Detection and characterization of chlorinated-dioxin ether cleavage function in the bacterium Geobacillus midousuji SH2B-J2.

Yuichiro Otsuka[1], Masaya Nakamura[2], Shojiro Hishiyama[2], Yoshihiro Katayama[3], Sadayori Hoshina[1]

[1]Department of Laboratory Medicine, Jikei University School of Medicine, Tokyo 105-8461, Japan
[2]Forestry and Forest Products Research Institute, Ibaraki 305-8687, Japan
[3]Tokyo University of Agriculture and Technology, Koganei 805-8588, Japan

Introduction

As of now, there are no dioxin degrading microorganism reported that can be applied to bioremediation. The reasons for this are that degrading function acquired from comprehensive screening of bacteria that can be grown with a single carbon source using non-chlorinated dioxin does not function against highly chlorinated dioxins, and that although white rot fungus capable of degrading lignin, a plant polyphenol substance, have been reported to reduce chlorinated dioxins, degrading enzyme remain unclear.

Geobacillus midousuji SH2B-J2 (J2 strain) that have been separated by Hoshina *et al*. have shown to reduce highly chlorinated dioxins in incineration fly ash, as well as octa-chlorinated dioxins (OCDD). However, details of its degrading mechanisms remain unclear. Since the J2 strain is capable of reducing even OCDD, it was hypothesized that the initial degradation reaction is intra-molecular ether bond cleavage, so J2 strain dioxin degradation mechanism was analyzed for verification.

Material and Methods

Powerful fluorescence assay system

In order to analyze faint dioxin degradation of the J2 strain, substance shown in Fig. 1 was synthesized for examination of ether bond cleavage within dioxin molecules. This substance (DDES) emits powerful fluorescence when even a single ether bond is severed. Since this fluorescence can be detected at picomol level, it allows highly sensitive examination.

Dioxin aryl ether cleavage assay in *Geobacillus midousuji* SH2B-J2

15 ml of agar prepared from Tripticase Soy liquid medium with yeast extracted added (TSB+YE) to a concentration of 0.3% was placed in 50 ml conical tube, and J2 strain was inoculated. 50 µg of DDES dissolved with Dimethyl sulfoxide (DMSO) was added to this bacterial emulsion, and cultured with a rotation at 20 rpm, 65 . After culturing, it was extracted three times with ethyl acetate, and then analyzed with thin-layer chromatography.

Next, reaction by coarse enzyme solution was conducted. 500 ml of TSB+YE was prepared in 3 l flask, inoculated with J2 strain, and cultured for three hours. All bacteria were recovered by a centrifuge, and bacterium was pulverized by a supersonic pulverizer. Pulverized solution was centrifuged to remove residual bacteria that were not pulverized. Resulting supernatants was further separated to cellular membrane and cellular matter using a centrifuge set at 150000 X g for 30 minutes. Cellular membrane (M) and cellular matter (C) were reacted with DDES, and analyzed under thin-layer chromatography.

Analysis of metabolite

Membranes were obtained in a manner similar to J2 strains cultured in 24 l TSB+YE liquid medium. All membranes were reacted with DDES. Reacted product was extracted with ethyl acetate, and separated/refined/concentrated by silica gel chromatography. Reacted product obtained was analyzed by GC-MS.

Results and Discussion

Fig 2A shows the analytical results for combined culturing of J2 strain and DDES. Results show that fluorescence was detected in culture with DDES added, in contrast to cultures with no DDES added.

Fig 2B shows the analytical results of cellular membrane and cellular matter of J2 strain reacted with fluorescent agar. Lanes 1, 2, 3, and 4 show the results of dioxin degradation induced by adding incineration flyash. Lanes 5, 6, 7, and 8 show the results with no flyash added. Lanes 1, 2, 5, and 6 are reactions of cellular membrane (M), and Lanes 3, 4, 7, and 8 are reactions of cellular matter (C). Even-numbered lanes are reactions with DDES added, and odd-numbered lanes are reactions of control with only DMSO, an DDES solvent, added. Results show that spots of fluorescent emission, supposedly indicating reaction substances, are detected only from membranes induced with incineration flyash additives. In reaction under similar conditions, reaction of chlorine with one or two fluorescent agar bonded showed spots indicating fluorescence emission. This suggests that the mechanism of dioxin-ether bond cleavage of J2 strain derives from membrane protein in cellular membrane.

Fig. 3 shows the GC-MS analysis of reaction products from fluorescent agar and membrane of mass-cultured J2 strain. Results show production of 4-methylumberriferone. This suggests that ether bond is cleaved by reductive reaction. Reductive detoxification of dioxins is reported in reaction by Glutathione-S-transferase for mammals. Fig. 4 shows the reaction pathway. Since mercapturic acid complex was detected as reactive product of J2 strain (Fig.3), it strongly suggests that detoxification mechanism of mammals are reacting against dioxins for J2 strains.

This study showed that J2 strain cleaves ether bonds of highly chlorinated dioxins, degrading them. The mechanism exist in the cellular membrane, and is a reductive degradation, not oxidative degradation as previously reported. Also, this reaction is similar to detoxification reaction seen in mammals, and is a totally new degradation mechanism for chlorinated dioxins in microorganisms.

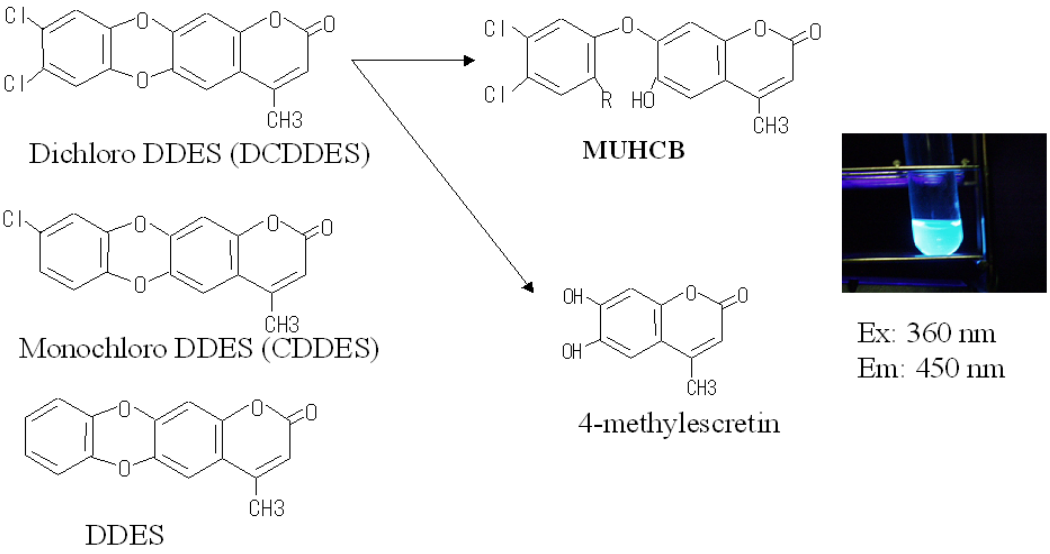

Fig.1 Structure of Escretin-type fluorescence dioxin aryl ether and it's cleavage assay system.

Scheme of 4-methylescretin released from chlorinated or no chlorinated DDES. Upon cleavage of the aryl ether linkage, 4-methylescretin is released and emits powerful fluorescence. Fluorescence of 4-methylescretin was measured with excitation at 360 nm and emission at 450 nm.

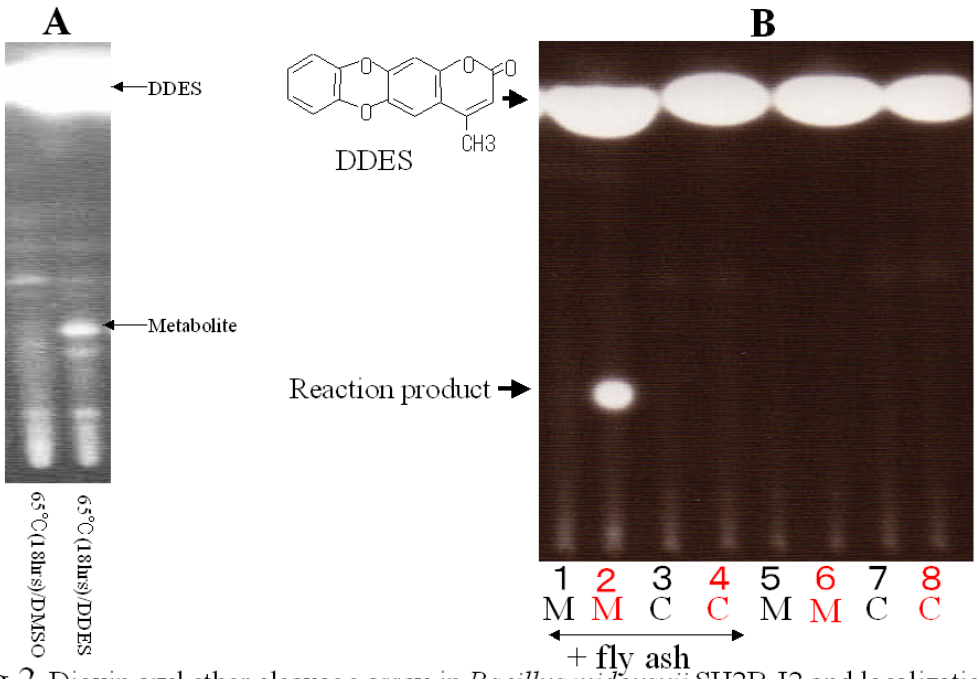

Fig.2 Dioxin aryl ether cleavage assay in *Bacillus midousuji* SH2B-J2 and localization of dioxin aryl ether cleavage function.

Fig.2B: Lane 1～4: add fly ash, Lane 5～8: none fly ash, Lane 1,3,5,7: add DMSO, Lane 2,4,6,8: add DDES+DMSO, M: membrane fraction, C: cytosol fraction

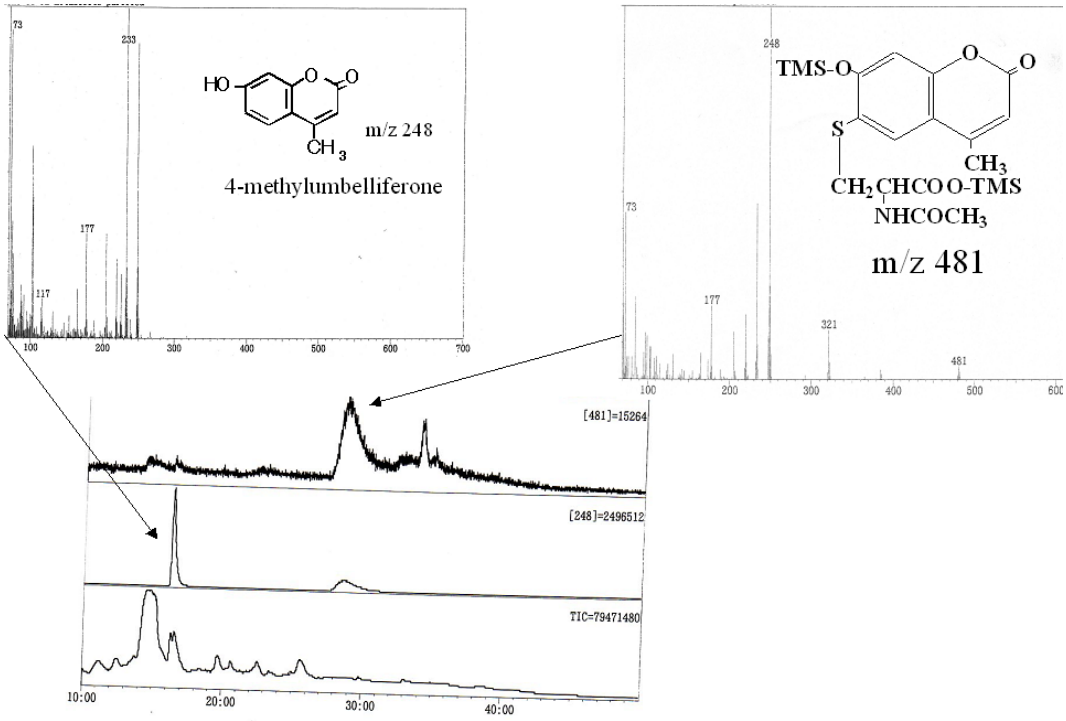

Fig.3 Mass chromatogram scanning of intermediate in reaction products of DDES.

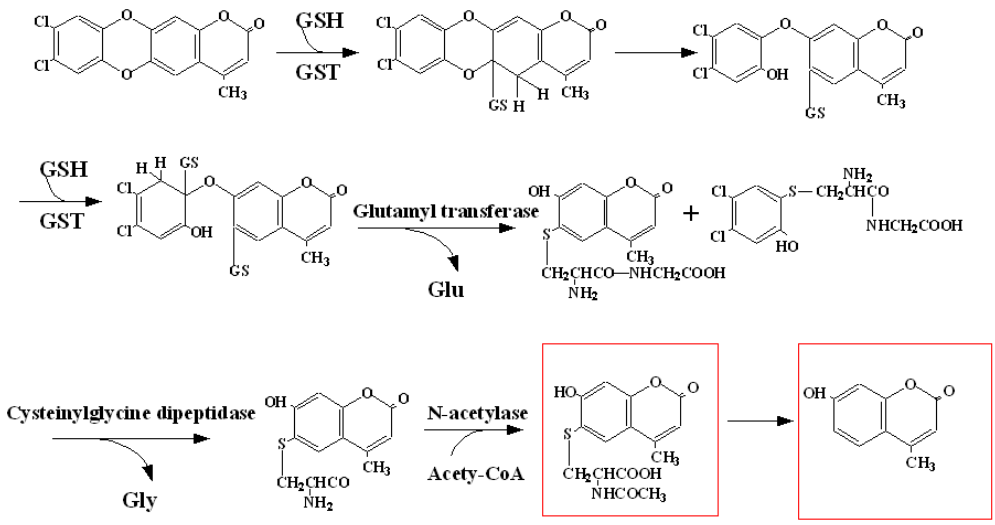

Fig.4 Proposed reaction pathway of dioxin aryl ether cleavage in *B. midousuji* SH2B-J2.

Reference
1. Sadayori Hoshina, Kazuo Miyaji, Hiroshi Goda, Kazuei Ishii and Tohru Furuichi. Biodegradation of dioxins in fly ash by thermophilic Bacillus midousuji. Organohalogen Compounds 58, 137-140, 2002.