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Dioxin Reductive Etherase DreE Reductively Cleaves Two Diaryl Ether Bonds of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a Sequential Fashion

Yuzo Suzuki[1], Masaya Nakamura[1]*, Yuichiro Otsuka[1], Nao Suzuki[2], Keisuke Ohyama[2], Takeshi Kawakami[3], Kanna Sato-Izawa[4], Shojiro Hishiyama[1], Kouya Inoue[5], Toshiji Kameyama[5], Atsushi Takahashi[3] and Yoshihiro Katayama[6]

[1] *Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305–8687, Japan*
[2] *Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture & Technology, Koganei, Tokyo 184–8588, Japan*
[3] *Takasago Thermal Engineering Co. Ltd., Shinjyuku, Tokyo 160–0022, Japan*
[4] *Department of Bioscience, Faculty of Life Sciences, Tokyo University of Agriculture, Setagaya, Tokyo 156–8502, Japan*
[5] *Kantteku Co. Ltd., Bunkyo, Tokyo 112–0004, Japan*
[6] *College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252–0880, Japan*
* TEL: +81–29–829–8282 FAX: +81–29–873–3797
* E-mail address: nmasaya@ffpri.affrc.go.jp

We have previously reported cloning and characterizing of the gene encoding the dioxin reductive etherase DreE for the reductive cleavage of diaryl ether bond of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a dioxin with the highest toxicity, in *Geobacillus thermodenitrificans* UZO 3. DreE expressed in *Escherichia coli* could reductively cleave diaryl ether bond of 2,3,7,8-TCDD to produce 3',4',4,5-tetrachloro-2-hydroxydiphenyl ether (TCDE) as the reaction product. In this study, we have investigated whether TCDE is first produced as a reaction intermediate which is then converted to 3,4-dichlorophenol (DCP) from 2,3,7,8-TCDD degradation mediated by DreE.

Key words: Bioremediation, Dioxin, 2,3,7,8-TCDD, TCDE, DreE

Abbreviations: 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDE, 3',4',4,5-tetrachloro-2-hydroxydiphenyl ether; DCP, 3,4-dichlorophenol; *dreE*, dioxin reductive etherase; PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, dibenzofurans; CoA, coenzyme A; CoASSCOA, coenzyme A disulfide; BSTFA, N,O-Bis(trimethyl silyl)trifluoroacetamide; Km, kanamycin.

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Dioxins consisting polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) have been formed not only naturally but also unintentionally as a by–products during the bleaching of pulp and the manufacture of pesticides [5]. PCDDs and PCDFs contamination has led to serious social problem because of their carcinogenic and genotoxic properties [11]. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is the most toxic among the dioxin isomers [4,6,12]. Microbial degradation of model compounds of PCDDs and PCDFs has been studied using many microorganisms [3,18]. However, despite numerous studies on the microbial degradation of PCDDs and PCDFs, no report has demonstrated the degradation of 2,3,7,8-TCDD at the molecular level.

We have previously reported cloning the gene encoding the dioxin reductive etherase DreE from the genome library of *G. thermodenitrificans* UZO 3 and showed that DreE expressed in *E. coli* could reductively cleave diaryl ether bond of 2,3,7,8-TCDD to produce TCDE as the reaction product [16]. Furthermore, DreE was found to have amino acid homology to RibT, a protein with yet unknown function that is encoded as part of the riboflavin biosynthesis operon of *Bacillus subtilis* [13,19]. On the other hand, no significant amino acid homology was found between DreE and a known reduction cleavage enzyme, β-etherase (LigE, LigF) of *Sphingobium* sp. strain SYK-6, that reductively cleaves the β-aryl ether bond that is the main intramolecular bond of lignin using GSH as electron donor [7–10]. These results suggest that the 2,3,7,8-TCDD degradation reaction is a novel reducing reaction different from known variations that require electron donors, such as GSH. We also showed that the deduced amino acid sequence of DreE possesses a coenzyme A (CoA) binding site and presumed that it is involved in 2,3,7,8-TCDD degradation by DreE. Previously, delCardayre et al reported that CoA, a coenzyme in various acyl group transfer reactions, also functions as a GSH analog and maintains a reductive environment in gram-positive bacteria [1,2,14]. Many gram-positive bacteria do not produce GSH, instead these organisms produce CoA at high levels by CoA disul-

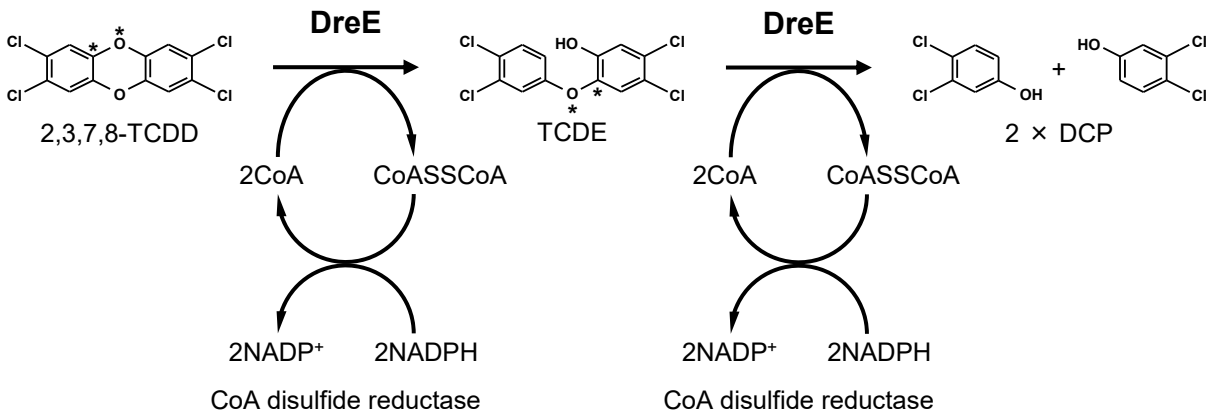

Fig. 1. Proposed degradation pathways for 2,3,7,8-TCDD by DreE. Enzyme: DreE, dioxin reductive etherase. Abbreviations: 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDE, 3',4',4,5-tetrachloro-2-hydroxydiphenyl ether; DCP, 3,4-dichlorophenol; CoA, coenzyme A; CoASSCOA, coenzyme A disulfide. Asterisks indicate the positions where hydrogen is added.

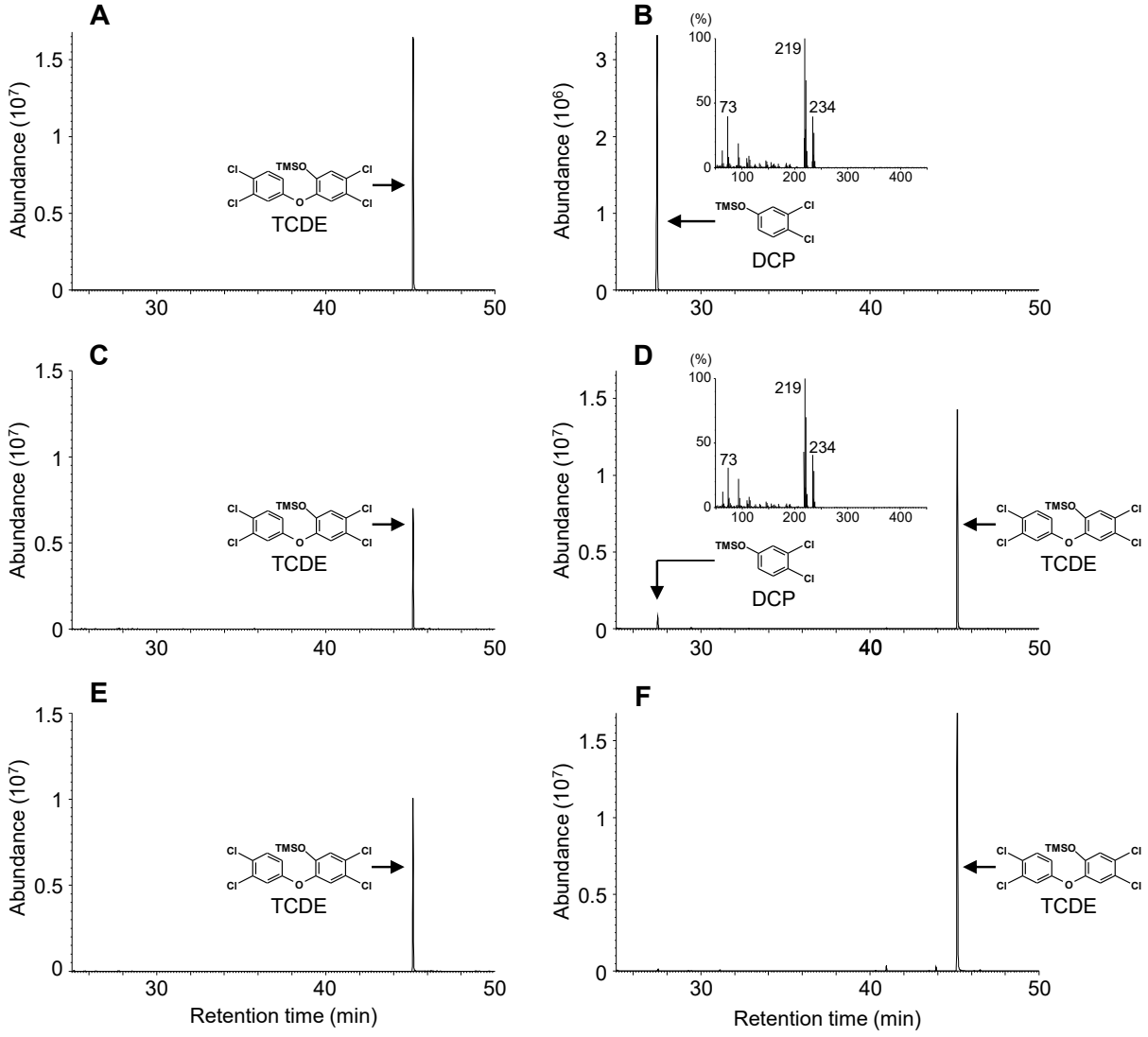

Fig. 2. GC-MS analyses of the reaction mixtures for TCDE degradation mediated by DreE. The selected ion monitoring (SIM) chromatograms of the substrate TCDE at *m/z*=396 (C), *m/z*=234 (D) and the detected product DCP at *m/z*=234 (D), and its corresponding MS spectrum were compared to authentic TCDE and DCP (A, B). The SIM chromatograms at *m/z*=396 (E) and at *m/z*=234 (F) represent the control chromatograms obtained when DreE was lacking from the enzyme assay.

fide reductases. Thus, it was suggested that CoA functions as a GSH analog in *G. thermodenitrificans* UZO 3 and is involved in 2,3,7,8-TCDD degradation by DreE. We therefore have proposed a molecular mechanism model for the reductive cleavage of diaryl ether bonds of 2,3,7,8-TCDD in *G. thermodenitrificans* UZO 3 (Fig. 1). DreE reductively cleaves diaryl ether bonds of 2,3,7,8-TCDD using two hydrogens from two molecules of CoA, which acts as an electron donor. Furthermore, CoASSCoA (CoA disulfide) produced from the reaction may be NADPH-dependently converted to two molecules of CoA by CoA disulfide reductase, which is predicted to exist in *G. thermodenitrificans* UZO 3.

However, contrary to our previous data with cell-free extract prepared from a wild strain of *G. thermodenitrificans* UZO [17], DCP was not generated as a final product from 2,3,7,8-TCDD degradation mediated by DreE. In this study, we have investigated whether TCDE is first produced as a reaction intermediate which is then converted to DCP from 2,3,7,8-TCDD degradation mediated by DreE (Fig. 1).

The TCDE was prepared following previous protocols [16,17]. DCP, N,O-Bis(trimethyl silyl)trifluoroacetamide (BSTFA), kanamycin (Km) and diethyl ether were purchased from Wako (Osaka). *E. coli* BL21 (DE3) was used as the host strain [15]. The expression plasmid pETE in which the *dreE* gene is inserted downstream of the T7 promoter of pET28b(+) (Novagen) was isolated as in a previous study [16]. Luria-Bertani medium was utilized for the cultivation of *E. coli* strains. For the culture of cells carrying antibiotic resistance markers, the media for *E. coli* transformants was supplemented with 25 mg of Km/liter. DreE preparation, the enzymatic reaction assays and GC-MS analysis were performed as described previously [16,17]. Briefly, 1 mL reaction mixture containing DreE (200 μg of protein/mL) and 15 μM TCDE dissolved in toluene and acetone (final concentration 0.5% and 2%, respectively) was prepared. The enzymatic reaction was performed at 65°C for 2 h inside a controlled incubator. Diethyl ether extracts from the TCDE reaction mixture were dried. The dried enzyme reactant was derivatized by BSTFA prior to GC-MS analysis.

We analyzed the TCDE-degrading activity of DreE to investigate whether it was a reaction intermediate during 2,3,7,8-TCDD degradation to DCP. GC-MS analysis of the diethyl ether extract of the complete reaction mixture in addition to the substrate TCDE showed peaks for DCP at retention times 27.45 min (Fig. 2D). The peaks of the substrate and products corresponded well with those of their respective authentic compounds (Fig. 2A, B). Diethyl ether extract of a reaction mixture that did not contain the DreE (control) was detected of TCDE, but showed no peak for DCP (Fig. 2E, F). These results show that DreE reductive cleaves of the two diaryl ether bonds of 2,3,7,8-TCDD in a sequential fashion. That is, TCDE is first produced by DreE as a reaction intermediate which is then converted to DCP (Fig. 1).

Acknowledgements

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References

1) delCardayre, S.B., K.P. Stock, G.L. Newton, R.C. Fahey, and J.E. Davies. 1998. Coenzyme A disulfide reductase, the primary low molecular weight disulfide reductase from *Staphylococcus aureus*. J. Biol. Chem. 273: 5744–5751.
2) delCardayre, S.B. and J.E. Davies. 1998. *Staphylococcus aureus* coenzyme A disulfide reductase, a new subfamily of pyridine nucleotide-disulfide oxidoreductase. J. Biol. Chem. 273: 5752–5757.
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) Hutzinger, O., M.J. Blumich, M. Vanderberg, and K. Olie. 1985. Sources and fate of PCDDs and PCDFs—An overview. Chemosphere. 14: 581–600.
6) Kaiser, J. 2000. Just how bad is dioxin? Science. 288: 1941–1944.
7) 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.
8) 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.
9) 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.
10) 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.
11) Meharg, A.A. and D. Osborn. 1995. Dioxins released from chemical accidents. Nature 375: 353–354.
12) 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.
13) Mironov, V.N, A.S. Kraev, M.L. Chikindas, B.K. Chernov, A.L. Stepanov, and K.G. Skryabin. 1994. Functional organization of the riboflavin biosynthesis operon from *Bacillus subtilis* SHgw. Mol. Gen. Genet. 242: 201–208.
14) Newton, G.L., K. Arnold, M.S. Price, C. Sherrill, S.B. Delcardayre, Y. Aharonowitz, G. Cohen, J. Davies, R.C. Fahey, and C. Davis. 1996. Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. J. Bacteriol. 178: 1990–1995.
15) Studier, F.W. and B.A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189: 113–130.
16) Suzuki, Y., M. Nakamura, Y. Otsuka, N. Suzuki, K. Ohyama, T. Kawakami, K. Izawa-Sato, R.R. Navarro, S. Hishiyama, K. Inoue, T. Kameyama, A. Takahashi, and Y. Katayama. 2018. Cloning and sequencing of the gene encoding the enzyme for the reductive cleavage of diaryl ether bonds of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in *Geobacillus thermodenitrificans* UZO 3. J. Biosci. Bioeng. 126: 488–496.
17) Suzuki, Y., M. Nakamura, Y. Otsuka, N. Suzuki, K. Ohyama,

T. Kawakami, Y. Umeka, J.S. Maninang, K. Izawa-Sato, S. Hishiyama, K. Inoue, T. Kameyama, A. Takahashi, and Y. Katayama. 2016. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) degradation by the thermophilic *Geobacillus* sp. UZO 3. J. Environ. Biotech. 15: 105–108.

18) Wittich, R.M. 1998. Degradation of dioxin-like compounds by microorganisms. Appl. Microbiol. Biotechnol. 49: 489–499.

19) Yakimov, A.P., T.A. Seregina, A.A. Kholodnyak, R.A. Kreneva1, A.S. Mironov, D.A. Perumov, and A.L. Timkovskii. 2014. Possible function of the *ribT* gene of *Bacillus subtilis*: Theoretical prediction, cloning, and expression. Acta. Nature. 6: 106–109.