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**CHARACTERIZATION AND POPULATION DYNAMICS OF  
TOLUENE-DEGRADING BACTERIA IN A  
CONTAMINATED FRESHWATER STREAM**

by

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## ABSTRACT

The impact of toluene contamination on the microbiology of the East Drainage Ditch was investigated. Toluene in this stream arises from a source of subsurface contamination, and streamwater concentrations range up to 6  $\mu\text{M}$ . Earlier stream studies had demonstrated biodegradation as the largest sink for toluene, with sediment- and rock surface-attached microorganisms accounting for most of the biodegradation.

Several aerobic toluene-degrading bacterial strains were isolated from rock surface biofilms, and four were selected for characterization. Strains T103 and T104 are Gram-positive and acid-alcohol-fast, with identical 16S rDNA sequences most similar to those of *Mycobacterium aurum* and *M. komossense*. They possess tuberculostearic acid, and fatty acid analyses indicate that they are not identical strains but related at the subspecies level. They constitute a new species of fast-growing mycobacteria. T101 and T102 are Gram-negative, produce yellow pigments, and can also degrade benzene. They share identical 16S rDNA sequences with *Xanthobacter autotrophicus*, and possess high levels of *cis*-11-octadecenoic acid and *cis*-9-hexadecenoic acid. Fatty acid analyses indicate that T101 and T102 are different but closely related strains.

Maximal velocity and half-saturation constant estimates revealed a fair diversity of toluene biodegradation kinetics among the four strains, although they were isolated under identical laboratory conditions. Comparisons with biodegradation kinetics of rock biofilms under batch conditions suggest that T102 may be a major contributor to toluene biodegradation in the stream.

Relative distributions of the toluene-degrading *X. autotrophicus* and *Mycobacterium sp.* were assessed in rock surface biofilms sampled over a period of one year. Quantitative PCR and slot-blot hybridization results revealed that these indigenous species are significantly more abundant in a contaminated reach than in a pristine reach, and more abundant in both reaches in summer months than in winter months. These results are consistent with earlier studies which showed higher biodegradation rates in contaminated stream reaches in summer months than in winter months, and higher plate counts and MPNs of toluene-degraders in contaminated reaches than in pristine reaches. Populations of these toluene-degrading bacterial species in the stream were observed to correlate with toluene presence, and with warmer temperatures.

Thesis Supervisor: Prof. Harold F. Hemond  
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## TABLE OF CONTENTS

ABSTRACT .....	3
ACKNOWLEDGMENTS .....	4
INTRODUCTION .....	11
References .....	14
CHAPTER ONE:           LITERATURE REVIEW .....	15
Toluene in the environment .....	16
Microbial communities in contaminated ecosystems .....	18
Molecular analyses of natural microbial communities .....	20
References .....	24
CHAPTER TWO:    TWO NEW <i>MYCOBACTERIUM</i> STRAINS .....	27
AND THEIR ROLE IN TOLUENE DEGRADATION	
IN A CONTAMINATED STREAM	
Abstract .....	28
Introduction .....	29
Materials and methods .....	30
Results .....	38
Discussion .....	43
Acknowledgments .....	50
References .....	66
CHAPTER THREE:  IMPORTANCE OF <i>XANTHOBACTER</i> .....	73
<i>AUTOTROPHICUS</i> IN TOLUENE BIODEGRADATION	
WITHIN A CONTAMINATED STREAM	
Abstract .....	74
Introduction .....	75
Materials and methods .....	76
Results .....	81
Discussion .....	85
Acknowledgments .....	89
References .....	102
CHAPTER FOUR:   DISTRIBUTION AND POPULATION .....	107
DYNAMICS OF TOLUENE-DEGRADING SPECIES	
IN A CONTAMINATED STREAM ASSESSED BY	
QUANTITATIVE PCR	

Abstract .....	108
Introduction .....	109
Materials and methods .....	111
Results .....	119
Discussion .....	123
Acknowledgments .....	128
References .....	141
CHAPTER FIVE: CONCLUDING REMARKS .....	147
<i>Mycobacterium</i> and <i>Xanthobacter</i> in the environment .....	149
Future research .....	152
References .....	156
APPENDIX A: ANALYSIS OF RESTRICTION FRAGMENT LENGTH POLYMORPHISMS FROM STATION D5 .....	162
Results .....	163
APPENDIX B: USE OF SLOT-BLOT HYBRIDIZATIONS TO QUANTIFY SPECIES-SPECIFIC NUCLEIC ACIDS IN NATURAL SAMPLES .....	169
Methods .....	170
Results .....	171
References .....	172

## LIST OF FIGURES

### CHAPTER ONE

- Figure 1. Catabolic pathways for the aerobic degradation of toluene. ....23

### CHAPTER TWO

- Figure 1. Scanning electron micrograph of toluene degrading .....52  
strain T103 grown on solid media (bar, 1  $\mu\text{m}$ ).
- Figure 2. Dendrogram of selected *Mycobacterium* strains generated .....54  
by cluster analysis of fatty acid profiles.
- Figure 3. Unrooted evolutionary distance tree based on the .....56  
16S rDNA sequences of strains T103 and T104, representative  
members of the *Mycobacterium* genus, and other high  
G+C Gram-positive bacteria.
- Figure 4. Toluene biodegradation kinetics for strains T103 and T104. ....58
- Figure 5. Scanning electron micrographs of biofilms of East Drainage .....60  
Ditch rocks.
- Figure 6. Bacterial counts (sampled on September 7 1993) and .....62  
toluene levels (sampled on September 19 1993) along  
the East Drainage Ditch.

### CHAPTER THREE

- Figure 1. Scanning electron micrograph of toluene-degrading .....91  
strain T101 grown on solid media (bar, 1  $\mu\text{m}$ ).
- Figure 2. Dendrogram of selected *Xanthobacter* strains generated by .....93  
cluster analysis of fatty acid profiles to produce unweighted  
pair matchings.
- Figure 3. Unrooted evolutionary distance tree based on the 16S rDNA .....95  
sequences of strains T101 and T102, representative members  
of the *Xanthobacter* genus, and other representative members  
of the alpha subdivision of *Proteobacteria*.
- Figure 4. Toluene biodegradation kinetics for strains T101 and T102. ....97

## CHAPTER FOUR

- Figure 1. Time profiles of toluene concentration, dissolved organic carbon content, and streamwater temperature at stations U50 and D5. ....130
- Figure 2. Amplification rates of DNAs. (A) Amplification rates of target (*X. autotrophicus*) and control (*M. thermoautotrophicum*) DNAs in standard and environmental mixtures; (B) Amplification rates of target (*Mycobacterium sp.*) and control (*M. thermoautotrophicum*) DNAs in standard and environmental mixtures. ....132
- Figure 3. Standard curve of QPCR of *Mycobacterium sp.* 16S rRNA genes. The relative masses of amplification products corresponding to target (*Mycobacterium sp.*) and control (*M. thermoautotrophicum*) were quantified by liquid scintillation counting and used to construct the standard curve. [MY], counts per minute associated with *Mycobacterium sp.* amplification products; [MT], counts per minute associated with *M. thermoautotrophicum* amplification products; [Input MY], pg of *Mycobacterium sp.* nucleic acid standard in PCR reaction. ....135
- Figure 4. Quantitative slot-blot hybridization and QPCR results (A) Mass equivalents of *X. autotrophicus* and *Mycobacterium sp.* nucleic acids at stations U50 and D5 estimated from QPCR, normalized to total nucleic acids estimated from quantitative slot-blot hybridization. (B) Cell densities of *X. autotrophicus* and *Mycobacterium sp.* at stations U50 and D5, normalized to rock surface area. Cell densities were derived after taking into account losses from extraction and purification. ....137

## APPENDIX A

- Figure 1. Restriction patterns of PCR-amplified 16S rRNA genes digested with *Pst*I and *Rsa*I. ....165
- Figure 2. Distribution of different types of restriction fragment length polymorphisms of 16S rRNA genes. ....167

## APPENDIX B

- Figure 1. Schematic diagram depicting the layout of nucleic acid extracts of standards and station D5 samples. ....174

Figure 2. Determination of  $T_d$  for the MY1003 probe using total nucleic acids extracted from pure cultures of *Mycobacterium sp.* .....176

Figure 3. Determination of  $T_d$  for the X1260 probe using total nucleic acids extracted from pure cultures of *X. autotrophicus.* .....178

Figure 4. Profiles of amounts of nucleic acids detected with the MY1003, X1260, and Eub338 probes at station D5. ....180

## LIST OF TABLES

### CHAPTER TWO

Table 1.	Characteristics of toluene degrading strains T103 and T104. ....	63
Table 2.	Whole-cell fatty acid compositions of strains T103, T104, ..... <i>M. komossense</i> , and <i>M. aurum</i> .	64
Table 3.	16S rRNA identity/distance matrix for T103/T104 and ..... related taxa.	65

### CHAPTER THREE

Table 1.	Characteristics of toluene degrading strains T101 and T102. ....	98
Table 2.	Whole-cell fatty acid compositions of strains T101, T102, ..... and <i>Xanthobacter autotrophicus</i> .	99
Table 3.	16S rRNA identity/distance matrix for T101/T102 ..... and related taxa.	100
Table 4.	Toluene biodegradation kinetics of pure bacterial cultures. ....	101

### CHAPTER FOUR

Table 1.	QPCR primers. ....	138
Table 2.	Cell counts in the East Drainage Ditch. ....	139
Table 3.	Total nucleic acids, <i>X. autotrophicus</i> nucleic acids, and ..... <i>Mycobacterium sp.</i> nucleic acids at stations U50 and D5.	140

### APPENDIX A

Table 1.	RFLP analysis of PCR-amplified 16S rRNA genes from ..... station D5.	168
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## **INTRODUCTION**

From a quantitative point of view, microorganisms play an important role in the degradation of organic compounds in the environment. Microorganisms are particularly suitable agents for such biodegradation because of their small size, ubiquitous distribution, high specific surface area, potentially high rate of metabolic activity, genetic malleability, potentially rapidly growth rate, and unrivaled enzymatic and nutritional versatility (Madsen, 1997). The types of microorganisms that may reside at any one place and time are dependent on the conditions of their environment. Microorganisms may perceive the introduction of organic chemicals into the environment as either new nutritional opportunities or toxic threats, and it is expected that microbial communities will adapt in response to unfamiliar compounds in their milieu. The physiological and genetic studies that comprise this thesis have been designed to obtain information on the types and distributions of toluene-degrading microorganisms living in the toluene-contaminated East Drainage Ditch, a freshwater stream located in the Aberjona Watershed.

Chapter One of this thesis is a literature review, and discusses relevant background information regarding toluene in the environment, microbial communities in contaminated ecosystems, and the use of molecular tools to study microbial ecology.

An earlier study had demonstrated that biodegradation is the most significant sink for toluene in the stream (Kim, 1995). We successfully isolated several strains of toluene-degrading bacteria from a contaminated reach of the stream, and these strains have been identified from 16S rDNA analyses as belonging to *Xanthobacter autotrophicus* and a novel *Mycobacterium sp.* Chapter Two details the isolation and

characterization of *Mycobacterium sp.* strains T103 and T104, while Chapter Three describes *X. autotrophicus* strains T101 and T102.

Chapter Four describes the use of quantitative PCR and slot-blot hybridization assays to assess the relative distributions of the two indigenous species of toluene-degrading bacteria in the stream. The findings were consistent with earlier studies, in which plate counts and most probable numbers of the toluene-degrading population were more significant at contaminated reaches of the stream than at pristine reaches (DeJesus, 1994), and in which biodegradation rates were higher in summer than in winter months (Kim *et al.*, 1995). The results lead us to a better understanding of the factors that influence the natural distributions of these bacteria, and give us a deeper appreciation of the intrinsic capacity of the indigenous microbial community to adapt to the introduction of contaminants in their environment.

Appendix A details the construction and analysis of a clone library of 16S rRNA gene sequences obtained from a contaminated reach of the stream. One of the clones had a restriction pattern identical to those of T103 and T104. Appendix B describes an unsuccessful attempt to quantify distributions of the *X. autotrophicus* and *Mycobacterium sp.* using quantitative hybridization; the hybridization signals detected were at or below the background levels present in the assay.

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## **Chapter One**

### **LITERATURE REVIEW**

## TOLUENE IN THE ENVIRONMENT

Toluene is among the 50 largest-volume industrial chemicals produced, with production figures of the order of millions of tons per year (Smith, 1990). It is a clear and colorless liquid, and has a sweet smell. It is produced from petroleum refining, and as a by-product in styrene production and coke-oven operations. Toluene has many industrial uses, and it is widely used in refining gasoline, in chemical manufacturing, in printing and leather tanning, and in the manufacture of paints, lacquers, rubber, and adhesives. Many consumer products also contain toluene, and these include gasoline, nail polish, cosmetics, rubber, cement, paint brush cleaners, stain removers, fabric dyes, and inks. In addition, cigarette smoke and automobile exhaust are sources of toluene emission to the atmosphere.

Because toluene has many industrial applications, it has also been found to be a common water contaminant in the vicinity of chemical waste sites. Toluene is usually disposed of at hazardous waste sites as a used solvent, and it occurs at measurable levels in about 54% of groundwater samples and 28% of surface water samples (U.S. Public Health Service, 1989). The mean concentrations of toluene in groundwater and surface water samples were 0.2 and 0.1  $\mu\text{M}$ , respectively. Drinking water can pose a potential health hazard if it is contaminated with toluene. The most important health concern for humans from exposure to toluene is its harmful effects on the nervous system. Short-term exposures to moderate amounts of toluene can result in fatigue, confusion, general weakness, drunken-type actions, memory loss, nausea, and loss of appetite. Long-term

exposures to high amounts of toluene have been shown to lead to permanent brain damage. Other effects such as loss of memory, loss of muscle control, and problems with speech, vision, and hearing have also been reported. Although toluene has not been shown to be carcinogenic by itself, it is an enhancing agent in skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene (Dean, 1978).

Degradation of toluene in water occurs primarily by microbial action. The microbial degradation of toluene has the potential to be rapid, provided that a suitable terminal electron acceptor is available for its oxidation. Half-lives of less than one day under favorable conditions have been reported (Wakeham *et al.*, 1983). Toluene is aerobically biodegraded by both ring attack and methyl-group hydroxylation (Smith, 1990). Figure 1 shows the five different degradation pathways exist for toluene (Zylstra, 1994). For example, toluene is degraded via catechol and subsequently the meta pathway by several strains of *Pseudomonas* by enzymes encoded on TOL plasmids (Nakazawa *et al.*, 1980; Franklin *et al.*, 1981). These plasmids often contain two catabolic operons. The ‘upper’ pathway operon encodes enzymes for the successive oxidation of toluene to the corresponding alcohol, aldehyde, and carboxylic acid derivatives. The ‘lower’ or meta-cleavage pathway operon encodes enzymes for the conversion of the carboxylic acids to catechols, whose aromatic rings are then cleaved (meta-fission) to produce the corresponding semialdehydes, which are in turn catabolised through the TCA cycle (Ramos *et al.*, 1987). *Burkholderia sp.* strain JS150 is unique in its ability to use multiple pathways for toluene degradation. It has been reported to synthesize four ring fission (lower) pathways and three distinct dioxygenases for the initial oxidation of substituted benzenes (Johnson and Olsen, 1997). This multiplicity of pathways enables strain JS150

to grow, not just with toluene, but also with benzene, ethylbenzene, halogen-substituted benzenes, and naphthalene as sole carbon sources.

Although many pure cultures that can degrade toluene have been isolated and extensively studied, it is not often apparent whether the microorganisms that have been isolated in the laboratory are actually carrying out the degradation reactions in the environment. It is also not clear how these microorganisms are distributed in their natural environments, and what factors influence their occurrence. A particular bacterium may dominate an enrichment culture or grow more easily in pure culture than the microorganisms responsible for the degradation process. Identifying the microorganisms involved in the degradation process in-situ will lead us to the most active cultures for potential bioremediation studies.

## **MICROBIAL COMMUNITIES IN CONTAMINATED ECOSYSTEMS**

Degradation of organic chemicals at hazardous waste sites by the indigenous microflora is an important process in the removal of contaminants. In some cases, biodegradation may be the only important process that can completely remove the chemical pollutants. Observations in the field suggest that natural assemblages of indigenous microorganisms possess an innate capacity for bioremediation. In some cases, adaptation of microorganisms to pollutants may be required before degradation can occur. For example, Spain *et al.* (1984) found that increases in the p-nitrophenol-degrading communities in a freshwater pond correlated with increases in biodegradation rates even though the total bacterial community did not change. They suggested that adaptation was

the result of selection of organisms able to grow at the expense of p-nitrophenol. During a long exposure period, it seems possible that a change in the community may occur that selects for a certain portion of the community and allows the number of degraders to increase.

Pignatello *et al.* (1983) studied the fate of pentachlorophenol (PCP) in man-made channels at a field site on the Mississippi River near Monticello, Minnesota. The PCP was degraded by both abiotic (primarily photolysis) and biotic (microbial degradation) processes. The biotic removal required a moderately long adaptive response by the aquatic microflora, but eventually became the predominant mechanism of PCP removal from the system. This adaptation in the streams was attributed to the time necessary for selective enrichment of an initially low population of PCP degraders on surface compartments (Pignatello *et al.*, 1985a). The extent of biodegradation in the streams increased with increasing PCP input, and this correlated with increasing numbers of PCP degrading microorganisms. Most of the PCP-mineralizing microorganisms that developed in the channels were either attached to surfaces (e.g. rocks and macrophytes), or associated with sediments. The contributions of different stream compartments (or microbial habitats) to microbial degradation of PCP were also assessed (Pignatello *et al.*, 1985b). Contributions to PCP loss were determined for rock (epilithic) surfaces, macrophyte (epiphytic) surfaces, sedimentary, and water column communities by measuring rates of PCP disappearance in stream water containing ambient concentrations of PCP in contact with the respective compartmental samples. Results indicated that the rock surface compartment was considerably more efficient at removing PCP than the other compartments.

## MOLECULAR ANALYSES OF NATURAL MICROBIAL COMMUNITIES

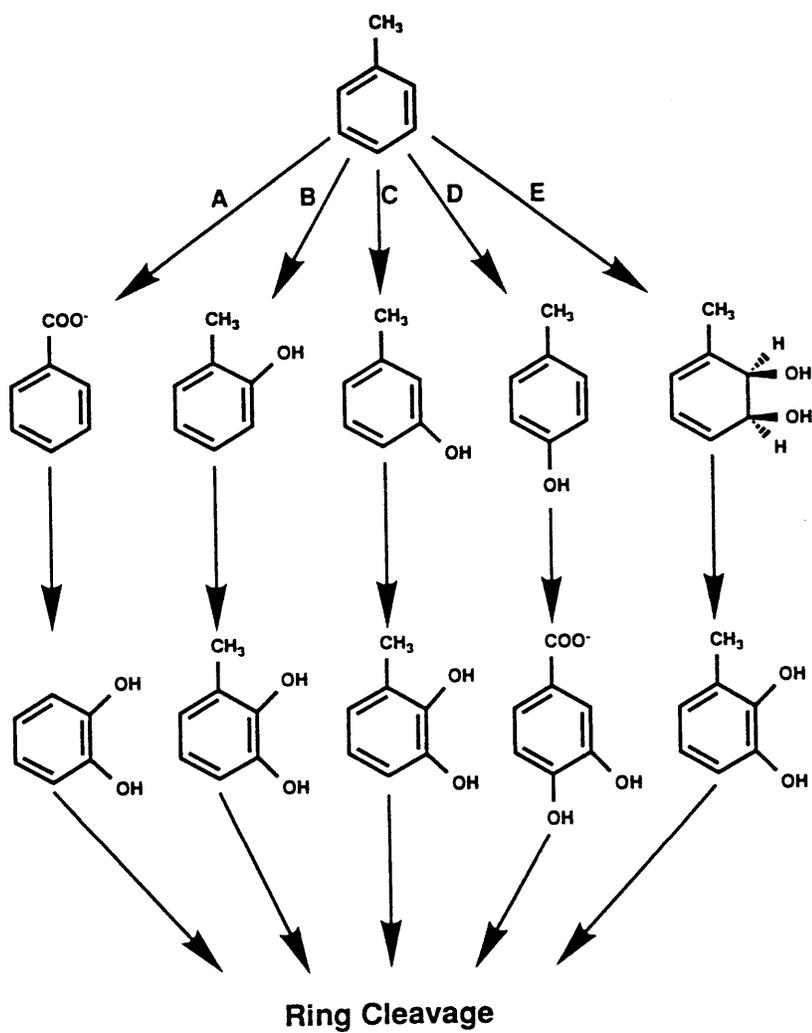
Our knowledge of the community structure of natural microbial ecosystems is limited because the majority of the organisms present cannot be recovered in culture. The isolation and study of pure cultures of microorganisms may provide a glimpse at the diversity of the microbial community in the environment, but many viable microorganisms resist cultivation. Discrepancies between direct counts and plate counts are typically several orders of magnitude and raise doubts as to whether cultivated bacteria are actually representative of the microbial communities from which they are isolated. The widespread view is that microbial ecosystems contain numerous novel and uncultivated species, and it has been estimated that only 20% of the bacterial species are actually known (Ward *et al.*, 1990). Culture methods alone may therefore be inadequate for studying microbial communities.

As an alternative to reliance on cultivation, molecular approaches based on phylogenetic analyses of rRNA sequences have been used to determine the species composition of microbial communities (Pace *et al.*, 1986). Molecular approaches can provide genetic markers for the dominant bacterial species in natural microbial populations (Giovannoni *et al.*, 1990). Although any gene may be used as a genetic marker, rRNA genes offer distinct advantages (Britschgi and Giovannoni, 1991). 16S rRNA genes are highly conserved and can therefore be used to examine distant phylogenetic relationships with accuracy. The extensive use of 16S rRNAs for studies of

microbial systematics and evolution has resulted in large computer data bases of 16S rRNA sequence information.

The analysis of extracted rRNA has been performed to identify bacteria in marine bacterioplankton (Giovannoni *et al.*, 1990; Schmidt *et al.*, 1991), in terrestrial hot springs (Stahl *et al.*, 1985; Ward *et al.*, 1990; Weller *et al.*, 1991), and in endosymbiotic associations (Amann *et al.*, 1991). Results from these studies revealed large numbers of community members that have not been cultured, and supported the widespread view that microbial ecosystems contain novel, uncultivated species. For example, the 55°C cyanobacterial mat of Octopus Spring, Yellowstone National Park, has been well characterized with respect to component microorganisms by microscopic and culture methods. Nevertheless, 16S rDNA sequences retrieved from the Octopus Spring mat revealed eight distinct community members which have not been cultivated from this community (Weller *et al.*, 1991). Indeed, the use of such culture-independent methods can complement, and in some cases circumvent, the bias of culture-dependent techniques and provide a more objective approach by which to understand the composition and character of microbial communities.

**Figure 1.** Catabolic pathways for the aerobic degradation of toluene.



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## Chapter Two

### TWO NEW *MYCOBACTERIUM* STRAINS AND THEIR ROLE IN TOLUENE DEGRADATION IN A CONTAMINATED STREAM

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## ABSTRACT

Two toluene degrading strains, T103 and T104, were independently isolated from rock surface biomass in a freshwater stream contaminated with toluene. The two strains exhibit different capacities for degradation of toluene and a range of aromatic compounds, and have major characteristics of the genus *Mycobacterium*. Both strains are aerobic, rod-shaped, Gram-positive, non-motile, acid-alcohol-fast, and produce yellow pigments. Their fatty acids include mainly straight-chain saturated and monounsaturated fatty acids with 10 to 20 carbon atoms, and large amounts of 10-methyloctadecanoic acid. Fatty acid analyses indicate that T103 and T104 are not identical strains but are related at the subspecies level. They have identical 16S rDNA sequences that are most similar to *Mycobacterium aurum* and *Mycobacterium komossense*, and they constitute a new species of fast-growing mycobacteria. Ecological studies reveal that toluene contamination within the stream has selectively enriched for toluene degrading bacteria in the epilithic microbial community, and indicate that strains T103 and T104 and other microorganisms are involved in the biodegradation. Within a library of 16S rDNA clones constructed from DNA from rock biofilms, a clone was found with a 16S rDNA sequence identical to that of strains T103 and T104.

## INTRODUCTION

In the United States, toluene is ranked 27th among the top 50 chemical products by production volume, with 931 million gallons ( $3.5 \times 10^9$  liters) of toluene manufactured in 1994 (22). Industry uses toluene in refining gasoline, in chemical manufacturing, in the manufacture of paints, lacquers, and adhesives, and in some printing and leather tanning processes. Toluene is listed as a priority pollutant (50) as toluene contamination of drinking water is a potential health hazard. In a survey conducted by the U.S. Environmental Protection Agency in 1988, toluene contamination was detected in groundwater, surface water, or soil at 29% of the hazardous waste sites surveyed. The average amounts of toluene detected were 0.2  $\mu\text{M}$  in groundwater, 0.1  $\mu\text{M}$  in surface water, and 77  $\mu\text{g}/\text{kg}$  in soil (50).

Since toluene is ubiquitous in the environment, it is not surprising that microorganisms capable of degrading toluene have been isolated from a variety of environments (41, 52, 55). For example, an assortment of *Pseudomonas* species that can grow on toluene as the sole source of carbon and energy have been isolated from polluted topsoil from different sites in the Netherlands (11). *Pseudomonas* sp. strains T2 and T3 were isolated from oil tanker ballast water that was contaminated with toluene (4). *Mycobacterium vaccae* strain JOB5, originally isolated from soil inoculum on plates incubated with 2-methylbutane (30), is able to grow on toluene (5).

From an ecological perspective, we are concerned with understanding the microbial communities that degrade toluene in nature. One expects to find toluene-polluted

environments enriched in toluene-degrading microorganisms. However, little is known about the abundance and the relative importance of these microorganisms in the conversion of toluene in the environment. Therefore, studies that address the response of stream bacteria to effects of anthropogenic chemical impact are important in enhancing our understanding of microbial diversity.

In this paper, we describe the isolation and characterization of two closely related toluene degrading strains of a novel *Mycobacterium* species from rock surface biofilms in a toluene-contaminated reach of a small freshwater stream. We also present ecological data to assess the role of this novel *Mycobacterium* species in degrading toluene in the rock surface biofilms, and to determine the impact of toluene contamination on the stream's microbial community. Earlier field studies had demonstrated that biodegradation is the most significant sink for toluene in this stream throughout the year, accounting for 40% to 70% of the toluene sink in spring and summer (20). Microorganisms attached to stream sediments and rock surfaces are responsible for most of the biodegradation (7).

## MATERIALS AND METHODS

**Study site.** The East Drainage Ditch is a small freshwater stream in an industrial area of Wilmington and Woburn, Massachusetts (7, 20), and forms part of the Aberjona Watershed, a 90 km<sup>2</sup> area 20 km north of Boston. Toluene in the stream arises from a source of subsurface contamination below a 80 m long culvert (10) located 1,600 m upstream from the confluence of the East Drainage Ditch with the Halls Brook Storage

Area. Toluene levels typically range from 0.6 to 4.2  $\mu\text{M}$  in the streamwater (20).

Sampling stations are 50 m (U50) and 100 m (U100) upstream of the upstream end of the culvert, and 5m (D5) and 50 m (D50) downstream of the downstream end of the culvert.

**Collection and isolation of bacterial strains.** Rocks were collected in July 1992 from the streambed at station D5 of the East Drainage Ditch. Rock biomass was scraped with sterile spatulas, serially diluted, and plated onto a mineral salts (MS) agar medium (38) supplemented with trace elements (54). The plates were incubated in a desiccator which contained a beaker of saturated toluene solution (15 ml), which was replaced every two to three days. The toluene partitioned via the air into the agar. Calculations based on Henry's Law (39) indicate a theoretical toluene concentration of approximately 110  $\mu\text{M}$  in the agar, although the actual concentration may be affected by compounding factors such as hydrophobic interactions between toluene and organic constituents of the agar. Plates were monitored for colonies for periods up to seven weeks. Colonies were picked and restreaked on fresh plates. Several colonies were obtained which grew only in the presence of toluene, and four were selected for further characterization. Colonies were numbered sequentially starting from T101. Strains T103 and T104 possessed traits typical of mycobacteria, and results of their characterization are presented in this paper.

**Phenotypic characterization of isolates.** Tests for Gram stain, oxidase activity, catalase activity, carbon source utilization, nitrate reduction, and acid-fastness (Ziehl-Neelsen method) were performed on strains T103 and T104 as previously described (27, 40). To test for growth on aromatic compounds, colonies were transferred onto plates of MS media and incubated in the same manner as with toluene; substrates were supplied by

diffusion from a reservoir in the dessicator that resulted in theoretical concentrations in the agar of 10 mg/l of benzene, o-xylene, m-xylene, p-xylene, phenol, or chlorobenzene. The reservoirs were replenished with the relevant substrates every two to three days and plates were monitored for up to four weeks. Visible colonies were picked and restreaked on fresh plates that were further incubated either in the presence or absence of the relevant substrate.

**SEM.** Cells on agar plates incubated with toluene were observed by scanning electron microscopy (SEM), as described elsewhere (28). SEM was also employed to view rock surfaces with intact biofilms and rock surfaces after extraction of nucleic acids. Rocks were broken up with a hammer, and small fragments of rock surfaces were prepared as for the cells before viewing with SEM.

**Toluene biodegradation kinetics.** The rates of toluene degradation were determined with a headspace gas chromatography method described previously (7). Cells were initially grown in 1100-ml teflon-stoppered glass bottles with 600 ml of MS medium initially containing 110  $\mu$ M toluene. Toluene levels were monitored daily and replenished when depleted. After every five toluene feedings, the headspace was flushed with air to replenish the oxygen. Cells were harvested by centrifugation during exponential phase, and resuspended in 100 ml of fresh MS medium. The kinetic experiments were performed in 60 ml serum bottles with 19 ml of medium and 0.5 ml of the concentrated cell suspension (230  $\mu$ g protein/ml for strain T103 and 185  $\mu$ g protein/ml for strain T104). The bottles were capped with teflon coated stoppers (The West Co., Phoenixville, Pennsylvania). Toluene was injected at approximately 1.1  $\mu$ M,

2.2  $\mu\text{M}$ , 5.4  $\mu\text{M}$ , and 10.9  $\mu\text{M}$  (aqueous concentration). Control experiments were performed with autoclaved cell suspensions. All bottles were shaken in the dark at a temperature of 20°C on a rotary shaker at 150 rpm, and assayed hourly for toluene until little or no toluene remained. Initial rates of toluene disappearance with time were determined and used to establish the Michaelis-Menten kinetic parameters. Protein was measured with the BCA protein assay, according to manufacturer's specifications (Pierce, Rockford, Illinois).

**Fatty acid analyses.** The bacteria were grown on Middlebrook 7H10 agar (9) for 7 days at 28°C. Cells were then harvested and saponified, and fatty acid methyl esters were prepared as described previously (36). The analysis was performed at Microbial ID Inc. (MIDI, Newark, Delaware) using an HP5890 series II gas chromatograph (Hewlett-Packard Co.) and the MIDI Microbial Identification System software for identification of fatty acids. Strains T103 and T104, together with *M. komossense* type strain (ATCC 33013), were analyzed in duplicate; the profiles obtained were compared with profiles from the Microbial Identification System (MIS) library (37).

**16S rDNA sequencing.** For total genomic DNA preparation, strains T103 and T104 were cultured in nutrient broth (NB) (Difco Laboratories, Detroit, Michigan) in teflon-stoppered serum bottles. The bottles were shaken at 20°C on a rotary shaker at 150 rpm. Cells were harvested by centrifugation after two to three days. Genomic DNA was extracted from pure cultures of the two strains using a miniprep for extraction of genomic DNA from bacteria, as described previously (1). The nearly full-length 16S rRNA gene was amplified from genomic DNA by PCR using a forward primer Eubac27F and a

reverse primer Universal 1492R (23). PCR reaction mixtures contained 2 mM MgCl<sub>2</sub>, 10 mM TrisHCl (pH 8.3), 50 mM KCl, 200 μM deoxynucleotide triphosphates, 2.5 U/100μl of *Thermus aquaticus* DNA polymerase, 0.2 μM each of oligonucleotide primer, and DNA template at 1 ng/μl. Acetamide (5%, wt/vol) was added to the reactions to enhance denaturation during the amplification reaction (32). All reactions were overlain with mineral oil and run in triplicate. Thermal cycling was carried out in a PTC-100 Peltier-effect thermal cycler (MJ Research, Inc.) as follows: denaturation at 95°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 1.5 min for a total of 30 cycles (8). Following amplification, the PCR product was purified using the Wizard PCR Prep purification kit (Promega Corp., Madison, Wisconsin) and resuspended in sterile H<sub>2</sub>O. Both strands of the purified PCR products were sequenced by automated dye dideoxy terminator sequencing at Michigan State University Sequencing Facility with a 373A DNA sequencing system (Applied Biosystems, Foster City, California). Sequences for oligonucleotides complementary to the conserved regions of the eubacterial 16S rRNA were kindly provided by Debra J. Lonergan (United States Geological Survey, Reston, Virginia); these oligonucleotides were chosen to prime the sequencing reactions. Sequencing reaction mixes consisted of 12 pmoles of sequencing primer, and 50 to 250 ng of PCR template in a total volume of 20 μl of sterile H<sub>2</sub>O.

**Phylogenetic analyses.** The 16S rDNA secondary structures of strains T103 and T104 were constructed manually using templates published in the Ribosomal Database Project (RDP) (25) to aid in the identification of homologous sequence positions. Sequence alignments were performed manually in the Genetic Data Environment (GDE)

(42). All reference sequences and the basic alignment to which the sequences of strains T103 and T104 were added were obtained from the RDP. Only homologous sites at which the 16S rDNA sequences of strains T103 and T104 could be aligned unambiguously with the reference sequences were included for further analyses. Phylogenetic distance analyses including bootstrap were performed using the programs DNADIST with the Jukes-Cantor correction, SEQBOOT with 100 replicates, and FITCH with input randomization and global rearrangement, all contained in the PHYLIP 3.5 package (13) implemented through the GDE. Parsimony analysis, including bootstrap analysis, was performed using PAUP 3.1 (45). Bootstrap values are shown if they were greater than 50%. Maximum likelihood analysis was performed with the fastDNAmI program available from the RDP (12, 29). Trees were constructed by allowing jumbled addition of taxa and global rearrangement of the branches.

**Nucleotide sequence accession numbers and strain designations.** The sequences for strains T103 and T104 have been deposited in the Genbank database under accession numbers U62889 and U62890. The Genbank accession numbers of the other sequences used in the analyses are as follows: *Mycobacterium aurum*, X55595; *Mycobacterium chelonae* subsp. *abscessus* str. L948, M29559; *Mycobacterium chitae*, X55603; *Mycobacterium chlorophenolicus* str. PCP-I, X79094; *Mycobacterium diernhoferi* str. SN 1418, X55593; *Mycobacterium fortuitum* subsp. *fortuitum*, X52933; *Mycobacterium gilvum*, X55599; *Mycobacterium komossense* str. Ko2, X55591; *Mycobacterium neoaurum*, M29564; *Mycobacterium sphagni* str. Sph29, X55590; *Mycobacterium thermoresistibile*, X55602; *Mycobacterium vaccae*, X55601; *Mycobacterium asiaticum* str. N61H, X55604; *Mycobacterium avium* str. serovar 1, M29573; *Mycobacterium*

*haemophilum*, L24800; *Mycobacterium tuberculosis* str. H37/Rv, X52917; *Mycobacterium xenopi*, X52929; *Corynebacterium xerosis*, M59058; *Gordona terrae*, X79286; *Nocardia otitidiscaviarum*, M59056; and *Rhodococcus equi*, M29574. The primary literature references for these sequences can be found in the RDP (25).

**Extraction and purification of genomic DNA from rock biofilms.** Rocks were collected in autoclaved 500 ml polypropylene bottles from station D5 in July 1993. The extraction protocol was based on a rapid freeze-thaw method developed by Tsai and Olson for direct DNA extraction from soil and sediments (49). The crude nucleic acids extract was further purified by gel electrophoresis. Polyvinylpyrrolidone (2% wt/vol) (PVP) was added to low-gel-temperature agarose (1.25% wt/vol) (Mallinckrodt Inc., Paris, Kentucky) to eliminate comigration of humic acids with nucleic acids by retarding the electrophoretic mobility of the phenolic groups of humic acids (53). Electrophoresis was performed overnight in PVP-low melt agarose at low voltage (5V/cm of gel length) to prevent overheating. TAE buffer (40mM Tris-acetate, 1mM EDTA) was used. The genomic DNA separated in the PVP-low melt agarose gel was visualized by ethidium bromide staining (35), and excised and purified with GeneClean II (Bio 101, Inc., La Jolla, California).

**Construction of environmental 16S rDNA clone library.** The 16S rRNA genes were amplified by PCR from the extracted environmental genomic DNA sample. These genes were cloned into the PCR II vector (Invitrogen, San Diego, California) and transformed into competent cells according to manufacturer's instructions. 46 colonies were picked for plasmid isolation and restriction analysis. Plasmid isolation was performed with an alkali lysis plasmid mini-prep, based on a method described

previously (1). The extracted plasmid DNA of individual clones were separated on a agarose gel and purified with GeneClean II (Bio 101, Inc., La Jolla, California). The 16S rRNA gene inserts were amplified by PCR from the purified plasmid DNA of individual clones. All reactions were performed in quadruplicate. Following amplification, the PCR products of each clone were pooled and purified using a Wizard PCR prep purification kit, and resuspended in sterile H<sub>2</sub>O.

**Restriction fragment length polymorphism (RFLP) analysis.** Aliquots of purified PCR products from the individual clones as well as from strains T103 and T104 were simultaneously digested with the 6-base specific *Pst*I and the 4-base specific *Rsa*I restriction endonucleases (5 U per reaction), according to manufacturer's specifications (Promega Corp., Madison, Wisconsin). Digested DNA was analyzed by gel electrophoresis in 2% agarose, carried out at 9 V/cm for 1 hr. Gels were stained in an aqueous solution of ethidium bromide (1 µg/ml) and photographed under UV illumination. One of the environmental clones possessed an identical RFLP pattern to those of strains T103 and T104. The 16S rDNA sequence of this clone was determined by automated dye dideoxy terminator sequencing, as described earlier.

**Bacterial counts.** To determine viable counts of heterotrophic bacteria, rock samples were collected from stations U100, U50, D5, and D50 along the East Drainage Ditch on September 7, 1993. Rock biomass was scraped with sterile spatulas, suspended in 10 ml of MS medium, and the resulting cell suspension successively diluted to obtain 10<sup>-2</sup> to 10<sup>-7</sup> g biomass/ml dilutions. Petri dishes containing 1% PTYG agar (3) were inoculated in duplicate with 100 µl of each dilution. After inoculation, the plates were

incubated at 20°C for up to three weeks. Heterotrophic colonies were counted on the plates every three days, until no new colonies were observed. Since counts were based on the highest dilution plates, which contained few colonies, colony overgrowth on the plates was not a problem.

Toluene degrading bacteria were enumerated with MS agar medium inoculated in duplicate with 100 µl of each dilution. After inoculation, the plates were placed in dessicators and toluene was supplied to the plates as described earlier. After several weeks of incubation, colonies growing on the highest dilution plates were picked and transferred to new plates for a second incubation. These colonies were incubated both in the presence or absence of toluene to confirm the abilities of these isolates to grow with toluene as an energy source. Colonies that grew only in the presence of toluene were counted as toluene-degrading colonies.

**Toluene levels in the stream.** Duplicate water samples were collected at stations U100, U50, D5, and D50 on September 19, 1993, using 40 ml EPA vials provided with hollow screw caps and teflon-coated silicone septa. Mercuric chloride was added to the samples to a final concentration of 15 mg/l. Toluene was quantitated using headspace gas chromatography as described previously (7).

## RESULTS

**Enrichment and isolation.** Two mycobacterial toluene degrading strains, T103 and T104, were independently isolated from the East Drainage Ditch. Strains T103 and

T104 originated from two yellow colonies 1 to 2 mm in diameter that appeared between three and eight days on toluene-incubated MS plates inoculated with  $10^6$  g biomass, indicating that these pure cultures were present in the rock surface biomass at a density of  $10^6$  cells/g biomass; biomass scrapings averaged 0.018 g fresh weight/cm<sup>2</sup> of rock surface. Both strains grew slowly (relative to other non-mycobacterial toluene-degrading strains similarly isolated) on solid media when incubated with toluene. Mycobacteria-like colonies were not detected on plates with smaller amounts of biomass.

**Morphological and phenotypic characteristics.** Strains T103 and T104 had morphologically similar rod-shaped cells when grown on solid media (Figure 1). Cells of both strains were nonmotile, and there was no evidence of flagella. The strains were aerobic, and tested Gram-positive (Table 1). The strains also tested positive for acid-alcohol-fastness, a defining characteristic of the mycobacteria (51).

Strain T104 grew better when incubated with o-, m-, or p-xylene than with toluene (Table 1); visible yellow colonies appeared on plates within three days. Strain T103 did not show any capacity to utilize the xylenes. Neither strain was able to grow on benzene, phenol, or chlorobenzene.

**Toluene biodegradation kinetics.** Strains T103 and T104 had maximal velocities ( $V_{max}$ ) of  $1.0 \pm 0.1$  and  $6.0 \pm 1.3$   $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$  respectively; their half-saturation constants ( $K_s$ ) were  $0.6 \pm 0.4$  and  $3.8 \pm 1.9$   $\mu\text{M}$  respectively (Figure 4).

**Fatty acid analyses.** Strains T103 and T104 contained mainly straight-chain saturated and monounsaturated fatty acids, as well as substantial amounts of tuberculostearic (10-methyloctadecanoic) acid (Table 2). Such high levels of

tuberculostearic acid as are present in these strains are typical of mycobacteria (16). Eleven fatty acids, including tuberculostearic acid, accounted for more than 80% of the total fatty acid composition. The coefficient of variation ([sample standard deviation/mean]  $\times$  100) for each fatty acid that accounted for more than 5.1% of the total fatty acid content in each case was less than 11%.

Strains T103 and T104 did not show any species match with existing mycobacterial entries in the MIS library. *Mycobacterium aurum* was the most closely related species in the MIS library to the two strains, giving weak similarity indices of  $0.31 \pm 0.01$  and  $0.24 \pm 0.05$  with strains T103 and T104, respectively. Multiple analyses of the same strain result in linkages among samples of that strain at a level of 2 Euclidean distances or lower; the Euclidean distance scale also permits determination of the relatedness of samples at the genus, species, and subspecies levels (approximately 25, 10, and 6 Euclidean distances respectively) (37). The slight phenotypical differences between T103 and T104 were confirmed by fatty acid analysis. T103 and T104 link at a Euclidean distance of 6.9 (Figure 2), indicating that they are not identical strains but are related at the subspecies level. T103 and T104 link with *Mycobacterium komossense* and *Mycobacterium aurum* at Euclidean distances of 10.5 and 14.3 respectively, indicating that they belong to the *Mycobacterium* genus, but are different species from *Mycobacterium komossense* and *Mycobacterium aurum*.

**16S rDNA sequence analyses.** The sequences of approximately 1425 nucleotide bases, corresponding to the *E. coli* 16S rDNA sequence from nucleotide 55 to 1501, were obtained in both directions for the two strains. The sequences of the two strains were

identical. The secondary structure of the T103/T104 sequence was identical to the secondary structure of other fast-growing mycobacteria. The T103/T104 sequence contains the shortened stem structure bounded by positions 455 to 477 (*E. coli* numbering) that typically distinguishes the fast-growing mycobacteria from the slow-growing ones (44).

Phylogenetic analyses performed on the final data set of 1,165 nucleotides revealed that the T103/T104 sequence was very similar to the 16S rDNA sequences of the other mycobacteria. An identity matrix constructed with the aligned sequences obtained from the RDP (Table 3) showed that the level of identity between the T103/T104 sequence and the sequences of the representative mycobacteria was greater than 96%. The T103/T104 sequence was most identical to the sequences of *Mycobacterium aurum* (identity 99.0%) and *Mycobacterium komossense* (identity 98.9%). The levels of identity between the T103/T104 sequence and the sequences of the fast-growing mycobacteria, the slow-growing mycobacteria, and other non-mycobacterial nocardioform bacteria ranged from 96.9 to 99.0%, 96.1 to 97.3%, and 93.0 to 95.7%, respectively.

Distance and bootstrap analyses also showed that strains T103 and T104 belong to the fast-growing members of the *Mycobacterium* genus (Figure 3). In the distance analysis, all the mycobacteria fell into a closely related, coherent group, distinct from the other high G+C Gram-positive bacteria examined; the bootstrap value was 81%. Within the genus, the fast-growing mycobacteria are set apart from the slow-growing species; the slow-growing mycobacteria define a distinct line of evolutionary descent, with a bootstrap value of 87%. Although strains T103 and T104 are positioned within the group of fast-growing mycobacteria, the precise relationships among the fast-growing species

remained unresolved because of low bootstrap values. Similar tree topologies were also observed in the parsimony analyses (data not shown). An examination of nine most parsimonious trees revealed that the mycobacteria clustered together in these trees, with the slow-growing mycobacteria forming a distinct clade. However, the mycobacteria cluster was not supported in the bootstrap results. Maximum likelihood analysis also showed that strains T103 and T104 fell within the fast-growing mycobacteria (data not shown), although three of the four other nocardioform bacteria (with the exception of *Corynebacterium xerosi*) also formed a distinct sub-cluster within the fast-growing group of mycobacteria. In these analyses, the slow-growing mycobacteria also constituted a distinct clade.

**Extraction of genomic DNA.** Scanning electron micrographs demonstrate the presence of bacteria on rock biofilms. Rod-shaped bacteria appear to predominate, although it was difficult to distinguish coccoid-shaped bacteria from mineral particles on the rock surfaces. Many of the rod-shaped bacteria were interconnected by an extracellular matrix (Figure 5A). There was little evidence of remaining biological material on rock surfaces after DNA extraction (Figure 5B).

**RFLP analysis.** The amplified 16S rDNAs of 46 environmental clones from station D5 produced single bands of about 1,500 bp (data not shown), corresponding to the expected size of the 16S rRNA genes. From the 46 clones, 33 unique RFLP types were detected. The maximum number of clones associated with the same RFLP type was 6. Of the remaining clones, 27 possessed unique restriction patterns. The restriction pattern of one of these clones was identical to those of strains T103 and T104. The 16S

rRNA gene in this clone was sequenced and found to be identical to the 16S rRNA gene sequences of strains T103 and T104.

**Bacterial counts and toluene levels.** No toluene has been detected at station U100 (Figure 6), although very low levels of toluene (0.04  $\mu\text{M}$ ) have been measured at station U50. Downstream of the culvert, stations D5 and D50 had high toluene concentrations of 2.6 and 1.7  $\mu\text{M}$ , respectively, in September 1993. The total heterotrophic bacterial plate counts at these stations ranged from  $2 \times 10^8$  to  $7.7 \times 10^8$  cells/g biomass. Toluene degrading bacteria were not detected for station U100, where no toluene was present. The counts of toluene degrading bacteria for the other three stations increased with increasing toluene concentration, and made up 0.03, 1.09, and 0.35 % of the total heterotrophic plate counts at stations U50, D5, and D50, respectively.

## DISCUSSION

**Taxonomic and phylogenetic considerations.** Strains T103 and T104 are both capable of degrading toluene and have major characteristics of the genus *Mycobacterium*. Strains T103 and T104 exhibit some differences in physiology. Unlike strain T103, strain T104 is able to grow on the xylenes. Also, their fatty acid profiles are sufficiently different for them to be considered as different strains. Fatty acid analysis appears to be useful to distinguish closely-related strains, especially when 16S rDNA sequences cannot tell such strains apart.

T103 and T104 are thus different strains of a novel species of fast-growing mycobacteria capable of growth with toluene as the sole carbon source. Compared to the slow-growing mycobacteria, many of which are human and animal pathogens, the fast-growing mycobacteria are common saprophytes in natural habitats (18). They have been isolated from a diverse array of habitats and are able to survive and multiply under a wide range of pH, temperature, and other environmental conditions. The fast-growing mycobacteria are able to biotransform a variety of xenobiotic compounds and pollutants, including polycyclic aromatic hydrocarbons (15) and groundwater pollutant mixtures (5). There is a long history of the isolation from oil-contaminated soils of mycobacteria that have the capacity to degrade the aromatic fraction of the oil (47, 48).

Phylogenetic analysis of strains T103 and T104 indicates that they are most similar to *Mycobacterium aurum* and *Mycobacterium komossense*. *Mycobacterium aurum* is a fast-growing species commonly isolated from soil (51). *Mycobacterium aurum* strain MO1, isolated from a mixed culture in a laboratory reactor, utilized morpholine as the main source of carbon, nitrogen, and energy (6, 26), while *Mycobacterium aurum* L1 metabolized vinyl chloride under aerobic conditions (17). *Mycobacterium komossense* is a fast-growing non-pathogenic species that has been repeatedly isolated from *Sphagnum* vegetation of moors in south Sweden and the Atlantic coastal area of Norway (19). It is unable to utilize benzoate and benzamide; it is not known if it possesses the ability to utilize other aromatic hydrocarbons or xenobiotic compounds.

Other closely related species have been found to degrade a range of aromatic hydrocarbons. Other than strains T103 and T104 however, *Mycobacterium vaccae* is the only *Mycobacterium* species known to grow on toluene. *Mycobacterium vaccae* strain

JOB-5 can also degrade acetone, cyclohexane, styrene, benzene, ethylbenzene, propylbenzene, dioxane, and 1,2-dichloroethylene (5). A *Mycobacterium* sp., isolated from soil of a former coal gasification site, is able to degrade the polycyclic aromatic hydrocarbons phenanthrene, pyrene, and fluoranthene (2); this isolate is closely related to *Mycobacterium gilvum* (16S rDNA identity of > 99.8%) (14).

**Biodegradation kinetics.** Toluene contamination of the East Drainage Ditch appears to selectively enrich for toluene degrading bacteria within the epilithic microbial community. That  $K_s$  values for strains T103 and T104 lie within the range of toluene concentrations observed in the stream suggests that these strains are adapted to the ambient level of toluene contamination in the stream. Non-carbon nutrients are unlikely limiting in this case, as these are present in the stream at high levels (46).

Maximal velocities and half-saturation constants have also been reported for other aerobic toluene-degrading bacteria. Robertson and Button (34) reported toluene degradation by a marine *Pseudomonas* sp. strain T2 and by a terrestrial *Pseudomonas putida* strain PpF1 with Michaelian kinetics. Uptake for strain T2 was characterized by a maximal velocity of 14 mg toluene/g cells-hr (or 0.30  $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$ , assuming that 50% of a typical cell's dry weight is protein), and a half-saturation constant of 0.48  $\mu\text{M}$ . Corresponding values for strain PpF1 are 20 mg toluene/g cells-hr (or 0.43  $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$ ) and 0.68  $\mu\text{M}$ , respectively. Values reported for strain PpF1 are component values for the conversion of toluene to  $\text{CO}_2$  only.

In order to assess the role that strains T103 and T104 may play in toluene biodegradation in intact biofilms, we compared toluene biodegradation rates obtained in

the laboratory for rock biofilms and for pure cultures of our mycobacteria. Toluene biodegradation rates for East Drainage Ditch rocks with their natural biofilms under summer conditions were determined by Cohen *et al.* (7). The natural biofilm rate was observed to be first order for toluene concentrations up to 2.2  $\mu\text{M}$ , and approached zero order for toluene concentrations greater than 4.3  $\mu\text{M}$ , with a  $V_{\text{max}}$  of 2.0  $\text{nmol}/\text{cm}^2$  of rock surface-hr. These rates were determined in batch studies similar to those described in the Materials and Methods section, except that whole rocks were used instead of cell suspensions.

Assuming mass transport to not be limiting, the portion of the toluene biodegradation on the rock biofilm that may be attributed solely to strains T103 and T104 can be estimated from the cell densities of strains T103 and T104 on the rock surfaces and their individual kinetic parameters (Figure 4). The cell density (CD), estimated from plate counts of the mycobacterial isolates ( $10^6$  cells/g biomass) and biomass density (0.018 g biomass/ $\text{cm}^2$  of rock surface), was determined to be  $1.8 \times 10^4$  cells/ $\text{cm}^2$  of rock surface. Since the biofilm data are most reliable for toluene concentrations greater than 4.3  $\mu\text{M}$  (7), comparisons of biofilm rates and pure culture rates are performed for toluene concentrations greater than or equal to 4.3  $\mu\text{M}$ . Assuming a typical cell protein weight of 0.2 pg (24), and assuming that the mycobacteria are uniformly distributed throughout the biofilm, the relative contributions of strains T103 and T104 to the toluene biodegradation by the biofilm can be estimated as follows:

$$(\text{CD} \times \text{cell protein weight} \times k_{\text{strain T103 or T104}} \times 100 \%) / V_{\text{max, biofilm}}$$

where:

$$k_{\text{strain T103/T104}} = V_{\text{max, strain T103/T104}} \times S / (K_{\text{s, strain T103/T104}} + S); \text{ and}$$

S = toluene concentration.

At a toluene concentration of 4.3  $\mu\text{M}$ , strains T103 and T104 are estimated to account for 0.2% and 0.6%, respectively, of the toluene biodegradation that occurs on the rock surfaces. These numbers should be interpreted as upper bounds on the relative contributions of the pure cultures to toluene degradation in the biofilms; to the extent that diffusion limitation occurs, these numbers may be lower, especially in the case of strain T104. Strain T103, having a  $K_s$  much lower than 4.3  $\mu\text{M}$ , would be much less affected. The relative contributions are relatively low, and suggest that, although strains T103 and T104 are involved in toluene biodegradation on the rock surfaces, other bacterial species may play a larger role. We expect that these results provide a reasonable estimate of the importance of strains T103 and T104 to toluene degradation in the East Drainage Ditch itself. Cohen *et al* (7) showed that constantly shaken flasks provided a reasonable microcosm to represent the fast-flowing stream. Kim *et al.* (21) found that mass transport of the substrate to the biofilm surface is not a limiting factor in the turbulent flow regime of this stream, although biofilms themselves were not always free of transport limitation.

**Microbial ecology.** The 16S rRNA gene insert of clone C16 (RFLP type 14) was identical in sequence to strains T103 and T104. These strains were isolated from rock biofilms collected in July 1992 while clone C16 was independently recovered from rock biofilms collected in July 1993. This suggests that this *Mycobacterium* species is a permanent member of the microbial community in the stream.

Although our library of 46 environmental clones is not a large one, the variety of RFLP types detected hints at the broad diversity of the microbial community in the East Drainage Ditch. This diversity is expected; the stream is not an extreme environment that might select for a less diverse and more specialized community, e.g. as found for the high temperature outflow from Octopus Spring in Yellowstone National Park (33), where only three phylogenetic types were recovered.

Toluene levels in the stream do not appear to have a significant effect on total heterotrophic bacterial counts. On the other hand, counts of toluene degrading bacteria in samples from the contaminated stations are about an order of magnitude higher than those from pristine stations and suggest that toluene in the contaminated reaches of the stream have caused the epilithic bacterial communities to adapt by selectively enriching for toluene degrading bacteria. Other studies of freshwater environments (31, 43) also reported viable heterotrophic counts to be unaffected by the presence of a xenobiotic contaminant. This suggested that the contaminant was not toxic to most members of the bacterial community, although suppressed strains may be simply replaced by tolerant ones. Spain *et al* (43) studied acclimation of microbial communities in a freshwater pond exposed to *p*-nitrophenol on Santa Rosa Island near Pensacola, Florida, and reported increases of three orders of magnitude of the numbers of organisms capable of degrading

the contaminant of interest. Pignatello *et al* (31) examined the fate of pentachlorophenol in man-made channels at a field station on the Mississippi River near Minticello, Minnesota, and reported that the pentachlorophenol degrading population increased up to two orders of magnitude after adaptation, as compared to a background population. The increased counts in the East Drainage Ditch are not as high as those observed in these studies, and this is probably because of temperature effects in which microbial activity can be greatly reduced at lower temperatures (31). The East Drainage Ditch study was conducted in the fall when stream temperatures averaged 11°C. In the other studies, pond temperatures averaged 18°C (43) while channel temperatures ranged from 19°C to 30°C (31).

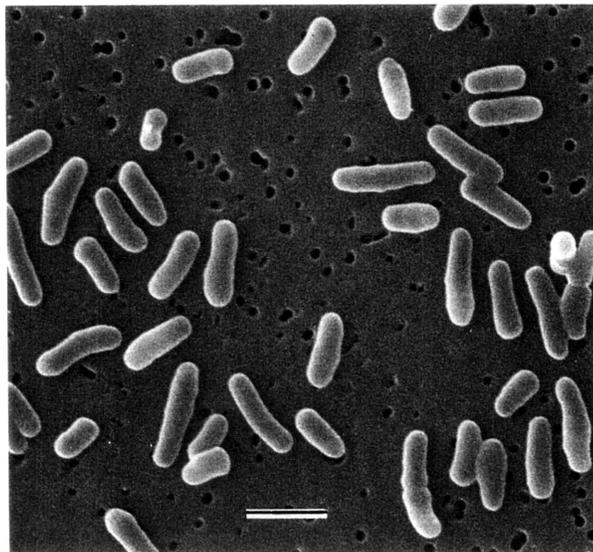
In summary, we describe two closely related toluene-degrading mycobacterial strains isolated from rock surface biofilms from a toluene-contaminated freshwater stream. These strains constitute a novel fast-growing *Mycobacterium* species and extend our knowledge of the list of *Mycobacterium* species that can grow on toluene. Fast-growing mycobacteria are seen to play an important role in the environment, as evidenced by increasing discoveries of members within this group that can degrade a wide range of xenobiotic compounds. Our ecological experiments show a stream community response to the presence of low levels of toluene. This community response appears to be more complex than the stimulation of a single toluene-degrading microorganism; other microorganisms must also be present that are also involved in toluene degradation in the stream.

## **ACKNOWLEDGMENTS**

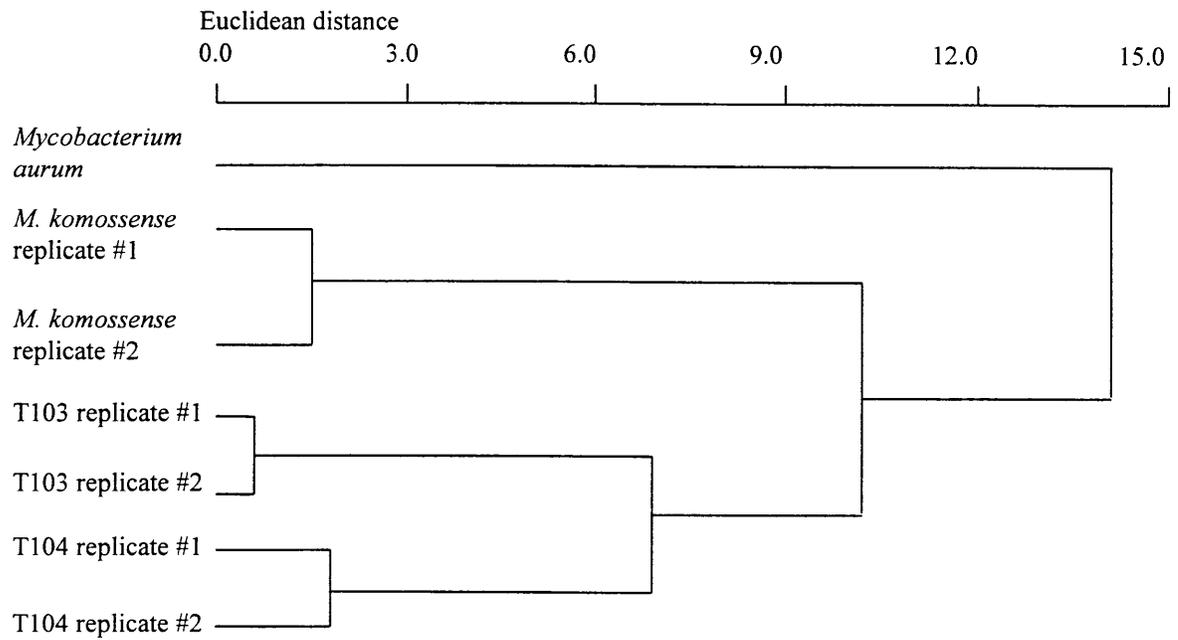
This work was supported by NIEHS Superfund Basic Research Program Grant 5P42ES04675-06 and Office of Naval Research Grant N00014-91-J-1489.

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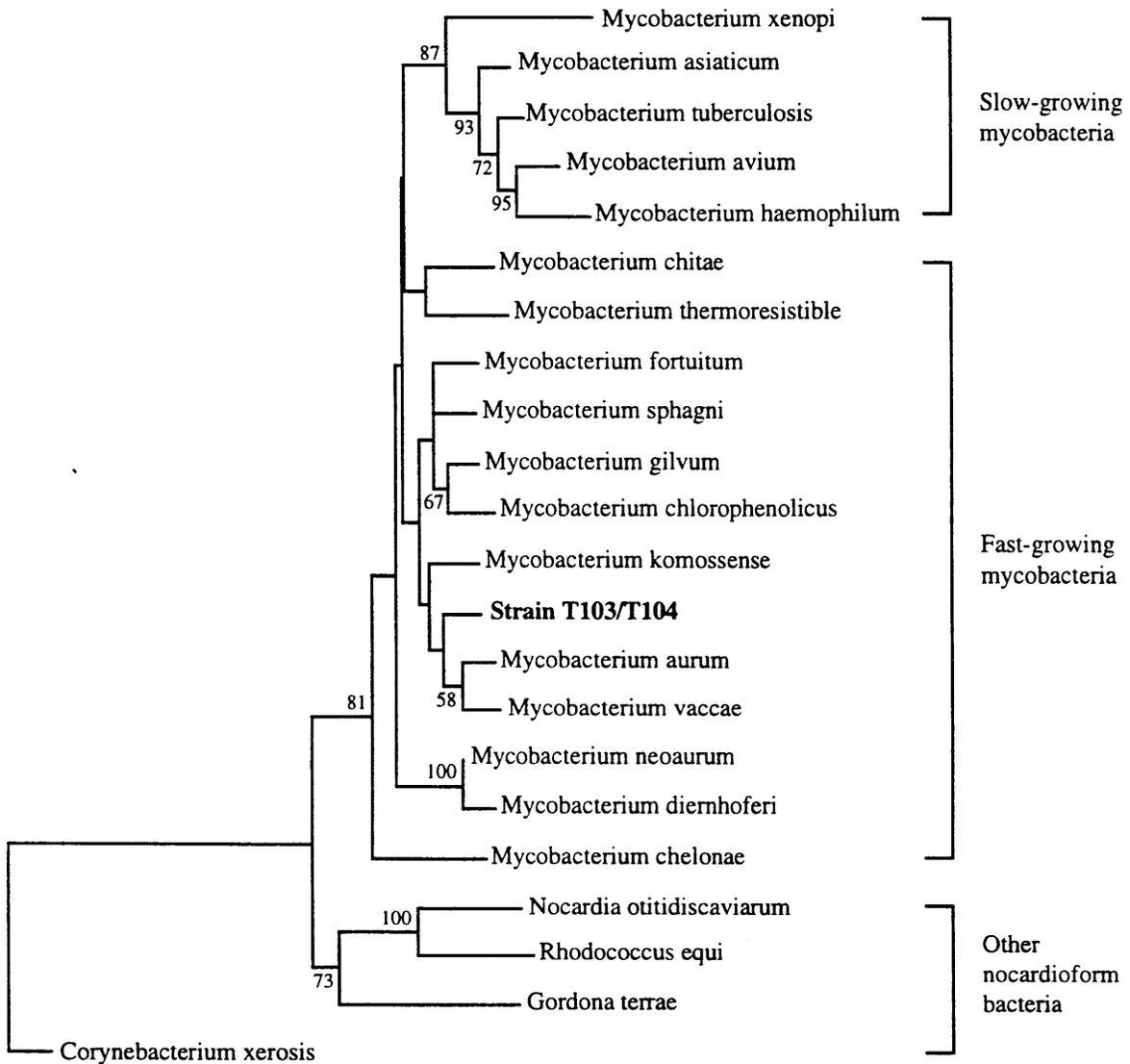
**Figure 1.** Scanning electron micrograph of toluene degrading strain T103 grown on solid media (bar, 1  $\mu\text{m}$ ).



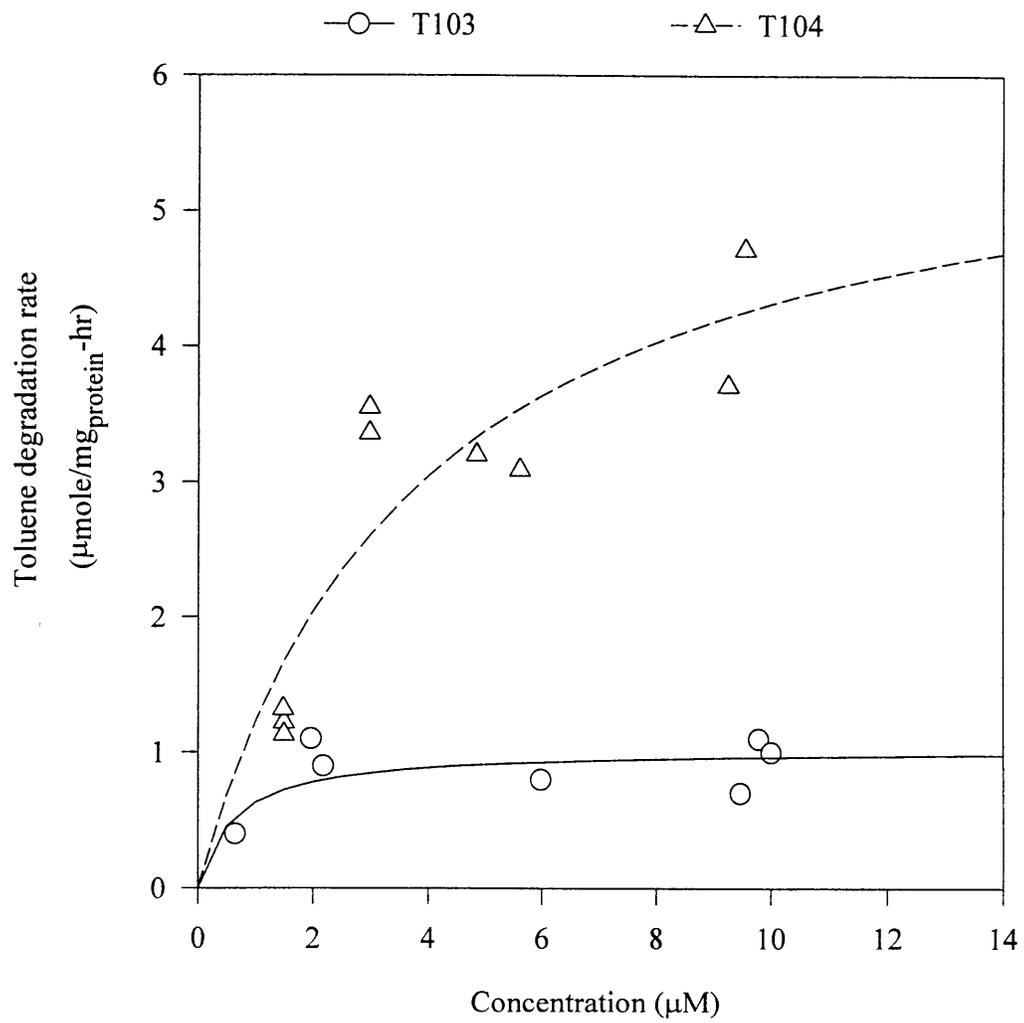
**Figure 2.** Dendrogram of selected *Mycobacterium* strains generated by cluster analysis of fatty acid profiles.



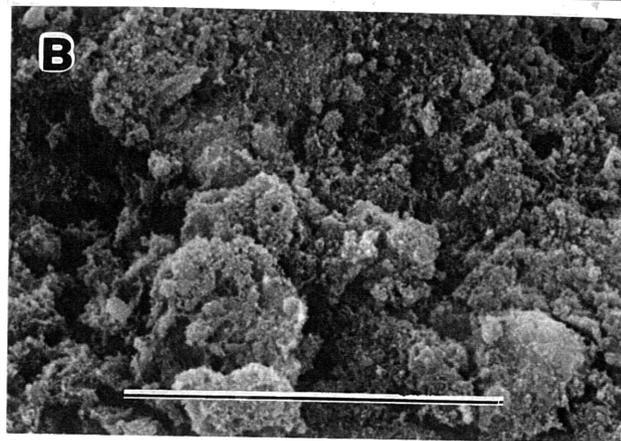
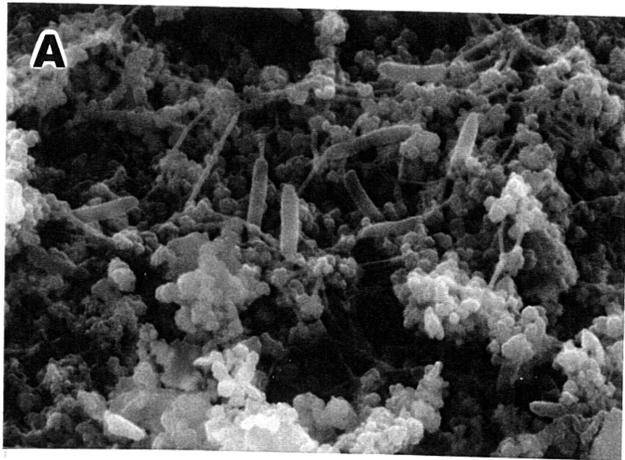
**Figure 3.** Unrooted evolutionary distance tree based on the 16S rDNA sequences of strains T103 and T104, representative members of the *Mycobacterium* genus, and other high G+C Gram-positive bacteria. Bootstrap values greater than 50% are shown at the nodes. Bar = 0.01 nucleotide difference per sequence position.



**Figure 4.** Toluene biodegradation kinetics for strains T103 and T104.

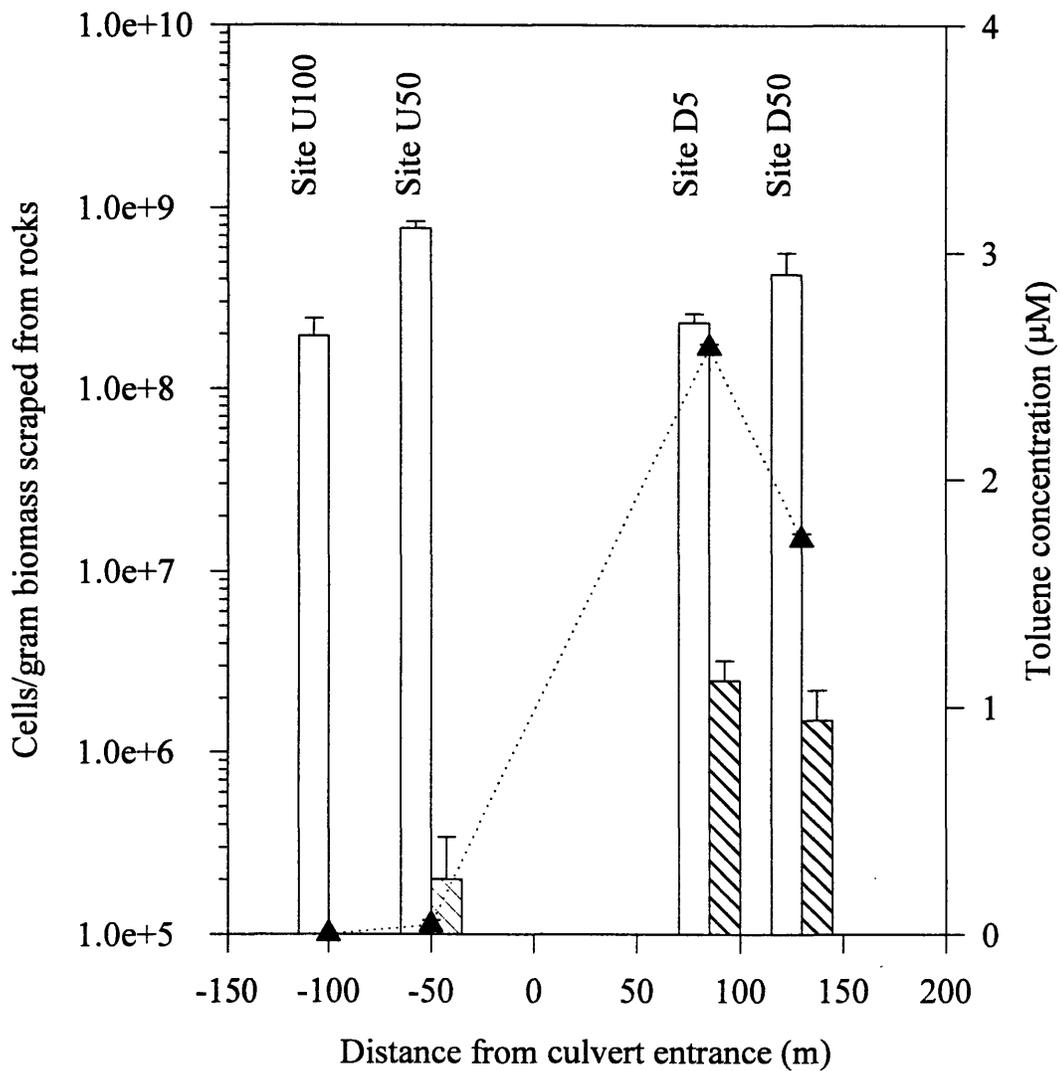


**Figure 5.** Scanning electron micrographs of biofilms of East Drainage Ditch rocks: (A) before DNA extraction; (B) after DNA extraction (bars, 10  $\mu\text{m}$ ).



**Figure 6.** Bacterial counts (sampled on September 7 1993) and toluene levels (sampled on September 19 1993) along the East Drainage Ditch. Error bars represent standard deviations of duplicate samples.

-  Viable count of heterotrophic bacteria on 1% PTYG plates incubated at 20°C for up to four weeks in the dark.
-  Plate count of toluene degrading bacteria on minimal salts agar with 110 μM toluene. Incubations were performed at 20°C for up to four weeks in the dark.
-  Toluene concentration.



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-  Toluene concentration (September 19, 1993)

**Table 1.** Characteristics of toluene degrading strains T103 and T104

	T103	T104
Morphology	Rod	Rod
Flagella	-	-
Pigmentation	Yellow	Yellow
Gram stain	+ <sup>a</sup>	+
Acid fast	+	+
Nitrate reduction	-	-
Catalase	+	+
Oxidase	-	-
Citrate	+	+
Growth on sugars:		
Glucose	+	+
Lactose	-	-
Maltose	+	+
Sucrose	-	-
Growth on aromatic compounds:		
Toluene	+ <sup>b</sup>	+
Benzene	-	-
Chlorobenzene	-	-
o-xylene	-	++
m-xylene	-	++
p-xylene	-	++
Phenol	-	-

<sup>a</sup> +, good growth or activity; -, poor/no growth or activity.

<sup>b</sup> For growth on aromatic compounds, cultures were streaked onto agar plates containing a minimal salts media. Plates were then incubated in dessicators with substrate concentrations of approximately 10 mg/l; incubation temperature was 20°C. Each compound was tested twice. ++, very good growth within three days; +, good growth after three days; -, poor/no growth.

**Table 2.** Whole-cell fatty acid compositions of strains T103, T104, *M. komossense*, and *M. aurum*

Fatty acid	% of total in:			
	Strain T103	Strain T104	<i>M. komossense</i> (ATCC 33013)	<i>M. aurum</i> <sup>a</sup>
Decanoic acid	1.98 (1.1) <sup>b</sup>	1.49 (3.8)	N.D. <sup>c</sup>	N.D.
Dodecanoic acid	2.11 (1.0)	1.64 (5.1)	0.25 (2.9)	N.D.
Tetradecanoic acid	6.58 (0.4)	7.25 (4.1)	8.18 (2.4)	5.93
Pentadecanoic acid	0.79 (5.4)	0.45 (1.6)	0.34 (8.3)	N.D.
<i>cis</i> -7-hexadecenoic acid	0.70 (1.0)	0.76 (15.9)	0.95 (9.7)	2.19
<i>cis</i> -9-hexadecenoic acid	2.63 (0.0)	2.36 (2.7)	2.34 (1.2)	N.D.
<i>cis</i> -10-hexadecenoic acid	4.70 (4.1)	5.09 (10.4)	8.11 (0.5)	5.58
Hexadecanoic acid	22.93 (0.0)	23.02 (0.5)	27.2 (0.6)	30.12
Summed feature 1 <sup>d</sup>	0.78 (12.7)	1.14 (3.1)	0.85 (2.5)	N.D.
Summed feature 2 <sup>e</sup>	14.68 (3.2)	19.69 (0.1)	12.15 (6.1)	10.01
Heptadecanoic acid	N.D.	N.D.	0.24 (9.0)	N.D.
<i>cis</i> -9,12-octadecadienoic acid	0.85 (5.0)	0.89 (14.3)	1.00 (2.1)	N.D.
<i>cis</i> -9-octadecenoic acid	20.93 (0.6)	17.98 (5.9)	16.0 (3.8)	27.35
<i>cis</i> -11-octadecenoic acid	0.61 (3.5)	0.25 (141.4)	0.54 (6.6)	N.D.
Octadecanoic acid	0.87 (12.3)	1.35 (6.3)	3.46 (0.6)	2.66
10-methyloctadecanoic acid (tuberculostearic acid)	10.96 (2.2)	12.11 (1.7)	14.98 (1.9)	9.09
Summed feature 3 <sup>f</sup>	7.23 (1.0)	4.05 (3.5)	3.18 (8.4)	3.74
Eicosanoic acid	0.72 (18.8)	0.51 (8.3)	0.28 (5.1)	2.23

<sup>a</sup>From Microbial Identification System library.

<sup>b</sup>The numbers in parentheses are coefficients of variation, N = 2.

<sup>c</sup>N.D., not detected.

<sup>d</sup>Summed feature 1 contains 8-methylhexadecanoic acid and 10-methylhexadecanoic acid.

<sup>e</sup>Summed feature 2 contains *cis*-10-heptadecenoic acid, *cis*-11-heptadecenoic acid,  $\omega$ cyclopropane 7-8 hexadecanoic acid, and 2-octadecanol.

<sup>f</sup>Summed feature 3 contains  $\omega$ cyclopropane 8-9 octadecanoic acid,  $\omega$ cyclopropane 10-11 octadecanoic acid, and 2-eicosanol.

Table 3. 16S rRNA identity/distance matrix for T103/T104 and related taxa

Organism <sup>a</sup>	% nucleotide identity/evolutionary distance <sup>b</sup>																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 Strain T103/T104		1.0	2.5	3.2	2.7	2.4	1.7	2.2	1.1	2.1	1.6	3.2	2.0	3.2	3.3	3.5	2.8	4.1	7.3	5.2	4.4	4.9
2 <i>Mycobacterium aurum</i>	99.0		2.6	3.5	1.8	2.9	2.2	1.6	2.0	2.5	2.7	3.4	1.0	3.2	3.2	3.4	2.7	4.4	7.8	5.4	4.9	5.7
3 <i>Mycobacterium chelonae</i>	97.5	97.4		4.4	4.1	3.2	3.2	3.7	2.8	2.6	3.6	3.6	3.6	4.2	3.9	4.6	4.2	5.0	8.5	4.7	5.7	5.2
4 <i>Mycobacterium chitae</i>	96.9	96.6	95.7		2.4	3.0	1.8	2.1	2.5	2.4	1.8	2.1	3.0	2.6	3.8	4.3	2.9	3.4	7.5	5.7	6.2	6.1
5 <i>Mycobacterium chlorophenicus</i>	97.3	98.2	96.1	97.7		3.2	1.8	1.0	2.4	2.8	1.4	3.0	1.6	2.3	3.5	4.1	2.6	4.1	7.6	5.9	5.4	6.1
6 <i>Mycobacterium diernhoferi</i>	97.6	97.2	96.9	97.1	96.8		2.5	2.4	2.9	0.5	2.7	2.6	2.4	2.8	3.6	4.0	3.5	4.1	8.4	5.0	6.2	5.7
7 <i>Mycobacterium fortuitum</i>	98.3	97.9	96.9	98.2	98.2	97.5		1.1	1.5	2.0	1.2	2.4	2.4	2.7	3.7	4.2	2.8	3.8	7.5	5.5	4.9	5.3
8 <i>Mycobacterium gilvum</i>	97.9	98.4	96.4	97.9	99.0	97.6	98.9		2.1	2.1	1.4	2.5	1.5	2.4	3.8	4.3	2.9	4.1	7.5	5.5	5.4	5.9
9 <i>Mycobacterium komossense</i>	98.9	98.0	97.3	97.5	97.6	97.2	98.5	97.9		2.4	1.4	3.1	2.6	3.0	3.1	3.6	2.2	3.6	6.9	5.2	4.7	5.0
10 <i>Mycobacterium neoaurum</i>	97.9	97.5	97.4	97.6	97.3	99.5	98.0	97.9	97.7		2.2	2.1	2.1	2.5	3.3	3.7	3.2	3.7	8.1	4.6	5.6	5.2
11 <i>Mycobacterium sphagni</i>	98.4	97.3	96.5	98.2	98.6	97.3	98.8	98.6	98.6	97.9		2.5	2.4	2.3	3.6	4.1	2.7	3.7	7.0	5.3	4.6	4.9
12 <i>Mycobacterium thermoresistibile</i>	96.9	96.7	96.5	97.9	97.1	97.4	97.7	97.5	97.0	97.9	97.5		2.5	2.6	3.8	4.3	3.5	3.7	8.0	5.9	6.4	6.3
13 <i>Mycobacterium vaccae</i>	98.0	99.0	96.5	97.1	98.4	97.6	97.7	98.5	97.4	97.9	97.6	97.5		3.1	3.8	4.0	3.2	4.7	8.2	5.5	5.2	5.9
14 <i>Mycobacterium asiaticum</i>	96.8	96.8	95.9	97.4	97.8	97.3	97.3	97.6	97.1	97.5	97.8	97.4	97.0		1.5	1.8	1.3	3.3	6.9	5.7	5.7	6.1
15 <i>Mycobacterium avium</i>	96.7	96.8	96.2	96.3	96.6	96.5	96.4	96.3	97.0	96.7	96.5	96.3	96.3	98.5		1.6	1.2	3.7	6.9	5.5	6.5	6.7
16 <i>Mycobacterium haemophilum</i>	96.6	96.7	95.5	95.8	96.1	96.1	95.9	95.8	96.5	96.4	96.0	95.8	96.1	98.2	98.5		1.6	4.2	7.9	6.3	6.5	7.1
17 <i>Mycobacterium tuberculosis</i>	97.3	97.3	95.9	97.2	97.4	96.6	97.3	97.2	97.9	96.9	97.3	96.6	96.8	98.7	98.8	98.5		2.7	6.4	5.7	5.8	6.2
18 <i>Mycobacterium xenopi</i>	96.1	95.7	95.2	96.7	96.0	96.1	96.3	96.0	96.5	96.4	96.4	96.4	95.5	96.7	96.4	95.9	97.3		7.8	6.6	6.6	6.4
19 <i>Corynebacterium xerosis</i>	93.0	92.6	91.9	92.9	92.8	92.0	92.9	92.9	93.4	92.4	93.3	92.4	92.3	93.4	93.4	92.5	93.8	92.6		7.8	8.6	7.1
20 <i>Gordona terrae</i>	94.9	94.8	95.5	94.5	94.3	95.2	94.7	94.7	95.0	95.5	94.8	94.3	94.7	94.5	94.7	94.0	94.5	93.6	92.6		5.5	4.8
21 <i>Nocardia otitidiscaviarum</i>	95.7	95.3	94.5	94.1	94.8	94.1	95.3	94.8	95.5	94.6	95.5	93.9	94.9	94.5	93.7	93.7	94.4	93.6	91.8	94.7		3.1
22 <i>Rhodococcus equi</i>	95.3	94.5	94.9	94.2	94.2	94.5	94.8	94.3	95.2	95.0	95.3	94.0	94.3	94.2	93.6	93.2	94.1	93.8	93.2	95.4	97.0	

<sup>a</sup> Species 2-13, fast-growing mycobacteria; species 14-18, slow-growing mycobacteria; species 19-22, other nocardioform bacteria.

<sup>b</sup> The values on the lower left are nucleotide percent identities, and the values on the upper right are Jukes-Cantor corrected evolutionary distances. A total of 1,165 nucleotides were used in the analysis.

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## **Chapter Three**

### **IMPORTANCE OF *XANTHOBACTER AUTOTROPHICUS* IN TOLUENE BIODEGRADATION WITHIN A CONTAMINATED STREAM**

## ABSTRACT

Toluene degrading strains T101 and T102 were isolated from rock surface biomass in a toluene-contaminated freshwater stream. Based on the results of direct plating of rock surface biomass, these organisms were present at a density of  $5.5 \times 10^6$  cells/g biomass. The two strains can also degrade benzene. Both are aerobic, rod-shaped, Gram-negative, non-motile, catalase-positive, oxidase-positive, and produce yellow pigments. They have high levels of *cis*-11-octadecenoic acid and *cis*-9-hexadecenoic acid; 3-hydroxyhexadecanoic acid is the major hydroxy fatty acid present. Phylogenetic analyses show that T101 and T102 have 16S rDNA sequences identical to *Xanthobacter autotrophicus*. Based on fatty acid analyses, T101 and T102 link at a Euclidean distance of 2.8, indicating that they are different strains belonging to the same species *Xanthobacter autotrophicus*. Toluene biodegradation kinetics experiments show that T101 and T102 had maximal velocities ( $V_{max}$ ) of  $3.8 \pm 0.5$  and  $28.3 \pm 2.2$   $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$ , and half-saturation constants ( $K_s$ ) of  $0.8 \pm 0.5$  and  $11.5 \pm 2.4$   $\mu\text{M}$ , respectively. T102 has a higher capacity than T101 to degrade toluene, and kinetic calculations suggest that T102 may be a major contributor to toluene biodegradation in the stream. These two strains, together with *Xanthobacter autotrophicus* GJ10, are the only *Xanthobacter* strains reported to degrade toluene.

## INTRODUCTION

Toluene is a widely used synthetic organic chemical and is ranked 27th among the top 50 chemical products by production volume in the United States, with 931 million gallons ( $3.5 \times 10^9$  liters) of toluene manufactured in 1994 (Kirschner, 1995). Toluene is widely used in industry, and is usually disposed of at hazardous waste sites as used solvents. In a 1988 survey, toluene was detected in groundwater, surface water, or soil at 29% of the hazardous waste sites surveyed. The average amounts measured were  $0.2 \mu\text{M}$  in groundwater,  $0.1 \mu\text{M}$  in surface water, and  $77 \mu\text{g/kg}$  in soil (USPHS, 1989).

Since toluene is ubiquitous, it is not surprising that microorganisms capable of degrading toluene have been isolated from a range of environments (Worsey *et al.*, 1975; Zeyer *et al.*, 1986; Smith, 1990). Several *Pseudomonas* species that can grow on toluene as the sole source of carbon and energy have been isolated from contaminated topsoil from multiple sites in the Netherlands (Duetz *et al.*, 1994), and from oil tanker ballast water (Button *et al.*, 1981). Two strains of a novel toluene degrading *Mycobacterium* species (Tay *et al.*, submitted) were isolated from rock surface biofilms in a toluene-contaminated freshwater stream (Kim *et al.*, 1995b; Cohen *et al.*, 1995). These reports support the ecological view that toluene-contaminated environments should be enriched in toluene degrading microorganisms. However, little is known about the diversity, abundance, and importance of cultured microorganisms in the conversion of toluene in the natural environment. Therefore, studies that couple the isolation of indigenous

bacteria with their response to anthropogenic chemical contamination are important in enhancing our understanding of microbial diversity.

We describe the isolation and characterization of two new toluene degrading strains of *Xanthobacter autotrophicus* isolated from the East Drainage Ditch, a small freshwater stream in Wilmington, Massachusetts; toluene in the stream arises from subsurface contamination (Durant, 1991). Toluene biodegradation kinetics of the pure cultures are compared with batch toluene biodegradation rates determined previously in the laboratory for biofilm material growing on East Drainage Ditch rocks (Cohen *et al.*, 1995) to assess the potential importance of these microorganisms in degrading toluene on the rock biofilms.

## MATERIALS AND METHODS

**Isolation of bacterial strains.** Rocks were collected in July 1992 from the streambed of the East Drainage Ditch. Rock biomass was scraped with sterile spatulas, serially diluted, and plated onto a mineral salts (MS) agar medium (Schraa *et al.*, 1986) supplemented with trace elements (Zehnder *et al.*, 1980). The plates were incubated in a desiccator with a beaker of water (15 ml) previously equilibrated with toluene; the water was replaced every two to three days. The toluene partitioned via the air into the agar. Calculations based on Henry's Law (Schwarzenbach *et al.*, 1993) indicate a theoretical toluene concentration of approximately 110  $\mu\text{M}$  in the agar, although the actual concentration may be affected by factors such as hydrophobic interactions between

toluene and organic constituents of the agar. Plates were monitored for up to seven weeks. Colonies were picked and restreaked on fresh plates. Several colonies were obtained which grew only in the presence of toluene, and four, numbered sequentially starting from T101, were selected for further characterization. Strains T101 and T102 are described in this paper.

**Phenotypic characterization of isolates.** Tests for Gram stain, oxidase activity, catalase activity, carbon source utilization, nitrate reduction, and acid-fastness (Ziehl-Neelsen method) were performed on T101 and T102 as previously described (Murray *et al.*, 1994; Smibert and Krieg, 1994). To test for growth on aromatic compounds, colonies were transferred onto plates of MS medium and incubated in the same manner as with toluene; substrates were supplied by diffusion from a reservoir in the dessicator that resulted in theoretical concentrations in the agar of 10 mg/l of benzene, o-xylene, m-xylene, p-xylene, phenol, or chlorobenzene. Plates were monitored for four weeks. Visible colonies were picked and restreaked on fresh plates that were further incubated either in the presence or absence of the relevant substrate.

**SEM.** Cells on agar plates incubated with toluene were observed by scanning electron microscopy (SEM) as described elsewhere (Newman *et al.*, 1997). The cells were fixed with glutaraldehyde, dehydrated in a graded series of ethanol solutions, and dried using a critical point dryer. The specimens were sputter coated with gold and palladium, and examined with an Amray model 1000A scanning electron microscope (Amray, Bedford, Massachusetts) at operating voltages of 20 and 30 kV.

**Toluene biodegradation kinetics.** Rates of toluene degradation were determined with a headspace gas chromatography method (Cohen *et al.*, 1995). Cells were grown in

1.1 L teflon-stoppered glass bottles with 600 ml of MS medium initially containing 110  $\mu\text{M}$  toluene. Toluene levels were monitored daily and replenished when depleted. After every five toluene feedings, the headspace was flushed with air to replenish the oxygen. Cells were harvested by centrifugation during exponential phase and resuspended in 100 ml of fresh MS medium. Kinetic experiments were performed in 60 ml teflon-stoppered serum bottles (The West Co., Phoenixville, PA) with 19 ml of medium and 0.5 ml of the concentrated cell suspension (158  $\mu\text{g}$  protein/ml for strain T101 and 109  $\mu\text{g}$  protein/ml for strain T102). Toluene was injected at approximately 1.1  $\mu\text{M}$ , 2.2  $\mu\text{M}$ , 5.4  $\mu\text{M}$ , and 10.9  $\mu\text{M}$  (aqueous concentration). In the case of T102, toluene was also injected at a concentration of approximately 54.3  $\mu\text{M}$ . Control experiments were performed with autoclaved cell suspensions. Bottles were shaken in the dark at a temperature of 20°C on a rotary shaker at 150 rpm, and assayed hourly for toluene. Initial rates of toluene disappearance with time were determined and used to establish Michaelis-Menten kinetic parameters. Protein was measured with the BCA protein assay (Pierce, Rockford, IL).

**Fatty acid analyses.** The bacteria were grown on trypticase soy broth agar (Difco Laboratories, 1985) at 28°C for six days, harvested, and saponified. Fatty acid methyl esters were prepared as described previously (Sasser, 1990a). The analysis was performed at Microbial ID Inc. (MIDI, Newark, DA) using the MIDI Microbial Identification System software for identification of fatty acids. T101 and T102 were analyzed in duplicate and compared with profiles of other closely related microorganisms from the Microbial Identification System (MIS) library (Sasser, 1990b).

**16S rDNA sequencing.** T101 and T102 were cultured in nutrient broth (NB) (Difco Laboratories, 1985) in teflon-stoppered serum bottles, shaken at 20°C on a rotary shaker at 150 rpm. Cells were harvested by centrifugation and genomic DNA extracted using a miniprep (Ausubel *et al.*, 1992). The nearly full-length 16S rRNA gene was amplified from by PCR using forward primer Eubac27F and reverse primer Universal 1492R (Lane, 1991). PCR reaction mixtures contained 2 mM MgCl<sub>2</sub>, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 200 μM deoxynucleotide triphosphates, 2.5 U/100μl of *Taq* DNA polymerase, 0.2 μM each of oligonucleotide primer, and DNA template at 1 ng/μl. Acetamide (5%, wt/vol) was added to the reactions to enhance denaturation during the amplification reaction (Reysenbach, 1992). All reactions were overlain with mineral oil and run in triplicate. Thermal cycling was carried out in a PTC-100 Peltier-effect thermal cycler (MJ Research, Inc.) as follows: denaturation at 95°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 1.5 min for a total of 30 cycles (DeLong, 1992). Following amplification, the PCR product was purified using the Wizard PCR Prep purification kit (Promega Corp., Madison, Wisconsin) and resuspended in sterile H<sub>2</sub>O. Both strands of the purified PCR products were sequenced by automated dye dideoxy terminator sequencing at Michigan State University Sequencing Facility with a 373A DNA sequencing system (Applied Biosystems, Foster City, California). Sequencing primers complementary to the conserved regions of the eubacterial 16S rRNA were kindly provided by Debra J. Lonergan (United States Geological Survey, Reston, Virginia). The 16S rDNA sequences of 1392 nucleotide bases (corresponding to

the *E. coli* 16S rDNA sequence from nucleotide 44 to 1500) were obtained in both directions for the two strains.

**Phylogenetic analyses.** The 16S rDNA secondary structures of T101 and T102 were constructed manually using templates published in the Ribosomal Database Project (RDP) (Maidak *et al.*, 1997) to aid in the identification of homologous sequence positions. Sequence alignments were performed manually in the Genetic Data Environment (GDE) (Smith *et al.*, 1992). All reference sequences and the basic alignment to which the sequences of strains T101 and T102 were added were obtained from the RDP. Only homologous sites at which the 16S rDNA sequences of strains T101 and T102 could be aligned unambiguously with the reference sequences were included for further analyses, resulting in a final data set of 1,205 nucleotide positions. Phylogenetic distance analyses including bootstrap were performed using the programs DNADIST with the Jukes-Cantor correction, SEQBOOT with 100 replicates, and FITCH with input randomization and global rearrangement, all contained in the PHYLIP 3.5 package (Felsenstein, 1989) implemented through the GDE. Parsimony analysis, including bootstrap analysis, was performed using PAUP 3.1 (Swofford, 1991). Bootstrap values are shown if they were greater than 50%. Maximum likelihood analysis was performed with the fastDNAmI program available from the RDP (Felsenstein, 1981; Olsen *et al.*, 1994). Trees were constructed by allowing jumbled addition of taxa and global rearrangement of the branches.

**Nucleotide sequence accession numbers and strain designations.** The sequences for strains T101 and T102 have been deposited in the Genbank database under accession numbers U62887 and U62888. The Genbank accession numbers of the other sequences

used in the analyses are as follows: *Xanthobacter autotrophicus* str. 7C, X94201; *Xanthobacter flavus* str. 301, X94199; *Xanthobacter agilis* str. SA35, X94198; *Aquabacter spiritensis* str. SPL-1, ATCC 43981; *Azorhizobium caulinodans* str. ORS571, X94200; *Ancylobacter aquaticus*, M62790; *Thiobacillus novellus*, D32247; *Rhodopseudomonas viridis*, D25314; *Rhodoplanes elegans* str. AS130, D25311; *Rhodoplanes roseus* str. 941, D25313; *Beijerinckia indica* subsp. *indica*, M59060; *Afipia felis*, M65248; *Rhizobium leguminosarum*, X67233; *Bradyrhizobium japonicum*, X66024; *Bartonella quintana* str. Fuller, M11927; *Blastobacter denitrificans*, X66025; *Brucella abortus* str. 11/19, X67233; *Methylobacterium extorquens*, ATCC 43645; *Agrobacterium tumefaciens*, D12784; *Methylosinus trichosporium* str. OB3b, M29024; and *Rhodomicrobium vannielii* str. EY33, M34127.

## RESULTS

**Enrichment and isolation.** Two toluene degrading *Xanthobacter* strains, T101 and T102, were independently isolated from yellow colonies 1 to 2 mm in diameter that appeared within eight days on toluene-incubated MS plates inoculated with  $10^{-6}$  and  $10^{-7}$  g biomass, indicating that these strains were present in the rock surface biomass at an average density of  $5.5 \times 10^6$  cells/g biomass; biomass scrapings averaged 0.018 g fresh weight/cm<sup>2</sup> of rock surface. Both strains grew fairly rapidly on solid media incubated with toluene relative to other non-*Xanthobacter* toluene degrading strains similarly

isolated. *Xanthobacter*-like colonies were not detected on plates with smaller amounts of biomass.

**Morphological and phenotypic characteristics.** T101 and T102 had a pleomorphic appearance with slightly irregular rod-shaped cells when grown on solid medium (Figure 1). Cells of both strains were nonmotile, and there was no evidence of flagella. The strains were aerobic, catalase-positive, oxidase-positive, and tested Gram-negative (Table 1). Both strains grew well with toluene, and were also able to grow on benzene, but not on phenol or the xylenes. While both strains displayed similar morphological and phenotypic characteristics, a few differences were observed. T102 was able to reduce nitrate while T101 was not.

**Fatty acid analyses.** Whole-cell fatty acid compositions are shown in Table 2. T101 and T102 contained mainly straight-chain saturated and unsaturated, hydroxy, and cyclo fatty acids. *cis*-11-octadecenoic acid was by far the most abundant, accounting for more than 80% of the total; *cis*-9-hexadecenoic acid is the second most abundant, accounting for more than 5% of the total fatty acid composition. The main hydroxy acid present is 3-hydroxyhexadecanoic acid. The fatty acid profiles were highly reproducible, having coefficients of variation ( $[\text{sample standard deviation}/\text{mean}] \times 100$ ) for the major fatty acids (those that accounted for more than 5.1% of the total fatty acid content) of less than 17%.

T101 and T102 showed good species match with *Xanthobacter autotrophicus* in the MIS library, giving high similarity indices of  $0.72 \pm 0.04$  and  $0.54 \pm 0.12$ , respectively. The dendrogram in Figure 2 was generated using cluster analysis to produce unweighted

pair matchings based on fatty acid compositions. Using the criteria adopted by MIDI (Sasser, 1990b), multiple analyses of identical strains should result in linkages of 2 Euclidean distances or lower; the relatedness at the genus, species, and subspecies levels should correspond to 25, 10, and 6 Euclidean distances, respectively. The slight phenotypical differences between T101 and T102 were confirmed by fatty acid analysis. T101 and T102 link at a Euclidean distance of 2.8, indicating that they are different strains belonging to the same species *Xanthobacter autotrophicus*.

**16S rDNA sequence analyses.** T101 and T102 had identical 16S rDNA sequences. The secondary structure of the T101/T102 sequence was similar to the secondary structure of other bacteria in the alpha subdivision of the *Proteobacteria*, as determined by manual reconstruction. The T101/T102 sequence contains the octanucleotide GAUUUAUCG within the shortened helix bounded by positions 198 to 219 (*E. coli* numbering); this octanucleotide sequence is found in 80% of all bacterial catalogs of the alpha subdivision of *Proteobacteria* (Woese, 1987), while the shortened helix typically distinguishes the alpha subdivision from the other subdivisions of *Proteobacteria* (Woese, 1987).

The T101/T102 sequence was identical to the sequence of *Xanthobacter autotrophicus* and was very similar to the sequences of *Xanthobacter flavus* (identity 97.6%), *Xanthobacter agilis* (identity 96.8%), *Aquabacter spiritensis* (identity 97.3%), and *Azorhizobium caulinodans* (identity 96.8%) (Table 3). However, the levels of identity between the T101/T102 sequence and the sequences of other members of the alpha subdivision of the *Proteobacteria* fell below 94.5%.

The high 16S rDNA identities observed above confirms the close association within the *Aquabacter*, *Azorhizobium*, and *Xanthobacter* genera that was first reported by Rainey and Wiegel (1996). Distance and bootstrap analyses also support this finding. The *Aquabacter-Azorhizobium-Xanthobacter* cluster was recovered in all of the distance trees calculated in a bootstrap analysis; the bootstrap value was 100%. Rainey and Wiegel (1996) also pointed out that the *Aquabacter-Azorhizobium-Xanthobacter* cluster consists of two subclusters (Figure 3). One subcluster comprising T101/T102, *Xanthobacter autotrophicus*, and *Aquabacter spiritensis* was recovered in 74% of the trees analysed. The other subcluster comprising *Azorhizobium caulinodans*, *Xanthobacter agilis*, and *Xanthobacter flavus* was recovered in 71% of the trees analysed. Rainey and Wiegel (1996) noted that members of the genera *Aquabacter*, *Azorhizobium*, and *Xanthobacter* are intermixed, and that there is a lack of phylogenetic coherence among the *Xanthobacter* species. Similar tree topologies were also observed in the parsimony and maximum likelihood analyses (data not shown). Some slight rearrangements in the branching orders of other clusters within the alpha subdivision were observed with the different methods of phylogenetic analyses. The *Aquabacter-Azorhizobium-Xanthobacter* cluster was recovered in the parsimony and maximum likelihood analyses, although the *Azorhizobium caulinodans*, *Xanthobacter agilis*, and *Xanthobacter flavus* subcluster was not significantly supported in the parsimony bootstrap analyses.

**Toluene biodegradation kinetics.** Strains T101 and T102 had maximal velocities ( $V_{\max}$ ) of  $3.8 \pm 0.5$  and  $28.3 \pm 2.2$   $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$  respectively; their half-

saturation constants ( $K_s$ ) were  $0.8 \pm 0.5$  and  $11.5 \pm 2.4 \mu\text{M}$  respectively. Table 4 shows a comparison of these two strains to some other known toluene degrading organisms.

## DISCUSSION

**Taxonomy and phylogeny.** T101 and T102 are both capable of degrading toluene and have major characteristics of the genus *Xanthobacter*. Although their 16S rDNA sequences are identical, the fatty acid profiles of T101 and T102 are sufficiently different for them to be considered as different strains of *Xanthobacter autotrophicus*. There are also some slight phenotypical differences between the two strains. Unlike T101, T102 is able to reduce nitrate. In addition, T102 has a higher capacity for degrading toluene than T101.

The *Xanthobacter* genus forms a distinct phylogenetic cluster with *Aquabacter* and *Azorhizobium* within the alpha subdivision of *Proteobacteria*. Nevertheless, members within each genera possess unique characteristics that allow them to be clearly differentiated from members of the other two genera (Dreyfus *et al.*, 1988; Irgens *et al.*, 1991; Wiegel, 1992; Rainey and Wiegel, 1996). *Aquabacter spiritensis*, isolated from Spirit Lake, Washington, differs from *Azorhizobium* and *Xanthobacter* species because it has gas vacuoles, it is unable to fix nitrogen, and its colonies have a chalky white appearance (Irgens *et al.*, 1991). *Azorhizobium caulinodans* can be differentiated from *Xanthobacter* by the absence of pleomorphy, and by the ability to nodulate the stems of the tropical legume *Sesbania rostrata* (Dreyfus *et al.*, 1988). *Xanthobacter* species are

pleomorphic on certain media and contain the water-insoluble yellow pigment zeaxanthin dirhamnoside (Wiegel, 1992); presently, no other organism is known to produce this pigment.

Within the *Aquabacter-Azorhizobium-Xanthobacter* cluster, only T101, T102, and *Xanthobacter autotrophicus* strain GJ10 (Janssen *et al.*, 1985) are known to degrade toluene. *Aquabacter spiritensis* is unable to utilize benzoate or benzylamine (Irgens *et al.*, 1991), and it has not been tested for toluene utilization. *Azorhizobium caulinodans* can utilize a variety of organic compounds, including *m*-hydroxybenzoate; however, it is not known if *Azorhizobium caulinodans* strains can utilize toluene (Dreyfus *et al.*, 1988).

**Biodegradation kinetics.** Since toluene concentrations in the East Drainage Ditch vary temporally as well as spatially, the different  $K_s$  values for toluene biodegradation may affect how successfully each strain survives in the natural environment. The maximal velocity and half-saturation constant for T101 are similar to those reported for two *Mycobacterium* strains that were also isolated from the East Drainage Ditch (Table 4). However, the maximal velocity of T102 is approximately seven times greater than that of strain T101, and the half-saturation constant for strain T102 is approximately fourteen times greater than reported for strain T101.

These kinetic results indicate that T102 may be more effective at degrading toluene when it is present at higher concentrations. Furthermore, T102 appears to be remarkably efficient at toluene degradation relative to other isolates whose kinetics have been studied. Robertson and Button (1987) reported toluene degradation by a marine *Pseudomonas* sp. strain T2 and by a terrestrial *Pseudomonas putida* strain PpF1 (Table 4). Uptake for strain T2 was characterized by a maximal velocity of 14 mg toluene/g

cells-hr (or 0.30  $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$ , assuming that 50% of a typical cell's dry weight is protein), and a half-saturation constant of 0.48  $\mu\text{M}$ . Corresponding values for strain PpF1 are 20 mg toluene/g cells-hr (or 0.43  $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$ ) and 0.68  $\mu\text{M}$ , respectively (values reported for strain PpF1 are component values for the conversion of toluene to  $\text{CO}_2$  only).

**Comparisons between culture and biofilm kinetics.** In order to assess the contribution of T101 and T102 to toluene biodegradation in intact biofilms, we compared toluene biodegradation rates obtained in the laboratory for rock biofilms and for pure cultures of T101 and T102. Toluene biodegradation rates for East Drainage Ditch rocks with their natural biofilms under summer conditions were determined by Cohen *et al.* (1995). These rates were determined in batch studies similar to the pure culture studies, except that whole rocks were used instead of cell suspensions. The natural biofilm rate was observed to be first order for toluene concentrations up to 2.2  $\mu\text{M}$ , and approached zero order for toluene concentrations greater than 4.3  $\mu\text{M}$ , with a  $V_{\text{max}}$  of 2.0  $\text{nmol}/\text{cm}^2$  of rock surface-hr.

We estimated the portion of the toluene biodegradation by the rock biofilm attributable solely to T101 and T102 from the cell densities of these strains on the rock surfaces and their individual kinetic parameters (Figure 4), assuming mass transport to not be limiting in these experiments. The cell density, estimated from plate counts of the *Xanthobacter* isolates ( $5.5 \times 10^6$  cells/g biomass) and areal biomass density (0.018 g biomass/ $\text{cm}^2$  of rock surface), was determined to be  $9.9 \times 10^4$  cells/ $\text{cm}^2$  of rock surface. Since the biofilm data are most accurate at toluene concentrations greater than 4.3  $\mu\text{M}$

(Cohen *et al.*, 1995), comparisons of biofilm rates and pure culture rates are shown for 4.3  $\mu\text{M}$ . Concentrations in the stream typically range from 2.2 to 5.4  $\mu\text{M}$  (Tay *et al.*, submitted). Assuming a cell protein weight of 0.2 pg (Lodish *et al.*, 1995), the relative contributions of strains T101 and T102 were estimated as follows:

% toluene degradation attributable to T101 or T102 =

$$(\text{Cell density} \times \text{cell protein weight} \times k_{\text{strain T101 or T102}} \times 100 \%) / V_{\text{max, biofilm}}$$

where:

$$k_{\text{strain T101/T102}} = V_{\text{max, strain T101/T102}} \times S / (K_{\text{s, strain T101/T102}} + S); \text{ and}$$

S = toluene concentration.

At a toluene concentration of 4.3  $\mu\text{M}$ , T101 and T102 may account for 3.8% and 28.0%, respectively, of the toluene biodegradation that occurs on the rock surfaces. To the extent that diffusion limitation occurs, these numbers may be lower, and should be considered upper bounds, especially in the case of strain T102. T101, having a  $K_s$  much lower than 4.3  $\mu\text{M}$ , would be less affected by diffusion limitation. Nonetheless, these calculations suggest that T102 may be a major contributor to toluene biodegradation on the rock surfaces. Similar calculations for the two *Mycobacterium* strains isolated from the same stream indicate, however, that they account for less than 2% of the toluene biodegradation on the rock surfaces (Tay *et al.*, submitted). We expect that these results

provide a reasonable estimate of the importance of *Xanthobacter autotrophicus* to toluene degradation in the East Drainage Ditch itself. Cohen *et al* (1995) showed that constantly shaken flasks provided a reasonable microcosm to represent the fast-flowing stream. Kim (1995a) found that mass transport of the substrate to the biofilm surface is not a limiting factor in the turbulent flow regime of this stream, although biofilms themselves were not always free of transport limitation.

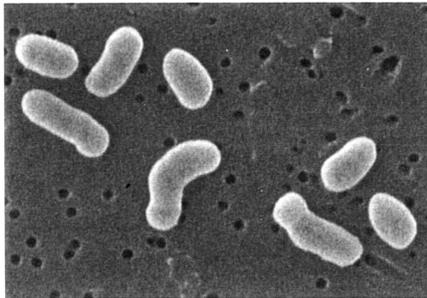
In summary, this study describes two closely related toluene degrading *Xanthobacter autotrophicus* strains that were isolated from rock surfaces from a toluene-contaminated freshwater stream. The results of our study extend our knowledge of the list of *Xanthobacter* strains that can grow on toluene. Our calculations suggest that T102 may be a major contributor to toluene biodegradation in the stream.

## ACKNOWLEDGMENTS

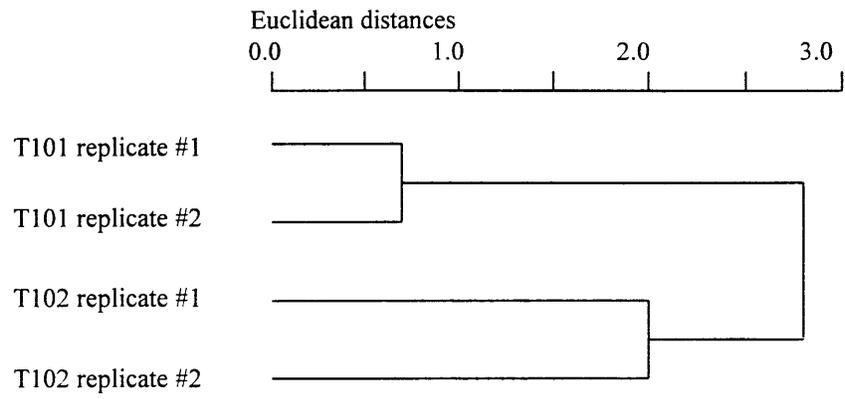
This work was supported by NIEHS Superfund Basic Research Program Grant 5P42ES04675-06 and Office of Naval Research Grant N00014-9I-J-1489.

We thank Michael Collins for carrying out the kinetics experiment, Karen Dohrman for performing the fatty acid analysis, Sue Lootens for performing 16S rDNA sequencing, Debra Lonergan for advising on aspects of 16S rDNA sequencing and analyses, and Dianne Newman and Edward Seling for performing scanning electron microscopy.

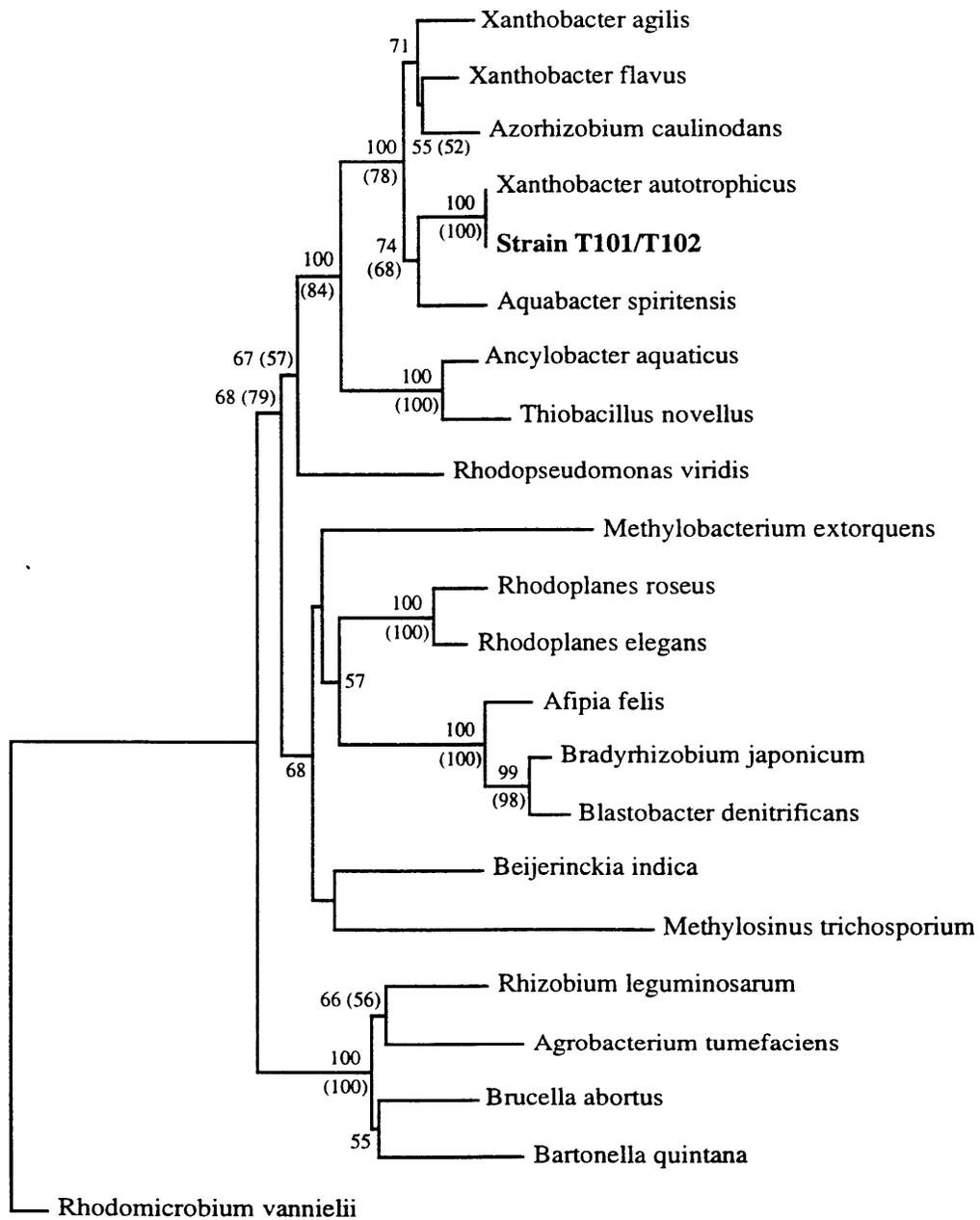
**Figure 1.** Scanning electron micrograph of toluene-degrading strain T101 grown on solid media (bar, 1  $\mu\text{m}$ ).



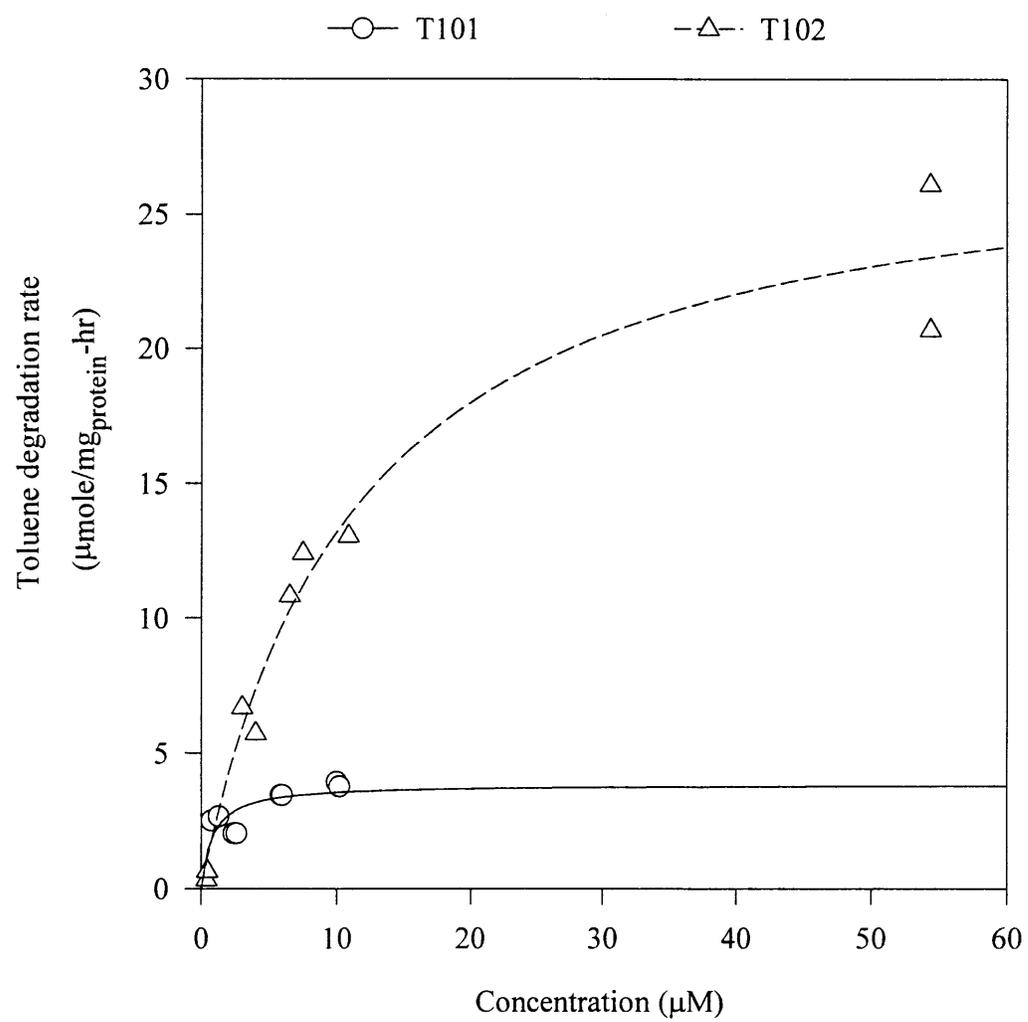
**Figure 2.** Dendrogram of selected *Xanthobacter* strains generated by cluster analysis of fatty acid profiles to produce unweighted pair matchings.



**Figure 3.** Unrooted evolutionary distance tree based on the 16S rDNA sequences of strains T101 and T102, representative members of the *Xanthobacter* genus, and other representative members of the alpha subdivision of *Proteobacteria*. Bootstrap values greater than 50% are shown at the nodes. Bootstrap values are shown for both distance (numbers without brackets) and parsimony (numbers within brackets) analyses. Bar = 1 nucleotide difference per 100 nucleotide positions.



**Figure 4.** Toluene biodegradation kinetics for strains T101 and T102.



**Table 1.** Characteristics of toluene degrading strains T101 and T102

	T101	T102
Morphology	Rod	Rod
Flagella	-	-
Pigmentation	Yellow	Yellow
Gram stain	<i>-<sup>a</sup></i>	-
Acid fast	-	-
Nitrate reduction	-	+
Catalase	+	+
Oxidase	+	+
Citrate	-	-
Growth on sugars:		
Glucose	+	+
Lactose	-	-
Maltose	+	+
Sucrose	-	-
Growth on aromatic compounds:		
Toluene	+++ <sup>b</sup>	++
Benzene	+	++
Chlorobenzene	-	-
o-xylene	-	-
m-xylene	-	-
p-xylene	-	-
Phenol	-	-

*a* +, good growth or activity; -, poor/no growth or activity.

*b* For growth on aromatic compounds, cultures were streaked onto agar plates containing a minimal salts media. Plates were then incubated in dessicators with substrate concentrations of approximately 10 mg/l; incubation temperature was 20° C. Each compound was tested twice. ++, very good growth within three days; +, good growth after three days; -, poor/no growth.

**Table 2.** Whole-cell fatty acid compositions of strains T101, T102, and *Xanthobacter autotrophicus*

Fatty acid	% of total in:		
	Strain T101	Strain T102	<i>X. autotrophicus</i> <sup>a</sup>
2-hydroxydodecanoic acid	N.D. <sup>b</sup>	0.69 (43.0) <sup>c</sup>	N.D.
<i>cis</i> -9-hexadecenoic acid	6.02 (5.6)	5.18 (16.4)	5.04
hexadecanoic acid	3.97 (3.6)	4.87 (5.2)	4.83
17:0 $\omega$ cyclopropane 7-8 hexadecanoic acid	N.D.	N.D.	2.45
3-hydroxyhexadecanoic acid	1.20 (2.5)	1.94 (26.2)	1.32
<i>cis</i> -11-octadecenoic acid	82.31 (0.2)	80.18 (1.1)	76.81
octadecanoic acid	2.98 (1.7)	3.27 (2.8)	1.98
19:0 $\omega$ cyclopropane 8-9 octadecanoic acid	N.D.	N.D.	5.99
<i>cis</i> -8,11,14-eicosatrienoic acid	N.D.	1.03 (24.1)	N.D.
<i>trans</i> -11-eicosenoic acid	3.53 (6.4)	2.86 (10.9)	1.55

<sup>a</sup> From Microbial Identification System library.

<sup>b</sup> N.D., not detected.

<sup>c</sup> The numbers in parentheses are coefficients of variation, N = 2.

Table 3. 16S rRNA identity/distance matrix for T101/T102 and related taxa

Organism	% nucleotide identity/evolutionary distance <sup>a</sup>																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 Strain T101/T102		0.0	2.4	3.2	2.7	3.3	5.7	6.1	6.3	7.3	7.4	7.4	8.6	9.1	9.4	9.6	9.7	9.5	10.3	10.7	11.3	10.6
2 <i>Xanthobacter autotrophicus</i>	100		2.4	3.2	2.7	3.3	5.7	6.1	6.3	7.3	7.4	7.4	8.6	9.1	9.4	9.6	9.7	9.5	10.3	10.7	11.3	10.6
3 <i>Xanthobacter flavus</i>	97.6	97.6		1.8	3.0	1.8	4.9	5.2	6.7	7.6	7.7	8.2	9.0	8.3	9.8	8.4	9.6	8.3	10.3	9.9	11.9	10.1
4 <i>Xanthobacter agilis</i>	96.8	96.8	98.2		3.1	2.4	5.1	5.8	6.6	7.4	7.3	7.5	8.7	7.7	9.6	8.4	9.5	8.0	9.5	9.8	10.7	9.0
5 <i>Aquabacter spiritensis</i>	97.3	97.3	97.1	96.9		3.0	6.1	6.9	5.7	7.7	8.2	8.6	8.6	8.1	9.3	9.2	9.3	8.1	10.7	9.5	11.5	9.9
6 <i>Azorhizobium caulinodans</i>	96.8	96.8	98.3	97.6	97.1		5.2	5.5	6.1	7.0	7.5	8.1	8.4	8.7	9.4	9.5	9.4	8.4	10.0	9.6	11.0	10.0
7 <i>Ancylobacter aquaticus</i>	94.5	94.5	95.3	95.1	94.1	94.9		2.0	6.2	7.7	8.1	7.4	8.5	8.6	9.5	8.7	9.7	8.5	9.5	8.9	11.0	9.8
8 <i>Thiobacillus novellus</i>	94.1	94.1	95.0	94.4	93.4	94.7	98.0		7.0	8.3	8.8	8.5	9.8	9.1	10.6	9.9	10.4	9.5	10.0	9.7	11.9	10.3
9 <i>Rhodopseudomonas viridis</i>	93.9	93.9	93.6	93.7	94.5	94.1	94.0	93.4		5.5	5.9	7.6	8.4	8.5	8.8	10.0	8.5	8.6	9.9	9.7	10.8	10.0
10 <i>Rhodoplates elegans</i>	93.0	93.0	92.8	92.9	92.7	93.3	92.7	92.1	94.7		1.8	6.6	6.9	10.0	6.4	10.4	6.0	8.5	9.3	9.7	9.7	9.3
11 <i>Rhodoplates roseus</i>	92.9	92.9	92.7	93.0	92.2	92.9	92.4	91.7	94.4	98.3		6.5	7.7	10.1	7.3	10.6	7.1	9.2	9.5	9.8	10.4	10.0
12 <i>Beijerinckia indica</i>	92.9	92.9	92.2	92.9	91.9	92.4	92.9	92.0	92.8	93.7	93.8		7.2	9.2	7.5	9.5	8.3	9.1	8.5	8.7	9.2	10.7
13 <i>Afipia felis</i>	91.9	91.9	91.5	91.8	91.9	92.0	92.0	90.8	92.0	93.4	92.7	93.1		9.6	2.0	10.4	2.9	9.4	8.8	9.8	11.5	10.4
14 <i>Rhizobium leguminosarum</i>	91.5	91.5	92.1	92.7	92.4	91.8	91.9	91.5	92.0	90.6	90.5	91.4	91.0		10.7	5.7	10.4	4.2	11.2	4.7	12.0	9.8
15 <i>Bradyrhizobium japonicum</i>	91.2	91.2	90.8	91.0	91.3	91.2	91.1	90.1	91.7	93.9	93.0	92.9	98.0	90.0		11.3	1.2	10.0	9.4	11.1	11.9	11.0
16 <i>Bartonella quintana</i>	91.0	91.0	92.0	92.0	91.4	91.0	91.8	90.7	90.6	90.3	90.1	91.0	90.3	94.5	89.5		11.4	4.8	11.8	5.9	12.6	10.9
17 <i>Blastobacter denitrificans</i>	90.9	90.9	91.0	91.1	91.3	91.2	90.9	90.3	92.0	94.2	93.2	92.1	97.2	90.3	98.8	89.5		9.7	9.3	10.7	12.4	11.1
18 <i>Brucella abortus</i>	91.1	91.1	92.1	92.4	92.4	92.0	92.0	91.1	91.9	92.0	91.4	91.5	91.2	95.9	90.6	95.4	90.9		10.9	5.2	11.7	9.9
19 <i>Methylobacterium extorquens</i>	90.4	90.4	90.4	91.0	90.0	90.6	91.1	90.6	90.7	91.3	91.0	92.0	91.7	89.6	91.2	89.0	91.3	89.9		11.6	11.9	11.1
20 <i>Agrobacterium tumefaciens</i>	90.0	90.0	90.7	90.8	91.1	91.0	91.6	90.9	90.9	90.9	90.8	91.8	90.8	95.4	89.7	94.4	90.0	94.9	89.3		11.5	10.4
21 <i>Methylosinus trichosporium</i>	89.5	89.5	89.0	90.0	89.4	89.8	89.8	89.0	90.0	90.9	90.3	91.4	89.4	88.9	89.0	88.4	88.5	89.2	89.0	89.4		12.2
22 <i>Rhodomicrobium vanniellii</i>	90.1	90.1	90.5	91.5	90.7	90.6	90.8	90.4	90.6	91.3	90.6	90.0	90.3	90.8	89.8	89.9	89.7	90.7	89.7	90.3	88.7	

<sup>a</sup> The values on the lower left are nucleotide percent identities, and the values on the upper right are Jukes-Cantor corrected evolutionary distances. A total of 1,205 nucleotides were used in the analysis.

**Table 4.** Toluene biodegradation kinetics of pure bacterial cultures

Organism	Maximal velocity $V_{\max}$ ( $\mu\text{moles toluene}/\text{mg}_{\text{protein}}\text{-hr}$ )	Saturation constant $K_s$ ( $\mu\text{M}$ )
<i>Xanthobacter autotrophicus</i> str. T101	$3.8 \pm 0.5$	$0.8 \pm 0.5$
<i>Xanthobacter autotrophicus</i> str. T102	$28.3 \pm 2.2$	$11.5 \pm 2.4$
<i>Mycobacterium spp.</i> str. T103 <sup>a</sup>	$1.0 \pm 0.1$	$0.6 \pm 0.4$
<i>Mycobacterium spp.</i> str. T104 <sup>a</sup>	$6.0 \pm 1.3$	$3.8 \pm 1.9$
<i>Pseudomonas sp.</i> str. T2 <sup>b</sup>	0.30	0.48
<i>Pseudomonas putida</i> str. PpF1 <sup>b, c</sup>	0.43	0.68

<sup>a</sup> From Tay *et al.*, 1997 (submitted).

<sup>b</sup> From Robertson and Button, 1987; maximal velocity values are derived assuming that 50% of a typical cell's dry weight is protein (Lodish *et al.*, 1995).

<sup>c</sup> Values reported for str. PpF1 are component values for the conversion of toluene to CO<sub>2</sub> only.

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## **Chapter Four**

# **DISTRIBUTION AND POPULATION DYNAMICS OF TOLUENE DEGRADING SPECIES IN A CONTAMINATED STREAM ASSESSED BY QUANTITATIVE PCR**

## ABSTRACT

The population dynamics of previously isolated species of toluene-degrading *Xanthobacter autotrophicus* and *Mycobacterium sp.* were compared between a contaminated and a pristine reach of a freshwater stream using quantitative molecular techniques. Total nucleic acids were sampled from rock surface biofilm communities over a period of one year. Quantitative slot-blot hybridization with the universal S-D-Bact-0338-a-A-18 probe indicated higher total rRNA content of the biofilm community in the spring and early summer months. Quantitative PCR using species-specific 16S rDNA primers indicated that these indigenous species were significantly more abundant in the rock biofilms from a contaminated reach than from a pristine reach, and that they were present in both reaches in greater numbers during the summer months than the winter months. At the contaminated reach, a brief bloom of *X. autotrophicus* was detected in the summer, while the *Mycobacterium sp.* population persisted for approximately 5 months. Populations of these toluene-degrading bacterial species in the stream were observed to correlate with toluene presence, and with warmer temperatures. These results are consistent with earlier studies which demonstrated higher in-situ biodegradation rates in contaminated reaches of the stream in the summer months than in the winter months, and higher CFU counts and MPNs of toluene-degrading populations in contaminated reaches than in pristine reaches.

## INTRODUCTION

Toluene is among the 50 largest-volume industrial chemicals produced in the United States, with 931 million gallons ( $3.5 \times 10^9$  liters) of toluene manufactured in 1994 (Kirschner, 1995). It has many applications in chemical manufacturing, in the manufacture of paints, lacquers, and adhesives, and in some printing and leather tanning processes, and is a significant component of gasoline. Toluene is usually disposed of at hazardous waste sites as a used solvent, and has been detected in surface and groundwaters in the vicinity of one-third of hazardous waste sites surveyed by the U.S. Environmental Protection Agency in 1988 (U.S. Public Health Service, 1989). Drinking water can pose a potential health hazard if it is contaminated with toluene. Toluene is a depressant of the central nervous system, and is an enhancing agent in skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene (Dean, 1978).

Because toluene contamination can impact human health, it is important to understand the role microorganisms play in degrading toluene in the environment. Many microorganisms capable of degrading toluene have been isolated from a variety of environments (Worsey and Williams, 1975; Zeyer *et al.*, 1986; Smith, 1990), although it is not often clear whether these microorganisms are the major degraders in the environment. In general, a particular bacterium may grow more easily in pure culture than do the microorganisms mainly responsible for the degradation process in the field (Dunbar *et al.*, 1997). One reason is that toluene-degrading strains are typically isolated under higher toluene concentrations than those existing in the field (Massol-Deya *et al.*, 1997).

Other studies that examined the response of microbial populations to anthropogenic disturbance have reported increases in contaminant-degrading populations and their relative proportions within microbial communities in contaminated environments. Such studies typically enumerate degraders using culture-based methods such as most probable

number (MPN) techniques or colony hybridizations (Spain *et al.*, 1984; Pignatello, *et al.*, 1986; Song and Bartha, 1990; Lemke *et al.*, 1997). The abundance of degraders and the ratios of their numbers to total heterotrophs are useful indicators of microbial community adaptation to contamination. However, culture-based methods may be inefficient and biased, as microbial communities may contain numerous novel and uncultured species (Ward *et al.*, 1990). Some species may be difficult to culture, while other species may enter a viable but nonculturable state (Huq and Colwell, 1996; Lee and Ruby, 1995).

An alternative approach to culture-based enumeration of contaminant degraders is the application of 16S rRNA-based techniques to measure concentrations of specific nucleic acid sequences that are unique to the bacterial groups of interest. Comparing the relative concentrations of nucleic acids specific to degraders within total community nucleic acids may provide a more reliable indicator of the presence of specific bacterial populations in the community. In quantitative slot-blot hybridization, target nucleic acid sequences are detected in a nucleic acid mixture by binding with a homologous complementary probe sequence. Probes can be tailored to monitor a particular bacterial species by having a specific sequence, or a broader group of microorganisms by targeting less variable sites on the 16S rRNA molecule (Stahl *et al.*, 1988; Raskin *et al.*, 1994; Polz and Cavanaugh, 1995). However, a large number of targets is needed to detect a signal, and this approach would not be likely to detect species in low abundances (Amann *et al.*, 1995). To overcome the limited sensitivity of the quantitative hybridization method, quantitative PCR (QPCR) can be used to amplify a target DNA sequence *in vitro* and significantly enhance the probability of detecting rare DNA sequences in heterologous mixtures of DNA (Lee *et al.*, 1996). This strategy has been used for bacterial quantifications of *Synechocystis* 6803 in Baltic Sea sediment (Moller and Jansson, 1997), an uncultured bacterial strain in soil (Lee *et al.*, 1996), and *Pseudomonas sp.* from marine water and sediments (Leser, 1995; Leser *et al.*, 1995), where between 10 to 30

*Pseudomonas* cells were successfully quantified against a background of  $4 \times 10^6$  cells per ml of seawater.

The purpose of the work reported here was to determine whether two bacterial species involved in the degradation of toluene were in fact enriched in rock biofilms in a freshwater stream that was contaminated with toluene. Quantitative hybridization was employed to measure the total bacterial community present on rock surface biofilms in the stream, while QPCR was used to measure the presence of the two species. The toluene-degrading species of *Xanthobacter autotrophicus* and *Mycobacterium sp.* have previously been isolated from the study site and characterized (Chapters 2 and 3). These species belong to genera whose members have been isolated from a diverse array of habitats, and which have the metabolic versatility to degrade a variety of xenobiotic compounds and pollutants (Wiegel, 1992; Hartmans and de Bont, 1992).

## MATERIALS AND METHODS

**Study site.** The East Drainage Ditch is a small freshwater stream in an industrial area of Wilmington and Woburn, Massachusetts (Cohen *et al.*, 1995; Kim *et al.*, 1995), and forms part of the Aberjona Watershed, a 90 km<sup>2</sup> area 20 km north of Boston. Toluene in the stream arises from a source of subsurface contamination below a 80 m long culvert located 1,600 m upstream from the confluence of the East Drainage Ditch with the Halls Brook Storage Area. Two sampling stations were selected for this study. Station D5 (the contaminated site) is located 5 m downstream of the culvert, and toluene concentrations at this location typically range from 2.2 to 5.4  $\mu\text{M}$ . Station U50 (the pristine or control site) is located 50 m upstream of the culvert and toluene concentrations here are typically not detectable or present at very low levels ( $< 0.2 \mu\text{M}$ ). Toluene-degrading *X. autotrophicus* strains T101 and T102, and *Mycobacterium sp.* strains T103

and T104 have previously been isolated and characterized from rock surface biofilms from station D5 (Chapters 2 and 3).

**Stream measurements.** Stream measurements of temperature, toluene concentrations, and dissolved organic carbon (DOC) were conducted periodically from October 1995 to September 1996. Streamwater temperatures were measured in triplicate at the sampling stations with a low-temperature range thermometer (VWR Scientific Products Corp., West Chester, PA). Streamwater samples were collected in duplicate from each sampling station in autoclaved glass bottles for toluene and DOC measurements. The bottles were completely filled to minimize degassing of the volatile toluene. Mercuric chloride was added to the streamwater samples to a final concentration of 15 mg/l. Toluene was measured using a purge-and-trap headspace gas chromatography method as described by Kim *et al.* (1995). 80 ml water samples were purged by helium for 30 minutes and trapped in a Supelco Volatile Purge Trap packed with 60/80 Chromosorb (U.S. E.P.A. Method 601). Toluene was thermally desorbed at 180°C for 4 min and transferred to the analytical column by carrier gas flow. Analysis was performed using a Perkin-Elmer 3920B Gas Chromatograph. Streamwater samples for DOC measurements were prepared by filtering with a 0.45  $\mu$ M glass fiber filter (Gelman Scientific), acidifying with 50% phosphoric acid, and sparging with helium for 15 minutes. DOC measurements were performed with a Shimadzu TOC-5000 Analyzer (Shimadzu Corporation, Columbia, MD).

**Cell counts.** Cell counts were determined for rocks collected from sampling station D5 on 4 different days in July 1992. Biomass was scraped off the rocks with an autoclaved spatula, weighed, suspended in 10 ml of a minimal salts medium, and serially diluted as described previously (Tay *et al.*, 1998). Total cell counts on the rock biofilms were determined with DAPI staining using an epifluorescence microscope as described previously (Cohen *et al.*, 1995). At least 20 grids with a total of 300 cells were counted for each sample. All samples were run in duplicate, and cells that were visualized as

attached to particles were counted twice, assuming that the same number of cells would also be attached beneath the particle. Viable counts of heterotrophic bacteria were determined by plating on PTYG agar (Tay *et al.*, 1998).

**Rock sampling and nucleic acid extraction.** Rocks were collected periodically in roughly two- to four-week intervals from October 1995 to September 1996 from the streambed of the East Drainage Ditch at sampling stations U50 and D5. Between 40 to 45 rocks were collected per sampling. Total nucleic acids from the rock surface biofilms were extracted using a rapid freeze-thaw method (Tsai and Olson, 1991). Total area of rock surfaces per sampling was measured by fitting rock surfaces to graph paper, and were  $1610 \pm 174 \text{ cm}^2$  and  $1630 \pm 140 \text{ cm}^2$  for stations U50 and D5, respectively. Rock samples in autoclaved 500 ml nalgene bottles were mixed with 80 ml of lysis solution (0.15 M NaCl, 0.1 M Na<sub>2</sub>EDTA [pH 8], 15 mg/ml lysozyme) and incubated in a 37°C water bath for 2 h with agitation at 20- to 30-min intervals. 80 ml of 0.1 M NaCl-0.5 M Tris-HCl (pH 8)-10% sodium dodecyl sulfate was added. Three cycles of freezing in a -70°C dry ice-ethanol bath and thawing in a 65°C water bath were performed to release nucleic acids from the microbial cells in the rock surface biofilms. 60 ml of 0.1 M Tris-HCl (pH 5)-saturated phenol was then added to the rocks and shaken at 150 rpm for 30 min. The liquid was decanted off the rocks and centrifuged at  $6,000 \times g$  for 10 min. The aqueous layer was collected and mixed with phenol/chloroform/isoamyl alcohol. The resulting supernatant was extracted with chloroform, and precipitated with ice-cold ethanol. The pellet of crude nucleic acids was obtained by centrifugation at  $10,000 \times g$  for 10 min, dried, and resuspended in DEPC-treated water. The crude nucleic acids extract was further purified with between one to three passes through Chroma Spin columns according to manufacturer's specifications (Clontech Laboratories Inc., Palo Alto, CA).

Nucleic acid extraction and purification efficiencies and nucleic acid content per cell need to be quantified in order to estimate the actual cell densities on the rock surface

biofilms from the QPCR data. Lysis efficiency was not determined but scanning electron micrographs of rock surfaces showed little remaining biological material after nucleic acid extraction and indicated near complete lysis (data not shown). Extraction efficiency was estimated as the ratio of the volume of aqueous solution remaining at the end of the extraction process (75 ml) to the volume of aqueous solution at the start of the extraction process (160 ml). The extraction efficiency was estimated as 47%. Purification efficiency was estimated as  $18 \pm 3\%$  (N=4) and was determined by comparing the amounts of *X. autotrophicus* and *Mycobacterium sp.* nucleic acids present before and after purification. Total nucleic acids for use as controls or standards in quantitative hybridization and QPCR were extracted from cell suspensions of pure cultures of *X. autotrophicus* strain T101, *Mycobacterium sp.* strain T104, and *Methanobacterium thermoautotrophicum* strain Marburg. The numbers of *X. autotrophicus* and *Mycobacterium sp.* cells in the standards were calculated after adjusting for the nucleic acid content per cell. This was determined by counting bacteria in the cell suspensions with DAPI staining, and relating the counts to yields of extracted nucleic acids.

**Quantitative slot-blot hybridizations.** Quantitative slot-blot analysis was performed with the universal probe S-D-Bact-0338-a-A-18 (Stahl and Amann, 1991; Wheeler *et al.*, 1996) to obtain a measure of the total bacterial community present in the purified nucleic acid extracted from the rock surface biofilms from stations U50 and D5. Quantitative slot-blot analysis was similarly performed to measure the amounts of *X. autotrophicus* and *Mycobacterium sp.* nucleic acids present in the nucleic acids extracted from the rock surface biofilms from station D5. Probes specific to the 16S rDNA of *X. autotrophicus* (X1260), and *Mycobacterium sp.* (MY1003) were designed with the following sequences: X1260, 5'-TTGCTAGGGGTCGCCCTTT-3'; MY1003, 5'-TACCTATCTCTAGGCACGTC-3'. The mid-point dissociation temperatures ( $T_d$ ) of the probes were determined as described previously (Polz and Cavanaugh, 1997) to be 60.5°C and 50.1°C, respectively.

For measuring total bacterial community, three replicates of each purified extract were blotted on Zeta-Probe membranes (Bio-Rad, Hercules, California), hybridized, and quantified as described by Polz and Cavanaugh (1995). A dilution series of nucleic acids from *X. autotrophicus* strain T101 used as standards were blotted in duplicate on the same membrane as the samples for hybridization. The universal probe S-D-Bact-0338-a-A-18 was labeled with polynucleotide kinase to an approximate specific activity of  $5 \times 10^8$  cpm  $\mu\text{g}^{-1}$ . Hybridizations were performed overnight at 40°C with 1 pmol of probe per slot. This was followed by two 15-min low temperature washes and a final 15-min specific wash at 60°C, which was the  $T_d$  of the probe. Quantification of probe remaining bound to the target was performed by exposure of the membrane to a Fuji BAS-III imaging plate which was analyzed with a Fujix BAS2000 phosphor imager and BAS 2000 Image File Manager 2.1 analysis software. Species-specific nucleic acids were similarly assayed, using duplicate blots of standard nucleic acids extracted from pure cultures of *Xanthobacter autotrophicus* strain T101 and *Mycobacterium sp.* strain T104. Hybridizations were performed with the MY1003 probe, the X1260 probe, and the S-D-Bact-0338-a-A-18 probe. The membrane was stripped between hybridizations by immersing in washing buffer twice for 20 minutes at 80°C, and checked for complete removal of radiolabelled products by overnight autoradiography.

**QPCR.** Quantification of specific 16S rDNA targets of *X. autotrophicus* and *Mycobacterium sp.* in the purified nucleic acid extracts from the rock surface biofilms was conducted using a QPCR approach (Lee *et al.*, 1996). In QPCR, a constant amount of an internal control DNA is added to the PCR mixture, and competes with the target DNA for amplification reagents. Standard curves are generated from known ratios of target PCR products to control PCR products, and known starting masses of target nucleic acids in the PCR mixture. The measured target:control PCR product ratio permits interpolation from standard curves to the starting mass of the target nucleic acids present

in each environmental extract. The control chosen for this study was the 16S rDNA sequence of *Methanobacterium thermoautotrophicum* strain Marburg.

Primer pairs specific to the 16S rDNA of *X. autotrophicus* strains T101 and T102 (X848F and X1258R), *Mycobacterium sp.* strains T103 and T104 (MY508F and MY1099R), and the control *M. thermoautotrophicum* strain Marburg (MT780F and MT1432R) were designed and are listed in Table 1. These primer sequences were checked against the Ribosomal Database Project by using CHECK-PROBE (Maidak *et al.*, 1997) to ensure that there were no matches with other existing sequences in the database. These primer pairs resulted in PCR amplification products that were 450 bp, 636 bp, and 692 bp in length, respectively. Their different lengths allow for easy separation of the PCR products by gel electrophoresis. The specificities of these primers were also checked by amplification of environmental nucleic acids. Sequencing of the amplification products obtained from environmental nucleic acids with the first two primer pairs confirmed that only *X. autotrophicus* and *Mycobacterium sp.* sequences were amplified. No amplification products were detected with the *M. thermoautotrophicum*-specific primers.

To establish amplification conditions that minimized potential PCR inhibition by environmental contaminants, all samples were spiked with fixed amounts of *M. thermoautotrophicum* DNA, and serially diluted. PCR was then performed using the *M. thermoautotrophicum*-specific primers. The PCR products were visualized by agarose gel electrophoresis with ethidium bromide staining, and the range of dilutions within which PCR product accumulation was observed to be proportional to the amount of target present was determined for each sample. A working dilution was selected within this range of dilutions for each sample and this dilution was subsequently used for QPCR. Based on this, all environmental nucleic acid extracts were eventually diluted between 16-fold to 64-fold for use in QPCR.

PCR amplification efficiency may decrease over successive cycles because of decreasing concentrations of primers and dNTPs, kinetic bias, or decreasing enzyme activity (Sardelli, 1993; Suzuki *et al.*, 1996). This is represented by a plateau phase, in which the amount of amplification product starts to level off at higher amplification cycles. In order to ensure that the PCR process did not enter this plateau phase, and that accumulation rates of target and control DNA were similar in environmental samples as well as in the standard mixtures, the accumulation of target and control DNA was determined from 15 to 40 PCR cycles in 5 cycle-intervals. The plateau phase defines the upper range of the quantitative PCR response, and as such defines the upper limit for a reliable quantitative PCR assay. Environmental mixtures were prepared by adding known amounts of control nucleic acids to environmental samples. Standard mixtures were prepared by adding known amounts of target and control nucleic acids. The environmental mixtures and standard mixtures were designed to contain amounts of standard and control nucleic acids expected in the mixtures eventually used in QPCR.

Because target and control amplification products were of different lengths, they could be easily separated by gel electrophoresis. The separated bands with incorporated label could then be excised from the gel and the amount of radioactivity in each band was quantified by liquid scintillation. In order to easily visualize the amplification products on the gel, nonlabeled amplification products obtained from separate PCR reactions were added to the samples prior to gel electrophoresis. The additional nonlabeled DNA served only to locate the appropriate bands on the gel, and did not interfere with quantification of the radiolabeled bands by scintillation counting (Lee *et al.*, 1996). Two replicates of each experiment were performed.

PCR amplification was conducted in a total volume of 25  $\mu$ l containing 1.25 mM  $MgCl_2$ , 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 200  $\mu$ M each of dATP, dCTP, dGTP, and dTTP, 10  $\mu$ Ci [ $\alpha$ - $^{32}P$ ]dCTP, 2.5 U/100 $\mu$ l of DNA polymerase, and 0.2  $\mu$ M each of oligonucleotide primer. Acetamide (5%, wt/vol) was added to the reactions to enhance

denaturation during the amplification reaction (Reysenbach *et al.*, 1992). All reactions were overlain with mineral oil. A single set of standard samples containing known amounts of target DNA was prepared. All reaction mixtures were spiked with a constant amount of control, and all samples were amplified in triplicate. PCR was performed on a DNA thermal cycler (Ericomp). The PCR mixtures were first subjected to a denaturation step for 5 min at 94°C. The subsequent 35 cycles consisted of denaturation at 94°C for 1.5 min, annealing at 61°C for 4 min, and extension at 72°C for 1.5 min. The longer annealing time was chosen to ensure saturation of each target sequence with the annealed primers during each cycle. Longer annealing time improves the ability of primers to find the correct complementary annealing sites (Zhou *et al.*, 1997).

Each set of PCR amplifications involved triplicates of three environmental samples, five standard mixtures, and a negative control, in which the PCR aliquot contained no target but the same constant amount of control DNA as in the standard and environmental mixtures. A standard curve was constructed for each amplification set by plotting the logarithm of the ratio of the radioactivity (as counts per minute) in the target DNA band to that in the control DNA band against the logarithm of known masses of total nucleic acids. Least-squares linear regression analysis of the standard curves yielded  $r^2 > 0.95$  in all cases.

**Analysis of quantification data.** Mass equivalents of *X. autotrophicus* or *Mycobacterium sp.* nucleic acids (obtained from the QPCR experiments) were normalized to amounts of total nucleic acids in the extracts (obtained from the quantitative hybridization experiments) to provide an indicator of enrichment of the sequences within the community, regardless of whether the total community nucleic acids increases or decreases as a result of environmental factors. Use of the normalized mass equivalents also eliminates variability due to differences in nucleic acid extraction and purification efficiency among samples. On the other hand, areal cell densities represent the absolute concentrations of *X. autotrophicus* and *Mycobacterium sp.* on rock surface

biofilms in the stream, and could be calculated as (amount of nucleic acids per  $\mu\text{l}$  of extract)  $\times$  (volume of extract per sampling) / [ (nucleic acid content per cell)  $\times$  (surface area of rocks per sampling)  $\times$  (extraction efficiency)  $\times$  (purification efficiency) ].

## RESULTS

**Stream measurements.** Toluene levels, DOC concentrations, and streamwater temperatures at stations D5 and U50 from October 1995 to September 1996 are shown in Figure 1. Toluene levels were typically below detection or present at very low levels ( $< 0.2 \mu\text{M}$ ) at station U50, and ranged from 2.2 to 5.4  $\mu\text{M}$  at station D5. No distinct seasonal variations in toluene levels were observed at either sampling station. DOC levels ranged from 10.0 to 20.4  $\text{mg of C}\cdot\text{L}^{-1}$  at both stations. There were no discernible differences in DOC levels at both stations. DOC levels tended to be lower during the winter months, and increased during the spring and summer months. pH, nitrate, ammonia, and phosphorus concentrations measured in the summer were also similar for the two stations (data not shown).

**Cell counts.** The cell count data are presented in Table 2, together with other relevant cell count data from previous studies (DeJesus, 1994). At station D5 in July 1992, the same period during which the toluene-degrading isolates were obtained, cell counts of total bacteria by DAPI staining averaged  $6.0 \times 10^9$  cells/g biomass ( $10.8 \times 10^7$  cells/cm<sup>2</sup> rock surface), while viable counts of heterotrophic bacteria averaged  $3.0 \times 10^8$  cells/g biomass ( $5.4 \times 10^6$  cells/cm<sup>2</sup> rock surface), or 5% of the total count. Based on the appearance of the isolates on agar plates containing serial dilutions of environmental inoculum, *X. autotrophicus* and *Mycobacterium sp.* were previously estimated to be present in the rock biofilms at station D5 at densities of  $5.5 \times 10^6$  cells/g biomass and  $1.0 \times 10^6$  cells/g biomass respectively (Chapters 2 and 3). *X. autotrophicus* constituted 0.09% of the total bacteria, and 1.8% of the heterotrophic bacteria, while *Mycobacterium*

*sp.* accounted for 0.02% of the total bacteria, and 0.3% of the heterotrophic bacteria. In the September 1993 studies, the densities of toluene-degrading bacteria were generally an order of magnitude higher at station D5 than at station U50 (Table 2). Toluene-degrading bacteria as determined by colony counts comprised 0.02% of total bacteria and 1.1% of heterotrophic bacteria at station D5, and 0.0007% of total bacteria and 0.03% of heterotrophic bacteria at station U50. Using the MPN approach, toluene-degrading bacteria made up 0.15% of total bacteria and 10.0% of heterotrophic bacteria at station D5, and 0.005% of total bacteria and 0.17% of heterotrophic bacteria at station U50.

**PCR accumulation rates.** To ensure that the PCR accumulation rates of target or control DNA are similar in sample mixtures and standard mixtures, we performed PCR over a range of cycles. Sample mixtures consisted of working dilutions of environmental nucleic acids to which 2.5 pg of control nucleic acids was added. Standard mixtures consisted of approximately the same concentrations of the two templates. Figures 2A and 2B show that between cycle 30 and cycle 40, *X. autotrophicus* and *Mycobacterium sp.* DNA purified from rock biofilms amplified at approximately the same rate as that obtained directly from laboratory culture. This demonstrates that the PCR amplification of *X. autotrophicus* DNA from environmental samples was not significantly affected by PCR-inhibiting contaminants that might have originally been present. Similarly, the accumulation rates for the control DNA are similar in both the environmental samples and standard mixtures (Figures 2A and 2B). Based on these PCR accumulation results, QPCR experiments were performed over 35 cycles.

**Quantitative slot-blot hybridization and QPCR.** Results of quantification of total bacterial nucleic acids by slot-blot hybridization are shown in Table 3. Slot-blot hybridizations with the universal probe Eub338 yielded positive signals for all samples. Total nucleic acids ranged from 23 to 28,800 ng at station U50 and from 470 to 23,900 ng at station D5. There was no significant difference in the amounts of total nucleic acids between both stations (F test,  $P < 0.05$ ). Not counting the significant loss of nucleic acids

at station U50 on 7/7/96 that likely arose during extraction, there is a 100-fold variation in the amounts of total nucleic acids obtained from either station over the one-year period. Although total nucleic acids seemed high during the spring and early summer months, it was not established if there was any correlation with seasonal or weather patterns such as nutrient inputs to the stream from spring melt or rainfall runoff. Quantitative hybridizations with the species-specific MY1003 and X1260 probes revealed that the species-specific nucleic acids were in all cases present at concentrations in the environmental nucleic acids that were too low to be quantified reliably (Appendix B).

Because of the insufficient sensitivity of the hybridization assay, QPCR was employed to quantify the amounts of species-specific nucleic acids in the environmental samples. Figure 3 illustrates the analysis involved and was used to quantify three samples from station U50. PCR was performed in the same run on the standard mixtures and triplicate aliquots of the three samples. Working solutions of the 4/21/96, 5/26/96, and 6/23/96 samples were prepared by diluting them 16-fold, 16-fold, and 32-fold respectively. Dilutions were determined earlier for all samples at which PCR was not inhibited by environmental contaminants. Standard mixtures consisted of 2.5 pg *M. thermoautotrophicum* nucleic acids and 1.25, 0.25, 0.125, 0.025, or 0.0125 pg *Mycobacterium sp.* nucleic acids. The background level was determined from a PCR aliquot containing no target but the same constant amount of control DNA as in the standard mixtures. The log [(target products)/(control products)] values for appropriately diluted 4/21/96, 5/26/96, and 6/23/96 samples from station U50 averaged -0.655, -0.556, and -0.855 respectively, giving input target nucleic acid quantities of 0.032, 0.055, and 0.010 pg respectively (in a volume of 2.5  $\mu$ l). These concentrations were multiplied by the appropriate dilution factor and volume of extract and divided by the extraction and purification efficiencies to give target *Mycobacterium sp.* nucleic acid contents of 450, 390, and 210 pg in the original samplings.

Target nucleic acids were quantified in the same way for the rest of the samples (Table 3). *X. autotrophicus* nucleic acids per sampling ranged from 120 to 300 pg at station U50, and from 230 to 11,400 pg at station D5. *Mycobacterium sp.* nucleic acids per sampling ranged from 210 to 770 pg at station U50, and from 740 to 5,290 pg at station D5. Target PCR products were not detected in several cases, as the log [(target products)/(control products)] value was less than the corresponding background value. The quantification data is further presented in two ways: (1) mass equivalents of *X. autotrophicus* or *Mycobacterium sp.* nucleic acids per nanogram of total nucleic acids (Figure 4A), and (2) cell densities on rock surfaces of *X. autotrophicus* and *Mycobacterium sp.* (Figure 4B).

Figure 4A profiles the mass equivalents of *X. autotrophicus* and *Mycobacterium sp.* nucleic acids normalized to total nucleic acids. The normalized mass equivalents of the species-specific nucleic acids were higher at station D5 than at U50. Normalized *X. autotrophicus* mass equivalents ranged from 0.03 to 0.70 pg per ng of total nucleic acids at station D5, compared to from 0.004 to 0.056 pg per ng of total nucleic acids at station U50. Normalized *Mycobacterium sp.* mass equivalents ranged from 0.07 to 0.46 pg per ng of total nucleic acids at station D5, compared to from 0.034 to 0.081 pg per ng of total nucleic acids at station U50. There is a peak of normalized *X. autotrophicus* mass equivalents in the summer months, and fairly constant normalized *Mycobacterium sp.* mass equivalents averaging 0.36 pg per ng of total nucleic acids from March 1996 to July 1996.

Cell densities of *X. autotrophicus* and *Mycobacterium sp.* were also significantly higher at station D5 than at station U50 (Figure 4B). *X. autotrophicus* cell densities ranged from 22 to 1100 cells per cm<sup>2</sup> of rock surface at station D5, compared with 12 to 29 cells per cm<sup>2</sup> of rock surface at station U50. *X. autotrophicus* cell densities at station D5 were highest in the summer months, with a pronounced peak of 1100 cells per cm<sup>2</sup> of rock surface occurring on 6/23/96. *Mycobacterium sp.* cell densities ranged from 49 to

350 cells per cm<sup>2</sup> of rock surface at station D5, compared with 14 to 52 cells per cm<sup>2</sup> of rock surface at station U50. *Mycobacterium sp.* cell densities at station D5 appeared high throughout the year, and were also measurable in the winter month of January 1996, although the densities were generally lower in the winter than in the warmer months (Figure 4B).

## DISCUSSION

The overall approach adopted for this investigation is important for several reasons. The use of molecular techniques to quantify microbial populations in natural environments should overcome the bias that is inherent in culture-based and DAPI staining techniques. Also, monthly observations allows for a study of microbial population dynamics under changing environmental conditions that would not be possible if only one time point was examined. Because variations in microbial community structure may exist among rocks collected from the same station, a minimum of 40 rocks were collected from each sampling for nucleic acid extraction. The microbial community is expected to be well represented by the use of a large sample size. Although nucleic acids were lost during the extraction and purification steps, the approximate losses can be determined and permit accurate enough measures of species distributions to draw conclusions about population trends within the microbial communities as a result of changing environmental conditions. QPCR was employed to detect the toluene-degrading species, as quantitative hybridization was shown to be too insensitive for the purpose of this study. QPCR was able to detect as little as  $0.4 \times 10^{-3}\%$  by mass of species-specific nucleic acids in a mixture of total nucleic acids (Table 3), while quantitative hybridization with species-specific probes required at least 0.2% by mass of target to be present for successful detection (Appendix B).

The presence of toluene in the stream is associated with higher abundances of the two toluene-degrading species. The relative distributions of *X. autotrophicus* at station D5 were between 2 to 42 times higher than the corresponding values at station U50. For *Mycobacterium sp.*, the relative distributions at station D5 were between 4 to 11 times higher than the corresponding values at station U50. Stations D5 and U50 were similar in many respects and were differentiated only by the presence or absence of toluene. Other physical and chemical parameters such as temperature and DOC concentrations measured over a year (Figure 1), as well as pH, nitrate, ammonia, and phosphorus concentrations measured in the summer (data not shown) were similar for the two stations. The abundances measured for the two indigenous toluene-degrading species using 16S rRNA-based molecular methods are consistent with an earlier study which demonstrated an order of magnitude increase in culturable toluene-degrading populations in the contaminated reaches compared to the pristine reaches of the stream (DeJesus, 1994). The enrichment by toluene of the stream bacteria is also consistent with the view that natural populations are well-suited to adapting to pollutant contamination in their environment (Massol-Deya *et al.*, 1997).

Similar observations of microbial adaptation to the presence of pollutants have been reported previously in a variety of environments, including ponds, streams and soils (Mu and Scow, 1994; Pignatello *et al.*, 1986; Spain *et al.*, 1984; Wiggins *et al.*, 1987). Increases in MPNs of the degrading communities correlated with increases in biodegradation rates. Lemke *et al.* (1997) adopted a rRNA-based colony hybridization approach and found higher numbers of *Acinetobacter calcoaceticus* in a stream with thermal, radioactive, and chemical pollution, than in a pristine, upstream site, which suggested the ability of this species to attain larger populations by utilizing compounds present in the polluted site. These studies demonstrate that adaptation of microbial communities was the result of selection of organisms able to grow at the expense of the contaminant.

The toluene-degrading species of *X. autotrophicus* and *Mycobacterium sp.* were also detected at very low numbers at station U50, where there was little or no toluene contamination (Figure 4). This suggests that the toluene-degrading population at station D5 developed from a small resident population that already existed in the stream prior to toluene contamination. These species need not rely solely on toluene for growth, but are heterotrophs that can presumably survive on the dissolved organic material present in the stream (Chapters 2 and 3). The adaptation process thus appears to involve the selection of organisms able to grow at the expense of toluene, although the recruitment of plasmids that encode for toluene degradation cannot be discounted. It would be interesting to determine the distributions in the stream of the genes that encode for the different pathways of toluene degradation (Zylstra, 1994). Competition among pathways for toluene in the natural environment is not well understood, although several studies have been described that examined competition in laboratory ecosystems (Duetz *et al.*, 1994; Massol-Deya *et al.*, 1997).

The observed increases in the toluene-degrading populations in the warmer months indicates that the higher summer biodegradation rates reported in a previous study (Kim *et al.*, 1995) is due not only to higher per-cell activity from rising temperatures but also to higher populations. Temperature influences the activity of biological systems, and rates of natural biodegradation can generally be estimated for different temperatures by assuming that the rate doubles for each 10°C rise in temperature (Atlas and Bartha, 1993; Bradley and Chapelle, 1995). For example, the toluene mineralization rate at 11°C (the in situ temperature) was shown to average 35% of that at 25°C for groundwater at a hazardous waste site containing elevated levels of toluene (Armstrong *et al.*, 1991). Toluene biodegradation rates in the East Drainage Ditch were 1.73 hr<sup>-1</sup> in summer and 0.20 hr<sup>-1</sup> in winter, where water temperatures averaged 19°C and 6°C, respectively (Kim *et al.*, 1995). Although there was a strong dependence of in-situ biodegradation rates on stream temperatures, the effect of temperature alone is not sufficient to account for the

difference between the summer and winter rates; based on temperature effects alone, the summer biodegradation rate should be 2.6 times the winter biodegradation rate, or  $0.52 \text{ hr}^{-1}$ . The actual summer rate of  $1.73 \text{ hr}^{-1}$  is much higher than that calculated based on temperature effects alone, and this increased rate is consistent with the observed increases in the toluene-degrading populations in summer.

Although general trends in the abundances of the two species were observed, the patterns of abundances were not simple. The complex population dynamics indicates that changes in the bacterial assemblages in the stream are influenced not only by temperature and toluene presence, but also by other environmental factors (e.g. nutrient availability, predation, or competition). It also suggests that different bacterial species could dominate toluene biodegradation in the stream at different times of the year. The occurrence of peaks or blooms of the two species did not occur at the same times. In addition, there were noticeable differences in the population dynamics of the two species. For example, there was a minor peak of *Mycobacterium sp.* cells at station D5 on 1/1/96 and 1/14/96 that was not observed for *X. autotrophicus* (Figure 4B). *X. autotrophicus* cell densities at station D5 peaked sharply at 530 and 1100 cells per  $\text{cm}^2$  of rock surface on 5/26/96 and 6/23/96, respectively, but fell abruptly to below 150 cells per  $\text{cm}^2$  of rock surface at other times of the year. Similar short-lived bacterial blooms lasting 2-3 weeks have also been observed in a marine ecosystem study, where 16S rRNA-based probes with degeneracies were used (Rehnstam *et al.*, 1993). *Mycobacterium sp.* populations appeared to be more evenly distributed over the spring and summer months, with cell densities that ranged from 200 to 350 cells per  $\text{cm}^2$  of rock surface from 3/17/96 to 6/23/96. The brief summer bloom of *X. autotrophicus* emphasizes the importance in ecological studies of sampling over adequate time intervals in order to detect possible transients in population densities. This approach should be more useful than one with only one sampling period.

*X. autotrophicus* and *Mycobacterium sp.* respond favorably to toluene presence in the stream, although the molecular quantifications in this study indicate that these two

species occupy a small fraction of the community. Their relative abundances averaged 0.06% and 0.04%, respectively, in the summer months. Previous calculations comparing toluene biodegradation kinetics of biofilms and pure cultures suggest, however, that *X. autotrophicus* strain T102 may account for up to 28.0% of the biodegradation occurring on the rock surfaces. T102 possessed kinetic parameters indicative of greater competitiveness for toluene compared to the other toluene-degrading strains studied (Chapters 2 and 3). This ability of a small subpopulation to make a disproportionately large contribution to toluene degradation within a microbial community has also been found in an investigation of biofilters for waste gas treatment (Moller *et al.*, 1996). A strain of *Pseudomonas putida* was present in the biofilm with a relative abundance of 4%, yet accounted for 65% of the toluene removal performed by the biofilm community.

Although molecular methods were employed in this study to assess the presence of bacterial populations in the East Drainage Ditch, the trends observed were similar to those reported in earlier studies using culture-based methods. Greater abundances of toluene-degrading populations and the toluene-degrading species were found at station D5 than at station U50. MPNs of toluene degraders in September 1993 constituted 0.15% of total bacteria and 10.0% of heterotrophic bacteria at station D5, and 0.005% of total bacteria and 0.17% of heterotrophic bacteria at station U50. Based on the molecular measures, *X. autotrophicus* in September 1996 constituted 0.047% of total bacteria at station D5, and was not measurable at station U50; *Mycobacterium sp.* was not measurable at either station in September 1996, although throughout the year, it was generally present in higher numbers at station D5 than at station U50. It should be noted that the above relative MPN and molecular abundance measures are not entirely comparable. The sampling periods in question were different. In addition, MPNs were normalized to total cell counts while the molecular abundances were normalized to rRNA content. Furthermore, MPN overestimates might arise if the serial dilutions of

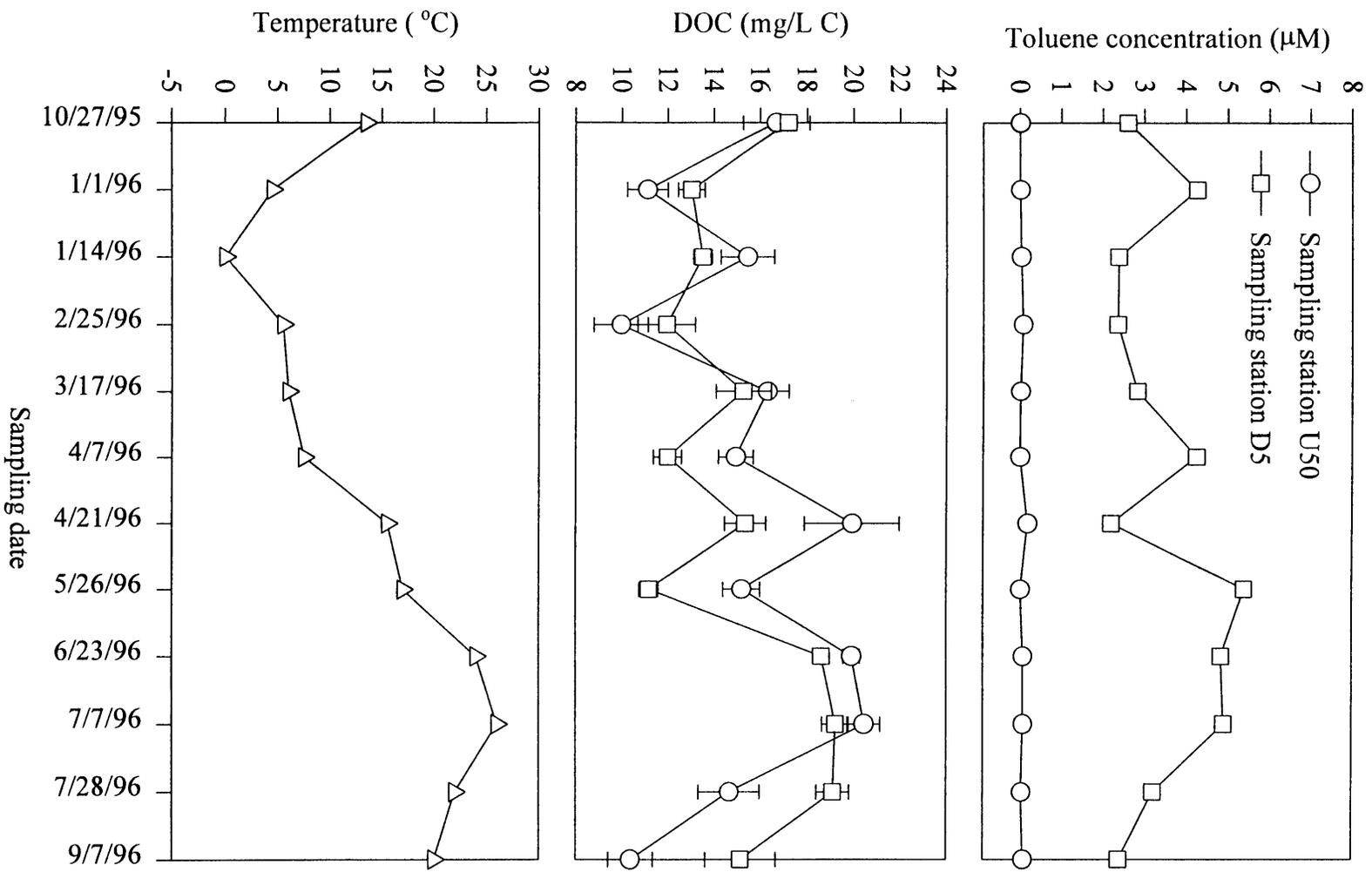
environmental inocula were not sufficiently mixed, resulting in the carrying-over of clumps of biomass to the higher dilutions before plating.

In summary, this study is one of the first efforts to employ rRNA-based methods to monitor specific bacterial species in a freshwater stream over a long time period. Changes in the distributions of the two indigenous toluene-degrading species were associated with the presence or absence of toluene, as well as the effect of temperature. Differences in the distribution patterns of the two species suggest a complex community response, in which toluene degradation could involve different groups of microorganisms in the stream at different times of the year.

#### **ACKNOWLEDGMENTS**

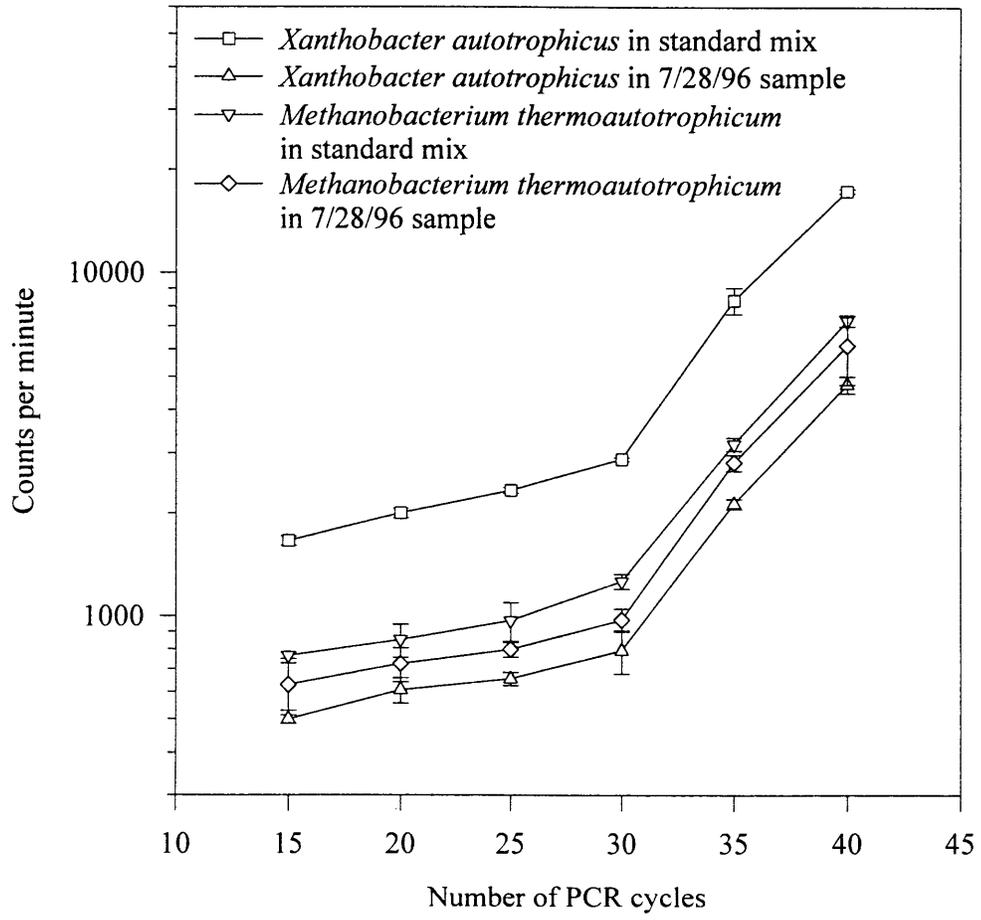
This work was supported by NIEHS Superfund Basic Research Program Grant 5P42ES04675-06 to H.F.H. and an NSF grant to C.M.C.

**Figure 1.** Time profiles of toluene concentration, dissolved organic carbon content, and streamwater temperature at stations U50 and D5.

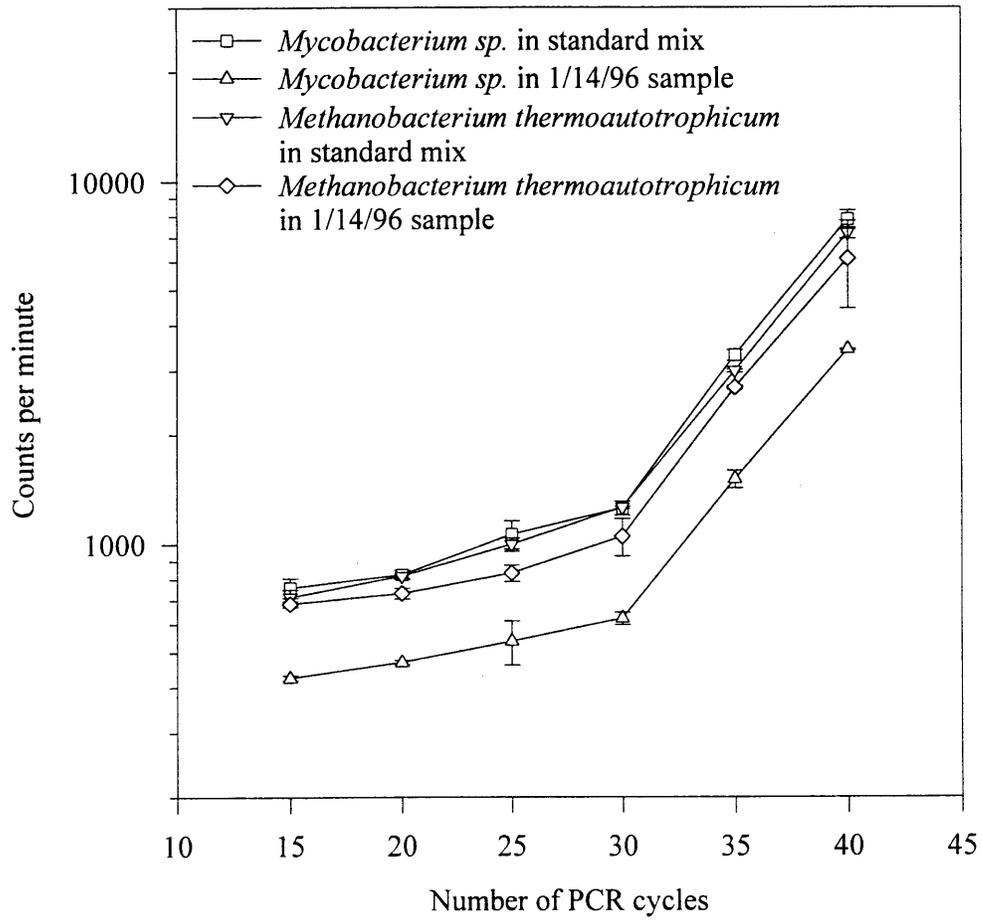


**Figure 2.** Amplification rates of DNAs. (A) Amplification rates of target (*X. autotrophicus*) and control (*M. thermoautotrophicum*) DNAs in standard and environmental mixtures; (B) Amplification rates of target (*Mycobacterium sp.*) and control (*M. thermoautotrophicum*) DNAs in standard and environmental mixtures.

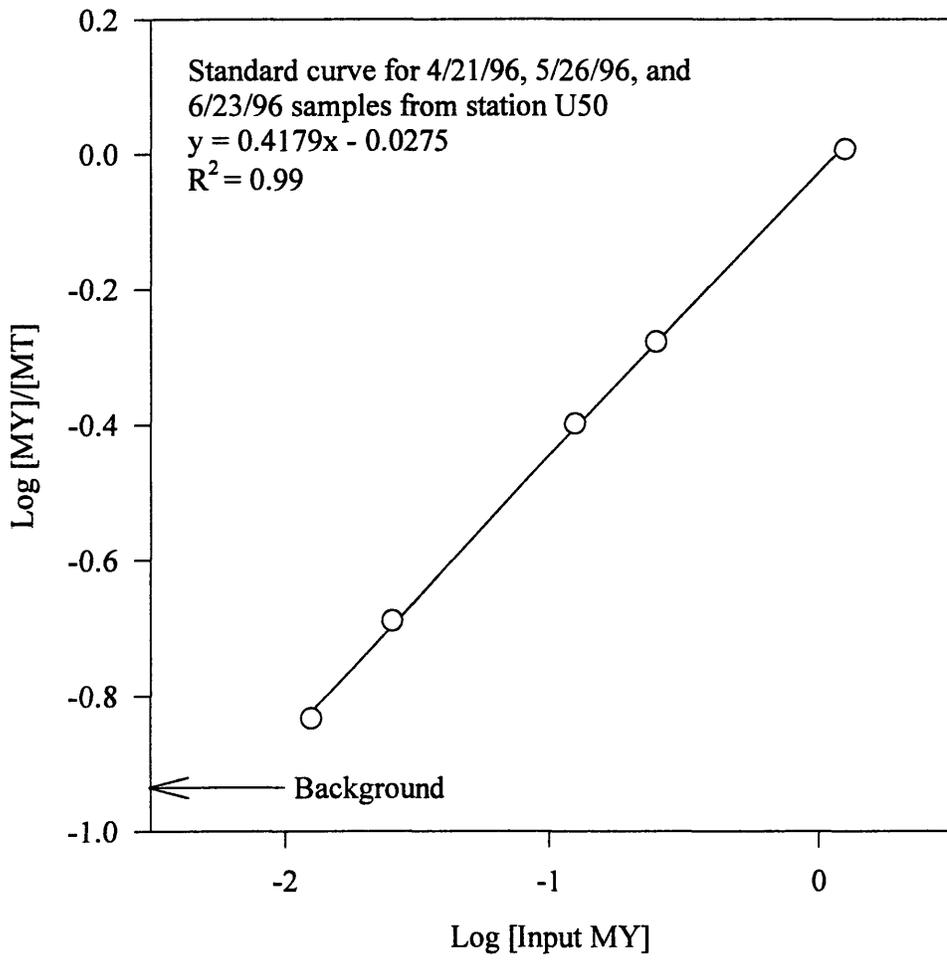
**A**



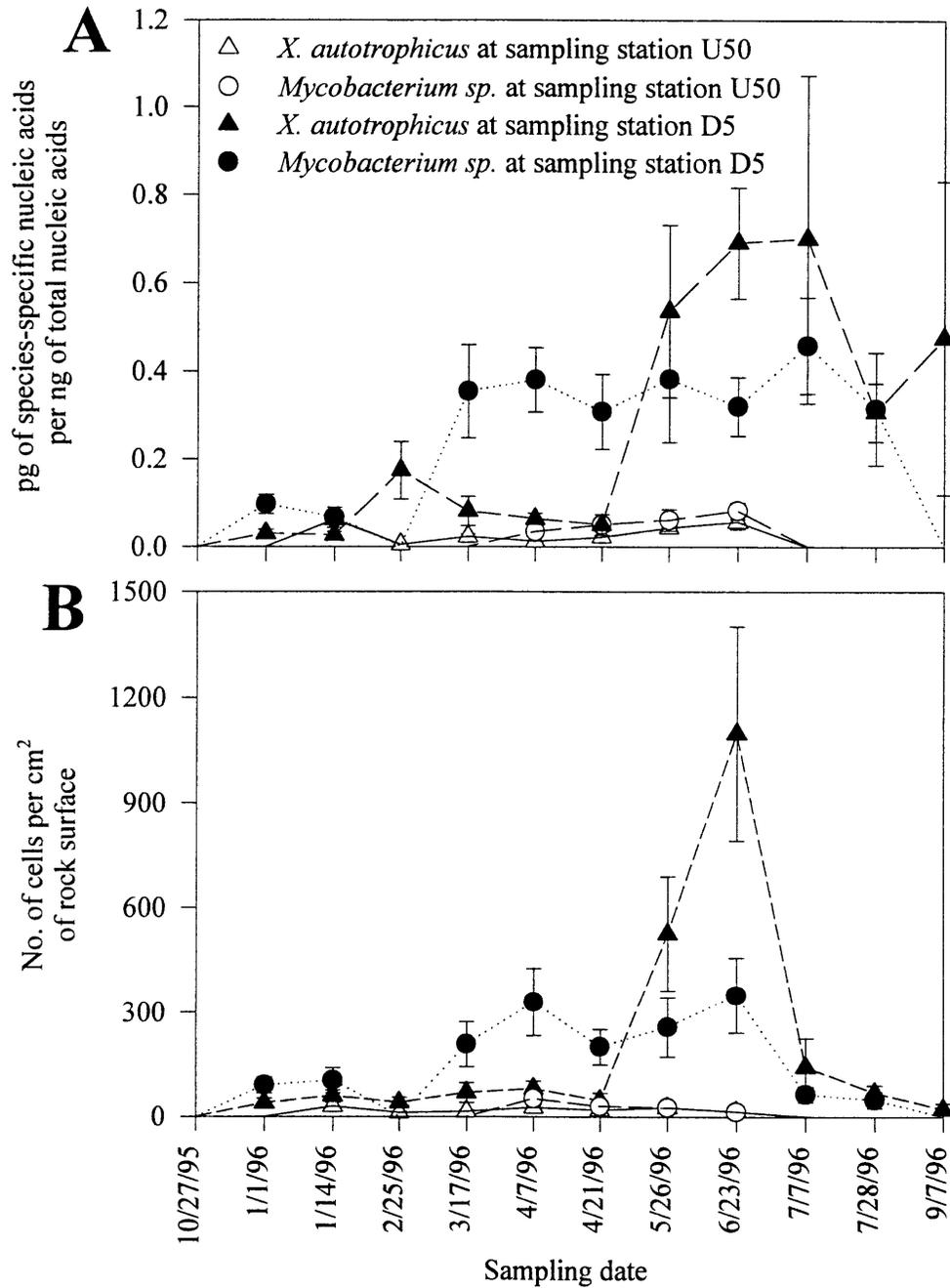
# B



**Figure 3.** Standard curve of QPCR of *Mycobacterium sp.* 16S rRNA genes. The relative masses of amplification products corresponding to target (*Mycobacterium sp.*) and control (*M. thermoautotrophicum*) were quantified by liquid scintillation counting and used to construct the standard curve. [MY], counts per minute associated with *Mycobacterium sp.* amplification products; [MT], counts per minute associated with *M. thermoautotrophicum* amplification products; [Input MY], pg of *Mycobacterium sp.* nucleic acid standard in PCR reaction.



**Figure 4.** Quantitative slot-blot hybridization and QPCR results. (A) Mass equivalents of *X. autotrophicus* and *Mycobacterium sp.* nucleic acids at stations U50 and D5 estimated from QPCR, normalized to total nucleic acids estimated from quantitative slot-blot hybridization. (B) Cell densities of *X. autotrophicus* and *Mycobacterium sp.* at stations U50 and D5, normalized to rock surface area. Cell densities were derived after taking into account losses from extraction and purification.



**Table 1.** QPCR primers.

Species/strain	Primer name <sup>a</sup>	Sequence
<i>Xanthobacter autotrophicus</i> str. T101/T102	X848F	5'-GCCGTTAGGCAGCTTGCTGC-3'
<i>Xanthobacter autotrophicus</i> str. T101/T102	X1258R	5'-GCTAGGGGTCGCCCTTTGC-3'
<i>Mycobacterium</i> sp. str. T103/T104	MY508F	5'-CCTGTAGAAGAAGGACCGGCC-3'
<i>Mycobacterium</i> sp. str. T103/T104	MY1099R	5'-CGCGCTGGCAACATAAGATAAGGG-3'
<i>Methanobacterium</i> <i>thermoautotrophicum</i> str. Marburg	MT780F	5'-GGACGAAAGCTAGGGGCGCG-3'
<i>Methanobacterium</i> <i>thermoautotrophicum</i> str. Marburg	MT1432R	5'-CAGCAAATACTGTGGCCTC-3'

<sup>a</sup> The number used in the primer name corresponds to the number of the *E. coli* position to which the 3' end of the primer anneals. F refers to a forward primer, and R refers to a reverse primer. Forward primers are 16S rRNA-like, while reverse primers are complementary to the 16S rRNA. By convention, position number 1 is the 5' terminal nucleotide of the 16S rRNA of *E. coli*. All sequences are listed in 5'-3' direction.

**Table 2.** Cell counts in the East Drainage Ditch<sup>a</sup>

Station	Reference	Total DAPI counts	Heterotrophic bacteria	Toluene degraders (plate counts)	Toluene degraders (MPN <sup>b</sup> )	<i>Xanthobacter autotrophicus</i> (plate counts)	<i>Mycobacterium sp.</i> (plate counts)
D5	This study <sup>c</sup>	$6.0 \pm 3.2 \times 10^9$ (N=8)	$3.0 \pm 1.7 \times 10^8$ (N=8)	N.D. <sup>e</sup>	N.D.	N.D.	N.D.
D5	Chapter Two <sup>c</sup>	N.D.	N.D.	N.D.	N.D.	N.D.	$1.0 \pm 0.0 \times 10^6$ (N=3)
D5	Chapter Three <sup>c</sup>	N.D.	N.D.	N.D.	N.D.	$5.5 \pm 5.2 \times 10^6$ (N=4)	N.D.
D5	DeJesus, 1994 <sup>d</sup>	$1.5 \pm 0.1 \times 10^{10}$ (N=2)	$2.3 \pm 0.3 \times 10^8$ (N=2)	$2.5 \pm 0.7 \times 10^6$ (N=2)	$2.3 \times 10^7$ (N=2)	N.D.	N.D.
U50	DeJesus, 1994 <sup>d</sup>	$2.8 \pm 0.1 \times 10^{10}$ (N=2)	$7.7 \pm 0.7 \times 10^8$ (N=2)	$2.0 \pm 1.4 \times 10^5$ (N=2)	$1.3 \times 10^6$ (N=2)	N.D.	N.D.

<sup>a</sup> All counts are expressed in cells per g of biomass  $\pm$  one standard deviation; N is the number of samples.

<sup>b</sup> In the MPN experiments, incubated vials were scored as positive if the activity of <sup>14</sup>CO<sub>2</sub> evolved exceeded 20% of the activity originally added as <sup>14</sup>C-toluene (DeJesus, 1994).

<sup>c</sup> Sampling in July 1992.

<sup>d</sup> Sampling in September 1993.

<sup>e</sup> Not determined.

**Table 3.** Total nucleic acids, *X. autotrophicus* nucleic acids, and *Mycobacterium sp.* nucleic acids at stations U50 and D5<sup>a</sup>.

Sampling date	Sampling station U50			Sampling station D5		
	Total nucleic acids (ng)	<i>X. autotrophicus</i> nucleic acids (pg)	<i>Mycobacterium sp.</i> nucleic acids (pg)	Total nucleic acids (ng)	<i>X. autotrophicus</i> nucleic acids (pg)	<i>Mycobacterium sp.</i> nucleic acids (pg)
10/27/95	320 ± 60	B.D. <sup>b</sup>	B.D.	1060 ± 180	B.D.	B.D.
1/1/96	670 ± 100	B.D.	B.D.	14200 ± 3200	410 ± 100	1370 ± 60
1/14/96	4920 ± 380	300 ± 90	B.D.	23900 ± 6100	620 ± 80	1580 ± 370
2/25/96	28800 ± 870	120 ± 20	B.D.	2420 ± 520	420 ± 130	B.D.
3/17/96	6700 ± 690	150 ± 30	B.D.	8880 ± 2070	720 ± 250	3140 ± 590
4/7/96	22900 ± 770	270 ± 40	770 ± 140	13100 ± 1500	830 ± 130	5000 ± 770
4/21/96	8970 ± 990	190 ± 10	450 ± 120	9850 ± 2720	490 ± 190	3030 ± 100
5/26/96	6390 ± 750	270 ± 70	390 ± 150	10200 ± 3100	5480 ± 1110	3900 ± 860
6/23/96	2600 ± 120	150 ± 40	210 ± 50	16500 ± 1700	11400 ± 1670	5290 ± 950
7/7/96	23 ± 3	B.D.	B.D.	2130 ± 110	1490 ± 790	970 ± 230
7/28/96	740 ± 170	B.D.	B.D.	2340 ± 150	720 ± 150	740 ± 300
9/7/96	210 ± 3	B.D.	B.D.	470 ± 40	230 ± 170	B.D.

<sup>a</sup> The amounts of total nucleic acids were determined using slot-blot hybridization, while the amounts of species-specific nucleic acids were determined from QPCR. Standards used in slot-blot hybridization were total nucleic acids of *X. autotrophicus*. Standards used in QPCR were total nucleic acids of *X. autotrophicus* and *Mycobacterium sp.* All quantities are total estimates for each sampling. Losses due to extraction and purification have been taken into account.

<sup>b</sup> Below detection.

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## **Chapter Five**

### **CONCLUDING REMARKS**

The impact of toluene contamination on the microbiology of the East Drainage Ditch was investigated. Four aerobic toluene-degrading bacterial strains were isolated from rock surface biofilms and characterized using physiological and molecular techniques. Strains T103 and T104 are Gram-positive and acid-alcohol-fast, with identical 16S rDNA sequences most similar to those of *Mycobacterium aurum* and *M. komossense*. They possess tuberculostearic acid, and fatty acid analyses indicate that they are not identical strains but related at the subspecies level. They constitute a new species of fast-growing mycobacteria. Strains T101 and T102 are Gram-negative, produce yellow pigments, and can also degrade benzene. They share identical 16S rDNA sequences with *X. autotrophicus*, and possess high levels of *cis*-11-octadecenoic acid and *cis*-9-hexadecenoic acid. Fatty acid analyses indicate that T101 and T102 are different but closely related strains.

Maximal velocity and half-saturation constant estimates revealed a fair diversity of toluene biodegradation kinetics among the four strains, although they were isolated under identical laboratory conditions. Comparisons of biodegradation kinetics of pure cultures and rock biofilms under batch conditions suggest that T102 may be a major contributor to toluene biodegradation in the stream.

Relative distributions of the toluene-degrading *X. autotrophicus* and *Mycobacterium sp.* were assessed in rock surface biofilms sampled over a period of one year. Quantitative PCR and slot-blot hybridization results revealed that these indigenous species are significantly more abundant in a contaminated reach than in a pristine reach, and more abundant in both reaches in summer months than in winter months. These results are consistent with earlier studies which showed higher biodegradation rates in

contaminated stream reaches in summer months than in winter months, and higher plate counts and MPNs of toluene-degraders in contaminated reaches than in pristine reaches. There appears to be a selection in the stream for these species based on the presence or absence of toluene, and this selection is modified by temperature.

### ***MYCOBACTERIUM AND XANTHOBACTER IN THE ENVIRONMENT***

The toluene-degrading species of *Mycobacterium sp.* and *Xanthobacter autotrophicus* isolated from the East Drainage Ditch belong to genera which are increasingly recognized as important players in the recycling of chemicals in the environment. A discussion of the metabolic diversity of these groups of bacteria is presented below.

**Metabolic versatility of fast-growing mycobacteria.** Toluene-degrading strains T103 and T104 belong to a novel species of fast-growing mycobacteria. Compared to the clinically important slow-growing mycobacteria, many of which are human and animal pathogens, the fast-growing mycobacteria have received far less attention (Hartmans and de Bont, 1992b). Fast-growing mycobacteria are common saprophytes in natural habitats. They have been isolated from a diverse array of habitats, including sputum, soil, sediments, fresh and salt water, dust, and activated sludge. They have the capacity to survive and multiply under a wide range of pH, temperature, and other environmental conditions. They can grow on common substrates such as sugars, alcohols, and organic acids, and a large variety of hydrocarbons including branched-chain, unsaturated, aromatic, and cyclic hydrocarbons. Involvement of the fast-growing mycobacteria has

been reported in the biotransformations of a variety of xenobiotic compounds and pollutants, including polycyclic aromatic hydrocarbons (Heitkamp and Cerniglia, 1989; Boldrin *et al.*, 1993; Kelley *et al.*, 1993; Guerin and Jones, 1988), chlorinated phenolic compounds (Hagglblom *et al.*, 1988; Hagglblom *et al.*, 1994), trichloroethylene (Wackett *et al.*, 1989; Vanderberg *et al.*, 1995), groundwater pollutant mixtures (Burbach and Perry, 1993), ethene (Hartmans *et al.*, 1991), propane (Vanderberg and Perry, 1993), and toluene (Burbach and Perry, 1993).

The fact that fast-growing *Mycobacterium* species have been isolated from a wide variety of environments and have different biocatalytic capabilities suggests that the organisms within this group may be important in pollutant biodegradation, and therefore may be useful in the bioremediation of contaminated environments. For example, inoculation of sediments with a pyrene-degrading *Mycobacterium* strain resulted in an enhancement of the mineralization rates of several polycyclic aromatic hydrocarbons (Heitkamp and Cerniglia, 1989).

**Metabolic versatility of *Xanthobacter*.** The metabolic versatility of the *Xanthobacter* genus makes its members ideal candidates for consideration in the bioremediation of contaminated environments. The genus *Xanthobacter* consists of three species, *Xanthobacter agilis* (Jenni and Aragno, 1987), *Xanthobacter autotrophicus* (Wiegel *et al.*, 1978), and *Xanthobacter flavus* (Jenni *et al.*, 1987; Malik *et al.*, 1979; Reding *et al.*, 1992). These organisms are Gram-negative, pleomorphic rods, can obtain energy from oxidizing hydrogen, and can fix nitrogen under chemolithoautotrophic, as well as under chemoheterotrophic conditions (Wiegel, 1992). *Xanthobacter* species are widespread due to their metabolic diversity. They have been isolated from a diverse array

of habitats, including marine sediments (Lidstrom-O'Connor *et al.*, 1983), soils of flooded rice fields and rhizospheres of wetland rice (Reding *et al.*, 1991; Reding and Wiegel, 1993; Oyaizu-Masuchi and Komagata, 1988), street ditches and wet meadow soil in Europe, South Africa, North America, and Asia (de Bont and Leijten, 1976; Wiegel and Schlegel, 1976, 1984; Oyaizu-Masuchi and Komagata, 1988), and sewage samples (White *et al.*, 1987; Jenni and Aragno, 1987). Many *Xanthobacter* strains have been reported that are able to degrade a variety of xenobiotic compounds and pollutants. For example, *Xanthobacter autotrophicus* strain GJ10 (Janssen *et al.*, 1985) is able to utilize a number of halogenated short-chain hydrocarbons (including 1,2-dichloroethane) and halogenated carboxylic acids as sole carbon source for growth. Apart from halogenated compounds, a few other organic compounds such as toluene could also support growth of strain GJ10, although phenol and benzene could not. *Xanthobacter flavus* strain 14p1 (Spiess *et al.*, 1995) was isolated from river sludge using 1,4-dichlorobenzene as the sole source of carbon and energy. However, it has an extremely limited substrate range and is not able to utilize other aromatic or chloroaromatic compounds as growth substrates. *Xanthobacter* sp. strain Py2, originally isolated on propene, is also able to grow on 1-pentene (van Keulen and Da Fonseca, 1994). Industrial interest in microbial utilization of alkenes is motivated by the formation of optically pure epoxides for possible biotechnological applications (Hartmans *et al.*, 1989a; Wiegel, 1992). Propene-grown *Xanthobacter* sp. strain Py2 cells have also been shown to continuously degrade trichloroethylene (Reij *et al.*, 1995). *Xanthobacter* sp. strain 124X, isolated from an enrichment culture with sewage as the inoculum and styrene as the carbon source (van

den Tweel *et al.*, 1986), can also metabolize styrene oxide, which is a potential and more toxic intermediate of styrene degradation (Hartmans *et al.*, 1989b).

Because of their ubiquity and metabolic versatility, members of the *Xanthobacter* genus have been considered in schemes to bioremediate contaminated environments. For example, Hartmans *et al.* have suggested that *Xanthobacter* sp. strain 124X can be used as biocatalysts in biofilters to remove styrene from industrial waste gases (Hartmans *et al.*, 1989). It is anticipated that more novel fast-growing *Xanthobacter* species possessing interesting biocatalytic capabilities will be isolated and described.

## FUTURE RESEARCH

A few areas of further research are discussed in this section.

**Distribution of toluene biodegradation pathways.** Toluene biodegradation has been shown to proceed by five different pathways (Smith, 1990). We have not yet elucidated the pathways that are present in our toluene-degrading strains. It is also not clear how these pathways are distributed in natural environments, and whether each different pathway might be preferred under selective environmental conditions that might give it a competitive advantage in nature. Researchers are starting to address these issues by studying competition in laboratory-constructed ecosystems. Duetz *et al.* (1994) compared the competition behaviors in a chemostat of four strains of bacteria that can degrade toluene through different pathways. Under conditions of toluene or oxygen limitation, there was an apparent superiority of strains that start degradation of toluene by hydroxylation of the aromatic nucleus. On the other hand, Massol-Deya *et al.* (1997)

found that a methyl-group oxidizing strain outcompeted two other strains carrying toluene-hydroxylation pathways in a fluidized bed reactor. It is therefore still not apparent how different toluene degradation pathways are selected for in nature, and more work can be done to elucidate the mechanisms of selection.

Another approach to studying pathway distributions in natural communities is to identify the genes that encode for contaminant degradation, and to develop catabolic gene probes to hybridize to community DNA (Atlas *et al.*, 1992). Bacterial genes encoding degradation of aromatic hydrocarbons, such as benzene, toluene, naphthalene, and biphenyl, have in many cases been cloned, or at least localized to specific plasmids such as those belonging to the TOL and NAH families (Burlage *et al.*, 1989). DNA probes for such catabolic genes have been used to monitor the occurrence of specific degradative genotypes in environmental populations. However, there are difficulties with this molecular approach. In one study, TOL plasmids were used to probe for toluene degradative genes in microbial populations from freshwater sediments (Sayler *et al.*, 1985). Unexpectedly, it was found that 98.6% of the microbial population from cultured cells that tested positive for TOL probe hybridization were incapable of toluene catabolism and growth. This may be due to the low specificity of the catabolic gene probe prepared from TOL plasmid DNA, which resulted in cross-hybridization with selected bacteria harboring other catabolic genes. The use of specific catabolic gene probes may therefore not always accurately reflect the ability of the community to metabolize the given compound, and this is especially true for compounds such as toluene, for which multiple degradation pathways are known to exist. This approach is

also complicated by the recent finding that multiple degradation pathways can also exist within a single bacterial strain (Johnson and Olsen, 1997).

**Biofilm structure and function in natural environments.** The physiological activity and growth rates of organisms present in multispecies biofilms are still poorly understood. For example, it is not clear how the physiological state of individual cells vary in a multispecies biofilm community versus in pure culture. In assessing the contributions of individual strains to toluene degradation by rock biofilms, it was assumed in this thesis that the kinetic parameters of the individual cultures also applied to the respective strains residing in the rock biofilms. In a study involving in situ hybridization and image analysis, the sulfate-reducing bacterial population of a multispecies biofilm was found to grow faster in young biofilms than in old, established biofilm communities (Poulsen *et al.*, 1993). Measurements of growth-related parameters for *Pseudomonas putida* in a biofilter for waste gas treatment showed reduced rRNA content and cell size relative to that in batch culture, indicating that the *P. putida* population was not degrading toluene at a maximum rate in the biofilm environment (Moller *et al.*, 1996).

Biofilms have been envisioned as uniform structures, where active bacteria are randomly distributed in the biofilm matrix (Rittmann and McCarthy, 1980). However, the use of nondestructive methods such as scanning confocal laser microscopy (SCLM) has resulted in a more detailed picture, showing that biofilms consist of cell aggregates or microcolonies embedded in exopolysaccharide matrices (Lawrence *et al.*, 1991). The microcolonies are interspersed with voids and water channels, where liquid flow has been demonstrated (Stoodley *et al.*, 1994), indicating that molecular diffusion is not the only

process governing mass transport in biofilms. Such channels have been shown to supply as much as half of the oxygen consumed by the biofilms (DeBeer *et al.*, 1994).

A better understanding of biofilm function can be gained from information about the activity and spatial distribution of microbial community members in the biofilm. However, this is still poorly understood. A recent study (Moller *et al.*, 1996) used specific 16S rRNA probes and SCLM to examine a single toluene-degrading species as a representative of the toluene-degrading population in a multispecies microbial consortium from a laboratory-scale biological filter for waste gas treatment. This species was present in dense cell clusters (microcolonies) and as individual cells throughout the biofilm, and it was suggested that toluene could penetrate the biofilm to a higher degree than if the mass transport were governed by diffusion alone. The toluene substrate could have been available to the cells residing deep inside the biofilm, and the transport of toluene could have been facilitated by the channel structures described earlier.

Biofilm physiology can also be examined for biofilms in natural environments such as the East Drainage Ditch using techniques involving quantitative *in situ* hybridization and SCLM. Ribosomal content, cell size, and other physiological parameters can be measured for individual cells in the biofilms to establish the physiological state of subpopulations of microorganisms in the biofilm communities, and this can provide a deeper understanding of structure-function relationships in the biofilm matrix..

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**Appendix A**

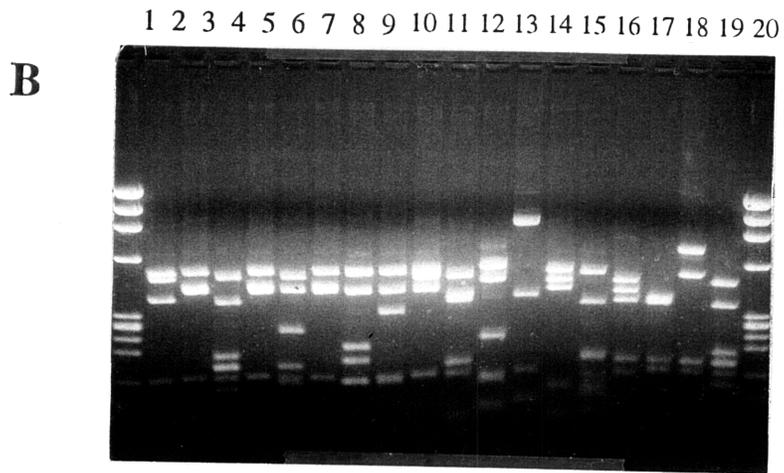
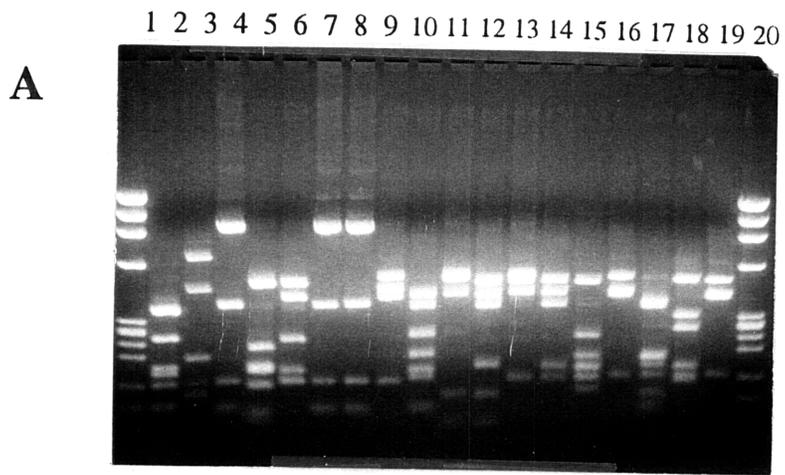
**ANALYSIS OF  
RESTRICTION FRAGMENT LENGTH POLYMORPHISMS  
FROM STATION D5**

Chapter 2 referred to an investigation into the genetic diversity of the natural microbial community on rock biofilms at station D5 in the East Drainage Ditch. The detailed results of that investigation are presented here.

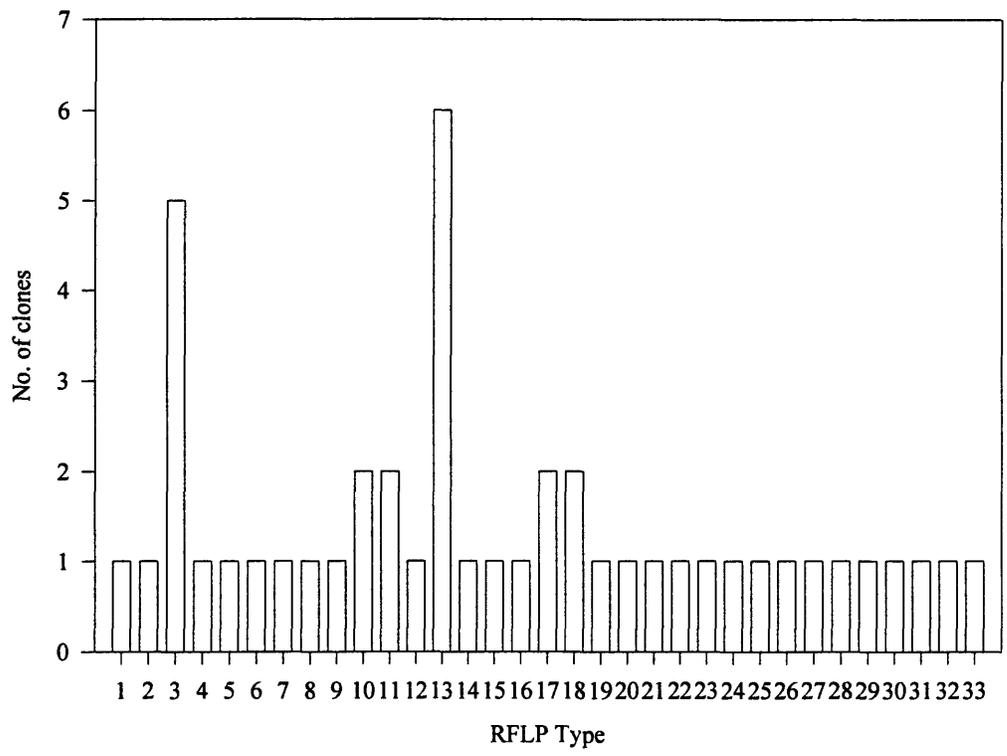
## RESULTS

**RFLP analysis.** 46 environmental clones were recovered from station D5 in July 1993. The amplified 16S rDNAs of the 46 environmental clones produced single bands of about 1,500 bp, corresponding to the expected size of the 16S rRNA genes. From the 46 clones, 33 unique RFLP types were detected with the two endonucleases used (Figures 1 and 2; Table 1). The maximum number of clones associated with the same RFLP type (RFLP type 13) was 6. Of the remaining 40 clones, 27 possessed unique restriction patterns. The restriction pattern (RFLP type 14) of one of these clones (clone C16) was identical to those of strains T103 and T104. The 16S rRNA gene in this clone was sequenced and found to be identical to the 16S rRNA gene sequences of strains T103 and T104.

**Figure 1.** Restriction patterns of PCR-amplified 16S rRNA genes digested with *Pst*I and *Rsa*I. (A) Lanes 1, 20, *Hind*III-digested  $\phi$ X DNA ladder; lanes 2 to 19, clones C1 to C18. (B) Lanes 1, 20, *Hind*III-digested  $\phi$ X DNA ladder; lanes 2 to 19, clones C19 to C36. (C) Lanes 1, 12, 16, *Hind*III-digested  $\phi$ X DNA ladder; lanes 2 to 11, clones C37 to C46; lanes 14, 15, strains T103, T104.



**Figure 2.** Distribution of different types of restriction fragment length polymorphisms of 16S rRNA genes.



**Table 1.** RFLP analysis of PCR-amplified 16S rRNA genes from station D5

Clone no.	RFLP type no.	Restriction fragment lengths (bp) of individual bands								Total length (bp)
C1	1	343	327	230	157	145	124	92		1418
C2	2	671	441	180	134	113				1539
C3	3	985	365	133	90					1573
C4	4	487	209	166	159	128	90	84		1323
C5	5	490	406	232	155	133				1416
C6	3	979	363	133	90					1565
C7	3	979	363	133	90					1565
C8	6	522	435	384	120					1461
C9	7	417	352	239	181	144	129	85		1547
C10	8	538	507	423	105	70				1643
C11	9	493	408	352	155	104	71			1583
C12	10	538	489	426	134					1587
C13	11	500	413	349	143	121				1526
C14	12	481	220	165	141	140	121	100		1368
C15	13	504	413	397	125					1439
C16	14	354	341	166	163	147	127	96	78	1472
C17	15	500	304	255	143	127	120			1449
C18	13	507	413	394	128					1442
C19	16	508	464	353	125					1450
C20	13	511	415	392	126					1444
C21	17	480	348	170	149	146	127	107		1527
C22	13	515	415	392	126					1448
C23	18	501	412	244	154	136	124			1571
C24	13	511	415	392	126					1444
C25	19	506	391	184	151	112				1344
C26	20	525	421	302	121					1369
C27	10	532	495	418	127					1572
C28	21	506	391	363	152	130	72			1614
C29	22	573	473	219	122	84				1471
C30	3	1035	369	127	87					1618
C31	23	540	470	412	98	63				1583
C32	24	519	339	150	110	97	92	67		1374
C33	11	492	408	350	149	122				1521
C34	25	364	361	342	149	129				1345
C35	26	736	503	145	129					1513
C36	17	462	326	166	141	138	121	104		1458
C37	27	464	395	348	153	120	81			1561
C38	28	533	411	365	133	100				1542
C39	29	381	355	160	138	119	78			1231
C40	18	480	403	238	151	126	117			1515
C41	30	501	350	152	147	118	102			1370
C42	13	512	417	397	126					1452
C43	31	409	350	238	148	127	116			1388
C44	3	978	357	124	82					1541
C45	32	445	340	228	155	142	98	60		1468
C46	33	426	345	185	154	138	124	76		1448
T103	14	357	342	168	166	145	125	95	77	1475
T104	14	357	342	168	166	145	125	95	77	1475
T101	-	773	516	123						1412
T102	-	773	516	123						1412

**Appendix B**

**USE OF SLOT-BLOT HYBRIDIZATIONS TO QUANTIFY  
SPECIES-SPECIFIC NUCLEIC ACIDS  
IN NATURAL SAMPLES**

Chapter 4 referred to an investigation into the genetic diversity of the natural microbial community on rock biofilms at station D5 in the East Drainage Ditch. An attempt was made to quantify the amounts of species-specific nucleic acids in the environmental extracts by slot-blot hybridization. The detailed results of that investigation are presented here.

## METHODS

**Probe design.** Probes specific to the 16S rDNA of *X. autotrophicus* strains T101 and T102 (X1260), and *Mycobacterium sp.* strains T103 and T104 (MY1003) were designed with the following sequences: MY1003, 5'-TACCTATCTCTAGGCACGTC-3'; X1260, 5'-TTGCTAGGGGTCGCCCCTTT-3'. The midpoint dissociation temperatures ( $T_d$ ) of the probes were determined as described previously (Polz and Cavanaugh, 1997).

**Quantitative hybridization.** Nucleic acid blotting and hybridizations were performed as described previously (Polz and Cavanaugh, 1997). Nucleic acids from station D5 were spotted in triplicate on a Zetaprobe nylon membrane (Bio-Rad, Hercules, California) together with duplicate standards based on nucleic acids extracted from pure cultures of *Xanthobacter autotrophicus* and *Mycobacterium sp.* The spotting scheme is depicted in Figure 1. Hybridizations were performed in turn with the MY1003 probe, the X1260 probe, and the Eub338 probe (Stahl and Amann, 1991), which is nearly universal for all members of the domain *Bacteria*. The membrane was washed between

hybridizations by immersing in washing buffer twice for 20 minutes at 80°C, and checked for complete stripping of radiolabelled products by overnight autoradiography.

## RESULTS

**Determination of  $T_d$ .** Figures 2 and 3 show plots of amount of probe remaining on the blots against wash temperature for the MY1003 and X1260 probes.  $T_d$  is determined as the wash temperature at which half the original probe amount remains on the membrane. It is at this temperature that the hybridization assay offers good sensitivity and specificity.  $T_d$  for MY1003 and X1260 were determined to be 50.1°C and 60.5°C, respectively.

**Slot-blot hybridizations.** Least-squares linear regression analysis of the standard curves of hybridization signals versus known amounts of nucleic acids yielded  $r^2 > 0.95$  in all cases. The hybridization results are summarized in Figure 4, and the amounts of species-specific nucleic acids detected are normalized to the total amount of *Bacteria* detected. Background levels in the assay were determined for the MY1003 and X1260 probes by quantifying the activity present on the slots containing *X. autotrophicus* and *Mycobacterium sp.* standards, respectively. The results indicate that the amounts of species-specific nucleic acids were in all cases less than the maximum background level detected with the relevant probe. Nucleic acids of *X. autotrophicus* and *Mycobacterium sp.*, if present in the samples, are present at levels that are too low to be detected with any confidence by the hybridization assay.

## REFERENCES

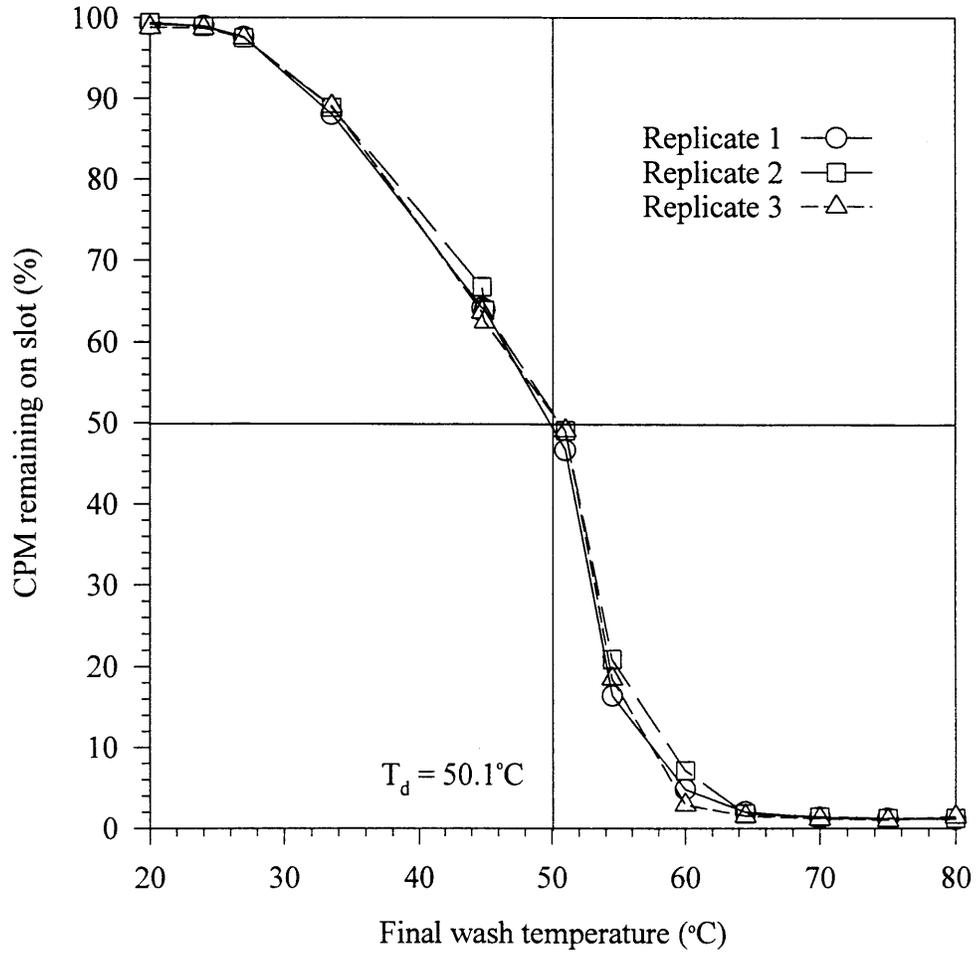
1. **Polz, M. F., and C. M. Cavanaugh.** 1997. A simple method for the quantification of uncultured microorganisms in the environment based on in vitro transcription of 16S rRNA. *Appl. Environ. Microbiol.* **63**:1028-1033.
2. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes, p. 205-248. *In* E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, Chichester, United Kingdom.

**Figure 1.** Schematic diagram depicting the layout of nucleic acid extracts of standards and station D5 samples.

		Xanthobacter standards	
10/27/95	○ ○ ○		○ ○ 3000 ng
12/17/95	○ ○ ○	9/7/96	○ ○ 1000 ng
1/1/96	○ ○ ○	○	○ ○ 100 ng
1/14/96	○ ○ ○	○	○ ○ 10 ng
2/25/96	○ ○ ○	○	○ ○ 1 ng
3/17/96	○ ○ ○		○ ○ 0.1 ng
4/7/96	○ ○ ○	0.1 ng	○ ○
4/21/96	○ ○ ○	1 ng	○ ○
5/26/96	○ ○ ○	10 ng	○ ○
6/23/96	○ ○ ○	100 ng	○ ○
7/7/96	○ ○ ○	1000 ng	○ ○
7/28/96	○ ○ ○	3000 ng	○ ○
			Mycobacterium standards

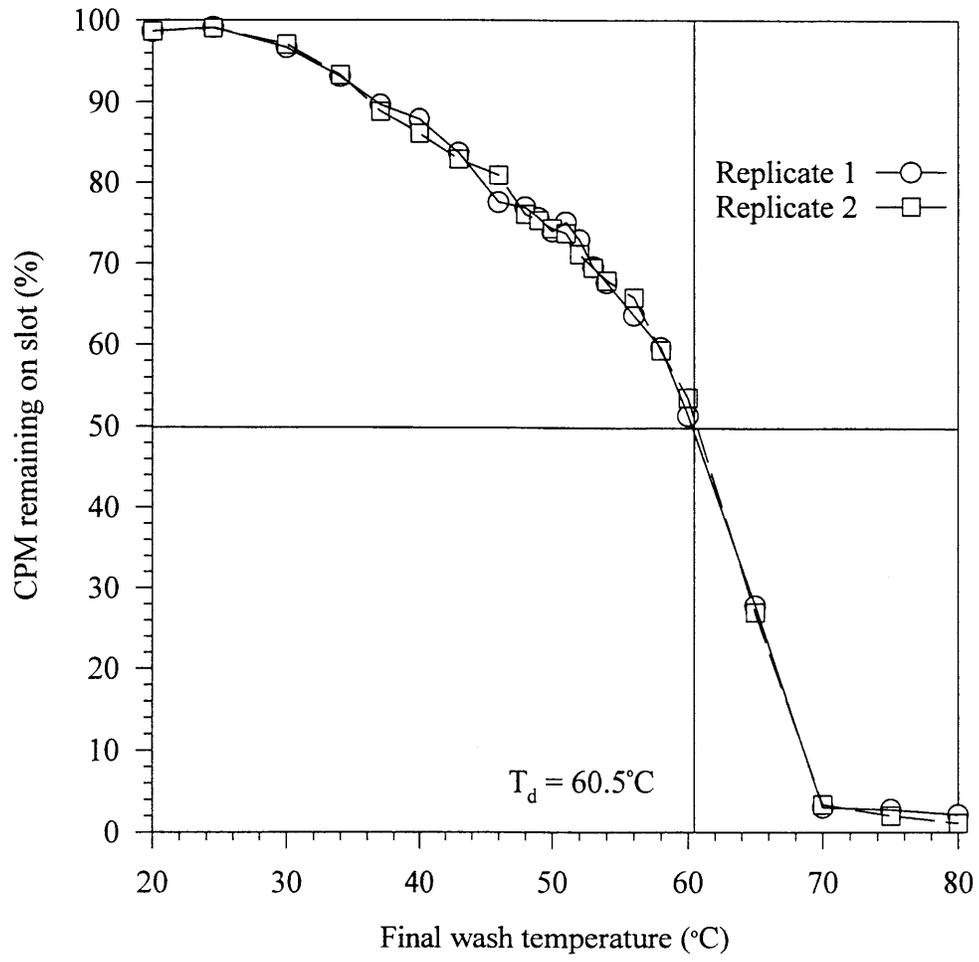
**Figure 2.** Determination of  $T_d$  for the MY1003 probe using total nucleic acids extracted from pure cultures of *Mycobacterium sp.*

### Probe MY1003

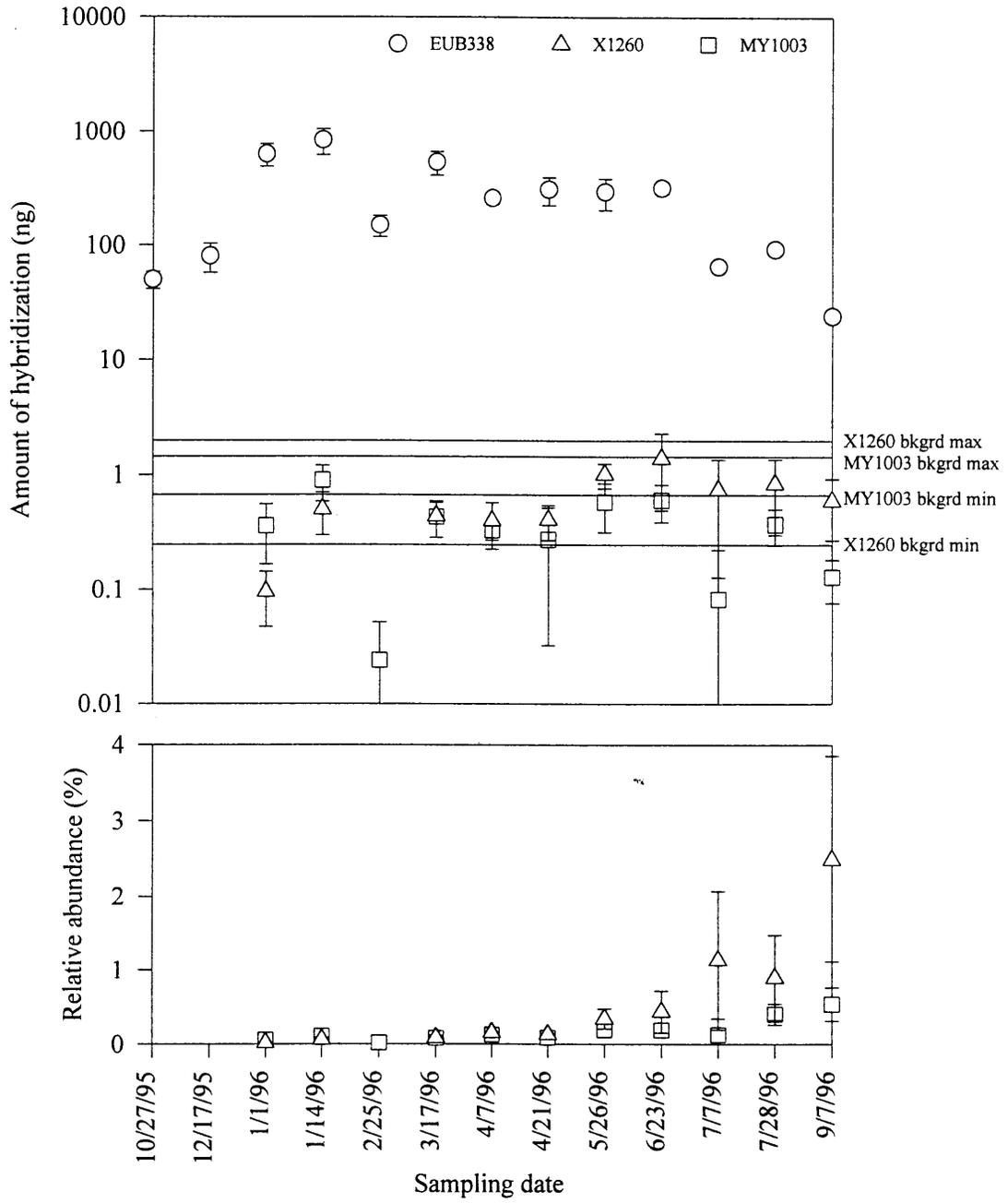


**Figure 3.** Determination of  $T_d$  for the X1260 probe using total nucleic acids extracted from pure cultures of *X. autotrophicus*.

### Probe X1260



**Figure 4.** Profiles of amounts of nucleic acids detected with the MY1003, X1260, and Eub338 probes at station D5. Relative abundances are based on amounts of species-specific nucleic acids detected with the MY1003 and X1260 probes expressed as a percentage of total nucleic acids detected using the Eub338 probe.



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