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2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) degradation by the thermophilic *Geobacillus* sp. UZO 3

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2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is the most toxic among the dioxin isomers. Despite previous breakthroughs, the global quest to discover bioremediation agents for this pollutant remains unabated. In this study, we characterize the 2,3,7,8-TCDD-degrading activity of the *Geobacillus* sp. UZO 3. Cell-free extracts of *Geobacillus* sp. UZO 3 were incubated for 18 h at 65°C in a reaction milieu that contained 2,3,7,8-TCDD as substrate. Reaction products in the milieu were dissolved in ethyl acetate and directly analyzed by GC-MS. GC-MS data revealed peaks that corresponded to 3',4',4,5-tetrachloro-2-hydroxydiphenyl ether (TCDE). Moreover, performing a similar assay that instead contained TCDE as substrate showed that the cell-free extract may also play a catalytic role in the subsequent conversion of TCDE to dichlorophenol (DCP). Our present results suggest that this thermophilic specie catalyzes the reductive cleavage of the diaryl ether bonds of 2,3,7,8-TCDD to TCDE as intermediate by-product which is then converted to DCP. We hereby demonstrate the nouveau 2,3,7,8-TCDD-degrading activity by a bacterial cell-free extract.

Key words: Dioxin, 2,3,7,8-TCDD, Microbial degradation, *Geobacillus* sp. UZO 3

1. Introduction

Dioxins continue to pose serious threat to the environment. Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), consisted of 75 and 135 isomers, respectively, are stable hydrophobic contaminants which persist in the environment. PCDD/Fs are introduced into the biosphere on a large scale not only as by-products from the manufacture of chlorinated phenols as pesticides in the 1930s, but also by incineration of wastes[6,7,12]. They induce a broad spectrum of biochemical and toxic effects to mammals and other organisms, such as teratogenesis, immunosuppression and tumor promotion. Rapid advances in the field of molecular toxicology revealed that these effects by PCDD/Fs are mediated by aryl hydrocarbon receptors (AhR). Among the PCDD/F isomers, 2,3,7,8-TCDD has been found highly toxic because it has the highest binding affinity to AhR[4,13,14].

From the mid-1980s, attention has been directed towards the development of technology for the clean-up of PCDD/Fs by bioremediation, marking the beginning of such kind of research[3,19]. The aerobic bacterium *Sphingomonas wittichii* RW1 is one of the most well studied dioxin-degrading bacteria[17,18]. Armengaud et al.[1] succeeded in cloning a dioxin dioxygenase gene *dxnA1A2* from *Sphingomonas wittichii* RW1 and demonstrated that DxnA1A2 introduces two atoms of oxygen at angular positions neighboring the diaryl ether bonds for subsequent degradation through an unstable intermediate acetal structure. Nam et al.[5,15] revealed that resting cells from *Sphingomonas wittichii* RW1 degradated 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD), 1,2,3-trichlorodibenzo-*p*-dioxin, 1,2,3,4-tetrachlorodibenzo-*p*-dioxin, and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin to their corresponding chlorocatechols. On the other hand, in an anaerobic bacterium, Bunge et al.[2] reported that *Dehalococcoides* sp. strain CBDB1 is capable of converting 1,2,3,4-tetrachlorodibenzo-*p*-dioxin to 2-monochlorodibenzo-*p*-dioxin, and 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin to 2,7- or 2,8-DCDD

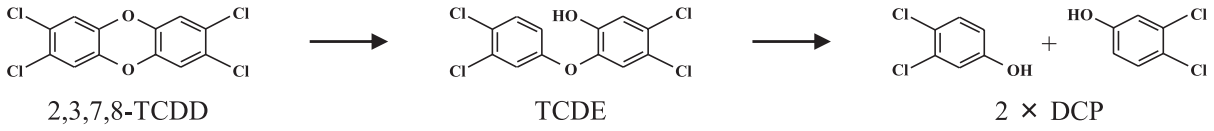

Fig. 1. Proposed degradation pathways for 2,3,7,8-TCDD by the *Geobacillus* sp. UZO 3 cell-free extract.
Compound definitions: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD); 3',4',4,5-tetrachloro-2-hydroxydiphenyl ether (TCDE); and dichlorophenol (DCP).

by dechlorination. Despite numerous studies on the microbial degradation of PCDD/Fs, no report has demonstrated a 2,3,7,8-TCDD-degrading activity of bacterial origin.

We have investigated the 2,3,7,8-TCDD-degrading activity of the *Geobacillus* sp. UZO 3 for several years. As preliminary step, we have previously reported 2,7-DCDD-degrading activity of the cell-free extract from *Geobacillus* sp. UZO 3 and demonstrated that it reductively cleaves diaryl ether bonds of 2,7-DCDD in a sequential fashion producing 4',5-dichloro-2-hydroxydiphenyl ether (DCDE) as the intermediate, and 4-chlorophenol as the final reaction product[16]. The structure of intermediate DCDE was identified by comparing it to the chemically-synthesized authentic compound in GC-MS analysis. Moreover, the detection of DCDE implicated the discovery of an unprecedented dioxin degradation enzyme that reductively cleaves the diaryl ether bonds similar to glutathione-*S*-transferase, a reduction cleavage enzyme[8–11].

To investigate the degradation activity of the cell-free extract from *Geobacillus* sp. UZO3 on 2,3,7,8-TCDD *in vitro*, we chemically synthesized the authentic compound of putative intermediate 3',4',4,5-tetrachloro-2-hydroxydiphenyl ether (TCDE) (Fig. 1). It appears difficult to demonstrate degradation of persistent organic pollutants like 2,3,7,8-TCDD by existing analytical methods which are based on substrate quantitative assay. To overcome this problem, structural identification of the intermediate that demonstrates degradative reaction was employed in this study. We monitored the production of the reaction intermediate TCDE in the degradation of 2,3,7,8-TCDD mediated by *Geobacillus* sp. UZO 3 cell-free extract.

2. Materials and Methods

2.1. Chemicals

2,3,7,8-TCDD was purchased from Cerilliant (Round Rock, Texas, USA). DCP, $Cu(OAc)_2$, CH_2Cl_2, $CDCl_3$, tetramethylsilane and N,O-Bis(trimethyl silyl)trifluoroacetamide (BSTFA) were purchased from Wako Pure Chemical (Osaka, Japan). 4,5-dichlorocatechol and 3,4-dichlorophenylboronic acid was purchased from Sigma-Aldrich (Steinheim, Germany). Purities of these chemicals range from 96.6 to 100%. All other chemicals used were of analytical grade and of the highest purity available.

2.2. Synthesis of TCDE

TCDE synthesis from 4,5-dichlorocatechol and 3,4-dichlorophenylboronic acid was adapted from the method for DCDE synthesis described previously[16]. Analysis by GC-MS showed that the synthesized TCDE has a molecular ion peak of $m/z=396$ (Fig. 2d) that corresponds to the estimated molecular weight of TCDE. Further analyses by ^{1}H-NMR and ^{13}C-NMR have consistently confirmed the identity of TCDE. The ^{1}H-NMR spectrum δ (ppm, $CDCl_3$) of TCDE is as follows: 5.51 (H, s, OH), 6.89 (1H, dd, $J=2.4$ Hz), 6.94 (1H, d, $J=8.5$ Hz), 7.17 (1H, d, $J=8.5$ Hz), 7.26 (1H, d, $J=8.5$ Hz), 7.44 (1H, dd, $J=8.5$ Hz). The ^{13}C-NMR spectrum δ (ppm, $CDCl_3$) of TCDE is as follows: 154.7, 146.4, 142.0, 133.8, 131.4, 128.6, 128.2, 123.6, 120.2, 120.0, 118.1, 117.6.

2.3. Preparation for cell-free extracts, enzymatic reaction assays and analytical methods

The preparation for cell-free extract of *Geobacillus* sp. UZO 3 using french pressure cell press and the enzymatic reaction assays were performed as described previously[16]. *Geobacillus* sp. UZO 3 was maintained in tryptic soy agar plates (Difco). *Geobacillus* sp. UZO 3 and routinely cultivated in liquid cultures of tryptic soy broth medium (Difco) at 65°C with vigorous shaking. When the optical density at 600 nm reached 1.2, cells were harvested by centrifugation, washed twice with 100 mM phosphate buffer (pH 7.0), and resuspended in a reduced volume of the same buffer. Cell free extracts was prepared from the disrupted bacterial cells using the french pressure cell press (Ohtake Co. Ltd., Tokyo, Japan). The disrupted cells were centrifuged at 25,000 g for 30 min (25°C) to remove intact cells and the supernatant was collected as cell free extracts. Briefly, the 1 mL reaction mixture contained 7.5 μM 2,3,7,8-TCDD dissolved in dimethyl sulfoxide (final concentration 5%) and the cell-free extract (about 2 mg of protein). The enzymatic reaction was performed at 65°C for 18 h. Ethyl acetate extracts from 2,3,7,8-TCDD reaction mixture were dried over anhydrous sodium sulfate and the solvent was volatilized by nitrogen gas. The dried enzyme reactant was derivatized by N,O-Bis(trimethyl silyl)trifluoroacetamid prior to GC-MS analysis. GC-MS and NMR analyses were performed as described previously[16].

3. Results and Discussion

3.1. 2,3,7,8-TCDD-degrading activity producing TCDE by *Geobacillus* sp. UZO 3 cell-free extract

We analyzed the 2,3,7,8-TCDD-degrading activity of

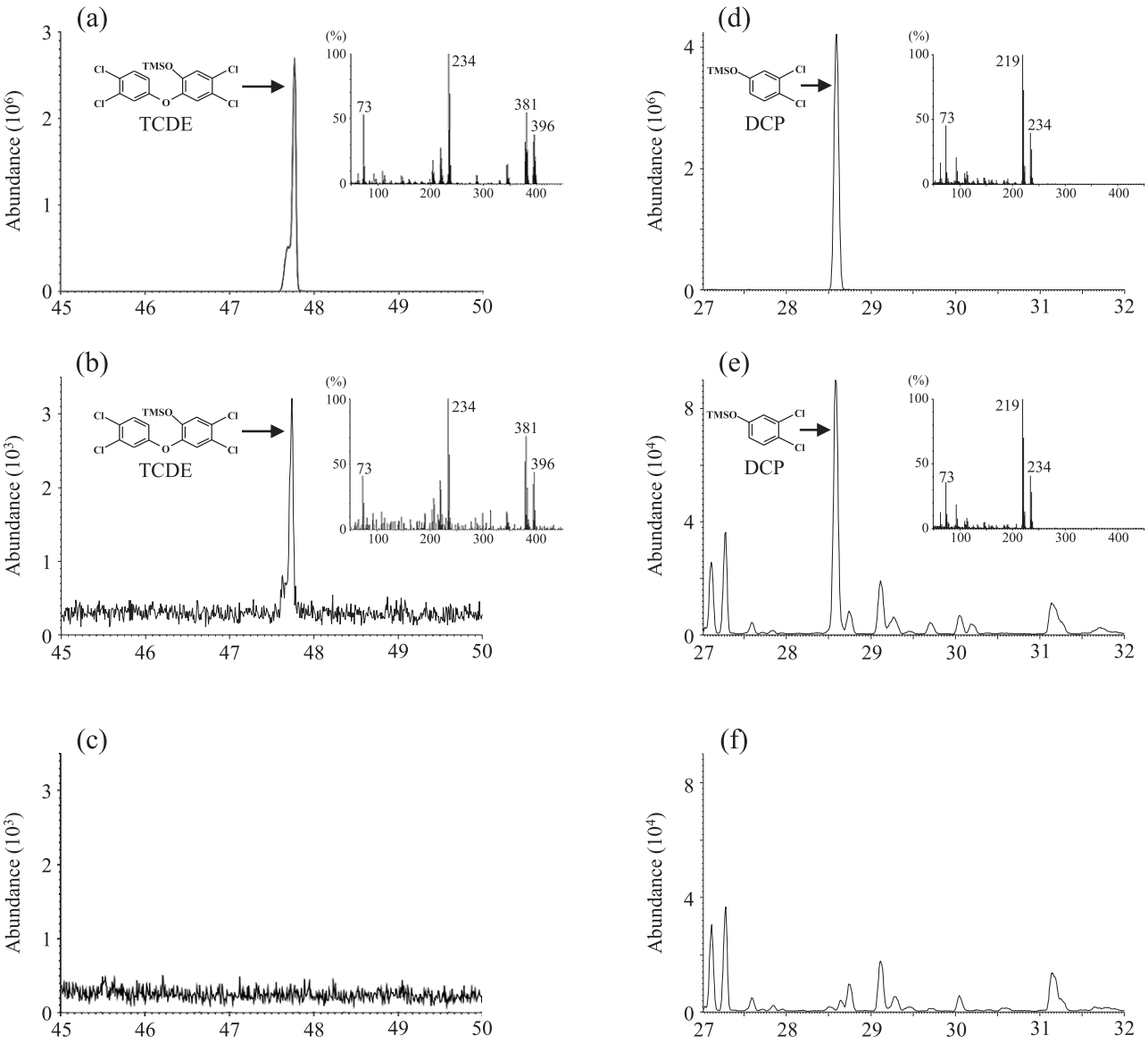

Fig. 2. GC-MS analyses of the reaction mixtures for 2,3,7,8-TCDD and TCDE degradations mediated by *Geobacillus* sp. UZO 3 cell-free extract.
The MS chromatograms of the detected intermediates TCDE at m/z=396 (b) from 2,3,7,8-TCDD degradation, and DCP at m/z=234 (e) from TCDE degradation, and their corresponding MS spectrums. The MS chromatograms at m/z=396 (c) and at m/z=234 (f) represent the control chromatograms obtained when the cell-free extract was lacking from the enzyme assay. The MS chromatograms of the authentic compounds TCDE at m/z=396 (a) and DCP at m/z=234 (d), and their corresponding MS spectrums.

Geobacillus sp. UZO 3 cell-free extract after 18 h of incubating the reaction milieu at 65°C. GC-MS analysis of the ethyl acetate extract of the complete reaction milieu in addition to the substrate 2,3,7,8-TCDD showed peak for TCDE at retention time 47.75 min (Fig. 2b). The peaks of the substrate and product corresponded well with those of their respective authentic compounds (Fig. 2a, b). Ethyl acetate extract of a reaction mixture that did not contain the cell-free extract (control) was detected of 2,3,7,8-TCDD, but showed no peak for TCDE (Fig. 2c). These results suggest that the chlorinated dioxin degradation enzymes contained in *Geobacillus* sp. UZO 3 cell-free extract reductively cleaves diaryl ether bonds of 2,3,7,8-TCDD to produce TCDE.

3.2. *Geobacillus* sp. UZO 3 cell-free extract degrades TCDE to DCP

We further investigated the TCDE-degrading activity of *Geobacillus* sp. UZO 3 cell-free extract. We likewise analyzed by GC-MS the ethyl acetate extract of a similar reaction milieu that instead contained TCDE as substrate. DCP was detected in the ethyl acetate extract (Fig. 2e) implying that the *Geobacillus* sp. UZO 3 cell-free extract catalyzes the reductive cleavage of the two diaryl ether bonds in 2,3,7,8-TCDD in a sequential fashion. That is, TCDE is first produced as a reaction intermediate which is then converted to DCP.

The results of this study provide strong evidence that *Geobacillus* sp. UZO 3 cell-free extract reductively cleaves the diaryl ether bonds of 2,3,7,8-TCDD (Fig. 1). This is the first report to demonstrate a 2,3,7,8-TCDD-degrading activity of bacterial origin. Work is in progress to clone the genes of the enzymes involved in the reductive cleavage of the diaryl ether bonds of 2,3,7,8-TCDD. Further enzymological characterization of the response mechanism towards 2,3,7,8-TCDD is envisioned to contribute in the remediation of PCDD/Fs-contaminated areas.

Acknowledgments

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References

1) Armengaud, J., B. Happe, and K.N. Timmis. 1998. Genetic analysis of Dioxin Dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. J. Bacteriol. 180: 3954–3966.
2) Bunge, M., L. Adrian, A. Kraus, M. Opel, W.G. Lorenz, J.R. Andreesen, H. Gorisch, and U. Lechner. 2003. Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium. Nature. 421: 357–360.
3) Field, J.A. and R. Sierra-Alvarez. 2008 Microbial degradation of chlorinated dioxins. Chemosphere. 71: 1005–1018.
4) Gibbons, A. 1993. Dioxin tied to endometriosis. Science. 262: 1373.
5) Hong, H.B., Y.S. Chang, I.H. Nam, P. Fortnagel, and S. Schmidt. 2002. Biotransformation of 2,7-dichloro- and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* RW1. Appl. Environ. Microbiol. 68: 2584–2588.
6) Hutzinger, O., M.J. Blumich, M. Vanderberg, and K. Olie. 1985. Sources and fate of PCDDs and PCDFs—An overview. Chemosphere. 14: 581–600.
7) Kaiser, J. 2000. Just how bad is dioxin? Science. 288: 1941–1944.
8) Masai, E., Y. Katayama, S. Nishikawa, M. Yamasaki, N. Morohoshi, and T. Haraguchi. 1989. Detection and localization of a new enzyme catalyzing the beta-aryl ether cleavage in the soil bacterium (*Pseudomonas paucimobilis* SYK-6). FEBS Lett. 249: 348–352.
9) Masai, E., Y. Katayama, S. Kawai, S. Nishikawa, M. Yamasaki, and N. Morohoshi. 1991. Cloning and sequence of the gene for a *Psedomonas paucimobilis* enzyme that cleaves β-aryl ether. J. Bacteriol. 173: 7950–7955.
10) Masai, E., Y. Katayama, S. Kubota, S. Kawai, M. Yamasaki, and N. Morohoshi. 1993. A bacterial enzyme degrading the model lignin compound beta-etherase is a member of the glutathione-*S*-transferase superfamily. FEBS Lett. 323: 135–140.
11) Masai, E., A. Ichimura, Y. Sato, K. Miyauchi, Y. Katayama, and M. Fukuda. 2003. Roles of the enantioselective glutathione *S*-transferases in cleavage of β-aryl ether. J. Bacteriol. 185: 1768–1775.
12) Meharg, A.A. and D. Osborn. 1995. Dioxins released from chemical accidents. Nature. 375: 353–354.
13) Mimura, J., K. Yamashita, K. Nakamura, M. Morita, T.N. Takagi, K. Nakao, M. Ema, K. Sogawa, M. Yasuda, M. Katsuki, and Y. Fujii-Kuriyama. 1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells. 2: 645–654.
14) Mimura, J. and Y. Fujii-Kuriyama. 2003. Functional role of AhR in the expression of toxic effects by TCDD. Biochim. Biophys. Acta. 1619: 263–268.
15) Nam, I.H., Y.M. Kim, S. Schmidt, and Y.S. Chang. 2006. Biotransformation of 1,2,3-tr- and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* RW1. Appl. Environ. Microbiol. 72: 112–116.
16) Suzuki, Y., M. Nakamura, Y. Otsuka, N. Suzuki, K. Ohyama, T. Kawakami, K. Sato, S. Kajita, S. Hishiyama, T. Fujii, A. Takahashi, and Y. Katayama. 2011. Novel enzymatic activity of cell-free extract from thermophilic *Geobacillus* sp. UZO 3 catalyzes reductive cleavage of diaryl ether bonds of 2,7-dichlorodibenzo-*p*-dioxin. Chemosphere. 83: 868–872.
17) Wilkes, H., R.M. Wittich, K.N. Timmis, P. Fortnagel, and W. Francke. 1996. Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. Appl. Environ. Microbiol. 62: 367–371.
18) Wittich, R.M., H. Wilkes, V. Sinnwell, W. Francke, and P. Fortnagel. 1992. Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. Appl. Environ. Microbiol. 58: 1005–1010.
19) Wittich, R.M. 1998. Degradation of dioxin-like compounds by microorganisms. Appl. Microbiol. Biotechnol. 49: 489–499.