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Degradation of polychlorinated naphthalenes by the lignin-degrading basidiomycete *Phlebia lindtneri*

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Introduction

Polychlorinated naphthalenes (PCNs) are ubiquityous environmental pollutants that are structurally similar to other polychlorinated diaromatic hydrocarbons, such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls[1]. PCNs are a group of compounds composed of two fused benzene rings (naphthalene) with one to eight chlorine substitusions. There are 75 possible PCN congeners with unique combinations of numbers and positions chlorine. Besides, they are hydrophobic waxy solids with high thermal stability and inertness. Technical mixtures of the 75 possible PCN congeners have been used in dieletric fluids, engine oil additives, electroplating masking compounds, wood preservatives, lubricants, and dye production[2]. Global production of PCN mixtures has been estimated to be approximately 150,000 tons[1]. Potential environmental exposures to PCNs may be cause for concern. Occupational exposures to PCNs has been linked to serious health problems in humans, including chlorance and liver disease. PCNs have also been identified as causative agents of X-disease in cattle. The toxicity of PCN mixtures is ascribed mainly to penta- to heptachlorinated naphthalenes, which have been shown experimentally to exhibit dioxin-like effects on mammalian liver cell line[3]. The hazard due to PCNs is enhanced by their potency to bioaccumulate.

White-rot basidiomycetous fungi are primarily responsible for initiating the depolymerization of lignin, which is a key step in the earth's carbon cycle. To utilize the superior fungal ability to degrade aromatic compounds, many applied studies have been attempted. Among them, the degradation of environmentally persistent pollutants such as dioxin, biphenyl derivatives, and polyaromatic hydrocarbons (PAHs) has been well documented[4].

The lignin degrading fungus, *Phlebia lindtneri* can metabolize dioxins, such as 2,7-dichlorodibenzo-*p*-dioxin and 2,8-dibenzofuran[5]. In this study, we demonstrated the degradation of CNs (chloronaphthalenes) and characterized metabolites from the degradation of CNs by *P. lindtneri*. We also discussed the mechanisms of the degradation of naphthalene (NAP) and CNs by *P. lindtneri*.

Materials and methods

Culture conditions. Stock cultures of *P. lindtneri* strain GB-1027 was grown from mycelial inocuram at 30℃ in stationary culture (10 ml of medium) under air. The medium (pH 4.5) used in this study was that previously described[6], with 1.0% glucose and either 1.2 or 12 mM ammonium tartrate as the carbon and nitrogen sources in media LN and HN, respectively. In addition, cultures for the metabolism of NAP and PCNs contained 0.1% Tween 80.

Metabolic experiments. After pre-incubation for 5days, the substrates [NAP, 2-chloronaphthalene (2-CN), 1,4-dichloronaphthalene (1,4-DCN), and 2,7-dichloronaphthalene (2,7-DCN) in 25 mM *N,N'*-dimethlformamide] were added to the cultures to final concentration

of 0.1 mM. After additional incubation, the recovery of substrates was determined by high performance liquid chromatography (HPLC) after homogination in acetone (equal volumes of acetone and medium), centrifugation (3,000*g* for 10 min) and filtration (0.45 cm). The metabolic products were analyzed by gas-chromatography mass-spectrometry (GC/MS) after extraction with ethyl acetate (20 ml × 3) at pH 2, drying over Na_2SO_4 , evaporation under reduced pressure, and derivatization using *N,O*-bis(trimethylsilyl)trifluoroacetimide /pyridine (4:1, v/v) or acetic anhydride/pyridine (1:1, v/v).

Cytochrome P-450 inhibitor experiments. Aliquots (approximately 1ml) of the homogenate of *P. lindtneri* were used to inoculate 10ml LN and were incubated for 5 days at 30℃. The cytochrome P-450 inhibitors 1-aminobenzotriazole or pyperonyl butoxide (PB) were dissolved in MeOH and add to the cultures to final concentration of 0, 0.1, 0.01 and 1.0 mM at the same time as substrate feeding. The cultures were incubated for 1day or 5 days, and recovery of substrates was measured using the methods described for the metabolic experiments.

Instrumentation. HPLC analysis was carried out using a Waters 626 pump with a Waters 996 photodiode array detector fitted with an inertsil ODS-3 column, 150 × 4.6 mm inner diameter (GL Science) with 60% acetonitrile in 0.1%trifluoro acetic acid (TFA) at flow rate of 1 ml/min (NAP, 2-CN), and with 70% acetonitrile in 0.1% trifluoro acetic acid (TFA) at flow rate of 1 ml/min (1,4-DCN, 2,7-DCN).

GC/MS was performed on a HP6890 GC system linked to a HP 5973 mass selective detector and 30-m fused DB-5MS column (0.25 mm inside diameter, 0.25μm film thickness ; J & W). The oven temperature was programmed to increased from 70℃ to 300℃ at 20℃ /min. Products were identified by comparison of their retention times on GC and of mass fragmentation patterns with chemically prepared standards or otherwise indicated.

Results and discussion

Fungal metabolism of NAP, 2-CN, 1,4- and 2,7-DCN. NAP and 2-CN were completely transformed for a 5-day incubation period by *P. lindtneri* (data not shown). Under LN conditions, NAP converted into five hydroxylated products (Table 1). 1-Naphthol, 2-naphthol, and 1,2-NAP-diol were identified from the culture extracts at pH 2 on GC/MS analysis using authentic standards. After acetylation, NAP-diol and NAP-dihydrodiol were suggested as previous reported[6]. 2-CN was converted into four hydroxylated products (3-chloro-2-naphtol, 6-chloro-1-naphtol and other two chloronaphtols) by *P. lindtneri*. After acetylation, seven additional products were obtained. Three of these showed a molecular ion peak at m/z 280, and remarkable fragment ions at m/z 220 (M^+ -CH_3CO_2H) and m/z 178 (M^+ -CH_3CO_2H, CH_2CO), suggesting the introduction of a dihydrodiol group to 2-CN. Four other products showed a molecular ion peak at m/z 278, and remarkable fragment ions at m/z 236 (M^+ -CH_2CO) and m/z 194 (M^+ -$(CH_2CO)_2$), suggesting the introduction of two hydroxyl groups to 2-CN. From these results, it was estimated that these compounds were the diacetate of CN-dihydrodiol and CN-diol. HPLC analysis showed that *P. lindtneri* exhibited an ability to degrade 1,4-DCN (95%) at 5 days incubation period (Table 2). In contrast, lower activity was observed in the degradation of 2,7-DCN (50%). It is estimated that the introduction of a hydroxyl group to substrate was limited by positions of chlorine atom substituted. When 1,4-DCN or 2,7-DCN need to add to cultures, three metabolites were suggested from their characteristic mass fragmentation pattern to be 1,4-dichloro-hydroxy-NAP (Product A, C), 1,4-DCN-dihydrodiol (Product B), 2,7-dichloro-hydroxy-NAP (Product D, F), and 2,7-DCN-dihydrodiol (Product E), respectively. Product A, C, D and F showed a molecular ion peak at m/z 212, and remarkable fragment ions at m/z 183 (M^+ -COH) and m/z 148 (M^+ -COHCl), m/z 113 (M^+ -$COHCl_2$), suggesting the

introduction of a hydroxyl group to 1,4-and 2,7-DCN. Product B and E showed a molecular ion peak at m/z 230, and remarkable fragment ions at m/z 212 (M^+ -H_2O), suggesting the introduction of a dihydrodiol group to 1,4-and 2,7-DCN. These results were supported by a shift of a GC/MS peak of these products with derivatization.

Effect of a cytochrome P450 inhibitor on fungal metabolism of 1,4- and 2,7-DCN. In Table 2, the effect of PB on fungal metabolism of 1,4- and 2,7-DCN was investigated and showed a concentration-dependent inhibition of degradation, respecively. The oxidation reaction path was efficiently shut off by adding PB exogenously, that is to say, as a PB concentration was lowerd, the hydroxylated products (A~F) more accumulated. Interestingly, the hydroxylation of 2,7-DCN was more inhibited by PB than one of 1,4-DCN.

Table 1. Mass spectra and GC retention time of the fungal metabolites of NAP and 2-CN.

Substrate or metabolite		GC retention time (min)	Mass spectram *m/z* (relative intensity [%])
NAP		10.32	
1-naphtol		17.98	145 (11), 144 M^+ (100), 116 (16), 115 (66)
2-naphtol		17.76	145 (11), 144 M^+ (100), 116 (47), 115 (96)
NAP-dihydrodiol		18.73	162 M^+ (19), 144 (31), 131 (25), 116 (100), 115 (80)
NAP-dihydrodiol	(diacetoxy)	22.11	246 M^+ (trace), 186 (5), 145 (18), 144 (100), 128 (9), 127 (7), 115 (18), 114 (12)
1,2-NAP-diol	(diacetoxy)	24.35	244 M^+ (6), 202 (11), 161 (11), 160 (100), 131 (17), 103 (8)
NAP- diol	(diacetoxy)	25.37	244 M^+ (7), 202 (11), 161 (8), 160 (100), 131 (9), 103 (4)
2-CN		14.77	
3-chloro-2-naphtol		18.54	180 (33), 178 M^+ (100), 151 (2), 149 (7), 116 (20), 115 (54), 114 (14)
chloronaphtol		21.95	180 (27), 178 M^+ (85), 151 (5), 149 (16), 116 (10), 115 (100), 114 (14)
6-chloro-1-naphtol		21.86	180 (30), 178 M^+ (88), 151 (5), 149 (15), 116 (10), 115 (100), 114 (13)
chloronaphtol		22.12	180 (33), 178 M^+ (100), 151 (4), 149 (13), 116 (5), 115 (55), 114 (11)
CN- dihydrodiol	(diacetoxy)	24.14	280 M^+ (trace), 222 (1), 220 (3), 180 (35), 178 (100), 164 (2), 162 (7)
CN- dihydrodiol	(diacetoxy)	25.32	280 M^+ (trace), 222 (trace), 220 (6), 180 (37), 178 (100), 164 (2), 162 (8)
CN- diol	(diacetoxy)	25.65	280 (trace), 278 M^+ (1), 238 (3), 236 (10), 196 (32), 194 (100), 167 (4), 165 (11), 159 (13)
CN- diol	(diacetoxy)	27.79	280 (trace), 278 M^+ (5), 238 (4), 236 (11), 196 (33), 194 (100), 167 (trace), 165 (3), 159 (9)

Conclusion

In this study, NAP, 2-CN, 1,4- and 2,7-DCN were transformed into several hydroxylated metabolites by *P. lindtneri.* The formation of CN-dihydrodiol indicated that *P. lindtneri* init-

ially oxidize NAP via a cytochrome P450 monooxygenase and an epoxide hydrolase rather than a dioxygenase. The cytochrome P450 mechanism of oxidation was further supported by inhibition of 1,4- and 2,7-DCN degradation by the specific cytochrome P450 inhibitors PB (Table 2). To our knowledge, this is the first report showing metabolism of CNs to their hydroxylated products by fungal hydroxylation. The proposed degradation mechanism of CNs mirrors PAHs degradation pathways that are widely distributed in the fungal kingdom[7]. This reaction seems quite essential for detoxication of PCNs. Based on these facts, it is expected that fungi can degrade CNs via oxidation by cytochrome P450 monooxygenase system (Figure 1), although, it might be impossible to make this system responsive to CN replaced all by chlorine atoms.

Table 2 Inhibitory effect of piperonyl butoxide (PB) on fungal metabolism of 1,4-DCN and 2,7-DCN[a]

PB[b] (mM)	Remaining substrate (mM)		GC-MS peak area of products ($x10^6 ml^{-1}$)					
			1,4-DCN			2,7-DCN		
	1,4-DCN	2,7-DCN	Product A	Product B	Product C	Product D	Product E	Product F
0	0.005	0.05	1.34	3.7	0.85	1.05	1.67	0.18
0.01	0.04	0.06	0.69	3.81	0.64	0.45	0.70	0.08
0.1	0.07	0.07	0.17	0.95	0.24	trace	trace	trace
1	0.08	0.08	0	0	0	trace	0	0

[a] Transformation of 1,4-DCN , 2,7-DCN by cultures of *P. lindtneri* for 5-day incubation.
[b] PB was dissolved in MeOH and add to the culture at the same time as substrate feeding; 50 µl MeOH solution was add to make the final concentrations listed in the table

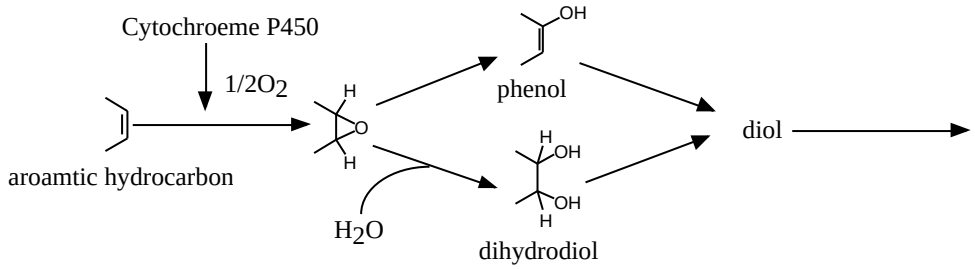

Figure 1 Possible pathways for the fungal metabolism of PCNs.

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