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Aerobic Mineralization of Trichloroethylene, Vinyl Chloride, and Aromatic Compounds by *Rhodococcus* Species

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Two *Rhodococcus* strains which were isolated from a trichloroethylene (TCE)-degrading bacterial mixture and *Rhodococcus rhodochrous* ATCC 21197 mineralized vinyl chloride (VC) and TCE. Greater than 99.9% of a 1-mg/liter concentration of VC was degraded by cell suspensions. [1,2-^{14}C]VC was degraded by cell suspensions, with the production of greater than 66% $^{14}CO_2$ and 20% ^{14}C-aqueous phase products and incorporation of 10% of the ^{14}C into the biomass. 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 the biomass. VC degradation was oxygen dependent and occurred at a pH range of 5 to 10 and temperatures of 4 to 35°C. Cell suspensions degraded up to 5 mg of TCE per liter and up to 40 mg of VC per liter. Propane competitively inhibited TCE degradation. Resting cell suspensions also degraded other chlorinated aliphatic hydrocarbons, such as chloroform, 1,1-dichloroethylene, and 1,1,1-trichloroethane. The isolates degraded a mixture of aromatic and chlorinated aliphatic solvents and utilized benzene, toluene, sodium benzoate, naphthalene, biphenyl, and *n*-alkanes ranging in size from propane to hexadecane as carbon and energy sources. The environmental isolates appeared more catabolically versatile than *R. rhodochrous* ATCC 21197. The data report that environmental isolates of *Rhodococcus* species and *R. rhodochrous* ATCC 21197 have the potential to degrade TCE and VC in addition to a variety of aromatic and chlorinated aliphatic compounds either individually or in mixtures.

Aromatic and chlorinated hydrocarbons, such as benzene, toluene, ethylbenzene, xylene, chloroform, vinyl chloride (VC), and trichloroethylene (TCE), contaminate subsurface aquifers (6, 33, 38), soils (27), and sediments (27, 32) in the United States. Organic wastes can enter the environment by various routes, including industrial discharge into water systems, accidental spills, and leaching from disposal sites (28). The majority of groundwater, soil, and sediment contamination most likely involves mixtures of organic toxicants. Contamination of the groundwater with aromatic and chlorinated aliphatic compounds poses serious health problems because of the toxic and carcinogenic nature of the compounds (7, 16, 35).

Pure and mixed cultures of bacteria have demonstrated the ability to degrade chlorinated aliphatic and aromatic hydrocarbons. Representative bacteria that aerobically degrade chlorinated aliphatic hydrocarbons in pure cultures have been reviewed recently by Ensley (8) and include methanotrophs (18, 24, 31), *Mycobacterium aurum* L1 (12), *Nitrosomonas europaea* (2), *Pseudomonas putida* F1 (37), *Pseudomonas fluorescens* (34), *Pseudomonas mendocina* (40), propane-oxidizing bacteria (5, 36), and a type IV actinomycete, which was isolated from a TCE-degrading bacterial mixture (25). These organisms are believed to contain mono- and/or dioxygenases that are capable of biotransforming chlorinated aliphatic hydrocarbons.

The purpose of this investigation was to examine the ability of *Rhodococcus* sp. strains Sm-1 and Wrink, which were isolated from a TCE-degrading bacterial mixture, and *Rhodococcus* rhodochrous ATCC 21197 to degrade aromatic, aliphatic, chlorinated aliphatic, and polyaromatic hydrocarbons (PAHs). Actinomycetes are known to biotransform a broad variety of substrates, including aliphatic and aromatic hydrocarbons (23, 30). *Mycobacterium* spp. have demonstrated the ability to degrade VC (13, 36) and TCE (5, 36). This study demonstrates the capability of *Rhodococcus* strains to mineralize VC and TCE, to degrade other chlorinated aliphatic hydrocarbons, and to degrade a mixture of aromatic and chlorinated aliphatic hydrocarbons.

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 Laboratories (Detroit, Mich.). VC dissolved in methanol was obtained from Supelco (Bellefonte, Pa.). VC in nitrogen gas (10%, vol/vol) was obtained from Matheson (East Rutherford, N.J.). [1,2-^{14}C]TCE dissolved in toluene (10 mCi/mmol; >98% purity) and [1,2-^{14}C]VC dissolved in toluene (0.53 mCi/mmol; >98% purity) were purchased from New England Nuclear Corp. (Boston, Mass.). Propane was supplied by Holston Gases (Knoxville, Tenn.).

Bacterial cultures, cultural media, and growth conditions. *Rhodococcus* sp. strains Sm-1 (ATCC 51239) and Wrink (ATCC 51240) were derived from a TCE-degrading bacterial mixture which originated from TCE-contaminated sediments at the Savannah River site (9). The propane oxidizers were obtained by incubating the TCE-degrading bacterial mixture on minimal salts agar plates under a propane atmosphere. Two different types of orange colonies were isolated from the minimal salts agar plates for further studies, namely, Sm-1,

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which formed smooth opaque colonies, and Wrink, which formed wrinkled opaque colonies. *R. rhodochrous* ATCC 21197 was obtained from the American Type Culture Collection (Rockville, Md.).

The phosphate-buffered medium (PBM) contained the following (per liter): $MgSO_4 \cdot 7H_2O$, 0.055 g; $CaCl \cdot 2H_2O$, 0.050 g; NH_4Cl, 1.48 g; trace mineral solution, 10 ml (19), and vitamin solution, 1 ml (19); plus 2.0 mM phosphate-bicarbonate buffer solution. Resazurin (0.2%, wt/vol) was added to monitor the redox status of the media. The pH of the medium was 7.2 unless stated otherwise. Cultures were also maintained on tryptic soy agar.

Cells were grown in 25-ml serum vials (Bellco Glass, Inc., Vineland, N.J.), which contained 5 ml of PBM plus propane (5%, vol/vol; headspace), for carbon source utilization experiments. Different aromatic and aliphatic compounds were tested as carbon and energy sources by placing an inoculum of cells in 5 ml of PBM plus one of the following compounds: benzene, 0.11 mM; toluene, 0.09 mM; phenol, 0.09 mM; sodium benzoate, 8.7 mM; *o-,m-,p*-xylene mixture, 0.08 mM; ethylbenzene, 0.08 mM; naphthalene, 0.1 mM; phenanthrene, 9.0 μM; biphenyl, 49 μM; or *n*-alkanes (C_5 to C_{16}; 1%, vol/vol). Cell growth was monitored spectrophotometrically (A_{660}). The vials were placed in a clamp, and an injection of either a gas or the aqueous phase of a water-saturated organic mixture was made via a syringe between the septa and the wall of the glass vial, avoiding puncture of the septa. PAHs were added as solids to the growth medium. Vials were sealed with Teflon-lined septa (Pierce, Rockford, Ill.) and crimp tops and incubated statically at 24°C in the dark for 1 to 4 weeks. Cell suspension biomass was determined gravimetrically at the end of each experiment.

For cell suspension studies, cells were grown in loosely capped 1-liter bottles containing 500 ml of PBM supplemented with 200 mg of tryptic soy broth per liter. After 48 h, the bottles were sealed with a rubber stopper, and a mixture of 3% (vol/vol) propane and 20% (vol/vol) oxygen (headspace gases) was added to each bottle unless stated otherwise. After 7 days, the cells were harvested by centrifugation, washed, and resuspended in 5 ml of PBM, which was amended with an appropriate compound or mixture of compounds as stated in the text. Controls consisted of uninoculated vials and vials containing cells inhibited with 0.1% formalin–sodium azide.

For certain experiments, PBM was made anaerobic by adding 1% (vol/vol) Oxyrase (Oxyrase, Ashland, Ohio) and 0.01 mM sodium lactate and by purging with nitrogen. The dissolved oxygen concentration was measured with a dissolved oxygen probe (Microelectrodes, Londonderry, N.H.).

Mycolic acid and menaquinone analysis. Pentafluorobenzyl esters of mycolic acids from freeze-dried cells were prepared as described by Minnikin (20). The mycolic acid pentafluorobenzyl esters were purified by thin-layer chromatography and analyzed by high-performance liquid chromatography (HPLC). A Gilson 305 pump was used isocratically with a Chrompack Li Chrosorb RP-18 column (200 by 3 mm). The eluent was acetonitrile-tetrahydrofuran (3:2 [vol/vol]) at 0.8 ml/min.

Menaquinones were extracted from freeze-dried cells and purified by thin-layer chromatography as described by Minnikin et al. (21). The purified menaquinones were analyzed by mass spectrometry and HPLC. Mass spectra were determined on a Kratos MS 80 instrument by electron impact with a direct insertion probe. HPLC was carried out as described above with an eluent of acetonitrile-isopropanol (75:25 [vol/vol]) at 2 ml/min.

Physiological and presumptive degradation tests. The isolates were tested for reduction of nitrate by using nitrate broth and for reduction of sulfate by using SIMs agar (Difco). Oxygen requirements were determined by inoculating the cells into tubes that contained semisolid agar supplemented with 1% glucose and 0.05% resazurin. Cells were inoculated on tryptic soy agar plates, which were then sprayed with a 2.5% solution (wt/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. After the headspace gases were analyzed for radioactive $^{14}CO_2$ in the VC mineralization experiments, the cells were fractionated to extract lipids and proteins. Lipids were extracted by using a modified version (39) of the Bligh-Dyer chloroform-methanol extraction (3). Total cell proteins were precipitated by using trichloroacetic acid and collected on Whatman glass filters (11). Radioactivity was determined with a model 1212 scintillation counter (LKB Instruments, Inc., Gaithersburg, Md.).

Analytical procedures. Headspace gas sampling was used for all volatile compound analysis. Total carbon dioxide and radioactive carbon dioxide were determined after acidification of the aqueous phase with 6 N HCl (4%, vol/vol) by gas chromatography-gas proportional counting (9, 22). A Shimadzu GC 8A gas chromatograph equipped with a thermal conductivity detector or a Shimadzu GC 9A gas chromatograph equipped with a photo-ionization detector was connected to a model 894 gas proportional counter (Packard Instruments Co., Inc., Downers Grove, Ill.). The radiolabeled purity of VC and TCE was verified by adding 5 μl of the compound to a scintillation vial and to a 25-ml EPA vial containing 5 ml of distilled water. After 2 h of equilibration, 0.5 ml of the headspace was injected into the gas chromatograph-gas proportional counter. The radiochemical purity was calculated by comparing the radioactivity in the observed gas-proportional counter peaks with the radioactivity determined from scintillation counting. Values of >98% purity were achieved typically with contamination by $^{14}CO_2$ of <1%. The amount of radiolabel added to the vials was based on scintillation counting of control vials that received identical aliquots of the radiolabeled compound. VC, vinylidine chloride, and *cis*- and *trans*-1,2-dichloroethylene (*cis*- and *trans*-1,2-DCE) were quantified by using a photo-ionization detector (HNU Systems, Newton, Mass.) as described previously (25). Dichloromethane, chloroform, carbon tetrachloride, tetrachloroethylene (PCE), 1,1,1-trichloroethane (1,1,1-TCA), and 1,1,2-TCA were analyzed with a Hewlett-Packard 5890 gas chromatography equipped with a capillary column as described previously (9). Benzene, toluene, xylene, and ethylbenzene were analyzed with a Shimadzu GC-9A gas chromatograph 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; detector temperature, 235°C. The data were analyzed with a Nelson Analytical 2600 chromatography software system (Perkin-Elmer). Packed columns were injected with 0.5 ml of headspace while capillary columns were injected with 5.0 μl of headspace. The limits of detection for sampled compounds were as follows (each per liter): dichloromethane, 15 μg; chloroformin, 80 μg; carbon tetrachloride, 0.8 μg; VC, 1.0 μg; *cis*-1,2-DCE, 1.0 μg; *trans*-1,2-DCE, 1.0 μg; 1,1-DCE, 1.0 μg; TCE, 11 μg; PCE, 0.15 μg; 1,1,1-TCA, 44 μg; 1,1,2-TCA, 45 μg; benzene, 0.7 μg; toluene, 0.5 μg; xylene, 6 μg; and ethylbenzene, 1.5 μg.

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

Sample[b]	% VC remaining[c]	% ^{14}C (±SD) found in fraction				% of total products recovered
		CO_2	VC gas	Aqueous	Biomass	
Sm-1 a	ND[d]	68 ± 5	0	25 ± 1	10 ± 2	103
Sm-1 b	ND	66 ± 1	0	26 ± 1	8 ± 1	99
Wrink a	ND	78 ± 5	0	19 ± 5	8 ± 2	106
Wrink b	ND	83 ± 4	0	21 ± 2	4 ± 2	107
ATCC a	ND	73 ± 5	0	24 ± 1	12 ± 1	108
ATCC b	ND	76 ± 2	0	25 ± 4	9 ± 2	110
Uninoculated	100	0	65	20	0	85
Killed	100	0	57	19	0	76

[a] Vials were assayed after 7 days, and the biomass was determined to be 400 to 800 mg (dry weight) per liter.
[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–sodium azide-killed cell suspension.
[d] ND, not detected.

RESULTS AND DISCUSSION

Identification of isolates. Preliminary identification by using the Microbial Identification System, which identifies microbes by fatty acid composition, indicated that the environmental isolates Sm-1 and Wrink had similarity indexes of 0.264 and 0.337, respectively, to the genus *Rhodococcus* (10). The similarity index is a mathematical expression of the extent to which the fatty acid profile of the environmental isolates matches the mean profile for the organism named. A similarity index between 0.3 and 0.5 usually indicates that the sample is from a species that is different from, but closely related to, the one named. These results suggested that isolates Sm-1 and Wrink were likely related to the genus *Rhodococcus*. *R. rhodochrous* ATCC 21197, which is orange with a dry wrinkled colonial appearance, was used as a taxonomic standard. HPLC profiles indicated that strains Sm-1 and Wrink produced mycolates and menaquinone similar to those from the standard ATCC strain, indicating that the strains are in the *Rhodococcus* genus. All three strains have physiological characteristics identical to those previously described for strain Sm-1 (25).

Biodegradation of radiolabeled VC. Cell suspensions of the three organisms were incubated with 0.55 μCi of [1,2-^{14}C]VC (0.53 mCi/mmol), and the results of the VC mineralization experiment are shown in Table 1. The percentage of the radiolabel detected in the CO_2, aqueous, and biomass fractions was calculated by comparing the percentage of radiolabel in each fraction with the original 0.55 μCi added per vial. The radioactivity in the aqueous phase of the controls was estimated by using Henry's Law calculations, which were used to account for the loss of the radiolabel during separation of the biomass from the aqueous phase. The three isolates degraded greater than 99.9% of the VC in 7 days, as determined by comparison with uninoculated and 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 and killed cell suspensions did not produce $^{14}CO_2$. Cell-free supernatants contained between 19 and 26% of the total radioactivity, while the biomass incorporated about 10% of the radioactivity. The uninoculated and killed controls had a total of 85% of the radioactivity recovered, with 65% of the radioactivity determined by gas-proportional counting and 20% estimated from the partitioning of VC into the aqueous phase. Mass balance calculations revealed that between 76 and 110% of the total label was recovered (Table 1), indicating that the loss of the highly volatile VC from the Teflon-sealed vials was small. The three organisms 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 (10%), proteins (2 to 22%), and other cellular products (42 to 70%) was calculated by comparing the radioactivity of each fraction with the radioactivity of unfractionated biomass for each isolate (data not shown). 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 (data not shown). Formalin-azide-inhibited 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.

A time course study of VC degradation by cell suspensions of the three organisms is shown in Fig. 1. Triplicate experimental vials and separate controls containing 1 mg of VC per ml were used for each time point. *R. rhodochrous* and isolate Sm-1 started to degrade the VC within the first 2 h, while isolate Wrink appeared to have a 2-day lag. All three isolates

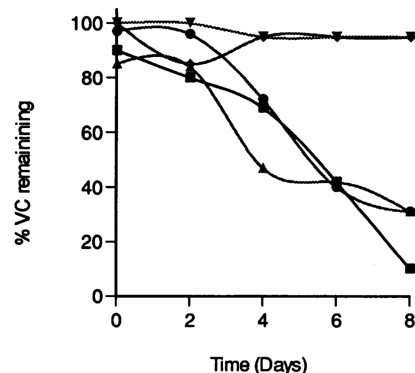

FIG. 1. VC degradation. Symbols: ■, Sm-1; ●, Wrink; ▲, *R. rhodochrous*; ▼, uninoculated; ◆, killed. Triplicate vials containing cell suspensions of each organism were sacrificed at each time point. The percent loss of VC (1 mg/liter) was determined by comparing differences in the VC concentrations in the cell suspensions with those of an uninoculated vial and a 0.1% formalin–sodium azide-killed cell suspension.

TABLE 2. Mineralization of [1,2-^{14}C]TCE by isolates Sm-1 and Wrink and *R. rhodochrous* ATCC 21197[a]

Sample	% TCE remaining (±SD)[b]	% ^{14}C (±SD) found in fraction				% Of products recovered
		CO_2	TCE gas	Aqueous	Biomass	
Sm-1	40 ± 16	28 ± 7	17 ± 7	58 ± 8	7 ± 0	110
Wrink	ND[c]	36 ± 1	<0.5	48 ± 0.5	10 ± 0	94
ATCC	8 ± 3	33 ± 2	<0.5	51 ± 5	10 ± 1	94
Uninoculated	100 ± 1	0	42 ± 5	63 ± 2	2.7 ± 0.3	112
Killed	100 ± 1	0	47 ± 2	71 ± 3	2.1 ± 0.1	128

[a] Vials were assayed after 4 weeks, and the biomass was determined to be 200 ± 30 mg (dry weight) per liter.
[b] Values represent the percent difference between averages of triplicate sample vials and averages of an uninoculated vial and a 0.1% formalin–sodium azide-killed cell suspension.
[c] ND, not detected.

degraded between 70 and 90% of the VC in 8 days. The rate of VC degradation for the three isolates proceeded, between days 4 through 8, at 5 to 20 mmol of VC g (dry weight) of biomass^{-1} day^{-1}. After the 8-day incubation, <10% of the VC had escaped from the control vials, demonstrating retention of the volatile toxicant. Repeated-measure statistical analysis using the Hotelling-Trace Test (SAS; SAS Institute, Cary, N.C.) indicated that the three isolates did not significantly differ in the rate of VC degradation at the 95% significance level (F = 0.7160).

Cell suspensions of strain Sm-1 were incubated under different environmental conditions to assess the effects of oxygen, pH, and temperature on the degradation of VC. After 1 week of incubation, 1 mg of VC per liter was degraded by >99.9% aerobically (dissolved oxygen, 13%) but not anaerobically (dissolved oxygen, 0%). Greater than 70% of a 1-mg/liter concentration of VC was degraded at pH 5 to 10, with optimal degradation of >99.9% occurring at pH 6 to 7, while <40% was degraded at pH 3, 4, and 11. VC degradation did not occur at a temperature of 55°C; >99.9% was degraded between 4 and 35°C. These results demonstrate that the cells degrade VC under a wide range of environmental conditions. Anaerobic conditions, temperature, and pH extremes may limit VC degradation by killing or damaging cells, while anaerobic conditions may also affect VC degradation since oxygen may be required for epoxidating VC.

Biodegradation of radiolabeled TCE. Strains Sm-1 and Wrink and *R. rhodochrous* were grown with 5% propane plus 0.3 μCi of [1,2-^{14}C]TCE (10 mCi/mmol), and the results of the TCE mineralization experiment are given in Table 2. The percentage of the radiolabel in the CO_2, aqueous, and biomass fractions was calculated by comparing the percentage of radiolabel in each fraction with the original 0.3 μCi added per vial. The radioactivity in the aqueous phase of the controls was estimated by using Henry's Law calculations, which were used to account for loss of the radiolabel during separation of the biomass from the aqueous phase. Sm-1, Wrink, and *R. rhodochrous* were typically capable of degrading 60 to 99.9% of [1,2-^{14}C]TCE in 4 weeks, as determined by comparison with killed and uninoculated controls. The isolates mineralized between 28 and 36% of [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 detect the presence of volatile chlorinated TCE transformation products such as VC, vinylidine chloride, or *cis*- or *trans*-1,2-DCE. This is in contrast to the anaerobic degradation of TCE, which results in the production of those compounds (4, 17, 29). The controls did not produce detectable amounts of $^{14}CO_2$. Sm-1, Wrink, and *R. rhodochrous* produced ^{14}C-aqueous products, which accounted for 48 to 58% of the total radiolabel. The controls had a total of 67% of the radiolabel in the aqueous phase, with 10% of the radioactivity determined from scintillation counting and 57% estimated from the partitioning of the TCE into the liquid phase. Cells grown on propane incorporated 7 to 10% of the radioactivity into biomass compared with 3% for the killed cell suspensions. An overall mass balance indicated that between 94 and 128% of the total ^{14}C was recovered. Radiolabel was likely incorporated into, rather than absorbed to, the cells since the propane-grown cells had three times more radioactivity than the inhibited cell suspensions.

Upper concentration limits of VC and TCE degradation. Cell suspensions of strain Sm-1 were incubated with increasing concentrations of either VC or TCE for a 1-week period (Fig. 2). Strain Sm-1 was not able to degrade greater than 10 mg of TCE per liter, but it was able to degrade up to 20 mg of VC per liter. In a separate experiment, cells were incubated for 2 weeks, during which time they degraded 30% of a 40-mg/liter concentration of VC (data not shown). These results indicate that strain Sm-1 more readily degrades higher concentrations of VC than TCE. This may be because of TCE toxicity or because VC has fewer chlorines than TCE.

Propane inhibition studies. Cell suspensions of strain Sm-1 were incubated with 1 mg of TCE per liter and with either 0, 4, 40, or 80% (vol/vol; headspace) propane. Concentrations of greater than 40% propane caused a competitive inhibition of

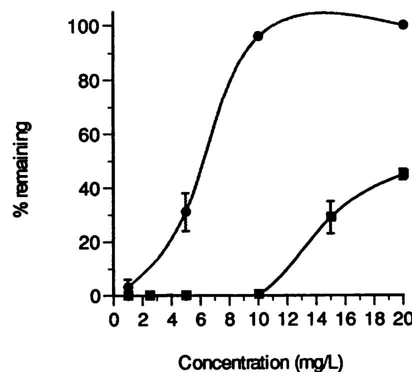

FIG. 2. Degradation of different concentrations of VC and TCE by cell suspensions of Sm-1. Symbols: ■, VC; ●, TCE. The percent loss of the compound was determined by comparing differences in the VC concentrations in the cell suspensions with those of an uninoculated vial and a 0.1% formalin–sodium azide-killed cell suspension.

TABLE 3. Degradation of chlorinated aliphatic hydrocarbon solvents by cell suspension of isolates Sm-1 and *R. rhodochrous*[a]

Compound	Initial concn (mg/liter)	% Organic solvent remaining (±SD)[b]	
		Sm-1	*R. rhodochrous*
Methylene chloride	3.0	4 ± 4	6 ± 10
Chloroform	5.0	7 ± 4	7 ± 2
1,1-DCE	1.0	61 ± 8	37 ± 6
cis-1,2-DCE	1.0	<5	<5
trans-1,2-DCE	1.0	100 ± 14	100 ± 8
1,1,1-TCA	5.0	13 ± 12	5 ± 2
1,1,2-TCA	5.0	ND[c]	ND
Tetrachloroethylene	1.0	100 ± 15	100 ± 13

[a] Vials were assayed after 7 days, and the biomass was determined to be 1,400 ± 200 mg (dry weight) per liter.

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

[c] ND, not detected.

TABLE 4. Degradation of mixed-organic solvents by cell suspensions of isolates Sm-1 and *R. rhodochrous*[a]

Compound	Initial concn (mg/liter)	% Organic solvent remaining (±SD)[b]	
		Sm-1	*R. rhodochrous*
Benzene	0.7	ND[c]	71 ± 16
Toluene	0.5	ND	8 ± 11
Ethylbenzene	0.2	ND	23 ± 4
Xylene	6.0	ND	100
Chloroform	8.0	19 ± 7	17 ± 15
VC	1.0	ND	55 ± 0
1,1-DCE	0.4	ND	23 ± 1
cis-1,2-DCE	0.8	ND	17 ± 17
trans-1,2-DCE	0.6	100	100
TCE	1.1	85 ± 10	89 ± 1
PCE	0.2	100	100
1,1,1-TCA	4.4	85 ± 13	100
1,1,2-TCA	4.5	35 ± 21	78 ± 1

[a] Cells for the cell suspensions were grown with 30% propane and 20% oxygen. Vials were assayed after 14 days, and the biomass was determined to be 1,600 ± 600 mg (dry weight) per liter.

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

[c] ND, not detected.

TCE (23% ± 7% degraded) degradation compared with vials lacking propane (87% ± 3% degraded). These results demonstrate that a propane monooxygenase is most likely involved in TCE degradation (5, 36).

Utilization of alkanes. Cell suspensions of Sm-1 degraded >92% of a 5-mg/liter concentration of butane, pentane, or hexane in 7 days. The ability to utilize *n*-alkanes as a carbon and energy source was tested by inoculating the three organisms into 5 ml of PBM containing separate mixtures (1%, vol/vol) of C_5 to C_{16} *n*-alkanes. Vials containing *n*-alkanes typically obtained a biomass with an optical density at 660 nm (OD_{660}) of >0.4, while control vials lacking a carbon source obtained a biomass with an OD_{660} of <0.02. It was observed that the cells tended to grow at the water-hydrocarbon interface. More biomass was obtained on *n*-alkanes ranging in size from C_{11} to C_{16}, probably because of the lower solubility, which resulted in a lower toxicity of the longer-chain hydrocarbons than of the shorter-chain hydrocarbons.

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 1-week incubation periods (Table 3). The organisms generally were able to degrade chlorinated hydrocarbons containing three or fewer chlorine groups. Greater than 93% of a 3-mg/liter concentration of chloroform and of a 5-mg/liter concentration of methylene chloride was degraded by both isolates. Neither of the cultures degraded PCE or carbon tetrachloride, which are believed to be degraded by reductive dechlorination (4). 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. The isolates readily degraded *cis*-1,2-DCE but were not capable of degrading *trans*-1,2-DCE. The ability to degrade aliphatic compounds with three or fewer chlorine groups appears consistent with data obtained for methanotrophs, pseudomonads, and propane-oxidizing bacteria (36, 37).

Utilization of aromatic hydrocarbons. Cell suspensions of Sm-1 degraded >99.8% of a 5-mg/liter concentration of benzene or toluene in 7 days. The ability of the three organisms to utilize various aromatic hydrocarbons as energy and carbon sources was tested. Biomass increased for the three isolates when phenol or benzoate was used as a carbon source (OD, >0.4) compared with the biomass of vials lacking a carbon source (OD, <0.1). Only isolates Sm-1 and Wrink exhibited growth on toluene or benzene (OD, 0.3). None of the isolates exhibited significant growth on a mixture of xylene isomers or ethylbenzene (OD, <0.1). 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 xylene and ethylbenzene, as growth substrates but are able to cometabolize the alkyl substituent to an oxidized derivative (23).

The degradation of PAHs and chlorinated aromatics was assessed by streaking the isolates on a tryptic soy agar plate and spraying with a solution of the compound. Within 48 h, the three organisms degraded phenanthrene and 4-chlorobiphenyl, but after 4 weeks of incubation, none of the organisms degraded anthracene or 4,4′-dichlorobiphenyl. The degradation of 4-chlorobiphenyl was accompanied by the production of a yellow 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 (14).

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 PBM 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 utilize naphthalene and biphenyl (OD, >0.6) but not phenanthrene.

Biodegradation of a mixture of organic compounds. The ability of cell suspensions of isolates Sm-1 and *R. rhodochrous* to degrade mixtures of aromatic and chlorinated aliphatic compounds was investigated. Table 4 shows the results of a mixed-organic solvent degradation experiment. Toluene and ethylbenzene were readily degraded by the two organisms.

Greater than 99.9% of a 6-mg/liter concentration of xylene and a 0.7-mg/liter concentration of benzene were degraded by isolate Sm-1, while *R. rhodochrous* degraded less than 29% of these compounds. Chlorinated ethenes containing two or fewer chlorine groups were generally degraded to a greater extent (>70%) than compounds containing three or more chlorines. PCE and *trans*-1,2-DCE were not degraded. Greater than 72% of an 8-mg/liter concentration of chloroform was degraded by the isolates. 1,1,2-TCA was degraded to a greater extent than 1,1,1-TCA by both isolates. Isolate Sm-1 appeared to be able to degrade a greater percentage of several compounds in comparison with *R. rhodochrous*. In replicate experiments, the same trends were observed, but *R. rhodochrous* degraded >99.9% of xylene. 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. 1,1-DCE and *cis*-1,2-DCE were degraded both in the mixed-waste degradation experiments and in the individual degradation experiments, while *trans*-1,2-DCE was not degraded in either experiment. Perhaps the geometry of *trans*-1,2-DCE is not conducive for enzyme attack by the *Rhodococcus* species. Several bacterial species degrade both *cis*- and *trans*-1,2-DCE (12, 24, 37), although *N. europaea* oxidized *trans*-1,2-DCE slower than *cis*-1,2-DCE (2). The ability of isolate Sm-1 to degrade 11 of 13 compounds is comparable to results obtained with the bacterial mixture from which the isolate was derived (9). These results have significant implications for the bioremediation of mixed-organic waste sites. The concentrations of organics used in these experiments is indicative of that found at subsurface contaminated sites, and the U.S. Department of Energy has stated that research is needed for the evaluation of the degradation of mixed-organic wastes (27).

There are significant differences between the bacterial mixture and the pure culture isolates Sm-1 and Wrink. The bacterial mixture was catabolically more versatile than the isolates since the bacterial mixture degraded small amounts of PCE and *trans*-1,2-DCE and utilized methane as a growth substrate (26), while the isolates could not degrade PCE or *trans*-1,2-DCE or utilize methane. The isolates could not degrade TCE at concentrations higher than 10 mg/liter, while the bacterial mixture degraded TCE at concentrations as high as 150 mg/liter (9). Correspondingly, the bacterial mixture had higher rates of TCE degradation than the isolates (26). These differences may indicate that microbial consortia are more efficient at degrading mixtures of chlorinated hydrocarbons than are pure cultures. However, isolates Sm-1 and Wrink may be responsible for many of the catabolic reactions in the bacterial mixture.

Rhodococcus sp. strains Sm-1 and Wrink have demonstrated the potential to degrade a variety of U.S. Environmental Protection Agency priority pollutants including aromatic and aliphatic hydrocarbons and PAHs, both singularly and in a mixture. The three *Rhodococcus* isolates appeared to be similar and degraded TCE and VC at similar rates and to similar extents (Tables 1 and 2 and Fig. 1). Strains Sm-1 and Wrink, which were isolated from contaminated sediments, appeared to exhibit a broader degradative capacity than *R. rhodochrous* ATCC 21197. Strains Sm-1 and Wrink could utilize toluene, benzene, naphthalene, and biphenyl for growth substrates, whereas *R. rhodochrous* could not. As noted in Table 4, the environmental isolate previously exposed to chlorinated aliphatic hydrocarbons was able to degrade greater percentages of the 13 components than was *R. rhodochrous*. Similar to the bacterial mixture described above, previous acclimation of strains Sm-1 and Wrink to chlorinated aliphatic hydrocarbon contamination may have resulted in their being more catabolically versatile than the *R. rhodochrous* obtained from the American Type Culture Collection.

Despite minor differences in catabolic versatility, carbon from VC and TCE was incorporated into cellular biomass to a similar extent by the three *Rhodococcus* strains. All three organisms grew on propane, and studies have shown that growth on propane involves monooxygenases, although other mechanisms for propane utilization may exist (15). The three organisms also utilized certain aromatic hydrocarbons as growth substrates. Propane monooxygenase (36) and toluene monooxygenase (40) are among the enzymes responsible for the initial incorporation of oxygen into chlorinated alkenes. It is tempting to speculate that the ability to degrade TCE and other chlorinated aliphatic hydrocarbons may be a characteristic of several *Rhodococcus* sp. due to the presence of a monooxygenase(s).

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