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AEROBIC DEGRADATION OF TRICHLOROETHYLENE, VINYL CHLORIDE, AND AROMATIC COMPOUNDS BY *RHODOCOCCI* SPECIES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Kenneth Jay Malachowsky

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DEDICATION

This thesis is dedicated to my parents

Mr. Harry Malachowsky

and Mrs. Goldie Malachowsky

and in addition I would like to dedicate this thesis to my ever loving wife, Leslie, without whose patience, support, love, and understanding would not have allowed me to accomplish this piece of work.

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ABSTRACT

Aromatic and chlorinated aliphatic hydrocarbons are frequent contaminants of ground waters, which often are found together in mixtures contaminating the environment. Contamination of the ground water with these compounds poses serious health threats due to their carcinogenic and mutagenic properties. Recently, studies have described the ability of microbes to biotransform aromatic and chlorinated aliphatic hydrocarbons into harmless compounds, such as carbon dioxide.

The purpose of this investigation was to characterize the ability of two type IV actinomycetes isolated from a trichloroethylene degrading consortium and *Rhodococcus rhodochrous* to degrade aromatic and aliphatic hydrocarbons. Trichloroethylene and vinyl chloride were mineralized by the three isolates. Other chlorinated aliphatic hydrocarbons, such as methylene chloride, vinylidine chloride, and 1,1,1-trichloroethane were degraded by the isolates. In addition, a variety of aliphatic, aromatic, and polyaromatic hydrocarbons were also degraded by the isolates and many of these hydrocarbons served as an energy and carbon source for the isolates. The ability to degrade a mixture of aromatic and chlorinated aliphatic hydrocarbons was tested and the isolates degraded 11 out of 13 compounds. The isolates were shown

to be catabolically versatile since they degraded many different types of hydrocarbons.

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PART I

BACKGROUND

GENERAL INTRODUCTION

Scope of the organic pollution problem. Contamination of the environment with organic wastes is a major problem in the United States. In 1979, the Environmental Protection Agency (EPA) listed 129 compounds, which included chlorinated hydrocarbons, aromatic hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyl's (PCBs), pesticides, insecticides, and heavy metals as priority pollutants due to their toxicity, suspected toxicity, and because they persist in the environment (104). Industrial sites have been shown to contain mixtures of aromatic and chlorinated aliphatic wastes (57). The majority of ground water, soil, and sediment contamination involves mixtures of different types of organic wastes (29,38,77,83).

Occurrence of chlorinated aliphatic hydrocarbons. Chlorinated hydrocarbons, such as methylene chloride (MC), chloroform (CF), carbon tetrachloride, vinyl chloride (VC), dichloroethylenes (DCE), trichloroethylene (TCE), and tetrachloroethylene (PCE) contaminate subsurface aquifers (20,23,37,62,72,74,83,87,89,101,102,112), soils (83), and sediments (31,92,84) in the U.S. These environmental pollutants

often contaminate aquifers that are used for drinking water (55). These compounds are produced in industry in large amounts and are used in applications such as degreasing, plastic production, dry cleaning, refrigeration, shoe polishing, and they are common industrial solvents (48,91). TCE was used in amounts exceeding 178 million pounds in 1985, explaining why it is the most prevalent ground water contaminant in the U. S. (55,102,112). Chlorinated aliphatic compounds can enter ground water by various routes which include: spill sites, leaching from disposal sites, and industrial waste waters. Industrial waste waters have been documented to have organic waste concentrations exceeding 1000 mg/L (5,18,84,100,103). These contaminants are easily transported through the soil by ground water movement, which can result in the spreading of a contamination plume (85). Contamination of the ground water with chlorinated aliphatic compounds, such as VC, poses serious health problems due to the toxic and carcinogenic nature of the compounds (21,45,61,81,97-99,105).

The half-life of chlorinated hydrocarbons in a particular environment depends upon the physical and chemical properties of that environment (17,104). Abiotic half-lives of chlorinated hydrocarbons, due to oxidation, hydrolysis, or sorption to particulate matter in aquatic systems, are on the order of 0.7-to-0.9 years (104). In an open aquatic system, volatilization and photolysis are the major fates for these compounds (32), but in subsurface aquifers volatilization and photolysis probably occur at insignificant rates or

do not occur at all. Sites have been documented that were contaminated with chlorinated hydrocarbons for many years (83). It is thus necessary to implement remediation strategies due to the persistence of the toxic organics in the environment.

Remediation of contaminated ground waters or soils can involve physical methods, such as air-stripping or adsorption to activated carbon (55). Air-stripping takes advantage of the volatile nature of the organic compound. The efficacy of removing an organic compound by air-stripping generally increases with an increasing Henry's constant (55). Air-stripping has the disadvantage of not destroying the toxicant but merely displacing it to the atmosphere. The use of granular activated carbon is based on the ability of activated carbon to adsorb organic compounds. Granular activated carbon usage is theoretically problematic since the adsorbed compounds can deadsorb when influent concentrations of the contaminant compounds decline. A second problem with the use of granular activated carbon is that the contaminated activated carbon needs to be disposed in a safe manner (36,55). A remediation method that does not displace the toxicant from one site to another nor risks the further contamination of the site could prove to be beneficial in environmental and economic terms. Biological remediation of chlorinated aliphatic contaminated soils and ground waters offers a way to mineralize the toxicants to harmless end products such as carbon dioxide (33,54,79).

Aerobic biodegradation of chlorinated aliphatic hydrocarbons. Methanotrophs were the first group of bacteria that were shown to have the ability to degrade chlorinated aliphatic compounds. While it was known since 1977 that methanotrophs were capable of degrading alkenes (19), Wilson and Wilson first demonstrated in 1985 the aerobic degradation of TCE in soils (115). These soils were treated with natural gas to stimulate the activity of the methanotrophs since it was hypothesized that the methanotrophs would be able to biotransform chlorinated alkenes (115). Fogel et al showed that a methanotrophic mixed-culture could aerobically mineralize TCE as well as degrade VC and VDC, but could not degrade PCE (31). The methanotrophic mixed- culture degraded TCE at a rate of 2 x 10^{-6} μmol TCE $h^{-1}\mu g^{-1}$ of protein. At the end of the mineralization experiment, 23% of the radiolabel appeared as $^{14}CO_2$, 34% was incorporated into biomass, and 43% remained in solution as nonvolatile or nonchlorinated molecules.

Little et al first demonstrated mineralization of TCE by pure cultures of Type 1 methanotrophs isolated from a TCE contaminated aquifer and also demonstrated the presence of possible metabolites of TCE degradation, such as gloxilic acid and dichloroacetic acid (54). A greater percentage (72%) of the radiolabel appeared as $^{14}CO_2$ when [1,2 ^{14}C]TCE was incubated with mixed-ground water cultures than with the methanotrophic strain 46-1, which mineralized only 41% of the radiolabel to $^{14}CO_2$ (54). The heterotrophs in the mixed-ground water cultures were apparently

further metabolizing the methanotrophic TCE breakdown products. This suggests that mixed cultures are more efficient in mineralizing TCE than pure cultures.

Other studies involving soils or sediments exposed to methane demonstrated the degradation of TCE (30), CF (92), or mixes of halocarbons (42). Methanotrophs are able to degrade a wide variety of compounds, such as alkenes, chlorinated aliphatic hydrocarbons, and aromatic compounds, due to the expression of methane monooxygenase (MMO) (31,43,53-54,70,96). The degradation of chlorinated aliphatic compounds by methanotrophs would be considered a cometabolic process since the methanotrophs are not able to assimilate the epoxidation breakdown products for energy.

Other groups of bacteria containing mono- or dioxygenases also demonstrate the ability to degrade chlorinated aliphatic hydrocarbons. The ammonia-oxidizing bacterium *Nitrosomonas europaea* degraded 10μM TCE at rates of 1.1nmol mg^{-1} min^{-1} of protein. The organism is believed to degrade TCE due to the presence of an ammonia monooxygenase (2,82). *Pseudomonas putida* F1 mineralized TCE at a rate of 1.8 nmol mg^{-1} min^{-1} of protein after exposure to toluene or phenol, which induced a toluene dioxygenase (65-67,108). *P. putida* F1 did not degrade PCE, VC, or ethylene. The involvement of an aromatic pathway in the degradation of TCE was proven by genetically engineering the toluene dioxygenase genes from *P. putida* F1 into *E. coli*. Wildtype *E. coli* did not have the ability to degrade TCE but the *E. coli* containing the cloned toluene dioxygenase

genes proved capable of degrading TCE (119). Similiar genetic experiments using *Psuedomonas mendocina* implicated a toluene monooxygenase in the degradation of TCE (117). *Psuedomonas flurorescens* degraded a variety of chlorinated aliphatic compounds without the need to be induced by an aromatic compound (106). VC mineralization was also reported in unamended samples of ground water (24). After 108 days, 65% of [1,2 ^{14}C]VC appeared as $^{14}CO_2$.

Propane-oxidizers, which utilize propane as an energy and carbon source (7,9), also have demonstrated the ability to degrade chlorinated aliphatic compounds (11,109). Propane oxidizers are similar to methanotrophs since both contain monoxygenase(s). However, propane oxidizers are heterotrophs and are able to utilize a variety of substrates, such as propane, hexadecane, or glucose for carbon and energy. The methanotrophs are limited to C_1 metabolism for energy. The majority of propane oxidizers are in the Actinomycetales family. Wackett et al surveyed hexane, prococene, cyclohexanone, and propane monooxygenases and found that only propane oxidizers degraded TCE (109).

A heterotrophic consortium, which was isolated from a site contaminated with short-chain hydrocarbons, aerobically degraded greater than 91% of 50 mg/L of TCE when grown on a variety of substrates such as methane, propane, methanol, acetate, glucose, or trypticase. The consortium degraded up to 150 mg/L of TCE during a three week incubation (30), which is the highest concentration of TCE reported to be degraded biologically. A type IV actinomycete,

which was isolated from the same TCE degrading consortium, mineralized VC when grown on propane and also degraded VC when grown on propane or glucose (79).

Bioreactor studies. Bioreactors have been utilized as a tool for chlorinated aliphatic degradation studies. Niedzielski and Phelps designed a continuous-recycle expanded-bed bioreactor that allowed for direct headspace sampling of volatile compounds (69,78). The bioreactors were inoculated with a TCE degrading consortium derived from a TCE contaminated site (30). The aerobic consortia utilized methane and propane as energy sources and degraded 95% of 20 mg/L TCE in five days. A mixture of organic wastes, which included aromatic and chlorinated aliphatic hydrocarbons, was degraded in the bioreactor, thus demonstrating the catabolic versatility of the consortium (77). Other types of bioreactors designed to degrade chlorinated aliphatic hydrocarbons include fixed-film, packed-bed bioreactors (93), recirculating bioreactors (32), two-stage biofilm bioreactors (28), and single-pass packed-bed bioreactors (51).

Anaerobic biodegradation of chlorinated aliphatic hydrocarbons. Bower and McCarty (8) demonstrated that under methanogenic conditions highly chlorinated aliphatic compounds, such as PCE, were reductively dechlorinated to TCE and then eventually to VC (107). Other studies have also shown the reductive

dechlorination of compounds such as PCE and TCE resulting in harmful end products such as DCE and VC (6,49,71,94). Thus reductive dechlorination of chlorinated alkenes can serve as a source of VC contamination in ground water (59). Limited anaerobic biodegradation of VC has been reported at rates of less than .05 μmol/day (6,35,107). The rate limiting step in the reductive dechlorination of PCE to ethylene appears to be the conversion of VC to ethylene (35). Fathepure and Vogel designed a two-stage bioreactor to overcome the problems of producing toxic, recalcitrant molecules from reductive dechlorination of highly chlorinated compounds. The first stage had an anaerobic column that was used for reductive dechlorination, which was followed by an aerobic column that oxidized the chlorinated compounds produced by reductive dechlorination to carbon dioxide and water soluble products (28).

Occurrence and biodegradation of aromatic hydrocarbons. Aromatic compounds, such as benzene, toluene, ethylbenzene, and xylene are ground water contaminants that are released from petroleum hydrocarbon spills (44,47,114). These compounds can enter the environment following the same modes of entry as chlorinated aliphatic compounds. Physical cleanup would be similar to that of chlorinated aliphatic compounds since both types of compounds are volatile and not very soluble in water (104). Aromatic hydrocarbons are toxic and persist in the environment

(104). Both aerobic (16,29,34,50,52,95) and anaerobic degradation (26,44,46) have been reported for aromatic hydrocarbons.

PAHs can enter the environment from coal tar creosote sites (68), oil refineries, coal gasification sites, coal liquefaction sites, and from accidental spilling of hydrocarbons and oils (13). These compounds are of environmental concern due to their toxicity, carcinogencity, and potential for trophic bioaccumulation (56,80). The compounds are recalcitrant, especially in the case of four- and five-ring PAHs, which have very low solubilities, adsorb onto surfaces (104), and are very stable due to resonance structures (41). Studies have demonstrated that PAHs can be degraded by indigenous microbes at PAH contaminated sites (58,63,64). Pure cultures of psuedomonads and actinomycetes aerobically degrade naphthalene (22,86), phenanthrene (27,39,73), and anthracene (14,27).

Subsurface microbiology studies. The exact qualitative and quantitave nature of subsurface microbes was questioned in early studies due to the small populations of microbes detected there and due to the methodology involved in the collection of subsurface specimens (90,110-111). While early studies gave evidence that microbes in the subsurface were responsible for various geomicrobiological cycles (12,118), it was thought that subsurface microbes were not responsible for significant biotic processes. Recent studies indicate that subsurface microbiota are responsible for various transformations of subsurface compounds,

such as organics and compounds containing sulfur, that effect ground water quality (15,25,113,116). Other studies show a diverse population of active microbes in sediment samples associated with the water table and vadose zones (4,5,33,75-76,88). At subsurface sites contaminated with organic wastes, it would be important to determine whether indigenous microbes were present that were capable of degrading the toxic wastes in order to facilitate bioremediation of the area. The U. S. Department of Energy (DOE) manages sites contaminated with mixtures of aromatic and chlorinated aliphatic compounds in the subsurface, which could eventually affect ground water quality (83). Different strategies of remediation have been investigated by the DOE at these sites. The DOE has also been responsible for the investigation of subsurface microbes at the Savannah River Site (SRS) in Aiken, South Carolina (30,83). Fliermans et al. have shown that indigenous microbes from a TCE contaminated area at SRS were capable of degrading greater than 150 mg/l TCE (30). While this study was not able to demonstrate pure cultures capable of reproducibly degrading 100 mg/L TCE (30), further work with the isolated TCE degrading consortia resulted in the isolation of a type IV actinomycete, Sm-1, that was able to mineralize VC (79).

This investigation identified isolate Sm-1 and a new isolate, Wrink, as belonging to the *Rhodococcus* genus. This research demonstrated the ability of the *Rhodococci* species to mineralize the EPA priority pollutants TCE and VC. This information is significant

since there is a dearth of information concerning the ability of *Rhodococci* to degrade chlorinated ethenes. This study also demonstrates the potential feasibility of utilizing *Rhododcocci* to degrade mixtures of aromatic and chlorinated aliphatic solvents. Mixed-organic waste degradation data is important since the majority of contaminated sites contain more than one type of toxicant (29,38,77,83).

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PART II

AEROBIC DEGRADATION OF TRICHLOROETHYLENE, VINYL CHLORIDE, AND AROMATIC COMPOUNDS BY *RHODOCOCCI* SPECIES

ABSTRACT

Two *Rhodococci* species, which were isolated from a trichloroethylene-degrading consortium, and *Rhodococcus rhodochrous* mineralized vinyl chloride and trichloroethylene. Greater than 99.9% of 1 mg/L of [1,2-^{14}C]VC was degraded by cell suspensions with the production of greater than 66% $^{14}CO_2$, 25% ^{14}C-aqueous phase products, and 10% of the ^{14}C incorporated into the biomass. Growth cultures that utilized propane as a substrate were able to mineralize greater than 28% of [1,2-^{14}C]TCE to $^{14}CO_2$, with approximately 40% appearing in ^{14}C-aqueous phase products, and another 10% of ^{14}C incorporated into biomass. Cell suspensions of the isolates also degraded other chlorinated aliphatic hydrocarbons, such as chloroform, vinylidine chloride, and 1,1,1-trichloroethane. The isolates were able to degrade a mixture of aromatic and chlorinated aliphatic solvents as well as utilize benzene, toluene, sodium benzoate, naphthalene, biphenyl, and n-alkanes ranging in size from propane-to-hexadecane as carbon and energy sources. The data suggest that *Rhodococci* species are catabolically versatile and may have the potential to degrade a variety of organic pollutants.

INTRODUCTION

Aromatic and chlorinated hydrocarbons, such as benzene, toluene, ethylbenzene, xylene, chloroform (CF), vinyl chloride (VC), and trichloroethylene (TCE), contaminate subsurface aquifers (7,38,43), soils (31), and sediments (31,37) in the U.S. Organic wastes can enter the environment by various routes including industrial discharge into water systems, spill sites, and disposal sites (32). The majority of ground water, soil, and sediment contamination most likely involves mixtures of organic toxicants. Contamination of the ground water with aromatic and chlorinated aliphatic compounds poses serious health problems due to the toxic and carcinogenic nature of the compounds (8,19,39).

Pure and mixed-cultures of bacteria have demonstrated the ability to degrade chlorinated aliphatic and aromatic hydrocarbons. Representative bacteria that aerobically degraded chlorinated aliphatic hydrocarbons in pure cultures include methanotrophs (11,22,28,36), *Nitrosomonas europaea* (2), *Psuedomonas putida* F1, (41), *Pseudomonas fluorescens* (40), *pseudomonas mendocina* (45), propane-oxidizing bacteria (6,42), and a type IV actinomycete, which was isolated from a TCE degrading consortium (30). These organisms are believed to contain mono- and/or dioxygenases that are capable of biotransforming chlorinated aliphatic hydrocarbons.

Anaerobic (9,20) and aerobic (30,35) degradation of aromatic compounds have been reported.

The purpose of this investigation was to examine the ability of aerobic type IV actinomycete isolates Sm-1, Wrink, and *Rhodococcus rhodochrous* (ATCC #21197) to degrade aromatic, aliphatic, chlorinated aliphatic, and polyaromatic hydrocarbons. Studies included radioisotope mineralization experiments, mixed-waste degradation studies, and individual organic waste degradation studies. This report describes and gives mass balances for the aerobic mineralization of TCE and VC by the isolates and demonstrates the ability of the isolates to degrade aromatic and chlorinated aliphatic compounds in mixed-organic waste experiments.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent grade and were obtained from Mallinckrodt, Inc. (Paris, KY.), Aldrich Chemical Co. (St. Louis, MO), or Difco (Detroit, MI.). VC dissolved in methanol was obtained from Supelco (Bellefonte, PA.). VC in nitrogen gas (10% [vol/vol]) was obtained from Matheson (East Rutherford, NJ). [1,2-^{14}C]TCE dissolved in toluene (10mCi/mmol), and [1,2-^{14}C]VC dissolved in toluene (0.53 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA.). Propane was supplied by Holston Gases (Knoxville, TN).

Bacterial cultures, cultural media, and growth conditions. Bacterial isolates Sm-1 and Wrink were derived from a a TCE-degrading consortium, which originated from TCE contaminated sediments at Savannah River Site (30). The TCE-degrading consortium was placed on minimal salts agar plates and incubated under a propane atmosphere. Orange colonies predominately grew and two different types were isolated for further studies: Sm-1, which formed smooth opaque colonies, and Wrink, which formed wrinkled opaque colonies. *Rhodococcus rhodochrous* (ATCC #21197) was purchased from the American Type Culture Collection (Rockville, MD).

The phosphate-buffered medium contained the following (per liter): $MgSO_4$ $7H_2O$, .055 g; CaCl $2H_2O$, .050 g; NH_4Cl 1.48 g; trace mineral solution, 10 mL, and vitamin solution, 1 mL (25); 2.0 mM phosphate-bicarbonate buffer solution and either glucose, propane, benzene, toluene, phenol, benzoate, xylene, ethylbenzene, naphthalene, phenanthrene, biphenyl, raw diesel fuel, or n-alkanes (C_5 to C_{16}) were added as carbon and energy sources. Resazurin was added (1 mL of a 0.2% solution per liter media) to monitor the aerobic status of the media. The pH of the medium was 7.2. Cultures were also maintained on tryptic soy agar (TSA).

For growth experiments, cells were grown in 25-mL serum vials (Bellco Glass, Inc., Vineland, NJ) that had 5 ml of phosphate-buffered medium and a carbon and energy source. For cell suspension studies, cells were grown in 1-liter bottles containing 500 mL of phosphate-buffered medium supplemented with 200 mg/L tryptic soy broth. After 48 hours, 3% propane was added to each bottle. At day 7, the cells were harvested and resuspended in 5 mL of phosphate-buffered medium, which was amended with an appropriate compound or mix of compounds as stated in the text. Controls consisted of uninoculated vials and vials containing formalin/sodium azide killed cell suspensions. Vials were sealed with Teflon-lined septa (Pierce, Rockford, IL) and were incubated statically at 24 °C in the dark for 1-4 weeks, unless otherwise stated. All manipulations involved syringe techniques for the transfer of solutions.

Mycolic acid analysis. Pentaflurobenzyl esters of mycolic acids from freeze-dried cells were prepared according to Minnikin (23). The mycolic acid pentaflurobenzyl esters were purified by thin layer chromatography (TLC) and analysed by high performance liquid chromatography (HPLC). A Gilson 305 pump was used isocratically with a Chrompack Li Chrosorb RP-18 column (200 x 3 mm). The eluent was acetonitrile/tetrahydrofuran (3:2) at 0.8 mL/min.

Menaquinone identification. Freeze-dried cells were extracted and purified by TLC according to Minnikin et al (24). The purified menaquinones were analysed by mass spectrometry and HPLC. Mass spectra were determined on a Kratos MS 80 instrument by electron impact, using a direct insertion probe. HPLC was carried out isocratically using a Gilson 305 pump and a Chrompack Li Chrosorb RP-18 column (200 x 3 mm). The eluent was acetonitrile/isopropanol (75:25) at 2 mL/min.

Physiological tests. The isolates were tested for reduction of nitrate using nitrate broth and for reduction of sulfate using SIMs agar (Difco, Detroit, Michigan). Oxygen requirements were determined by inoculating the cells into semi solid agar butts containing 1% glucose and 0.05% resazaurin. Cells were inoculated on TSA plates, which were then sprayed with a 2.5% solution (w/vol) of either anthracene, phenanthrene, biphenyl, 4-chlorobiphenyl, or

4,4'-dichlorobiphenyl dissolved in acetone. The degradation of the respective PAH was determined by observing clearing of the PAH by the growing colony.

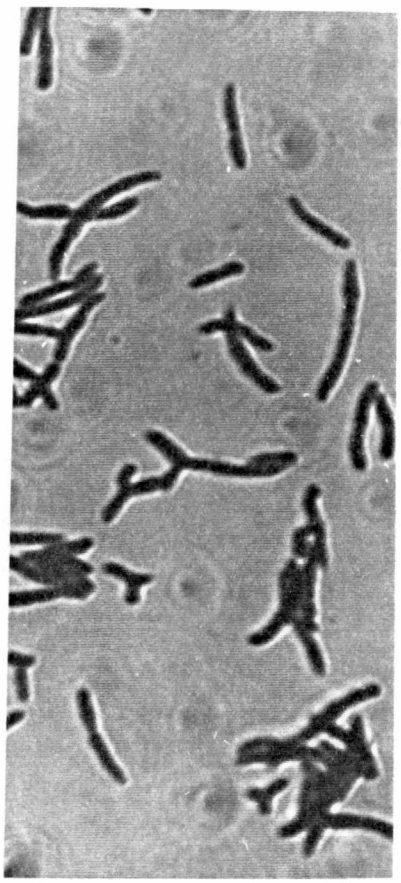
Cell fractionation. Figure A1 shows a flow chart for the VC mineralization experiment. After the headspace gases were analyzed for radioactivity in the VC mineralization experiment, the cells were fractionated to extract lipids and proteins. Lipids were extracted using a modified (44) Bligh-Dyer chloroform methanol extraction (3). Total cell proteins were precipitated using trichloroacetic acid and were collected on Whatman glass filters (14). Radioactivity was determined by a LKB-Wallac model 1212 scintillation counter (LKB Instruments, Inc., Gaithersburg, MD).

Analytical procedures. Headspace sampling was used for all volatile compound analysis. Total and radioactive carbon dioxide were determined, after acidification of the aqueous phase with 6 N HCl, by gas chromatography-gas proportional counting (10,26). A Shimadzu GC 8A gas chromatograph equipped with a thermal conductivity detector was connected to a Packard 894 gas proportional counter (Packard Instruments Co., Inc., Downers Grove, Ill.) The gas chromotograph operated at 110 °C; the helium carrier gas flow was 45 mL/min, with a stainless steel column (0.3175 cm by 2 m) packed with Carbosieve (80/100 mesh). Carbon dioxide formation from VC and TCE was confirmed by gas chromatography-

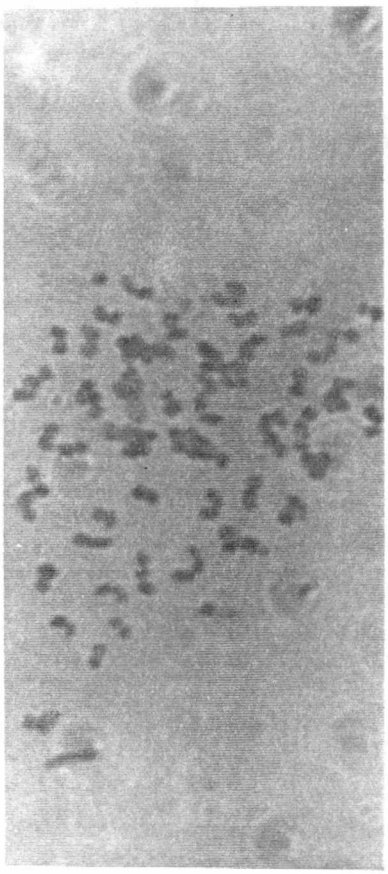
gas proportional counting techniques. VC, VDC, *cis*- and *trans*- 1,2 dichloroethylene were quantified using a photo-ionization detector (HNU Systems, Newton, Mass.). The gas chromatograph was equipped with a 2.5-m-long, 3.2-mm-diameter Poropak T column (30). Short-chain chlorinated hydrocarbons were analyzed on a Hewlett-Packard 5890 capillary gas chromatograph with a split-splitless injector, a 50-m methyl silicone-fused silica capillary column, an electron capture detector, and was operated isothermally at 60 °C with hydrogen as the carrier gas (10). Benzene, toluene, xylene, and ethylbenzene were analyzed with a Shimadzu GC-9A GC equipped with a 2.4-m-long, 3.2-mm-diameter Poropak N-packed column and a flame ionization detector. The following conditions were used: oven temperature, 220 °C; injector temperature, 230 °C; and detector temperature, 235 °C. The data were analyzed with a Nelson Analytical 2600 chromatograph system (Perkin Elmer). The limits of detection for sampled compounds were as follows: methylene chloride, 15 µg/L; CF, 80 µg/L; carbon tetrachloride, 0.8 µg/L; VC, 0.1 µg/L; *cis* -DCE, 0.08 µg/L; *trans* -DCE, 0.06 µg/L; VDC, 0.04 µg/L; TCE, 11 µg/L; PCE, 0.15 µg/L; 1,1,1-TCA, 44 µg/L; 1,1,2-TCA, 45 µg/L; benzene, 0.07 µg/L; toluene, 0.05 µg/L; xylene, 6 µg/L; and ethylbenzene, 1.5 µg/L.

RESULTS

Preliminary identification using the Microbial Identification System (MIS), which identifies microbes by fatty acid composition, indicated that isolates Sm-1 and Wrink had similarity indexes of 0.264 and 0.337, respectively, to the genus *Rhodococcus*. *Rhodococcus rhodochrous* (ATCC #21197), which is orange with a dry wrinkled colonial appearance. This culture was used as a taxonomic standard. Figure A2 shows the common characteristics of isolates Sm-1, Wrink, and *R. rhodochrous*. All three isolates are gram-positive, non-acid fast, asporogenous rods that exhibit branching during growth. Stationary-phase cells exhibit a rod-to-coccus morphogenesis (Plate 1). The organisms are obligately aerobic, non-motile, catalase positive and utilize sucrose as a carbon and energy source. Cellular fatty acids consisted of normal 14-to-22-carbon long saturated and unsaturated fatty acids plus the branch-chain 10-methyl octadecanoic acid. HPLC profiles indicated that Sm-1 and Wrink produced mycolates of a similiar chain length to those from the standard ATCC strain. Menaquinone analysis showed the presence of a single component corresponding to the MK-8 (H_2) menaquinone. This assignment was confirmed for the ATCC standard strain by mass spectrometry (molecular ion m/z 718). Propane, but not methane, was used as a carbon and energy source by all three



Sm-1 at 2 days of growth



Sm-1 at 14 days of growth

PLATE 1. Phase-contrast photomicrographs of isolate Sm-1 at 2 days and 14 days of growth.

isolates.

Biodegradation of radiolabeled VC. Cell suspensions of isolates Sm-1, Wrink, and *R. rhodochrous* were incubated with 1 mg/L of [1,2-^{14}C]VC (0.55 μCi) and the results of the VC mineralization experiment are given in Table 1. The percent of the radiolabel in the CO_2, aqueous, and biomass fractions was calculated by comparing the percent of radiolabel in each fraction to the orginal 0.55 μCi added per vial. The radioactivity in the aqueous phase of the controls was estimated with Henry's Law calculations, using 1.6 as a Henry's Law constant for VC. These calculations were used to account for loss of the radiolabel during separation of the biomass from the aqueous phase. The three isolates degraded greater than 99.9% of 1 mg/L [1,2-^{14}C]VC in seven days as determined by comparison to uninoculated & killed controls. Between 68 and 83% of the label was transformed into $^{14}CO_2$ as determined by headspace analysis after acidification with 6 N HCl. Uninoculated & killed cell suspensions did not produce $^{14}CO_2$. Cell free supernatants contained between 19-26% of the total radioactivity while the biomass incorporated about 10% of the radioactivity. The uninoculated & killed controls had a total of 20% of the radioactivity with 0.5% of the radioactivity as determined by scintillation counting and 20% as estimated from the partioning of VC into the aqueous phase. Mass balance calculations revealed between 99 and 110% of the total label recovered (Table 1), indicating that loss of the highly volatile VC from the Teflon-sealed

TABLE 1. Mineralization of [1,2-^{14}C]VC by cell suspensions of isolates Sm-1, Wrink, and *Rhodococcus rhodochrous* (ATCC #21197)[a]

sample[b]	% VC loss[c]	% $^{14}CO_2$	% [^{14}C]-VC Gas	% [^{14}C]-aqueous	% [^{14}C]-biomass	% Total [^{14}C]-products recovered
Sm-1 a	>99.9	68±5	0	25±1	10±2	103
Sm-1 b	>99.9	66±1	0	26±1	8±1	99
Wrink a	>99.9	78±5	0	19±5	8±2	106
Wrink b	>99.9	83±4	0	21±2	4±1	107
ATCC a	>99.9	73±5	0	24±1	12±1	108
ATCC b	>99.9	76±2	0	25±4	9±2	110
Controls		0	65	20	0.03	85

[a]Cells were grown in 500 ml of phosphate-buffered medium supplemented with 200 mg/L of tryptic soy broth and 3% propane. After 7 days of growth, the cells were harvested, washed and suspended in 5 ml of phosphate-buffered medium plus 1.0 mg/L of VC (0.55 µCi) dissolved in toluene (0.53 mCi/mmol). Vials were assayed after 7 days, and the biomass was determined to be 400-800 mg/L (dry weight).

[b]Results given are of replicates "a" and "b".

[c]Values represent percent differences between averages of triplicate sample vials and averages of an uninoculated vial and a 0.1% formalin and sodium azide killed cell suspension.

vials was insignificant. The three isolates did not show any significant differences in the amount of VC degraded or in the distribution of the radiolabel between CO_2, aqueous products, or biomass.

The radiolabeled biomass was fractionated to determine the incorporation of ^{14}C into different cellular macromolecules. The fraction of radiolabel incorporated into lipids, proteins, and other cellular products was calculated by comparing the radioactivity of each fraction to the radioactivity of unfractionated biomass for each isolate. Lipids, which were extracted using chloroform:methanol, contained about 10% of the radioactivity (Table 2). Radioactivity within the trichloroacetic acid precipitated proteins varied from 2% for isolate Wrink to 22% for isolate Sm-1. The residual biomass contained 42 to 70% of the radioactivity. Mass balance calculations revealed 93±12% recovery of the biomass radioactivity for isolates Sm-1 and *R. rhodochrous*, while 49±8% recovery was obtained for isolate Wrink.

A time course study of VC degradation by cell suspensions of the three isolates is shown in Figure 1. Triplicate experimental vials and separate controls containing 1 mg/L VC were used for each time point. *R. rhodochrous* and Sm-1 started to degrade the VC within the first two hours while Wrink appeared to have a 2 day lag. All three isolates degraded between 70 and 90% of the VC in eight days. The rate of VC degradation for the three isolates proceeded, between days four through eight, at 5-to-20 mmol of VC g^{-1} (dry

TABLE 2. Fractionation of radiolabeled cells incubated in the presence of [1,2-^{14}C]VC[a]

Sample	% [^{14}C]-lipids[b]	% [^{14}C]-proteins	% Remainder of [^{14}C]-cellular products[c]	% Total [^{14}C]-biomass recovered
Sm-1	11±5	22±12	59±13	92±13
Wrink	5±8	2±1	42±8	49±8
ATCC[d]	10±3	13±5	70±12	93±12

[a]Cells were grown as stated in Table 1. The cells were incubated with 1 mg/L VC (0.55 μCi). Lipids were extracted using methanol/chloroform. Proteins were extracted using trichloroacetic acid precipitation.

[b]The percent incorporation of radiolabel into lipids, proteins, and remainder of cellular products was calculated by comparing the activity of incorporated radiolabel in unfractionated biomass for each isolate (Data is in Table 1). All data are averages of triplicate samples.

[c]Values represent radiolabel activity in biomass that was not extracted by the methanol/chloroform or trichloroacetic acid precipitation.

[d]ATCC=*Rhodococcus rhodochrous* (ATCC #21197).

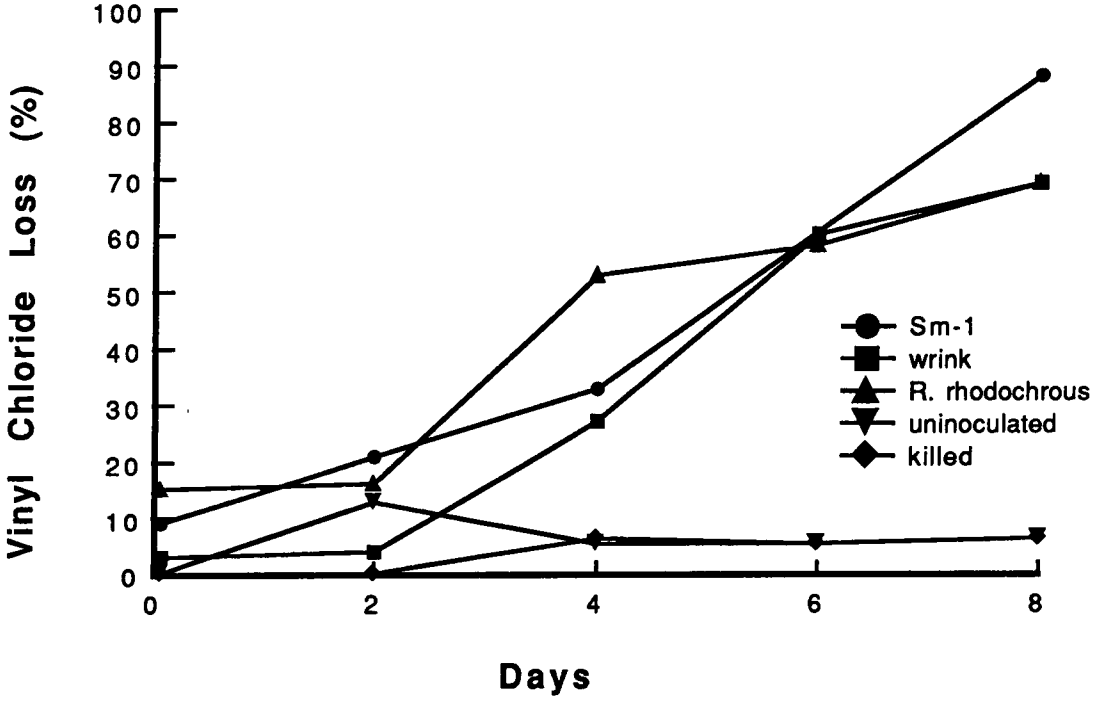


Figure 3. Time course degradation of VC by cell suspensions of Sm-1, Wrink, and *R. rhodochrous* (ATCC #21197)

Triplicate vials containing cell suspensions of each isolate were sacrificed at each time point. The percent loss of VC was determined by comparing differences in the VC concentration in the cell suspensions to an uninoculated vial and a 0.1% formalin and sodium azide killed cell suspension.

weight biomass) day^{-1}. After the eight day incubation, <10% of the VC had escaped from the control vials, demonstrating retention of the volatile toxicant. Repeated measures statistical analysis using the Hotelling-Trace (SAS, SAS Institute, Cary, NC) indicated that the three isolates did not significantly differ in the rate of VC degradation at the 95% significance level (F statistic=0.7160).

The three isolates were grown in phosphate-buffered medium supplemented with 5% propane plus 1 mg/L VC for four weeks and consistently degraded >99% of VC during successive subcultures (Table A1).

Biodegradation of radiolabeled TCE. Isolates Sm-1, Wrink, and *R. rhodochrous*, were grown with 5% propane plus 1 mg/L of [1,2- ^{14}C]TCE (0.3 μCi) and the results of the TCE mineralization experiment are given in Table 3. The percent of the radiolabel in the CO_2, aqueous, and biomass fractions was calcuated by comparing the percent of radiolabel in each fraction to the orginal 0.3 μCi added per vial. The radioactivity in the aqueous phase of the controls was estimated using Henry's Law calculations with a Henry's Law constant of 0.36 for TCE. These calculations were used to account for loss of the radiolabel during separation of the biomass from the aqueous phase. Isolates Sm-1, Wrink, and *R. rhodochrous* were typically capable of degrading 60-to-99.9% of 1 mg/L [1,2-^{14}C]TCE in four weeks as determined by comparison to killed & uninoculated controls. The isolates mineralized between 28 to 36% of

Table 3. Mineralization of [1,2-^{14}C]TCE by isolates Sm-1, Wrink, and *Rhodococcus rhodochrous* (ATCC #21197)[a]

Sample	% Total TCE loss[b]	% $^{14}CO_2$	% [^{14}C]-TCE gas	% [^{14}C]-aqueous	% -[^{14}C]-biomass	% [^{14}C]-products recovered
Sm-1	60±16	28±7	17±7	32±8	7±0	84±7
Wrink	>99.9	36±1	<.5	48±.5	10±0	94±1
ATCC	92±3	33±2	<.5	51±5	10±1	94±5
controls		0	45±3	53±3	3±0	101±3

[a]Cells were grown in 25-ml vials containing 5 ml of phosphate-buffered medium and 5% propane plus 1 mg/L of TCE (0.3 µCi) dissolved in toluene (10 mCi/mmol) and were incubated for 4 weeks at 25 °C. The biomass was determined to be 200±30 mg/L (dry weight). The percent of the radiolabel in each fraction was calculated by comparing the percent of radiolabel in each fraction to the original 0.3 µCi added per vial.

[b]Values represent percent differences between averages of triplicate sample vials and averages of an uninoculated vial and a 0.1% formalin and sodium azide killed cell suspension.

[1,2-^{14}C]TCE. Vials with isolate Sm-1 contained 17% of the radiolabel in the form of gaseous TCE, while isolates Wrink and *R. rhodochrous* did not reveal any detectable residual TCE. Gas chromatography analysis did not demonstrate the accumulation of volatile chlorinated TCE transformation products such as VC, VDC, *cis* or trans DCE were produced. The controls did not produce detectable amounts of $^{14}CO_2$. Isolates Sm-1, Wrink, and *R. rhodochrous* produced ^{14}C-aqueous products, which accounted for 32-51% of the radiolabeled. The controls had a total of 53% of the radiolabel in the aqueous phase with 8% of the radioactivity as determined from scintillation counting and 45% as estimated from the partioning of the TCE into the liquid phase. Cells grown on propane incorporated 7-10% of the radioactivity into biomass as compared to 3% for the killed cell suspensions. An overall mass balance indicated between 84 and 94% of the total ^{14}C recovered.

Isolate Sm-1 was consistently capable of degrading 1 mg/L TCE during successive subcultures grown in phosphate-buffered medium supplemented with 5% propane and incubated for four weeks. While isolate Sm-1 degraded 87±3, 92±0, and 82±13 during three successive subcultures, isolates Wrink and *R. rhodochrous* degraded <30% of the TCE under these conditions (Table A1).

Utilization of alkanes. Cell suspensions of isolate Sm-1 degraded >92% of 5 mg/L of butane, pentane, or hexane in seven days. The ability to utilize n-alkanes as a carbon and energy source was

tested by inoculating isolates Sm-1, Wrink, *R. rhodochrous* into 5 mL of phosphate-buffered medium containing either 5% propane or 1% (vol/vol) of n-alkanes (C_5 to C_{16}). Vials containing n-alkanes typically obtained biomass > .4 $O.D._{660nm}$, while control vials lacking a carbon source obtained biomass < .02 $O.D._{660nm}$. Cells tended to grow at the water-hydrocarbon interface.

Biodegradation of other chlorinated aliphatic hydrocarbons. Cell suspensions of Sm-1 and *R. rhodochrous* were tested for their ability to degrade various chlorinated ethenes and ethanes during one week incubation periods (Table 4). The isolates generally were able to degrade chlorinated hydrocarbons containing three or less chlorine groups. Greater than 93% of 3 mg/L of chloroform and of 5 mg/L methylene chloride were degraded by both isolates. Neither of the cultures were able to degrade PCE or carbon tetrachloride. Isolates Sm-1 and *R. rhodochrous* were generally similar in the percentage of each compound degraded. However, vinylidine chloride was degraded to a greater extent by *R. rhodochrous* than by Sm-1 (95% significance level by Student's *t* test). 1,1,2-TCA was degraded to a greater extent than 1,1,1-TCA. It is curious to note that the isolates readily degraded *cis* DCE but were not capable of degrading *trans* DCE.

Utilization of aromatic hydrocarbons. Cell suspensions of Sm-1 were able to degrade >99.8% of 5 mg/L benzene or toluene in

Table 4. Degradation of chlorinated aliphatic solvents by cell suspensions of isolates Sm-1 and *Rhodococcus rhodochrous* (ATCC #21197)[a]

Compound	% Organic solvent loss[b]	
	Sm-1	*R. rhodochrous*
methylene chloride	96±4	94±10
chloroform	93±4	93±2
carbon tetrachloride	0	0
vinylidine chloride	39±8	63±6
cis-1,2-dichloroethylene	5<	5<
trans-1,2-dichloroethylene	0	0
1,1,1-trichloroethane	87±12	95±2
1,1,2-trichloroethane	100	100
perchloroethylene	0	0

[a]Cells were grown in 500 ml of phosphate-buffered medium supplemented with 200 mg/L of tryptic soy broth and 3% propane. After 7 days of growth, the cells were harvested, washed, and suspended in 5 ml of phosphate-buffered medium, which was then amended with one of the following organic solvents: methylene chloride, 3 mg/L; chloroform, 5 mg/L; carbon tetrachloride, 1 mg/L; vinylidine chloride, 1 mg/L; *cis*-1,2-dichloroethylene, 1 mg/L; *trans*-1,2-dichloroethylene, 1 mg/L; 1,1,1-trichloroethane, 5 mg/L; 1,1,2-trichloroethane, 5 mg/L; and perchloroethylene, 1 mg/L. Vials were assayed after 7 days, and the biomass was determined to be 1400±200 mg/L (dry weight).

[b]Values represent percent differences between averages of triplicate sample vials and averages of an uninoculated and a 0.1% formalin and sodium azide killed cell suspension.

seven days. The ability of isolates Sm-1, Wrink, and *R. rhodochrous* to utilize various aromatic hydrocarbons as energy and carbon sources was tested. An inoculum of cells was placed in 5 mL phosphate-buffered medium plus one of the following compounds: benzene, 0.11 mM; toluene, 0.09 mM; xylene, 0.08 mM; ethylbenzene, 0.08 mM; phenol, 0.09 mM; or sodium benzoate, 8.7 mM. Biomass increased for the three isolates using phenol or benzoate, as compared to vials lacking a carbon source. Only isolates Sm-1 and Wrink had growth on toluene or benzene. None of the isolates exhibited significant growth on xylene or ethylbenzene. Significant growth was determined by having an $O.D._{660nm} \geq 0.4$. The degradation of PAHs and chlorinated aromatics was assessed by streaking the isolates on a TSA plate and spraying with a solution of the compound. Within 48 hours, isolates Wrink, Sm-1, and *R. rhodochrous* degraded phenanthrene, but after four weeks of incubation, none of the isolates were able to degrade anthracene. The three isolates were able to degrade 4-chlorobiphenyl within 48 hours, but none of the isolates were able to degrade 4,4'-dichlorobiphenyl after four weeks of incubation. The degradation of 4-chlorobiphenyl was accompanied by the production of a yellow-colored product that subsequently disappeared within a few days.

After the isolates demonstrated the ability to degrade PAHs, they were then tested for their ability to utilize naphthalene, phenanthrene, and biphenyl as growth substrates. An inoculum of the isolates was added to vials containing phosphate-buffered medium

plus 10 mg of the test compound and 20% O_2. *R. rhodochrous* was not able to utilize any of the compounds as an energy and carbon source. However, isolates Sm-1 and Wrink were able to grow using naphthalene, which was accompanied by the production of a brownish colored soluble product. Isolates Sm-1 and Wrink utilized biphenyl but not phenanthrene as a carbon and energy source.

Colorimetric tests for mono- and dioxygenases. Cell suspensions of Sm-1, Wrink, and *R. rhodochrous* were incubated with naphthalene for 90 minutes and then tetrazotized O-Dianiside (t-OD) was added, which resulted in the immediate production of a purple color indicating the production of α-napthol from naphthalene (5). However, no color was produced in uninoculated vials and 0.1% formalin and sodium azide killed cell suspensions. The production of α–napthol from naphthalene is indicative of a monooxygenase reaction (5). The presence of dioxygenases was tested by incubating cell suspensions with indole. After 24 hours, a pink color was produced, which indicated the presence of indirubin (14). The production of indirubin, which is an isomer of indigo, from indole demonstrates the presence of dioxygenase(s) (14). Control vials did not produce a pink color.

Biodegradation of a mixture of organic compounds. The ability of cell suspensions of isolates Sm-1 and *R. rhodochrous* to degrade mixtures of organic compounds was investigated. Table 5

Table 5. Degradation of mixed-organic solvents by cell suspensions of isolates Sm-1 and *Rhodococcus rhodochrous* (ATCC #21197)[a]

Compound	% Organic solvent loss[b]	
	Sm-1	*R. rhodochrous*
benzene	100±0	29±16
toluene	100±0	92±11
ethylbenzene	100±0	77±4
xylene	100±0	0
chloroform	81±7	83±15
vinyl chloride	100±0	45±0
VDC	100±0	76±1
cis-1,2-DCE	100±0	83±17
trans-1,2-DCE	0	0
TCE	15±10	11±1
PCE	0	0
1,1,1-TCA	15±13	0
1,1,2-TCA	65±21	22±1

[a]Cells were grown in 500 ml of phosphate-buffered medium supplemented 30% propane (p). After 7 days of growth, the cells were harvested, washed, and suspended in 5 ml of phosphate-buffered medium, which was amended with a mixture of organic compounds: benzene, .7 mg/L; toluene, .5 mg/L; ethylbenzene, .15 mg/L; xylene, 6 mg/L; chloroform, 8 mg/L; vinyl chloride, 1 mg/L; vinylidine chloride (VDC), .4 mg/L; *cis*-1,2-dichloroethylene (DCE), .8 mg/L; *trans*-1,2-dichloroethylene (DCE), .6 mg/L; trichloroethylene (TCE), 1.1 mg/L; perchloroethylene (PCE), .15 mg/L; 1,1,1-trichloroethane (TCA), 4.4 mg/L; and 1,1,2-trichloroethane (TCA), 4.5 mg/L. Vials were assayed after 14 days, and the biomass was determined to be 1600±600 mg/L (dry weight).

[b]Values represent percent differences between averages of triplicate sample vials and averages of an uninoculated and a 0.1% formalin and sodium azide killed cell suspension.

shows the results of a mixed-organic solvent degradation experiment. Toluene and ethylbenzene were readily degraded by the two isolates. Greater than 99.9% of 6 mg/L xylene and .7 mg/L benzene were degraded by isolate Sm-1, while *R. rhodochrous* degraded less than 29% of these compounds. Chlorinated ethenes containing 2 or fewer chlorine groups were generally degraded to a further extent (>70%) than compounds containing 3 or more chlorines. PCE and *trans* DCE were not degraded. Greater than 72% of 8 mg/L chloroform was degraded by the isolates. 1,1,2-TCA was degraded to a further extent than 1,1,1-TCA by the two isolates. Isolate Sm-1 appeared to be able to degrade a greater percentage of each compound as compared to *R. rhodochrous*. In replicate experiments the same trends were observed but *R. rhodochrous* degraded >99.9% of xylene.

Isolates Sm-1, Wrink, and *R. rhodochrous* were able to utilize 0.06% (vol/vol) raw diesel as a growth substrate.

DISCUSSION

Pure cultures of three different bacterial isolates have demonstrated the ability to aerobically mineralize VC and TCE. Two of the isolates, Sm-1 and Wrink, were derived from a TCE contaminated site. The third isolate, *Rhodococcus rhodochrous*, came from the American Type Culture Collection. The isolates also degraded n-alkanes, aromatic hydrocarbons, PAHs, chlorinated aliphatic hydrocarbons, chlorinated aromatic hydrocarbons, and a mixture of aromatic and chlorinated aliphatic hydrocarbons. Physiological and morphological identification of isolates Sm-1 and Wrink indicated that they are type IV actinomycetes, which includes species belonging to the *Corneybacterium*, *Mycobacterium*, *Rhodococcus*, and *Nocardia* genera (13). Menaquinone identification and mycolic acid analysis definitively identified the unknown isolates to the *Rhodococcus* genus. Actinomycetes can biotransform a broad variety of substrates including aliphatic and aromatic hydrocarbons (27,34). *Mycobacterium* sp. have demonstrated the ability to degrade VC (16,42) and TCE (6,42). This study demonstrated the ability of *Rhodococcus* species to degrade chlorinated aliphatic hydrocarbons and a mixture of aromatic and chlorinated aliphatic hydrocarbons.

Cell suspensions mineralized VC without a requirement for exogenous carbon, such as propane, during the degradation process probably since endogenous metabolism was providing energy and reducing power for the cells. The degradation of 1 mg/L [1,2-^{14}C]VC was accompanied by the quantitative production of $^{14}CO_2$, water soluble products, and biomass. The killed cell suspensions did not have ^{14}C associated with the supernatant or biomass indicating that the live cells released transformation products of VC into the aqueous phase and incorporated, rather than adsorbed, the radiolabel into biomass. The radiolabeled biomass had radioactivity in lipids and proteins indicating that a fraction of the radiolabel possibly was shunted through a metabolic pool, which was incorporated into cellular macromolecules.

Cells grown on 5% propane mineralized TCE. The degradation of [1,2-^{14}]TCE by the isolates resulted in the release of $^{14}CO_2$, aqueous products, and biomass. Radiolabel was incorporated rather than absorbed to the cells since the propane grown cells had three times more radioactivity as compared to killed cell suspension. TCE mineralization did not result in the production of volatile chlorinated compounds such as VC, *cis* or *trans* DCE, or VDC. This is in contrast to anaerobic degradation of TCE, which results in the accumulation of those compounds (4,22,33).

The degradation of VC and TCE resulted in significant percentages of the radiolabel appearing as $^{14}CO_2$, ^{14}C-aqueous phase products, and ^{14}C biomass. About 2 times more $^{14}CO_2$ was produced

during VC degradation (70%) than by TCE degradation (30%). This difference may be explained by the fact that the VC degradation experiment had 3 times more biomass, used cell suspensions as opposed to growing cells, and/or the ability to mineralize VC may be metabolically easier for the cells than TCE. The $^{14}CO_2$ may be either derived form 1) the direct metabolic oxidation of VC or TCE or 2) the flow of the radiolabel from VC or TCE into a common metabolic pool of the organisms, which is eventually oxidized to $^{14}CO_2$.

A wide range of chlorinated aliphatic hydrocarbons were aerobically degraded by the isolates including chlorinated ethanes, ethylenes, and chlorinated C_1 compounds containing three or fewer chlorine groups. However, compounds such as PCE or carbon tetrachloride, which are believed to be degraded by reductive dechlorination (4), were not degraded by the isolates. These results, concerning the ability to degrade aliphatic compounds with three or fewer chlorine groups, are consistent with data obtained for methanotrophs, psuedomonads, and propane-oxidizing bacteria (11,41,42).

R. rhodochrous, Sm-1, and Wrink are catabolically versatile since they utilized aliphatic, aromatic, and polyaromtic hydrocarbons as growth substrates (Figure A3). Aliphatic hydrocarbons ranging in size from C_3-to-C_{16} were utilized as energy and carbon sources. Better growth was obtained on n-alkanes ranging in size from C_{11}-C_{16}, probably due to the lower solubility, which resulted in lower toxicity of the longer-chain hydrocarbons as

compared to the shorter-chain hydrocarbons. Benzene, toluene, benzoate, and phenol were used as sources of energy and carbon but ethylbenzene and xylene were not utilized as growth substrates, even though ethylbenzene and xylene were degraded by cell suspensions. Actinomycetes typically are not able to utilize aromatics with short alkyl substitutions, such as xlyene and ethylbenzene, as growth substrates but are able to co-metabolize the alkyl substitutient to an oxidized derivative (27). Only certain three ring PAH isomers could be degraded since phenanthrene but not anthracene could be degraded.

The isolates are similar to F199, a bacterium isolated from the subsurface. F199 is a type IV actinomycete that degraded and utilized many of the same aromatic compounds as carbon and energy sources that the isolates biotransformed (12). There are significant differences between the isolates and F199. F199 is yellow, not able to degrade VC or TCE under the same conditions as isolates Sm-1 and Wrink (data not shown), and was not able to utilize propane as a carbon and energy source. Overall, the ability of the isolates to biotransform various aromatic hydrocarbons are very similar to results obtained with other actinomycetes.

4-chlorobiphenyl was degraded with the subsequent production of a yellow-colored product, which may indicate a meta cleavage reaction (1). The isolates are typical of many other bacteria since they degraded a monochlorinated biphenyl but were not capable of

degrading a more highly chlorinated biphenyl, such as 4',4-dichlorobiphenyl (16).

A complex mixture of aromatic and chlorinated aliphatic hydrocarbons was degraded by cell suspensions of isolates *R. rhodochrous* and Sm-1. The aromatics were degraded to a greater extent than the aliphatics: possibly the aromatics were preferred since some of them could serve as an energy source and because there was generally less of a given aromatic in the mixture than of a given chlorinated aliphatic hydrocarbon. It is unusual that *cis* DCE was degraded to a lesser extent than TCE, since less chlorinated compounds are generally degraded to a greater extent than highly chlorinated compounds under aerobic conditions (11). VDC and *cis* DCE were degraded both in the mixed-waste degradation experiments and in the individual degradation experiments while *trans* DCE was not degraded in either experiment. Further experimentation needs to be done to address the reasons why *trans* DCE was not degraded while the other compounds were degraded. Perhaps the geometry of *trans* DCE is not conducive for enzyme attack. The ability of isolate Sm-1 to degrade 11 out of 13 compounds is comparable to results obtained with the consortium from which the isolate was derived (29).

There are significant differences between the consortium and the pure culture isolates, Sm-1 and Wrink. The consortium was catabolically more versatile than the isolates since the consortium degraded PCE and utilized methane as a growth substrate (30), while

the isolates could not degrade PCE or utilize methane. The isolates could not degrade TCE concentrations higher than 10 mg/L (data not shown), while the consortium degraded TCE concentrations as high as 150 mg/L (10). Correspondingly, the consortium had higher rates of TCE degradation than the isolates (30). These differences may indicate that microbial consortia are more efficient at degrading chlorinated hydrocarbons than are pure cultures. However, isolates Sm-1 and Wrink may be responsible for the majority of catabolic reactions in the consortium.

Isolates Sm-1 and Wrink have demonstrated the potential to degrade a variety of EPA priority pollutants including aromatic, aliphatic, and polyaromatic hydrocarbons, singularly and in a mixture. The site from which isolates were derived is contaminated with short-chain chlorinated aliphatic hydrocarbons (10). *R. rhodochrous*, which was not isolated from a TCE contaminated site, also degraded similar environmental toxicants as isolates Sm-1 and Wrink. The isolates grew on propane and studies have shown that growth on propane involves monooxygenases (18). All three isolates also utilized certain aromatic hydrocarbons as growth substrates. The isolates probably contained 1) monooxygenase(s) as determined by the production of α–napthol from naphthalene (5) and 2) dioxygenase(s) as determined by the production of a compound that is most likely indirubin from indole (15). Propane monooxygenase (42), toluene monooxygenase (45), and toluene dioxygenase (41) were implicated to be key enzymes involved in epoxidating chlorinated

alkenes. It is tempting to speculate that the ability to degrade chlorinated aliphatic hydrocarbons may be a general characteristic of the *Rhodococcus* genus due to the presence of mono- and dioxygenase(s). More studies on other *Rhodococci* sp. will be necessary to verify this hypothesis. Further studies are planned to determine whether propane monooxygenases and/or toluene dioxygenases are involved in TCE degradation and also whether the enzymes are constitutive or inducible.

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PART III

SUMMARY

CONCLUSIONS

Isolates Sm-1, Wrink, and *Rhodococcus rhodochrous* have demonstrated the ability to degrade aliphatic, chlorinated aliphatic, aromatic, polyaromatic, and chlorinated aromatic hydrocarbons. While the isolates were capable of degrading a variety of compounds, the isolates were not able to degrade every compound tested. The isolates were able to degrade chlorinated aliphatics containing three or less chlorine substituents but were never able to degrade compounds such as tetrachloroethylene or carbon tetrachloride. The isolates were able to degrade phenathrene but not anthracene indicating that the shape of anthracene may not be conducive to enzymatic attack by these isolates. The isolates are similar to methanotrophs (4) since the isolates degrade the same types of chlorinated aliphatics as the methanotrophs but the isolates are also different since the isolates are able to utilize a variety of compounds such as sugars, aromatic, and polyaromatic hydrocarbons as growth substrates. In terms of versatility, the isolates are quite similar to the pseudomonads (5,7) since both groups are capable of utilizing aromatic hydrocarbons as growth substrates and both are able to degrade chlorinated aliphatic hydrocarbons.

RECOMMENDATIONS

While this study investigated the different types of hydrocarbons that the isolates could degrade, it did not address the issue of what types of enzyme(s) are responsible for the degradation and how the enzyme(s) are regulated. The isolates can either degrade the chlorinated aliphatics by a propane monooxygenase (6) and/or by mono- or dioxygenases involved in aromatic metabolism (5,8). Further work should be performed to determine whether the ability to degrade chlorinated aliphatics, such as TCE, is constitutive or inducible, and if the isolates are inducible, then to determine the inducer(s). Colorimetric tests for mono- and dioxygenases should be performed concurrently to determine the type of oxygenase that is involved in chlorinated aliphatic degradation (1,3). The specificity of the enzyme can be determined by competitive inhibition studies. Once the potential inducers and competitive inhibitors are known, the information can be used to help formulate an engineering plan to stimulate chlorinated aliphatic degradation at contaminated sites by microbes similar to the isolates. The ability of *R. rhodochrous* to degrade the same types of compounds as the environmental isolates, Sm-1 and Wrink, was unexpected. Possibly, the ability to degrade the wide variety of compounds is a property of *Rhococci* species. *Mycobacterium* sp.

have demonstrated the ability to degrade chlorinated aliphatic compounds (6,2) and now this study shows that certain *Rhodococcus* sp. also have this ability. Further studies need to be done to screen other *Rhodococci* species to test the hypothesis that the ability to degrade chlorinated aliphatic hydrocarbons is a general characteristic of this genera of bacteria. This study would be important since *Rhodococci* species may prove to have certain metabolic characteristics that would make them desirable for bioremediation.

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APPENDIX

Figure A1 shows a flow chart for the VC mineralization experiment. After the headspace gases were analyzed for radioactivity in the VC mineralization experiment, the cells were fractionated to extract lipids and proteins. Lipids were extracted using a modified Bligh-Dyer chloroform:methanol extraction. Total cell proteins were precipitated using trichloroacetic acid and were collected on Whatman glass filters. Radioactivity was determined by a LKB-Wallac model 1212 scintillation counter.

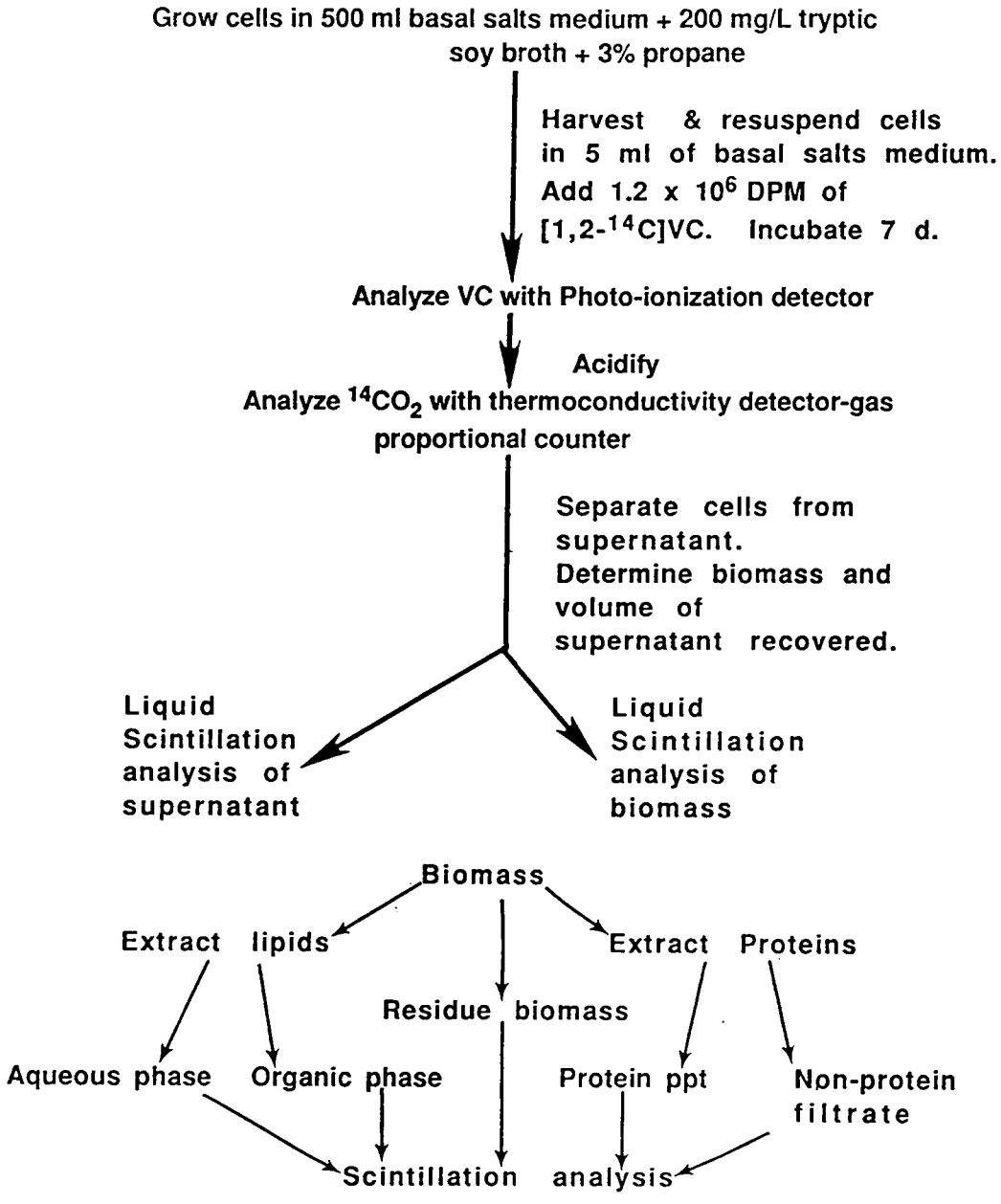


Figure 1. Protocol for VC Radiolabeled Studies

Common characteristics of the three isolates:

1. Asporogenous non-motile Gram-positive rods.
2. Statationary cells exhibit rod-to-coccus morphogenesis.
3. Obligately aerobic.
4. Catalase positive.
5. Fatty acids consist of normal 14-to-22 carbon-long saturated and unsaturated fatty acids plus branch-chain 10-methyloctodecanoic acid.
6. Phospholipids consist of phosphatidylinostiol, phosphatidylglycerol, and phosphatidylethanolamine.
7. Able to use sucrose as energy/carbon source.
8. Optimal temperature range of 30-to-35 °C.
9. Optimal pH of 7.0.
10. Do not use methane or H2/CO2 as carbon/energy source.
11. Do not perform anaerobic respiration or fermentation.
12. Orange pigmentation.
13. Plasmids were not detected.

Isolate Sm-1 has a smooth round colony morphology.
Isolate Wrink has a wrinkled colony morphology.
Isolate Rhodococcus rhodochrous ATCC #21197 has a rough wrinkled colony morphology.

Figure A2. Summary Characteristics of Sm-I, Wrink, and *Rhodococcus rhodochrous* (ATCC #21197)

Aromatics	Polyaromatics	Chlorinated aromatics
Benzene*	Naphthalene*	4-Chlorobiphenyl
Toluene*	Phenanthrene	
Phenol*	Biphenyl*	
Xylene		
Ethylbenzene		
Benzoate*		
Raw diesel fuel*		

Alkanes	Chlorinated aliphatics	Surfactants
Propane*	Methylene chloride	Linear alkylbenzene-sulfonate
Butane	Chloroform	
C5-C16*	Vinyl Chloride	
	Vinylidine Chloride	
	Cis-1,2-Dichloroethylene	
	Trans-1,2-Dichloroethylene	
	Trichloroethylene	
	1,1,1-trichloroethane	
	1,1,2-trichloroethane	

*=Compound used as sole carbon source

Figure A3. Types of compounds degraded by Isolates Sm-I, Wrink, and *Rhodococcus rhodochrous* (ATCC #21197)

Figure A3 is a summary of the types of organic compounds that the isolates degrade. The compounds are either cometabolized or utilized as a carbon and energy source by the isolates

TABLE A1. Assessment of VC and TCE degradation by isolates Sm-1, Wrink, and *Rhodococcus rhodochorous* (ATCC #21197) during growth on propane for a month[a].

Date	VC loss (%)[b]			TCE loss (%)[b]		
	Atcc[c]	Sm-1	Wrink	Atcc	Sm-1	Wrink
10/28/90	>99.9	>99.9	N.G.[d]	N.D.[e]	N.D.	N.D.
12/17/90	>99.9	>99.9	>99.9	N.D.	N.D.	N.D.
1/19/91	>99.9	>99.9	>99.9	N.D.	N.D.	N.D.
2/23/91	N.G .	>99.9	>99.9	N.D	N.D.	N.D.
3/4/91	>99.9	>99.9	>99.9	N.D.	N.D.	N.D.
4/4/91	>99.9	>99.9	>99.9	N.D.	N.D.	N.D.
5/11/91	>99.9	N.G.	>99.9	N.D.	N.D.	N.D.
6/12/91	>99.9	>99.9	>99.9	N.D.	N.D.	N.D.
7/25/91	>99.9	>99.9	>99.9	N.D.	N.D.	N.D.
8/30/91	>99.9	>99.9	>99.9	96	68	0
10/4/91	>99.9	99.0	>99.9	95±2	96±3	25±7
11/9/91	94±11	>99.9	86±17	87±3	75±25	0
12/13/91	>99.9	>99.9	>99.9	92±0	30±23	15±15
1/15/92	>99.9	>99.9	>99.9	82±13	32±14	0

[a] Cells were grown in triplicate 25 ml pressure tubes that contained 5 mL of PBBM that was supplemented either with 3 or 5% propane. Transferring was performed by placing 0.1 ml of old culture into fresh media. VC in methanol was added to each tube at a concentration of 1mg/L. Vials were incubated 25 °C in the dark for 4 weeks.

[b]Values represent percent differences between averages of triplicate samples and averages of an uninoculated and a 0.1% formalin and sodium azide kelled cell suspension.

[c]Atcc=*Rhodococcus rhodochrous* (ATCC #21197).

[d]N.G.=No growth. If cells did not grow, they were not used to calculate percent degradation.

[e]N.D.=Not determined

VITA

Kenneth Jay Malachowsky was born in New York, New York on January 6, 1967. He attended elementary and junior high schools in the Co-op City district. He graduated from the Bronx High School of Science in June 1985 in the top ten percent of his class. The following August, he entered Cornell University on early decision, where he majored in microbiology. He graduated May, 1989 from Cornell with a Bachelor of Science in Microbiology degree with Honors and Distinction. He then moved to Knoxville to work as a technician for Dr. Tommy Phelps and at the end of the year, he entered the Master's program in Biotechnology at the University of Tennessee, Knoxville and continued the work he started as a technician.

He is presently working on his Ph.D. in Dr. Gary Sayler's lab at the University of Tennessee, Knoxville. He is a member of the American Society for Microbiology.