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Transformation of *ortho*-substituted biphenyls by *Methylosinus trichosporium* OB3b: substituent effects on oxidation kinetics and product formation

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Abstract The ability of *Methylosinus trichosporium* OB3b, expressing soluble methane monooxygenase, to oxidize a range of *ortho*-substituted biphenyls was examined to better understand how substituents affect both the rate and products of oxidation in comparison to biphenyl. Inhibition of oxidation was observed over the tested substrate range for both biphenyl and *ortho*-halogenated biphenyls (2-chloro-, 2-bromo-, and 2-iodobiphenyl). No inhibition was observed during the oxidation of 2-hydroxybiphenyl and 2-methylbiphenyl. Analysis of the products of oxidation showed that, depending on the substituent, ring hydroxylation, substituent oxidation, and elimination pathways could occur. The type and abundance of products formed along with the relatively high kinetic isotope effect observed for deuterated vs. nondeuterated biphenyl (k_h/k_d = 3.4±0.02) are consistent with mechanisms that include both hydrogen abstraction and NIH-shift pathways. Knowledge of these substituent-dependent reaction rates and mechanisms enhances our understanding of the methanotrophic aryl transformation potential and allows for better prediction of the formation of oxidized intermediates by methanotrophic bacteria.

Key words Methanotrophs · Soluble methane monooxygenase · Substituted biphenyls · Biodegradation

Abbreviations *sMMO* Soluble methane monooxygenase · *NMS* Nitrate mineral salts · *SIM* Selective ion monitoring · *TMSI N*-Trimethylsilylimidazone

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Introduction

Since the early 1970s, research on the environmental fate and degradation of polychlorinated biphenyls (PCBs) has focused on either hydroxylation and ring fission reactions under oxic and denitrifying conditions (Adriaens et al. 1991, 1999), or dechlorination under methanogenic or sulfate-reducing conditions (Bedard and Quensen 1995). This body of work has resulted in a mechanistic framework describing complementary heterotrophic dioxygenase-catalyzed ring hydroxylation and cleavage reactions which tend to favor (up to hexa-) chlorinated congeners, and reductive dechlorination of (di- to deca-) chlorinated congeners. Both systems tend to exhibit decreased activity against *ortho*-substituted congeners relative to *meta*- and *para*-substitution, although significant variation between strains and conditions has been reported. Dechlorination and oxidation of less chlorinated *ortho*-substituted congeners have been demonstrated in a limited number of sediments, sediment enrichments, and pure cultures (van Dort and Bedard 1991; Bedard and Quensen 1995; Cutter et al. 1998; Hrywna et al. 1999). The final product distribution is dependent on both the microbial community and the initial substitution pattern of the chlorobiphenyl congeners (Flanagan and May 1993; Bedard and Quensen 1995; Adriaens et al. 1999).

Methanotrophs, bacteria that utilize methane as their sole source of carbon and energy, have been shown to degrade aromatic hydrocarbons through the non-specific nature of the soluble form of the methane monooxygenase (sMMO). This form of MMO has been shown to oxidize a wide range of substrates, including aliphatic hydrocarbons with up to eight carbons as well as aromatic compounds including halogenated benzenes, toluene, and styrene (Colby et al. 1977; Burrows et al. 1984; Green and Dalton 1989). As methanotrophs are commonly found in many environments, their metabolic activity may affect the fate of PCBs. The role of methanotrophs in degrading PCBs however is poorly understood and has only recently been examined. Hydroxylated intermediates from *ortho*-

and *ortho-/para*-substituted congeners as well as evidence for an NIH-shift mechanism during methanotrophic oxidation of 2-chlorobiphenyl have been reported (Adriaens 1994; Adriaens and Grbić-Galić 1994). Furthermore, the products and kinetic isotope effect of the oxidation of deuterated ethylbenzene by purified sMMO indicated that not only was the NIH-shift involved during substrate oxidation, but multiple oxidative pathways also occurred (Dalton et al. 1981; Wilkins et al. 1994). This information led us to hypothesize that the NIH-shift mechanism observed using 2-chlorobiphenyl may not be the only pathway involved in the oxidation of other substituted aromatic compounds; and it is likely that a substituent-dependent hydroxylation mechanism operates. Here, we report on the ability of whole cells of *Methylosinus trichosporium* OB3b expressing sMMO to oxidize a range of *ortho*-substituted biphenyls and we interpret mechanistic pathways in the light of the products observed. As anaerobic degradation of highly chlorinated biphenyls typically transforms these compounds to *ortho*-substituted biphenyls, data on methanotrophic oxidation of these compounds will allow for better integration of both anaerobic and aerobic biodegradation processes for pollutant destruction.

Methods and materials

Culture conditions and experimental procedures

M. trichosporium OB3b was grown in nitrate mineral salts (NMS) at 30 °C as described previously (Lontoh and Semrau 1998), but with no added copper to allow the synthesis of sMMO. To verify that sMMO was responsible for the oxidation of aromatic substrates, *M. trichosporium* OB3b was also grown in the presence of 20 μM copper as $Cu(NO_3)_2$ to allow the synthesis of particulate (p)MMO. Biomass concentrations were measured as protein using the Biorad Protein Assay kit with bovine serum albumin as a standard (Life Science Research, Hercules, Calif.). The naphthalene assay specific for sMMO activity was used to monitor whole-cell expression of sMMO in all cell suspensions (Brusseau et al. 1990). For both oxygen-uptake and transformation studies, cells were collected by centrifugation (6000×*g* for 30 min) and resuspended to 0.2 g (wet wt) ml^{-1} in phosphate buffer.

Oxygen-uptake experiments

A 1.9-ml glass, water-jacketed reactor was used at a constant temperature of 30 °C to measure the rates of oxygen consumption at various initial substrate concentrations (n=5–8). An electrolyte and membrane-covered Clarke-type electrode (Instech Laboratories, Plymouth, Mass.) was inserted into the reactor using a ground glass port with two rubber o-rings and was connected to a biological oxygen monitor (Yellow Springs, Yellow Springs, Ohio). Monitor output was sent to an A/D converter board (DAS08-PGL, Computer Boards) for data collection using Labtech Notebook software (Wilmington, Mass.). In all assays, the reaction chamber was filled with phosphate buffer before the addition of cells or substrate. The electrode was calibrated daily (following manufacturer's instructions) after application of fresh electrolyte and membrane. Cells expressing sMMO were grown and harvested as described earlier. To further verify that sMMO was responsible for oxidation of biphenyl, cells expressing sMMO were inactivated with acetylene as described earlier (Lontoh et al. 1999) and oxygen uptake was measured in the presence of 125 μM biphenyl. As a second control, *M. trichosporium* OB3b expressing pMMO was also added to the oxygen-uptake reactor at a concentration of 0.2 g (wet wt) ml^{-1} and incubated with 125 μM biphenyl.

Oxygen uptake was monitored in the presence of methane or the substituted biphenyls. Methane was added by bubbling 4 ml of methane into the cell suspensions to obtain 1.4 mM methane in solution. The *ortho*-substituted biphenyls were added as saturated solutions of 1,4-dioxane, as most of these substituted biphenyls are sparingly soluble in water. 1,4-dioxane was chosen as the delivery solvent as it was found not to be oxidized by cells expressing sMMO, had no affect on the oxygen probe, and was mutually soluble with the substrates as found from the literature or predicted using the two-suffix Margules equation and the UNIFAC method (Lyman et al. 1982). Oxygen uptake for both chemical (substrate in solvent) and killed (autoclaved cells and substrate in solvent) controls was also measured to determine their effect on probe response and membrane stability. With the exception of 2-methylbiphenyl, these controls did not affect the stability of the oxygen-uptake probe. Fluctuations in the dissolved oxygen concentrations in the presence of 2-methylbiphenyl were observed, due to deterioration of the probe membrane by 2-methylbiphenyl itself. Such fluctuations were controlled by more frequent replacement of the probe membrane. The initial rates of oxygen uptake were measured after the substituted biphenyl was added.

During the assays, it was noticed that microbial activity decreased up to 50% over 8 h as measured by a decrease in oxygen uptake in the presence of 1.4 mM methane, possibly due to decay of active sMMO after harvesting the cells. To account for this variation when comparing oxygen uptake in the presence of different substituted biphenyls, the rates shown here are normalized to the rate of oxygen uptake in the presence of 1.4 mM methane (measured just prior to the measurement of oxygen uptake in the presence of a new concentration of aromatic substrate). The oxygen-uptake rates were also corrected for endogenous metabolism, i.e., the amount of oxygen utilized by *M. trichosporium* OB3b before the addition of any substrate.

Resting-cell transformation studies

Resting-cell incubations were conducted in sterile acid-rinsed 160-ml serum vials. Cells were grown in NMS medium with no added copper to allow the synthesis of sMMO. They were harvested in the late-exponential phase, pelleted, and resuspended in phosphate buffer to a density of 0.2 g ml^{-1}. Resuspended cells (100 μl) were removed to measure oxygenuptake activity in the presence of methane as previously described. The remaining resuspended cells were diluted with NMS medium to a concentration of 1.4–2.3 mg (wet wt) ml^{-1}. The vials were filled with 20–30 ml of this dilute cell suspension and supplemented with different *ortho*-substituted biphenyls, at the concentration where its observed oxygen-uptake rate was maximum. Three vials were sealed with teflon-lined red rubber septa and incubated at 30 °C and 270 rpm for 2 days. Another vial, serving as a time zero sample, was quenched with 20–30 ml of hexane and immediately frozen. Further, a killed control was prepared using autoclaved cells, and a chemical control was prepared using NMS medium and *ortho*-substituted biphenyl.

After sample incubation, a 5-ml aliquot was removed from each microcosm and subjected to three hexane extractions (1:1, v:v) to measure the amount of substituted biphenyl degraded. These extracts were pooled, dried over anhydrous $MgSO_4$ (EM Science, Gibbstown, N.J.), and concentrated to 0.1 ml using a rotoevaporator and a thermoevaporator (heated at 60 °C) with a gentle stream of N_2. To determine oxidative product(s), the remainder of each of the incubation mixtures was acidified to pH 2 with concentrated H_2SO_4 and then "salted out" with approximately 10 ml of a saturated NaCl solution. The resulting mixture was extracted three times with HPLC-grade ethyl acetate (Fisher Scientific, Pittsburgh, Pa.). These extracts were pooled and dried over anhydrous $MgSO_4$ and concentrated to 0.1 ml using rotoevaporation and thermoevaporation. If products were difficult to detect in these ex-

tracts, they were dried using thermoevaporation at 60 °C and then derivatized by the addition of *N*-trimethylsilyl-imidazone (TMSI; Alltech, Deerfield, Ill.) and acetonitrile, with subsequent heating at 60 °C (Kohler et al. 1988, 1993; Kitson et al. 1996).

Prepared samples were then analyzed on a mass spectrometer (Model MSD, Hewlett Packard, Palo Alto, Calif.) operated at 70 eV and interfaced to a gas chromatograph (GC; model 5890, Hewlett Packard). The GC column used was a 30-m DB-5 capillary column (J&W Scientific, Folsom, Calif.). The injection mode was split/splitless (split ratio = 10), and the injector and detector temperatures were held at 280 °C. The temperature program ran from 90 °C to 190 °C for 10 min at a rate of 5 °C min^{-1} and then from 190 °C to 280 °C at a rate of 20 °C min^{-1} for 10.5 min, with an initial and final holding time of 5 min. When standards were not available, the products of transformation were identified using a combination of linear (100–500 *m*/*z*) and selective ion-monitoring (SIM) analysis. During SIM analysis, masses in both the molecular ion cluster and the major fragments (loss of halogen, loss of TMSI, etc.) were used as fingerprints for the potential product(s).

Materials

All chemicals used in media preparation were of reagent grade or better. Highest purity methane (>99.99%) was obtained from Matheson Gas Company. Distilled-deionized water from a Corning Millipore D2 system was used for all experiments. The biphenyl substrates included electron-donating [hydroxy, methyl-] and electron-withdrawing [chloro-, bromo-, iodo-] *ortho*-substituents. Biphenyl was used as the unsubstituted reference compound.

Results

Mechanism and kinetics of biphenyl oxidation

As shown in Fig. 1, the oxidation rate of biphenyl by whole cells of *M. trichosporium* OB3b showed a clear maximum at 125 μM, with rates decreasing significantly as the biphenyl concentration increased above this value. Incubations of resting cells with 125 μM biphenyl resulted in approximately 10% of the added biphenyl being oxidized to all three of the possible (*ortho*-, *meta*-, and *para*-) hydroxybiphenyl isomers. As shown in Table 1, *para*-hydroxybiphenyl was the predominant monohydroxylated product (90%), followed by the *ortho*- (9%) and *meta*- (1%) isomers. Further oxidation of hydroxylated biphenyls was shown in the 2-hydroxybiphenyl incubation extracts, where two dihydroxybiphenyl isomers of equal abundance were detected. The dihydroxybiphenyls, however, could not be identified due to the lack of authentic standards for all possible isomers, although it could be concluded that the products were not 3, 4-, 2,5-, or 2,2′-dihydroxybiphenyls (for which standards exist).

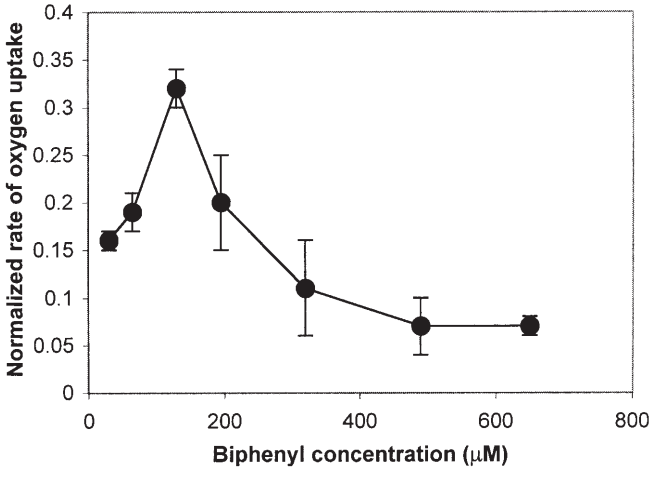

Fig. 1 Oxygen uptake in the presence of biphenyl normalized to oxygen uptake in the presence of methane. *Error bars* give the standard deviation for triplicate samples

Table 1 Products of substituted biphenyl oxidation by *Methylosinus trichosporium* OB3b

Substrate	Products	% Relative abundance
Biphenyl	2-Hydroxybiphenyl [a]	9
	3-Hydroxybiphenyl [a]	1
	4-Hydroxybiphenyl [a]	90
2-Hydroxybiphenyl	Dihydroxybiphenyls (2)	100
2-Methylbiphenyl	Hydroxymethylbiphenyls (2)	56
	2-Methylybiphenyl, α-hydroxy	44
2-Chlorobiphenyl	Hydroxychlorobiphenyls (3)	100
2-Bromobiphenyl	Hydroxybromobiphenyls (3)	41
	2-Hydroxybiphenyl	59
2-Iodobiphenyl	Hydroxyiodobiphenyls (4) [a]	18
	2-Hydroxybiphenyl [a]	82

[a]Derivatized samples

To better understand the mechanism(s) by which biphenyl is oxidized, kinetic isotope experiments were performed, as measured by the ratio of the maximum rate of oxygen uptake by whole cells of *M. trichosporium* OB3b in the presence of non-deuterated biphenyl to uniformly deuterated biphenyl (k_h/k_d). If this ratio is near unity, no isotope effect exists, indicating that a carbon–hydrogen bond is not broken during the rate-limiting step, i.e., oxygen is directly added to the aromatic ring via an arene oxide. If the ratio is greater than one, carbon–hydrogen bond breakage does occur, as observed in the formation of radical species in hydrogen abstraction reactions. For *M. trichosporium* OB3b, this ratio was found to be 3.4±0.02, indicating that a carbon–hydrogen bond in biphenyl is broken in the rate-limiting step.

Substituent effects on product distribution and oxidation kinetics

Since it was previously shown that 2-chlorobiphenyl was oxidized via an NIH-shift mechanism (Adriaens 1994) and it is demonstrated here that biphenyl undergoes direct hydroxylation of the aromatic ring, the effect of a range of *ortho*-substituents on product distribution and oxygen-uptake kinetics was evaluated. Concentration-dependent, protein-normalized oxygen-uptake rates for substituted biphenyls are shown in Fig. 2. With the exception of

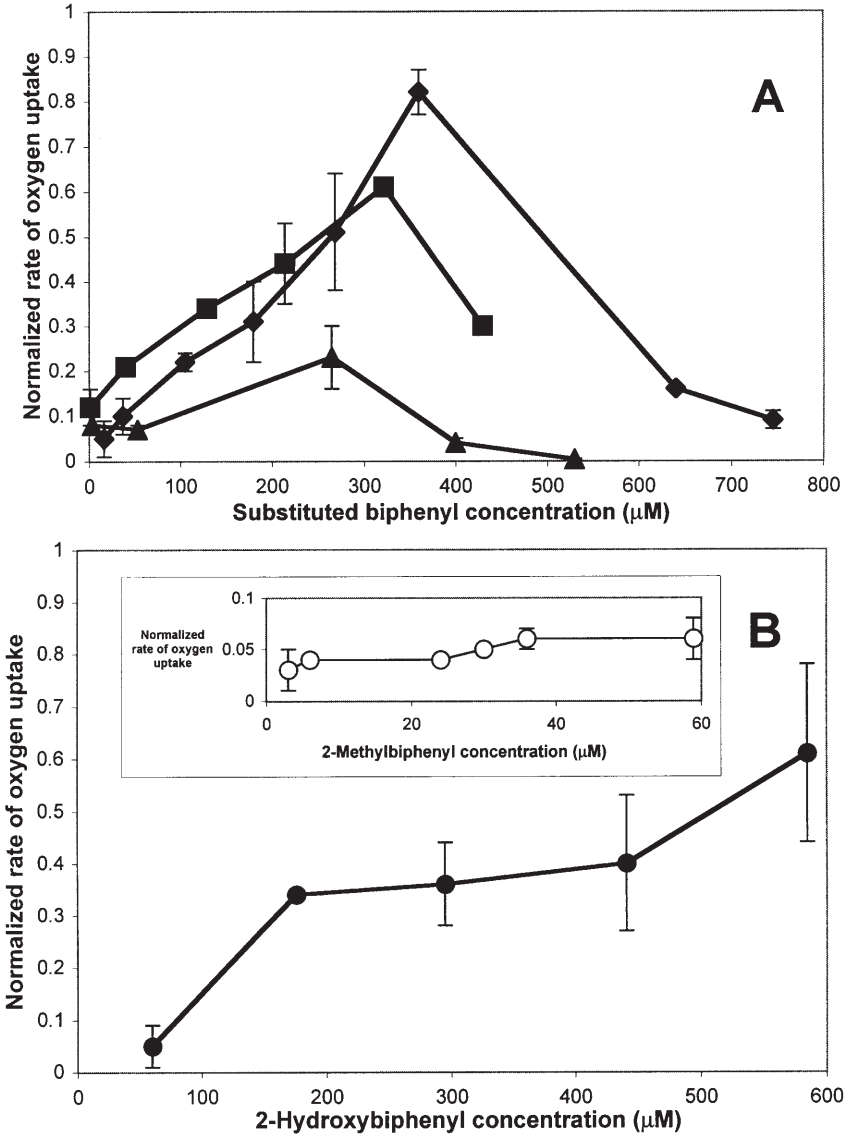

Fig. 2 Oxygen uptake in the presence of substituted biphenyls normalized to oxygen uptake in the presence of methane. **A** Oxygen uptake in the presence of 2-chlorobiphenyl (▲), 2-bromobiphenyl (■), and 2-iodobiphenyl (◆). **B** Oxygen uptake in the presence of 2-methylbiphenyl (❍), and 2-hydroxybiphenyl (●). *Error bars* give the standard deviation for triplicate samples

2-methyl- and 2-hydroxybiphenyl (shown in Fig. 2B), all substrates exhibited normalized rate curves with a clear maximum.

The products and their distribution are summarized in Table 1. Hydroxylation of the aromatic ring was the dominant reaction, followed by halogen elimination and hydroxylation of the alkyl side chain in the case of 2-methylbiphenyl. The products of oxidation of halogenated biphenyls included multiple, hydroxylated halobiphenyls (for 2-chloro-, 2-bromo-, and 2-iodo-biphenyl) and hydroxybiphenyl (i.e., the loss of the halogen substituent for 2-bromo- and 2-iodo-biphenyl). Counter to incubations with chlorobiphenyl (where three hydroxylated derivatives were observed), 2-hydroxybiphenyl accumulated as the primary product in the case of 2-bromo- and 2-iodobiphenyl.

Incubations with 2-methylbiphenyl offered the possibility of distinguishing between ring- and side-chain oxidation. The primary product was identified as the α-hydroxy derivative as shown in Fig. 3A, where oxidation of the methyl substituent to its benzyl alcohol derivative ($-CH_2OH$) was indicated by the appearance of prominent peaks at 165 *m/z* and 152 *m/z*, which correspond to the loss of $-H_2-Si(CH_3)_3$ and $-CH_2-O-Si(CH_3)_3$ from the TMSI-derivatized products. Ring oxidation also occurred, as two hydroxymethylbiphenyls were observed. As shown in Fig. 3B, the mass spectrum of one of these products does not have the prominent peaks in Fig. 3A, but rather one at 169 *m/z*, which corresponds to the loss of both a methyl- and the TMSI-derivative group. Since no isomer-specific identification of ring-hydroxylated products was possible with the methods employed, it could not be determined whether direct ring oxidation or NIH-shift rearrangements occurred during oxidation of these substrates.

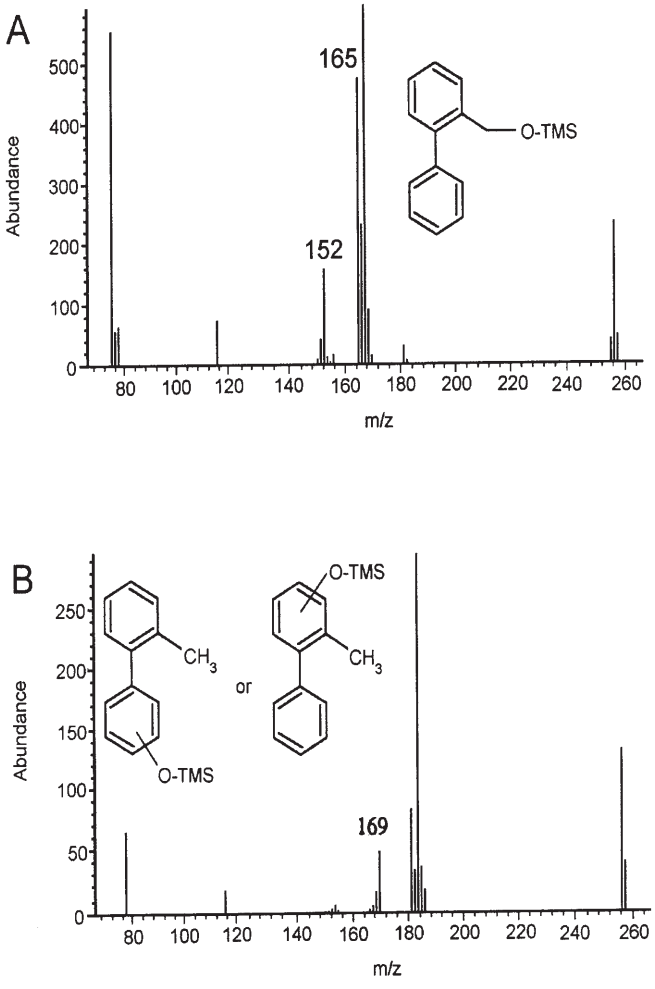

Fig. 3 Mass spectra of isolated products of 2-methylbiphenyl oxidation by *Methylosinus trichosporium* OB3b: **A** 2-methylbiphenyl, α-hydroxy-, and **B** hydroxylated methylbiphenyl product

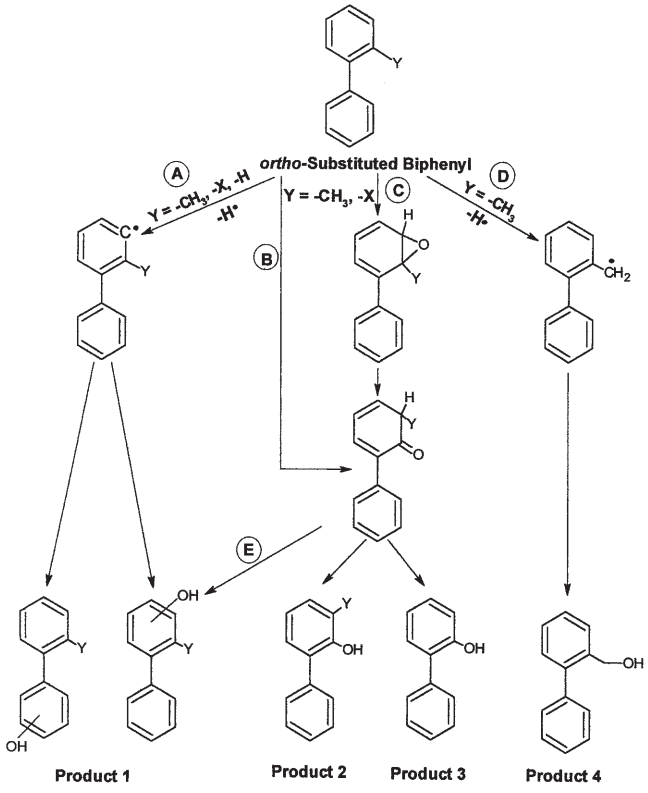

Fig. 4 Possible pathways followed in the oxidation of substituted biphenyls by *M. trichosporium* OB3b. Hydrogen abstraction pathways include substituent oxidation and ring oxidation to yield product 1 (the non-NIH-shifted, hydroxylated substituted biphenyl) or product 4 (the benzyl alcohol derivative of 2-methylbiphenyl). Product 1 may also be formed via a pathway with an arene oxide intermediate. Oxidation pathways involving the NIH-shift with rentention of the original substituent yield the NIH-shifted product (product 2). The same pathway with loss of the original substituent can also yield 2-hydroxybiphenyl (product 3)

Oxidation of biphenyl by cells either inactivated with acetylene or expressing pMMO

As negative controls to verify sMMO was responsible for the oxidation of the aromatic substrates, oxygen uptake in the presence of 125 μM biphenyl was measured for both *M. trichosporium* OB3b expressing sMMO inactivated with acetylene and *M. trichosporium* OB3b expressing pMMO. This concentration was chosen as the highest oxygen-uptake rates were observed for *M. trichosporium* OB3b expressing sMMO. No oxygen uptake was observed for either cells inactivated with acetylene or expressing pMMO, indicating that sMMO was responsible for the oxidation of the aromatic substrates.

Discussion

Previous research has shown that hydroxylated intermediates accumulate during methanotrophic oxidation of substituted aromatic compounds (Jeszequel and Higgins 1983; Dalton and Leak 1985; Adriaens 1994). Little information is available, however, on how strongly different substituents on the biphenyl ring affect degradation rates and product distribution. Considering that substrate oxidation by purified sMMO can follow multiple mechanisms (Wallar and Lipscomb 1996), it is important to determine the applicability of multiple pathways at the whole-cell level in order to assess the dominant transformation reactions resulting from methanotrophic activity in soils and sediments. From the product distribution of the oxidation of different substituted biphenyls by whole cells expressing sMMO, multiple oxidation mechanisms can be postulated as shown in Fig. 4. The mechanisms include both an NIH-shift and hydrogen abstraction, with the predominant mechanism dependent on the original *ortho*-substituent.

Specifically, from the data summarized in Table 1 and the high kinetic isotope effect for biphenyl, the stereospecificity for oxidation appears to favor the *para*-position. The phenyl ring is a weak *ortho*-, *para*-director, rendering both positions enriched in electrons which may be

favored by the electrophilic oxygen species generated by sMMO (Wallar and Lipscomb 1996). The kinetic isotope effect of 3.4 ± 0.02 measured with deuterated and non-deuterated biphenyl indicates that a C–H bond is broken during the rate-limiting step; and this would be consistent with a hydrogen abstraction mechanism (pathway A to yield Product 1 in Fig. 4). Furthermore, as dihydroxylated products are observed in the 2-hydroxybiphenyl (2HBP) extract, the initial products can be further hydroxylated, although it was not possible to determine where the oxidation occurred, due to the lack of authentic standards.

Oxidation of 2-chloro-, 2-bromo- and 2-iodo-biphenyl by whole cells of *M. trichosporium* OB3b yielded multiple halo-hydroxybiphenyls (products 1 and 2); however, the primary product from the 2-bromo- and 2-iodobiphenyl extracts was 2HBP (product 3). As shown in Fig. 4, the formation of these different products suggests that multiple oxidative pathways occurred. First, the 2-hydroxybiphenyl formed in the 2-bromo- and 2-iodo-biphenyl incubations may have resulted from an NIH-shift pathway (pathways B and C, with or without an arene oxide intermediate and with subsequent loss of the original substituent). Second, all three halogenated biphenyls may have undergone a hydrogen abstraction pathway (pathway A) resulting in a product with the original *ortho*-substituent intact (product 1). Third, it is possible that product 1 could also have formed from an intermediate arene oxide (pathway C followed by E). Fourth, whole cell degradation of these compounds could be initiated by attack at the substituted carbon resulting in the NIH-shifted product with or without the involvement of an arene oxide intermediate (product 2 from pathways B and C).

It should also be noted in Table 1 that the distribution of products was significantly different for the halogen-substituted biphenyls. The relative fraction of 2HPB increased with decreasing electronegativity of the halogen, suggesting that the iodo-substituent was not retained on the ring as easily as the bromo- or chloro-substituent. This is consistent with results from earlier studies on the pathways followed by cytochrome P450 in the presence of substituted benzenes (Jerina et al. 1967; Daly et al. 1972). This suggests that subsequent intramolecular rearrangement is influenced by the electronic properties of the aromatic substituent, regardless of whether the arene oxide intermediate is involved. If an arene oxide intermediate was formed, one could hypothesize that the more electronegative substituents (chlorine and bromine) created more positive carbocation intermediates in the NIH-shift pathway. Subsequently, these substituents would be attracted to the adjacent positively charged carbon, resulting in a greater retention than for the iodo-substituent (product 2). If the arene oxide was not formed, the relative ease of iodine to leave the ring compared to chlorine or bromine, combined with its bulky size, could result in less retention on the ring and a greater abundance of 2HBP produced. Such a finding indicates that multiple pathways are possible for the whole-cell oxidation of substituted biphenyls by methanotrophs expressing sMMO.

Evidence of other enzymatic mechanisms followed by sMMO with *ortho*-substituted biphenyls was observed in incubations of resting cells with 2-methylbiphenyl (2MBP). Oxidation of the methyl group on 2-methylbiphenyl to yield a benzyl alcohol (pathway D to form Product 4) indicates the substituent itself can be oxidized. For 2MBP, the mechanism is most likely to be the same used for methane oxidation, i.e., abstraction of a hydrogen by the highly electrophilic ferryl active site of sMMO to yield a radical and then an alcohol (Shu et al. 1997). Not only was the substituent oxidized, however, but also two hydroxymethylbiphenyls were detected. Again, these products could have been formed either from oxidation of the ring with no substituent rearrangements or via an NIH-shift in which the methyl group was transferred (products 1 and 2). It was not possible to determine which pathway is preferred, but since evidence for the NIH-shift (involving partial retention of the original substituent) has been observed in this study, these results support previous studies that no single mechanism operates during the catalytic oxidation by cells expressing sMMO (Dalton and Leak 1985; Wilkins et al. 1994; Wallar and Lipscomb 1996). The predominant mechanistic pathway however is strongly influenced by the type of substituent present on the ring.

The substituent effects on substrate oxidation kinetics are less readily apparent, partly due to the fact that for none of the tested substrates could concentration-dependent rates be fitted to a general model. For example, the rates of biphenyl oxidation (which is hydroxylated via hydrogen abstraction and thus radical formation, as inferred from relatively high kinetic isotope effects) exhibited a clear trend in which oxidation rates initially increased with increasing substrate concentrations followed by a decrease. The halogen series (2-chloro-, 2-bromo-, and 2-iodo-biphenyl) also exhibited a similar pattern of inhibition. The general substrate inhibition model, however, did not fit these data well. This may be due to the fact that these compounds were oxidized via multiple pathways and that intermediates of these pathways may have different levels of toxicity. For example, it is possible that toxicity during methanotrophic oxidation of the halogenated biphenyls is related to the stability of the corresponding halogenated aromatic radicals generated after hydrogen abstraction. Heterotrophic monooxygenases have been shown to be sensitive to aromatic halogens as they uncouple electron flow, resulting in the inhibition of the hydroxylation reaction (Kohler et al. 1988; Adriaens and Focht 1991). As chlorine is more electronegative than bromine or iodine, it is possible that the intermediates formed during 2-chlorobiphenyl oxidation were more reactive than those formed in 2-bromo- and 2-iodobiphenyl, thus yielding less stable intermediates. This conclusion is supported by confirming that the halogen substituent was more easily removed going down the halogen series as shown by the product distribution in Table 1 and that the overall rates of oxygen uptake increased with decreasing electronegativity of the halogen substituents.

In conclusion, this study extends earlier findings of the broad range of substrates that whole cells expressing

sMMO can degrade, by showing degradation of different *ortho*-substituted biphenyls. The product distribution of these compounds also shows that multiple pathways observed at the enzyme level are also apparent at the whole-cell level (Wallar and Lipscomb 1996). Such a result is important as it indicates that results from purified sMMO can be used to predict the fate of these compounds when whole-cell methanotrophic activity is stimulated. As *ortho*-substituted biphenyls can accumulate during anaerobic degradation of PCBs, these studies provide a foundation on how methanotrophs can be used in conjunction with anaerobic degradation for the removal of highly chlorinated biphenyls. Further work should be done to determine how the products of methanotrophic oxidation of *ortho*-substituted biphenyls are further oxidized by heterotrophic microorganisms to ensure complete mineralization.

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References

Adriaens P (1994) Evidence for chlorine migration during oxidation of 2-chlorobiphenyl by a type II methanotroph. Appl Environ Microbiol 60:1658–1662

Adriaens P, Focht D (1991) Evidence for inhibitory substrate interactions during cometabolism of 3.4-dichlorobenzoate by *Acinetobacter* sp. strain 4CB1. FEMS Microbiol Ecol 85:293–300

Adriaens P, Grbić-Galić D (1994) Cometabolic transformation of mono- and dichlorobiphenyls and chlorohydroxybiphenyls by methanotrophic groundwater isolates. Environ Sci Technol 28:1325–1330

Adriaens P, Huang C-M, Focht, DD (1991) Biodegradation of PCBs by aerobic microorganisms. In: Baker RA (ed) Organic substances and sediments in water, vol 1. Lewis, Chelsea, Mich., pp 311–327

Adriaens P, Barkovskii AL, Albrecht ID (1999) Fate of chlorinated aromatic compounds in soils and sediments, In: Bollag DCAM, Frankenberger, WT, Sims R (eds) Bioremediation of contaminated soils. Soil Science Society of America/American Society of Agronomy Monograph, Soil Science Society of America Press, Madison, Wis., pp 175–205

Bedard DL, Quensen JF III (1995) Microbial reductive dechlorination of polychlorinated biphenyls. In: Young LY, Cerniglia CE (ed) Microbial transformation and degradation of toxic organic chemicals. Wiley, New York, pp 127–217

Brusseau GA, Tsien H-C, Hanson RS, Wackett LP (1990) Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase. Biodegradation 1:19–29

Burrows KJ, Cornish A, Scott D, Higgins IJ (1984) Substrate specificities of the soluble and particulate methane monooxygenases of *Methylosinus trichosporium* OB3b. J Gen Microbiol 5:335–342

Colby J, Stirling DI, Dalton H (1977) The soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). Biochem J 165:395–402

Cutter L, Sowers KR, May HD (1998) Microbial dechlorination of 2,3,5,6-tetrachlorobiphenyl under anaerobic conditions in the absence of soil or sediment. Appl Environ Microbiol 64:2966–2969

Dalton H, Leak DJ (1985) Mechanistic studies on the mode of action of methane monooxygenase. In: Degn H, Cox RP, Toftlund H (eds) Gas enzymology. Reidel, Boston, Mass., pp 169–186

Dalton H, Golding BT, Waters BW, Higgins R, Taylor JA (1981) Oxidations of cyclopropane, methylcyclopropane, and arenes with the mono-oxygenase system from *Methylococcus capsulatus*. J Chem Soc, Chem Commun 10:482–483

Daly JW, Jerina DM, Witkop B (1972) Arene oxides and the NIH-shift: the metabolism, toxicity, and carcinogenicity of aromatic compounds. Experientia 28:1120–1164

Flanagan WP, May RJ (1993) Metabolite detection as evidence for naturally occurring aerobic PCB biodegradation in Hudson River sediments. Environ Sci Technol 27:2207–2212

Green J, Dalton H (1989) Substrate specificity of soluble methane monooxygenase J Biol Chem 264:17698–17703

Hrywna Y, Tsoi TV, Maltseva OV, Quensen JF III, Tiedje JM (1999) Construction and characterization of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. Appl Environ Microbiol 65:2163–2169

Jerina D, Daly J, Landis W, Witkop B, Udenfriend S (1967) Intramolecular migration of tritium and deuterium during nonenzymatic aromatic hydroxylation. J Am Chem Soc 89:3347–3349

Jezequel SG, Higgins IJ (1983) Mechanistic aspects of biotransformations by the monooxygenase system of *Methylosinus trichosporium* OB3b. J Chem Tech Biotechnol 33B:139–144

Kitson FG, Larsen BS, McEwen CN (1996) Gas chromatography and mass spectrometry: a practical guide. Academic Press, San Diego, Calif.

Kohler H-P E, Kohler-Staub D, Focht DD (1988) Degradation of 2-hydroxybiphenyl and 2, 2′-dihydroxybiphenyl by *Pseudomonas* sp. strain HBP1. Appl Environ Microbiol 54:2683–2688

Kohler H-PE, van der Maarel MJEC, Kohler-Staub D (1993) Selection of *Pseudomonas* sp. strain HPB1 Prp for metabolism of 2-propylphenol and elucidation of the degradative pathway. Appl Environ Microbiol 59:860–866

Lontoh S, Semrau JD (1998) Methane and trichloroethylene degradation by *Methylosinus trichosporium* OB3b expressing particulate methane monooxygenase. Appl Environ Microbiol 64:1106–1114

Lontoh S, DiSpirito AA, Semrau JD (1999) Dichloromethane and trichloroethylene inhibition of methane oxidation by the membrane-associated methane monooxygenase *of Methylosinus trichosporium* OB3b. Arch Microbiol 171:301–308

Lyman WJ, Reehl WF, Rosenblatt DH (1982) Handbook of chemical estimation methods. McGraw-Hill, New York

Shu L, Nesheim JC, Kauffmann K, Munck E, Lipscomb JD, Que L Jr (1997) An $Fe_2^{IV}O_2$ diamond core structure for the key intermediate Q of methane monooxygenase. Science 275:515–518

van Dort HM, Bedard DL (1991) Reductive *ortho*- and *meta*-dechlorination of a polychlorinated biphenyl congener by anerobic microorganisms. Appl Environ Microbiol 57:1576–1578

Wallar BJ, Lipscomb JD (1996) Dioxygen activation by enzymes containing binuclear non-heme iron clusters. Chem Rev 96: 2625–2657

Wilkins PC, Dalton H, Samuel CJ, Green J (1994) Further evidence for multiple pathways in soluble methane-monooxygenase-catalyzed oxidations from the measurement of deuterium kinetic isotope effects. Eur J Biochem 226:555–560