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Cometabolic Degradation of Chlorinated Alkenes by Alkene Monooxygenase in a Propylene-Grown *Xanthobacter* Strain

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Propylene-grown *Xanthobacter* cells (strain Py2) degraded several chlorinated alkenes of environmental concern, including trichloroethylene, 1-chloroethylene (vinyl chloride), *cis*- and *trans*-1,2-dichloroethylene, 1,3-dichloropropylene, and 2,3-dichloropropylene. 1,1-Dichloroethylene was not degraded efficiently, while tetrachloroethylene was not degraded. The role of alkene monooxygenase in catalyzing chlorinated alkene degradations was established by demonstrating that glucose-grown cells which lack alkene monooxygenase and propylene-grown cells in which alkene monooxygenase was selectively inactivated by propyne were unable to degrade the compounds. C_2 and C_3 chlorinated alkanes were not oxidized by alkene monooxygenase, but a number of these compounds were inhibitors of propylene and ethylene oxidation, suggesting that they compete for binding to the enzyme. A number of metabolites enhanced the rate of degradation of chlorinated alkenes, including propylene oxide, propionaldehyde, and glucose. Propylene stimulated chlorinated alkene oxidation slightly when present at a low concentration but became inhibitory at higher concentrations. Toxic effects associated with chlorinated alkene oxidations were determined by measuring the propylene oxidation and propylene oxide-dependent O_2 uptake rates of cells previously incubated with chlorinated alkenes. Compounds which were substrates for alkene monooxygenase exhibited various levels of toxicity, with 1,1-dichloroethylene and trichloroethylene being the most potent inactivators of propylene oxidation and 1,3- and 2,3-dichloropropylene being the most potent inactivators of propylene oxide-dependent O_2 uptake. No toxic effects were seen when cells were incubated with chlorinated alkenes anaerobically, indicating that the product(s) of chlorinated alkene oxidation mediates toxicity.

A variety of aerobic bacteria which contain broad-substrate-specificity oxygenases are capable of oxidizing halogenated aliphatic compounds. Bacteria possessing this ability include toluene-oxidizing bacteria (22, 35), methane-oxidizing bacteria (19, 24, 29), ammonia-oxidizing bacteria (2, 16, 26, 27, 32), and propane-oxidizing bacteria (34), and the enzymes which have been implicated in catalyzing halocarbon oxidations are toluene mono- and dioxygenase (41, 42), methane monooxygenase (5, 24, 29), ammonia monooxygenase (2, 26, 27, 32), and propane monooxygenase (34), respectively. Many of the halogenated aliphatic compounds which are substrates for these enzymes are recognized as priority pollutants and health hazards which are prevalent contaminants in certain soil and aquatic environments. Their generally recalcitrant nature has intensified interest in the potential of microbial systems to degrade these xenobiotics to nontoxic compounds. An important subdivision of these aliphatic halocarbons includes chlorinated alkenes such as trichloroethylene, which has found widespread application as a solvent and degreaser, and vinyl chloride, which is used in the production of polyvinylchloride.

Despite the proven ability of certain bacteria to biodegrade halogenated hydrocarbon pollutants via oxygenase attack, there are several potential obstacles to their exploitation in biological remediation schemes. First, since oxygenases require the input of reductant to catalyze the oxygenation of substrates, sustained oxidations in obligate lithotrophs are dependent upon the further oxidation of the product of the oxygenase reaction to supply reductant for the oxygenation of additional substrate molecules. The oxidation of nonphysiological substrates by broad-substrate oxygenases will generally not generate such a product, necessitating the presence of a cometabolite which can do so. Second, the initial oxidation product(s) of halocarbon oxidations is in many cases an irreversible inactivator of the oxygenase and other cellular proteins. This is most clearly manifest in the case of chlorinated alkene oxygenation, where one of the immediate oxidation products can be a reactive epoxide. For example, one product of trichloroethylene oxygenation is trichloroethylene epoxide (5, 19, 21). This short-lived epoxide likely gives rise to the compounds that covalently modify and inactivate cellular proteins (5, 24, 28, 36). Third, the final oxidation product resulting from oxygenation may itself be xenobiotic. An ideal remediation scheme will result in the complete mineralization of a xenobiotic to CO_2 and halide ions or at least to compound(s) of reduced or no toxicity to biological systems.

In an effort to identify bacteria capable of sustained biodegradation of chlorinated alkenes, we have considered a class of bacteria which are able to grow with simple olefins (ethylene, propylene, and butylene) as their sole carbon and energy source (8). These bacteria initiate the oxidation of alkenes via oxygenation to the corresponding epoxides (8). The epoxides then undergo further enzymatic oxidation to provide for the energy and carbon requirements of the cell (8, 38). The ability of alkene-oxidizing bacteria to enzymatically metabolize reactive epoxides could potentially circumvent a number of the barriers to sustained chlorinated alkene degradation discussed above. For example, one alkene-utilizing bacterium, *Mycobacterium* strain L1, was shown to grow with either ethylene or vinyl chloride as the sole carbon and energy source (9). Two isoprene (2-methyl-1,3-butadi-

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ene)-utilizing bacteria, *Alcaligenes denitrificans* subsp. *xylooxidans* JE75 and *Rhodococcus erthyropolis* JE77, were recently shown to cometabolize trichloroethylene, dichloroethylenes, and vinyl chloride and reportedly exhibited a higher degree of tolerance to trichloroethylene than has been observed for other trichloroethylene-degrading bacteria (4).

The alkene-utilizing bacterium we have chosen to investigate for its chlorocarbon-degrading capacity is a *Xanthobacter* strain (Py2) isolated with propylene as a carbon and energy source (30). In addition to propylene, *Xanthobacter* strain Py2 will grow on ethylene or 1-butene, as well as a variety of nonalkene carbon sources such as glucose, succinate, H_2-CO_2, C_1 to C_4 alcohols, propylene glycol, propionaldehyde, and propylene oxide, the immediate product of propylene oxygenation (30, 31). When grown with propylene as a carbon source, an NADH-dependent alkene monooxygenase activity was shown to be present in cell extracts (30). This monooxygenase activity did not oxygenate alkanes (30), in contrast to the monooxygenases of methane- and ammonia-oxidizing bacteria, which are able to oxygenate both alkenes and alkanes (3, 13). Another distinctive feature of the alkene monooxygenase activity of *Xanthobacter* strain Py2 was its relative insensitivity to acetylene, a potent inhibitor of the monooxygenases of methane- and ammonia-oxidizing bacteria (18, 25).

In this paper we demonstrate that several C_2 and C_3 chlorinated alkenes of environmental concern are oxidized by cell suspensions of *Xanthobacter* strain Py2 and demonstrate that alkene monooxygenase is the enzyme catalyzing these oxidations. We also demonstrate that several metabolites are able to stimulate chlorinated alkene oxidation and investigate the potential toxic effects associated with chlorinated alkene metabolism.

MATERIALS AND METHODS

Chemicals. Propylene, propylene oxide, propyne, 1,3-dichloropropylene (a mixture of *cis*- and *trans*-isomers), 2,3-dichloropropylene, tetrachloroethylene, trichloroethylene, 1,1-dichloroethylene, *cis*- and *trans*-1,2-dichloroethylene, 1,3-dichloropropane, 2,3-dichloropropane, 1-1-dichloroethane, and 1,2-dichloroethane were purchased from Aldrich Chemical Co., Milwaukee, Wis. 1,1,2,2-Tetrachloroethane, 1,1,2-trichloroethane, and chloroethane were purchased from Eastman Kodak Co., Rochester, N.Y. Vinyl chloride was purchased from Alltech Associates, Deerfield, Ill. Ethylene was purchased from Airco, Murray Hill, N.Y.

Growth and preparation of cells. *Xanthobacter* strain Py2 (30) was provided by J. A. M. de Bont, Agricultural University, Wageningen, The Netherlands. Cultures were grown in 1-liter shake flasks containing 0.5 liter of mineral medium (39) with propylene (10% [vol/vol] gas phase) or glucose (0.2% [wt/vol]) added as a carbon and energy source. Culture flasks containing propylene were sealed with rubber stoppers fitted with an inlet tube and rubber septum through which sterile propylene was added as an overpressure. The air and propylene in the culture flasks were replenished periodically during growth. Cells were harvested by centrifugation after reaching an optical density (A_{600}) of 0.8, and the sedimented cells were resuspended in buffer (50 mM sodium phosphate, pH 7.2). The cells were centrifuged again and suspended in buffer as described above at approximately 0.2 g (wet weight) per ml. Cell suspensions were stored on ice and used within a few hours of harvesting.

Analytical procedures. Hydrocarbons and chlorocarbons were quantified by using gas chromatographs (Shimadzu model GC-8A) equipped with Porapak Q columns (0.3 by 40 cm; Waters Associates, Farmington, Mass.) and fitted with either a flame ionization detector (FID) or an electron capture detector (ECD). The gas chromatographs were operated isothermally with detector temperatures of 220°C (ECD) or 200°C (FID) and column temperatures ranging from 65 to 170°C. The gas chromatographs were interfaced to a Spectra Physics model SP4270 integrator (ECD) or a Shimadzu model C-R3A integrator (FID). O_2 measurements were performed with a Clark-type O_2 electrode operated at 30°C. Protein concentrations of cell suspensions were determined by the biuret assay (6) after solubilization in 3 M NaOH for 30 min at 65°C. Bovine serum albumin was used as the standard. The partitioning of propylene and ethylene between the gas and liquid phases in incubation vials was calculated from published Bunsen coefficients for the gases (40).

Quantitation of propylene oxidation and propylene oxide utilization. The oxidation of propylene and utilization of propylene oxide by resting-cell suspensions was assayed by monitoring substrate depletion over time by gas chromatography. Assays were conducted in stoppered serum vials (9 ml) which contained 1 ml (total volume) of buffer (as described above) and propylene or propylene oxide (0.5 or 2 μmol). After equilibration of the gas and liquid phases at 30°C (180 shaking cycles per min) reactions were initiated by the addition of cells (0.2 to 0.5 mg of protein). Samples of the gas phase (for propylene quantitation; 25 to 50 μl) or liquid phase (for propylene oxide quantitation; 2 to 5 μl) were removed periodically with a microsyringe and analyzed by FID gas chromatography. Rates of propylene oxidation were derived from the slopes of plots of alkene concentration versus time. The specific activity of cell suspensions ranged from 50 to 90 nmol of propylene oxidized per min per mg of protein.

Propyne treatment of cell suspensions. Resuspended cells (10 to 20 mg of protein) were incubated with shaking at 30°C in stoppered serum vials (35 ml) containing 10 ml of buffer, 5 μmol of propylene, and 2.5 ml of propyne. Control cells were incubated identically but in the absence of propyne. After a 60-min incubation period, cells were sedimented by centrifugation, resuspended in 10 ml of buffer, and centrifuged a second time. The cells were then resuspended to a 2-ml final volume and stored on ice.

Degradation of chlorinated hydrocarbons. Chlorocarbon degradation assays were conducted in inverted serum vials (9 ml) sealed with Teflon-lined silicone septa (Alltech) and containing 2 ml of buffer and 500 nmol of chlorocarbon. Chlorocarbons (except chloroethane and vinyl chloride, which were added as gases) were transferred to sealed incubation vials from saturated aqueous solutions prepared at room temperature by using gas-tight syringes. The amount of substrate added was estimated from solubility tables (12). Incubations were performed with shaking at 30°C as described above for propylene oxidation assays. Samples of the gas phase (25 μl) were removed immediately after initiating assays and at the desired time points and analyzed by ECD or FID gas chromatography.

Inhibition of propylene and ethylene oxidation by chlorocarbons. Incubations were conducted as described above in serum vials (9 ml) sealed with Teflon-lined septa containing 1 ml of buffer, 500 nmol of propylene or ethylene, and 500 nmol of chlorocarbon. Assays were initiated by the addition of cells (0.19 mg of protein), samples of the gas phase (25 μl) were removed at 0, 10, 20, and 30 min, and propylene and ethylene were quantified by FID gas chromatography.

Effect of cometabolites on O_2 uptake and chlorocarbon degradation. The rates of O_2 uptake of cell suspensions in the presence of 1 mM propylene oxide, propionaldehyde, propylene glycol, 1-propanol, and glucose were measured by injecting 3.8 μl of a 500 mM stock solution of cometabolite into the O_2 electrode chamber in which buffer (1.9 ml) and cells (1.24 mg of protein) had been preequilibrated for 5 min. To measure propylene stimulation of O_2 uptake, the chamber was first equilibrated with 1.8 ml of buffer containing 0.9 mM propylene; cells (in a 100-μl volume) were then added to initiate the assay.

Chlorocarbon degradation assays in the presence of cometabolites were performed as described above. Cometabolites were added to 1 mM concentrations from 500 mM stock solutions prepared in buffer, with the exception of propylene, which was added as a gas to give 0.05, 0.5, or 2.5 μmol of propylene in the assay vials. Reactions were initiated by the addition of cell suspensions (0.19 mg of protein), and samples of the gas phase (25 μl) were removed at 0 and 60 min and analyzed by ECD gas chromatography.

Toxicity effects of chlorinated alkene oxidations. Possible toxic effects resulting from the oxidation of chlorinated alkenes were assessed as follows. Cell suspensions (2.9 mg of protein) were incubated with shaking at 30°C in serum vials (35 ml) sealed with Teflon-lined septa containing 5 ml of buffer, 1 mM glucose, and 1.25 μmol of chlorocarbon. After incubation for 60 min, remaining chlorocarbons were removed by sedimenting the cells by centrifugation and washing the cells twice by repeated resuspension in buffer followed by centrifugation. The cells were then resuspended in 0.5 ml of buffer. Alkene monooxygenase activity was assayed over a 60-min time course as described above with resuspended cells (0.35 mg of protein) and propylene (2 μmol). Propylene oxide-dependent O_2 uptake of resuspended cells was measured in the O_2 electrode. Cells (0.58 mg of protein) and buffer (1.9 ml) were preequilibrated in the electrode chamber for 10 min, and the rate of O_2 uptake supported by endogenous reductant was recorded. Propylene oxide (3.8 μl of a 500 mM stock solution; 1 mM final concentration) was then injected, and the rate of O_2 uptake was recorded until all O_2 had been depleted. The rates of propylene oxide-dependent O_2 uptake were obtained by subtracting the endogenous O_2 uptake rates from the rates obtained after propylene oxide addition.

Incubation vials designed to determine the effect of enzyme turnover on toxicity were prepared as described above with the following exceptions. Assay vials containing cells, buffer, and glucose were sealed with butyl rubber stoppers. Anaerobic vials were prepared by repeatedly evacuating the vials on a vacuum manifold followed by flushing with N_2. 1,1-Dichloroethylene, prepared as a saturated solution in O_2-free, N_2-saturated buffer, was then added to initiate assays. After incubation for 60 min with shaking at 30°C, the cells were washed and resuspended as described above. Control cells were incubated under the same conditions but in the presence of O_2.

RESULTS

Degradation of chlorinated alkenes by *Xanthobacter* strain Py2. The time courses for the degradation of vinyl chloride, trichloroethylene, and 1,3-dichloropropylene by resting-cell suspensions of propylene-grown *Xanthobacter* strain Py2 are shown in Fig. 1. With 500 nmol of chlorinated alkenes the initial rates were 33 (vinyl chloride), 27 (1,3-dichloropropylene), and 8.6 (trichloroethylene) nmol of substrate degraded per min per mg of cell protein. These rates are comparable to the rates observed when the cells were assayed with 500 nmol of propylene (92 nmol/min/mg) or ethylene (26 nmol/min/mg) as a substrate. Vinyl chloride and 1,3-dichloropropylene were completely degraded within the time frame of the experiment shown in Fig. 1, while 60% of the trichloroethylene was degraded.

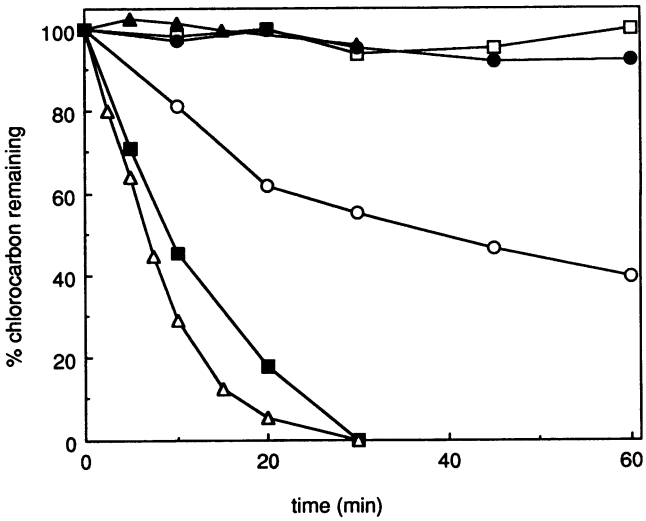

FIG. 1. Time course of chlorinated alkene degradations catalyzed by *Xanthobacter* strain Py2. Cell suspensions (1.1 mg of protein) were incubated with chlorinated alkenes (500 nmol) as described in Materials and Methods. Datum points represent the averages of duplicate measurements. Symbols: □, boiled cells plus 1,3-dichloropropylene; ■, active cells plus 1,3-dichloropropylene; ▲, boiled cells plus vinyl chloride; △, active cells plus vinyl chloride; ●, boiled cells plus trichloroethylene; ○, active cells plus trichloroethylene.

Requirements for the degradation of chlorinated alkenes. The ability of propylene-grown cells to degrade chlorinated alkenes at rates comparable to the rates of propylene and ethylene oxidation suggests that the degradations are catalyzed by alkene monooxygenase. Two methods were applied to firmly establish the role of alkene monooxygenase in catalyzing chlorinated alkene oxidations. First, cells were grown in the absence of propylene with glucose as a carbon and energy source in order to potentially repress the enzymes involved in alkene utilization. Second, an inhibitor of alkene monooxygenase was identified and used to selectively inhibit the alkene monooxygenase of propylene-grown cells. Acetylene has been characterized as a suicide substrate and irreversible inactivator of ammonia monooxygenase (18) and methane monooxygenase (25) and has recently been shown to be a potent inactivator of alkene monooxygenase in the ethylene-utilizing *Mycobacterium* strain E3 (10). Acetylene is, however, reported to be a poor inhibitor of the alkene monooxygenase of *Xanthobacter* strain Py2 (30). Since propylene is a better substrate for the *Xanthobacter* alkene monooxygenase than ethylene, by analogy we considered propyne a potential inhibitor. Propylene-grown cells were incubated for 60 min with 5 μmol of propylene in either the presence or absence of 110 μmol propyne and then washed and resuspended in propyne-free buffer. These cells and cells grown with glucose as a carbon source were then assayed for their ability to metabolize propylene and propylene oxide. The propylene-oxidizing activity of cells pretreated with propyne was dramatically decreased relative to

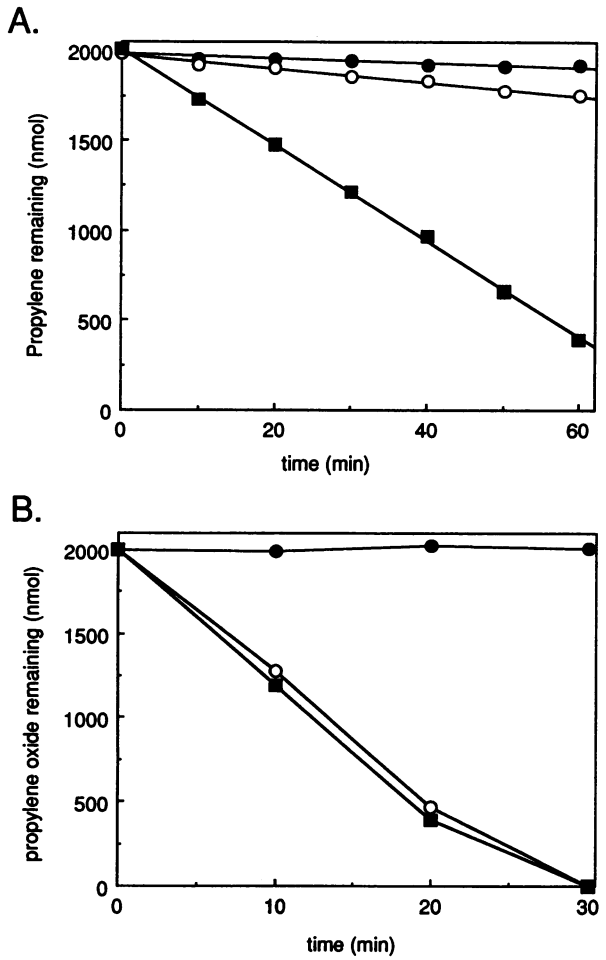

FIG. 2. Effect of propyne treatment of *Xanthobacter* cells on propylene oxidation and propylene oxide utilization. Datum points represent the averages of duplicate measurements. (A) Propylene oxidation. Symbols: ■, propylene-grown cells (0.48 mg of protein); ○, propylene-grown, propyne-treated cells (1.2 mg of protein); ●, glucose-grown cells (1.3 mg of protein). (B) Propylene oxide utilization. Symbols: ■, propylene-grown cells (1.2 mg of protein); ○, propylene-grown, propyne-treated cells (1.2 mg of protein); ●, glucose-grown cells (0.90 mg of protein).

that of control cells incubated without propyne (Fig. 2A). The specific activity of the propyne-treated cells, when compensated for propylene losses in buffer-only incubation vials over the same time course, was 4% of the activity of the control cells. No recovery of propylene-oxidizing activity was observed over the 60-min time course of Fig. 2A, demonstrating that propyne acts essentially as an irreversible inactivator. However, the ability of propyne-treated cells to metabolize propylene oxide, the product of propylene oxidation, was not affected, demonstrating that propyne inhibition is specific for the alkene monooxygenase (Fig. 2B). The specificity of propyne as an inhibitor of alkene monooxygenase was further confirmed by demonstrating that propyne-treated cells were able to consume O_2 with glucose or propylene oxide as a reductant at the same rate as cells not treated with propyne (data not shown). Cells grown with glucose as the carbon source were unable to metabolize either propylene or propylene oxide (Fig. 2), confirming that the enzymes of alkene utilization are repressed.

TABLE 1. Degradation of chlorinated C_2 and C_3 alkenes by *Xanthobacter* strain Py2 containing an active alkene monooxygenase[a]

Substrate	% Chlorocarbon remaining after 30-min incubation with the following cell suspensions:			
	Propylene grown			Glucose grown
	Active	Boiled	Propyne treated	
1,3-Dichloropropene	0	98	100	95
2,3-Dichloropropene	29	100	97	100
Tetrachloroethylene	99	100	98	97
Trichloroethylene	54	98	100	98
cis-1,2-Dichloroethylene	31	99	99	93
trans-1,2-Dichloroethylene	16	98	97	94
1,1-Dichloroethylene	94	104	98	100
Vinyl chloride	0	96	100	98

[a] Chlorocarbon degradation assays (500 nmol of chlorocarbon per assay) were conducted as described in Materials and Methods with propylene-grown suspension (1.1 mg of protein) or glucose-grown suspension (1.3 mg of protein). Values represent the averages of triplicate measurements. Standard errors for replicates were <5%.

Eight chlorinated alkenes were tested as substrates for propylene-grown, propyne-inhibited, and glucose-grown cells. Six of these compounds, 1,3-dichloropropylene, 2,3-dichloropropylene, trichloroethylene, *cis*- and *trans*-1,2-dichloroethylene, and vinyl chloride, were readily degraded by propylene-grown cells (Table 1). Tetrachloroethylene was not degraded, and 1,1-dichloroethylene was only slightly degraded. None of the chlorinated alkenes were degraded by glucose-grown cells or propyne-inhibited cells, confirming the role of alkene monooxygenase in catalyzing the degradations. Chlorinated alkanes, including 1,3-dichloropropane, 1,2-dichloropropane, 1,1,2-trichloroethane, 1,2-dichloroethane, and chloroethane, were not degraded by active-cell suspensions.

Inhibition of propylene and ethylene oxidation by chlorinated alkenes and alkanes. The eight chlorinated alkenes tested as substrates in Table 1, along with the corresponding saturated alkane analogs, were investigated as potential inhibitors of propylene and ethylene oxidation. Propylene-grown cells were incubated with 500 nmol of propylene or ethylene in the presence and absence of 500 nmol of the chlorinated hydrocarbons, and the depletion of propylene and ethylene was monitored by gas chromatography over a 30-min time course. The progress curves for alkene oxidation in the presence of the chlorinated hydrocarbons remained largely linear over this time course, but the rates of alkene oxidation were in many cases dramatically decreased. Table 2 quantitates the inhibition of propylene and ethylene oxidation exerted by the chlorinated alkenes and alkanes. The inhibitions were more dramatic versus ethylene oxidation than versus propylene oxidation, a result that most likely reflects the fact that ethylene is a poorer substrate for alkene monooxygenase and also the slightly decreased solubility of ethylene in aqueous solution relative to that of propylene. The six chlorinated alkenes shown to be good substrates for alkene monooxygenase inhibited alkene oxidation to various degrees, with the chlorinated propylenes being the most inhibitory. 1,1-Dichloroethylene, which is a poor substrate, was nevertheless a potent inhibitor of alkene oxidation. Tetrachloroethylene, although not a substrate for alkene monooxygenase, was an inhibitor, as were many of the chlorinated alkanes.

TABLE 2. Inhibition of alkene monooxygenase by chlorinated alkenes and alkanes[a]

Chlorocarbon	Estimated concn of dissolved chlorocarbon (μM)[b]	% Activity with[c]:	
		Propylene	Ethylene
None		100	100
1,3-Dichloropropene	330	9.3	1.8
2,3-Dichloropropene	260	29	2.0
Tetrachloroethylene	70	90	62
Trichloroethylene	78	88	50
cis-1,2-Dichloroethylene	170	43	7.2
trans-1,2-Dichloroethylene	90	96	64
1,1-Dichloroethylene	31	15	0
Vinyl chloride	25	54	17
1,3-Dichloropropane	ND	84	38
1,2-Dichloropropane	250	87	67
1,1,2,2-Tetrachloroethane	ND	18	19
1,1,2-Trichloroethane	ND	88	58
1,2-Dichloroethane	330	100	62
1,1-Dichloroethane	140	93	85
Chloroethane	71	98	94

[a] Rates of propylene and ethylene oxidation are expressed as percent activity relative to that in assays conducted in the absence of chlorocarbon and represent the averages of duplicate measurements. The correlation coefficients (r^2) for the progress curves of alkene concentration versus time were greater than 0.990.

[b] The dissolved concentrations of chlorocarbons were estimated experimentally by measuring the partitioning of chlorocarbons between the gas and liquid phases by gas-phase and liquid-phase injections on the FID gas chromatograph. ND, not determined.

[c] The dissolved concentrations of propylene and ethylene were estimated to be 8.4 and 5.9 μM, respectively, by using published Bunsen coefficients for the gases as described in Materials and Methods.

TABLE 3. Effect of cometabolites on the extent of 1,3-dichloropropylene oxidation by *Xanthobacter* cell suspensions

Cosubstrate	O_2 uptake rate (nmol of O_2 consumed min^{-1} mg^{-1})	1,3-Dichloropropene degraded (nmol in 60 min)
None	10.3	180
Propylene[a]	64.0	
0.05 μmol		230
0.5 μmol		160
2.5 μmol		85
Propylene oxide	63.4	400
Propionaldehyde	80.0	370
Propylene glycol	10.3	160
1-Propanol	36.8	190
Glucose	31.9	320

[a] O_2 uptake was measured in the presence of a single concentration (0.9 mM in the electrode chamber) of propylene. 1,3-Dichloropropylene degradation was measured in the presence of 0.05, 0.5, or 2.5 μmol of propylene; these amounts correspond to 0.94, 9.4, and 47 μM propylene in the aqueous phase, respectively.

Stimulation of chlorinated alkene degradation. The oxidation of chlorinated alkenes described in Fig. 1 and Table 1 was performed in the absence of an added reductant and was therefore dependent upon endogenous reductant pools initiating and sustaining the assays. In order to identify metabolites which can stimulate the oxidation of chlorinated alkenes, we considered both substrates and intermediates of the probable propylene oxidation pathway as well as alternate carbon sources not involved in alkene utilization. The immediate product of propylene oxidation is propylene oxide, but the identity of the product of propylene oxide metabolism has not been established (8). Two likely pathways could be utilized for the further metabolism of propylene oxide: hydration to propylene glycol or isomerization to propionaldehyde. Two lines of evidence suggest that propionaldehyde is an actual intermediate of propylene oxide oxidation. First, when the utilization of propylene oxide by cell suspensions was assayed by gas chromatography (Fig. 2), a transient peak eluting with a retention time identical to that of propionaldehyde appeared as the propylene oxide peak disappeared (data not shown). Second, propionaldehyde but not propylene glycol stimulated O_2 uptake by propylene-grown cell suspensions (Table 3).

The endogenous rate of O_2 uptake in resting cells was stimulated from three- to eightfold by the addition of glucose, 1-propanol, propylene, propylene oxide, and propionaldehyde (Table 3). Glucose, propylene oxide, and propionaldehyde also stimulated the extent of 1,3-dichloropropylene degradation (Table 3), demonstrating that the oxidation of these metabolites generates a reductant capable of donating electrons to alkene monooxygenase. Propylene, when present at a low concentration, stimulated the extent of 1,3-dichloropropylene degradation slightly but at higher concentrations became inhibitory, probably by competing with 1,3-dichloropropylene for binding to alkene monooxygenase. Surprisingly, propanol did not stimulate 1,3-dichloropropylene degradation despite stimulating O_2 uptake. Apparently the oxidation of propanol generates a reductant incapable of donating electrons to alkene monooxygenase. Propylene glycol, which did not stimulate O_2 uptake, also had no effect on the degradation of 1,3-dichloropropylene.

Effect of chlorinated alkene metabolism on propylene oxidation and propylene oxide-dependent O_2 uptake. In order to assess potential toxic effects associated with chlorinated alkene oxidations, cells were incubated with chlorinated alkenes for 60 min with glucose added as a cometabolite and were then washed to remove any remaining chlorinated alkenes or water-soluble oxidation products. These incubations were conducted with the same ratio of cell suspension to chlorinated alkene as the experiments shown in Fig. 1 and Table 1. The rates of propylene oxidation and propylene oxide-dependent O_2 uptake catalyzed by the resuspended cells were used as criteria for quantitating the toxic effects of the chlorinated alkenes. Cells incubated in the presence of chlorinated alkenes which are substrates for alkene monooxygenase had decreased propylene oxidation and propylene oxide-dependent O_2 uptake activities (Table 4). These toxic effects were most dramatically manifest with 1,1-dichloroethylene, where virtually all of the alkene monooxygenase activity and 80% of propylene oxide-dependent O_2 uptake activity were lost. Tetrachloroethylene, which is not oxidized by cells but is an inhibitor of alkene oxidation, had no effect on either activity, suggesting that toxicity may be mediated by an oxidation product(s) and not the chlorinated alkenes themselves. This hypothesis was tested by incubating cells with 1,1-dichloroethylene as described above but in the absence of O_2 to prevent enzyme turnover. After a 45-min incubation the cells were washed and resuspended in fresh buffer. As shown in Fig. 3, the cells incubated in the absence of O_2 were fully competent to catalyze propylene oxidation, while cells incubated under the same conditions in the presence of O_2 were largely inactive. The cells incubated anaerobically were also fully competent to catalyze propylene oxide-dependent O_2 uptake (data not shown). These results support the idea that a product of chlorinated alkene oxidation mediates toxicity. In addition, these results sug-

TABLE 4. Effect of chlorocarbon degradation on propylene oxidation and propylene oxide-dependent O_2 uptake[a]

Chlorocarbon	% Activity after incubation with chlorocarbon	
	Alkene monooxygenase[b]	Propylene oxide-dependent O_2 uptake[c]
None	100	100
1,3-Dichloropropene	57	26
2,3-Dichloropropene	52	28
Tetrachloroethylene	106	105
Trichloroethylene	31	68
cis-1,2-Dichloroethylene	81	66
trans-1,2-Dichloroethylene	85	98
1,1-Dichloroethylene	1.1	21
Vinyl chloride	69	110

[a] The preincubation of cells with chlorocarbons and glucose and the determination of alkene monooxygenase and O_2 uptake activities are described in Materials and Methods.

[b] Rates of propylene oxidation (determined over a 60-min time course) are expressed as percent activity relative to that in cell suspensions which had been incubated in the absence of chlorocarbon and represent the averages of duplicate measurements.

[c] The rates of propylene oxide-dependent O_2 uptake are expressed as percent activity relative to that in cell suspensions which had been incubated in the absence of chlorocarbon.

gest that the low extent of 1,1-dichloroethylene degradation relative to the other chlorinated alkenes (Table 1) is not a function of 1,1-dichloroethylene being a poor substrate for alkene monooxygenase but rather that it acts as a potent mechanism-based inactivator of the monooxygenase and other cellular proteins.

DISCUSSION

In this study eight chlorinated alkenes were tested as substrates for the alkene-oxidizing bacterium *Xanthobacter* strain Py2. Perhaps the most important of these in terms of environmental concern are the widely used industrial solvents trichloroethylene and tetrachloroethylene, which are prevalent groundwater pollutants and health hazards. Under anaerobic conditions trichloroethylene and tetrachloroethylene are biotransformed to dichloroethylenes (*cis*-, *trans*-, and 1,1-dichlorinated isomers) and vinyl chloride (33), which are themselves potential health hazards. The accumulation of vinyl chloride as a trichloroethylene and tetrachloroethylene transformation intermediate is of particular concern, since it is an especially potent mutagen and carcinogen. In addition to these chlorinated ethylenes we also considered two chlorinated propylenes, 1,3- and 2,3-dichloropropylene, as potential substrates. 1,3-Dichloropropylene, shown to be a mutagen (20), is widely used as a soil fumigant for agricultural pest control. 2,3-Dichloropropylene, which is a minor component (~5%) of the commercial 1,3-dichloropropylene fumigant preparation Telone, is also mutagenic (20).

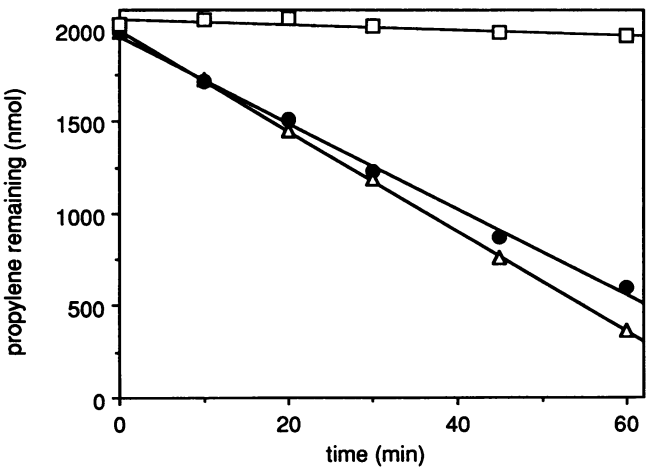

FIG. 3. Turnover dependence of 1,1-dichloroethylene toxicity. Cells (0.43 mg of protein) were treated with 1,1-dichloroethylene aerobically or anaerobically as described in Materials and Methods. Datum points represent the averages of duplicate measurements. Symbols: △, aerobically incubated cells without glucose or 1,1-dichloroethylene; □, aerobically incubated cells with glucose and 1,1-dichloroethylene; ●, anaerobically incubated cells with glucose and 1,1-dichloroethylene.

As shown in Fig. 1 and Table 1, whole-cell suspensions of propylene-grown bacteria efficiently removed six of the chlorinated alkenes discussed above. The role of alkene monooxygenase in the degradations was firmly established by showing that cells grown with glucose as an alternative carbon source and propylene-grown cells in which alkene monooxygenase was selectively inactivated with propyne were unable to catalyze the degradations. Similar methods have been applied to demonstrate the catalytic roles of other bacterial monooxygenases in catalyzing chlorinated hydrocarbon degradations without necessitating the fractionation and purification of the active cellular components and reconstitution of activity in vitro. For example, specific inhibitors of ammonia monooxygenase (acetylene and Cu chelators) have been used to show that ammonia monooxygenase catalyzes halogenated hydrocarbon oxidations in *Nitrosomonas europaea* cell suspensions (2, 26, 27, 32). The role of toluene dioxygenase from *Pseudomonas putida* F1 in catalyzing trichloroethylene degradation was established by showing that toluene dioxygenase-deficient mutant strains were unable to oxidize trichloroethylene and was further confirmed by cloning the toluene dioxygenase genes into *Escherichia coli*, thereby conferring trichloroethylene-degrading ability (35, 42). The role of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b in catalyzing chlorinated hydrocarbon degradations was established by correlating the biodegradation of trichloroethylene with the expression of soluble methane monooxygenase (24, 29). Purified soluble methane monooxygenase was subsequently shown to catalyze the oxidation of chlorinated alkenes, confirming its role in in vivo degradations (5).

Alkene monooxygenase in *Xanthobacter* strain Py2 oxidizes the same range of chlorinated ethylenes as ammonia monooxygenase and soluble methane monooxygenase, the two oxygenases which have been studied in the most detail with respect to alternative halogenated hydrocarbon substrates. Like other oxygenases capable of oxidizing chlorinated alkenes, *Xanthobacter* alkene monooxygenase is unable to degrade tetrachloroethylene. However, one marked difference between alkene monooxygenase and ammonia and methane monooxygenases is the unreactivity of the former towards saturated hydrocarbons. *N. europaea* ammonia monooxygenase is able to oxidize a variety of nonphysiological compounds, including alkanes (13, 15), alkenes (13, 15, 17), aromatics (14), and halogenated alkanes and alkenes (2, 26–28, 32). Soluble methane monooxygenase exhibits a similar broad substrate specificity for these compounds (3, 23, 24). Alkene monooxygenase from *Xanthobacter* strain Py2, in contrast, does not oxygenate alkanes (30)

and, as shown in this study, is also unreactive towards chlorinated alkanes. The inhibition of ethylene and propylene oxidation by chlorinated alkanes suggests, however, that they compete with alkenes for binding to the enzyme (Table 2). The differences in the substrate specificities of ammonia, methane, and alkene monooxygenases apparently reflect the differing physiological roles of the enzymes: ammonia and methane monooxygenases are intended to catalyze the hydroxylation of ammonia to hydroxylamine and methane to methanol, respectively, while alkene monooxygenase of *Xanthobacter* strain Py2 is designed to catalyze the epoxidation of alkenes. Alkene monooxygenase is thus a more specific enzyme and appears to be limited to catalyzing epoxidation reactions with olefinic substrates structurally related to its physiological substrates. Ammonia and methane monooxygenases, in contrast, are able to catalyze both hydroxylation and epoxidation reactions with a wide variety of structurally unrelated compounds.

Several metabolites of *Xanthobacter* strain Py2 stimulated the oxidation of chlorinated alkenes, presumably by increasing the amount of reductant available for alkene monooxygenase to catalyze substrate epoxidations. An attractive feature of *Xanthobacter* strain Py2 is its metabolic versatility, allowing it to use metabolites such as glucose, which are not involved as substrates or intermediates of propylene oxidation, as reductants for chlorinated alkene oxidation. This contrasts with bacteria which are restricted to using the physiological substrate of the monooxygenase which catalyzes the alternate substrate oxidations as a source of reductant. For example, the nitrifying bacterium *N. europaea*, an obligate autotroph which does not utilize carbon sources other than CO_2, is dependent upon the addition of the physiological monooxygenase substrate ammonia to sustain chlorinated hydrocarbon oxidations over an extended time period (2, 26, 27, 32). Since ammonia and the chlorinated hydrocarbon are competitive substrates for ammonia monooxygenase, the rate of oxidation of each substrate will be affected by the relative concentration of the other. A similar problem exists in the methane-oxidizing bacteria but can be partially circumvented by adding formate, an intermediate of methane oxidation, to stimulate the degradation of chlorinated hydrocarbons (1, 24).

One of the greatest obstacles to applying monooxygenase-containing bacteria for the degradation of chlorinated alkenes is toxicity resulting from the formation of reactive epoxide oxidation products which are potent alkylating agents. Trichloroethylene oxidation in toluene-, methane-, and ammonia-oxidizing bacteria has been shown to result in the covalent modification of a variety of cellular proteins, including the oxygenase catalyzing trichloroethylene oxidation (23, 28, 36). This covalent modification is accompanied by an irreversible loss of oxygenase activity and requires the de novo synthesis of new oxygenase proteins to restore activity (23, 28, 36). Since alkene-utilizing bacteria are designed to further metabolize the epoxide products of alkene oxidations, it can be envisioned that they might enzymatically metabolize and deactivate chlorinated epoxides before they are able to mediate the inactivation of cellular proteins. A precedent exists in *Mycobacterium* strain L1, which is able to grow with vinyl chloride as the sole carbon and energy source, implying that it is able to derive energy from the metabolism of vinyl chloride epoxide (9). The ability of alkene-utilizing bacteria to metabolize epoxides generated by trichloroethylene and 1,1-dichloroethylene oxidation, however, may be limited by how rapidly the epoxides react to alkylate cellular proteins or abiotically decompose to form other potentially toxic compounds (11).

As an initial step in determining the potential of *Xanthobacter* strain Py2 to deal with chlorinated alkene oxidation products, we examined the effect of chlorinated alkene oxidation on alkene monooxygenase activity and propylene oxide-dependent O_2 uptake activity under the conditions used for the degradations described in the legend to Fig. 1 and the footnote of Table 1. As shown in Table 4 and Fig. 3, a turnover-dependent partial loss of the activities was seen, with the magnitude of activity loss differing for the various chlorinated alkenes. Interestingly, incubation of cells with the chlorinated propylenes resulted in a greater loss of propylene oxide-dependent O_2 uptake activity relative to propylene oxidation activity, whereas with the chlorinated ethylenes the opposite result was generally seen. This result suggests that the propylene oxide-metabolizing enzyme(s) may be more sensitive to chlorinated propylene epoxide products than chlorinated ethylene epoxides.

It is apparent from the data presented in Table 4 that *Xanthobacter* strain Py2 is subject to some of the same toxicity problems as those described for toluene-, methane-, and ammonia-degrading bacteria. It will require further experiments to determine how *Xanthobacter* cells will deal with chlorinated alkenes under physiological conditions and in the presence of the growth substrate propylene. Isoprene-utilizing bacteria which oxidize trichloroethylene have recently been shown to tolerate the presence of relatively high concentrations of trichloroethylene during growth, leading to the conclusion that the bacteria have an increased resistance to trichloroethylene inactivation (4). However, under the conditions of those experiments little oxidation of trichloroethylene occurred until the isoprene in the growth media had been consumed and the cells had ceased growing (4). As a more rigorous criterion for measuring the toxic effects of trichloroethylene and other chlorinated alkenes, we have quantitated propylene oxidation and propylene oxide-dependent O_2 uptake activity following the incubation of cells with chlorinated alkenes under turnover conditions. The incubations were performed in the absence of propylene and with glucose as a noncompetitive cometabolite to ensure that propylene would not inhibit chlorinated alkene oxidation and hence protect against inactivation.

In summary, in this study we have defined several chlorinated alkenes of environmental concern as substrates for alkene monooxygenase in propylene-grown *Xanthobacter* cells, examined the inhibition of alkene oxidation exerted by these alternate substrates, identified suitable cometabolites for sustaining chlorinated alkene oxidations, and examined the toxicity resulting from the oxidations. On the basis of the results of previous studies with alkene-utilizing bacteria (7, 31, 37), it is likely that the immediate products of chlorinated alkene oxidation in *Xanthobacter* strain Py2 are the corresponding chlorinated epoxides. The ultimate fate of the carbon and chlorine atoms of chlorinated alkene oxidations and the possibility that chlorinated epoxides are substrates for an epoxide-utilizing enzyme(s) of propylene-grown *Xanthobacter* cells are questions that have not yet been addressed. The answers to these questions will further aid in determining the feasibility of applying alkene-utilizing bacteria to the remediation of groundwater and soil contaminated with trichloroethylene and other hazardous chlorinated alkenes.

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