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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 in *Geobacillus thermodenitrificans* UZO 3

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We have previously reported that a cell-free extract prepared from *Geobacillus thermodenitrificans* UZO 3 reductively cleaves diaryl ether bonds of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), a dioxin with the highest toxicity, in a sequential fashion producing 3′,4′,4,5-tetrachloro-2-hydroxydiphenyl ether (TCDE) as the intermediate, and 3,4-dichlorophenol (DCP) as the final reaction product. The detection of TCDE implicated the discovery of an unprecedented dioxin-degrading enzyme that reductively cleaves the diaryl ether bonds. In this study, we report the cloning and sequencing of the dioxin reductive etherase gene *dreE* which codes for the 2,3,7,8-TCDD-degrading enzyme. We showed that *dreE* was expressed in *Escherichia coli* and that the product of the expression could reductively cleave diaryl ether bonds of 2,3,7,8-TCDD to produce TCDE. Furthermore, we established that the amino acid sequence encoded by *dreE* was homologous to an enzyme with yet unknown function that is encoded by a gene located in the riboflavin (vitamin B2) biosynthesis operon in *Bacillus subtilis*. We also showed that the amino acid sequence possesses a coenzyme A (CoA) binding site that is conserved in the N-acyltransferase superfamily. For the first time, the degradation of 2,3,7,8-TCDD at the molecular level using a enzyme of bacterial origin has been demonstrated. A novel mechanism model for the reductive cleavage of diaryl ether bond of 2,3,7,8-TCDD was also proposed.

[**Keywords:** Bioremediation; Dioxin; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Degradation gene; Cloning]

Dioxin is a generic name for compounds consisting of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), which have 75 and 135 congeners, respectively. Dioxin is formed not only naturally but also artificially as a by-product of insecticides or herbicides production. The use of these chemicals has increased since the 1940s (1), mainly in western nations, causing serious environmental pollution and consequent damage to living organisms worldwide. The most toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), was massively formed as a by-product during the production of Agent Orange (trichlorophenoxyacetic acid), which was used for clearing jungles and thus inadvertently released into the environment (2). After the second world war, academic research on dioxins, represented by 2,3,7,8-TCDD, was actively conducted in many fields, including medicine, environmental chemistry, and microbiology. These studies revealed that dioxins are carcinogenic and genotoxic environmental chemicals that affect organisms over a long period and across generations (3). Dioxin research in the medical field significantly progressed to a point where it was shown that the toxicity of 2,3,7,8-TCDD is mediated by its receptor, aryl hydrocarbon receptor (AhR). Among dioxin congeners, 2,3,7,8-TCDD exhibited the highest affinity for AhR. Efforts to elucidate the molecular mechanisms for cancer development caused by AhR-mediated toxicity of 2,3,7,8-TCDD continued, and the molecular mechanism of oncogenesis in various reproductive cells has been actively investigated (4–6).

In the field of environmental bioremediation, researches on dioxin-degrading bacteria were very popular worldwide until the 2000s (7,8). For example, Wittich et al. (9) elucidated the dioxin degradation mechanism by the aerobic bacterium *Sphingomonas wittichii* RW1, and succeeded in cloning a dioxin dioxygenase gene *dxnA1A2* from this bacterium. DxnA1A2 transfers two atoms of oxygen to an angular position adjacent to the diaryl ether bond of dibenzo-*p*-dioxin and dibenzofuran and cleaves the diaryl ether bonds via an unstable acetal intermediate (9–11). Substrate specificity for dioxin congeners was investigated in *S. wittichii* RW1 and it was found that 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD), 1,2,3-TriCDD, 1,2,3,4-TCDD, and 1,2,3,4,7,8-HxCDD were converted to chlorocatechols (12,13). However, aerobic microbial activity toward 2,3,7,8-TCDD-degradation, particularly the identification of

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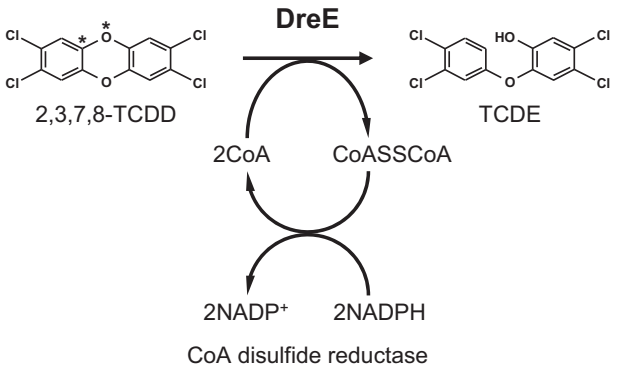

FIG. 1. Novel proposed model for the reductive cleavage of diaryl ether bond of 2,3,7,8-TCDD by *G. thermodenitrificans* UZO 3. Enzyme: DreE, dioxin reductive etherase. 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDE, 3′,4′,5-tetrachloro-2-hydroxydiphenyl ether; CoA, coenzyme A; CoASSCOA, coenzyme A disulfide. Asterisks indicate the positions where hydrogen is added.

degradation intermediates, has not been reported. On the other hand, it was reported that the anaerobe *Dehalococcoides* sp. strain CBDB1 could convert 1,2,3,4-TCDD to 2-MCDD and 1,2,3,7,8-PeCDD to 2,7- or 2,8-DCDD by dechlorination (14,15). Although the *Dehalococcoides* sp. strain CBDB1 was predicted by genomic analysis to possess 32 multiple reductive dehalogenase-homologous genes (16,17), the expression of an active form of dehalogenase in *Escherichia coli* has not been successfully accomplished (18,19). Therefore, it will be extremely difficult to analyze an anaerobe-derived dehalogenase at the molecular level. A question therefore arises as to whether it is possible to degrade the most potent dioxin congener, 2,3,7,8-TCDD, by various bacterial functions developed during biological evolution in the ecosystem? With the recent decline in dioxin research, fundamental questions about this group of highly toxic chemicals remain unanswered.

We have previously reported that a cell-free extract prepared from *Geobacillus thermodenitrificans* UZO 3 reductively cleaves diaryl ether bonds of 2,3,7,8-TCDD in a sequential fashion producing 3′,4′,4,5-tetrachloro-2-hydroxydiphenyl ether (TCDE) as the intermediate, and 3,4-dichlorophenol (DCP) as the final reaction product (Fig. 1) (20). The structure of the TCDE was identified by gas chromatography–mass spectrometry (GC–MS) analysis using a chemically-synthesized authentic compound. The detection of TCDE intermediate implicated the discovery of an unprecedented dioxin-degrading enzyme that reductively cleaves the diaryl ether bonds of 2,3,7,8-TCDD in a manner that is similar to glutathione *S*-transferase (GST), a reduction cleavage enzyme that uses GSH as electron donor (21–24).

The aim of this study was to elucidate the 2,3,7,8-TCDD degradation mechanism in *G. thermodenitrificans* UZO 3 as well as to demonstrate the degradation of 2,3,7,8-TCDD at the molecular level by cloning the gene encoding the degradation enzyme. The above fundamental question pertaining 2,3,7,8-TCDD degradability was clarified by presenting experimental facts that can be confirmed by scientific verification.

MATERIALS AND METHODS

Chemicals The 2,3,7,8-TCDD and 2,7-DCDD were purchased from Cerilliant (Round Rock, TX, USA) and Accu Standard (New Haven, CT, USA), respectively. The TCDE and 4′,5-dichloro-2-hydroxydiphenyl ether (DCDE) were prepared following previous protocols (20,25). DCP, isopropyl-β-etherase-D-thiogalactopyranoside (IPTG), *N,O*-bis(trimethyl silyl)trifluoroacetamide (BSTFA), coenzyme A (CoA) and glutathione (GSH) were purchased from Wako (Osaka). Purities of these chemicals range from 96.6% to 100%. All other chemicals used were of analytical grade and of the highest purity available.

Bacterial strain, plasmids and culture media The strains and plasmids used in this study are listed in Table 1. Luria–Bertani medium (LB) was utilized for the cultivation of *E. coli* strains. For the culture of cells carrying antibiotic resistance markers, the media for *E. coli* transformants were supplemented with 12.5 mg of chloramphenicol (Cm)/L, 100 mg of ampicillin (Ap)/L, or 25 mg of

TABLE 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristic	Reference or source
Strain		
G. thermodenitrificans UZO 3	Wild type	20,25
E. coli EPI300	F⁻ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) φ80d*lacZ* ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ⁻*rps*L *nup*G *trf*A	Epicentre
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r_K^- m_K^+), *e14*⁻ (*mcrA*⁻), *supE44*, *relA1*, Δ(*lac-proAB*)/ F′(*traD36*, *proAB*⁺, *lac I*^{q}, *lacZ*ΔM15)	30
E. coli BL21(DE3)	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm (DE3)*	31
Plasmid		
pCC1FOS	Fosmid vector, Cm^r	Epicentre
pBluescript II KS(+) and SK(+)	Cloning vectors, Ap^r	29
pET28b(+)	Expression vector, T7 promoter, Km^r	Novagen
pCDE1	pCC1FOS with approximately 36-kb fragment carrying *dreE*	This study
pCDE9	pCC1FOS with approximately 34-kb fragment carrying *dreE*	This study
pCDE13	pCC1FOS with a 6.3-kb BamHI fragment from pCDE1	This study
pCDE14	pCC1FOS carrying the same fragment as pCDE13 in the opposite direction	This study
pCDE15	pCC1FOS with a 8.4-kb BamHI fragment from pCDE1 carrying *dreE*	This study
pCDE16	pCC1FOS carrying the same fragment as pCDE15 in the opposite direction	This study
pCDE95	pCC1FOS with a 6.5-kb BamHI fragment from pCDE9	This study
pCDE96	pCC1FOS carrying the same fragment as pCDE95 in the opposite direction	This study
pBKSEN2	pBluescript II KS(+) with a 3.5-kb EcoRV-NheI fragment from pCDE15	This study
pBKSBLN	pBluescript II KS(+) with a 1.5-kb BlnI-NheI fragment from pBKSEN2	This study
pBSKBP	pBluescript II SK(+) with a 0.8-kb BlnI-PciI fragment from pBKSEN2	This study
pBKSEP	pBluescript II KS(+) with a 1.2-kb EcoRV-PciI fragment from pBKSEN2	This study
pBSKSE	pBluescript II SK(+) with a 1.0-kb SacII-EcoRV fragment from pCDE15	This study
pBSKSN	pBluescript II SK(+) with a 0.7-kb SacII-NheI fragment from pCDE15	This study
pBKSBN	pBluescript II KS(+) with a 3.0-kb BamHI-NheI fragment carrying *dreE* from pCDE15	This study
pBKSEN	pBluescript II KS(+) with a 0.8-kb EcoRV-NheI fragment carrying *dreE* from pBKSBN	This study
pBKSNN	pBluescript II KS(+) with a 0.3-kb NcoI-NheI fragment from pBKSEN	This study
pBKSEB	pBluescript II KS(+) with a 0.25-kb EcoRV-BstXI fragment from pBKSEN	This study
pETE	pET28b(+) with a 0.35-kb NdeI-HindIII fragment carrying *dreE*	This study

Cm^r, Ap^r and Km^r resistance to chloramphenicol, ampicillin and kanamycin, respectively.

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