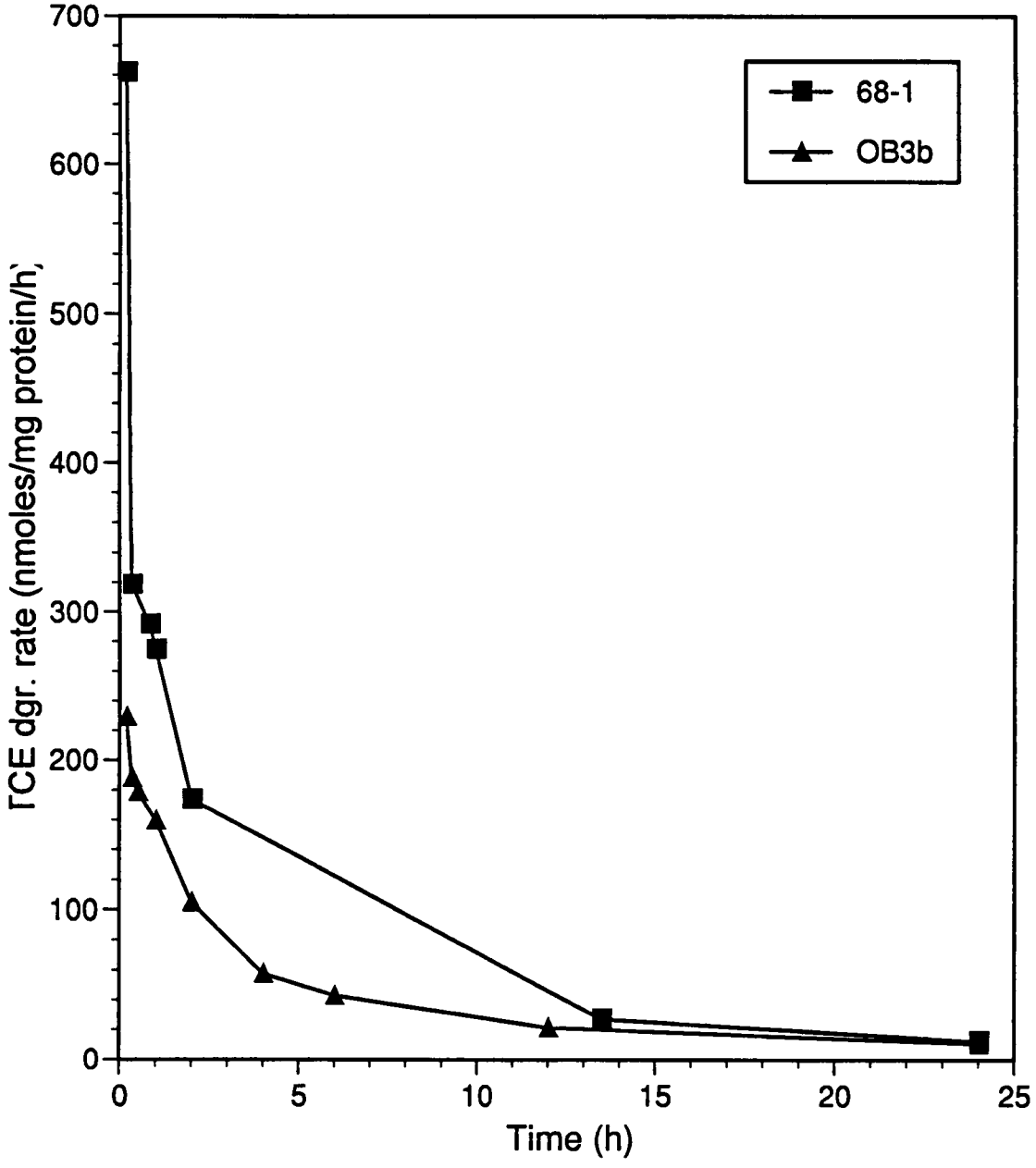


Figure IV-5. Change of TCE degradation rates of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b. It was assumed that no TCE degradation occurred at 0 h.

This appears to be due to the rapid TCE degradation rate of 68-1 as reflected by the higher V_{max} value of TCE degradation by 68-1. In heat-killed controls in both strains, at least about 80 % of initial TCE was maintained and thus extractable. Changes of TCE degradation rates of 68-1 and OB3b are also monitored (Figure IV-5). The TCE degradation of 68-1 decreased to 47 % of the initial degradation rate in 1 h whereas the degradation rate of OB3b decreased to the similar level in 2 h. The rapid decrease in TCE degradation rates of both strains may be attributed to the substrate depletion and sMMO inactivation.

Distribution of TCE degradation activity of cell fractions in strain 68-1

In order to confirm the attribution of TCE degradation by strain 68-1 to sMMO, cells of the strain were ruptured and the soluble and particulate fractions were assayed for TCE degradation activity based on a method modified from DiSpirito et al. (1992). As shown in the Figure IV-6, significantly higher TCE degradation (1092.2 ± 247.8 pmoles/h/mg protein) was observed in the soluble fraction of the 68-1 cells grown in the absence of copper (1.0 μM $CuSO_4$) while little TCE degradation (26.0 ± 3.2) occurred in the particulate fraction of the same cell preparation. However, in the presence of copper, the TCE degradation activity of the soluble fraction was reduced to a point where no significant difference in the activity between the soluble and particulate fractions was observed. This indicated that the sMMO was derepressed in the absence of copper and was responsible for the high rates of TCE degradation. The overall TCE degradation rates were lower than those of whole cells because MMO activity loss occurred during the preparation of cell fractions following the cell ruptureand the degradation rates were determined after 22 h incubation.

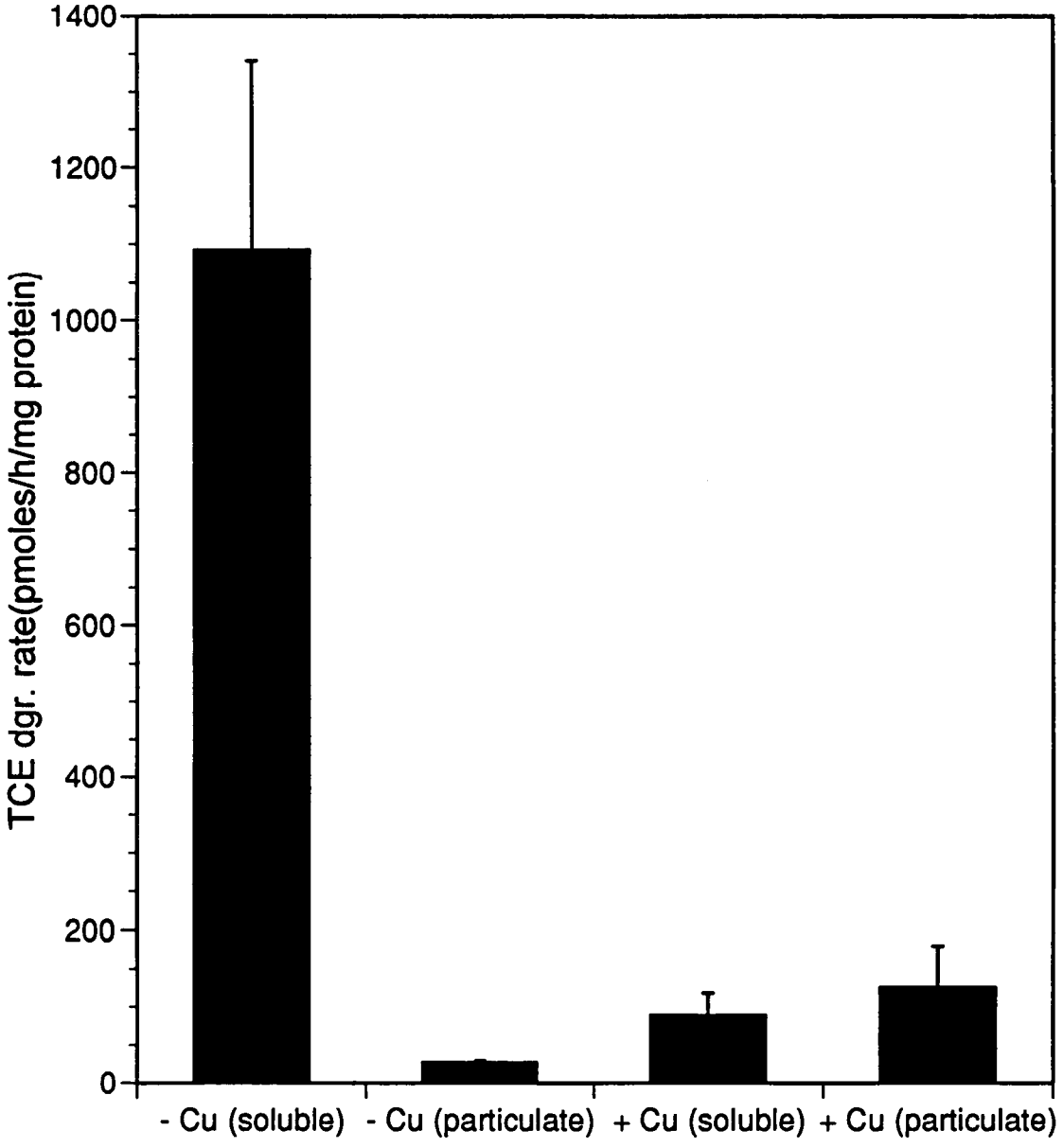


Figure IV-6. TCE degradation rate of soluble and particulate fractions of *Methylomonas methanica* 68-1 grown in the presence (+) or absence (-) of copper (1 μM $CuSO_4$)

E. Naphthalene oxidation and TCE degradation kinetics

The V_{max} value of the naphthalene oxidation by 68-1 was more than 1.5 times as high as that by OB3b while the TCE degradation V_{max} value of 68-1 was more than twice as high as that of OB3b. By contrast, however, strain 68-1 had a slightly lower affinity for naphthalene and TCE compared to OB3b (Table IV-4). These variations in oxidation rates and substrate affinities of sMMO in these strains may be linked to variations in the rate of electron transfer and the active site structure.

F. Genetic homology analysis of sMMO and MDH (methanol dehydrogenase) of strain 68-1 using sMMO MDH gene probes

Using low stringency hybridization conditions allowing approximately 40 % nucleotide mismatch, the sMMO component B gene probe (Tsien et al., 1992) bound non-specifically to the DNA's of a wide range of bacterial species, many not capable of producing either sMMO or pMMO (Figure IV-7, panel A). When more stringent conditions were applied allowing for 30 % nucleotide mismatch the probe only bound to the DNA of the strain of the probe origin (OB3b) and to *Methylosinus sporium* ATCC 35069 (Figure IV-7, panel B; Table IV-5). The probe, however, did not bind to the DNA of 68-1 in either the low or high stringency conditions.

The Southern blot hybridization performed in the stringent conditions has shown that the *Eco*RI fragments, 2.2 kb and 1.6 kb of OB3b and ATCC 35069, respectively, hybridized to the sMMO probe. The gene probe, however,

Table IV-4. Naphthalene oxidation and TCE degradation kinetic parameters of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b grown during copper-free conditions.

Parameters	*Methylomonas methanica* 68-1	*Methylosinus trichosporium* OB3b
Napthalene oxidation[A]:		
K_m (μM)	70 ± 4	40 ± 3
V_{max} (nmoles/h/mg protein)	551 ± 27	321 ± 16
TCE degradation[B]:		
K_m (μM)	225 ± 13	126 ± 8
V_{max} (nmoles/h/ mg protein)	2325 ± 260	995 ± 160

[A] The stationary phase culture was used. Naphthalene concentrations used were in the range of 7.3 to 234.0 μM.
[B] The stationary phase culture was used. TCE concentrations used were in the range of 5.0 to 500.0 μM.

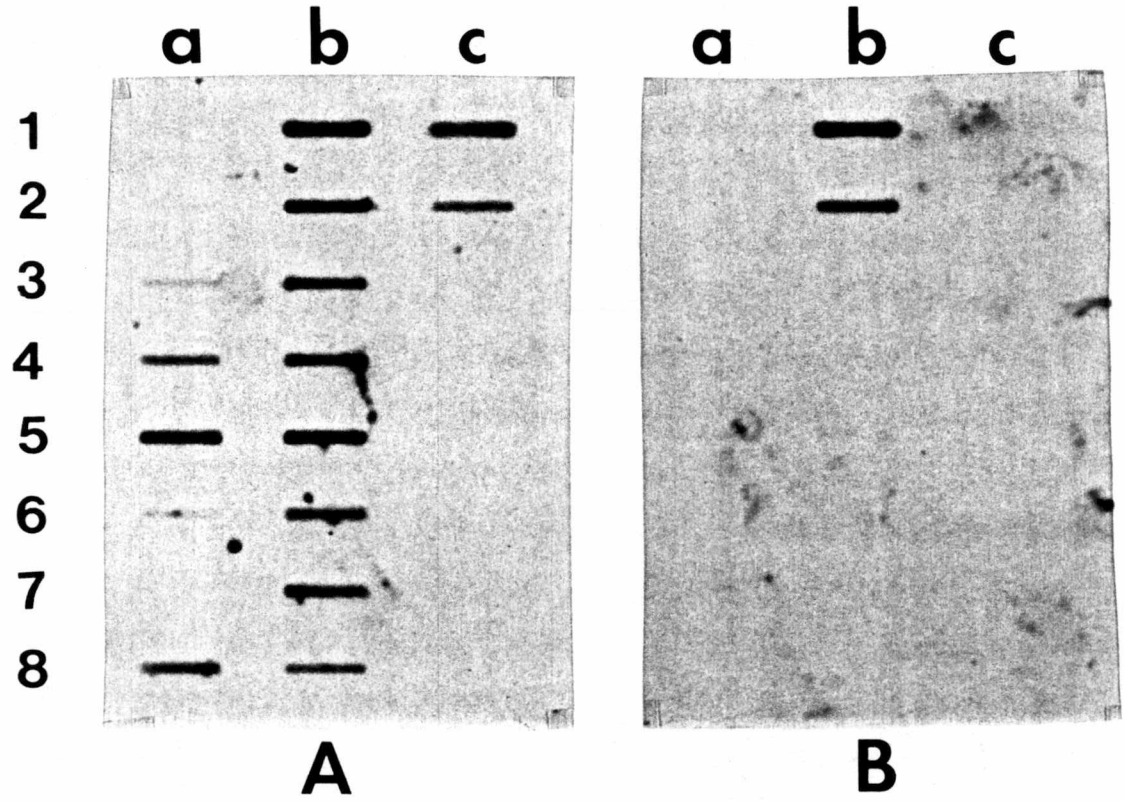


Figure IV-7. DNA slot blot hybridization of strain 68-1 and other methylotrophs with a sMMO (soluble methane monooxygenase) gene probe carrying *mmoB* and *mmoZ*. Hybridizations were performed at low stringency (A) and high stringency (B). a1, 68-1; a2, *Methylomonas methanica*; a3, *Methylomonas rubra*; a4, *Methylomonas alba* BG8; a5, *Methylomonas clara*; a6, *Methylomonas agile*; a7, *Methylophilus methylotrophus*; a8, *Methylobacterium rhodesianum*; b1, *Methylosiuns trichosporium* OB3b; b2, *Methylosinus sporium* 5; b3, *Methylocystis parvus* OBBP; b4, *Methylobacterium organophilum* XX; b5, *Methylobacterium extorquens* AM1; b6, *Methylococcus capsulatus* Bath; b7, *Methylococcus capsulatus* Texas; b8, *Hyphomicrobium* sp.; c1, *Rhizobium leguminosarum* 248; c2, *Agrobacterium tumefaciens*; c3, *Pseudomonas putida*; c4, *Bacillus subtilis*.

Table IV-5. DNA slot blot hybridization of strain 68-1 and other methanotrophs and methylotrophs with a sMMO gene probe and a methanol dehydrogenase gene probe.

strains	Hybridization to probe of sMMO gene (*mmoB* & *mmoZ*)[A]	MDH gene (*moxF*)[B]
68-1	–	–
Methylomonas methanica	–	–
Methylomonas rubra	–	+
Methylomonas alba BG8	–	+
Methylomonas clara	–	–
Methylomonas agile	–	–
Methylophilus methylotrophus	–	NT
Methylobacterium rhodesianum	–	–
Methanomonas methylovora	NT	+
Methylosiuns trichosporium OB3b	+	–
Methylosinus sporium 5	+	–
Methylocystis parvus OBBP	–	–
Methylobacterium organophilum XX	–	+
Methylobacterium extorquens AM1	–	+
Methylococcus capsulatus Bath	–	NT
Methylococcus capsulatus Texas	–	+
Hyphomicrobium sp.	–	+
Rhizobium leguminosarum 248	–	–
Agrobacterium tumefaciens	–	–
Pseudomonas putida	–	–
Bacillus subtilis.	–	–

[A] Hybridization allowing 30 % mismatch. [B] Hybridization allowing 40 % mismatch. NT Not tested.

did not hybridize to any *Eco*RI restriction fragment of the type I and X methanotrophs, and heterotrophs tested (e.g., 68-1, *Methylomonas methanica*, *Methylomonas rubra*, *Methylomonas alba*, *Methylomonas agile*, *Methylococcus capsulatus* Bath, *M. capsulatus* Texas, *Rhizobium leguminosarum*, and *Agrobacterium tumefaciens*) (data not shown).

A methanol dehydrogenase (MDH) structural gene probe from *Methylobacterium organophilum* XX (2.5 kb *Sma* I fragment carrying MDH V-C gene) non-specifically hybridized to genomic DNA's of various methanotrophs (types I, II, and III) and methylotrophs (both RuMP and Serine pathways) tested under low stringent hybridization conditions allowing 40 % mismatch (Table IV-5). The MDH gene probe did not hybridize to the DNA's of non-methanotrophs or methylotrophs while the sMMO component B gene probe did under similar hybridization conditions.

G. TCE resilience and TCE transformation capacity

The effect of repeated additions of TCE (0.064 mg/mg dry weight cells) on the degradation by 68-1 and OB3b cells was shown in the Figure IV-8. The TCE transformation capacity of 68-1 was similar to that of OB3b before second addition (at 2 h) but 50 % lower than OB3b after the second addition. This trend of lower transformation capacity of 68-1 was expected to more prominent after fifth addition of TCE. However, another independent experiment showed that TCE resilience and transformation capacity of 68-1 (0.095 mg TCE/mg cells) was higher than that of OB3b (0.081 mg TCE/mg cells) if lower TCE load (0.0065 mg/mg dry weight cells per TCE addition) as employed by Alvarez-Cohen et al. (1991a) were considered.

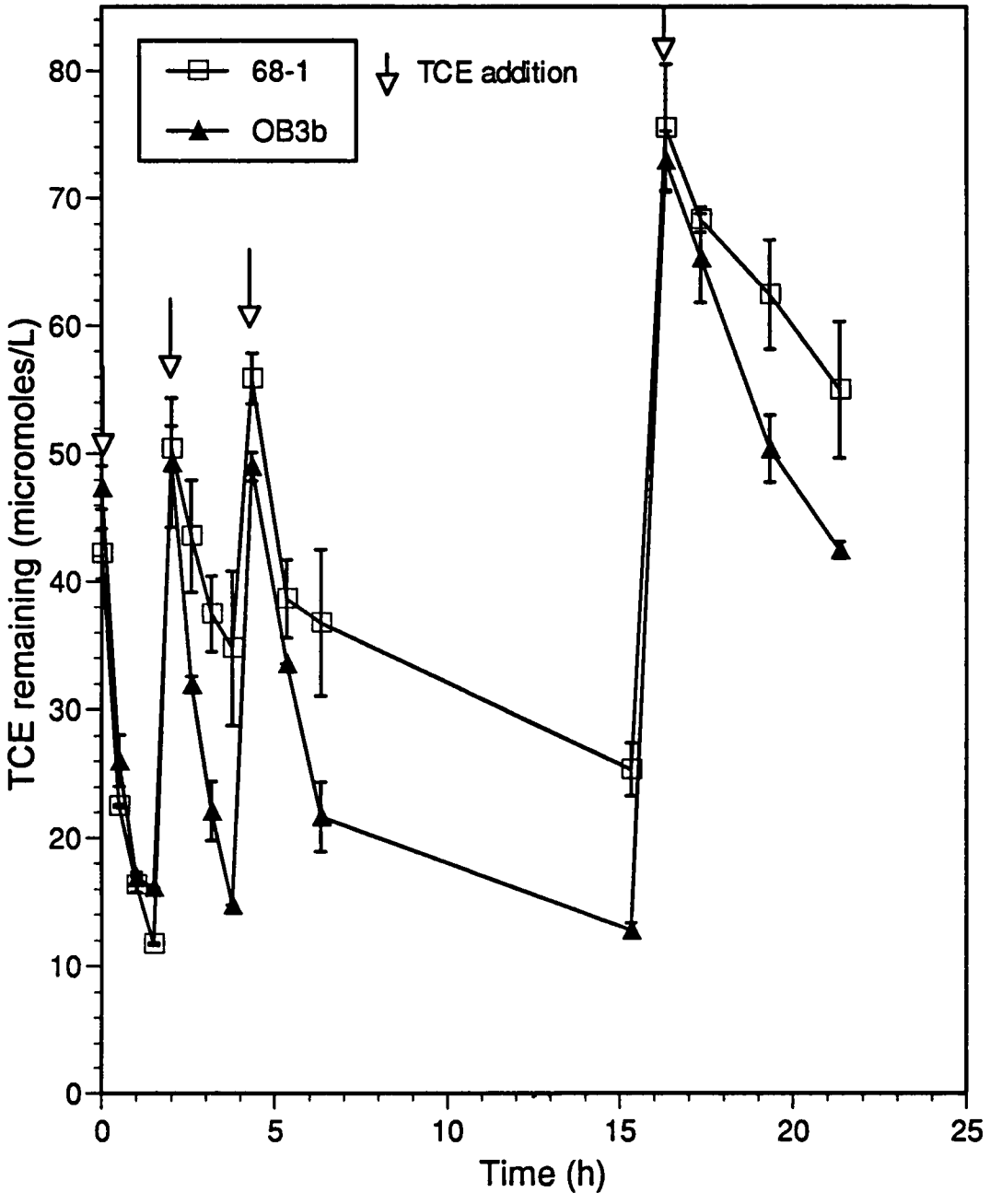


Figure IV-8. Effect of repeated addition of TCE (42 μM for each addition) on its degradation by *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b resting cells (0.25 mg/ml).

H. Substrate specificity of sMMO of *M. methanica* 68-1 in degradation of chlorinated aliphatic hydrocarbons

The ability of 68-1 to degrade several chlorinated aliphatic compounds was assessed in comparison with OB3b (Table IV-6). The degradation of these compounds was monitored using gas chromatography. As mentioned in the Section D, 68-1 proved to have higher specific rates of degradation for several of these compounds (dichloromethane, chloroform, 1,1-dichloroethane, and 1,2-dichloroethane) when compared to OB3b. On the other hand, 68-1 did not seem to be as effective as OB3b for the degradation of some chlorinated aliphatic compounds, including 1,1,1-and 1,1,2-trichloroethane, and 1,1,1,2- and 1,1,2,2-tetrachloroethane.

I. Construction of genomic DNA library and cloning

One of the major goals of this study was to develop a DNA probe coding for methane monooxygenase (soluble or particulate) of type I methantrophs. The DNA probe was expected to be used to detect and monitor type I methanotroph(s) from a mixed culture in a bioreactor and eventually from environmental samples. In this section the cloning strategy of a sMMO gene from a type I methanotroph, *Methylomonas methanica* 68-1, is described. The specificity of the cloned putative sMMO gene as a DNA probe is also examined.

Construction of genomic DNA library and screening

The cloning strategy of a gene coding soluble methane monooxygenase of *Methylomonas methanica* 68-1 is shown in Figure IV-9. The chromosomal DNA of 68-1 was completely digested with restriction endonuclease *Eco*RI,

Table IV-6. Chlorinated aliphatic compound degradation by *Methylomonas methanica* 68-1and *Methylosinus trichosporium* OB3b.

compound[A]	Degradation rate (nmol/h/mg protein)	
	Methylomonas methanica 68-1	*Methylosinus trichosporium* OB3b
Dichloromethane	1450 ± 89	899 ± 35
Chloroform	432 ± 35	227 ± 21
1,1-Dichloroethane	174 ± 18	110 ± 14
1,2-Dichloroethane	127 ± 28	89 ± 10
1,1-Dichloroethylene	4 ± 2	11 ±1
1,2-Dichloroethylene (*cis*:*trans* = 1:1)	14 ± 3	30 ± 2
1,1,1-Trichloroethane	2 ± 1	15 ± 2
1,1,2-Trichloroethane	48 ± 6	62 ± 20
1,1,1,2-Tetrachloroethane	0	8 ± 1
1,1,2,2-Tetrachloroethane	3 ± 1	15 ± 1

[A] compounds were tested at an initial concentration of 100 µM. No detectable degradation was observed for the compounds carbon tetrachloride and tetrachloroethylene.

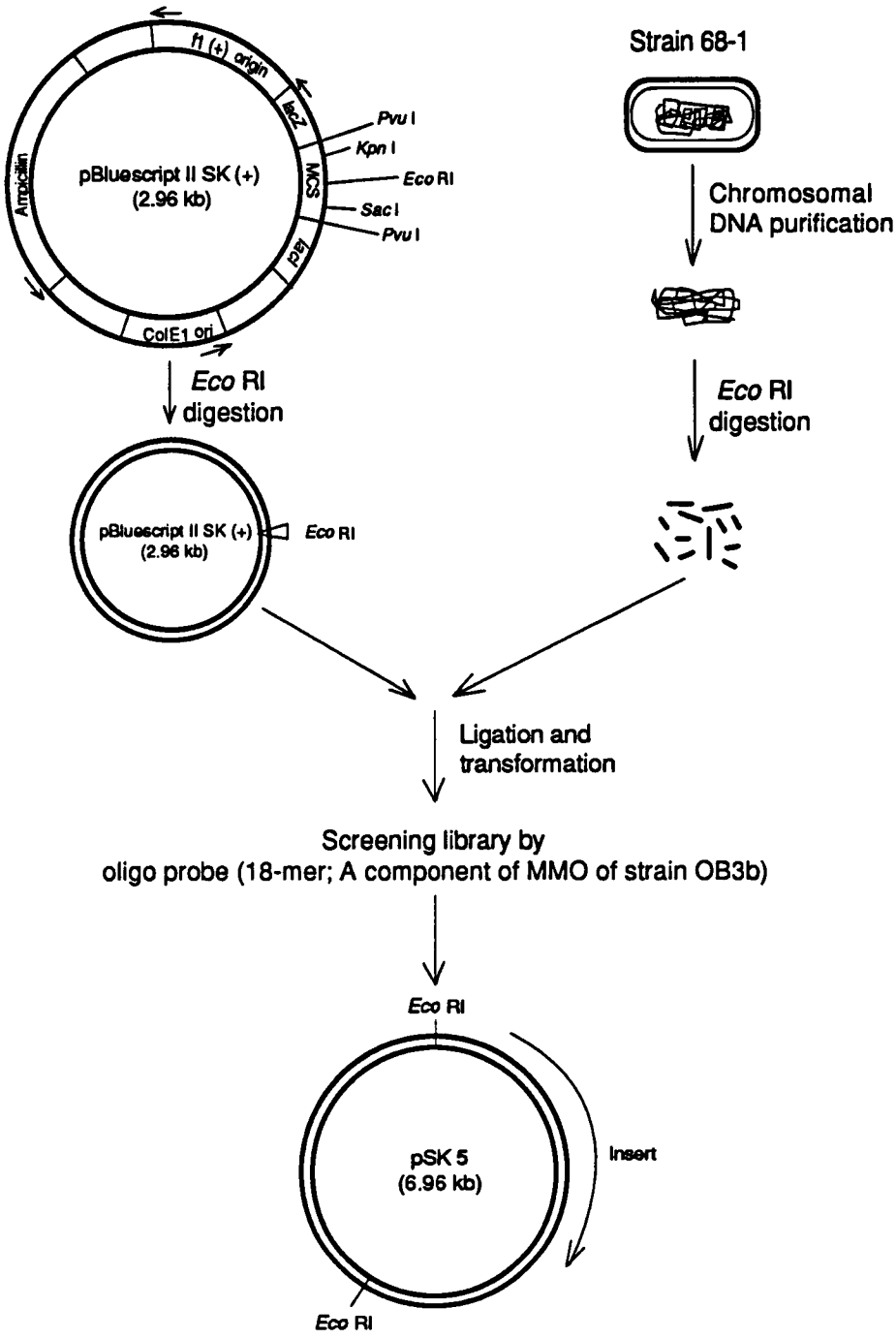


Figure IV-9. The shot-gun cloning of a gene coding soluble methane monooxygenase (sMMO) of *Methylomonas methanica* 68-1. The probe used for screening genomic DNA library was 18-mer deoxyoligonucleotides designed from sMMO (hydroxylase γ-subunit) of *Methylosinus trichosporium* OB3b.

and the restriction fragments were then cloned into the *Eco*RI restriction site of the vector pBluescript II SK(+) (Stratagene Cloning Systems, Inc., La Jolla, CA). The recombinant plasmids were used to transform *E. coli* XI1-Blue. A titer of approximately 2.8 X 10^5 CFU/μg DNA was obtained. The transformants were screened by the radiolabeled oligodeoxynucleotide probe (mixed degenerate oligodeoxynucleotide probes designed from amino terminal of sMMO γ-subunit of *Methylosinus trichosporium* OB3b; 18-mer each). To screen out false positive hybridizations, duplicate nylon filters for lifting clonies were prepared.

Southern blot hybridization

Among 10,000 colonies screened, a 4.0 kb insert of one recombinant plasmid, pSK5, was found to hybridize with the probe (Figure IV-10). The degenerate oligo probes designed from sMMO γ-subunit of *Methylosinus trichosporium* OB3b clearly hybridized to the 2.1 kb *Eco* RI fragment of the OB3b genomic DNA digest (arrow head in Figure IV-10B). The 2.1 kb fragment appeared to carry the genes encoding the sMMO hydroxylase γ-subunit, sMMO component B, and part of sMMO reductase of OB3b as shown by Cardy et al. (1991) and Tsien et al. (1992). In another independent Southern hybridization the 18-mer degenerate oligo probes strongly hybridized to the 2.2 kb *Eco* RI fragment (carrying the sMMO B component gene of OB3b) cloned by Tsien et al. (1992). The oligo probes, however, hybridized to multiple bands from the genomic DNA digest of 68-1 (7 bands seen from lane 3 of Figure IV-10B). Among them a 4 kb fragment appeared to be relatively more distinctive. The larger fragments (ca. 5.5 kb and 11 kb) could be products of incomplete

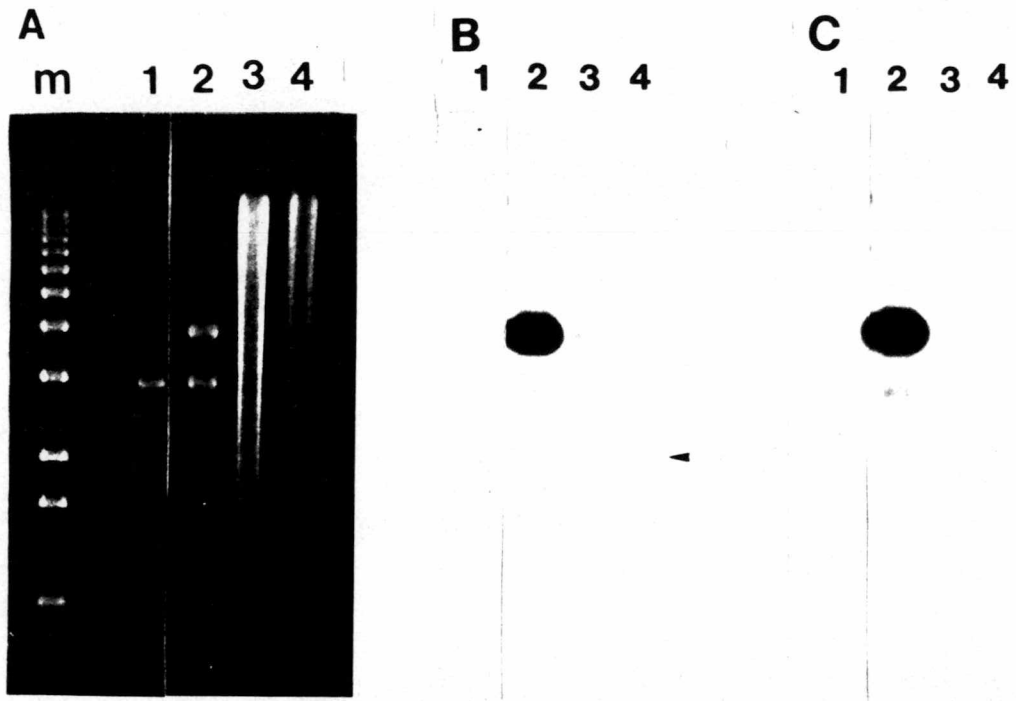


Figure IV-10. Southern blot hybridization of pSK5 using the 18-mer oligodeoxynucleotide and 4.0 kb insert of pSK5 as probes. (A) Samples were electrophoresed on an 1% agarose gel in TBE buffer and stained with ethidium bromide. Lanes: m, 1 kb DNA ladder (BRL); 1, pBluescript II SK(+) vector cut with *Eco*RI; 2, pSK5 cut with *Eco*RI; 3, 68-1 genomic DNA cut with *Eco*RI; 4, *M. trichosporium* OB3b genomic DNA cut with *Eco*RI; (B) Southern blot hybridization of panel (A) using the 18-mer probe; (C) Southern blot hybridization of panel (A) using the 4.0 kb insert of pSK5 as a probe.

digestion carrying the 4 kb fragment or come from a non-specific hybridization and the other fragments smaller than the 4 kb could come from the non-specific hybridization. These non-specific hybridizations appeared to be primarily derived from the final washing temperature (42°C) which was rather low compared with the potential washing temperature calculated from G+C content (% mol) of the mixed oligo probes (46-48°C). Southern hybridization using the putative sMMO gene of 68-1 (the 4 kb insert of pSK5) as a DNA probe showed that the probe clearly hybridized to the 4 kb *Eco* RI fragment of 68-1 genomic DNA digest but not the sMMO gene fragment of OB3b (2.1 kb Eco RI band indicated by the arrow head in Figure IV-10B) (Figure IV-10C).

PCR strategy

To confirm further that the oligo probe was specifically hybridizing to the target site of the insert in clone pSK5, polymerase chain reaction (PCR) using the oligo probe as a primer was attempted. The PCR strategy is shown in Figure IV-11. The primers were the oligo probe and M13 reverse sequencing primer (#1201, New England Biolabs, Inc., Beverly, MS) which were used as an insert-specific primer and a generic primer located on the vector respectively.

PCR amplification

The DNA thermal cycle was optimized based on a default file carried in DNA Thermocycler (Perkins Elmer Cetus Instrs., Norwalk, CT) as far as it could produce a distinct DNA band. Temperature cycling was melting at 95°C for 1 min, annealing at 42°C for 2 min, and polymerization at 72°C for 3 min. The

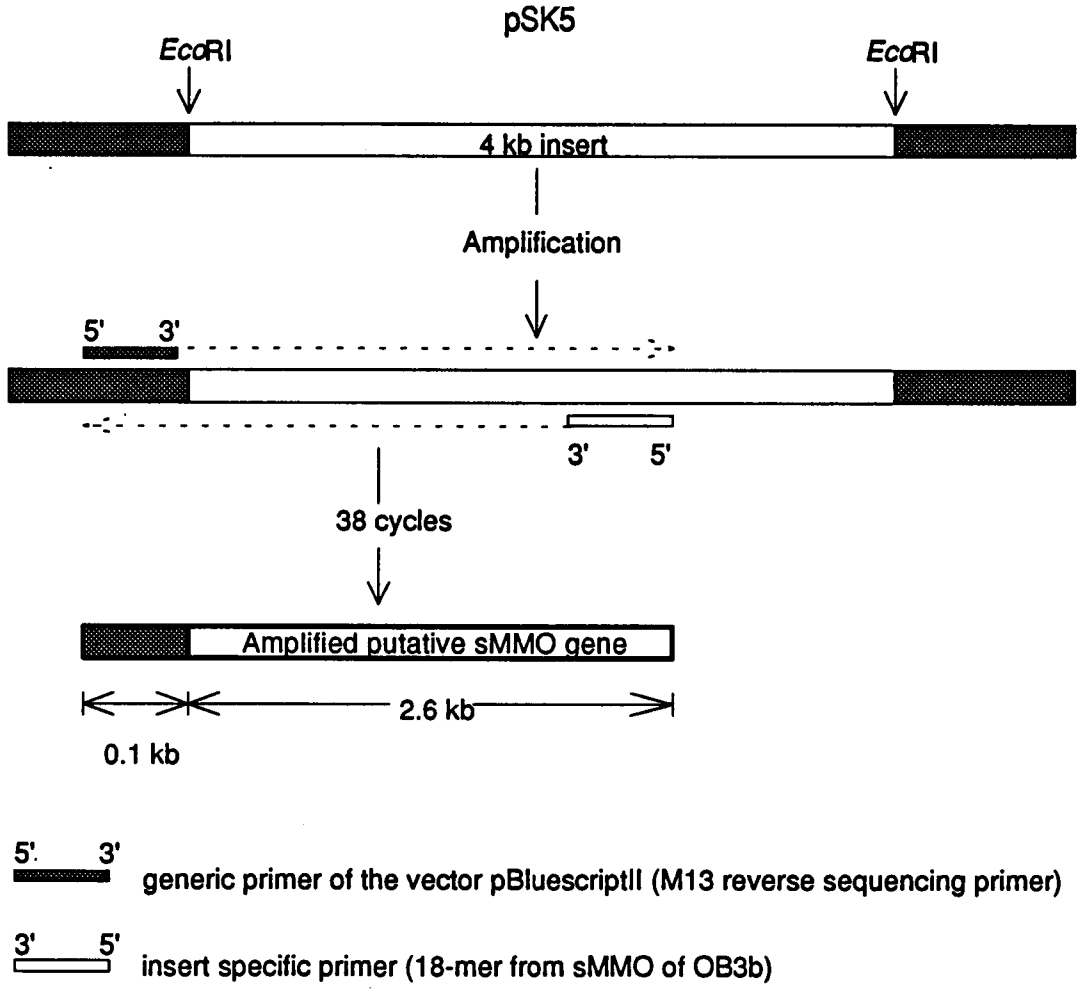


Figure IV-11. Schematic representation of amplification of the putative sMMO hydroxylase gene carrying of *Methylomonas methanica* 68-1 by a single-specific primer polymerase chain reaction (SSP-PCR) using pSK5 as a template DNA. The thermal cycling was as follows: melting at 95°C for 1 min, annealing at 42°C for 2 min, and polymeration at 72°C for 3 min.

amplified product was found to be a 2.7 kb fragment containing about 0.1 kb vector DNA by Southern blot hybridization in which the template DNA was used as probe (Figure IV-12). The vector DNA was used as a negative control. In the amplifications as shown in the figure, there was no vector amplification product detected (lane 2 of Figure IV-12).

Restriction map of pSK5

The restriction map of pSK5 is shown in Figure IV-13. In this map the location and priming orientation of primers were also shown (arrow marks). Pr1 is the M13 reverse sequencing primer located on the vector, Pr2 is the 18-mer primer designed from the sMMO γ-subunit of *M. trichosporium* OB3b, and Pr3 is an internal reverse primer available from DNA sequencing, respectively. Thus the putative sMMO gene from 68-1 was assumed to be located on the 0.9 kb *Hind* III-*Sph* I fragment. This restriction map was compared with the cloned and sequenced sMMO gene cluster of OB3b (Figure IV-14). It was assumed that the oligo probe could potentially hybridize to the N-terminal of a potential *mmoZ* the upstream of which was *mmoB.*

J. DNA sequencing of the putative sMMO gene carried on pSK5

The results of Southern blot hybridization and PCR amplification showed the necessity for DNA-sequencing of the putative sMMO gene to determine sequence homology to known sMMO genes and other genes of functional similarity. To do this, DNA sequence information from the putative gene was compared with several available databases.

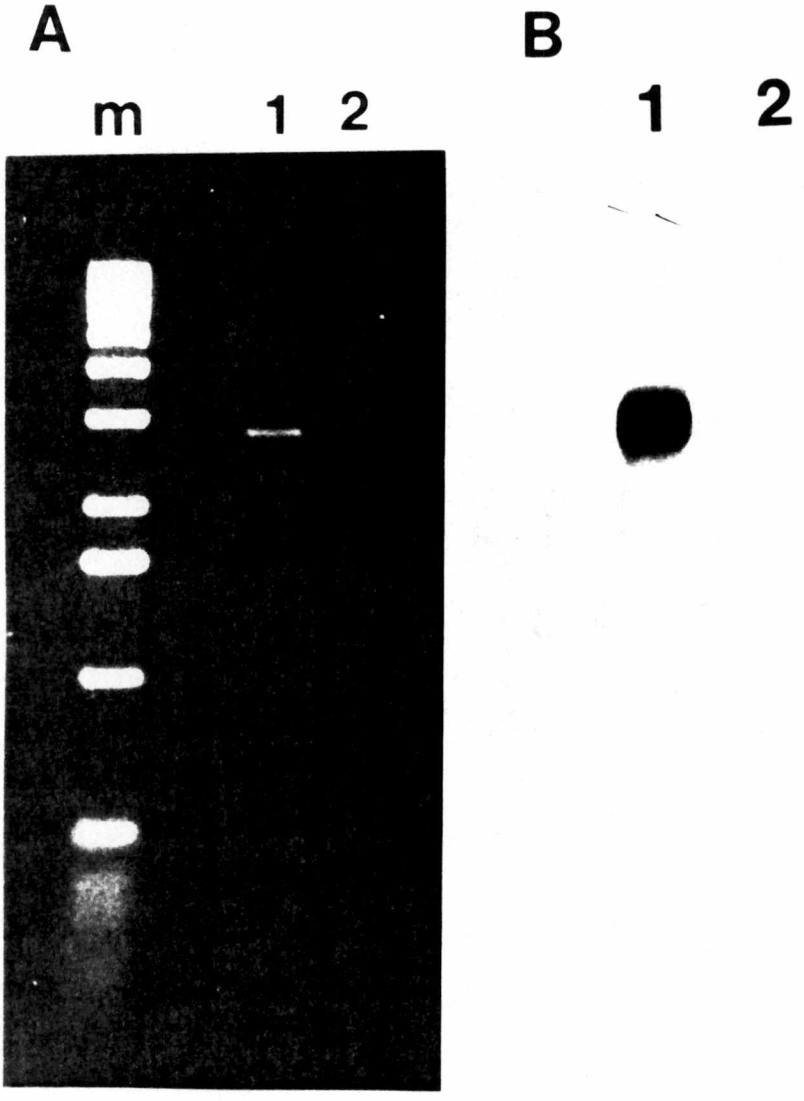


Figure IV-12. Amplification of the putative sMMO hydroxylase gene by single-specific-primer polymerase chain reaction (SSP-PCR). (A) 1% agarose gel electrophoresis in TBE buffer of amplified products. Lanes: m, 1 kb DNA ladder (BRL) ;1, amplified putative sMMO gene; 2, amplification of vector. (B) Southern blot of panel (A) using the 4.0 kb insert pSK5 as a probe.

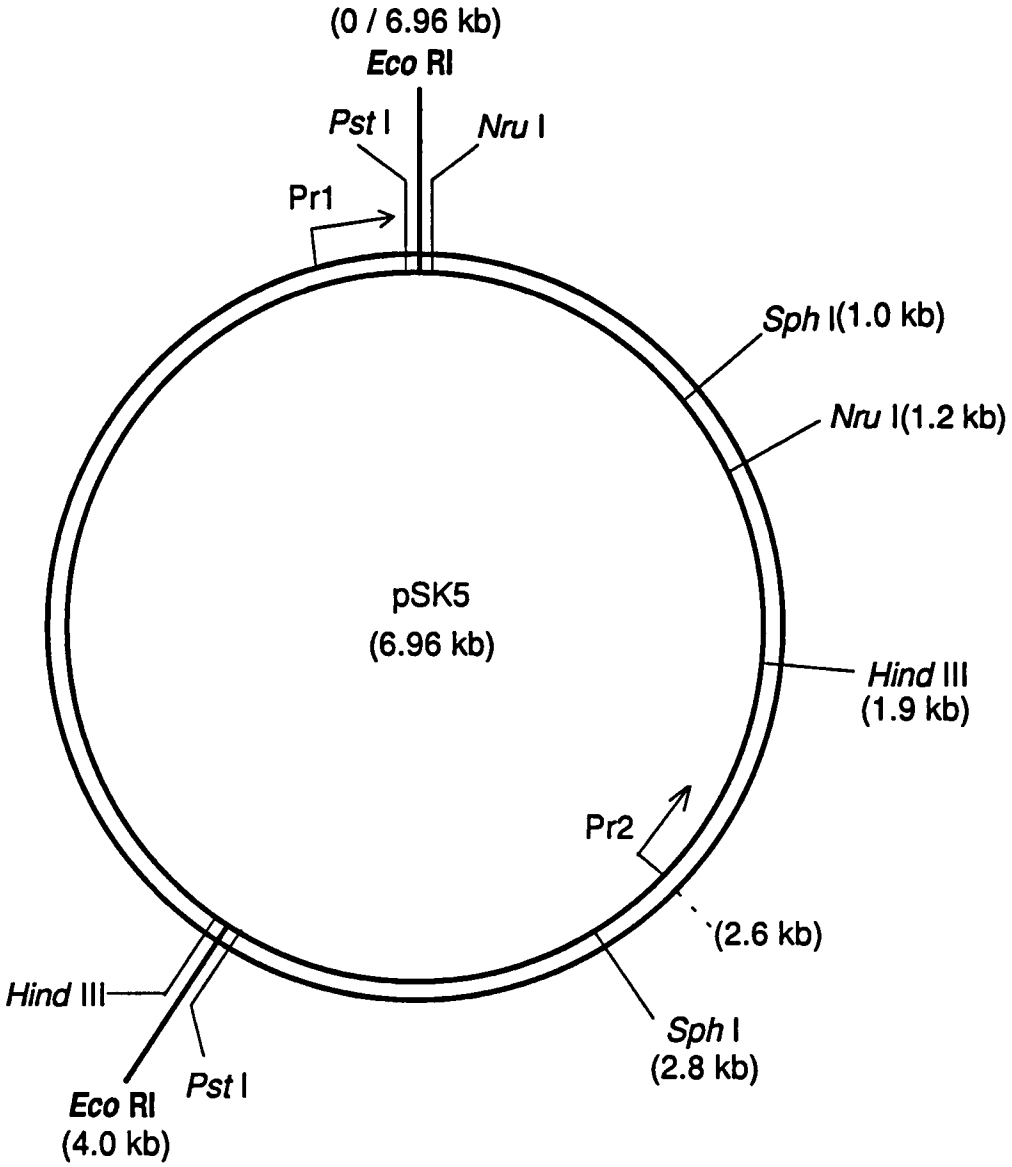
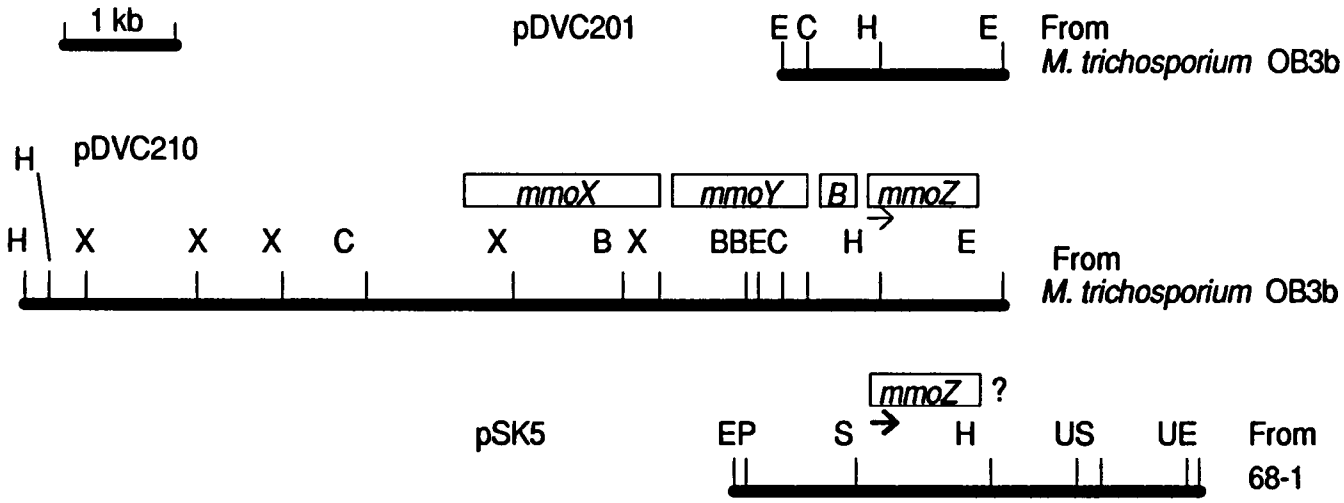


Figure IV-13. Restriction map of the recombinant plasmid pSK5. Pr1: M13 reverse sequencing primer on vector; Pr2: 18-mer oligodeoxynucleotide as a primer designed from sMMO (hydroxylase γ-subunit) of *Methylosinus trichosporium* OB3b.



→ The binding site of the 18-mer oligo probe designed from N-terminal of MMOZ of OB3b

→ A potential binding site of the 18-mer oligo probe (at the putative sMMO gene cloned)

Figure IV-14. Comparative analysis of restriction endonuclease maps of the pSK5, pDVC201, and pDVC210 inserts. Location of restriction sites are determined by vertical lines (C, *Cla* I; X, *Xho* I; *Bgl* II; E, *Eco* RI; H, *Hind* III; N, *Nru* I; S, *Sph* I; P, *Pst* I). The open boxes represent the limits of the structural genes *mmoX*, *mmoY*, *mmoB*, and *mmoZ*. pDVC201 and pDVC210 are adapted from Cardy et al. (1991).

Region of DNA sequencing

The first goal of the sequencing was to locate the binding site of the 18-mer (designed from sMMO hydroxylase γ-subunit of *M. trichosporium* OB3b) on the putative sMMO gene fragment from *Methylomonas methanica* 68-1. One of the approaches was to subclone the insert of pSK5 and then sequence the target primer region using a sequencing primer located on the vector (Figure IV-15). In this study the *Sph* I fragment (1.8 kb) of the 4.0 kb insert of pSK5 was subcloned into pUC18 and then the target region was sequenced using M13 forward sequencing primer (#1211) located on the *Hind* III site of the vector. There was about 200 bp distance between the vector primer site and the target site.

Sequence analysis

From the DNA sequencing strategy, the sense strand of the target fragment was sequenced several times using the double-stranded DNA template. The anti-sense strand was not sequenced in this study. The nucleotide sequence (241 bases) and their deduced amino acid sequences are shown in Figure IV-16. The nucleotide sequence can be aligned with the downstream region of the *mmoB* gene of *M. trichosporium* OB3b with little similarity. The nucleotide sequence was analyzed by GCG Sequence Analysis Software Package (PFASTA) running on VAX in University of Tennessee Computing Center. The nucleotide and amino acid sequences were also compared with the sequence data bases from GenBank and EMBL Data Library available at National Center for Biotechnology Information (National Institute of Health, Bethesda, MD), where the sMMO gene sequences of *M. capsulatus* Bath and *M. trichosporium*

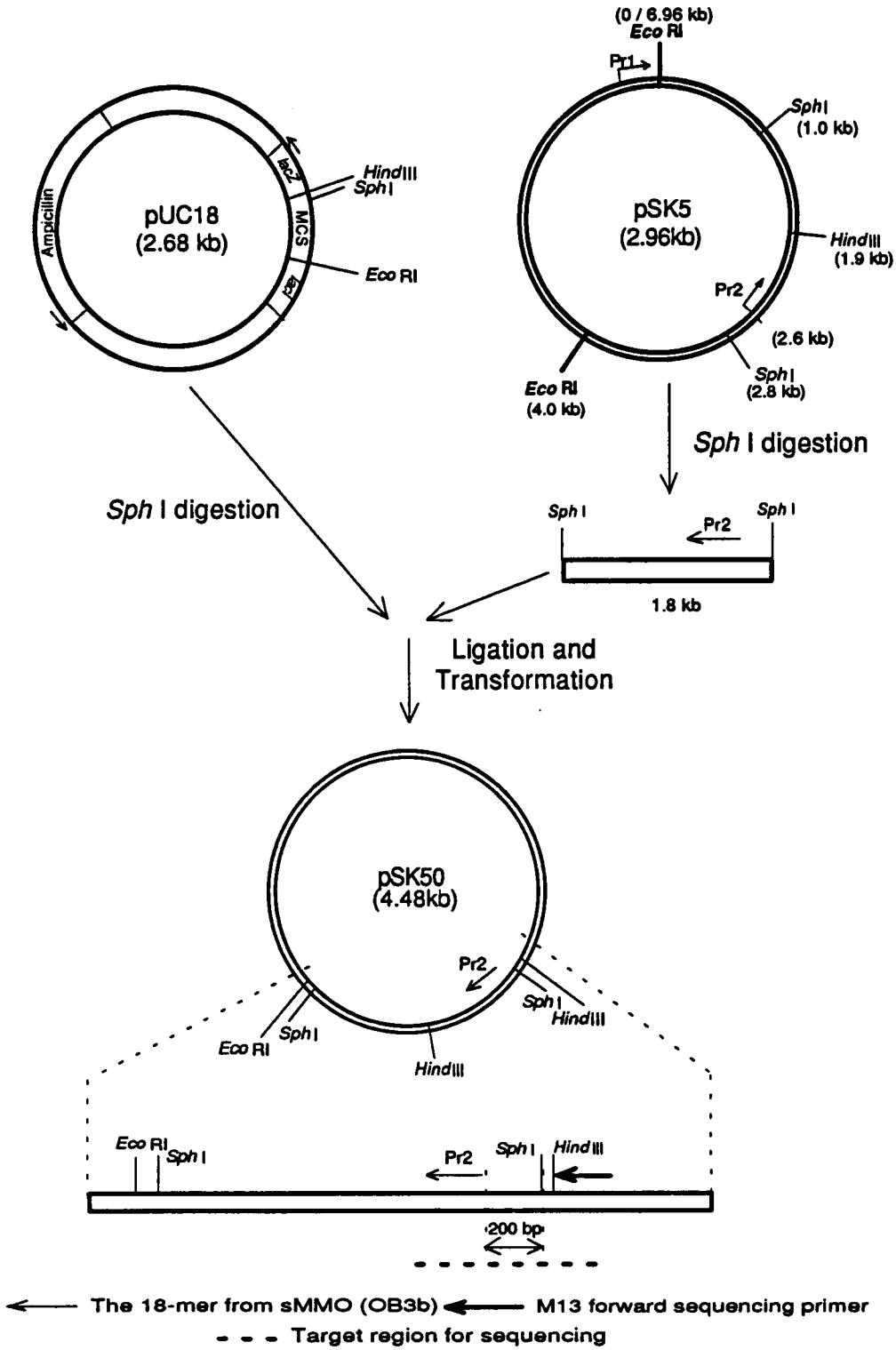


Figure IV-15. Subcloning of pSK5 and DNA sequencing strategy of the putative sMMO gene from *Methylomonas methanica* 68-1. The figure is not drawn in scale.

```
                                                                    60
ATGCGGCCTTCCTGTCATCGACGTTTTCGGTGTAAAAAATTAACTCGCCGTCGCTGTACT
 M  R  P  S  C  H  R  R  F  R  C  K  K  L  T  R  R  R  C  T
  C  G  L  P  V  I  D  V  F  G  V  K  N  *  L  A  V  A  V  L
   A  A  F  L  S  S  T  F  S  V  *  K  I  N  S  P  S  L  Y  S

                                                                   120
CGCTGAAGCGGCCGGCATGCGAATGTATCGGCATTTTGTTTATCCTGATGCATCAACTGC
 R  *  S  G  R  H  A  N  V  S  A  F  C  L  S  *  C  I  N  C
  A  E  A  A  G  M  R  M  Y  R  H  F  V  Y  P  D  A  S  T  A
   L  K  R  P  A  C  E  C  I  G  I  L  F  I  L  M  H  N  L  L

                                                                   180
TCGGTTTTGCTCCGCCCAGGGGCAACGAGCATCGATAGCGCCATGCCCGCCAGACTTAAC
 S  V  L  L  R  P  G  A  T  S  I  D  S  A  M  P  A  R  L  N
  R  F  C  S  A  Q  G  Q  R  A  S  I  A  P  C  P  P  D  L  T
   G  F  A  P  P  R  G  N  E  H  R  *  R  H  A  R  N  T  *  R

                                                                   240
GGTATTACCAGCATGAATATCGCCCGATAAATGGTAAACGGACGAATCGCCGCCATCTCT
 G  I  T  S  M  N  I  A  R  *  M  V  N  G  R  I  A  A  I  S
  V  L  P  A  *  I  S  P  D  K  W  *  T  D  E  S  P  P  S  L
   Y  Y  N  H  E  Y  R  P  I  N  G  K  R  T  N  R  R  H  L  C


G
```

Figure IV-16. Partial nucleotide and the deduced amino acid sequences of the 1.8 kb *Sph* I-*Sph* I fragment. The nucleotide sequence carries the upstream region of the putative sMMO hydroxylase (γ–subunit) gene of *Methylomonas methanica* 68-1.

OB3b were deposited . The nucleotide and amino acid sequence homology searches using these databases failed to detect any significant homology.

K. Microbial community analysis of environmental samples using gene probes

Although the putative sMMO gene fragment from strain 68-1 had not been completely characterized, it was selected as a cryptic DNA probe to detect a specific group of methanotrophs. In this section the potential applicability of the putative sMMO gene probe to specifically detect methanotroph(s) from a bioreactor mixed culture sample or TCE-contaminated environmental samples was evaluated along with other DNA probes such as the sMMO component B gene, methanol dehydrogenase structural gene, and toluene dioxygenase structural gene probes. To do this the specificity and sensitivity of the putative sMMO gene probe was first examined.

Probe specificity:

The putative sMMO DNA probe (4 kb) was cut from pSK5, labeled by [α-^{32}P]dCTP through nick-translation, and hybridized with dot-blotted chromosomal DNA (2 μg) of various methanotrophs and methylotrophs under high stringency conditions. The result is shown in Figure IV-17 and Table IV-7 . The probe hybridized with genomic DNAs of 68-1, *Methylomonas methanica* ATCC 35067, *Methylomonas rubra,* and *Methylococcus capsulatus* Bath ATCC 33009 , but not with type II methanotrophs or methylotrophs. This probe also did not hybridize with any of the heterotrophic bacteria tested in this study.

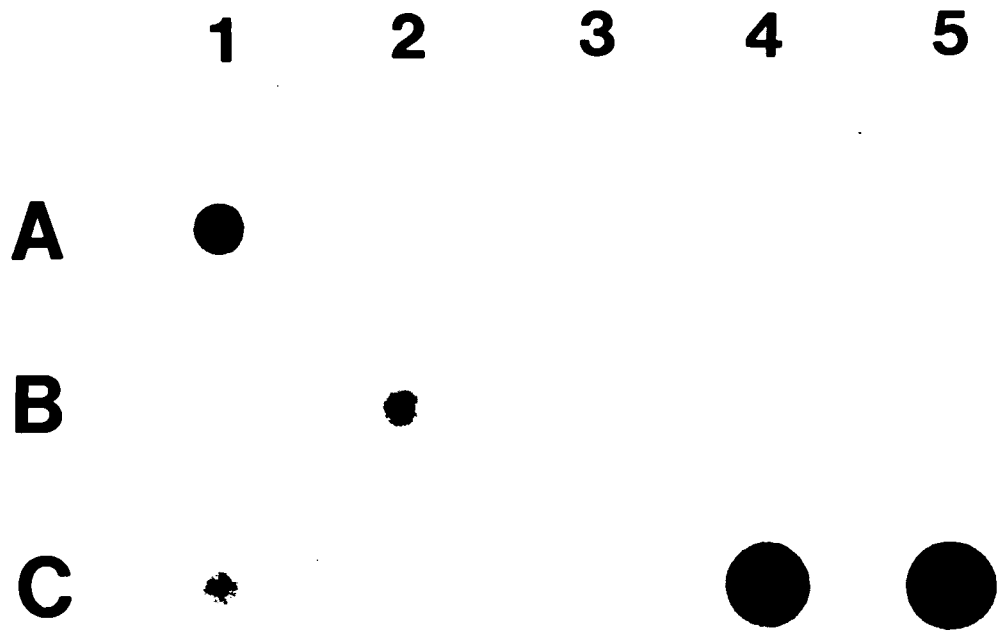


Figure IV-17. DNA dot blot hybridization of genomic DNA's from various methanotrophs and methylotrophs to examine specificity of the putative sMMO DNA probe from *Methylomonas methanica* 68-1. A1 *Methylomonas methanica* 68-1; A2 strain 46-1; A3 IAM012-A; A4 IAM012-B; A5 IAM026; B1 IAM030; B2 *Methylomonas methanica* ATCC 35067^{T}; B3 *Methylosinus trichosporium* OB3b; B4 *Methylobacterium organophilum* XX; B5 *Methylobacterium extorquens* AM1; C1 *Methylococcus capsulatus* Bath; C2 *Methylococcus capsulatus* ATCC 19069; C3 *Pseudomonas fluorescens* LOS1; C4 pSK5; C5 pUS325 carrying a methanol dehydrogenase (MDH) gene (*moxF*).

Table IV-7. Hybridization specificity of the clone pSK5 as a DNA probe to methanotrophs, methylotrophs, and heterotrophs.[A]

Type and species of bacterial strain	Source or reference	Hybridization specificity
Type I methanotrophs:		
Methylomonas agile	ATCC 35008	–
Methylomonas alba	ATCC 33003 (=BG8)	–
Methylomonas methanica	ATCC 35067^T	+
"Methylomonas rubra"	NCIMB 11913	+
Strain 68-1	Little et al., 1988	+
Strain 46-1	Little et al., 1988	–
Type X methanotrophs:		
Methylococcus capsulatus	ATCC 19069^T (=Texas)	–
Methylococcus capsulatus	ATCC 33009 (=Bath)	+
Type II methanotrophs:		
Methylocystis parvus	ATCC 35066 (= OBBP)	–
Methylosinus sporium	ATCC 35069 (=5)	–
Methylosinus trichosporium	ATCC 35070	–
RuMP pathway methylotrophs:		
"Methylomonas clara"	ATCC 31226	–
Methylophilus methylotrophus	ATCC 53528^T	–
Serine pathway methylotrophs:		
Hyphomicrobium sp.	ATCC 43129	–
Methylobacterium extorquens	ATCC 14718^T (=AM1)	–

Table IV-7 cont'd.

Type and species of baterial strain	Source or reference	Hybridization specificity
Methylobacterium organophilum	ATCC 27886^T (=XX)	–
Heterotrophs:		
Agrobacterium tumefaciens	ATCC 23308	–
Bacillus subtilis	ATCC 6051^T	–
Escherichia coli	HB101	–
Escherichia coli XL1-Blue	Stratagene Cloning System, Inc	–
Pseudomonas fluorescens	LOS1 (C. E. B. ; UTK)	–
Pseudomonas putida	strain F1 (Wackett and Gibson, 1988)	–
Rhizobium leguminosarum	strain 248 (Hirsch, 1979)	–

[A] High stringency hybridization allowing 30 % mismatch.

Sensitivity of the putative sMMO DNA probe for the detection of *Methylomonas methanica* 68-1 in pure or mixed culture

Various amounts of 68-1 DNA, DNA of an enriched culture of a methanotrophic consortium potentially containing 68-1 (Strandberg et al., 1989), DNA of *Pseudomonas fluorescens* LOS1 were immobilized on a nylon membrane and hybridized with the nick-translated pSK5 under high stringent conditions (Figure IV-18). The 4.0 kb probe could detect as little as 2 ng of the pure genomic 68-1 DNA, and up to 3 ng of the putative 68-1 DNA contained in 250 ng total DNA of the enriched methanotrophic consortium under the hybridization and detection conditions. The 3 ng DNA was equivalent to 9×10^5 cells of 68-1 given that each cell had a single genome carrying one copy of the putative sMMO gene and the DNA extraction efficiency was 100 %. As expected, no hybridization was observed with DNA of *P. fluorescens* LOS1 which was used as a negative control.

Analysis of Savannah River Site samples by DNA slot blotting

The bacterial densities overall ranged from 10^2 -10^8 cells/g fresh soil in the Savannah River Site with MHT1C site having the lowest bacterial densities. The densities did not significantly decrease or increase with depth.

The distribution of methanotroph-specific, methanol dehydrogenase (MDH), and toluene dioxygenase (TOD) genes in the contaminated sites were also examined. The results were shown in Tables IV-8, 9, and 10, and Figures 19, 20, 21, 22, and 23. The MHT9C site was known as the highest TCE-contaminated site and showed the lowest frequencies of the putative type I methanotroph-specific, MDH, and TOD genes and the less frequencies of the

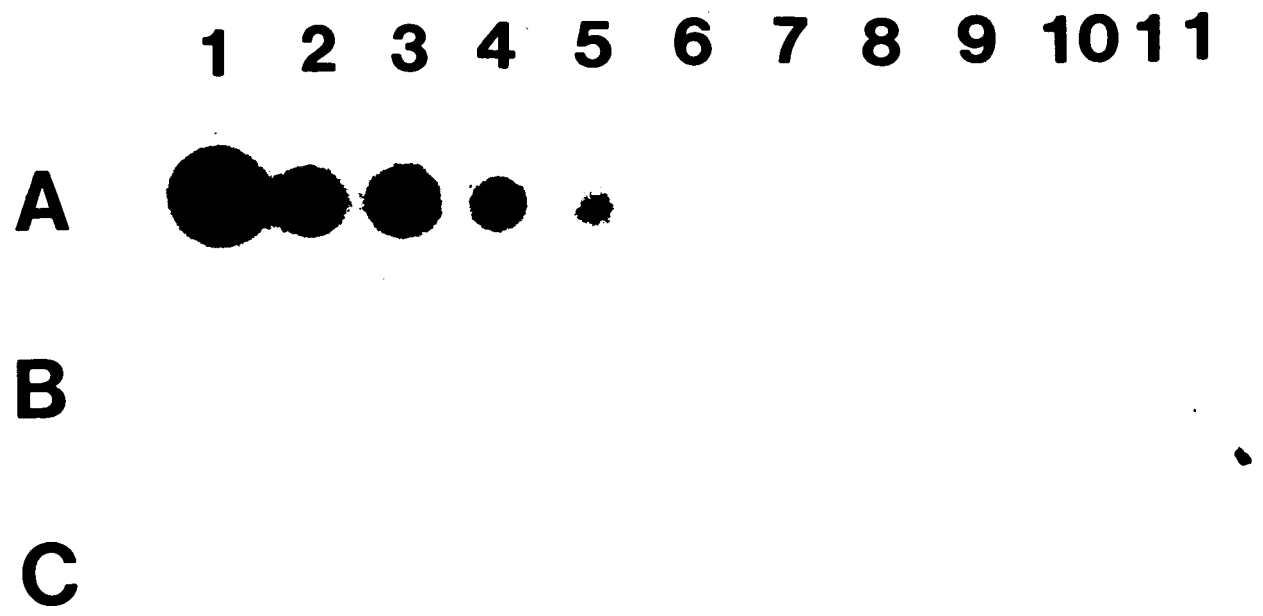


Figure IV-18. Sensitivity of specific detection of a type I methanotroph (potentially strain 68-1) in an enriched methanotrophic consortium (Strandberg et al., 1991) using the putative sMMO gene probe from *Methylomonas methanica* 68-1. Each row shows genomic DNA sample: A, *Methylomonas methanica* 68-1 (positive control); B, an enriched methanotrophic consortium; C, *Pseudomonas fluorescens* LOS1 (negative control). Various quantities of each DNA sample (ng) were hybridized with the nick-translated pSK5 plasmid. The DNA quantities in columns were: **1**, 2500; **2**, 1000; **3**, 500; **4**, 250; **5**, 125; **6**, 63; **7**, 32; **8**, 16; **9**, 8; **10**, 4; **11**, 2.

Table IV-8. Distribution of methanotrophic and methylotrophic oxidative genes, and toluene degradative gene in MHT 1C sites at Savannah River Plant, U. S. Department of Energy by direct DNA extration and slot blot hybridiztion.

Site	pSK5 (type I, X specific)[A]	sMMO B gene (type II specific)[B]	MDH gene (*moxF*)[C]	TOD gene[D]
MHT 1C 5	+	–	–	+
MHT 1C 11	+	+	+	+
MHT 1C 14	+	–	+	+
MHT 1C 15	+	–	–	–
MHT 1C 102	–	–	–	–
MHT 1C 104	–	–	+	–
MHT 1C 107	+	–	–	+

[A] 4 kb DNA fragment coding a putative sMMO (soluble methane monoxygenase) gene of *Methylomonas methanica* 68-1 (type I and type X methanotroph specific). [B] 2.1 kb DNA fragment coding sMMO gene (*mmoB* and *mmoZ*) of *Methylosinus trichosporium* OB3b (Tsien et al., 1992). [C] 2.5 kb DNA fragment coding methanol dehydogenase (MDH) gene (*moxF*) of *Methylobacterium organophilum*. XX (Machlin et al., 1988). [D] 2.5 kb DNA fragment coding toluene dioxygenase (TOD) gene (*todC$_2$C$_1$BA*) of *Pseudomonas putida* F1 (Zylstra and Gibson, 1989).

Table IV-9. Distribution of methanotrophic and methylotrophic oxidative genes, and toluene degradative gene in MHT 6C sites at Savannah River Plant, U. S. Department of Energy by direct DNA extration and slot blot hybridiztion.

Site	pSK5 (type I, X specific)	sMMO gene (type II specific)	MDH gene (*moxF*)	TOD gene
MHT 6C 336	–	–	–	–
MHT 6C 338	–	–	–	–
MHT 6C 340	–	–	–	–
MHT 6C 350	+	–	–	–
MHT 6C 352	–	–	–	–
MHT 6C 354	+	+	+	+
MHT 6C 366	–	–	–	–
MHT 6C 368	+	–	–	–
MHT 6C 370	–	–	–	–
MHT 6C 372	–	–	–	+
MHT 6C 374	–	–	–	+
MHT 6C 378	–	+	+	+
MHT 6C 380	+	+	+	+
MHT 6C 382	–	–	–	–
MHT 6C 384	+	+	+	+
MHT 6C 386	+	+	+	+
MHT 6C 390	+	+	–	+
MHT 6C 392	+	–	+	+

Table IV-10. Distribution of methanotrophic and methylotrophic oxidative genes, and toluene degradative gene in MHT 9C sites at Savannah River Savannah River Plant, U. S. Department of Energy by direct DNA extration and slot blot hybridiztion.

Site	pSK5 (type I , X specific)	sMMO gene (type II specific)	MDH gene (*moxF*)	TOD gene
MHT 9C 176	–	–	–	–
MHT 9C 178	–	–	–	–
MHT 9C 184	–	–	+	–
MHT 9C 186	+	+	+	+
MHT 9C 188	–	–	–	–
MHT 9C 190	–	–	–	–
MHT 9C 192	–	–	–	–
MHT 9C 194	–	–	–	–
MHT 9C 196	–	–	–	+
MHT 9C 276	–	–	–	–

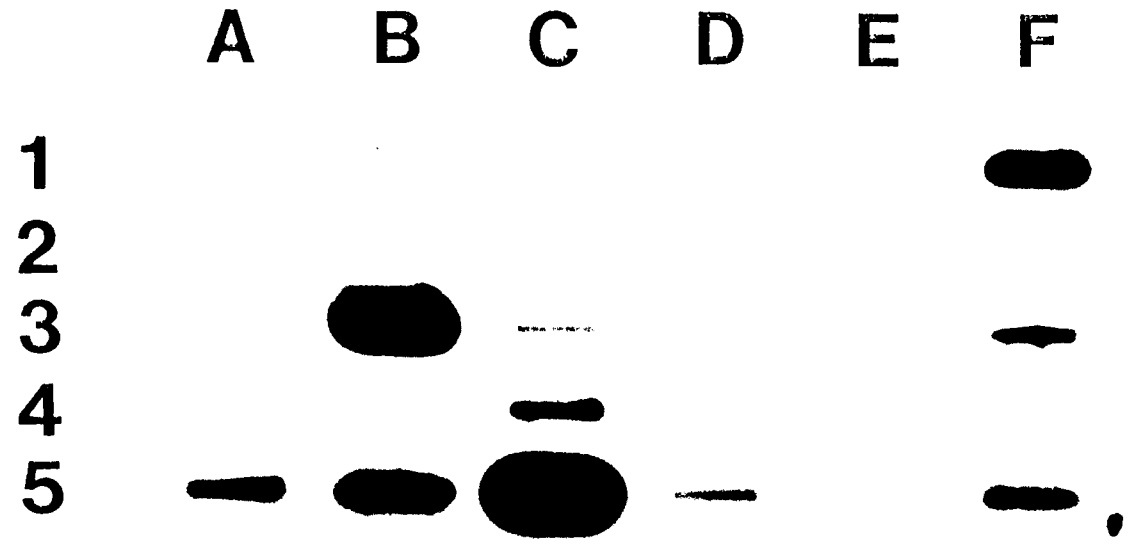


Figure IV-19. Detection of potential type I (or type X) methanotrophic bacterial populations in the various TCE-contaminated sites of Savannah River Plant, U. S. Department of Energy (Aiken, South Carolina) using the putative sMMO gene cloned from *Methylomonas methanica* 68-1. Each total DNA sample (100 ng) directly extracted from soils or sediments in the sites was subjected to a high stringent DNA:DNA hybridization using 2.6 kb DNA fragment amplified form pSK5 as a DNA probe. DNA's (750 ng) of other positive and negative control and vector were also loaded for reference. **1A - 1F**: MHT1C 15,102, MHT9C 176, 180, 184, and 186; **2A - 2F**: MHT9C 190, 192, 194, 276, MHT6C 336, and 340; **3A - 3F**: MHT6C 350, 354, 368, 372, 374, and 378; **4A -4F**: MHT6C 380, 382, 386, 390, 392, and MHT 178; **5A - 5F**: *nahA*, DNA of 68-1, putative sMMO gene (4 kb), sMMO gene, DNA of LOS1, and pBluescript II.

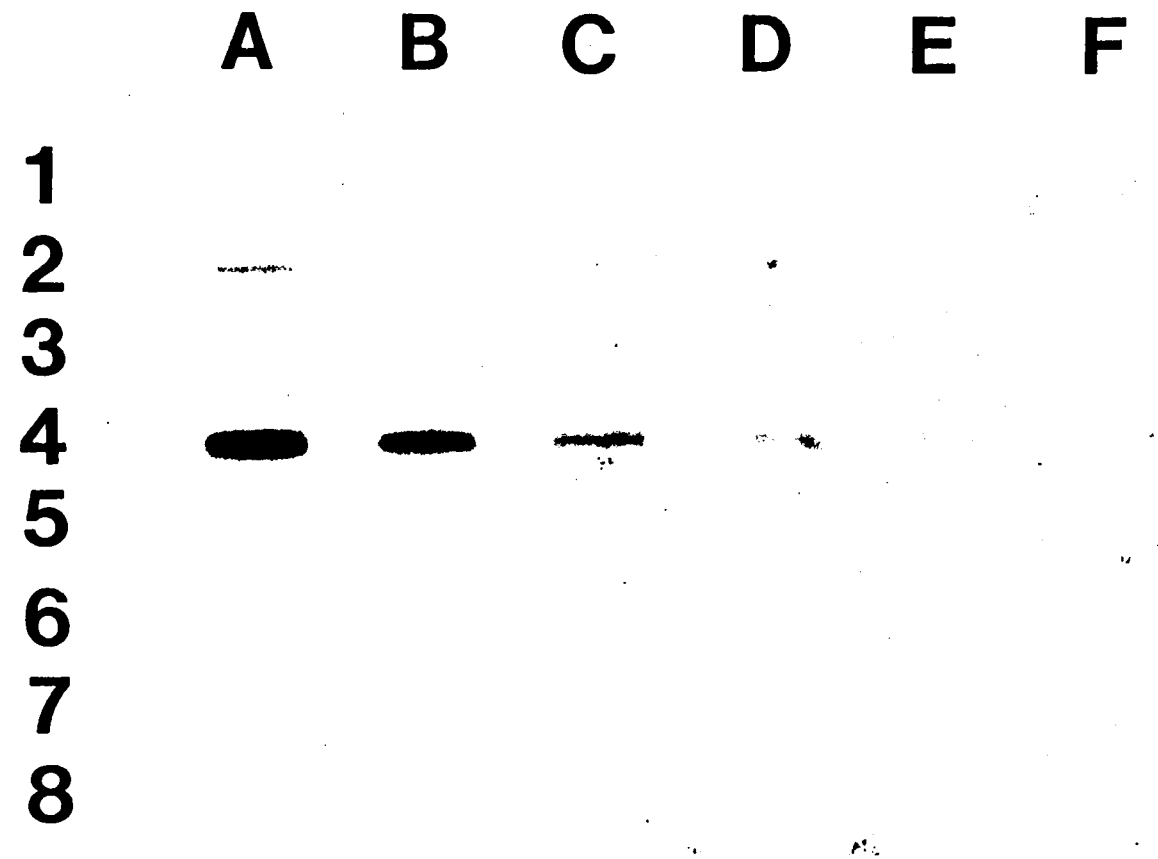


Figure IV-20. Detection sensitivity of type II methanotroph-specific soluble methane monooxygenase (sMMO) genes in the various TCE-contaminated sites of Savannah River Plant, U. S. Department of Energy (Aiken, South Carolina) using the sMMO gene (*mmoB and mmoZ*) probe from *Methylosinus trichosporium* OB3b. Each total DNA sample directly extracted from soils or sediments in the sites was subjected to a high stringent DNA:DNA hybridization. DNA quantity (ng) in each column A through F: 200, 100, 50, 25, 12.5, and 6.3; DNA sample in each row 1 through 8: MHT1C 11, MHT9C 186, MHT6C 350, 354, 376, 380, 384, and 392.

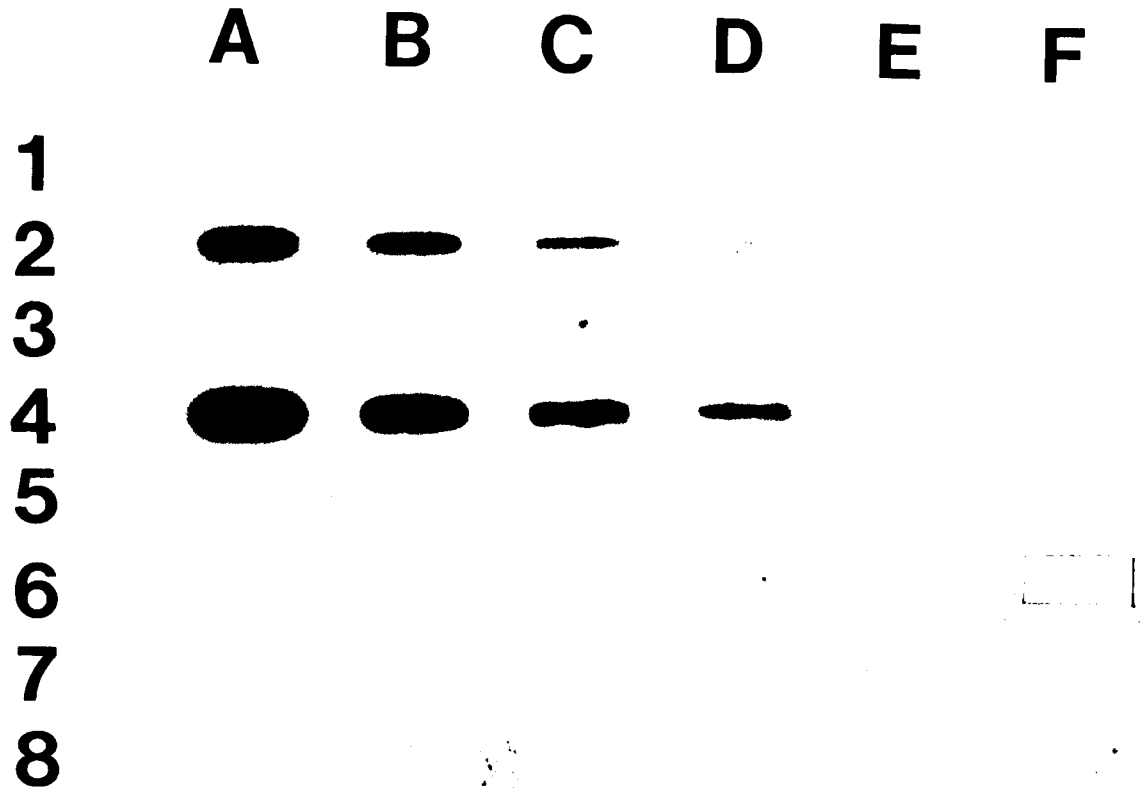


Figure IV-21. Detection sensitivity of methanol oxidation genes in the various TCE-contaminated sites of Savannah River Plant, U. S. Department of Energy (Aiken, South Carolina) using the methanol dehydrogenase (MDH) gene from *Methylobacterium organophilum* XX. Each total DNA sample directly extracted from soils or sediments in the sites was subjected to a high stringent DNA:DNA hybridization. DNA quantity (ng) in each column A through F: 200, 100, 50, 25, 12.5, and 6.3; DNA sample in each row 1 through 8: MHT1C 11, MHT9C 186, MHT6C 350, 354, 376, 380, 384, and 392.

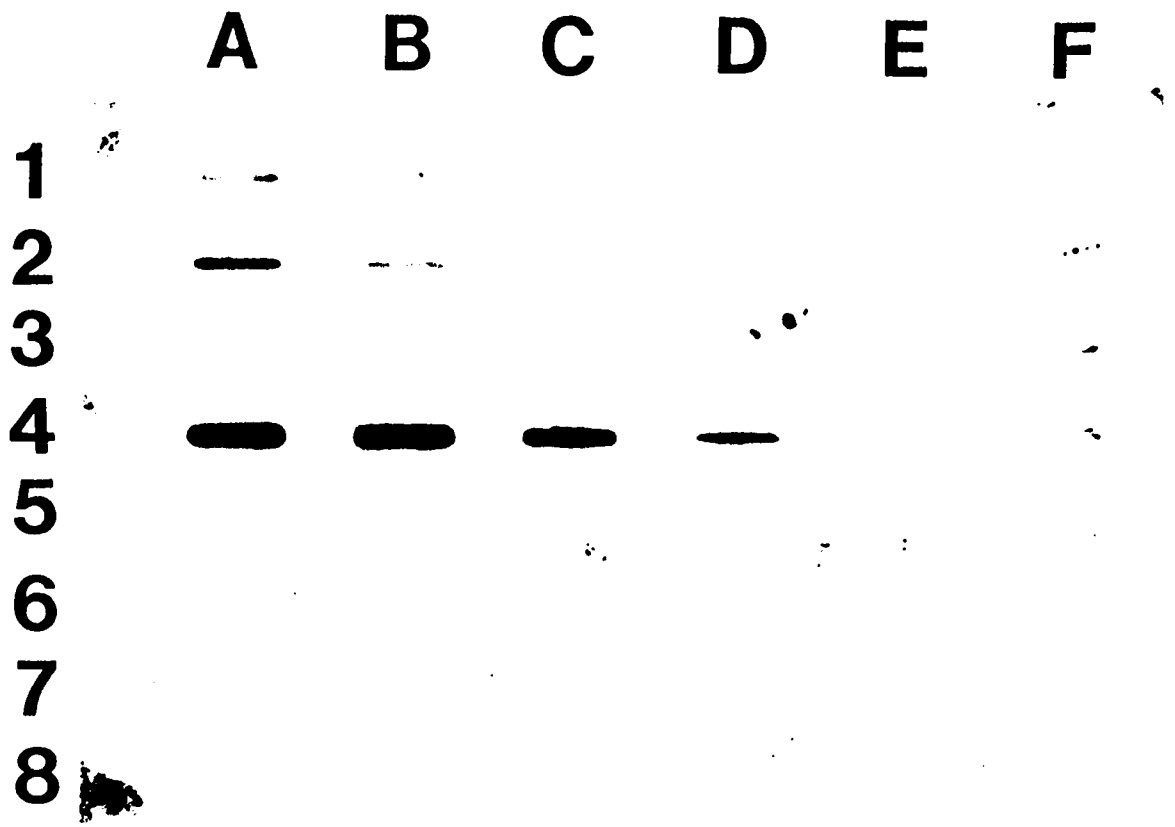


Figure IV-22. Detection sensitivity of toluene dioxygenase (TOD) genes in the various TCE-contaminated sites of Savannah River Plant, U. S. Department of Energy (Aiken, South Carolina) using the TOD gene from *Pseudomonas putida* F1. Each total DNA sample directly extracted from soils or sediments in the sites was subjected to a high stringent DNA:DNA hybridization. DNA quantity (ng) in each column A through F: 200, 100, 50, 25, 12.5, and 6.3; DNA sample in each row 1 through 8: MHT1C 11, MHT9C 186, MHT6C 350, 354, 376, 380, 384, and 392.

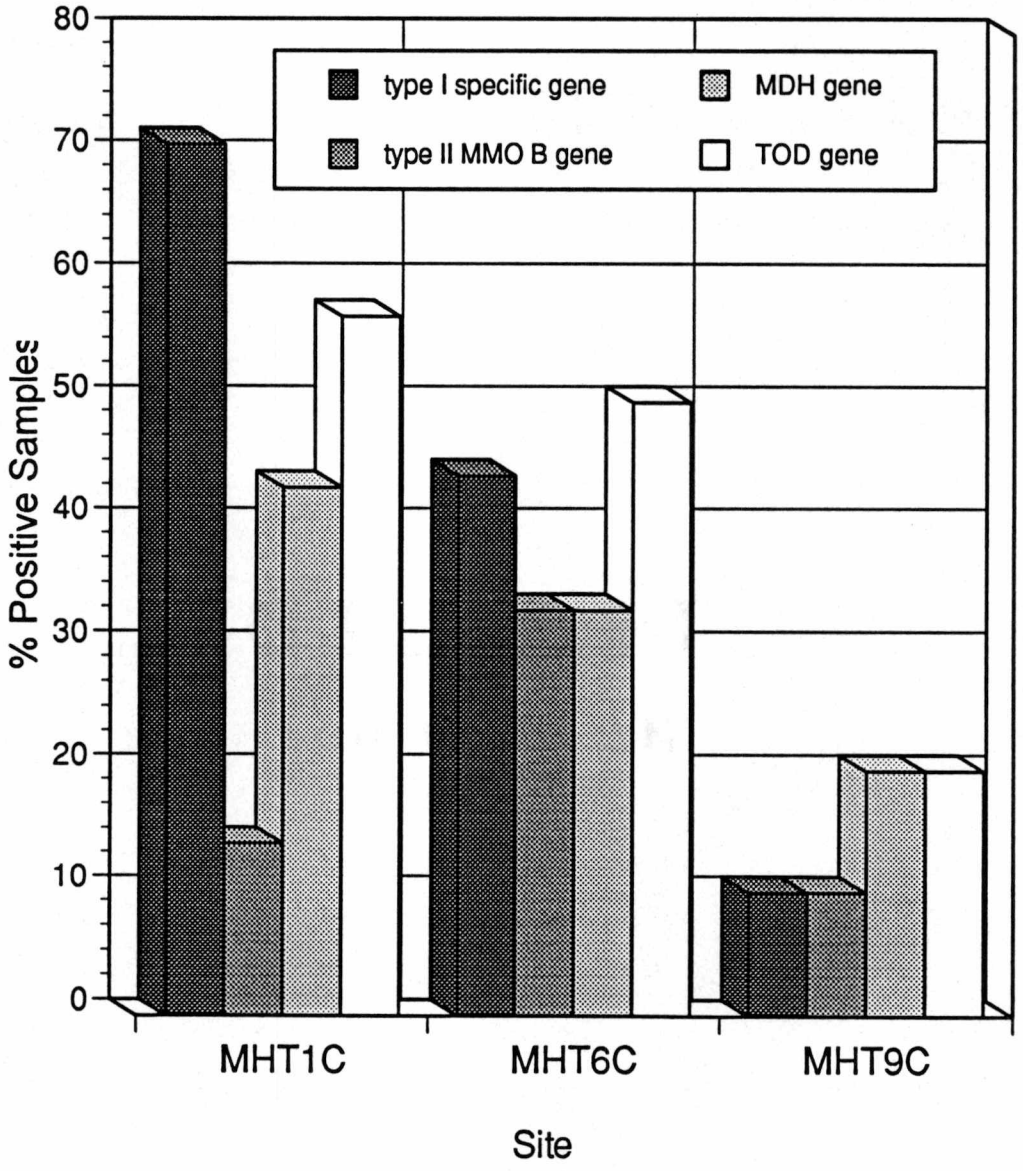


Figure IV-23. Distribution of methanotrophic, methylotrophic, and heterotrophic oxidative (or degradative) genes before methane injection in the TCE-contaminated sites at Savannah River Plant, Aiken, South Carolina. Type I methanotroph (pink-pigmented) specific gene, type II sMMO B gene, MDH gene, and TOD gene were derived from *Methylomonas methanica* 68-1, *Methylosinus trichosporium* OB3b, *Methylobacterium organophilum* XX, and *Pseudomonas putida* F1, respectively.

genes were observed in MHT6C and MHT9C in order. The type I (or type X) methanotroph-specific gene was 5 times as abundant as type II in the MHT1C site. Frequency of type II specific sMMO gene in MHT6C was 2.5-3 times as high as in MHT1C or MHT9C. Overall most of the methanotrophic DNA probe-specific samples showed MDH and TOD positive-reactions. The results of detection sensitivity of the sMMO, MDH, and TOD gene probes were shown in the Figures IV-19. 20, and 21. All the DNA probes allowed the detection of each relative target gene (at least 6.3 pg) carried on total DNA (200 ng) blotted. This amount of gene detected was equivalent to 2-3$\times 10^6$ cell number of each target cells given that each target gene (2.1 -2.5 kb in size) is a single copy and the DNA lysis and extraction efficiency is 100 % respectively.

L. Species competition of methanotrophic populations in flask culture and TCE degradation activity during the competition

To obtain preliminary monitoring data for bioreactor experiment, species competition in flask culture was performed. Then species competition was monitored by AODC, viable count, and DNA extraction and probing (using DNA probes and 16S rDNA-targeting oligodeoxynucleotide probes). The monitoring data were comparatively analyzed.

Monitoring of species competition in a mixed culture composed of 68-1 and OB3b and TCE degradation during the competition

Species competition of 68-1 and OB3b in flask competition studies were investigated by monitoring both species through AODC, viable count, and total DNA extraction and specific DNA probing (using pSK5 and sMMO B gene

probes; 16S rDNA-targeting oligodeoxynucleotide probes). The population density or the population ratio as to total population from each method were comparatively analyzed. TCE degradation rates during the competition were also monitored.

In Figure IV-24 the populations of 68-1 and OB3b (2.5×10^6 cells/ml each) were inoculated in 2 liter flasks containing 500 ml NMS medium with no added copper. Methane was replenished up to 30 % of the head space daily. Each specific population density was monitored by AODC and viable count. During the overall growth period the population of OB3b was about 1.6× as dominant as 68-1 based on AODC monitoring. In viable counts (VC; plate count) OB3b was 10-80 times as dominant as 68-1. This meant that there was a difference between AODC and VC by one order of magnitude. This discrepancy could be directly attributed to the fact that viable OB3b cells were more common than viable 68-1 cells. This assumption, however, may be only one part of the answer because there is a significant difference in viable counts between the two species even at the starting time point (0 h). Most of this discrepancy appeared to occur due to underestimation of 68-1 population during plate count analysis. It was possible that 68-1 population (particularly cells of early growth stage) could be out-competed somewhat by OB3b population because of slower growth of 68-1 cells on the plate culture than OB3b. This underestimation of 68-1 population was also proved by the fact that an equal inoculum of each culture was used for creating mixed culture population. Here the inoculum was counted by AODC but the count could be considered a viable count because the cells came from a late log phase culture. This assumption is supported by the fact that

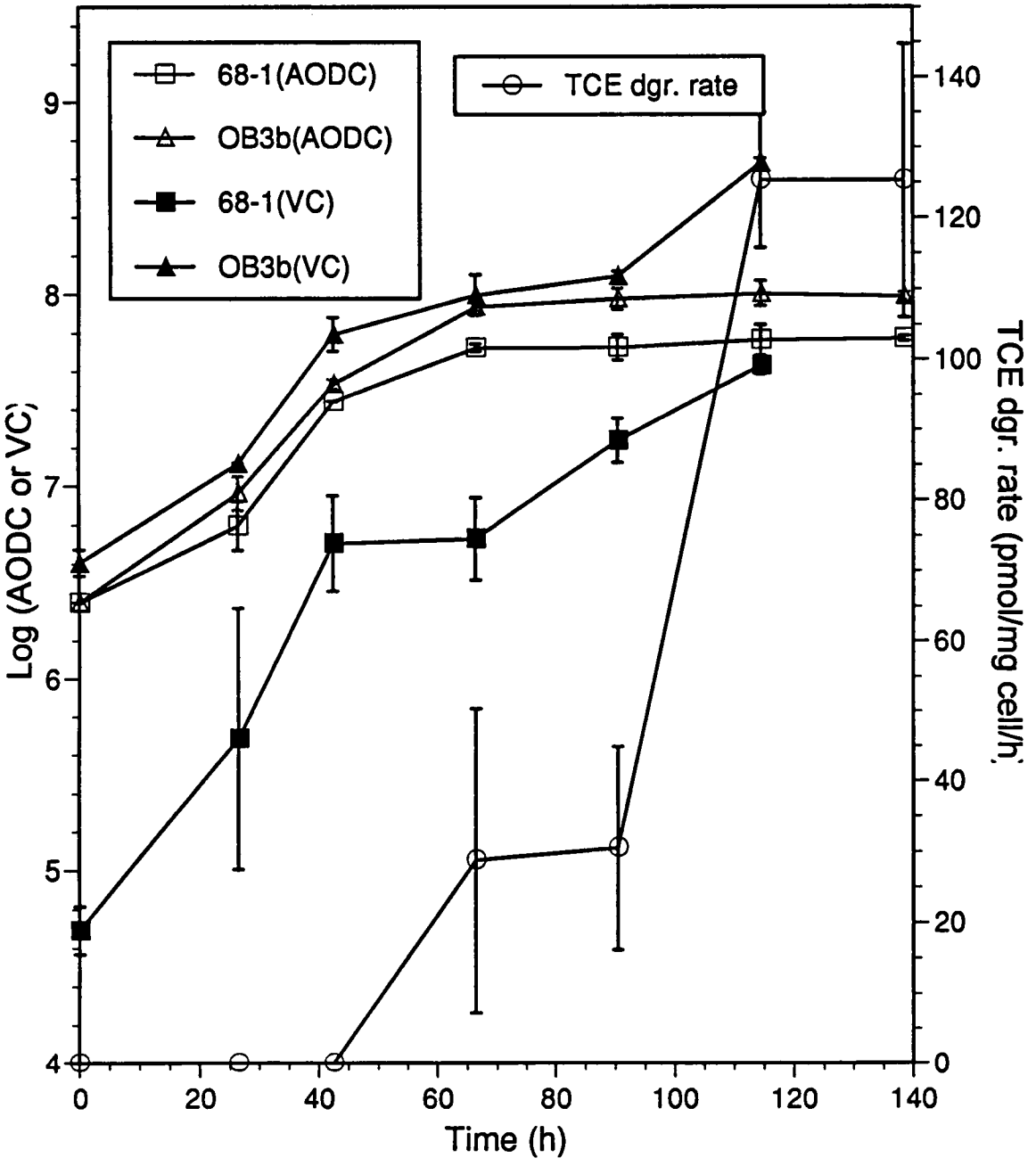


Figure IV-24. The species competition and TCE degradation in a mixed flask culture composed of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b. The populations were monitored by AODC and viable counts (VC= plate count). The medium contained no added copper. Methane was added up to 30 % of headspace daily.

cell density of OB3b population by AODC was similar to that determined by viable count.

One ml of the mixed culture was taken at each time point and analyzed for TCE degradation. Degradation was not observed until the late log phase and peaked at the late stationary phase.

The population monitoring data from AODC and viable count were also compared with population data obtained by using DNA probes (pSK5 and sMMO B component gene probe) and 16S rDNA-targeting oligodeoxynucleotide probes (1035-RuMP and 1034-Ser). The image of slot blot signals resulting from hybridization of the labeled DNA probes to the target genomic DNA's of 68-1 and OB3b is shown in Figure IV-25. The quantity of specific genes [the putative sMMO gene of 68-1 (panel A) and sMMO B component gene of OB3b (panel B)] was determined from quantification of these images and then population density of each species was then estimated using the conversion factor shown in Section N of Chapter III. The quantity of each specific gene per unit volume increased with time. This was a reflection of the increase of population density for each strain.

The species competition of 68-1 and OB3b was also monitored by quantifying the specific 16S rDNA gene of each species using 16S rDNA oligodeoxynucleotide probes (1035-RuMP for 68-1, 1034-Ser for OB3b, and eubacterial probe for total population) in terms of the image digitization value (OD value). The ratio of each population OD value as to total population OD value was used to monitor a specific population in the mixed culture. The total OD value could either be the OD value of eubacterial probe hybridization or the summed OD value of the OD values of the 68-1 and OB3b populations. It was

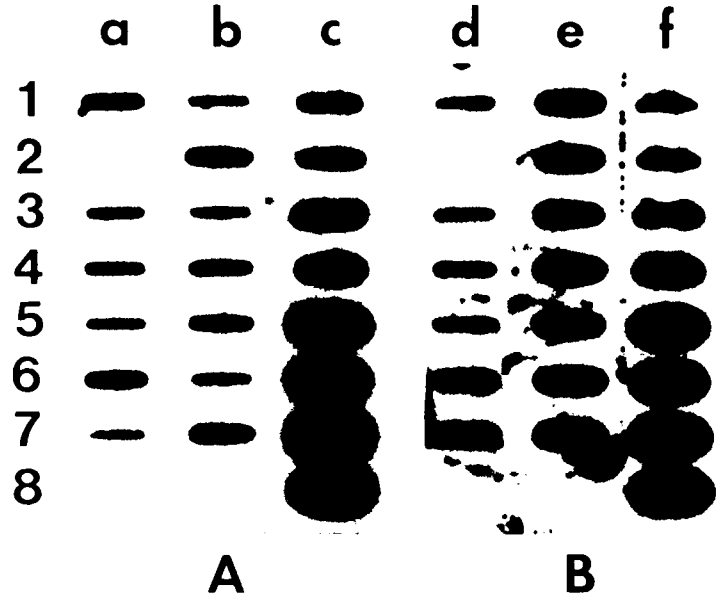


Figure IV-25. Monitoring of two species competition in a mixed culture composed of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b grown in a flask by total DNA extraction and hybridization using two specific DNA probes. The total genomic DNA of the mixed culture was extracted daily and prepared in duplicate for hybridization for each species-specific DNA probe [the putative sMMO gene from 68-1 at panel A and the sMMO gene (*mmoB* and *mmoZ*) from OB3b at panel B]. Two replicate volumes of the culture were sampled and subjected to DNA extraction and hybridization. The quantity of DNA loaded (ng) was adjusted to meet the hybridization signal range of each standard DNA. **0 h**: a1& a3 ; **26.5 h**: a4 & a5; **42.5 h**: a6; **66.5 h**: a7 & b1; **90.5 h**: b2 & b3; **114.5 h**: b4 & b5; **138.5 h**: b6 & b7. Standard DNA (ng): 68-1 and OB3b in columns c and f respectively (10, 10, 20, 20, 50, 50, 100, and 100 in each row 1 through 8).

assumed that each population ratio was directly related to each population density composing the total population density. The slot blot hybridization image was shown in Figure IV-26. Here standard DNA's for 68-1 and OB3b were not blotted and the absolute population density for each species was not determined as mentioned above.

The species competition was examined by population ratios as to the total population measured by AODC, viable count, DNA probes, and 16S rDNA oligo probes (Figures IV-27 and 28). The domination of OB3b population over 68-1 population was observed by all monitoring parameters. Viable count, DNA-probing count, and 16S rDNA-probing count appeared to correlate fairly well. However, a 30% underestimation was observed for the OB3b population measured by AODC after early stationary phase while approximately 250% overestimation was observed for 68-1 population assuming that data from all the other monitoring parameters were correct. These discrepancies decreased as early stationary growth stage approached with an exception of viable counts which did not reflect the data from the other monitoring parameters as well.

The TCE degradation activity of the mixed culture began to appear at early stationary phase and culminated at the late stationary phase (Figure IV-24). The overall degradation activity was lower by 2-3 orders of magnitude than that of pure culture of each strain shown in Section D of this Chapter. This could be mainly due to trace amount of copper present originated from distilled water (double distilled water was not used here) and minor contamination of non-methanotrophs in the mixed culture. The contribution of each population to the TCE degradation was considered to be directly proportional to the population density if all the viable cells were involved in TCE degradation.

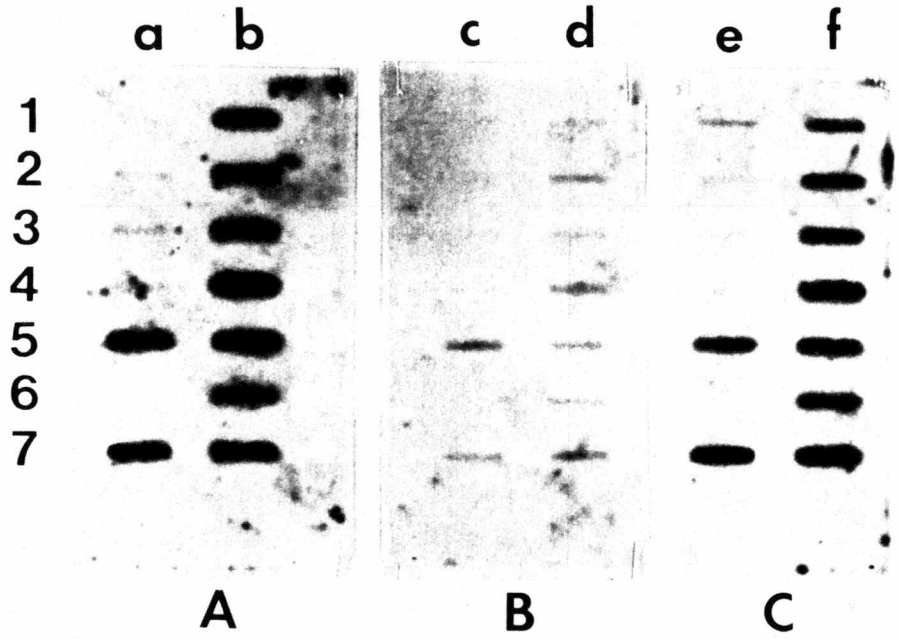


Figure IV-26. Monitoring of two species competition in a mixed culture composed of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b grown in a flask culture by total DNA extraction and hybridization using two specific DNA probes. The total genomic DNA of the mixed culture was extracted daily and prepared in triplicate for hybridization for each type- and kingdom- specific oligodeoxynucleotide probes (the eubacterial probe for at panel A, type I methanotroph-specific 1035-RuMP at panel B, and type II methanotroph-specific 1034-Ser at panel C). Two replicate volumes of the culture were sampled and subjected to DNA extraction and hybridization. **0 h**: a1, a2, c1, c2, e1, & e2; **26.5 h**: a3, a4, c3, c4, e3, & e4; **42.5 h**: a5, c5, &e5; **66.5 h**: a7, b1, c7, d1, e7, & f1; **90.5 h**: b2, b3, d2, d3, f2, & f3; **114.5 h**: b4, b5, d4, d5, f4, & f5; **138.5 h**: b6, b7, d6, d7, f6, & f7.

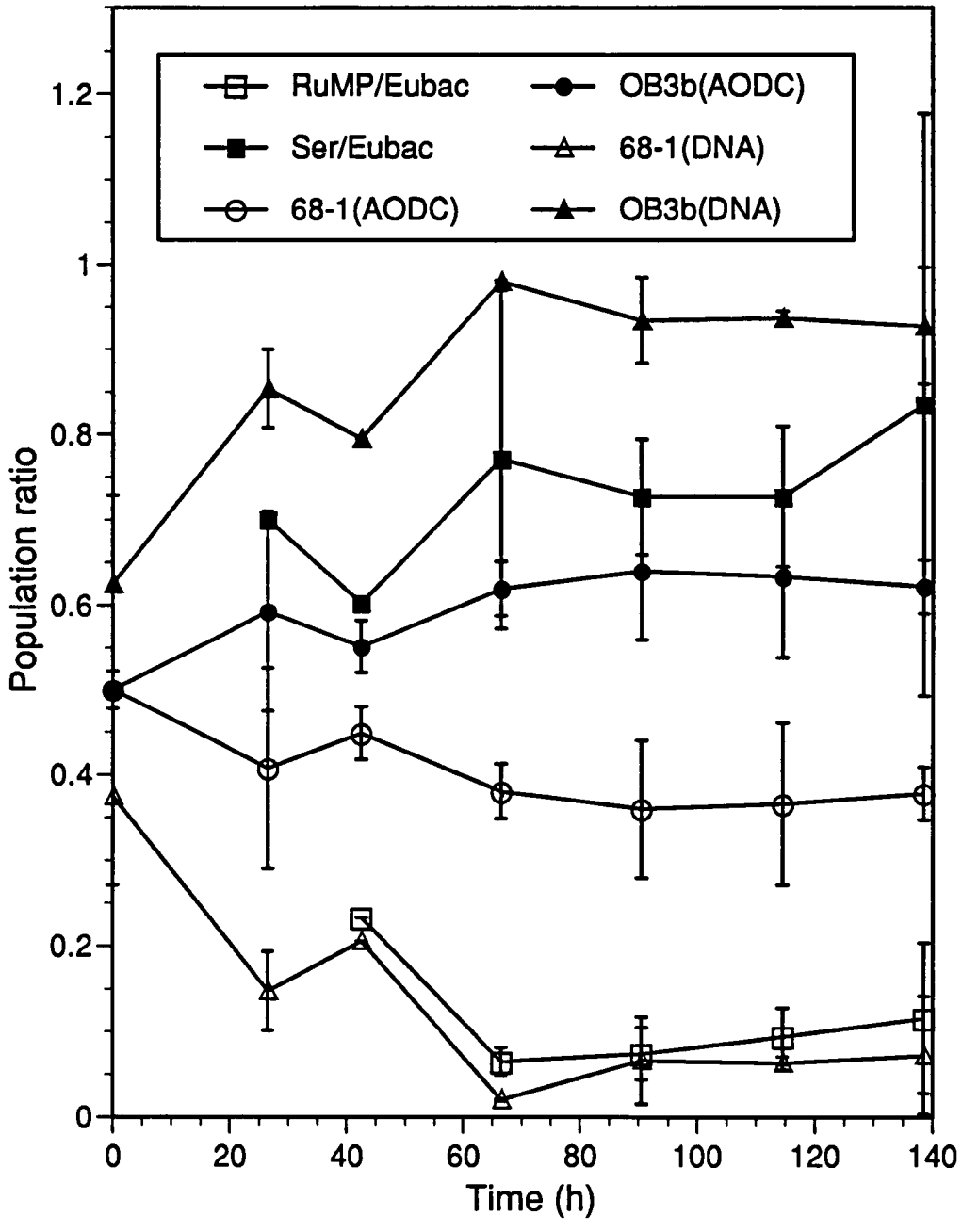


Figure IV-27. Competition of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b examined by population ratios as to total population measured by AODC, DNA probes, and 16S rRNA probes. The DNA probes were the 4 kb putative sMMO gene for 68-1 and 2.1 kb fragment carrying sMMO B gene for OB3b. The rRNA probes were 1035-RuMP for type I methanotroph, 1034-RuMP for type II methanotroph, and a eubacterial probe for total population.

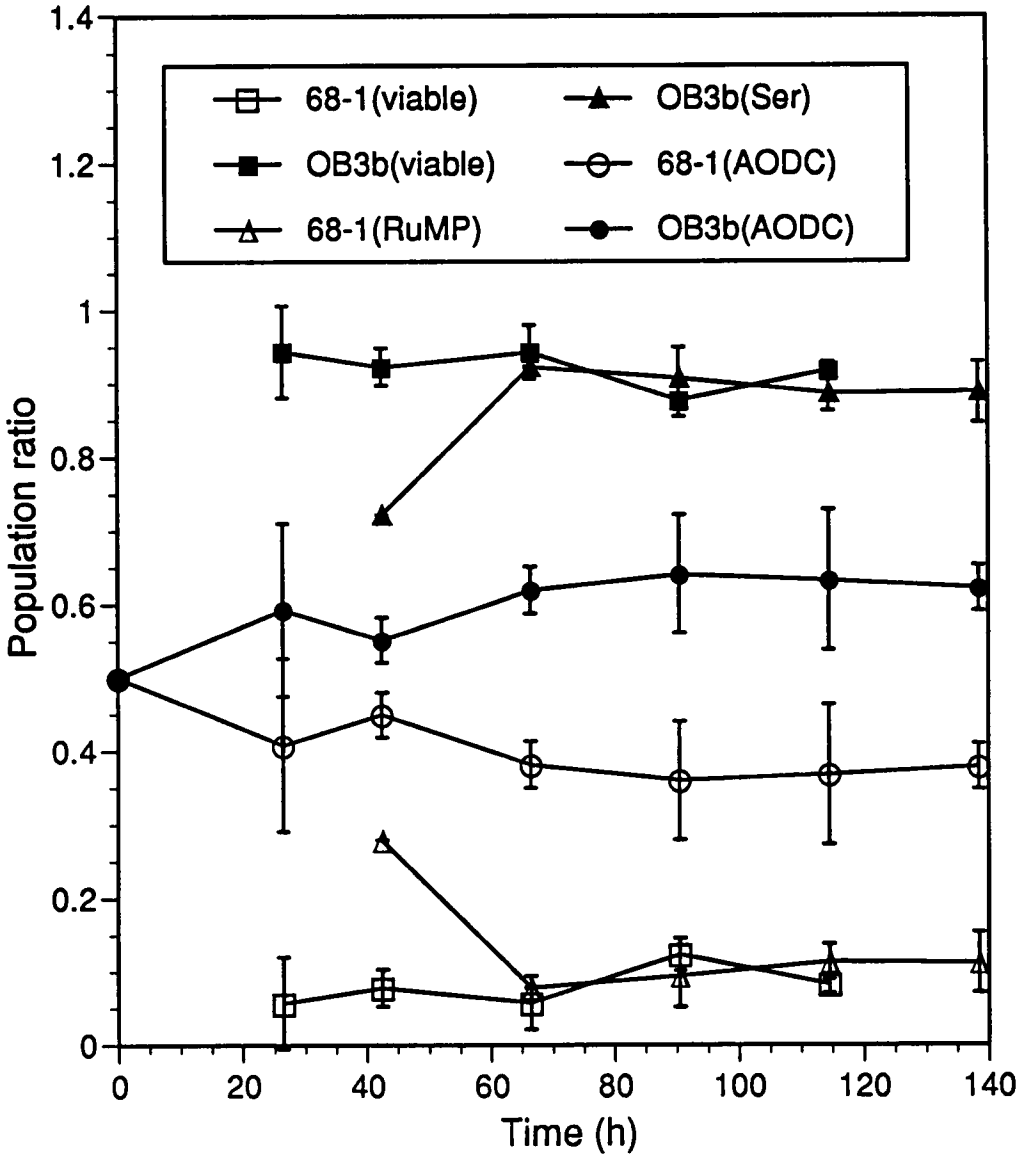


Figure IV-28. Competition of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b as examined by population ratios as to total population measured by AODC, 16S rDNA-targeting oligo probes, and viable count (= plate count). The oligo probes were 1035-RuMP for type I methanotroph and 1034-RuMP for type II methanotroph.

M. Species competition of methanotrophic populations in a continuous bioreactor

The last goal of this study was to monitor species competition of the two methanotrophic populations in continuous culture using gene probes and to investigate the consequent TCE degradation activity. The NMS medium components used in the flask culture were used in all bioreactor experiments. Optimum methane and air (or oxygen) flow rates were determined to maximize the specific growth rate of 68-1 pure culture in batch bioreactor and used for continuous bioreactor operation. Appropriate dilution rates were determined allowing maintenance of 68-1 population in continuous culture. These parameters were then used to routinely operate the continuous culture bioreactor for species competition in mixed culture.

Medium used in bioreactor experiment and the reactor operation

The medium used in the bioreactor studies was NMS (Cornish et al., 1984) containing phosphate buffer (10 mM), sodium nitrate (10 mM), other salts, and trace elements as major components. The concentration of iron was increased to 80 μM, as suggested by Park et al. (1991), and copper was not added to induce the sMMO production. Vitamins (refer Section N in Chapter III) and cycloheximide (30 mg/L; Sigma Chemical Co., St. Louis, MO) were also added to stimulate the cell growth of methanotroph(s) and to inhibit the growth of protozoa, respectively. Contamination of the bioreactor system was expected as the reactor could not be operated under aseptic conditions. The internal bioreactor surface, however, was sterilized between individual experiments by agitating for several hours with Clorox solution (final concentration 5-10% v/v).

Optimization of growth of *M. methanica* 68-1 in batch culture bioreactor

The objectives of growth of 68-1 pure culture in batch bioreactor was to find the optimum gas flow rates for methane and air (or oxygen) and to determine maximum specific growth rate which was an important parameter in determining dilution rate of the continuous culture bioreactor later. However, the optimum gas flow rates for OB3b were not determined as it could be assumed that the flow rates would be also near optimum for OB3b growth.

Growth of 68-1 in batch culture in the bioreactor is shown in Figure IV-29. Oxygen concentration in the medium maintained at least 10% (0.826 mg/L) by varying the air or oxygen flow rate and agitation. This level of oxygen was needed to stimulate the cell growth according to Park et al. (1991). The growth of 68-1

was monitored by AODC. There were a few bacterial types of contaminants observed but they did not significantly affect the 68-1 growth. Protozoa were readily controlled by adding cycloheximide once at start of the culture.

At the early growth period (0-67.5 h) fixed methane flow rate (56 ml/min) and agitation speed (173 rpm) were employed and the air flow rate was changed (17.5-50 ml/min). The bioreactor pressure was maintained at zero pressure. During this period significant cell growth was observed while dissolved oxygen concentration rapidly decreased (from 0.72 mg/L to 0.025 mg/L). After 67.5 h air and methane flow rates were increased to 95 and 68 (ml/min), respectively. The pressure and agitation were increased to 15 psi and 224 rpm, respectively, in order to increase growth rate. The cell density increased by 7 times until 115.5 h. At this time air was replaced by oxygen (40 at first and 20 ml/min later) since 15 psi was approximately a maximum pressure allowed for the air. The growth,

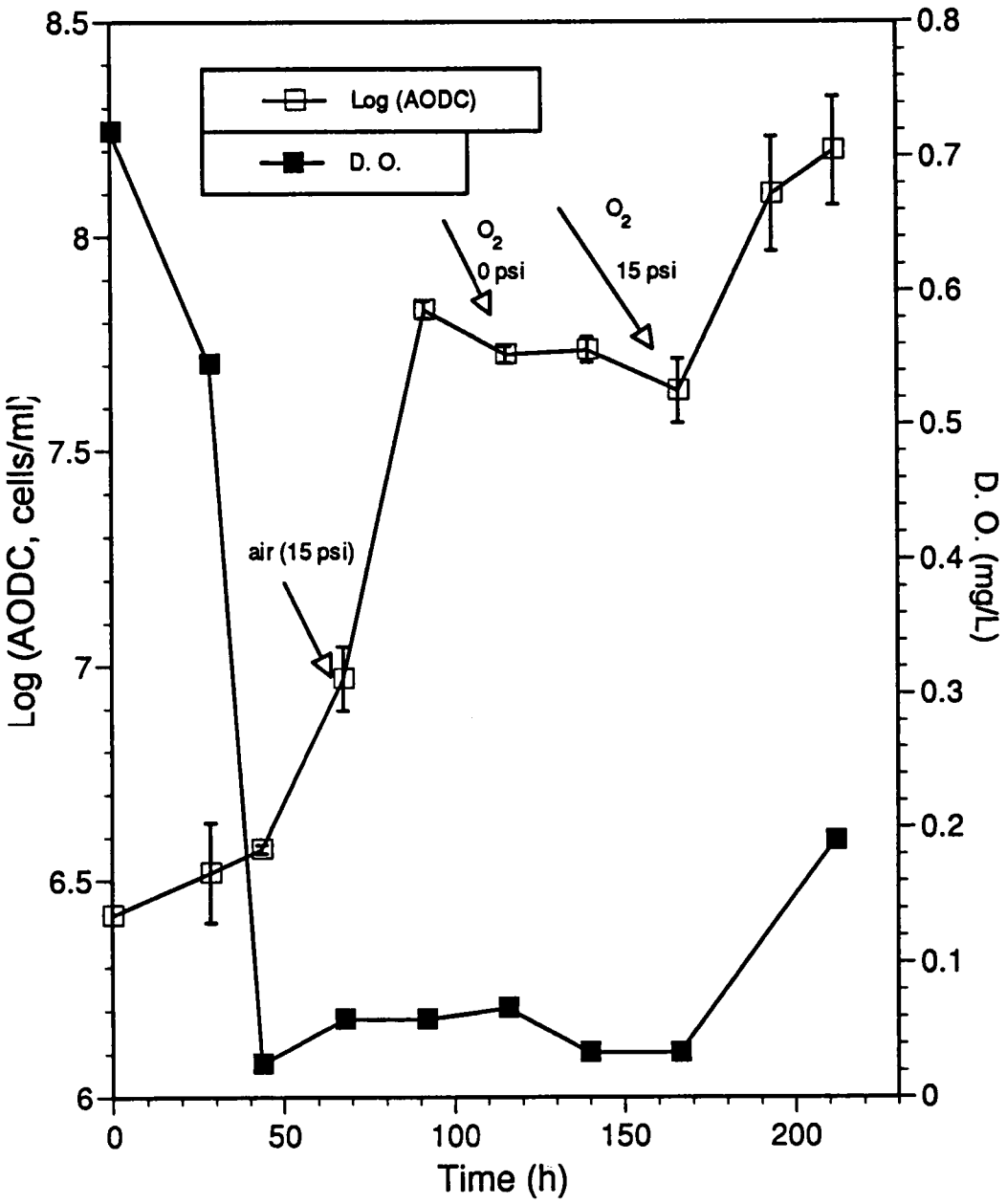


Figure IV-29. The growth of *Methylomonas methanica* 68-1 in the batch culture bioreactor. The medium contained 10 mM phosphate buffer, 10 mM sodium nitrate, 80 µM ferric chloride, no added copper, and other mineral salts. Methane and air (or oxygen) flow rates, bioreactor pressure, and agitation speed were frequently adjusted to increase the specific growth rates.

however, was retarded apparently due to the relative oxygen-depleted environment shortly after switching from air to oxygen. This retardation continued until oxygen flow rate fixed at 20 (ml/min) and methane flow rate increased to 100 (ml/min), and the bioreactor was operated under pressure again (15 psi) at 166 h. After this time point the cell density increased up to more than 10^8 (cells/ml). At 215.5 h the batch culture was switched to continuous culture mode with dilution rate of 0.022 h^{-1} (medium flow rate: 1 ml/min; residence time 45 h). Unfortunately a reverse medium flow occurred under this flow rate and pressure (15 psi) and the bioreactor stopped. Subsequently ambient pressure (0 psi) was employed and the cell density at continuous culture starting point was 10^7 (cells/ml) rather than 10^8 (cells/ml) in most cases.

The specific growth rates at the first exponential and second exponential phases were 0.048 h^{-1} and 0.029 h^{-1} respectively (Figures IV-30 and 31).

Effect of dilution rate on the growth of *M. methanica* 68-1 in continuous culture bioreactor

Based on the results from the batch culture experiments described above, the 68-1 cell density (10^8 cells/ml) were maintained at 0.017 h^{-1} dilution rate and the gas flow rates of methane (50 ml/min) and oxygen (2 ml/min) at zero pressure in a continuous culture bioreactor (Figure IV-32). Effect of dilution rate on the growth of 68-1 was examined in the similar growth conditions used in the above section. The dilution rates ranging from 0.011 h^{-1} to 0.024 h^{-1} allowed the growth of 68-1 cells ranging from 10^8 to 10^7 (cells/ml) (Figure IV-33).

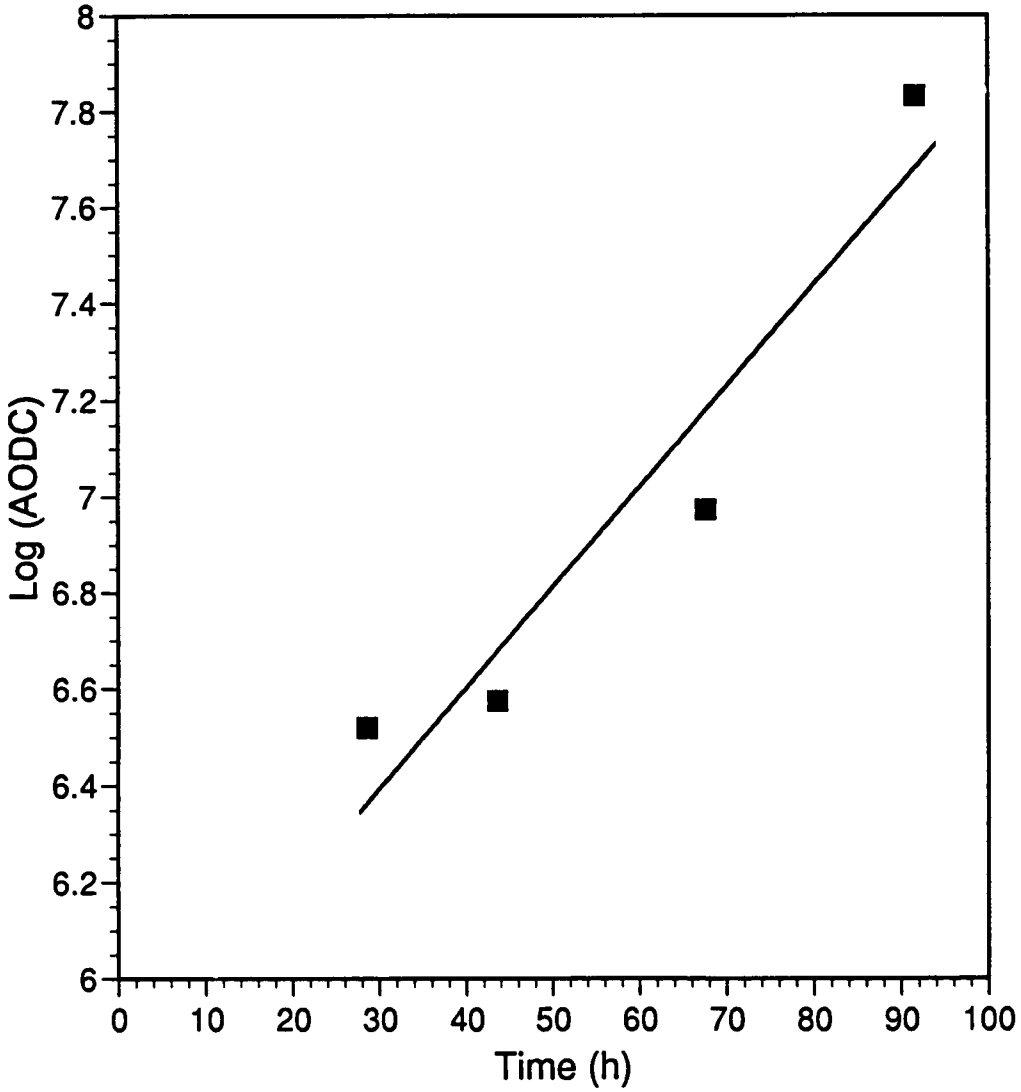


Figure IV-30. Growth of *Methylomonas methanica* 68-1 in the batch culture bioractor during the log phase and determination of specific growth rate. The equation of the linear fitting curve is $y = 0.0209x + 5.76$ (r^2 = 0.909). The specific growth rate was calculated as 0.048 h^{-1} (= 0.0209 x 2.30).

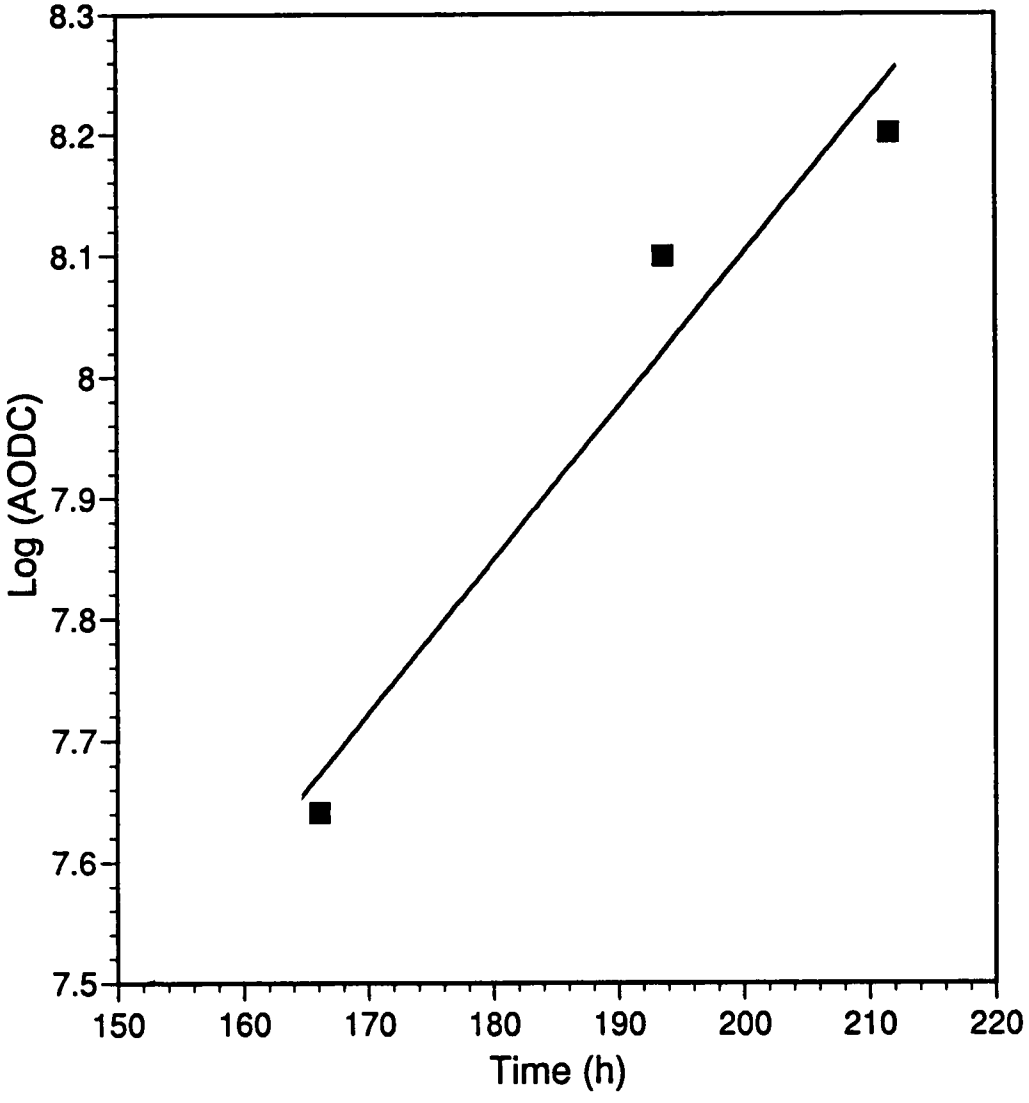


Figure IV-31. Growth of *Methylomonas methanica* 68-1 in the batch culture bioreactor during the log phase and determination of specific growth rate. The equation of the linear fitting curve is $y = 0.0126x + 5.56$ ($r^2 = 0.946$). The specific growth rate was calculated as 0.029 h^{-1} (= 0.0126 x 2.30).

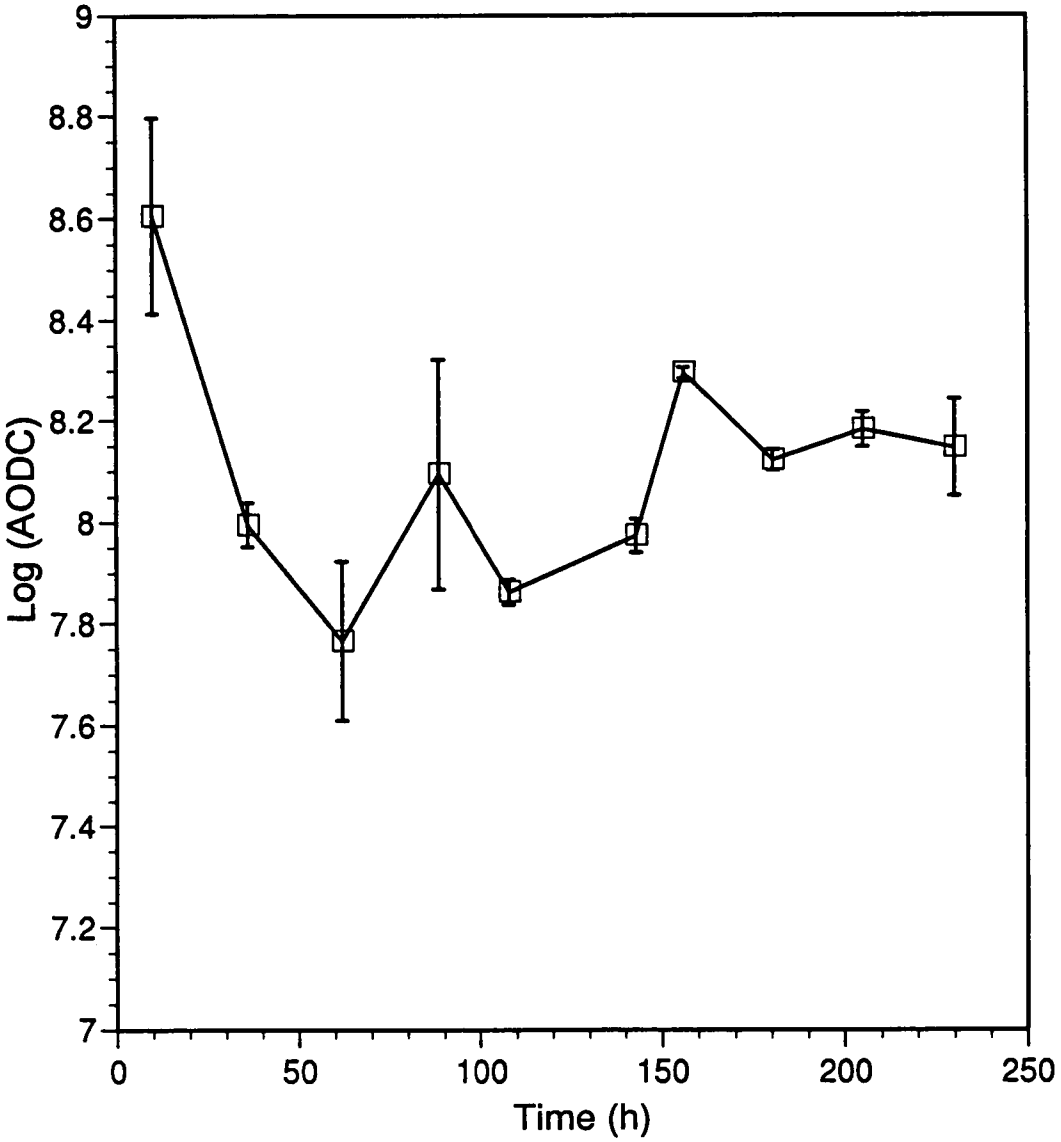


Figure IV-32. The growth of *Methylomonas methanica* 68-1 in the continuous culture bioreactor. The medium was NMS containing 10 mM phosphate buffer, 10 mM sodium nitrate, 80 µM ferric chloride, and no added copper. Methane and oxygen flow rates were 50 (ml/min) and 2 (ml/min) respectively. Dilution rate was 0.017 h^{-1} (medium flow rate: 0.77 ml/min).

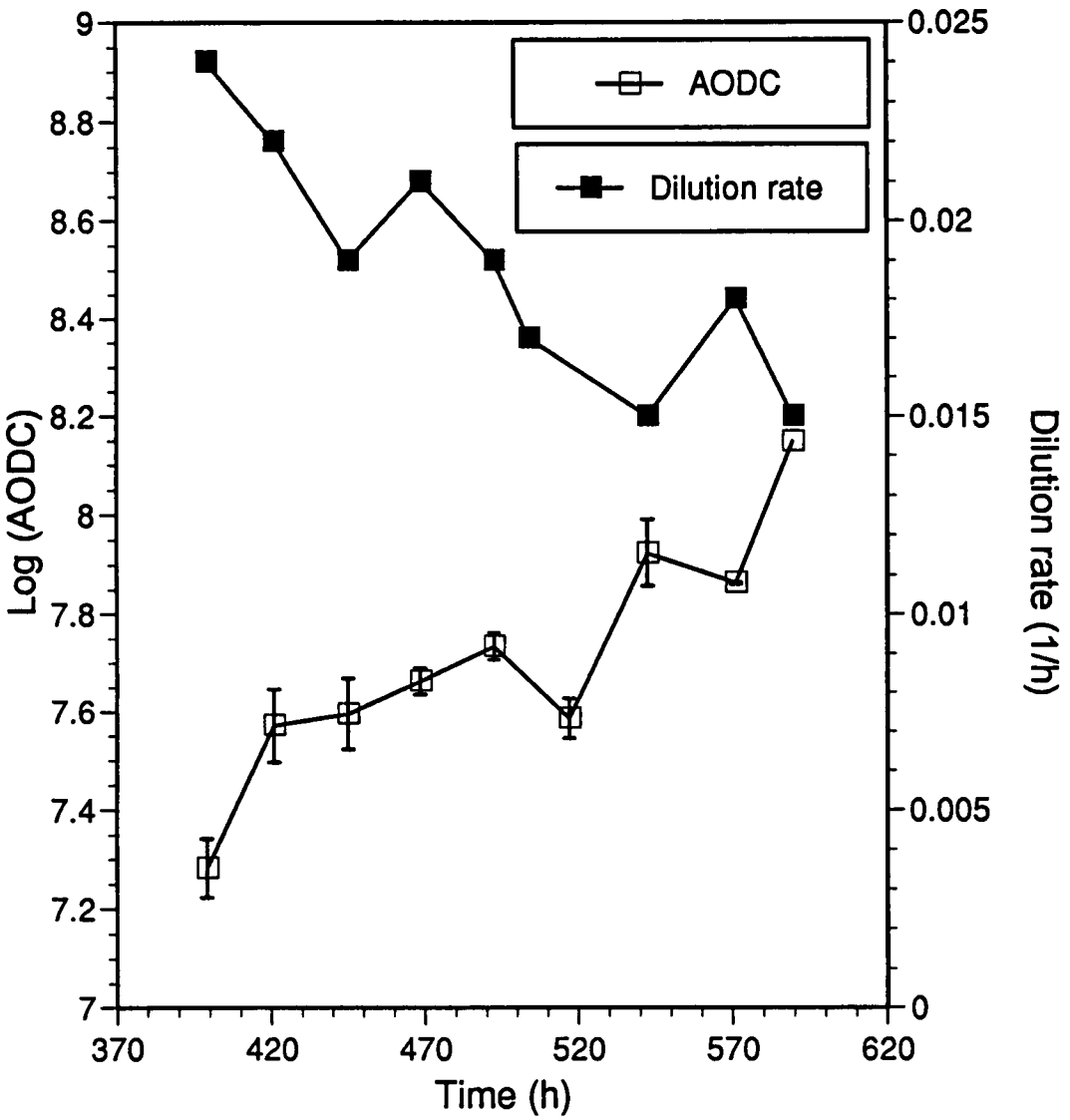


Figure IV-33. Effect of dilution rate on the growth of *Methylomonas methanica* 68-1 in the continuous culture bioreactor. The medium was NMS containing 10 mM phosphate buffer, 10 mM sodium nitrate, 80 mM ferric chloride, and no added copper. Methane and oxygen flow rates were 50 (ml/min) and 2 (ml/min) respectively.

Species competition of *M. methanica* 68-1 and *M. trichosporium* OB3b in continuous culture bioreactor

The two species competition of 68-1 and OB3b was examined in a continuous culture (Figure IV-34). Almost equal number of cells (68-1, 7.23×10^7 cells/ml; OB3b, 7.01×10^7 cells/ml) were inoculated into the bioreactor (2.7 liter full working volume) and the bioreactor was operated at 0.011 h^{-1} dilution rate to increase cell density until 60 h. The dilution rate was then switched to 0.022 h^{-1} (retention time 45 h). Until 33 h there was a slight increase in cell density of both species but decreased later on. The decrease was partly due to the accompanying increase in population of non-methanotrophic bacterial contaminants. After 33 h 68-1 population was out-competed by OB3b population with the two species in the mixed culture being maintained at 1.9×10^6 and 2.0×10^7 (cells/ml), respectively. This state of species competition was evident before the bioreactor reached a steady-state (possibly after 180-225 h or 4-5 times the 45 h reactor retention time). The dissolved oxygen was rapidly consumed at a early stage of growth (until 9 h) and then gradually increased until the populations were stabilized. Therefore the dissolved oxygen concentration could be used as an indicator of the population growth and stabilization. The TCE degradation rate was not determined.

Another similar competition experiment was performed as shown in Figures IV- 35 and 36. In this case the mixed culture was not subjected to low dilution rate ($0.011 h^{-1}$) to increase the cell density in contrast to the experiment described above (Figure IV-34). The 68-1 population was still out-competed by OB3b population when the populations were monitored by AODC. However, there was a significant discrepancy between 68-1 population monitored by

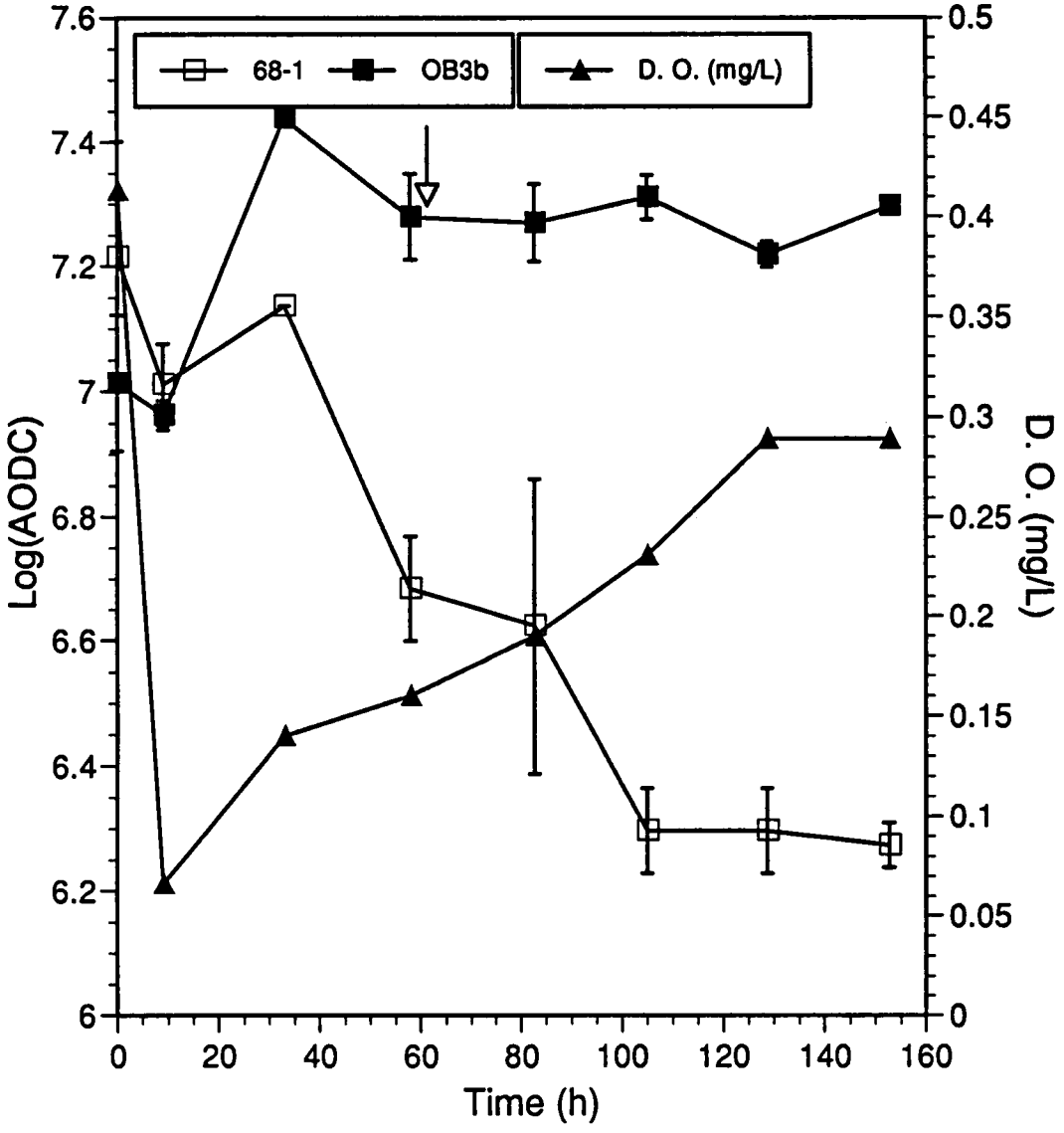


Figure IV-34. The two species competition of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b in the continuous culture bioreactor. The NMS medium contained 10 mM phosphate buffer, 10 mM sodium nitrate, 80 μM ferric chloride, and no added copper. Methane and oxygen flow rates were 50 (ml/min) and 2 (ml/min) respectively. The dilution rates were changed from 0.011 h^{-1} to 0.022 h^{-1} at 60 h (arrow mark).

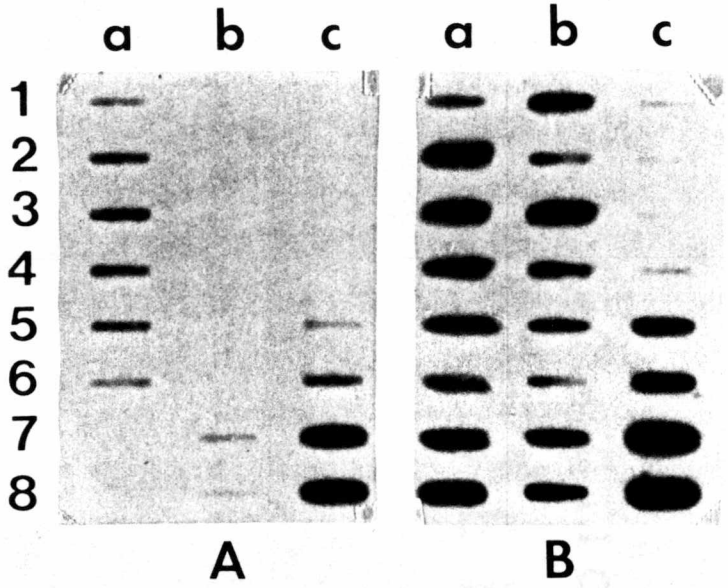


Figure IV-35. Monitoring of two species competition in a mixed culture composed of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b grown in a continuous culture bioreactor (dilution rate 0.011 h^{-1}) by total DNA extraction and hybridization using two specific DNA probes. The total genomic DNA of the mixed culture was extracted daily and prepared in duplicate for hybridization for each species-specific DNA probe [the putative sMMO gene from 68-1 at panel A and the sMMO gene (*mmoB* and *mmoZ*) from OB3b at panel B]. Two replicate volumes of the culture were sampled and subjected to DNA extraction and hybridization. The quantity of DNA loaded (ng) was adjusted to meet the hybridization siganal ranges of each standard DNA. **0 h**: a1& a2 ; **21 h**: a3 & a4; **49.5 h**: a5 &a6; **64.5 h**: a7 & a8; **88.5 h**: b1 & b2; **114.5 h**: b3 & b4; **136.5 h**: b5 & b6; **160.5 h**: b7 & b8. Standard DNA (ng): 68-1 and OB3b in columns c's respectively (10, 10, 20, 20, 50, 50, 100, and 100 in each row 1 through 8).

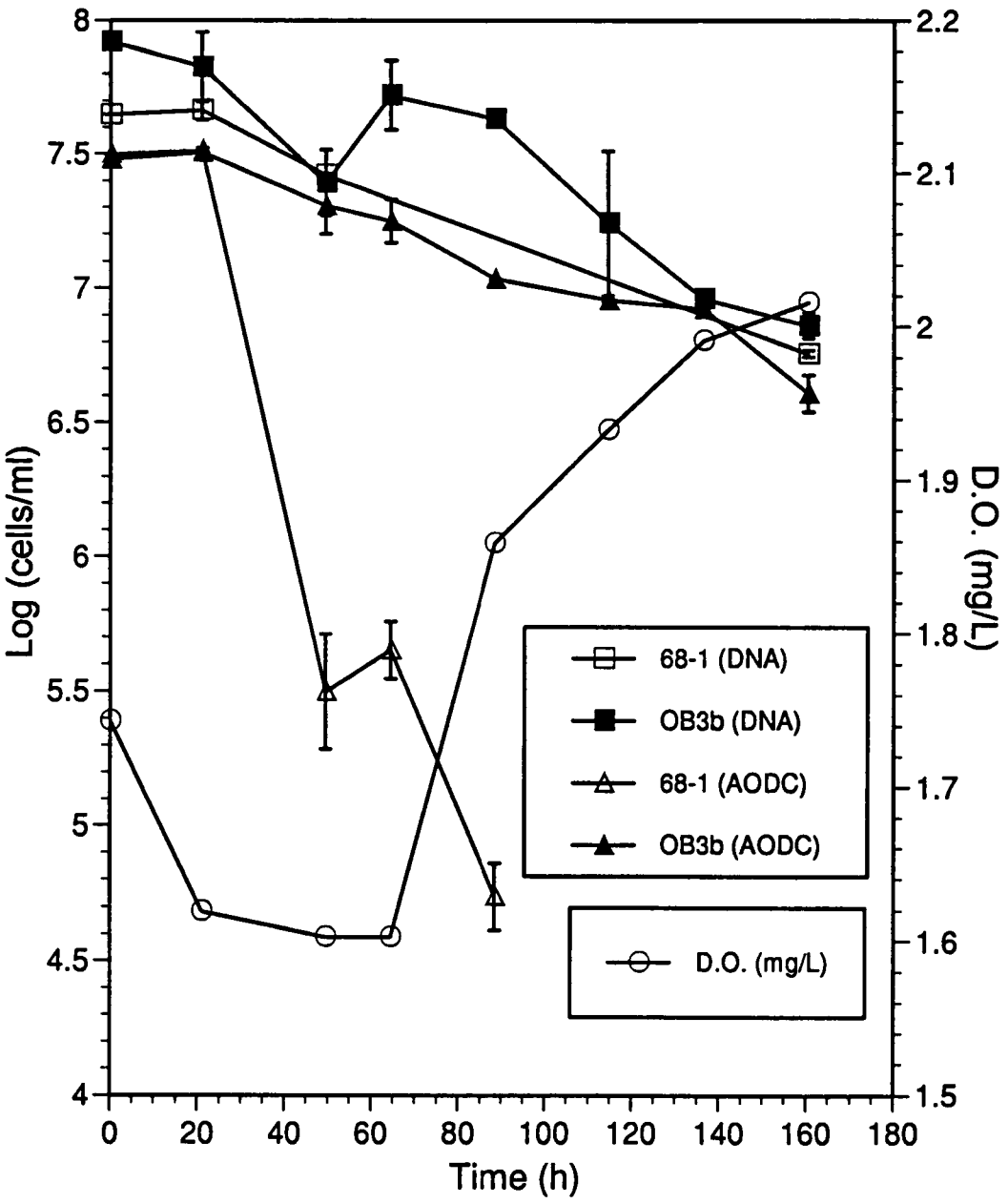


Figure IV-36. The two species competition of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b in the continuous culture bioreactor. The NMS medium contained 10 mM phosphate buffer, 10 mM sodium nitrate, 80 μM ferric chloride, and no added copper. Methane and oxygen flow rates were 50 (ml/min) and 2 (ml/min) respectively. The dilution rates was 0.011 h^{-1}.

AODC and the population by specific DNA probing while the OB3b populations by both monitoring methods correlated well with each other. The reason for the discrepancy could be that AODC method was not sensitive enough to count cellnumber less than 10^6 (cells/ml). This cell density was also a methanotrophic bacterial population detection limit as reported previously (Graham et al., 1993).

N. Effect of growth regimes on species competition of *M. methanica* 68-1 and *M. trichosporium* OB3b in flask culture

Important growth factors such as methane, nitrate, and copper were tested for their effect on the two species competition of 68-1 and OB3b. These factors have been previously reported to affect sMMO production and cell growth in mixed culture composed of *M. trichosporium* OB3b and *M. albus* BG8 (Graham et al., 1993).

Methane gradient

The methane (the sole carbon source) effect on the two species competition is shown in Figure IV-37. 68-1 was more competitive than OB3b at higher methane concentrations (25 % and 50 %) while the opposite was true at lower methane concentrations (5 % and 10 %). The specific growth rate of 68-1 was highest at 50 % methane. This indicated that environments of high methane concentration could be more favorable to type I methanotrophs like 68-1.

Copper gradient

The copper effect on the two species competition under a methane non-limiting condition was shown in Figure IV-38. The two species which had been

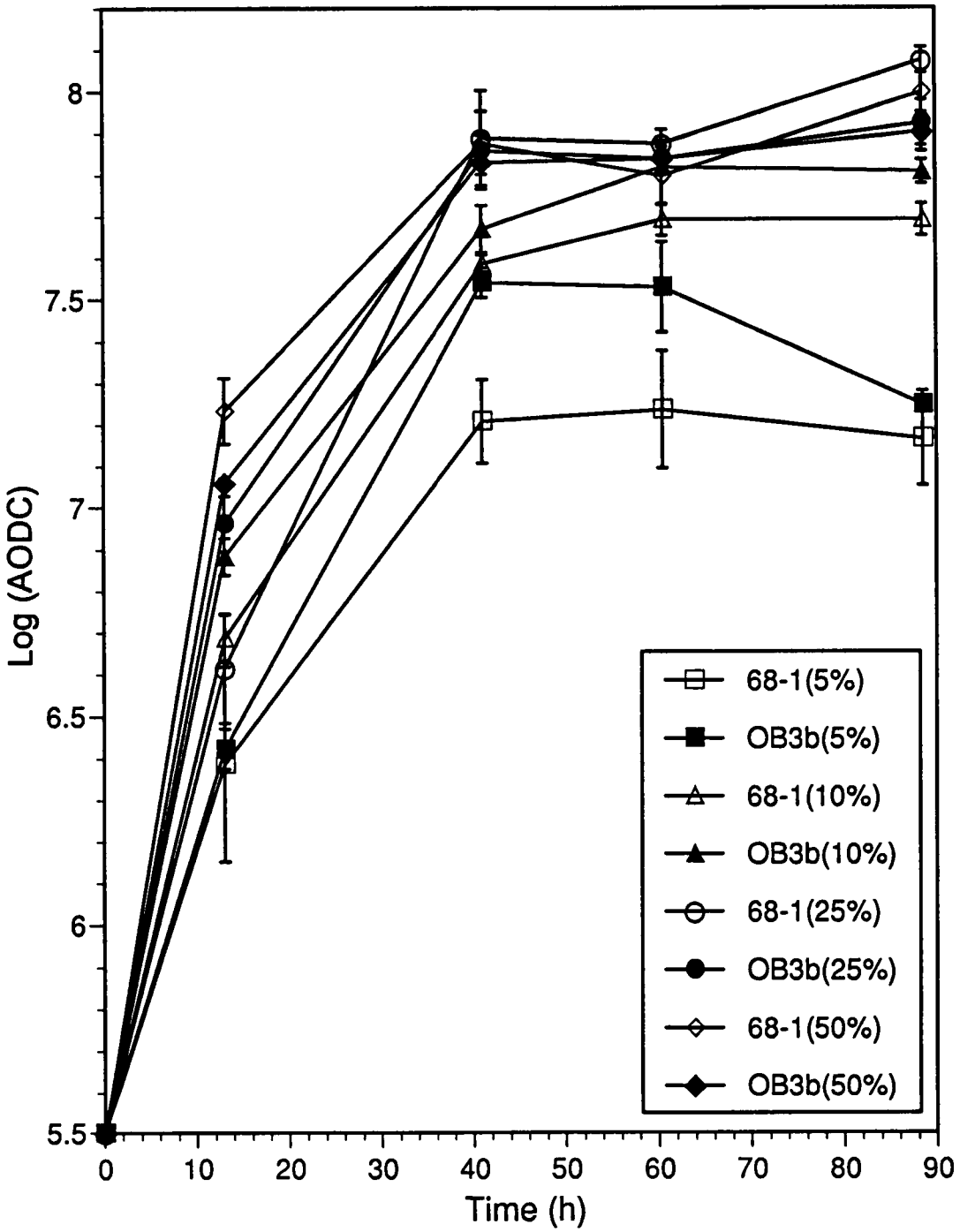


Figure IV-37. Effect of methane as a carbon source on the two species competition of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b in batch flask culture. The medium contained 10 mM phosphate buffer, 10 mM sodium nitrate, 80 µM ferric chloride, and no added copper. Methane was added up to each specified % (v/v) of the headspace once.

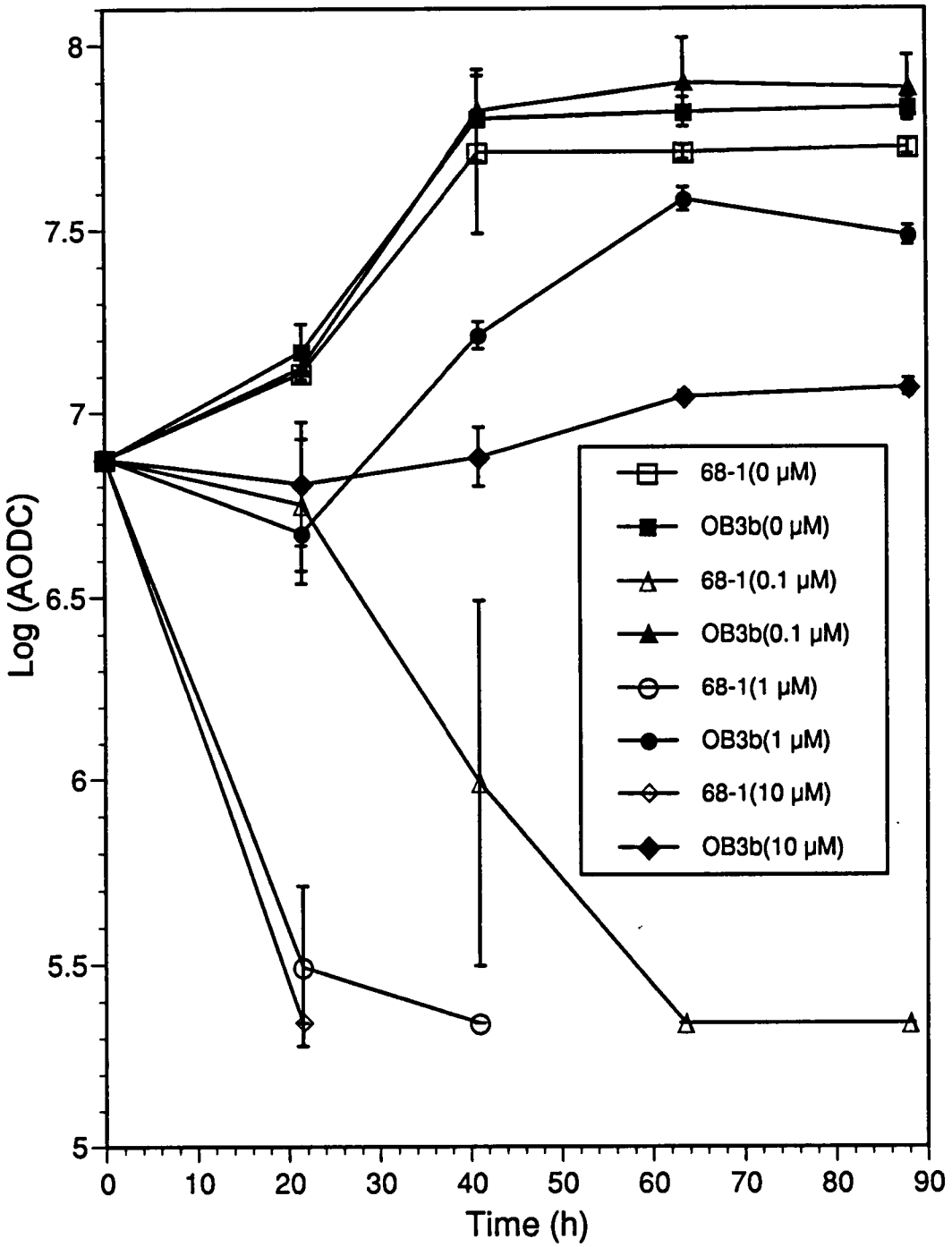


Figure IV-38. Effect of copper on the two species competition of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b in batch flask culture. The medium contained different concentrations of copper sulfate and methane was added up to 30 % of the headspace daily.

adapted under copper-depletion condition were sensitive to copper. The 68-1 cells showed copper sensitivity at 0.1 μM while OB3b cells showed sensitivity at 1 μM. Overall, the decreased competition of the 68-1 population by the OB3b population became more prominent as the copper concentration increased. Higher copper concentrations (1 μM and 10 μM) caused the population of 68-1 to decrease to threshold detection levels by 22 h. Interestingly, a lower concentration of copper (0.1 μM) actually stimulated the growth of OB3b cells. In the absence of copper the OB3b population was still slightly more competitive than the 68-1 population.

Nitrate gradient

The sodium nitrate (nitrogen source) effect on the two species competition under a methane non-limiting condition is shown in Figure IV-39. The 68-1 population appeared to be more sensitive to nitrate depletion than OB3b where the type I population only maintained the inoculum level of population density at stationary phase but OB3b population density was higher by one order of magnitude compared to 68-1. Moreover, the 68-1 population showed increased population density but could not compete with OB3b population even with an increasing concentration of nitrate. The OB3b population, however, also seemed to be more responsive to the increase of nitrate concentration.

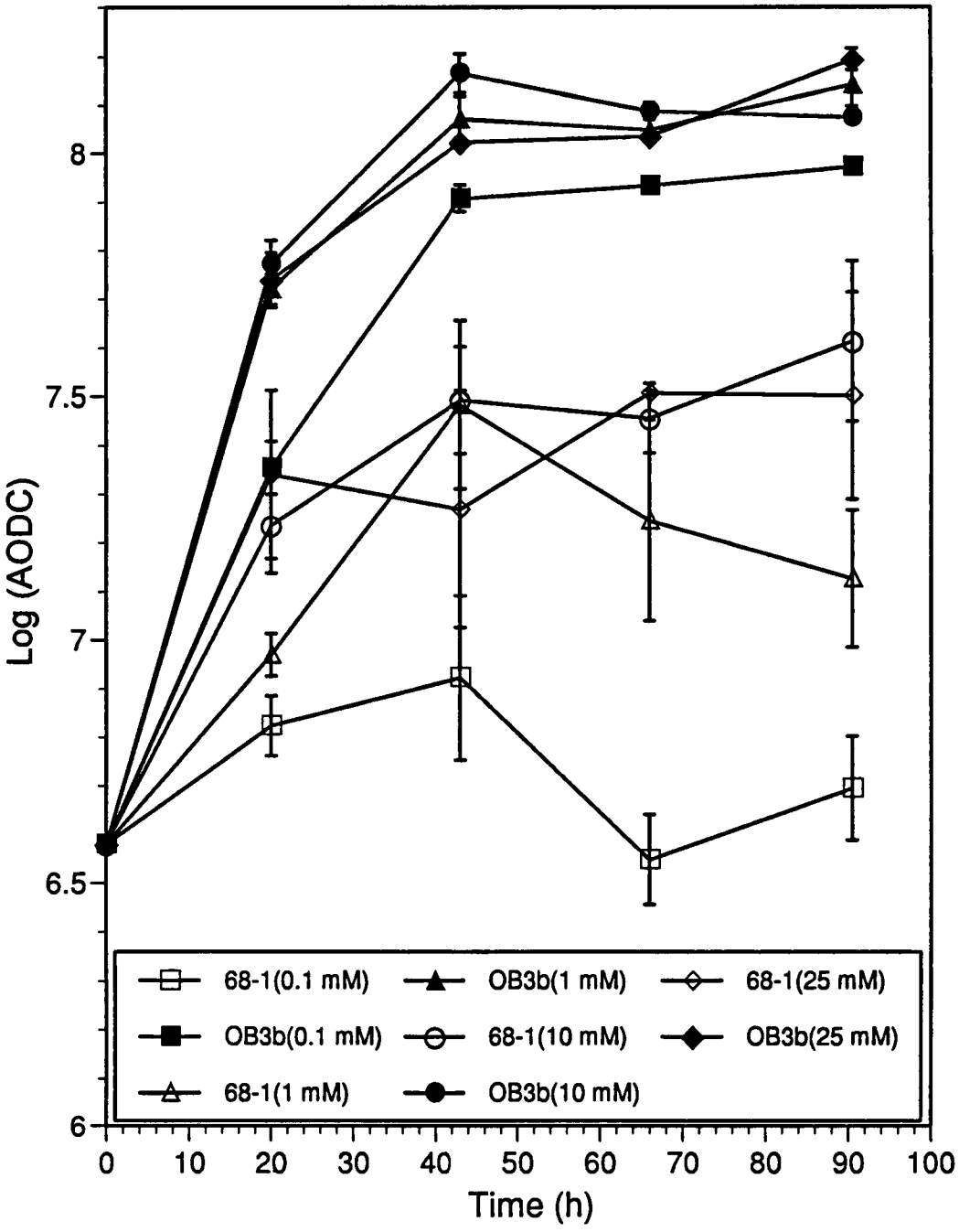


Figure IV-39. Effect of sodium nitrate as a nitrogen source on the two species competition of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b in batch flask culture. The medium contained different concentrations of sodium nitrate and no added copper. Methane was added up to 30 % of the headspace daily.

CHAPTER IV

DISCUSSION

In this study it was shown that the strain 68-1 produced a soluble methane monooxygenase (sMMO) under copper limitation. This is the first report presenting an evidence for a sMMO production and subsequent TCE degradation by a type I methanotroph. This strain was identified as *Methylomonas methanica* through morphological characteristics (e.g., pigment production, colony type, ultrastructure, etc.), 16S rRNA signature probe hybridization, and phospholipid fatty acid and lipopolysaccharide hydroxyl fatty acid profiles. The pink-pigmentation of 68-1 was more prominent in the medium containing copper than containing no added copper. The total RNA from the strain 68-1 also hybridized specifically to a type I methanotroph-specific oligodeoxynucleotide probe (1035-RuMP) not type II methanotroph-specific oligo probe (1034-Ser). The phospholipid fatty acid (PLFA) and lipopolysaccharide hydroxyl fatty acid (LPS-OHFA) profiles conclusively allowed the identification of 68-1 as a pink-pigment type I methanotroph.

Interestingly, another pink-pigment type I methanotroph, K25-1, recently isolated from a bioreactor effluent sample obtained from Oak Ridge National Laboratory (Oak Ridge, Tennessee) (unpublished data). The inoculum of the bioreactor was from a TCE-contaminated groundwater sample at the U. S. Department of Energy Kansas City Plant (personal communication from A. V. Palumbo). Strain K25-1 appears to be a *Methylomonas* spp., produced more slime than 68-1, produced sMMO (measured by naphthalene oxidation), and

could degrade TCE. This adds to the evidence that sMMO production by type I methanotroph occurs.

The absence of copper in the medium exerted a negative effect on the growth of strain 68-1 including significant decreases in maximum specific growth rate, growth yield for methane, and total biomass at early stationary phase (Table IV-3). This is unlike the effect of copper limitation on the type I methanotroph *Methylomonas alba* which only produces pMMO (Brusseau et al., 1990). In the absence of copper *M. alba* completely loses its ICM (intracytoplasmic membrane) and drastically decreases its specific growth rate (Collins et al., 1991). Similar phenomena were also observed in *Methylococcus capsulatus* Bath grown on methanol in the absence of copper (Prior and Dalton, 1985). This thermophilic methanotroph loses most of its ICM with the membranes only occurring at the cell periphery, however, a gradual increase in ICM formation occurred as the copper ion concentration increased to 4.8 μM where ICM were fully recovered. In the methanotroph "*Methanomonas margaritae*" ICM formation was evident during copper limitation, however, its arrangement was altered (Takeda and Tanaka, 1980). In this case the addition of copper ions strongly accelerated the growth of the cells. By contrast no change was observed in the ICM of *Methylomonas* sp. MM2 regardless of copper concentration (Henry and Grbic-Galic, 1990). The involvement of copper in formation of the stacked intracellular membranes was also supported by the mutants of *Methylosinus trichosporium* OB3b constitutively producing sMMO in the presence of high copper concentration (Phelps et al., 1992). The mutants lacked the ability to form the ICM and failed to show particulate MMO activity. The addition of copper to the mutant cultures had no noticeable effect on the

growth rate and sMMO expression. A possible mechanism behind this phenomenon was that the mutant phenotype resulted from defects in copper uptake and metabolism rather than from changes in sMMO expression or enzyme stability (Fitch et al., 1993).

It seems that sMMO activity in *Methylomonas methanica* is a strain-specific feature because no other type I methanotroph has shown the enzyme activity yet. It has been theorized that sMMO may be an adaptation to conditions in which there is a long term copper limitation (Dalton and Leak, 1985). Habitats lacking readily available copper but abundant methane and oxygen may provide a niche for sMMO-producing methanotrophs.

The decrease in TCE degradation rates in 68-1 and OB3b (Figures IV-5 and 8) could be associated with the sMMO enzyme inactivation by loss of iron from the hydroxylase component of the enzyme (Green and Dalton, 1989). Other possible reasons for the decrease in the rates could be toxic effects to the enzyme caused by TCE or its transformation products such as TCE epoxide and CO (Alvarez-Cohen and McCarty, 1991a; Alvarez-Cohen and McCarty, 1991b; Fox et al., 1990; Henry and Grbic-Galic, 1991a; Henry and Grbic-Galic, 1991b). The contribution of depletion of reducing power (NADH) to the decreasing degradation rates is unlikely because formate was added.

The TCE degradation rate shown as V_{max} (2325 nmoles/h/mg protein; equivalent to 995 mmoles/h/g cells) was higher than the degradation rates of OB3b reported by Oldenhuis et al. [(1989); 27 nmoles/min/mg cells = 1620 mmoles/h/g cells] and Tsien et al. [(1989); 725 mmoles/h/g cells]. The degradation rate of OB3b in this study was 1052.6 nmoles/h/mg protein (equivalent to 500 mmoles/h/g cells).

The sMMO component B gene probe from OB3b did not hybridize to the genomic DNA of various methanotrophs (types I, II, and X) except species from its own genus under high stringency conditions, as previously reported (Stainthorpe et al., 1990). The probe did not hybridize to the genomic DNA of 68-1 even under low stringency which allowed 40 % mismatch. Although studies have indicated that the amino acid sequence homology of sMMO between type X (*M. capsulatus* Bath) and type II (*M. trichosporium* OB3b) methanotroph is high (78-94 %) (Cardy et al., 1991; Murrell, 1992), codon usage variation (Murrell, 1992) critically affects gene probe hybridization results. Using conditions in which approximately 30 % nucleotide mismatch was accounted for, *Methylosinus trichosporium* OB3b sMMO gene probe did not hybridize to the homologous genes in *Methylococcus capsulatus* Bath (Stainthorpe et al., 1990b). Further studies will be necessary to determine exactly how the 68-1 differs from the more well studied sMMOs of *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath in terms of DNA and amino acid sequences. This will elucidate the evolutionary diversity of sMMOs in methanotrophs.

In this study a methanol dehydrogenase (MDH) structural gene probe from *M. organophilum* XX (Machlin et al., 1988) was found to hybridize to putative MDH genes from a variety of methanotrophs (types I, II, and X) and methylotrophs (utilizing RuMP and Serine pathways) and showed little or no genetic homology to non-methanotrophs or methylotrophs. This gene probe had a high DNA sequence homology with a well-characterized MDH gene, *moxF,* encoding the large subunit of the enzyme (Nunn and Lidstrom, 1986a, b) from *Methylobacterium extorquens* AM1. Stephens et al. (1988) also had a similar hybridization result by using the *moxF* gene probe.

The biodegradation spectrum of the various chlorinated hydrocarbons by the sMMO of OB3b was similar to that of the previous reports (Oldenhuis et al., 1989; Tsien et al., 1989). The difference in substrate specificity between 68-1 and OB3b may be related to variations in the active site structure of the sMMOs of these strains. The degree of substrate range of MMO (sMMO or pMMO) or other oxygenases was discussed in terms of regiospecificity (Harayama et al., 1992). The preferential hydroxylation of the aliphatic compounds, for example, is driven by the regiospecificity and may result from either the architecture of the substrate-binding site, chemical constraints imposed by the reaction mechanism, or a combination of the two (Green and Dalton, 1989). From this standpoint, 1,1,2-trichloroethane appeared to be a more preferred substrate for sMMOs of 68-1 and OB3b than 1,1,1-trichloroethane, although they were chlorinated to the same degree. Another possible explanation for this difference would be toxicity of these hydrophobic organic compounds exerted on lipid bilayers of the cell whose components are different between the two strains (Table IV-2).

The oligodeoxynucleotide probes (designed from sMMO γ-subunit of OB3b) used in screening the genomic DNA library of 68-1 were an equal mixture of two degenerate probes selected from a set of 16 degenerate probes (refer to Section I of Chapter II). This resulted in 8 times lower probability of picking up the true target gene of the sMMO. It is, therefore, worth while to use the available degenerate probes for future library screening.

DNA sequencing strategy (using pSK50 as a template and M13 forward sequencing primer generic on the cloning vector) led to the sequencing of the putative *mmoB* gene (the second half carrying C-terminal end of *mmoB*) of 68-1. DNA and amino acid sequence data base searches did not reveal any

sequences of significant homology to those of the putative sMMO gene. This result was not surprising because there is no significant homology between the *mmoB* gene of *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath although they have high homology (89.4 %) at the deduced amino acid sequence level (Cardy et al., 1991). The low DNA sequence homology among *mmoB* genes from the type I, II, and X methanotrophs was also supported by the slot and Southern blot hybridizations in this study and a previous report (Tsien et al., 1992) using the sMMO component B gene probe of OB3b. The results of the protein (peptide) sequence searches, however, seem to indicate that there is no peptide sequence similarity in the component B of sMMO between the type I and the type II (or X) methanotrophs. This may be likely because there is a significant variation in mass of MMO B protein components even within type II methanotrophs. *Methylocystis* sp. strain M (Nakajima et al., 1992), a type II methanotroph, has 35- to 40- kDa regulatory protein (component B) compared with that of OB3b (15- to 16 kDa) (Murrell, 1992). These issues, however, would be clarified by a complete sequence analysis of the putative sMMO gene of 68-1.

A more extensive study on the methanotrophic bacterial community analysis of a TCE-contaminated site at the Department of Energy Savannah River Site has been reported (Jimenez et al., 1992). In this study 12 wells, including the three wells examined for the type I specific gene, were subjected to distribution analysis of catabolic genotypes in the subsurface sites. The study showed that the overall distribution of sMMO component B, MDH, and TOD genes through the depth profile appeared random, indicating that widespread TCE degradation potential across most of SRS sites existed. The type I specific

genes may also be present in the 9 untested sites considering how the frequency of the type I gene corresponded to catabolic gene frequencies. Recently Bowman et al. (1993) reported that type I methanotrophs were not isolated from groundwater samples of the three contaminated sites examined for the type I methanotroph gene, however, type II methanotrophs (*Methylosinus* sp. and *Methylocystis* sp.) were predominantly isolated from SRS sites including the three sites examined with the putative type I sMMO gene probe. Most of the type II methanotrophs were found to be the sMMO probe-positive and showed TCE degradation activity (Bowman et al., 1993). Type I methanotrophs (*Methylobacter* spp.) were isolated from the sediments of the three sites (MHT1C, 6C and 9C) in less than 10 % of total isolates but *Methylomonas* spp. were not (personal communication from J. P. Bowman). These results indicate that type I methanotrophs may exist in a dormant state or occur at very lower population densities in comparison with type II methanotrophs. This might lead to domination of type I methanotrophs by the type II during the enrichment process employed by Bowman et al. (1993). The domination of type II methanotrophs in the SRS sites is not surprising as the SRS groundwaters are oligotrophic (potentially poor methane concentration indicated by lower organic carbon concentration and lower nitrate concentration) and all type II methanotrophs isolated could fix dinitrogen (Bowman et al., 1993). Under the oligotrophic conditions type II methanotrophs could be selected as supported by the data from Graham et al. (1992). Populations or genes of methanotrophs present in lower numbers could be detected by PCR technology. Recently McGowan et al. (1992) showed that *mmoB* gene could be amplified and identified from DNA's isolated from a variety of freshwater and sea water

environments using PCR. In this case the DNA samples used for amplification were prepared from bacterial cells isolated from the environments or from total DNA samples directly isolated from filtered water. In this study PCR could be used to detect genes of low abundance from DNA samples isolated from the contaminated soils, sediments, and groundwaters and thus be useful for assessing biodegradative potential.

Overall population density data determined by plate counts (viable counts), DNA probing, and 16S rDNA-targeting oligodeoxynucleotide probing appeared to correlate well with each other, however, the results did not always reflect the data obtained by AODC procedure. For example one experiment involving 68-1 and OB3b mixed culture populations, the 68-1 population was overestimated by AODC (250 % compared with data from the other three monitoring parameters) while the OB3b population was underestimated (30 %). These differences were more significant in the cultures measured in the stationary phase compared to cultures in the log phase. Graham et al. (1993) reported that AODC and the counts determined by fluorescently labeled probes were in agreement within 20 % during monitoring of two different methanotrophic populations (*Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3b) in competition studies. The fluorescently labeled nucleic acid probes used by Graham et al. (1993) were two 16S rRNA-targeting oligodeoxynucleotide probes specific to RuMP pathway and serine pathway methanotrophs and methylotrophs respectively (Tsien et al., 1990). Overall the methanotroph monitoring efficiency and population estimation by 16S rDNA-targeting oligo probes and AODC were relatively similar and similar to OB3b population monitoring results obtained by Graham et al. (1993). This indicates the feasibility

of monitoring methanotrophs in mixed culture using by 16S rDNA-targeting oligo probes and AODC. The significant overestimation of 68-1 population by AODC in this study appeared partially to come from mistakenly counting some of the OB3b population as 68-1 population as in stationary phase some OB3b and 68-1 cells seemed to show more morphological and staining similarity. The reason for increased orange color staining in OB3b cells was not clear but was suspected to be due to the increased staining time (from 3 min to 6 min). It was evident in this study that the increased staining time of OB3b cells could lead to more orange color. This is supported by a previous report (Jones, 1974). The traditional theory of acridine orange color reaction is that single-stranded nucleic acids (e.g., RNA's) emit orange to red fluorescence while those that are double-stranded tend to fluoresce green *in vivo* (Daily, 1979). It should, however, be born in mind that variations in the staining method (e.g., sample treatment and washing: Van Es and Meyer-Reil, 1982; AO concentration and contact time: Jones, 1974) and the level of bacterial physiological activity (e.g., bacterial growth rate and ribosome content: Moyer and Morita, 1989; McFeters et al., 1991) can influence the outcome of the acridine staining.

In both batch and continuous cultures grown under copper and/or methane limitations, 68-1 (type I methanotroph) always seemed to be out-competed by OB3b (type II) in this study (Figures IV-24, 34, and 36). However, 68-1, once adapted to the copper limitation, did not show stimulated growth when copper was added. Actually copper addition (at least 0.1 μM) inhibited the growth of 68-1 in batch culture probably due to toxicity (Figure IV-38). This toxicity may come from inactivation of the sMMO by copper ion (Cu^{++}). A previous report (Green et al., 1985) showed that trace concentrations of Cu^{+},

Cu^{++}, Ag^{+}, or Cd^{++} could cause irreversible *in vitro* inactivation of sMMO from *Methylococcus capsulatus* Bath. Four copper atoms have been reported to bind specifically to the reductase component of the sMMO, leading to disruption of the protein structure and loss of the iron-sulfur center and of flavin adenine dinucleotide (Colby and Dalton, 1979). This kind of copper toxicity was not detectable in OB3b cells that had been adapted to copper limitation (Figure IV-38). However, Graham et al. (1993) showed that a type I methanotroph (*Methylomonas albus* BG8) producing only pMMO could out-compete the strain OB3b (type II) under copper non-limiting conditions (at least 0.5 μM) in continuous culture. Therefore, it can be generally considered that the concentrations that derepress sMMO (i.e., copper limiting condition) tends to favor OB3b (potentially type II methanotrophs, especially *Methylosinus* spp.) over *Methylomonas* spp. (potentially type I methanotrophs).

High methane concentration (500-1000 μM) favored the growth of 68-1 over that of OB3b in batch flask cultures in this study. This appeared to be complemented by the higher yield coefficient of 68-1 for methane compared with that of OB3b, regardless of copper concentration (Table IV-3) and the higher TCE (or naphthalene or potentially methane) K_m value in absence of copper. This result indicated that the competitive success of the type I organism was attributed to higher growth yield and more efficient cell maintenance parameters between the two organisms. The growth yield variations are due to the fact that energy from RuMP pathway is higher than serine pathway (Anthony, 1986). In contrast, a competition study by Graham et al. (1993) showed that *M. albus* BG8 out-competed OB3b in a continuous culture running under methane limitation

(less than 6 μM) and copper non-limitation (2 μM) although methane half saturation constants (K_m) of the two competing organisms were same (25 μM).

The population of 68-1 was always dominated by OB3b regardless of the availability of nitrogen, i.e., sodium nitrate in absence of copper (Figure IV-39). OB3b can fix dinitrogen under nitrogen limiting conditions (Graham et al., 1993). The dinitrogen fixation of 68-1 is not examined. Previous reports showed that in general type II and X methanotrophs can actively fix dinitrogen while nitrogen fixation among the type I methanotrophs is less extensive (Murrell and Dalton, 1983; Oakley and Murrell, 1988; Bowman et al., 1993). A few strains of *Methylomonas methanica* and all the strains of *Methylomonas fodinarum* and *Methylomonas aurantiaca* tested can fix dinitrogen while all group II methanotrophs tested can (Bowman et al., 1993). The population of 68-1 is more likely to be out-competed by OB3b population under nitrogen-limiting conditions if it were assumed that 68-1 cannot fix dinitrogen.

The sMMO production has been reported only in type II and X methanotrophs so far. This study, however, shows that at least one species (*Methylomonas methanica* 68-1) in the type I methanotrophs as one important group among the three types of methanotrophs can also produce sMMO and degrade TCE. Moreover, the putative sMMO gene cloned from 68-1, if confirmed, will be useful in detecting and monitoring the homologous genes and populations in the environments. These results contribute to the fundamental knowledge elucidating the potential role of type I methanotrophs *in situ* TCE bioremediation.

CHAPTER VI

CONCLUSIONS

The goal of this study was to examine whether a type I methanotroph could produce sMMO and degrade TCE, and a DNA probe developed from the methanotroph could specifically detect and monitor the population in a defined mixed culture degrading TCE. The model type I methanotroph was strain 68-1, which was isolated from a TCE-contaminated well. The defined mixed culture was composed of 68-1 and *Methylosinus trichosporium* OB3b, a type II methanotroph whose sMMO was extensively characterized biochemically and genetically. The results in this dissertation provide good evidence for the potential role of type I methanotrophs in TCE biodegradation and *in situ* TCE bioremediation.

The strain 68-1 was identified as *Methylomonas methanica* by pattern of intracytoplasmic membrane ultrastructure, 16S rRNA signature probe hybridizations, and PLFA and LPS-OHFA profiles. 68-1 could produce sMMO under copper-limiting condition, and oxidize naphthalene and degrade TCE more rapidly than *M. trichosporium* OB3b at room temperature. There was little genetic homology between the sMMO genes of 68-1 and OB3b, indicating the diversity of sMMO genes in methanotrophs. However, substrate specificity indicated a potential functional similarity between of the sMMOs of the type I and II methanotrophs. The putative sMMO gene fragment (4 kb) of 68-1 was cloned and partially characterized. The DNA fragment cross-hybridized to the genomes of a few pink-pigment type I methanotrophs (not producing sMMO) and a type X methanotroph (producing sMMO), indicating the conservation of the putative

gene at least within the RuMP pathway methanotrophs. The putative sMMO DNA probe was successfully used to monitor the 68-1 population in the mixed culture containing OB3b. The monitoring data were supported by plate count and 16S rDNA-targeting oligo probe hybridization results while AODC data generally complemented the DNA probe results with certain levels of error. The 68-1 population was out-competed by OB3b population in both flask and continuous cultures under copper-limiting condition. Other flask competition studies showed that 68-1 population was dominant in higher methane concentrations while OB3b population was always dominant regardless of copper or nitrate concentrations. By virtue of this OB3b could be more competitive than 68-1 in the nutrient-depleted (oligotrophic) environments found in nature. The putative sMMO DNA probe was successfully used to detect the potential homologous methanotrophic populations in TCE-contaminated subsurface environments. Therefore, the putative sMMO gene from 68-1, if confirmed, could be used to monitor the populations of the potential type I sMMO producers in the environment.

The issues which need to be addressed in the future studies include: (i) the factors involved in the higher naphthalene oxidation and TCE degradation activities of 68-1 cells despite its higher K_m values for the substrates compared with those of OB3b cells; (ii) the purification of the sMMO components of 68-1 and comparative analysis with other characterized sMMOs to obtain the essential information facilitating the molecular genetic study of the sMMO of type I methanotrophs; (iii) the proof or disproof of the putative sMMO gene fragment (4 kb) from 68-1 as a sMMO gene by DNA sequencing; (iv) the cloning of sMMO gene by screening the genomic DNA library using new probes (antibody probe

or oligo probes designed from the amino acid sequence of sMMO components of 68-1 in case of disproof at (iii); (v) the increase of detection sensitivity of the type I sMMO homologous genes by new techniques (*i.e.*, quantitative PCR); and (vi) the detection and monitoring of the potential sMMO-producing methanotrophs in environments and the use of the population data to optimize *in situ* TCE bioremediation.

Considering the critical role of microorganisms in *in situ* bioremediation, the detection of specific degradative genes and organisms, and monitoring and prediction of the degradation activity will be an essential process to perform a successful bioremediation. Therefore, the finding of sMMO activity in type I methanotrophs, and their gene probe development and its application will make more feasible the methanotrophic TCE bioremediation.

LIST OF REFERENCES

LIST OF REFERENCES

Allen, L. N., A. D. Olstein, C. L. Haber, and R. S. Hanson. p. 236-243. In R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds. American Society for Microbiology, Washington, D. C.

American Standard Test Method (ASTM). 1985. Simultaneous enumeration of total and respiring bacteria in aquatic systems by microscopy. ASTM Designation 4454-85:650-653.

Alvarez-Cohen, L., P. L. McCarty, E. Boulygina, R. S. Hanson, G. A. Brusseau, and H. C. Tsien. 1992. Characterization of a methane-utilizing bacterium from a bacterial consortium that rapidly degrades trichloroethylene and chloroform. Appl. Environ. Microbiol. 58:1886-1893.

Alvarez-Cohen, L. and P. L. McCarty. 1991a. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. 57:228-235.

Alvarez-Cohen, L. and P. L. McCarty. 1991b. Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells. Appl. Environ. Microbiol. 57:1031-1037.

Abramochinka, F. N., L. V. Bezrukova, A. V. Koshelev, V. F. Gal'chenko, and M. V. Ivanov. 1987. Microbial oxidation of methane in a body of freshwater. Microbiol. 56:375-382.

Ando, S., S.-I. Kato, and K. Komagata. 1989. Phylogenetic diversity of methanol-utilizing bacteria deduced from their 5S ribosomal RNA sequences. J. Gen. Appl. Microbiol. 35:351-361.

Anthony, C. 1986. Bacterial oxidation of methane and methanol. Adv. Microbial Physiol. 27:113-210.

Arciero, D., T. Vannelli, M. Logan and A.B. Hooper. 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomnas europaea*. Biochem. Biophy. Res. Comm. 159:640-643.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current protocols in molecular biology.

John Wiley and Sons, New York.

Barkay, T., R. R. Turner, A. VandeBrook, and C. Liebert. 1991. The relationships of Hg (II) volatilization from a freshwater pond to the abundances of *mer* genes in the gene pool of the indigenous microbial community. Microb. Ecol. 21:151-161.

Barrio-Lage, G., F. Z. Parsons, R.S. Nassar, and P. A. Lorenzo. 1986. Sequential dehalogenation of chlorinated ethenes. Environ. Sci. Technol. 20:96-99.

Blackburn, J. W., R. K. Jain, and G. S. Sayler. 1987. The molecular microbial ecology of a naphthalene-degrading genotype in activated sludge. Environ. Sci. Technol. 21:884-890.

Bone, T. L. and D. L. Balkwill. 1986. Improved floatation technique for microscopy if *in situ* soil and sediment microorganisms. Appl. Environ. Microbiol. 51:462-468.

Boxrukova, L. V., Y. I. Nikolenko, A. I. Nestrov, V. F. Gal'chenko, and M. V. Ivanov. 1983. Comparative serological analysis of methanotrophic bacteria. Microbiology 52:639.

Bowman, J. P., L. Jimenez, I. Rosario, T. C. Hazen, and G. S. Sayler. 1993 Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. Appl. Environ. Microbiol. 59:2380-2387.

Bowman, J. P., J. H. Skerratt, P. D. Nichols, and L. I. Sly. 1991. Phospholipid fatty acid and lipopolysccharide fatty acid signature lipids in methane-utilizing bacteria. FEMS Microbiol. Ecol. 85:15-22.

Bowman, J. P., L. I. Sly, J. M. Cox and A. C. Hayward. 1990. *Methylomonas fodinarum* sp. nov. and *Methylomonas aurantica* sp. nov.: two closely related type I obligate methanotrophs. Syst. Appl. Microbiol. 131:155-164.

Bowman, J. P., L. I. Sly, P. D. Nichols, and A. C. Hayward. 1993. Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. Internatl. J. Syst. Bateriol. 43:735-753.

Broholm, K., B. K. Jensen, T. H. Christensen and L. Olsen. 1990. Toxicity of 1,1,1-tichloroethane and trichloroethene on a mixed culture of methane-oxidizing bacteria. Appl. Environ. Microbiol. 56:2488-2493.

Brusseau, G. A., H. C. Tsien, R. S. Hanson, and L. P. Wackett. 1990. Optimization of trichloroethylene oxidation of by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1:19-29.

Brusseau, G. A., E. S. Bulygina, and R. S. Hanson. 1994. Phylogenetic analysis and development of probes for differentiating methylotrophic bacteria. Appl. Environ. Microbiol. 60:626-636.

Burrows, K. J., A. Cornish, D. Sott, and I. J. Higgins. 1984. Substrate specificities of the soluble and particulate methane mono-oxygenases of *Methylosinus trichosporium* OB3b. J. Gen. Microbiol. 130:3327-3333.

Cardy, D. N. L., V. Laidler, G. P. C. Salmond, and J. C. Murrell. 1991. Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylosinus trichosporium* OB3b. Mol. Microbiol. 5:335-342.

Chan, S. I., H. T. Nguyen, A. K. Shiemke and M. E. Lidstrom. 1993. Biochemical and biophysical studies toward chracterization of the membrane-associated methane monooxygenase, p. 93-107. In J. C. Murrell and D. P. Kelly (ed.), Microbial growth on C1 compounds, Itercept Ltd., Andover, United Kingdom.

Chaudhry, G. R. and S. Chapalamadugu. 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. 55:59-79.

Church, G. M. and W. Gilbert .1984. Genomic sequencing. Proc. Natl. Acad. Sci. 81:1991-1995.

Cicerone, R. and R. S. Oremland. 1988. Biogeochemical aspects of atmospheric methane. Global Biogeochem. Cycles 2:299-327.

Colby, J. and H. Dalton. 1976. Some properties of a soluble methane mono-oxygenase from *Methylococcus capsulatus* strain Bath. Biochem. J. 157:495-497.

Colby, J. and H. Dalton. 1978. Resolution of the methane mono-oxygenaseof *Methylococcus capsulatus* into three components. Biochem. J. 171:461-468.

Colby, J. and H. Dalton. 1979. Characterization of the second prothetic group of the flavoenzyme NADH-acceptor reductase (Component C) of the methane mono-oxygenase from *Methylococcus capsulatus* (Bath). Biochem. J. 177:903-908.

Colby, J. and H. Dalton and R. Whittenbury. 1979. Biological and biochemical aspects of microbial growth on C_1 compounds. Ann. Rev. Microbiol. 33:481-517.

Collins, M. L. P., L. A. Buchholz, and C. C. Remsen. 1991. Effect of copper on *Methylomonas albus* BG8. Appl. Environ. Microbiol. 57:1261-1264.

Cornish, A., K. M. Nicholls, D. Scott, B. K. Hunter, W. J. Aston, I. J. Higgins, and J. K. M. Sanders. 1984. In vitro ^{13}C-NMR investigations of methanol oxidation by the obligate methanotroph *Methylosinus trichosporium* OB3b. J. Gen. Microbiol. 130:2565-2575.

Craun, C. F. 1984. Health aspects of groundwater pollution, p. 135-179. In G. Bitton and C. P. Gerba (ed.) Groundwater pollution microbiology John Wiley and Sons, New York.

Daily, J. R. 1979. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations and comparative accuracy, p. 29-45. In J. W. Costerton and R. R. Cowell (ed.) Native aquatic bacteria: enumeration, activity and ecology ASTM, Philadelphia.

Dalton, H. 1992. Methane oxidation by methanotrophs: Physiological and mechanistic implications, p. 85-113. In J. C. Murrell and H. Dalton (ed.), Methane and methanol utilizers Plenum Press, New York.

Dalton, H. and D. J. Leak. 1985. Methane oxidation by microorganisms, p. 173-200. In S. R. K. Poole and C. S. Dow (ed.), Microbial gas metabolism Academic Press, London.

Dalton, H., S. D. Prior, and S. H. Stanley. 1984. Regulation and control of methane monooxygenase, p. 75-82. In R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds American Society for Microbiology, Washington, D. C.

Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophy. Res. Commun. 23:641-646.

DiSpirito, A. A., J. Gulledge, A. K. Shiemke, J. C. Murrell, M. E. Lidstrom, and C. L. Krema. 1992. Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs. Biodegradation 2:151-164.

Drummonds, D. and H. Dalton. 1989. Solubilization of methane monooxygenase from *Methylococcus caspsulatus* (Bath). Eur. J. Biochem. 182:667-671.

Dunkleblum, E., S. H. Tan, and R. J. Silk. 1985. Double-bond location in monounsaturated fatty acids by dimethyl disulfide derivatization and mass spectrometry: application to analysis of fatty acids in pheromone glands of four Lepidoptera. J. Chem. Ecol. 11:265-277.

Fathepure, B. Z. and S. A. Boyd. 1987. Anaerobic bacteria that dechlorinate perchloroethylene. Appl. Environ. Microbiol. 53:2671-2674.

Fitch, M. W., D. W. Graham, R. G. Arnold, S. K. Agarwal, P. Phelps, G. E. Speitel, Jr., and G. Georgiou. 1993. Phenotypic charaterization of copper-resistant mutants of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. 59:2771-2776.

Fliermans, C. B., T. J. Phelps, D. Ringelberg, A. T. Mikell and D. C. White. 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. Appl. Environ. Microbiol. 54:1709-1714.

Fliermans, C. B. and D. Balkwill.1989. Microbial life in deep terrestrial subsurfaces. Bioscience 39:370-377.

Fogel, M. M., A. R. Taddeo and S. Fogel. 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. 51:720-724.

Folsom, B. R., P. J. Chapman and P. H. Prichard. 1990. Phenol and trichloroethylene deradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. 56:1279-1285.

Foster, J. W. and R. H. Davis. 1966. A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. J. Bacteriol. 91:1924-1931.

Fox, B. G., J. G. Borneman, L. P. Wackett, and J. D. Lipscomb. 1990. Haloalkane oxidation by the soluble methane monooxygenase from

Methylosinus trichosporium OB3b: Mechanistic and environmental implications. Biochemistry 29:6419-6427.

Fox, B.G., W. A. Froland, J. E. Dege and J. D. Lipscomb. 1989. Methane monooxygenase from *Methylosinus trichosporium* OB3b: Purification and properties of a three-component system with specific activity a type II methanotroph. J. Biol. Chem. 264:10023-10033.

Fox, B. G. and J. D. Lipscomb. 1988. Purification of a high specific activity methane monooxygenase hydroxylase component from a type II methanotroph. Biochem. Biophy. Res. Comm. 154:165-170.

Freedman, D. L. and J. M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. 55:2144-2151.

Gal'chenko, V. F., F. N. Abramochinkia, L. V. Bezrukova, E. N. Sokolov, and M. V. Ivanov. 1988. Microbiology 47:248-253.

Ghiorse, W. C. and J. T. Wilson.1988. Microbial ecology of subsurface. Adv. Appl. Microbiol. 33:107-172.

Gold, T. J. 1979. Terrestrial sources of carbon and earthquake outgassing. J. Petrol. Geol. 1:13-19.

Graham, D. W., J. A. Chaudhary, R. S. Hanson, and R. G. Arnold. 1993. Factors affecting competition between type I and typeII methanotrophs in two-organism, continuous-flow reactors. Microbial. Ecol. 25:1-17.

Green, P. N. 1992. Taxonomy of methylotrophic bacteria, p. 23-84. In In J. C. Murrell, and H. Dalton (ed.), Methane and methanol utilizers Plenum Press, New York.

Green, J. and H. Dalton. 1985. Protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). J. Biol. Chem. 260:15795-15801

Green, J. and H. Dalton. 1986. Steady-state kinetic analysis of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). Biochem. J. 236:155-162.

Green, P. N. and H. Dalton. 1989. Substrate specificity of soluble methane monooxygenase: mechanistic implications. J. Biol. Chem. 264:17698-

17703.

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.

Guckert, J. B., D. B. Ringelberg, D. C. White, R. S. Hanson, and B. J. Bratina. 1991. Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the proteobacteria. J. Gen. Microbiol. 137:2631-2641.

Hanson, R. S. 1980. Ecology and diversity of methylotrophic organisms. Adv. Appl. Microbiol. 26:3-39.

Hanson, R. S. 1992. Introduction, p. 1-21. In J. C. Murrell and H. Dalton (ed.), Methane and methanol utilizers. Plenum Press, New York.

Hanson, R. S. and E. V. Wattenburg. 1991. Ecology of methylotrophic bacteria, p. 328-348. In I. Goldberg and J. S. Rokem (ed.), Biology of methylotrophs. Butterworth-Heinemann, Boston.

Hanson, R. S., A. I. Netrusov, and K. Tsuji. 1991. The obligate methanotrophic bacteria; *Methylococcus*, *Methylomonas*, *Methylosinus*, p. 661-684. In A. Balows, H. G. Truper, M. Dworkin, H. Harder, and K. H. Schleifer (ed.), The Procaryotes. Springer-Verlag, New York.

Harayama, S., M. Kok, and E. L. Neidle. 1992. Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. 46:565-601.

Henry, S. M. and D. Grbic-Galic. 1990. Effect of media on trichloroethylene oxidation by aquifer methanotrophs. Microbial. Ecol. 20:151-169.

Henry, S.M. and D. Grbic-Galic. 1991a. Influence of endogeneous and exogeneous electron donors and trichloroethylene oxidation on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. Appl. Environ. Microbiol. 57:236-244.

Henry, S.M. and D. Grbic-Galic. 1991b. Inhibition of trichloroethylene oxidation by the transformation intermediate carbon monoxide. Appl. Environ. Microbiol. 57:236-244.

Henschler, D., W.R. Hoos, H. Fetz, E. Dallmeier and M. Metzler. 1979. Reactions of trichloroethylene epoxide in aqueous systems. Biochem. Pharm. 28:543-548.

Henson, J. M., M. V. Yates, J. W. Cochran, and D. L. Shackleford. 1988. Microbial removal of halogenated methanes, ethanes, ethylenes in an aerobic soil exposed to methane. FEMS Microbiol. Ecol. 53:193-201.

Henson, J. M., M. V. Yates and J. W. Cochran. 1989. Metabolism of chlorinated methanes, ethanes, and ethylenes by a mixed bacterial culture growing on methane. J. Ind. Microbiol. 4:29-35.

Heyer, J. 1977. Results of enrichment experiments of methan-assimilating organisms from an ecological point of view, p. 19-21. In G. A. Skryabin, M. B. Ivanov, E. N. Kondratjeva, G. A. Zavarzin, Y. A. Trotsenko, and A. I. Ntrosov (ed.), Microbial growth on C_1 compounds, U.S.S.R. Academy of Sciences, Rusching, USSR.

Heyer, J. and R. Suchow. 1985. Okologische Untersuchungen der Methanoxidation in einem sauren Moorsee. Limnologica 6:247-266.

Higgins, I. J., R. C. Hammond, F. S. Sariaslani, D. Best, M.M. Davies, S. E. Tryhorn and F. Taylor. 1979. Biotransformation of hydrocarbons and related compounds by whlole organism suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem. Biophy. Res. Comm. 89:671-677.

Hirsch, P. R. 1979. Pasmid-determined bacteriocin production by *Rhizobium leguminosarum*. J. Gen. Microbiol. 113:219-228.

Hou, C. T., R. Patel, A. I. Laskin and N. Barnabe. 1979. Microbial oxidation of gaseous hydrocarbons: epoxidation of C_2 to C_4 *n*-alkenes by methylotrophic bacteria. Appl. Environ. Microbiol. 38:127-134.

Holt, J. G., M. A. Bruns, B. J. Caldwell, and C. D. Pease. 1992. Stedman's Bergey's bacteria words Williams and Wilkins Co., Baltimore.

Jenkins, O. and D. Jones. 1987. Taxonomic studies on some Gram-negative methylotrophic bacteria. J. Gen. Microbiol. 133:453-473.

Jensen, T. E. and W. A. Corpe. 1991. Ultrastructure of methylotrophic microorgnisms. p. 39-78. In I. Goldberg and J. S. Rokem (ed.), Biology of methylotrophs.Butterworth-Heineman, Boston.

Jimenez, L., I. Rosario, J. P. Bowman, S.-C. Koh, and G. S. Sayler. 1992. Molecular environmental diagnostics of trichloroethylene (TCE)-contaminated subsurface environments, p. 392-396. In R. E. Hinchee, A. Leeson, L. Semprini, and S. K. Ong (ed.), Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds. Lewis Publishers, Boca Raton.

Jones, J. G. 1974. Some observations on direct counts of freshwater bacteria obtained with a fluorescence microscope. Limnol. Oceanogr. 19:540-543.

Kastner, M. 1991. Reductive dechlorination of tri- and tetrachloroethylenes depends on transition from aerobic to anaerobic conditions. Appl. Environ. Microbiol. 57:2039-2046.

Lang, B. F. and G. Burger. 1990. A rapid, high resolution DNA sequencing gel system. Analytical Biochemistry 188:176-180.

Lanzarone, N. A. and P. L. McCarty. 1990. Column studies on methanotrophic degradation of trichloroethylene and 1,2-dichloroethane. Ground Water 28:910-919.

Lee, M. D., J. M. Thomas, R. C. Borden, P. B. Bedient, C. H. Ward, and J. T. Wilson. 1988. Biorestoration of aquifers contaminated with organic compounds. CRC Critical Rev. in Environ. Control. 18:29-89.

Lidstrom, M. E. 1988. Isolation and characterization of marine methnotrophs Ant. van Leew. 54:189-199.

Lidstrom, M.E. and D.I. Stirling. 1990. Methylotrophs: Genetics and commercial applications. Annu. Rev. Microbiol. 4:27-58.

Little, C. D., A. V. Palumbo, S. E. Herbes, M.E. Lidstrom, R. L. Tyndall, and P. J. Gilmer. 1988. Trichloroethylene biodgradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. 54:951-956.

Luft, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophysic. Biochem. Cytol. 9:409-414.

Machlin, S. M., P. E. Tam, C. A. Bastien, and R. S. Hanson. 1988. Genetic and physical analyses of *Methylobacterium organophilum* XX genes encoding methanol oxidation. J. Bacteriol. 170:141-148.

Mancinelli, R. L. and M. R. White. 1991. Methylotroph activity in soil permeated with methane. Abstr. Gen. Meet. Am. Soc. Microbiol. 1992:I-45

Mayer, K. P., D. Grbic-Galic, L. Semprini, and P. L. McCarty. 1988. Degradation of trichloroethylene by methanotrophic bacteria in a laboratory column of saturated aquifer material. Water Sci.Technol. 20:175-178.

McFeters, G. A., A. Singh, S. Byun, P. R. Callis, and S. Williams. 1991. Acridine orange staining reaction as an index of physiological acitvity in *Escherichia coli*. J. Microbiol. Meth. 13:87-97.

Meyer, J., R. Haubold, J. Heyer, and W. Bockel. 1986. Contribution to the taxonomy of methanotrophic bacteria: correlation between membrane type and GC-value. Z. Allg. Mikrobiol. 26:155-160.

Morgan, P. and R. J. Watkinson. 1989. Microbiological methods for the cleanup of soil and ground water contaminated with halogenated organic compounds. FEMS Microbiol Rev. 63:277-300.

Moyer, C. L. and R. Y. Morita. 1989. Effects of growth rate and starvation survival on cellular DNA, RNA and protein of a psychrophilic marine bacterium. Appl. Environ. Microbiol. 55:2710-2716.

Mullens, I.A. and H. Dalton. 1987. Cloning of the gamma-subunit methane monooxygenase from *Methylococcus capsulatus*. Bio/Technol. 5:490-493.

Munkres, K. D. and F. M. Richard. 1965. The purification and properties of Neurospora malate dehydrogenase. Arch. Biochem. Biophys. 109:466-479.

Murrell, J. C. 1992. Genetics and molecular biology of methanotrophs. FEMS Microbiol. Rev. 88:233-248.

Murrell, J. C. 1992. The genetics and molecular biology of obligate methane-oxidizing bacteria, p. 115-148. In J. C. Murrell, and H. Dalton (ed.), Methane and methanol utilizers Plenum Press, New York.

Murrell, J. C. and H. Dalton. 1983. Purification and properties of glutamine sythetase from *Methylococcus capsulatus* (Bath). J. Gen. Microbiol. 129:1187-1196.

Nakajima, T., H. Uchiyama, O. Yagi, and T. Nakahara. 1992. Purification and properties of a soluble methane monooxygenase from *Methylocystis* sp. M. Biosci. Biotech. Biochem. 56:736-740.

National Research Council. 1993. *In situ* bioremediation: when does it work? National Academic Press, Washington, D. C.

Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey and P. H. Prichard. 1987. Biodegradation of trichloroetylene and involvement of an aormatic biodegradative pathway. Appl. Environ. Microbiol. 53:949-954.

Nelson, M. J. K., S. O. Montgomery, E. J. Oneill and P. H. Prichard. 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environ. Microbiol. 52:383-384.

Nelson, M. J. K., S. O. Montgomery and P. H. Prichard. 1988. Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. 54:604-606.

Nichols, P. D., G. A. Smith, C. P. Antworth, R. S. Hanson and D. C. White. 1985. Phospholipid and polysaccharide normal and hydroxy fatty acids as potential signatures for methane-oxidizing bacteria. FEMS Microbiol. Ecol. 32:327-335.

Nichols, P. D., J. B. Guckert, and D. C. White. 1986. Determination of monosaturated bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyldisulfide adducts. J. Microbiol. Meth. 5:49-55.

Nichols, P. D., J. M. Henson, C. P. Antworth, J. Parsons, J. T. Wilson and D. C. White. 1987. Detection of a microbial consortium, including type II methanotrophs, by use of phospholipid fatty acids in an aerobic halogenated hydrocarbon-degrading soil column enriched with natural gas. Env. Toxicol. Chem. 6:89-97.

Nunn, D. N. and M. E. Lidstrom. 1986a. Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. J. Bacteriol. 166:581-590.

Nunn, D. N. and M. E. Lidstrom. 1986b. Phenotypic characterization of 10 methanol oxidation mutant classes in *Methylobacterium* sp. strain AM1. J. Bacteriol. 166:590-597.

Oakley, C. J. and J. C. Murrell. 1988. *nifH* genes in the obligate methane-oxidizing bacteria. FEMS Microbiol. Lett. 49:53-57.

Oelmuller, U., N. Kruger, A. Steinbuchel, and C. G. Friedrich. 1990. Isolation of prokaryotic RNA and detection of specific mRNAm with biotinylated probes. J. Microbiol. Meth.11:73-84.

Ogram, A., G. S. Sayler and T. Barkay. 1987. The extraction and purification of microbial DNA from sediments. J. Microbiol. Meth. 7:57-66.

Ogram, A.V. and G.S. Sayler. 1988. The use of gene probes in the rapid analysis of natural microbial communities. J. Ind. Microbiol. 3:281-292.

Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt. 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55:2819-2826.

Oldenhuis, R., J. Y. Oedzes, J. J. van der Waarde and D. B. Jassen. 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. 57:7-14.

Oldenhuis, R., R. L. J. M. Vink, D. B. Jassen and B. Witholt. 1990. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55:2819-2826.

Park, S., M. L. Hanna, R. T. Taylor, and M. W. Droege. 1991. Batch cultivation of *Methylosinus trichosporium* OB3b. Biotechnol. and Bioeng. 38:243-433.

Patel, R.N., C.T. Hou, A.I. Laskin, A. Felix and P. Derelanko. 1979. Microbial oxidation of gaseous hydrocarbons. II. Hydroxylation of alkanes and epoxydation of alkenes by cell-free particulate fractions of methane-utilizing bacteria. J. Bacteriol. 139:675-679.

Patel, R. N., C. T. Hou, and A. I. Laskin. 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.

Patel, R. N. 1984. Methane monooxygenase from *Methylobacterium* sp. strain CRL-26, p. 83-90. In R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds. American Society for Microbiology, Washington, D. C.

Patel, R. N. and J. C. Savas. 1987. Purification and properties of the hydroxylase component of methane monooxygenase. J. Bacteriol. 169:2313-2317.

Pavlostathis, S G. and P. Zhuang. 1991. Transformation of trichloroethylene by sulfate-reducing cultures enriched from a contaminated subsurface soil. Appl. Microbiol. Biotechnol. 36:416-420.

Phelps, P. A., S. K. Agarwal, G. E. Speitel, Jr., and G. Georgiou. 1992. *Methylosinus trichopsorium* OB3b mutants having constitutive expression of soluble methane monooxygenase in the presence of high levels of copper. Appl. Environ. Microbiol. 58:3701-3708.

Pilkington, S.J., G.P.C. Salmond, J.C. Murrell and H. Dalton. 1990. Identification of the gene encoding the regulatory protein B of soluble methane monooxygenase. FEMS Microbiol. Lett. 72:345-348.

Pirt, S. J. 1975. Priciples of microbes and cell cultivation. Wiley and Sons, New York.

Prior, S. D. and H. Dalton.1985. The effect of copper ions on the membrane content and methane monooxygenase acitvity in methanol grown cells of *Methylococcus capsulatus* (Bath). J. Gen. Microbiol.131:155-163.

Putzer, K. P., L. A. Buchholz, M. E. Lidstrom, and C. C. Remsen. 1991. Separation of methanotrophic bacteria by using percoll and its application to isolation of mixed and pure cultures. Appl. Environ. Microbiol. 57:3656-3659.

Reed W. M. and P. R. Dugan. 1978. Distribution of *Methylomonas methanica* and *Methylosinus trichosporium* in Cleveland harbor as determined by an indirect fluorescent antibody-membrane filter technique. Appl. Environ. Micobiol. 35:422-430.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:202-212.

Richardson, K. L. and D. T. Gibson. 1984. A novel pathway for toluene oxidation in *Pseudomonas mendocina*. Abstr. Gen. Meet. Am. Soc. Microbiol. 1984:K54.

Rittman, B. E., B. E. Smets and D. A. Stahl. 1990. The role of genes in biological processes. Environ. Sci. Technol. 24:23-29.

Roberts, P. V., J. E. Schreiner, and G. D. Hopkins.1982. Field study of organic water quality changes during groundwater recharge in the Palo Alto baylands. Water Res. 16:1025.

Rudd, J. W. M. and R. D. Hamilton. 1978. Methane cycling in a eutrophic shield lake and its effects on whole lake water. Limnol. Oceanogr. 19:519-524.

Rudd, J. W. M. and C. D. Tayler. 1980. Methane cycling in aquatic environments. Adv. Aquat. Microbiol. 2:77-150.

Russell, P., M. G. Schell, K. K. Nelson, L. J. Halverson, K. M. Sirotkin, and G. Stacey. 1985. Isolation and characterization of the DNA region encoding nodulation functions in *Bradyrhizobium japonicum*. J. Bacteriol. 164:1301-1308.

Sambrook, J., E .F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc.Natl. Acad. Sci. 74:5463.

Saralov, A. I., I. N. Krylova, E. E. Saralova, S. I. Kuznetsov. 1985. Distribution and species composition of methane oxidizing bacteria in lake water. Microbiology 53:695-701.

Sayler, G. S., M. S. Shields, E. T. Tedford, S. W. Hooper, K. M. Sirotkin, and J. W. Davis. 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Appl. Environ. Microbiol. 49:1295-1301.

Schaumburg, F. D. 1990. Banning trichloroethylene: responsible reaction or overkill?. Environ. Sci. Technol. 24:17-22.

Scott, D., J. Brannan and I. J. Higgins. 1981. The effect of growth

conditions on intracytoplasmic membranes and methane mono-oxygenase activities in *Methylosinus trichosoprium* OB3b. J. Gen. Microbiol. 125:63-72.

Semprini, L., P. V. Robers, G. D. Hopkins and P. L. McCarty. 1990. A field evaluation of in-situ biodgradation of chlorinated ethenes:part 2, results of biostimulation and biotransformation experiments. Ground Water 28:715-727.

Shackleford, L. 1988. Microbial removal of halogenated methanes, ethanes, and ethylenesin an aerobic soil exposed to methane. FEMS Microbiol. Ecol. 53:193-201.

Shields, M. S., S. O. Montgomery, P. J. Chapman, S. M. Cuskey and P. H. Prichard. 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl. Environ. Microbiol. 55:1624-1629.

Smith, R. L., B. L. Howes, and S. P. Garabedian. 1991. *In situ* measurement of methane oxidation in groundwater by using natural-gradient tracer tests. Appl. Environ. Microbiol. 57:1997-12004.

Stainthorpe, A. C., V. Lees, G. P. C. Salmond, H. Dalton and J. C. Murrell. 1990a. The methane monooxygenase gene cluster of *Methylococcus capsulatus* (Bath). Gene 91:27-34.

Stainthorpe, A. C., J. C. Murrell, G. P. C. Salmond, H. Dalton and V. Lees. 1989. Molecular analysis of methane monooxygenase from *Methylococcus capsulatus* (Bath). Arch. Microbiol. 152:154-159.

Stainthorpe, A. C., G. P. C. Salmond, H. Dalton, and J. C. Murrell. 1990b. Screening of obligate methanotrophs for soluble methane monooxygenase genes. FEMS Microbiol. Lett. 70:211-216.

Stanley, S.H., S.D. Prior, D.J. Leak and H. Dalton. 1983. Copper stress underlies the fundamental changes in intracellular location of methane monooxygenase in methane-oxidiztion organisms: studies in batch and continuous cultures. Biotech. Lett. 5:487-492.

Steffan, R. J., A Breen, R. M. Atlas, and G. S. Sayler. 1989. Application of gene probe methods for monitoring specific microbial populations in freshwater ecosystems. Can. J. Microbiol. 35:681-685.

Stephens, R. L., M. G. Haygood, and M. E. Lidstrom. Identification of putative methanol dehydrogenase (*mox*F) structural genes in methylotrophs and cloning of moxf genes from *Methylococcus capsulatus* (Bath) and *Methylomonas albus* BG8. J. Bacteriol. 170:2063-2069.

Stirling, D. I., J. Colby and H. Dalton. 1979. A comparison of the substrate and electron-donor specificities of the methane mono-oxygenases from three strains of methane-oxidizing bacteria. Biochem. J. 177:361-364.

Stirling, D.I. and H. Dalton. 1977. Effect of metal-binding agenets and other compounds on methane oxidation by two strains of *Methylococcus capsualtus*. Arch. Microbiol. 114:71-76.

Strandberg, G.W., T. L. Donaldson, and L. L. Farr. 1989. Degradation of trichloroethylene and trans-1,2-dichloroethylene by a methanotrophic consortium in a fixed-film packed-bed bioreactor. Environ. Sci. Technol. 23:1422-1425.

Takeda, K. and K. Tanaka. 1980. Ultrastructure of intracytoplasmic membranes of *Methanomonas margaritae* cells grown under different conditions. Antonie van Leeuwenhoek 46:15-25.

Tonge, G. M., D. E. F. Harrison and I. J. Higgins. 1977. Purification and properties of the methane mono-oxygenase enzyme system from *Methylosinus trichosporium* OB3b. Biochem. J. 161:333-344.

Tonge, G. M., D. E. F. Harrison, C. J. Knowles and I.J. Higgins. 1975. Properties and partial purification of the methane-oxidizing enzyme system from *Methylococcus trichosporium*. FEBS Letters 58:293-299.

Tsien, H. C., B. J. Bratina, K. Tsuji, and R. S. Hanson. 1990. Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. Appl. Environ. Microbiol. 56:2858-2865.

Tsien, H. C., G. A. Brusseau, R. S. Hanson, and L. P. Wackett. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. 55:3155-3161.

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.

Tsuji, K., H.C. Tsien, R.S. Hanson, S.R. DePalma, R. Scholtz and S. LaRoche. 1990. 16S ribosomal RNA sequence analysis for determination for phylogenetic relationship among methylotrophs. J. Gen. Microbiol. 136:1-10.

Tyler, S. C. 1991. The global methane budget, p. 7-38. In J. E. Rogers and W. B. Whitman (ed.), Microbial production and consumption of greenhouse gases: methan, nitrogen oxides, and halomethanes. American Society for MIcrobiology, Washington, D. C.

Uchiyama, H., T. Nakajima, O. Yagi and T. Tabuchi. 1989. Aerobic degradation of trichloroethylene by a new type II methane-utilizing bacterium, strain M. Agric. Biol. Chem. 53:2903-2907.

U. S. Environmental Protection Agency. 1979. Status of toxic chemicals: trichloroethylene. EPA-600/2-79-210m U. S. Environmental Protection Agency, Washington, D. C.

U. S. Environmental Protection Agency. 1985a. Substances found at proposed and final NPL sites through update number three. Document NPL-U3-6-3 U. S. Environmental Protection Agency, Washington, D. C.

U. S. Environmental Protection Agency. 1985b. National primary drinking water regulations: volatile synthaetic organic chemicals:fianl rule. Fed. Reg. 50: 46880-46901.

Van Es, F. B. and L. A. Meyer-Reil. 1982. Biomass and metabolic activity of heterotrophic marine bacteria, p. 111-170. In K. C. Marshall (ed.) Advances in microbial ecology vol. 6, Plenum Press, New York.

Verschueren, K. 1977. Handbook of environmental data on organic chemicals Van Nostrand Reinhold Co., N. Y.

Vogel, T. and P. L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl clororide, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49:1080-1083.

Wackett, L. P., and D. T. Gibson. 1983. Rapid method for detection and quantitation of hydroxylated aromatic intermediates produced by microorganisms. Appl. Environ. Microbiol. 45:1144-1147.

Wackett, L. P. and D. T. Gibson. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1.

Appl. Environ. Microbiol. 54:1703-1708.

Wackett, L. P., G. A. Brusseau, S. R. Householder, and R. S. Hanson. 1989. Survey of microbial oxygenases: richloroethylene degradation by propane oxidizing bacteria. Appl. Environ. Microbiol. 55:2960-2964.

Wackett, L. P. and S. R. Householder. 1989. Toxicity of trichloroethylene to *Pseudomnas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol. 55:2723-2725.

Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals J. Biophys. Biochem. Cytol. 4:475-478.

Westrick, J. J., J. W. Mello, and R. F. Thomas. 1984. The groundwater survey. J. Amer. Water Works Assoc. 5:52-59.

White, D. C., R. J. Bobbie, J. S. Herron, J. D. King, and S. J. Morrison. 1979. Biochemical measurements of microbial biomass and activity from environmental samples, p. 69-81. In Native aquatic bacteria: enumeration, activity and ecology ASTM STP695, American Society for Testing and Material, Philadelphia.

Whittenbury, R., J. Colby, H. Dalton, and H. L. Reed. 1976. Biology and ecology of methane oxidizers, p. 281-292. In H. G. Schlegel, G. Gottschalk, and N. Pfennig (ed.), Microbial production and utilization of gases. E. Goltze KG, Gottingen, Germany.

Whittenbury, R. and N. R. Krieg. 1984. Methylococcaceae fam. nov., p. 256-261. In N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.

Whittenbury, R., K. C. Philips, and J. F. Wilkinson. 1970. Enrichment, isolation and some properties of methane utilizing bacteria. J. Gen. Microbiol. 61:205-218.

Williams, E. and B. W. Bainbridge. 1971. Genetic transformation in *Methylococcus capsulatus*. J. Appl. Bacteriol. 34:683-687.

Williams, E., M. A. Shimmin, and B. W. Bainbridge. 1977. Mutation in obligate methylotrophs *Methylococcus capsulatus* and *Methylococcus albus*. FEMS Microbiol. Lett. 2:293-296.

Wilson, J. T. and B. H. Wilson. 1985. Biotransformation of trichloroethylenein soil. Appl. Environ. Microbiol. 49:242-243.

Winter, R. B., K.-M. Yen, and B. D. Ensley. 1989. Efficient degradation of trichloroethylene by a recombinant *E. coli.* Bio/Technol. (March):282-285.

Woodland, M. P. and H. Dalton. 1984. Purification and characterization of the methane monooxygenase from *Methylococccus capsulatus* (Bath). J. Biol. Chem. 259: 53-59.

Zylstra, G.J. and D.T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in E. coli. J. Biol.Chem. 264:14940-14946.

Zylstra, G.J., W.R. McCombie, D.T. Gibson and B.A. Finette. 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. Appl. Env. Microbiol. 54:1498-1503.

Zylstra, G.J., L.P. Wackett and D.T. Gibson. 1989. Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. Appl. Environ. Microbiol. 55:3162-3166.

VITA

Sung-Cheol Koh was born on August 13, 1956 in Taegu, Republic of Korea. He attended Busock High School, where he graduated in 1975. In the spring of 1976, Sung-Cheol enrolled with high distinction at College of Agriculture, Seoul National University, Suwon, Republic of Korea. He graduated from the Seoul National University in 1980 with a Bachelor of Science degree in Agronomy. Following the undergraduate training, Sung-Cheol enrolled in the Plant Pathology major in the Department of Agricultural Biology, Seoul National University, Suwon, Republic of Korea, where he received the Mater of Science degree in Plant pathology in February 1983. Following academic training, he was employed by the Research and Technology Institute of Pacific Chemical Company, Inc., Seoul, Republic of Korea, where he served as a researcher in industrial microbiology until 1987. He joined the Department of Microbiology, University of Tennessee, Knoxville in the fall of 1987, and later the Graduate Program in Ecology at the University in the summer of 1992. Sung-Cheol was a member of the Korean Society of Plant Protection and the Korean Society of Applied Microbiology and Bioengineering. He is a member of the American Society for Microbiology and the Association of Korean Scientists and Engineers since 1988.