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Chloroform Cometabolism by Butane-Grown CF8, *Pseudomonas butanovora*, and *Mycobacterium vaccae* JOB5 and Methane-Grown *Methylosinus trichosporium* OB3b

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Chloroform (CF) degradation by a butane-grown enrichment culture, CF8, was compared to that by butane-grown *Pseudomonas butanovora* and *Mycobacterium vaccae* JOB5 and to that by a known CF degrader, *Methylosinus trichosporium* OB3b. All three butane-grown bacteria were able to degrade CF at rates comparable to that of *M. trichosporium*. CF degradation by all four bacteria required O_2. Butane inhibited CF degradation by the butane-grown bacteria, suggesting that butane monooxygenase is responsible for CF degradation. *P. butanovora* required exogenous reductant to degrade CF, while CF8 and *M. vaccae* utilized endogenous reductants. Prolonged incubation with CF resulted in decreased CF degradation. CF8 and *P. butanovora* were more sensitive to CF than either *M. trichosporium* or *M. vaccae*. CF degradation by all three butane-grown bacteria was inactivated by acetylene, which is a mechanism-based inhibitor for several monooxygenases. Butane protected all three butane-grown bacteria from inactivation by acetylene, which indicates that the same monooxygenase is responsible for both CF and butane oxidation. CF8 and *P. butanovora* were able to degrade other chlorinated hydrocarbons, including trichloroethylene, 1,2-*cis*-dichloroethylene, and vinyl chloride. In addition, CF8 degraded 1,1,2-trichloroethane. The results indicate the potential of butane-grown bacteria for chlorinated hydrocarbon transformation.

Chlorinated aliphatic hydrocarbons (CAHs) cause serious environmental problems through contamination of ground water, drinking water, and soil. In situ bioremediation of CAHs by aerobic cometabolism is a promising method for remediating contaminated sites. Degradation of target compounds occurs because of their fortuitous oxidation by enzymes which function physiologically to initiate the oxidation of growth substrates. Methane (5, 12, 13, 23, 26), propane (29), ammonia (18, 27), and toluene (or phenol) (6, 11, 30) are examples of growth substrates which also support the cometabolism of several CAHs. Trichloroethylene (TCE) is an example of a CAH which has received considerable attention and which is cometabolically degraded by a number of bacteria (5, 12, 13, 18, 23, 26, 27, 29, 30). Chloroform (CF), in contrast, is more recalcitrant. CF is used as an industrial solvent and can be produced in drinking water as a result of chlorination. Because of its toxicity, the presence of CF in drinking water is regulated under the Safe Drinking Water Act Amendments of 1986 (20).

Although CF has been recognized as a recalcitrant compound, some bacteria have been shown to dehalogenate it through aerobic cometabolic processes. The methane-utilizing bacterium *Methylosinus trichosporium* OB3b cometabolizes a number of CAHs, including CF and TCE (5, 12, 13, 23, 26). *M. trichosporium* OB3b synthesizes two types of methane monooxygenases (MMO): a particulate enzyme (pMMO) associated with the extensive internal membrane system and a soluble enzyme (sMMO). sMMO is expressed under conditions of copper limitation (24). Both types of enzymes catalyze dehalogenation (7). However, sMMO has a broader substrate specificity and much greater activity towards TCE (12, 26). Speitel et al. (23) studied the kinetics of CF and TCE degradation by *M. trichosporium* OB3b at initial concentrations of around 0.1 mg/liter for CF and 1 mg/liter for TCE. Their reported pseudo first-order rate constants ranged from 0.2 to 0.4 liter (mg of total suspended solids × day)$^{-1}$ for CF and from 0.5 to 3.3 liters (mg of total suspended solids × day)$^{-1}$ for TCE in the absence of methane. The major degradation product from CF was CO_2. Oldenhuis et al. (12) examined the kinetics of the degradation of TCE and other CAHs by *M. trichosporium* OB3b. Compounds that were readily degraded included CF, *trans*-1,1-dichloroethylene, and TCE, with V_{max} values of 550, 330, and 290 nmol min^{-1} mg of cells^{-1}, respectively. 1,1-Dichloroethylene was a very poor substrate. The ammonia-oxidizing bacterium *Nitrosomonas europaea* can degrade CF and other CAHs (18, 27). Rasche et al. (19) proposed that halogenated hydrocarbons fall into one of three classes based on their biodegradabilities and inactivating potentials: (i) compounds which were not biodegradable by *N. europaea* and which had no toxic effect on the cells; (ii) compounds which were cooxidized by *N. europaea* and had little or no toxic effect on the cells; and (iii) compounds which were cooxidized and produced a turnover-dependent inactivation of ammonia oxidation by *N. europaea*. Both CF and TCE were class III compounds. Rasche et al. (19) suggested that ammonia monooxygenase (AMO) catalyzed the dehalogenation. Recently, McClay et al. (11) reported CF degradation ability by seven toluene-oxidizing bacterial strains. Three *Pseudomonas* strains, *P. mendocina* KR1 and *Pseudomonas* sp. strains ENVPC5 and ENVBF1, mineralized CF to CO_2. The highest rate of CF oxidation was achieved with *Pseudomonas* sp. strain ENVBF1 (1.9 nmol [min × mg of cell protein]$^{-1}$). CF was also oxidized by

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P. mendocina KR1 (0.48 nmol [min × mg of cell protein]$^{-1}$) and strain ENVPC5 (0.49 nmol [min × mg of cell protein]$^{-1}$). CF oxidation by each strain was inhibited by TCE, and acetylene substantially inhibited TCE oxidation by *P. mendocina* KR1.

Kim (10) showed that butane and propane were effective cometabolic substrates to drive the transformation of CF. The study was performed with mixed cultures in microcosms enriched from aquifer solids from the Hanford DOE site in Washington. The transformation yields, representing the amount of CF transformed in response to the utilization of the growth substrate, were 0.01 mg of CF/mg of substrate for butane and propane utilizers. Potential advantages of butane or propane as growth substrates are that they are highly soluble in water, inexpensive, and readily available. From one of these microcosms, we obtained a highly enriched culture, CF8, which grows on butane. The predominant cells in CF8 cultures are coryneform and pleomorphic, typically show aggregation of several cells, and stain gram-positive. In this work, we compare the CF-degrading ability of CF8 cultures with those of methane-grown copper-limited cultures of *M. trichosporium* OB3b and butane-grown cultures of *Mycobacterium vaccae* JOB5 and *Pseudomonas butanovora*. Wackett et al. (29) reported that propane-grown *M. vaccae* JOB5 cooxidized a number of CAHs, including TCE. CF was not studied. Because *M. vaccae* is also capable of growth on butane (15), we included *M. vaccae* in our study of CF degradation by butane-grown bacteria. *P. butanovora* was isolated for its ability to grow on butane, although it can also grow on a variety of alkanes (C_2 to C_9) and alcohols (C_2 to C_4) but not alkenes or sugars (25). CAH degradation by *P. butanovora* has not been reported. In this work, we show that cultures of all four bacteria degrade CF. We also show that CF8 and *P. butanovora* can degrade other chlorinated ethanes and ethenes. This is the first report of CAH degradation by pure cultures of butane-grown bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. trichosporium* OB3b (ATCC 35070) was cultured at 30°C in a nitrate mineral salts medium (ATCC medium 1306) and grown under copper-limited conditions. Cultures were grown in sealed vials (150 ml) containing 50 ml of liquid medium and 100 ml of air, with 50 ml of methane gas added as an overpressure. Methane gas (99.99% purity) was purchased from Airco Gases, Murray Hill, N.J. The medium was buffered with phosphate (pH 6.8; 2 mM KH_2PO_4 and 2 mM $Na_2HPO_4 \cdot 7H_2O$). *P. butanovora* (ATCC 43655) was cultured in 150-ml sealed vials (50 ml of medium), with 7 ml of *n*-butane and 5 ml of CO_2 added as an overpressure. *n*-Butane gas (99.0%) was purchased from Airgas, Inc., Randor, Pa. The growth medium (50 ml) consisted of 2 mM $MgSO_4 \cdot 7H_2O$, 400 μM $CaCl_2 \cdot 2H_2O$, 0.01% (wt/vol) yeast extract, and the trace elements described previously (31). The medium was buffered with phosphate [pH 7.1; 60 mM $(NH_4)_2HPO_4$, 7 mM $Na_2HPO_4 \cdot 7H_2O$, and 15 mM KH_2PO_4]. *M. vaccae* JOB5 (ATCC 29678) was grown in the *Xanthobacter* Py2 medium described previously (31) except that NH_4NO_3 replaced $NaNO_3$, the yeast extract was removed, and the pH was adjusted to 7.5. Cultures (50 ml of medium) were grown in 150-ml sealed vials with 50 ml of *n*-butane and 40 ml of O_2 added as an overpressure. CF8 was enriched from Hanford core material by growth on *n*-butane as the only reduced carbon source. CF8 was grown in the same media as that for *M. vaccae*, with 50 ml of *n*-butane added as an overpressure. Cell growth was monitored by removing a portion of the cultures and measuring optical density at 600 nm (OD_{600}). When cells were harvested, typical OD values for *M. trichosporium* OB3b, *P. butanovora*, CF8, and *M. vaccae* were 0.21, 0.60, 0.56, and 1.13, respectively.

Chloroform degradation assay. Cells were harvested from cultures by centrifugation (6,000 × *g* for 10 min), washed twice with the same buffer as in the growth medium, and then resuspended to a constant cell density (based on OD). Assays were conducted in 10-ml serum vials sealed with Teflon-coated butyl rubber stoppers (Alltech Associates, Inc., Deerfield, Ill.). Chloroform was added as a diluted aqueous solution which was made fresh daily from a chloroform-saturated solution at room temperature (approximately 20°C). The amount of CF to be added was estimated from solubility tables (21). The concentrations of CF in the liquid phase were calculated from Henry's law constants (9). The reaction mixtures (800 μl) containing the same phosphate buffer as in the growth medium and CF solution were equilibrated at 30°C, with constant shaking for at least 30 min before the assay was started. The reactions were initiated by the addition of 200 μl of concentrated cell suspensions (approximately 0.35, 0.34, 0.71, and 0.29 mg of protein in *M. trichosporium*, CF8, *P. butanovora*, and *M. vaccae*, respectively). For time course assays, a sample (20 μl) of the gas phase was removed for analysis of CF by gas chromatography. The Shimadzu GC-8A gas chromatograph was equipped with an electron capture detector and a stainless steel column (0.25 by 20 in.) packed with Porapak Q (Alltech Associates, Inc.) and was run at a column temperature of 135°C and a detector temperature of 230°C. Experiments were repeated at least three times. Data for each figure were from a typical experiment.

To determine if O_2 was required for CF degradation, the reaction vials were made anaerobic by purging with N_2 gas for 5 min prior to the addition of the O_2-free CF solution. Sodium-dithionite solution (100 μl of an approximately 10 mM solution) was added to a diluted CF stock solution (10 ml) to remove the dissolved O_2. The small amount of dithionite transferred to the reaction mixture was not toxic to the cells. The reaction mixtures included 5 mM formate or 5 mM butyrate as an electron donor.

Effects of substrates on CF degradation. Various amounts of growth substrates (methane for *M. trichosporium* and butane for CF8, *P. butanovora*, and *M. vaccae*) were added into the reaction vials containing 34.5 nmol of CF. Percentages of gas volume to total vial volume (10 ml) are shown in Table 2. The concentrations of methane and butane in the liquid phase were calculated from Bunsen coefficients (22) at 30°C. One, 5, and 25% (vol/total vol) of methane correspond to liquid-phase concentrations of 6.6, 33, and 160 μM, respectively, and 0.01, 0.1, 1, and 5% (vol/total vol) of butane correspond to 0.1, 1, 10, and 50 μM, respectively.

Chloroform toxicity. To determine if CF degrading ability was lost during CF degradation, cells were allowed to degrade CF (69 nmol) for 60 min, and then additional CF was added to bring the total amount back to 69 nmol. CF degradation was monitored for another 60 min to compare the degradation patterns before and after respiking. The incubation medium initially contained 5 mM electron donor (formate or butyrate).

To determine if butane degrading ability was lost during CF degradation, butane-dependent O_2 uptake was measured for *P. butanovora* and CF8 cells (200 μl) that had been incubated with 69 nmol of CF alone or 69 nmol of CF and 5 mM butyrate for 60 min. As controls, cells were incubated in the absence of CF. Samples of the gas phase were removed at 0 and 60 min to measure CF by gas chromatography. After 60 min of incubation, cells from 1 ml of the assay mixture were harvested and washed twice with phosphate buffer (1 ml) and resuspended in 100 μl of buffer. O_2 uptake rates were measured in the reaction chamber (1.8 ml) of an O_2 electrode (Biological Oxygen Monitor model 5300; Yellow Springs Instrument Company, Incorporated). The chamber was filled with phosphate buffer, and then the 100 μl of washed cell suspension was added. O_2 uptake rates were measured after the addition of the cells to the chamber and again after the addition of butane-saturated solution (120 nmol).

Acetylene inactivation assay. To determine if acetylene inactivated CF degradation, cells were exposed to acetylene prior to the addition of CF. Cell suspensions (200 μl) as described previously were incubated for 10 min in sealed 10-ml vials which contained phosphate buffer, 5 mM butyrate, and 0.1% (vol/total vial vol) acetylene or 0.1% acetylene with 50% butane. For CF8, 0.05% (vol/total vial vol) acetylene was used. Control cells were preincubated in the phosphate buffer and 5 mM butyrate. After preincubation, acetylene and butane were removed from the vials by opening the cap and purging with air for 3 min. The vials were sealed again, and then the reactions were initiated by the addition of 34.5 nmol of CF to the reaction mixture.

Degradation of chlorinated ethenes and ethanes. Degradation of the additional chlorinated hydrocarbons was tested for CF8 and *P. butanovora*. The reaction mixtures (800 μl) contained phosphate buffer and chlorinated aliphatic compounds (amounts used are indicated in Table 4). Assays were initiated by the addition of 200 μl of cell suspensions (described previously), and suspensions were incubated with shaking for the times indicated in the table. Chlorinated aliphatic compounds were quantified by gas chromatography.

Chloride and protein determinations. Chloride released by cells into the assay medium was measured by using a colorimetric assay (3). Protein content was determined by using the biuret assay (8) after the cells were solubilized in 3 N NaOH for 30 min at 65°C. Bovine serum albumin was used as the standard.

RESULTS

Four bacterial cultures, *M. trichosporium* OB3b, *P. butanovora*, CF8, and *M. vaccae* JOB5 were grown on appropriate substrates and tested for CF degradation abilities. All four cultures readily degraded CF. After 60 min, the three cultures grown on butane had consumed from 61 to 97% of the 34.5 nmol of CF initially present (Table 1). Therefore, CF degradation by butane-grown cells is not limited to CF8. The extent of CF degradation was comparable to that of *M. trichosporium* which was previously shown to degrade CF (12, 13, 23). However, time courses revealed that *M. trichosporium* degraded CF

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TABLE 1. CF[a] degradation by methane- and butane-grown organisms[b]

Organism	Growth substrate	Electron donor (5 mM)	nmol of CF degraded in 60 min[c]
M. trichosporium	Methane	Formate	34.1 ± 0.3
CF8	Butane	NR[d]	34.4 ± 1.9
P. butanovora	Butane	Butyrate	21.0 ± 5.0
M. vaccae	Butane	NR	30.0 ± 3.1

[a] The initial amount of CF was 34.5 nmol for each organism.
[b] CF degradation by all four organisms required O_2.
[c] Data are expressed as means ± standard deviations.
[d] NR, not required.

more rapidly than did the three butane-grown bacteria (Fig. 1). These assays were carried out in the absence of the growth substrate to avoid competition between CF and the growth substrate. The assays were carried out in the presence of an appropriate electron donor (5 mM formate for *M. trichosporium* and 5 mM butyrate for CF8, *P. butanovora*, and *M. vaccae*). CF8 and *M. vaccae* exhibited a high rate of endogenous substrate oxidation and, therefore, did not require any exogenous reductant. In the absence of O_2, CF degradation was not detected for any of the bacteria. This result is consistent with the idea that monooxygenases catalyze the degradation of CF.

Monooxygenase reactions require an input of reductant to complete the reduction of O_2. For some bacteria which use a monooxygenase to harvest substrate, the products of the monooxygenase reaction can provide the reductant for the monooxygenase activity. For instance, hydroxylamine (NH_2OH), which is produced from ammonia (NH_3) by AMO, is further oxidized to nitrite to provide the reductant for the monooxygenase reaction (32). The products of methane oxidation were tested for their effects on CF degradation by *M. trichosporium*. The addition of methanol and formaldehyde inhibited CF degradation by *M. trichosporium* (data not shown). *M. trichosporium* could degrade CF without an electron donor. However, the presence of formate enhanced CF degradation in the first 20 min (Fig. 1). Expected intermediates of butane oxidation, butanol, butyraldehyde, and butyrate, were tested for their effects on CF degradation by butane-grown cells. Butanol and butyraldehyde inhibited CF degradation (data not shown), perhaps because of toxicity at high concentrations. However, butyrate enhanced CF degradation by *P. butanovora*. The presence of butyrate did not show any effect on CF degradation by CF8 and *M. vaccae*. For *P. butanovora*, the CF degradation rate in the first 20 min was comparable to those for other bacteria, but the degradation stopped after 40 min of incubation (Fig. 1).

Acetylene is a mechanism-based inhibitor for several monooxygenases, including MMO and AMO (2, 17). Therefore, the effects of acetylene on CF degradation by the three butane-oxidizing bacteria and *M. trichosporium* were tested. When acetylene (0.1% vol/total vial vol) was added to reaction mixtures along with the electron donor, CF, and the cell suspension, the CF degradation by all four bacteria was completely inhibited (Fig. 1). This result further supports the idea that a monooxygenase is responsible for CF degradation in all four bacteria. To distinguish inhibition from inactivation, cells were exposed to acetylene for 10 min, the acetylene was removed by purging with air, and then CF degradation was measured (Fig. 2). As expected, acetylene irreversibly inactivated CF degradation in all three bacteria. Inclusion of butane during preincubation protected the cells from losing their CF degrading ability. In CF8, butane oxidation was also inhibited by acetylene (data not shown). Again, this is consistent with acetylene acting as a mechanism-based inactivator of butane monooxygenase.

In cometabolic processes, the presence of the physiological substrate at sufficiently high concentrations is expected to inhibit the degradation of the cosubstrate. To test the effect of the growth substrate on CF degradation by *M. trichosporium*, *P. butanovora*, CF8, and *M. vaccae*, the cells were incubated with CF in the presence of their growth substrates and an electron donor. The electron donor was included to ensure that reductant depletion did not occur. Three or four substrate concentrations were tested for each bacterium. In all four bacteria, the substrate inhibited CF degradation to some extent (Table 2). Less CF was degraded when more substrate was present. The extent to which the substrate inhibited CF degradation varied among the bacteria. With 1% butane, CF degradation by CF8 was completely inhibited, while 5% butane was required to inhibit CF degradation by *P. butanovora* and *M. vaccae* by more than 90%. In contrast, 1 and 5% methane did not inhibit CF degradation ability by *M. trichosporium*. Even 25% methane inhibited CF degradation only partially. Inhibition of CF degradation by the growth substrate is ex-

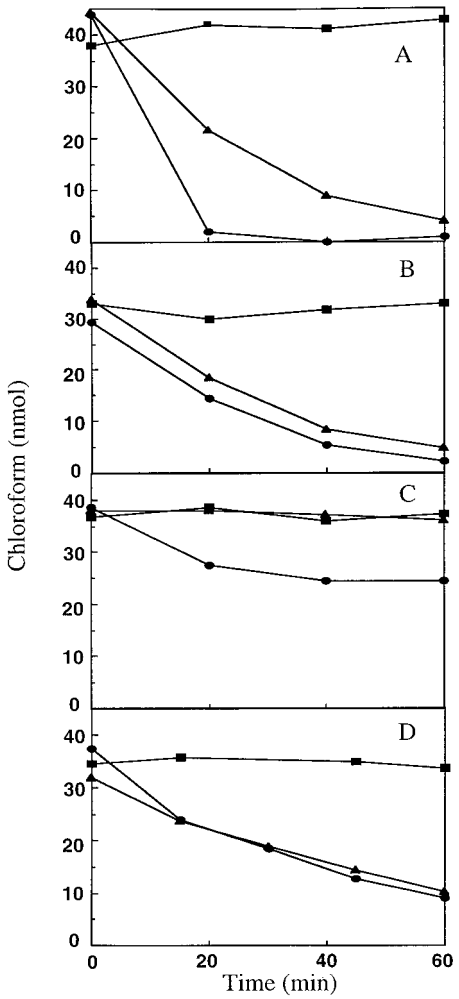

FIG. 1. Time course of CF degradation by *M. trichosporium* (A), CF8 (B), *P. butanovora* (C), and *M. vaccae* (D). Cells were incubated as described in Materials and Methods in the presence of 34.5 nmol of CF (▲), CF and 5 mM electron donor (formate for *M. trichosporium* or butyrate for CF8, *P. butanovora*, and *M. vaccae*) (●), and CF, electron donor, and 0.1% (vol/total vial vol) acetylene (■).

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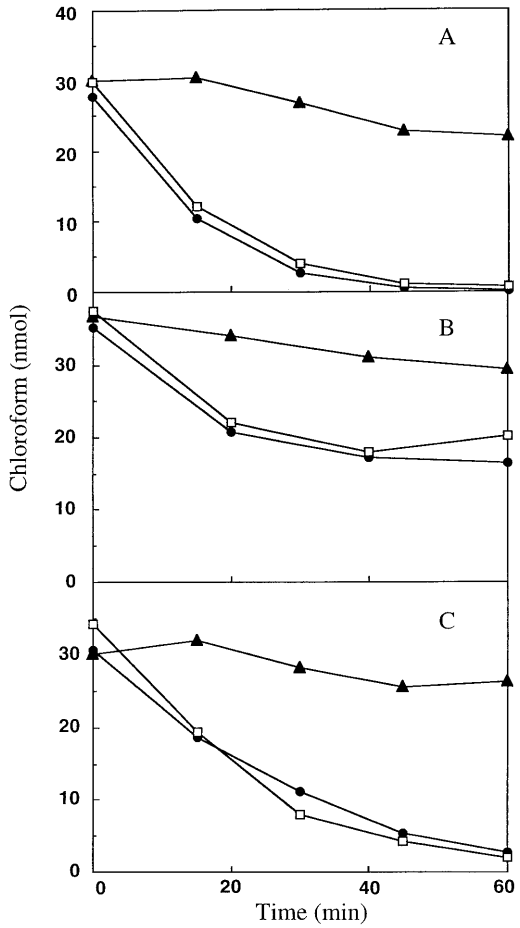

FIG. 2. Inactivation of CF degradation for CF8 (A), *P. butanovora* (B), and *M. vaccae* (C). Cells were preincubated for 10 min in vials containing phosphate buffer, 5 mM butyrate, and the following additions: none, (●); 0.05% (vol/total vial vol) acetylene for CF8 or 0.1% acetylene for *P. butanovora* and *M. vaccae* (▲); 0.05 or 0.1% acetylene and 50% butane (□). After 10 min, the vials were purged and the time course was initiated by the addition of 34.5 nmol of CF.

pected if both CF and the growth substrate compete to bind to the same enzyme (the monooxygenase). To confirm this idea, the effect of CF on butane degradation was tested for CF8. Butane oxidation was inhibited in the presence of CF. In the presence of 0.1% butane (1 μM in solution), the rate of butane consumption was inhibited 40% by 94 μM CF.

We determined the amount of CF degraded at different initial CF concentrations. The initial amounts of 34.5, 69, and 103.5 nmol of CF (12.9, 25.8, and 38.6 μM) were added to reaction mixtures, and the amounts of CF degraded in 20 and 40 min were compared (Table 3). The assays, except for that for that for CF8, were carried out in the presence of an appropriate electron donor (5 mM). Within the first 20 min, *M. trichosporium*, *P. butanovora*, and *M. vaccae* degraded more CF at higher CF concentrations. For *M. trichosporium*, the increase in the amount of CF degraded was nearly proportional to the increase in CF concentration, which indicates that the apparent K_m (appK_m) is well above 38.6 μM. For *P. butanovora* and *M. vaccae*, the increased CF degradation was not proportional to the increase in CF concentration, which suggests that the appK_m is in the range of the CF concentrations tested. CF8, which showed no increase in CF degradation with increasing CF concentrations, apparently has an appK_m of well below 12.9 μM. Between 20 and 40 min, *M. trichosporium* cells degraded CF completely at all three CF levels. In contrast, butane-grown cells were able to complete the degradation in only two cases. CF8 and *M. vaccae* consumed 34.5 nmol of CF but were unable to consume either 69 or 103.5 nmol of CF. *P. butanovora* consumed only half of the lowest CF concentration. Less degradation of CF from 20 to 40 min than from 0 to 20 min suggests a toxic effect of CF degradation on the cells.

To determine if the CF degradation capacity of cells decreased with time, CF degradation was monitored over a 2-h period. After 60 min, the CF concentration was adjusted to the initial value. An appropriate electron donor (5 mM) was included in the reaction mixture. In the first 60 min, *M. trichosporium*, CF8, *P. butanovora*, and *M. vaccae* degraded 92, 97, 71, and 56%, respectively, of the initial 69 nmol of CF. In the 60 min after respiking, 84, 11, 1, and 24%, respectively, of the CF was degraded (Fig. 3). *M. trichosporium* maintained nearly the same rate in CF degradation after the respike. CF8 degraded 69 nmol in the first 60 min, but most of the activity was lost during this period. Similarly, the CF degradation by *P. butanovora* that occurred during the first 60 min did not occur after the respike. Additional electron donor (5 mM) was added with CF after 60 min to determine whether these losses of CF degradation activity resulted from the reductant limitation (data not shown). However, the additional electron donor did not affect CF degradation after respiking, suggesting that the decreased CF degradation resulted from CF toxicity. *M. vaccae* retained some activity, though not as much as *M. trichosporium*. These results are consistent with those of Table 3 and show that CF8 and *P. butanovora* are more sensitive to CF toxicity than either *M. trichosporium* or *M. vaccae*. *M. trichosporium* seems to have the most resistance to CF toxicity.

The toxicity of CF to CF8 and *P. butanovora* was further examined by measuring butane-dependent O_2 uptake after exposure to CF. Cells of CF8 and *P. butanovora* were incubated with 69 nmol of CF for 60 min either in the presence or the absence of butyrate. As a control, cells were also incubated

TABLE 2. Inhibition of CF degradation by butane and methane

Organism	Amt of CF degraded in 60 min (nmol) with indicated amounts of substrates[a]					
	None	0.01%	0.1%	1%	5%	25%
M. trichosporium	34.3 ± 0.2	ND[b]	ND	34.3 ± 0.3	32.2 ± 1.2	19.0 ± 2.1
P. butanovora	22.5 ± 2.8	ND	18.1 ± 3.7	11.9 ± 2.0	2.1 ± 2.9	ND
CF8	33.0 ± 2.5	27.2 ± 7.4	19.2 ± 11.0	BD[c]	ND	ND
M. vaccae	25.8 ± 1.4	25.8 ± 1.9	24.2 ± 2.6	8.9 ± 1.3	1.4 ± 1.1	ND

[a] Amount of substrate added (vol/total vial vol). Methane was added to *M. trichosporium*, and butane was added to *P. butanovora*, CF8, and *M. vaccae*. Data are expressed as means ± standard deviations.
[b] ND, not determined.
[c] BD, below detection (<1 nmol).

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TABLE 3. CF degradation at different initial CF concentrations

Organism	Initial CF concn (μM)	Amt of CF (nmol)	Amt of CF degraded (nmol) during the indicated time periods[a]		
			0–20 min	20–40 min	0–40 min
M. trichosporium	12.9	34.5	30.1 ± 2.1	3.3 ± 1.3	33.4 ± 0.8
	25.8	69	61.1 ± 5.4	6.8 ± 3.8	67.8 ± 1.7
	38.6	103.5	86.2 ± 4.9	14.1 ± 2.8	100.3 ± 3.6
P. butanovora	12.9	34.5	13.6 ± 4.6	4.0 ± 1.8	17.6 ± 6.3
	25.8	69	22.3 ± 7.8	6.2 ± 2.9	28.5 ± 5.2
	38.6	103.5	25.8 ± 4.0	9.8 ± 2.4	35.6 ± 2.9
CF8	12.9	34.5	28.5 ± 2.3	6.0 ± 2.3	34.5 ± 0
	25.8	69	36.2 ± 4.9	11.5 ± 0.7	47.6 ± 4.9
	38.6	103.5	31.7 ± 7.2	8.7 ± 6.0	40.4 ± 6.2
M. vaccae	12.9	34.5	23.4 ± 2.1	7.2 ± 1.6	30.7 ± 2.8
	25.8	69	30.6 ± 6.3	15.1 ± 6.3	45.0 ± 11.3
	38.6	103.5	40.9 ± 4.7	23.8 ± 2.9	64.6 ± 3.7

[a] Data are expressed as means ± standard deviations.

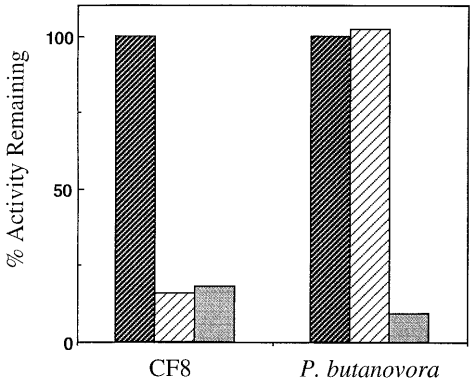

FIG. 4. O_2 uptake activity remaining in cells of CF8 and *P. butanovora* incubated in the presence or absence of CF (69 nmol) and an electron donor (5 mM). Cells were incubated for 60 min in phosphate buffer only (▨); buffer and 69 nmol of CF (◰); and buffer, 69 nmol of CF, and 5 mM butyrate (▤). The rate of O_2 uptake prior to the addition of butane (120 nmol) was subtracted from the rate of O_2 uptake after the addition of substrate. Butane-dependent O_2 uptake rates (19.9 and 9.4 nmol O_2/min for CF8 and *P. butanovora*, respectively) by the cells incubated in the absence of CF were considered as 100% activity remaining.

without CF. CF8 cells incubated with 69 nmol of CF in the presence and absence of butyrate degraded 79 and 99% of the CF in 60 min, respectively. O_2 uptake by both samples decreased (Fig. 4). *P. butanovora* incubated for 60 min with 69 nmol of CF in the presence of butyrate degraded 72% of the CF, but only 12% of the CF was degraded when cells were incubated without butyrate. O_2 uptake was similar to that for the control in the sample not exposed to butyrate but decreased in the sample which included butyrate (Fig. 4). Butane-dependent O_2 uptake decreased when cells were incubated under conditions which supported CF degradation.

We measured the amount of Cl^- and CO_2 released during CF degradation. The measurement of chloride release was particularly important to determine if CF degradation (defined as a loss of CF) resulted in the dechlorination of CF. Cells of *M. trichosporium*, *P. butanovora*, and CF8 were incubated with CF in the presence of an electron donor for 2 to 3 h. Chloride ion contents of control treatments containing acetylene were used to correct for the background chloride. The molar ratios ± standard deviations of chloride ions released to CF degraded were 2.1 ± 0.2 for *M. trichosporium*, 2.4 ± 0.3 for CF8, and 1.7 ± 0.5 for *P. butanovora*. While chloride release clearly accompanied CF degradation, complete dechlorination apparently did not occur, because the ratios were less than 3. This result differs from that for the butane microcosms (10), in which complete dehalogenation was observed. However, at least some portion of the degraded CF is completely dechlorinated, because CO_2 is a product. *M. trichosporium*, *P. butanovora*, or CF8 cells exposed to [^{14}C]chloroform converted 15 to 50% of the total ^{14}C into CO_2 (data not shown).

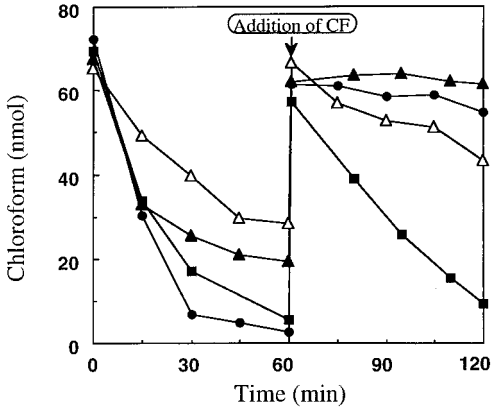

FIG. 3. CF toxicity. Degradation of CF (69 nmol) in the presence of a 5 mM concentration of appropriate electron donor was monitored for 60 min, and then CF was added to bring the total amount of CF to 69 nmol. CF degradation was monitored for an additional 60 min. Symbols: ■, *M. trichosporium*; ●, CF8; ▲, *P. butanovora*; △, *M. vaccae*.

It was of interest to determine if CAH degradation by butane-grown bacteria was limited to CF. *M. vaccae* was previously shown to degrade several CAHs (when grown on propane). Therefore, CF8 and *P. butanovora* were further studied for the ability to degrade other chlorinated ethenes and ethanes (Table 4). Both CF8 and *P. butanovora* were able to degrade trichloroethylene, 1,2-*cis*-dichloroethylene, and vinyl chloride. In addition, CF8 readily degraded 1,1,2-trichloroethane. 1,1,1-Trichloroethane and 1,2-*trans*-dichloroethylene were not degraded substantially by either CF8 or *P. butanovora*. It is interesting that 1,2-*cis*-dichloroethylene, but not 1,2-*trans*-dichloroethylene, was degraded by both bacteria. CF8 showed higher degradation activity for vinyl chloride and 1,1,2-trichloroethane than for other CAHs. In contrast, *P. butanovora* showed higher degradation activity for 1,2-*cis*-dichloroethylene than for other CAHs.

TABLE 4. Degradation of chlorinated ethenes and ethanes by *P. butanovora* and CF8

Compound	*P. butanovora*			CF8		
	Amt added (nmol)	Time (min)	Amt degraded (nmol)	Amt added (nmol)	Time (min)	Amt degraded (nmol)
Trichloroethylene	89	70	23	10	210	4.9
1,1,1-Trichloroethane	29	140	BD[a]	10	180	0.6
1,1,2-Trichloroethane	ND[b]	ND	ND	10	80	10
1,2-*cis*-Dichloroethylene	106	70	93	83	105	25
1,2-*trans*-Dichloroethylene	65	180	BD	70	130	BD
Vinyl chloride	100	85	24	100	130	65

[a] BD, below detection (<3% of the initial amount).
[b] ND, not determined.

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DISCUSSION

Kim et al. (10) demonstrated that butane was an effective substrate for cometabolism of CF and 1,1,1-trichloroethane in microcosms with Hanford aquifer core material. In this work, we extend this observation to two bacteria from the ATCC culture collection, *M. vaccae* JOB5 and *P. butanovora*, and an enrichment culture, CF8, derived from the Hanford microcosms. All three butane-grown microorganisms in this study were able to degrade CF. This is the first report of CF degradation by pure cultures of butane-oxidizing bacteria. In our study, CF degradation by CF8 was compared to CF degradation by two butane-oxidizing bacteria obtained from a culture collection and to that of a known CF degrader, *M. trichosporium* OB3b.

Our data indicate that these butane-grown bacteria possess a monooxygenase enzyme that initiates the oxidation of butane and CF. In this study, the involvement of butane monooxygenase in CF degradation was indicated by the following results: the requirement of O_2 for CF degradation, inhibition by acetylene, and inhibition by butane. *M. vaccae* is known to produce a propane monooxygenase which oxidizes butane by subterminal oxidation (15, 28). Most likely, the same monooxygenase is produced in propane- or butane-grown cells.

Competition between the growth substrates (butane or methane) and CF was observed (Table 2). With 1% butane (10 μM butane in solution), CF degradation (with 13 μM CF in solution) decreased to about half of the amount that occurred in the absence of butane for *P. butanovora* and *M. vaccae*. In contrast, CF degradation by CF8 was completely inhibited by 1% butane. In the microcosms from which CF8 was isolated, the presence of 2 mg of butane per liter in the liquid phase (38 μM) did not inhibit CF transformation (3 to 6 μM) in microcosms (10). However, at high CF concentration (10 μM), CF transformation appeared to accelerate as butane concentrations decreased, which may indicate that butane inhibits CF transformation. These differences between CF8 and the microcosms may indicate that CF8 was not the dominate butane oxidizer in the microcosms. Alternatively, these differences may reflect the differences between growth in the presence (microcosms) and absence (CF8) of CF. The effect of methane concentration on the CF kinetics of *M. trichosporium* has been studied. Speitel et al. (23) showed that the presence of methane caused significant enzyme competition at methane concentrations as low as 0.35 mg/l (22 μM), resulting in smaller CF rate constants. The rate constant in the presence of 0.35 mg of methane per liter was only 37% of that observed for the control. Competition between methane and other chlorinated aliphatics for sMMO has been observed with *M. trichosporium* OB3b (12).

Of the organisms tested, *M. trichosporium* OB3b and *M. vaccae* exhibit the potential to continuously degrade a high concentration (38.6 μM) of CF (Table 3 and Fig. 3). In contrast, *P. butanovora* and CF8 rapidly lost their CF degradation potential as a result of CF degradation. The mechanism of CF toxicity is not well understood. From the O_2 uptake assay, it is suggested that the cells were damaged as a result of oxidizing CF (Fig. 4). Butane-dependent O_2 consumption was not lost unless CF was degraded. Alvarez-Cohen and McCarty (1) studied CF and TCE toxicity with a mixed methane-utilizing culture of resting cells. Toxicities of CF, TCE, and their transformation products to whole cells were evaluated by comparing the formate oxidation activity of acetylene-treated cells to that of non-acetylene-treated cells with and without prior exposure to CF or TCE. The formate oxidation by cells exposed to either CF or TCE without acetylene significantly decreased compared to that with acetylene, suggesting that the solvents themselves were not toxic but that their transformation products were. Alvarez-Cohen and McCarty (1) have speculated that phosgene and TCE epoxide are responsible for the product toxicities of CF and TCE, since both compounds have been shown to exhibit irreversible binding to proteins thought to result in toxic behavior in mammalian systems (4, 16). A similar reaction may have caused the toxicity we observed with butane-grown bacteria. The results from our chloride release experiments suggested incomplete dehalogenation of CF. Perhaps not all of the CF proceeds through a pathway which leads to complete dehalogenation.

CF degradation by all three butane-grown bacteria and *M. trichosporium* was inactivated by acetylene. Acetylenes are known to inactivate a number of monooxygenases, including pMMO (17) and AMO (2), sMMO (17), and P450 monooxygenases (14). Acetylene inactivation required enzyme turnover, and the presence of butane protected the enzyme from inactivation (Fig. 2). These results suggest that acetylene is a mechanism-based inactivator of butane monooxygenase.

Besides CF, a number of other environmentally important chlorinated compounds were degraded by cells of CF8 and *P. butanovora* (Table 4). *M. trichosporium* OB3b and *M. vaccae* JOB5 also degrade various chlorinated aliphatic compounds (13, 29). *M. trichosporium* degraded 0.2 mM TCE, 1,2-*trans*-dichloroethylene, and 1,2-*cis*-dichloroethylene completely within 24 h. 1,1,1-Trichloroethane was partially degraded (13). In addition, vinyl chloride degradation by *M. trichosporium* was reported (5). Propane-grown *M. vaccae* degraded vinyl chloride (14 μM) completely within 2 h, and 1,1-dichloroethylene (20 μM) and 1,2-*cis*-dichloroethylene (17 μM) were degraded to a significant extent. At a starting concentration of 20 μM TCE, up to 99% was removed in 24 h. 1,2-*trans*-Dichloroethylene was only marginally degraded (29). The ability of CF8 and *P. butanovora* (Table 4) to degrade *cis*-dichloroethylene but not *trans*-dichloroethylene is similar to that of propane-grown *M. vaccae*. This study shows that butane-oxidizing bacteria can degrade chlorinated aliphatic hydrocarbons. Butane-grown bacteria may have potential in the bioremediation of these compounds.

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