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Biodegradation of dibenzo-*p*-dioxin, dibenzofuran, and chlorodibenzo-*p*-dioxins by *Pseudomonas veronii* PH-03

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Abstract

The dioxin-degrading strain *Pseudomonas veronii* PH-03 was isolated from contaminated soil by selective enrichment techniques. Strain PH-03 grew on dibenzo-*p*-dioxin and dibenzofuran as a sole carbon source. Further, 1-chlorodibenzo-*p*-dioxin, 2-chlorodibenzo-*p*-dioxin and other dioxin metabolites, salicylic acid, and catechol were also metabolized well. Resting cells of strain PH-03 transformed dibenzo-*p*-dioxin, dibenzofuran, 2,2′,3-trihydroxybiphenyl, and some chlorodioxins to their corresponding metabolic intermediates such as catechol, salicylic acid, 2-hydroxy-(2-hydroxyphenoxy)-6-oxo-2,4-hexadienoic acid, and chlorocatechols. The formation of these metabolites was confirmed by comparison of gas chromatography–mass spectrometry (GC–MS) data with those of authentic compounds. Although we did observe the production of 3,4,5,6-tetrachlorocatechol (3,4,5,6-TECC) from 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) with resting cell suspensions of PH-03, growth of strain PH-03 in the presence of 1,2,3,4-TCDD was poor. This result suggests that strain PH-03 is unable to utilize 3,4,5,6-TECC, even at very low concentration (0.01 mM) due to its toxicity. In cell-free extracts of DF-grown cells, 2,2′,3-trihydroxybiphenyl dioxygenase, 2-hydroxy-6-oxo-6-phenyl-2,4-hexadienoic acid hydrolase, and catechol-2,3-dioxygense activities were detected. Moreover, the activities of *meta*-pyrocatechase and 2,2′,3-trihydroxybiphenyl dioxygenase from the crude cell-free extracts were inhibited by 3-chlorocatechol. However, no inhibition was observed in intact cells when 3-chlorocatechol was formed as intermediate.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) belong to the class of two-ring aromatic compounds containing one to eight chlorine atoms. The structures of PCDD/Fs are chemically very stable due to the absence of reactive groups, and hence these compounds are degraded very slowly in the environment (Pollitt 1999). These halogenated compounds have never been produced for a specific purpose, yet they have become ubiquitous pollutants because they are formed during incineration and as byproducts of the synthesis of haloaromatic compounds (Meharg & Osborn 1995). In addition to being recalcitrant in the environment, these compounds are highly toxic and carcinogenic (Oh et al. 1999; Pollitt 1999). Hence, these compounds are of great concern to the environment and human health. These concerns have led to increased efforts to find effective detoxification and degradation processes.

The microbial degradation of dibenzo-*p*-dioxin (DD), its monochlorinated derivatives, and dibenzofuran (DF) has been studied by several groups (Cerniglia et al. 1979; Klecka et al. 1979, 1980); however, in those studies oxidation was performed cometabolically by bacteria that can grow on other aromatic compounds. Over the last decade, several aerobic bacterial strains that can utilize DD and DF as the sole source of carbon and energy, and co-oxidize their chlorinated derivatives, have been isolated and characterized (Fortnagel et al. 1990; Habe et al. 2001; Hong et al. 1999; Keim et al. 1999; Monna et al. 1993; Wilkes et al. 1992; Wittich et al. 1992). Several key enzymes of the degradative pathway have been investigated in detail (Bünz & Cook, 1993; Bünz et al. 1993). Some of these isolates are known to be effective biocatalysts, although for the compounds considered here their catabolic activity is limited to some di- and trichlorinated dibenzo-*p*-dioxins and dibenzofurans. In most cases, however, these microorganisms degrade specific isomers, and thus dioxin degradation is greatly affected by the chlorine substitution pattern (Bünz & Schmidt 1997; Keim et al. 1999; Parsons & Storms 1989; Schreiner et al. 1997; Wilkes et al. 1996).

Under anaerobic conditions PCDDs and PCDFs are converted into less chlorinated derivatives by methanogenic consortia (Adriaens et al. 1995; Beurskens et al. 1995). A recent study reported the isolation and characterization of anaerobes that could remove chlorines from chlorinated dioxins (Bunge et al. 2003). Although many dioxin degraders are capable of degrading low chlorinated derivatives, only very limited information is available regarding the aerobic bacteria that degrade tetrachloro- or higher chlorinated dioxins or dioxin-like compounds (Hong et al. 2002). Moreover, many strains described in previous studies showed co-oxidation potential on chlorinated dioxins. To date, no aerobic bacteria have been identified that are capable of utilizing chlorinated dioxins as a growth substrate. In the present study we characterize the degradation of two monochlorinated and one tetrachlorinated dioxins by a newly isolated bacteria *Pseudomonas veronii* PH-03, under conditions where the dioxins were the sole growth substrate. This is the first demonstration of aerobic transformation of these compounds by this strain.

Materials and methods

Isolation and identification of bacteria

A mixed culture obtained from soil samples collected near the incinerator of a chemical plant in the southern part of the Republic of Korea served as the starting material for the isolation of a pure culture. Soil samples were placed in sterilized 50-ml plastic centrifuge tubes. Samples were not refrigerated or frozen during transportation or storage. Soil samples (0.5 g) were added to 10 ml of phosphate buffer (pH 7.0). After vigorous shaking, particles were removed by centrifugation at $1000 \times g$ for 10 min. One hundred microliters of the clear suspension was then inoculated into 100 ml of nutrient broth and incubated overnight in a shaking incubator (160 rpm at 28 °C). One hundred microliters of the resulting liquid culture was streaked onto solid nutrient media. After 3 days of incubation, each colony was separately inoculated into 1 ml of minimal salt medium (MSM) containing 1 mM DD as the sole carbon source in a 15 ml centrifuge tube with a silicon stopper. In all experiments, MSM and solid MSM containing 1 mM DD or DF as the sole carbon source were prepared by the method described by Fortnagel et al. (1990). After 3 days of incubation, we observed the bacterial growth (based on an increase in turbidity) in one of the tubes. From this tube, 100 μl of the culture was streaked onto solid MSM containing 1 mM DD. Finally all colonies from solid media were screened for their ability to utilize dioxins in the same MSM containing 1 mM DD. Dioxin utilization was defined in terms of bacterial growth and substrate depletion in the growth culture. Growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}) in a spectrophotometer (Cary 3-bio, Varian, Victoria, Australia). Substrate depletion and dioxin concentration in the culture medium were measured using high-performance liquid chromatography (HPLC) after extraction three times with 5 ml of ethyl acetate. The extracts were dried under reduced pressure and redissolved in acetonitrile. In this experiment, 1 mM DF in acetone was added as the internal standard prior to the extraction. HPLC was performed using a liquid chromatograph (1100 series, Agilent, Waldbronn, Germany) equipped with an XDB-C18 column (125×30 mm, 5 μm) (Agilent) and diode array

detector. The aqueous solvent system (flow rate, 1.0 ml/min) contained 0.1% (wt/vol) phosphoric acid and 80% acetonitrile. Based on the screening results, we isolated a strain that could efficiently grow on DD and characterized that strain using a semiautomated microbial characterization test system (Biolog, Hayward, CA), fatty acid analysis, and comparative 16S rRNA gene analysis. The 16S rRNA gene was amplified by PCR from total DNA of this strain, and the nucleotide sequence was determined (Pearson & Lipman 1988). Nucleic acid sequence similarities were searched for the identification of isolated strain using the BLAST program on the EMBL and GeneBank databases. The gene sequence was deposited in the NCBI gene bank (Accession number AY091598). We named the isolated strain PH-03.

Chemicals

Catechol, 3,4,5,6-tetrachlorocatechol (3,4,5,6-TECC), salicylic acid, benzoic acid, and DF were purchased from Aldrich (St. Louis, MO). 3-Chlorocatechol was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). DD, 1-monochlorodibenzo-*p*-dioxin (1-MCDD), 2-monochlorodibenzo-*p*-dioxin (2-MCDD), and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) were purchased from Accustandard (New Haven, CT). 2,2′,3-Trihydroxybiphenyl (2,2′,3-THB) was obtained from Wako Pure Chemical (Tokyo, Japan). Agar, solvents and phosphoric acid were purchased from Merck (Darmstadt, Germany). All chemicals were of the highest grade commercially available.

Growth of the PH-03 strain on various substrates

To evaluate the ability of strain PH-03 to utilize dioxins and chlorinated dioxins, we examined growth of this strain in the presence of DD, 1-MCDD, 2-MCDD, 1,2,3,4-TCDD or DF (each at 1 mM). In addition to dioxins, several potential metabolic intermediates of dioxin degradation were also tested, namely catechol, salicylic acid, and 3,4,5,6-TECC. In the case of 1,2,3,4-TCDD, the substrate stock solution in acetone (10 mg/ml) was briefly heated in a 70 °C water bath to completely dissolve the 1,2,3,4-TCDD and then added to 25 ml of sterile MSM medium in a 250 ml Erlenmeyer flask to a final concentration of 1 mM. This medium was then inoculated with the PH-03 strain pregrown in MSM medium containing DF (1 mM) that had been washed three times ($8000 \times g$ at 4 °C for 20 min) in phosphate buffer (20 mM, pH 7.0). The flasks were maintained at 28 °C in a rotatory shaker (160 rpm). The OD_{600} and colony-forming units per milliliter were measured by the conventional plating method using nutrient agar every 4 or 6 h.

Biotransformation of dioxins and 2,2′,3-THB by resting cell suspensions

In order to find and identify metabolic intermediates, biotransformation studies were conducted using resting cells. The strain PH-03 was pregrown with 1 mM DF. After 3 days of incubation, the entire culture was inoculated into 250 ml of MSM containing 0.5 g of DF in a 2-l Erlenmeyer flask. After overnight incubation, the entire culture was harvested ($11{,}000 \times g$ for 10 min at 4 °C) and washed three times with 20 mM phosphate buffer. The optical density of the resting cell suspension was adjusted to 4.0 (OD_{600}) with 20 mM sterile sodium phosphate buffer. Then 10 ml of the cell suspension was added to sterile Erlenmeyer flasks (125 ml) containing DD, DF, chlorinated dioxins and 2,2′,3-THB. Control flasks received cells that had been inactivated by poisoning with 10 mM sodium azide and incubation at 75 °C for 1 h. The flasks were incubated at 28 °C under dark conditions in a rotatory shaker (160 rpm) for 5 days. In the case of 2,2′,3-THB, flasks were removed as soon as the culture turned into yellow; transformation of 2,2′,3-THB was also monitored spectrophotometrically. The flasks and the corresponding controls (prepared using the methods described below) were extracted four times with a one-fifth volume of chilled ethyl acetate. This procedure was repeated after acidification of the remaining aqueous phase to pH 3.5 with *ortho*-phosphoric acid. Extracts were combined, dried over anhydrous sodium sulfate, and ethyl acetate was evaporated under reduced pressure. Prior to the GC–MS measurements, samples were derivatized by using BSTFA [*N,O*-bis(trimethylsilyl)-trifluoroacetamide] to form trimethylsilyl derivatives by incubation at 60 °C for 1 h. The resulting derivatives were analyzed by HRGC ion-trap MS without further purification. GC–MS analyses were carried out on a Trace GC 2000 system (Thermoquest, San Jose, CA) linked to a mass

spectrometer (Finnigan Polaris Q, Thermoquest) by a 60 m DB-5 MS column. The initial temperature of 60 °C was maintained for 2 min, and then the temperature was increased to 290 °C over 10 min, after which it was held at 290 °C for 10 min. In all experiments, which were generally repeated three times, corresponding controls were employed using heat-inactivated (75 °C for 1 h) and poisoned (10 mM NaN_3) cultures. Metabolites were identified by comparison of their mass spectra and retention times with those of authentic compounds.

Determination of oxygen uptake rates

Oxygen uptake rates of strain PH-03 with various dioxin substrates were determined polarographically at 25 °C using a Clark-type electrode (Oxygraph, Hansatech, Norfolk, UK). Assays were performed with washed cells suspended in 20 mM phosphate buffer (pH 7.0) to an OD_{600} of about 1.0 in a volume of 1 ml. Stock substrate solutions were made up in DMF (100 mM). Final substrate concentrations of 0.1 mM were used for all assays, except for 1,2,3,4-TCDD, which was used at concentrations of 0.02 mM. Uptake rates were corrected for endogenous oxygen consumption.

Preparation of cell free extracts

Cells pregrown in the presence of 1 mm DF for 3 days at 28 °C were harvested by centrifugation (at 8000 × *g* for 20 min at 4 °C). The pellets were washed three times in sodium phosphate buffer (20 mM, pH 7.5) to remove traces of DF. The pellets were then resuspended in 50 mM sodium phosphate buffer (pH 7.5). For catechol 2,3-dioxygenase, the buffer was supplied with 10% acetone (v/v). Cell extraction was carried out by incubating the cell suspension in protein extracting reagents, as per the manufacturer's protocol (BugBuster™, Novagen, Darmstadt, Germany), followed by sonication at 300 W for 10 min under a pulsed 75%-duty-cycle condition (Sonosmasher, Ulsso-Tech, Seoul, Republic of Korea). Cell debris was removed by centrifugation at 17,000 × *g* for 1 h at 4 °C. The supernatant was immediately used in the enzyme activity measurements described in the next section. The protein content of the extract was measured by the Bradford method using bovine serum albumin as a standard (Bradford 1976).

Enzyme activities

Enzyme activities were determined spectrophotometrically using a UV–VIS spectrophotometer equipped with thermojacket cuvette holder and a water circulation system (Cary 3-Bio, Varian, Victoria, Australia). Extradiol dioxygenase (2,2′,3-trihydroxy biphenyl-2,3-dioxygenase) was determined by the method described by Happe et al. (1993) using 2,2′,3-trihydroxy biphenyl as a substrate. HOPDA hydrolase (2-hydroxy-6-oxo-6-2 (hydroxy phenyl)-hexa 2,4 dienioc acid hydrolase) activity was measured by the method of Omori et al. (1986) using HOPDA in 50 mM (phosphate buffer pH 7.5). Pyrocatechase (Catechol 1,2-dioxygenase activity) and metapyrocatechase were determined using catechol as a substrate by the methods described by Nokazawa & Nokazawa (1970) and Nozaki (1970), respectively. The enzyme activities were expressed as the amount of enzyme that converts one micromole of substrate/mg of protein/min. The inhibitory activity of 3-chlorocatechol, a well-known inhibitor of *meta*-cleavage enzymes, was also tested. The enzyme assay mixture was preincubated with 3-chlorocatechol (0.05 mM) for 1 min before initiation of the reaction with the appropriate substrates, as described previously (Wilkes et al. 1992).

Results

Identification of Pseudomonas *sp. PH-03*

A pure culture capable of growing in dioxins was isolated from a mixed culture obtained from contaminated soil. The strain was identified as Gram-negative, aerobic, and positive for oxidase and catalase. The isolated strain was identified as *Pseudomonas* sp. (89%) using the BIOLOG test system (Biolog, Hayward, CA). Further, analysis of 16S rRNA revealed that the strain was very close to *P. veronii* (99.6% homology). The gene sequence was deposited in the NCBI gene bank and the strain was named PH-03.

Growth of strain PH-03 with DD, DF, and other chlorinated dioxins

The ability of *P. veronii* PH-03 to utilize various dioxins, including DD, chlorinated congeners of

DD, and DF, as the sole growth substrate was tested in liquid MSM containing the substrate compound at a concentration of 1 mM. The growth and substrate utilization results are shown in Figures 1 and 2 respectively. The results show that the growth of strain PH-03, expressed as the increase in colony-forming units and turbidity (OD_{600}), correlates with the substrate depletion (Figure 2). Strain PH-03 grew on DD, DF, 1-MCDD, and 2-MCDD. The culture containing 1-MCDD and 2-MCDD became dark brown after 1–2 days of incubation, after which time no further growth was observed. The doubling time of the bacterial growth during the exponential growth phase ranged from 11.11 h for DD to 12.54 h for DF. Removal of DD, DF and chlorinated derivatives was directly associated with increase in microbial growth.

After 60 h of incubation, the initial substrate content of DD, DF, 1-MCDD, and 2-MCDD culture was reduced by 90.7%, 79.7%, 88.3%, and 78.6% respectively. In contrast, no significant growth was observed in the 1,2,3,4-TCDD culture, which exhibited a substrate reduction of only 10.6% (Figure 2). The inability of PH-03 to grow on 1,2,3,4-TCDD may be due to the low solubility of the compound (Shin et al. 1988), which leads to there being insufficient substrate in the culture broth to provide a carbon source for the bacteria or it may be due to the production of a toxic metabolic intermediate such as 3,4,5,6-TECC. Additional experiments with salicylic acid and catechol showed that these compounds could also serve as the carbon and energy sources for growth of strain PH-03 (data not shown); however, this strain could not grow on 3,4,5,6-TECC. In addition, when 1,2,3,4-TCDD was incubated with resting cell suspensions of strain PH-03 we did not observe the production of anticipated metabolic intermediates such as tetrachloro-*cis*-*cis*-2,4-hexadienoic acid that are typically formed during biodegradation of this compound.

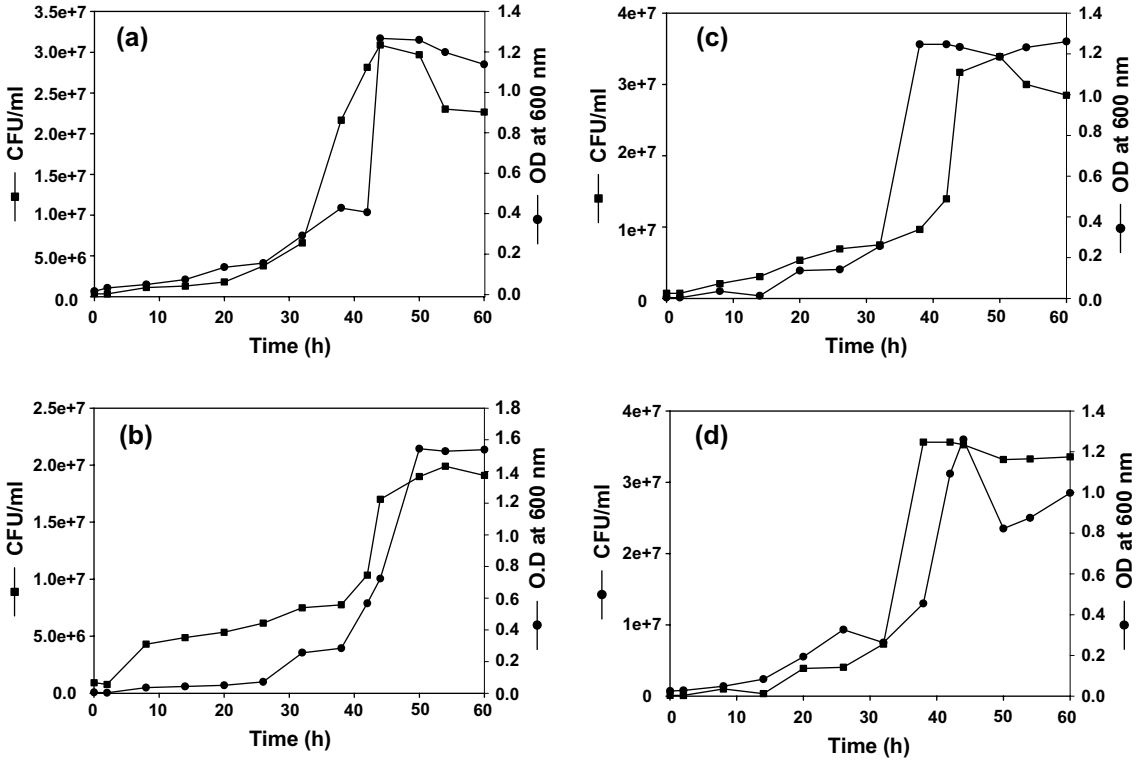

Figure 1. Growth of strain PH-03 with 1 mM of dibenzo-*p*-dioxin (a), dibenzofuran (b), 1-monochlorodibenzo-*p*-dioxin (c), and 2-monochlorodibenzo-*p*-dioxin (d) as the sole carbon source. Colony-forming unit/ml (-■-) and optical density at 600 nm (-●-) were shown.

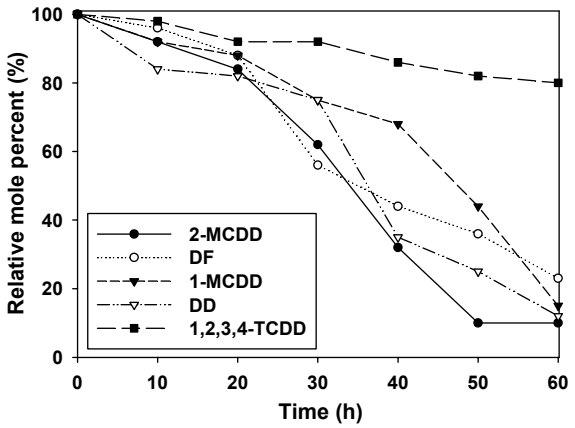

Figure 2. Depletion of DD, DF, 1-MCDD, 2-MCDD, and 1,2,3,4-TCDD according to growth or transform of PH-03. In order to measure the depletion rate of the substrates, the whole cultures of which initial concentration is 1 mM and total volume is 5 ml are sacrificed every 10 h.

Oxygen uptake rates of resting cell suspensions

The oxygen uptake rate of the washed cell suspension of PH-03 grown on DF was tested during growth on DD and DF, chlorinated congeners of DD, and some potential metabolites; the results are shown in Table 1. Among the different substrates tested, strain PH-03 showed a significant rate of oxygen uptake when grown on DD, DF, and monochlorinated dioxins such as 1-MCDD and 2-MCDD, and even greater oxygen uptake when grown on catechol and salicylic acid. Although the rate of oxygen consumption for 1,2,3,4-TCDD was higher than for its metabolite 3,4,5,6-TECC, the rate of uptake was less than that of the other compounds tested.

Table 1. Specific oxygen uptake rates of DD, DF, 1-MCDD, 2-MCDD, 1,2,3,4-TCDD and prospective metabolic intermediates by washed cells of *Pseudomonas veronii* PH-03

Assay substrate	Specific oxygen uptake rate [a]
DD	59.2
DF	62.9
1-MCDD	29.6
2-MCDD	25.9
1,2,3,4-TCDD	11.1
Catechol	88.8
Salicylic acid	92.5
3,4,5,6-TECC	< 1

[a] Results represent means of two independent experiments. The oxygen uptake rates are expressed as specific activities nanomoles of O_2 consumption per minute per mg of protein and are corrected for endogenous respiration. The cells were pre-grown with DF to an optical density of 1.0.

Determination of enzyme activities of crude cell extracts

The crude enzyme extracts from DF grown cells of strain PH-03 were used for the activities of catabolic enzymes involved in dioxin degradation. Polarographic experiments were conducted with cell extracts to test the ability of DF to serve as a substrate for angular dioxygenase, which catalyzes hydroxylation of aromatic rings. We observed the appearance of a yellow color in the reaction mixture during the incubation of DF with the crude enzyme and NADH, indicating the occurrence of angular dioxygenation resulting in the formation of 2,2′,3-THB. The activities of extradiol dioxygenase, the second enzyme involved in the dioxin catabolic pathway, and other enzymes involved in the lower pathway were measured using the respective substrates. Specific activities of 1.2, 7.2, and 6.2 μmols/min/mg of protein were detected for 2,2′,3-THB dioxygenase, 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoic acid (HOPDA) hydrolase, and catechol-2,3-dioxygenase respectively. However, the strain PH-03 did not produce catechol-1,2-dixoygenase under the conditions employed in our study. We additionally tested the effect of 3-chlorocatechol, a potential meta-cleaving enzyme inhibitor, on the activity of 2,2'3-THB dioxygenase and catechol-2,3-dioxygenase. When preincubated with 3-chlorocatchol, the crude enzyme showed no activity of 2,2′3-THB dioxygenase and catechol 2,3-dioxygenase. However, in whole cell suspension, we observed the cleavage 2,2′,3-THB in cultures incubated with 3-chlorocatechol.

Biotransformation of dioxins and 2,2′,3-THB

The biotransformation of DF, DD, its chlorinated derivatives, and 2,2′,3-THB was studied in resting cell suspensions of strain PH-03. The biotransformation of DD by strain PH-03 gave rise to a polar metabolite, which was identified as catechol in the mass spectrometric analysis [diagnostic peaks at m/z 254 (M^+), m/z 239 (M^+–CH_3), and

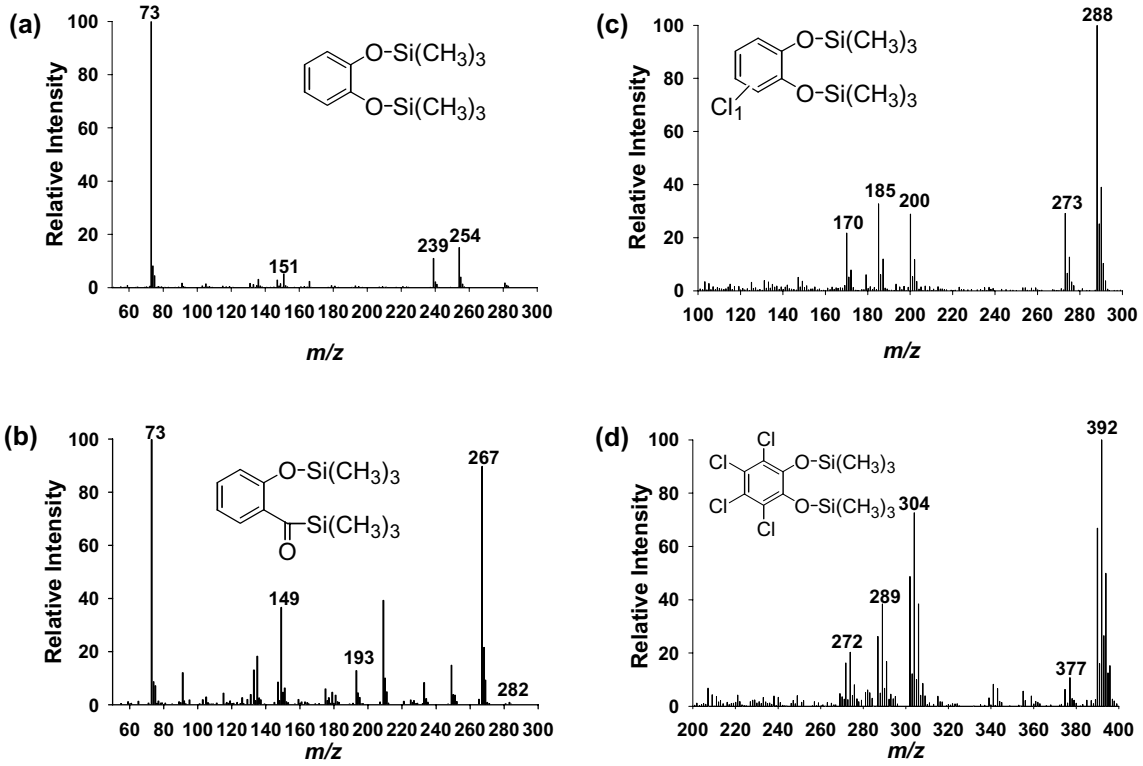

Figure 3. Identification of catechol (a), salicylic acid (b), monochlorocatechol (c), and 3,4,5,6-tetrachlorocatechol (d) as metabolites (as TMS derivative) produced from the corresponding substrates DD, DF, MCDDs and 1,2,3,4-TCDD by strain PH-03 by mass spectrometry.

m/*z* 73 ($Si(CH_3)_3$) after trimethylsilyl derivatization] (Figure 3a). Although the biotransformation of DF produced several other polar metabolites, the exact assignment of the mass peaks not due to salicylic acid was not completed due to a lack of authentic compounds (Figure 3b). The biotransformation of 1-MCDD and 2-MCDD by strain PH-03 also gave rise to the production of polar metabolites, which were identified as 3 and 4 chlorocatechols. Diagnostic peaks were observed at *m*/*z* 288 (M^+), *m*/*z* 273 (M^+–CH_3), *m*/*z* 200 (M^+–CH_3–$Si(CH_3)_3$), *m*/*z* 185 (M^+–CH_3–Si $(CH_3)_3$ –CH_3), and *m*/*z* 170 (M^+–CH_3–Si $(CH_3)_3$–CH_3–CH_3) (Figure 3c). In the case of 1,2,3,4-TCDD, one major metabolite accumulated which was characterized as 3,4,5,6-TECC [diagnostic peaks at *m*/*z* 392 (M^+), *m*/*z* 377 (M^+–CH_3), *m*/*z*304 (M^+–CH_3–$Si(CH_3)_3$), *m*/*z* 289 (M^+–CH_3–$Si(CH_3)_3$–CH_3), and *m*/*z* 274 (M^+–CH_3–$Si(CH_3)_3$–CH_3–CH_3)] (Figure 3d). Transformation of 2,2′,3-THB was determined spectrophotometrically. Formation of a yellow product with the maximum absorbance at 434 nm was observed during the incubation (data not shown).

The metabolic intermediates that accumulated during the biotransformation tests are listed in Table 2. The results indicated that the quantity of each metabolic intermediate was not stoichiometrically related to the amount of the substrate

Table 2. The metabolic intermediates detected by the resting cells of *Pseudomonas veronii* PH-03 after 5 days incubation

Substrate	Substrate remaining (mM)	Major metabolites	Amount (mM)
DD	0.25	Catechol	0.028
DF	0.10	Salicylic acid	0.043
1-MCDD	0.24	3-chlorocatechol	0.021
2-MCDD	0.26	4-chlorocatechol	0.034
1,2,3,4-TCDD	0.82	3,4,5,6-TECC	0.029

depleted. In addition, we were unable to identify or measure other metabolic intermediates due to a lack of authentic compounds.

Discussion

The present study was undertaken to isolate and characterize soil microorganisms capable of utilizing chlorinated dioxins. By selective enrichment techniques, *Pseudomonas veronii* PH-03, capable of utilizing DD and DF, was isolated from soil contaminated with incinerator wastes from a chemical plant. Study of the growth of *P. veronii* PH-03 with DD and DF as the sole carbon source in MSM showed distinct growth corresponding to decreased levels of the substrate in the growth medium. In addition, strain PH-03 grew well on 1- and 2-MCDDs, exhibiting growth rates similar to those observed for growth on DD and DF. In previous studies of the biodegradation of dioxins, chlorinated dioxins were degraded by co-oxidation in resting cells (Hong et al. 2002; Klecka & Gibson, 1979, 1980; Schreiner et al. 1997; Wilkes et al. 1996). In this study, we tested the ability of strain PH-03 to grow in the presence of 1- and 2-MCDD and 1,2,3,4-TCDD as the carbon source. We found that this strain grew well on both MCDDs, causing their rapid degradation. In the case of 1,2,3,4-TCDD, however, the rates of growth and substrate reduction were very low in liquid culture. The oxidation rate of chlorinated dioxins depends on the number of substituted chlorines (Kimura & Urushigawa 2001; Klecka & Gibson 1980; Schreiner et al. 1997; Wilkes et al. 1996). Consistent with this result, we observed little depletion of 1,2,3,4-TCDD in resting cell suspensions of PH-03. The degradation behavior of chlorinated dioxins also depends on the chlorine substitution patterns because certain substitution patterns prevent the attack by oxidizing enzymes. Thus, complete degradation of chlorinated dioxins may be inhibited by the formation of chlorinated metabolites that are resistant to enzyme attack. Further, the low degradation rate of 1,2,3,4-TCDD may be due to the formation of toxic chlorinated metabolites such as tetrachlorocatechol or to the concentration of 1,2,3,4-TCDD used in the experiments (1 mM) being too high.

Klecka & Gibson (1979, 1980) previously showed that DD was hydroxylated by lateral dioxygenation in *Pseudomonas* sp NCIB 9816, resulting in *cis*-1,2-dihydroxy[1,4]-dioxin as the initial product. Previous studies in *S. wittichii* RW1 and *P. resinovororans* have revealed a novel degradative pathway of DF and DD involving a new type of oxidative attack characterized by high regioselectivity for the angular positions of the two carbon atoms adjacent to the ether bridge (Bünz & Cook 1993; Habe et al. 2001; Hong et al. 2002; Keim et al. 1999; Nojiri et al. 2001). This reaction, termed angular dioxygenation, generates an unstable hemiacetal structure that rearomatizes spontaneously, prompting cleavage of the ether bond resulting in 2,2′,3-THB from DF and 2,2′,3-trihydroxydiphenyl ether from DD. This angular dioxygenation reaction occurs in both the aromatic ring of dioxins and fission of the ether bond (Kohler et al. 1993). In the present study, GC-MS analysis revealed accumulation of catechol and salicylic acid as a result of degradation of DD and DF, respectively, by the strain PH-03. This pattern indicates that the degradation pathway in strain PH-03 is analogous to that in the dioxin degraders of *Sphingomonas wittichii* RW1 (Wittich et al. 1992) and *Pseudomonas resinovorans* (Nojiri et al. 2001).

It has been reported that *Sphingomonas wittichii* RW1 can convert 1-MCDD to 3-chlorocatechol (Wilkes et al. 1996) and 1,2,3,4-TCDD to 3,4,5,6-tetrachlorocatechol (Hong et al. 2002). In the present study of the biodegradation of chlorinated dioxins by strain PH-03, we detected metabolites such as monochlorocatechols and 3,4,5,6-tetrachlorocatechol; the formation of these metabolites confirms that the catabolic enzyme system of strain PH-03 is similar to those of other well-known dioxin degraders (Halden & Dwyer 1997; Nojiri et al. 2001; Omori et al. 1986). In a study of the biodegradation of 210 PCDD/DF congeners by pure aerobic bacterial strains, Schreiner et al. (1997) demonstrated that the ability of the bacteria to degrade these compounds depended on the chlorine substitution pattern. Specifically, they found that *Pseudomonas* sp. DSM No 6708 and *Sphingomonas* sp DSM No 7135 showed high potential for isomer-specific degradation of mono- to tetra-chlorinated DD/DF congeners including the most toxic congener, 2,3,7,8-TCDD. They also reported the complete degradation of 1- and 2-MCDD and 2,3,7,8-TCDD by *Pseudomonas* sp. DSM 6709 within

25 days. However, other tetra, penta, and higher chlorinated dioxins were not significantly degraded. In our study, strain PH-03 was able to utilize 88.3% and 78.6% of MCDDs, and 10.7% of TCDD within 60 h when these compounds were used as substrates for growth. These results were obtained from experiments in which each compound was present as the sole carbon source, and hence confirm that these chlorinated substrates can serve as the sole carbon source for strain PH-03.

The consumption of oxygen and formation of 2,2′,3-THB during the incubation of DF with the crude enzyme indicate that angular dioxygenation has taken place. Further, the enzyme activities of 2,2′,3-THB dioxygenase, HOPDA hydrolase, and catechol 2,3-dioxygenase confirm that our strain has a catabolic pathway similar to that of *S. wittichi* RW1. 3-Chlorocatechol is a well-known inhibitor of *meta*-cleavage enzymes (Bartels et al. 1984; Klecka & Gibson, 1981). We also observed inactivation of 2,2′,3-THB and catechol-2,3-dioxygenase in the crude enzyme assay of strain PH-03. Interestingly, no inhibition was observed during the formation of HOPDA from 2,2′,3-THB in the resting cell suspension. These results suggest that the strain PH-03 may contain chlorocatechol-2,3-dioxygenase, and that this enzyme may be involved in the degradation of 3-chlorocatechol through *meta*-cleavage. However, this degradation pathway would be expected to generate chloromuconate products, and we observed no such products in our GC-MS analysis, although this may be due to such products undergoing further degradation or being formed in very small quantities. Previously, it has been reported that some chlorobenzene-degrading bacteria also effectively degrade 3-chlorocatechol by chlorocatechol 2,3-dioxygenase (Kaschabek et al. 1998; Mars et al. 1997, 1999).

The catabolic activity of several bacteria on chlorinated dioxins and furans has been studied since the late 1970s (Habe et al. 2001; Parsons & Storms 1989; Schreiner et al. 1997; Wilkes et al. 1996). However, only limited information is available regarding the biotransformation of highly chlorinated dioxins. Previous findings suggest that almost complete degradation of such compounds can only be achieved by a consortium consisting of the DF-mineralizing strain *S. wittichii* RW16 and *Burkholderia* sp. strain JWS (Armann et al. 1997). The initial attack by strain RW1 on the halogenated DF proceeded via attacks on the halogenated as well as the non-halogenated aromatic rings (Wilkes et al. 1996). Thus, the potential of DF-degrading bacterial strains such as *S. wittichii* RW1 for the conversion of PCDDs or PCDFs is known to be restricted to low-halogenated congeners because of the relatively narrow substrate range of the initial dioxygenase. Parsons & Storms (1989) also attempted to degrade 1,2,4-trichlorodibenzo-*p*-dioxin and 1,2,3,4-TCDD with strain JB1; however, they detected no evidence of degradation, even though these chlorodioxins have completely unsubstituted aromatic rings that would be expected to be susceptible to enzymatic attack. However, recent studies have shown that 1,2,3,4-TCDD and 2,7-dichlorodibenzo-*p*-dioxin could be biotransformed by the RW1 strain (Hong et al. 2002). Interestingly, they found accumulation of 3,4,5,6-TECC and 2,3,4,5-tetrachloro-6-methoxyphenol in the case of 1,2,3,4-TCDD metabolism. Our strain also produced 3,4,5,6-TECC during incubation with 1,2,3,4-TCDD, however, it failed to further degrade 3,4,5,6-TECC and the production of 2,3,4,5-tetrachloro-6-methoxyphenol was not detected.

Conclusions

In this study the bacterial strain *Pseudomonas veronii* PH-03 was isolated from contaminated soil by enrichment techniques using DD as the sole carbon source. In addition to DD, this strain effectively metabolized the low chlorinated dioxins, 1-MCDD and 2-MCDD. However, although the PH-03 strain was able to grow on 1,2,3,4-TCDD, growth on this substrate was poor, presumably due to the production of a toxic metabolite, 3,4,5,6-TECC. Given that 3-chlorocatechol did not inhibit the activity of *meta*-cleavage enzymes in intact cells, it is possible that the PH-03 strain could degrade chlorocatechols via the *meta*-cleavage pathway. Since the production of chlorocatechols, resistant to further degradation, is the main factor for limiting the mineralization of chlorinated dioxins, it will be interesting to elucidate the enzymes and genes encoding for chlorocatechol degradation from this strain. Further studies are currently in progress to investigate the biochemical and molecular char-

acteristics of this strain for the degradation of higher chlorinated dioxins.

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