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Description of Toluene Inhibition of Methyl Bromide Biodegradation in Seawater and Isolation of a Marine Toluene Oxidizer That Degrades Methyl Bromide

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Methyl bromide (CH_3Br) and methyl chloride (CH_3Cl) are important precursors for destruction of stratospheric ozone, and oceanic uptake is an important component of the biogeochemical cycle of these methyl halides. In an effort to identify and characterize the organisms mediating halocarbon biodegradation, we surveyed the effect of potential cometabolic substrates on CH_3Br biodegradation using a $^{13}CH_3Br$ incubation technique. Toluene (160 to 200 nM) clearly inhibited CH_3Br and CH_3Cl degradation in seawater samples from the North Atlantic, North Pacific, and Southern Oceans. Furthermore, a marine bacterium able to co-oxidize CH_3Br while growing on toluene was isolated from subtropical Western Atlantic seawater. The bacterium, Oxy6, was also able to oxidize *o*-xylene and the xylene monooxygenase (XMO) pathway intermediate 3-methylcatechol. Patterns of substrate oxidation, lack of acetylene inhibition, and the inability of the toluene 4-monooxygenase (T4MO)-containing bacterium *Pseudomonas mendocina* KR1 to degrade CH_3Br ruled out participation of the T4MO pathway in Oxy6. Oxy6 also oxidized a variety of toluene (TOL) pathway intermediates such as benzyl alcohol, benzylaldehyde, benzoate, and catechol, but the inability of *Pseudomonas putida* mt-2 to degrade CH_3Br suggested that the TOL pathway might not be responsible for CH_3Br biodegradation. Molecular phylogenetic analysis identified Oxy6 to be a member of the family *Sphingomonadaceae* related to species within the *Porphyrobacter* genus. Although some *Sphingomonadaceae* can degrade a variety of xenobiotic compounds, this appears to be the first report of CH_3Br degradation for this class of organism. The widespread inhibitory effect of toluene on natural seawater samples and the metabolic capabilities of Oxy6 indicate a possible link between aromatic hydrocarbon utilization and the biogeochemical cycle of methyl halides.

Methyl bromide (CH_3Br) and methyl chloride (CH_3Cl) are significant components of the tropospheric halogen burden. When transported into the stratosphere these gases are photolysed, releasing halogen atoms that contribute to catalytic ozone depletion. The global sources and sinks of these ozone-depleting substances are not well known, and their atmospheric budgets remain unbalanced (35, 76). Methyl bromide is produced anthropogenically for use as a fumigant, and it is a natural algal product (2, 38, 56, 57). The sources of CH_3Cl are primarily natural with some anthropogenic contributions, particularly from man-made fires (5, 25). The net global flux to the ocean reflects a balance between uptake of atmospheric CH_3Br and CH_3Cl and internal cycling (production and consumption) in the ocean distributed over large geographic regions. The ocean is a net sink for atmospheric CH_3Br (33), where it is degraded by chemical (17, 32) and biological (19, 34, 65) processes. Methyl chloride is also produced and destroyed in the oceans, although the oceans represent a net source in the atmospheric budget (46, 47, 77). Oceanic uptake of methyl chloride is almost entirely biological, as chemical reactions are too slow to play a significant role in the process (17, 64, 67).

Biodegradation of CH_3Br and CH_3Cl has been demonstrated in a variety of waters and soils (10, 19, 26, 34, 43, 51, 64–67, 72), but the organisms responsible have not been positively identified. Investigations with specific inhibitors demonstrated that methanotroph/nitrifier co-oxidation was partially responsible for CH_3Br removal in samples from certain soils (50) and a freshwater lake (19). However, methanotroph/nitrifier involvement was not detected in certain agricultural soils (26, 43) or in coastal marine and estuarine waters (19); therefore, other groups of bacteria consumed CH_3Br in those environments. Inhibitors of CH_3Br degradation apparently have not been tested in open ocean waters.

A variety of bacteria can cometabolize methyl halides in the laboratory. These include methane and ammonia oxidizers (13, 24, 54, 61), dimethyl sulfide and methylamine utilizers (29), and propane oxidizers (62). In addition, several bacteria have been isolated that are able to metabolize CH_3Br and CH_3Cl (11, 20, 39, 41, 58, 59, 68). For example, a marine methylotroph isolated from seawater, *Leisingera methylohalidivorans* MB2^T, utilizes CH_3Br supplied as a sole source of carbon and energy (~100 μM) (58), and it can consume CH_3Br supplied at near ambient concentrations (~2 pM) (20). Terrestrial bacteria known to utilize methyl halides include *Methylobacterium chlo-*

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romethanicum CM4^T, *Hyphomicrobium chloromethanicum* CM2^T, *Aminobacter* strain IMB-1, and *Aminobacter* strain CC495. Those terrestrial bacteria contain the *cmuA* gene, which is involved in the degradation of methyl halides and has methyltransferase and corrinoid functions (41). However, genes homologous to *cmuA* have not been identified in the marine bacterium *L. methylohalidivorans* (59a), suggesting the existence of a different CH_3Br degradation pathway.

Alkylated aromatic hydrocarbons such as toluene enter the sea as a pollutant or from natural oil seeps. Bacterial oxidation of toluene can be carried out by a variety of oxygenase enzymes (22, 70), and bacteria equipped with oxygenases are known to cometabolize a variety of halogenated substrates (3, 16, 36, 40, 48). The ability of both trihalomethanes and hydrocarbons to support marine oligobacteria has been suggested (8). However, degradation of methyl halides (MeX; X = Br, Cl, I) by utilizers of aromatic hydrocarbons has not been previously described. Here we report that toluene inhibits biodegradation of CH_3Br and CH_3Cl in natural seawater samples and describe a marine isolate that degrades CH_3Br while growing on toluene or *o*-xylene.

MATERIALS AND METHODS

Halocarbon degradation in seawater. Methyl bromide and methyl chloride loss rate constants were measured using a stable isotope incubation technique. Experiments utilized surface seawater collected from the subtropical Western Atlantic, the North Atlantic, the North Pacific, and the Southern Ocean. Surface seawater was collected from the following locations: (i) Bear Cut, Virginia Key, Miami, Florida (connects Biscayne Bay and the Atlantic Ocean) between November 1998 and March 1999; (ii) a North Pacific cruise track ranging in latitude from 11 to 58°N and longitude from 168°E to 125°W between September and October 1999 aboard the R/V *Ron Brown* (65); (iii) a buoy station in the Bedford Basin, Nova Scotia, Canada, from December 1999 to July 2000 (64); and (iv) a Southern Ocean cruise track ranging in latitude from 46 to 67°S and longitude from 138 to 145°E between October and December 2001 aboard the R/V *Aurora Australis* (67).

Samples of seawater were incubated in 100- or 150-ml glass syringes without headspace. Syringes were kept in the dark in a circulating water bath set within 1°C of the temperature of the water at the time of collection. Samples were paired, and one was amended with a compound to test whether it inhibited halocarbon biodegradation. Both samples received approximately 400 pM $^{13}CH_3Br$ (approximately 40 to 100 times ambient $^{12}CH_3Br$ concentrations) or 1 nM $^{13}CH_3Cl$ (approximately 10 to 20 times ambient $^{12}CH_3Cl$ concentrations). The following compounds were tested for the effect on halocarbon biodegradation: dimethyl sulfide (100 nM), methanesulfonic acid (50 nM), trimethylamine (2.5 to 500 nM), toluene (150 to 200 nM), and xylene (mixed isomers, 150 to 200 nM).

The concentration of $^{13}CH_3Br$ or $^{13}CH_3Cl$ was measured at 2-h intervals over the time course of the experiment (<14 h) by purge and trap extraction with gas chromatography-isotope dilution-mass spectrometric detection. The precision for an individual $^{13}CH_3Br$ measurement was ±0.2%. A complete description of the stable isotope technique, including instrumentation and data analysis, is detailed by King and Saltzman (34) and Tokarczyk and Saltzman (66). Additional details for the $^{13}CH_3Cl$ isotope dilution technique are given by Tokarczyk et al. (64).

The loss of $^{13}CH_3Br$ or $^{13}CH_3Cl$ in these experiments is described by first-order kinetics according to the equation $\ln(C/C_0) = -kt$, where C_0 is the initial concentration at the start of the experiment, C is the concentration at time t, and k is the first-order loss rate constant calculated from the slope of the least-squares regression. Typically, five to six points were used to calculate the slope of the regression line. The abbreviations k_{tot}, k_{totinh}, and k_{filt} refer to the rate constants for an unfiltered sample, an unfiltered sample amended with a potential inhibitor, and a 0.2-μm-pore-filtered sample, respectively. The abbreviation k_{chem} refers to the CH_3Br rate constant for a filtered or autoclaved sample calculated according to the temperature-dependent rate expression of King and Saltzman (34) for chemical loss of CH_3Br in filtered seawater. This equation has been verified for a variety of ocean waters (65, 66), and the relative uncertainty is estimated as ±7% of the calculated rate at the 95% confidence interval (66). Methyl bromide loss in filtered or autoclaved seawater is due to chemical mechanisms such as nucleophilic substitution and hydrolysis (17, 78). Loss due to volatilization from the syringes has been shown to be negligible (34).

The uncertainty in k_{tot} and k_{totinh} is given as the 95% confidence interval for the slope, calculated using the appropriate Student's t value (n-2 distribution) and the standard error of the slope (34). The relative uncertainty for k_{tot} ranged from 5 to 24% of the rate, with absolute values ranging from 0.005 to 0.03 day^{-1}. The uncertainty of k_{totinh} ranged from 0.008 to 0.04 day^{-1}. Differences between k_{tot} and k_{totinh} were considered significant if the 95% confidence intervals did not overlap. Rate constants were compared only when significant biological activity was observed: that is, when k_{tot} differed significantly from k_{chem} or k_{filt}. This requirement was met for 30 out of 46 experiments.

Culture isolation and maintenance. An enrichment culture was established from surface seawater collected from Bear Cut off Virginia Key, Miami, Florida, with toluene as the carbon source in an artificial seawater medium ("Lev marine") having the following components (per liter): NaCl (13.4 g), $MgCl_2 \cdot 6H_2O$ (2.6 g), KCl (0.17 g), $CaCl_2 \cdot 2H_2O$ (0.74 g), $MgSO_4 \cdot 7H_2O$ (3.4 g), NH_4Cl (0.30 g), KH_2PO_4 (0.05 g), Tris-HCl, pH 8.0 (7.9 g; 50 mM final concentration), SL-10 trace metals (10 ml) (73), and vitamins with B_{12} (1 ml) (52). Cultures were grown while shaking (125 rpm) at room temperature in 500-ml screw-cap bottles with periodic additions of toluene (2 μl/100 ml medium; ~100 μM). An enrichment culture was established that degraded CH_3Br while growing on toluene. Isolated bacterial colonies were obtained by streaking on Lev marine agar plates (1.5% Bacto agar; Difco) stored in a closed container in which a couple of drops of toluene or *o*-xylene were added to provide vapor to the growing cells. A pure culture was obtained by replacing toluene with *o*-xylene in the Lev marine medium and by repeated alternation between growth in liquid medium and streak plating. The culture, Oxy6, was maintained on marine agar (2216; Difco) and on Lev marine broth amended with 212 μM toluene (7 μl toluene/100 ml medium in 1-liter crimp-sealed vials), which was shaken (200 rpm) at room temperature under ambient light.

Pseudomonas putida mt-2 was grown on toluene or *m*-xylene (~100 μM) in screw-cap bottles using medium of the following composition (per liter): KCl (0.17 g), $CaCl_2 \cdot 2H_2O$ (0.021 g), $MgSO_4 \cdot 7H_2O$ (0.20 g), NH_4Cl (0.30 g), KH_2PO_4 (0.05 g), Tris-HCl, pH 8.0 (7.9 g), and SL-10 trace metals (10 ml). *Pseudomonas mendocina* KR1 was grown on 100 μM toluene in either LB broth or a mineral salt medium (40). *L. methylohalidivorans* was grown on 100 μM CH_3Br in MAMS medium (58), and *Aminobacter* strain IMB-1 was grown on 100 μM CH_3Br on a modified Doronina medium (11). All cultures were grown at room temperature, under room light, and shaken at 200 rpm.

Halocarbon degradation by cultures. Degradation of halomethanes was measured using pure cultures or suspensions of washed cells. Cultures were placed in 37-ml serum vials and capped with blue butyl stoppers for methyl halides and Teflon-lined gray butyl stoppers for polyhalogenated halocarbons. Halocarbon was added aseptically to achieve a final concentration of 1 to 10 μM, depending on the experiment. Halocarbon aqueous concentrations at 23°C were calculated using the following dimensionless partition coefficients: CH_3Br = 0.248 (14), methyl iodide (CH_3I) = 0.197 (31), methyl chloride (CH_3Cl) = 0.341 (21), dichloromethane (CH_2Cl_2) = 0.087 (21), dibromomethane (CH_2Br_2) = 0.032 (69), chloroform ($CHCl_3$) = 0.146 (21), bromoform ($CHBr_3$) = 0.021 (49), and toluene = 0.235 (15). Henry's constants (H) with dimensions of atm · m^3/ml were converted to dimensionless partition coefficients ($H_{no\ dim}$) by the equation $H_{no\ dim} = H/[R \cdot T_{(K)}]$ (45), where $R = 8.21e^{-5}$ atm · m^3/(mol · K) and $T_{(K)}$ = temperature in degrees kelvin. The salinity of Lev marine medium (16 g $liter^{-1}$) was used in equations that provided a salinity term (14, 15): otherwise, the equation for freshwater was used.

Halomethanes were measured on a Hewlett-Packard 5890 series II gas chromatograph equipped with electron capture detection (GC-ECD) using a Restek RTX-624 wide-bore capillary column (30 m, 0.53-μm inside diameter, 3.0-μm df [film thickness]) with injector and detector temperatures of 240°C and 325°C, respectively. Chromatography of CH_3Br, CH_3Cl, and CH_3I was performed isothermally at 50°C, and analysis of CH_2Br_2, $CHCl_3$, and $CHBr_3$ employed a temperature ramp (50°C, 20°C/min, 160°C, 1 min). Loss of halomethane over time in live samples was compared to loss in sterile medium or autoclaved controls. Cell counts were determined by acridine orange direct count (AODC) (28).

Methyl bromide degradation by Oxy6 was tested in the presence of 1% (vol/vol) acetylene. Acetylene was generated by adding 0.2 g calcium carbide (CaC_2) to a 20-ml vial. The vial was sealed and evacuated prior to adding 5 ml of distilled water via a needled syringe. The generated acetylene was collected in a 60-ml syringe and transferred to a 30-ml evacuated vial.

Substrate oxidation. The oxidation of a variety of aromatic compounds by whole cells grown on Lev marine broth with toluene was tested by measuring oxygen uptake at 30°C in a 5-ml chamber with a YSI oxygen electrode. Endogenous rates were measured prior to adding substrate (0.5 to 5 μl; typically 0.1 μmol) and were subtracted from the substrate rate. Rates were normalized by Klett reading and by protein concentration to compare rates from different batches of culture.

Pigment analysis and spectroscopy. Pigment analysis was performed using a Hewlett Packard 1100 series high-performance liquid chromatograph equipped with a diode array detector using a 10-cm path length. Cells were extracted in acetone, and the method used was similar to that described by Van Heukelem et al. (71), except that columns were kept at 35°C and three C_{18} columns were used in series to enhance pigment separation (53). The first column was a Rainin monomeric Microsorb-MV column (0.46 × 10 cm, 3-μm packing) followed by two polymeric Vydac 201TP columns (0.46 × 25 cm, 5-μm packing). A binary solvent system was used to separate pigments (solvent A, 80:20 methanol–0.5 M ammonium acetate; solvent B, 80:20 methanol-acetone). Retention times and peak areas were compared to standard pigments including a β-β-carotene standard obtained from the VKI Water Quality Institute (Agern Allé 11, Hørsholm, Denmark). Spectroscopy was also performed on whole cells grown in Lev marine medium and on the yellow supernatant remaining after cell centrifugation (HP8452 spectrophotometer).

Electron microscopy. Scanning electron microscopy (SEM) on samples of Oxy6 culture was performed by the University of Miami Dauer Environmental SEM Laboratory using a Philips XL30 ESEM-FEG (environmental scanning electron microscope field emission gun). Samples were filtered onto a 0.2-μm Nuclepore filter, and a small portion of the dried filter was excised and mounted on a standard SEM stub using double-sided carbon tape. Samples were gold coated in a sputter deposition coater (Cressington 108auto).

DNA extraction, amplification, and restriction fragment length polymorphism. Total genomic DNA was isolated by a spin kit according to the manufacturer's instructions (Sigma GenElute Genomic G-1N-10). The 16S rRNA was amplified by PCR using two methods. The first method used the universal primer 27F and eubacterium-specific primer 1492R (37). Amplifications were performed using the reagents supplied with GeneAmp Gold *Taq* (PE Biosystems) using a 50-μl reaction mixture containing 1× PCR Gold buffer, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 10 pmol of each primer, and 1.25 U of *Taq* polymerase. The reactions were amplified using a GeneMate thermal cycler (ISC Bioexpress) programmed for a hot start (40°C for 2 min, 94°C for 30 s) with 25 cycles of 1 min of denaturing at 94°C, 1 min of annealing at 55°C, and 1.5 min of extension at 72°C followed by a final 5 min of extension at 72°C. Amplifications also were performed using the eubacterial primers Unifor (5′-ACTCCTACGGGAGGCAGC-3′) (1) and Unirev (5′-GACGGGCGGT GTGTACAA-3′) (79). PCR reagents (MJ Research) and primers (Integrated DNA Technologies, Coralville, IA) were added to yield a 50-μl reaction mixture containing 20 pmol of each primer and 20 μl Eppendorf MasterMix (final concentration of 1.5 mM Mg^{2+}, 0.2 mM of each dNTP, and 1.25 U *Taq* polymerase). The reactions were amplified using a PTC-100 Hotbonnet thermocycler (MJ Research) programmed for 30 cycles of 1 min of denaturing at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C, with a final 8-minute extension at 72°C.

The PCR products were ligated into the pCR2.1 cloning vector and transformed into *E. coli* INVαF′ competent cells according to the manufacturer's instructions (TA cloning kit; Invitrogen). Plasmids were isolated (Promega Wizard Plus SV Minipreps), and 27 colonies containing F27 and R1492 amplicons were amplified by PCR for restriction fragment length polymorphism analysis (12). Restriction digests were performed overnight at 37°C using the enzymes MspI, Sau3A, and HhaI. Digests were resolved on 3% high-resolution agarose gels (Sigma).

DNA sequencing and phylogenetic analysis. The 16S rRNA inserts from 17 clones were sequenced. Two clones containing inserts from the 27F/1492R amplification were sequenced at the University of Miami DNA Core Laboratory using a Perkin-Elmer ABI 373 DNA sequencer with XL upgrade and Big Dye chemistry. Initial sequencing utilized primers designed to the M13 forward and M13(−20) reverse segments of the pCR2.1 vector. Based on those results, sequencing of the inserts was completed using primers designed by eye and with aid of the GeneRunner program (Hastings Software, Inc.) (5′-TAACCCAACA TCTCACGAC-3′ and 5′-GCGTGAGTGATGAAGGC-3′). In addition, 15 clones containing inserts from the Unifor/Unirev amplification were sequenced on an ABI 3730 capillary sequencer using the BigDye 3.1 sequencing kit (Applied Biosystems). The sequence reaction volume was 10 μl and contained 50 ng of purified amplicon, 1.075× buffer, 0.32 pmol of primer [M13 forward or M13(−20) reverse], and a 1/16 dilution of BigDye 3.1 mix (0.5 μl/reaction).

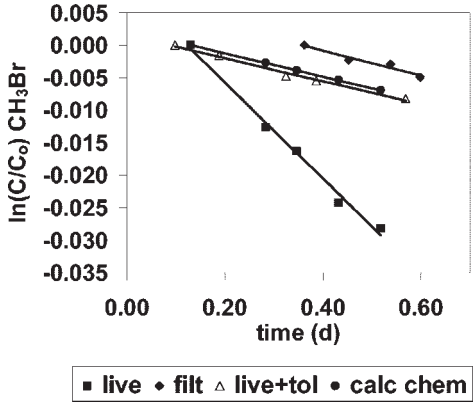

FIG. 1. Inhibition of $^{13}CH_3Br$ (400 pM) biodegradation by toluene (200 nM) in open ocean samples collected from the Gulf of Alaska, North Pacific Ocean. filt, 0.2-μm filtered; tol, toluene; calc chem, calculated chemical loss.

Sequence data were analyzed using the ContigExpress module of the Vector NTI software (version 9.0, Invitrogen). Related sequences were obtained by BLAST search of the GenBank nucleotide database. Multiple sequence alignment was performed using the default settings of the AlignX module of the Vector NTI software package, which is based on the Clustal W algorithm. Phylogenetic trees (neighbor joining, parsimony, maximum likelihood) and bootstrap analysis using 1,000 replicates (for neighbor joining and parsimony) were performed with the PAUP* package (63). Maximum-likelihood analysis in conjunction with the HKY-gamma substitution model employed a heuristic search with model parameters set by those estimated from the tree score of the parsimony analysis. In addition, Bayesian analysis was carried out with MrBayes V3.0B using the general time-reversible model with a gamma distribution for rate variation across sites (23, 30). Bayesian posterior probabilities were computed by running four Markov chain Monte Carlo chains for 1,000,000 generations. Trees were sampled every 100 generations, and 2,000 trees were discarded as "burn-in."

RESULTS

The effect of toluene on methyl halide biodegradation in natural seawater. In an attempt to identify the types of organisms responsible for CH_3Br biodegradation observed in seawater (19, 34, 65, 66), several possible inhibitors of CH_3Br degradation were tested. Dimethyl sulfide (100 nM), methanesulfonic acid (50 nM), trimethylamine (2.5–500 nM), and toluene (150 nM) were briefly surveyed using coastal subtropical Western Atlantic seawater. Out of these compounds, only toluene appeared to inhibit CH_3Br biodegradation in preliminary experiments. Toluene was thus chosen for a more detailed investigation using a variety of ocean waters.

Toluene (160 to 200 nM) was found to inhibit degradation of CH_3Br (400 pM) in seawater samples from the North Atlantic (coastal), the North Pacific, and the Southern Ocean. Toluene significantly inhibited CH_3Br loss, producing loss rate constants similar to those calculated for chemical loss or measured in filtered samples (Fig. 1). Experiments were conducted in waters ranging in temperature from 0.4 to 18.5°C and salinity from 25 to 34 ppt. Addition of toluene inhibited CH_3Br loss by 37 to 100% ($n = 17$) and CH_3Cl loss by 80 to 98% ($n = 3$) compared to live samples with no amendments (Table 1).

Based on results with a bacterial isolate (see below), xylene (200 nM) was tested using North Atlantic and Southern Ocean seawater. In contrast to toluene, addition of xylene did not

TABLE 1. Inhibition of biodegradation of $^{13}CH_3Br$ (400 pM) or $^{13}CH_3Cl$ (1 nM) in seawater by amendment with toluene (160 or 200 nM)

Methyl halide	Location	Latitude	Longitude	Temp (°C)	S (ppt)	$K_{tot} \pm$ SE (day^{-1})	$k_{totinh} \pm$ SE (day^{-1})	% Inhibition
Methyl bromide								
	Western Atlantic[a]	25.7 N	80.2 W	23.3	32.3	0.210 ± 0.020	0.150 ± 0.040	29[b]
	North Atlantic	48.1 N	66.4 W	18.5	30.0	0.097 ± 0.013	0.061 ± 0.011	37
	North Atlantic[a]	48.1 N	66.4 W	7.1	29.0	0.038 ± 0.005	0.016 ± 0.011	58
	North Atlantic[a]	48.1 N	66.4 W	7.0	25.0	0.126 ± 0.021	0.011 ± 0.006	91
	North Atlantic[a]	48.1 N	66.4 W	6.4	29.5	0.034 ± 0.007	0.012 ± 0.005	96
	North Pacific	49.2 N	132.0 W	12.0	32.3	0.068 ± 0.005	0.027 ± 0.006	60
	North Pacific	50.5 N	164.9 W	10.2	32.3	0.071 ± 0.009	0.018 ± 0.01	75
	North Pacific	50.2 N	147.5 W	10.1	32.6	0.053 ± 0.008	0.019 ± 0.006	64
	North Pacific	55.0 N	146.5 W	9.4	32.8	0.032 ± 0.006	0.014 ± 0.009	56
	North Pacific	52.1 N	156.7 W	9.3	32.5	0.038 ± 0.009	0.014 ± 0.009	63
	North Pacific	52.6 N	157.1 W	9.2	32.6	0.071 ± 0.008	0.016 ± 0.006	77
	North Pacific[a]	57.7 N	152.1 W	8.0	31.6	0.161 ± 0.008	0.075 ± 0.005	53
	Southern Ocean	48.3 S	144.5 E	7.3	34.1	0.055 ± 0.007	0.018 ± 0.014	67
	Southern Ocean	53.7 S	141.9 E	4.8	33.8	0.052 ± 0.005	0.008 ± 0.028	85
	Southern Ocean	53.6 S	141.2 E	3.7	33.7	0.054 ± 0.009	0.004 ± 0.016	93
	Southern Ocean	54.5 S	141.4 E	2.5	33.8	0.113 ± 0.009	0.005 ± 0.010	96
	Southern Ocean	58.8 S	139.8 E	1.4	33.8	0.034 ± −0.008	−0.003 ± 0.008	100
	Southern Ocean	60.8 S	139.9 E	0.4	33.9	0.025 ± −0.005	0.007 ± 0.010	72
Methyl chloride								
	North Atlantic	48.1 N	66.4 W	15.7	31.0	0.170 ± 0.019	0.004 ± 0.011	98
	Southern Ocean	52.7 S	142.4 E	4.7	33.8	0.166 ± 0.011	0.033 ± 0.011	80
	Southern Ocean	53.7 S	141.8 E	3.6	33.8	0.045 ± 0.013	0.001 ± 0.007	98

[a] Coastal water samples: W. Atlantic, Florida; N. Atlantic, Halifax; N. Pacific, off Kodiak Island, Alaska. All other samples were collected from the open ocean.
[b] 95% confidence intervals overlap; 150 nM toluene used.

inhibit the rate of biological degradation in every type of marine water tested. Xylene inhibited CH_3Br biodegradation for two of five samples in which there was significant biological activity. These samples were from the Southern Ocean (1.4°C and 0.4°C; inhibition of 100% and 44%, respectively). The three samples in which xylene did not inhibit degradation were from the coastal North Atlantic (17.5 and 16.0°C) and the Southern Ocean (4.8°C).

Isolation and characterization of a marine bacterium that cometabolizes CH_3Br while growing on toluene. To further investigate the inhibitory effect of toluene on CH_3Br biodegradation, an enrichment culture was established from seawater that was able to degrade CH_3Br while growing aerobically on toluene. A pure bacterial culture was obtained by repeated streak plating on Lev marine agar amended with *o*-xylene. The bacterium, termed Oxy6, was a motile rod of dimensions 0.4 to 0.5 by 0.3 μm that exhibited some pleomorphism, showing thread-like cells, similar to that observed with *Sandaracinobacter sibiricus* (75). Gram staining was indeterminate, but phylogeny (see below) indicated that it was gram negative.

Oxy6 grew on Lev marine, a minimal artificial seawater medium, with toluene or *o*-xylene supplied as the sole source of carbon and energy. The bacterium also grew on Difco 2216 marine medium with no additional amendments. AODC measurements were used to calibrate optical density at 600 nm (OD_{600}) spectrophotometer readings (Eppendorf Biophotometer), yielding the following equation: cells $ml^{-1} = 6.3 \times 10^{10} \cdot OD_{600} + 9.1 \times 10^7$ ($r^2 = 0.999$). No growth was observed if salt was not added to the medium, showing that this was an organism of marine origin (80). Growth was similar (~4.5×10^8 cells ml^{-1} day^{-1}), with salt concentrations of 3.4, 6.7, 13.4, or 26.7 g $liter^{-1}$ in Lev marine medium amended with toluene (223 μM toluene per day for 3 days).

A single colony type grew on agar plates, whether toluene, *o*-xylene, or complex marine medium was used for growth. Colonies were bright yellow, shiny, and punctiform. Growth of Oxy6 turned liquid media yellow. Absorption spectra and pigment analysis did not indicate the presence of bacteriochlorophyll (BChl) (27, 74, 75); however, cultures were grown under conditions that may have inhibited synthesis (75). Light intensities as low as 20 μE m^{-2} s^{-1} can inhibit BChl synthesis (75), and light intensities of 21.5 μE m^{-2} s^{-1} were measured in the laboratory (Licor LI 250 light meter, 2π sensor, 400- to 700-nm range). If this organism can produce BChl, it did not appear to be necessary for CH_3Br degradation. Pigment analysis of cell extracts produced spectra with peaks at 450 and 478 nm and an overall shape similar to that of β-β-carotene.

Aromatic hydrocarbon degradation can follow a variety of complex pathways (6, 20, 22, 70). A variety of substrates were tested in order to gather information regarding the possible oxidation pathway used by Oxy6 to metabolize toluene and cometabolize CH_3Br. Oxy6 did not oxidize the toluene 4-monooxygenase (T4MO) pathway substrates *p*-cresol, *p*-hydroxybenzoate, and protocatechuate (Table 2). Acetylene had no affect on CH_3Br degradation (Fig. 2), providing further evidence against a T4MO pathway in this organism (40). Oxy6 did oxidize a number of substrates consistent with the TOL pathway (Table 2) (70). In addition, the culture oxidized *o*-

TABLE 2. Oxy6 and *P. putida* mt-2 oxidation of toluene, xylenes, and variety of intermediates indicative of degradative pathways

Substrate	Oxygen uptake (μmol O_2 mg protein^{-1})	
	Oxy6	*P. putida* mt-2
Toluene	3.5	15.9
o-Xylene	2.7	0.0
m-Xylene	3.1	35.2
p-Xylene	3.3	37.9
TOL pathway		
Benzyl alcohol	3.1	30.7
Benzaldehyde	1.1	30.7
Benzoate	1.1	10.3
Catechol	27.2	52.8
T2MO pathway		
2-Hydroxytoluene (*o*-Cresol)	0.6	1.8
T3MO pathway		
3-Hydroxytoluene (*m*-Cresol)	0.6	0.0
T4MO pathway		
4-Hydroxytoluene (*p*-Cresol)	0.5	0.0
4-Hydroxybenzoate	0.0	0.0
Protocatechuate	0.0	0.0
XMO pathway		
2-Methylbenzyl alcohol	1.7	0.0
3-Methylcatechol[a]	13.7	25.4

[a] Also an intermediate in the T3MO pathway.

xylene and 2-methylbenzyl alcohol, indicating methyl group oxidation via a xylene monooxygenase.

Halocarbon degradation by Oxy6 and other cultures. Oxy6 grew with either toluene or *o*-xylene as the sole carbon source and degraded CH_3Br under those growth conditions ($1.0 \times 10^{-6} \pm 8.1 \times 10^{-7}$ pmol CH_3Br cells^{-1} day^{-1}, $n = 5$). However, Oxy6 did not grow if CH_3Br was supplied as the sole source of carbon. Oxy6 grew on marine broth (Difco 2216), but toluene addition was necessary for CH_3Br degradation to occur (Fig. 3). Oxy6 also degraded CH_3I ($8.5 \times 10^{-7} \pm 6.0 \times 10^{-7}$ pmol CH_3I cells^{-1} day^{-1}, $n = 2$) but was unable to degrade CH_3Cl, CH_2Br_2, $CHCl_3$, or $CHBr_3$ (Fig. 4, except $CHBr_3$ not shown).

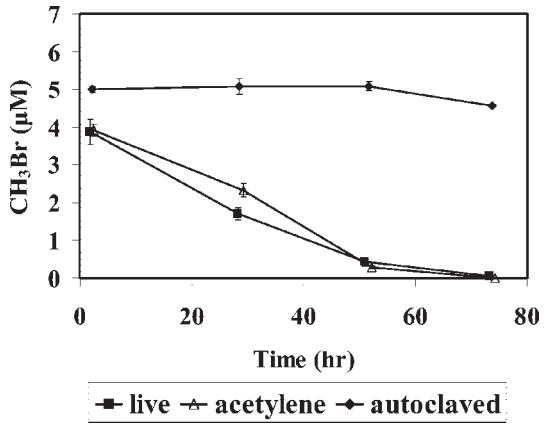

FIG. 2. Methyl bromide degradation by Oxy6 with and without addition of acetylene (1% [vol/vol]).

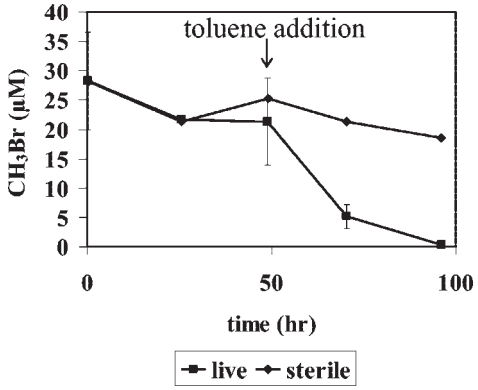

FIG. 3. Methyl bromide degradation by Oxy6 grown on marine broth before and after addition of 100 μM toluene.

Laboratory cultures were tested to see if they could help explain the observed effect of toluene on CH_3Br degradation in seawater and to test whether co-oxidation of MeBr occurs in well-characterized toluene utilizers. *L. methylohalidivorans* $MB2^T$ is a marine bacterium that can utilize CH_3Br as a sole source of carbon and energy (58) and is known to consume CH_3Br supplied at ambient levels (20). Addition of toluene did not inhibit CH_3Br degradation by *L. methylohalidivorans* (3.9 μM CH_3Br h^{-1} with or without toluene; cell counts not available). *Aminobacter* strain IMB-1, a terrestrial bacterium that utilizes CH_3Br (11) and consumes similarly low levels of CH_3Br (20), was tested similarly. Again, toluene was not found to inhibit metabolism of CH_3Br by this bacterial isolate (0.01 pmol CH_3Br cells^{-1} day^{-1} with or without toluene). The toluene-oxidizing bacteria *P. mendocina* KR1 and *P. putida* mt-2 were tested for CH_3Br degradation, but neither exhibited the ability to degrade CH_3Br.

Molecular phylogenetic analysis of Oxy6. Restriction digests (MspI, Sau3A, HhaI) of 27 plasmids containing inserted 16S rRNA sequences yielded identical banding patterns. Fifteen readable sequences were obtained from 10 clones yielding a contig of 1,450 bp (deposited into GenBank under accession no. AY858797). Coverage ranged from 2 to 9× except for the first 278 bp, which had 1× coverage of high quality sequence. Molecular phylogenetic analysis of the 16S rRNA sequence of Oxy6 grouped this bacterium within the family *Sphingomonadaceae* of the α-*Proteobacteria*, related to species of *Porphyrobacter*. The most closely related organisms were *Porphyrobacter tepidarius*, *Porphyrobacter donghaensis*, *Porphyrobacter* sp. KK348, *Porphyrobacter* sp. KK351, *Porphyrobacter* sp. MBIC3936, *Erythrobacter gaetbuli*, and unidentified bacteria UBA505839, AB105441, and AF235995, all with 97% similarity as computed by the Vector NTI AlignX module. Phylogenetic tree topology was not readily resolved by neighbor-joining, parsimony, or maximum-likelihood analysis. Bayesian analysis was performed in order to obtain confidence values for the tree topology. The Bayesian analysis revealed weak resolution of the *Erythrobacter* and *Porphyrobacter* clusters within the *Sphingomonadaceae*, with clade probabilities typically

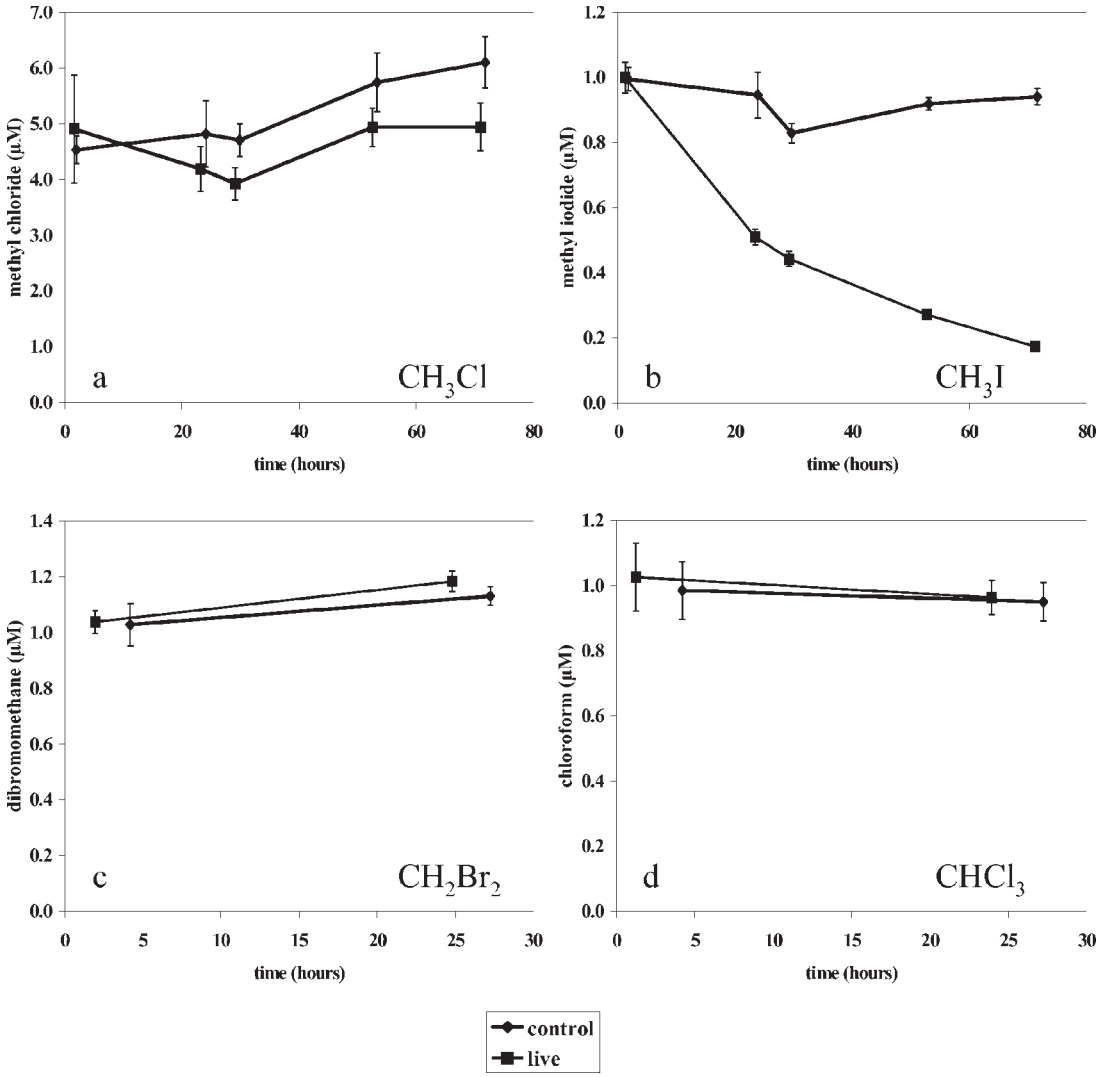

FIG. 4. Loss of (a) CH_3Cl, (b) CH_3I, (c) CH_2Br_2, and (d) $CHCl_3$ over time in vials containing live Oxy6 versus controls (sterile medium).

around 55%. The Bayesian tree (not shown) was consistent with visual inspection of the sequence alignment which showed that Oxy6 shared characteristics with both the *Porphyrobacter* and *Erythrobacter* clusters.

DISCUSSION

Given the broad geographic distribution of seawater sampling, it is noteworthy that toluene inhibited CH_3Br and CH_3Cl biodegradation to some extent in every sample (Table 1). These findings indicate that cometabolism of methyl halides by aromatic hydrocarbon utilizers may be widespread. The inhibition experiments performed with seawater were carried out at near-ambient concentrations of methyl halides (10 to 100× above ambient). Previous studies using the ^{13}C method have demonstrated that degradation at these concentrations displays first-order kinetics, without evidence of induction or nonlinearities (64, 65). In these studies, toluene was also added at concentrations approaching levels observed in seawater (100 to 200× above ambient) (7). Overall, these findings suggest that toluene can inhibit biological uptake of ambient CH_3Br in the ocean.

The pattern of substrate oxidation (Table 2) and CH_3Br degradation provides some insight into the metabolic pathways by which Oxy6 degrades CH_3Br. For example, Oxy6 did not oxidize *p*-cresol, 4-hydroxybenzoate, or protocatechuate, indicating that Oxy6 did not utilize the T4MO pathway (70). Acetylene did not inhibit CH_3Br degradation (Fig. 2), and *P. mendocina* KR1 was unable to degrade CH_3Br, providing further evidence against T4MO pathway involvement (40). The lack of inhibition by acetylene was also consistent with experiments performed with coastal seawater (19). The fact that *P. putida* mt-2, which uses the TOL pathway, did not degrade CH_3Br suggests that the TOL pathway also does not explain CH_3Br degradation in Oxy6, although it is unknown whether *P. putida* can transport CH_3Br into cells. Oxy6 appears to be equipped with a xylene monooxygenase that allows it to oxidize *o*-xylene and 2-methyl benzyl alcohol (Table 2). Perhaps this relatively uncommon ability to oxidize *o*-xylene (60) also confers the ability to degrade CH_3Br.

Several members of the *Sphingomonadaceae*, many of which have been isolated from contaminated terrestrial sediments,

have interesting degradative capabilities (4, 9, 18, 55). The isolation of Oxy6 from seawater highlights the potential of marine environments to harbor organisms with degradative abilities with potential use in bioremediation. It also suggests the possibility that such organisms may contribute to oceanic halocarbon uptake. Cometabolism of CH_3Br by a member of the *Sphingomonadaceae* that utilizes aromatic hydrocarbons appears to be a heretofore unrecognized mechanism of CH_3Br degradation.

Although Oxy6 did not degrade CH_3Cl, we have observed a lack of correlation between CH_3Br and CH_3Cl rate loss constants in the open ocean (64, 77), suggesting that these processes are not necessarily linked. In addition, stable isotope probing in soils revealed that different populations of bacteria assimilate CH_3Br and CH_3Cl as primary carbon sources (44). That work also indicated that a variety of bacteria mediate methyl halide degradation in soils and the cultured methyl halide utilizers poorly represent that diversity. This is likely the case in the oceans as well. Although we observed widespread toluene inhibition of methyl halide biodegradation, it was seldom complete, indicating that there are multiple pathways by which methyl bromide is metabolized in seawater. In addition, oceanic biodegradation of CH_3Br and CH_3Cl is widespread, with degradation rate constants (k_{biol}) of up to 20% day^{-1} observed in our studies in both subtropical and polar regions (64, 65, 66). With temperatures ranging from 30°C to −2°C, it is unlikely that the same organism is responsible in all these waters. Perhaps stable isotope and gene probing of environmental samples (44) will yield more information regarding the identities of the organisms responsible for oceanic biodegradation of halocarbons.

Bacterial models are needed to study the enzymatic and molecular mechanisms that ultimately control the global biological sink of halocarbons. Prior work on the environmental degradation of methyl halides has focused on C_1 utilizers because they are known to consume CH_3Br under standard laboratory conditions (24, 42, 50). The finding that *Leisingera methylohalidivorans* $MB2^T$ could uptake ambient levels of CH_3Br (20) indicated that it might provide an adequate bacterial model for CH_3Br biodegradation in seawater. However, toluene caused widespread inhibition of oceanic CH_3Br degradation, but it did not inhibit degradation of CH_3Br by MB2, suggesting that cometabolizers, rather than metabolizers, are perhaps the main consumers of CH_3Br in the open oceans. This hypothesis is supported by the idea that the pM concentrations of methyl halides found in the oceans should offer little sustenance to methyl halide metabolizers (29). In total, the evidence suggests that multiple organisms are involved in methyl halide biodegradation and this will complicate the search for a suitable bacterial model to bridge the gap between laboratory studies and environmental processes.

Conclusions. Toluene inhibited biodegradation of CH_3Br and CH_3Cl in coastal and open ocean seawater samples representing large areas of the marine environment ranging from the North Atlantic, North Pacific, subtropical Western Atlantic, and the Southern Ocean. The data suggest that cometabolism of methyl halides with aromatics is geographically widespread. A marine bacterium capable of cometabolic degradation of CH_3Br while growing on toluene or *o*-xylene was isolated. Sequencing identified the bacterium as being within the family *Sphingomonadaceae*, related to species within the *Porphyrobacter* genus. Substrate oxidation patterns and comparisons to other toluene oxidizers suggested that the T4MO pathway was not involved in CH_3Br degradation by this bacterium and that perhaps the ability to utilize *o*-xylene also conferred the ability to degrade CH_3Br. This appears to be the first report showing a link between aromatic hydrocarbon utilization and oceanic methyl halide biodegradation.

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