# MICROBIAL DEGRADATION OF CHLORINATED DIBENZO-*p*-DIOXIN BY A GRAM-POSITIVE BACTERIUM, *RHODOCOCCUS OPACUS* SAO101

Nobutada Kimura, and Yoichi Kamagata

Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

## Introduction

The biodegradation of dibenzo-*p*-dioxin (DD) and dibenzofuran (DF) by bacterial strains has been studied using several bacterial strains that are able to degrade these compounds; these strains were isolated and characterized (1-9). However, most of these strains oxidize DF and DD co-metabolically, a process that allows bacterial growth on other compounds and not DF and DD (1,3-6,9). Recently, *Sphingomonas* sp. strain RW1, which can utilize DD as a carbon source, has been studied. In this report, the metabolic pathway of DD was initiated by dioxygenation at the angular position of two carbon atoms adjacent to the ether bridge. The resulting hemiacetal compound was spontaneously transformed to a trihydroxy compound, and the hydroxylated aromatic rings of the trihydroxy compound were subsequently *meta*-cleaved by angular dioxygenase (8). Furthermore, the ability of strain RW1 to oxidize chlorinated derivatives of DD was analyzed. Strain RW1 degraded several mono-CDDs and di-CDDs, but it could not degrade the more highly chlorinated DD (10). Until now, there is little information regarding the potential microbial degradation of DD. In addition, studies on the microbial degradation of CDD are rare (5,10). Therefore, an understanding of DF-/DD-degrading bacteria is necessary for their application to environmental engineering in order to eliminate contaminants, and for understanding the fate of CDDs and CDFs in the environment. In this session, we report the isolation and characterization of a novel gram-positive bacterium, *Rhodococcus opacus* SAO101, which can utilize DF and DD as the sole carbon source. Some intermediates from DD degradation are identified and the metabolic pathway of DD is proposed. The co-oxidation ability of mono-, di- and tri-CDD congeners is also investigated.

## Materials and Methods

*Media*

A mineral salt medium (MM) was used containing (per liter):4.1 g of $Na_2HPO_4 \cdot 2H_20$; 0.4 g of $KH_2PO_4$; 0.5 g of $(NH_4)_2SO_4$; 0.1 g of $MgCl_2 \cdot 6H_2O$; 50 mg of $Ca(NO_3)_2 \cdot 4H_2O$; 20 mg of $Fe(NH_4)_2$-citrate, and 0.1 ml of a trace element solution without EDTA. For preparation of the liquid medium, DF dissolved in ethanol was added to cell suspensions in 100-ml Erlenmeyer flasks with Teflon seal screw caps. For preparation of solid medium, DF was supplied in the vapor form by adding it to the lids of inverted petri dishes.

*Identification and characterization of bacterial strain*

Genomic DNA of an isolated strain was purified according to the method described by Ausbel et al. The DNA was amplified by polymerase chain reaction (PCR) using specific primers corresponding to bases 8-27 and 1542-1525 (*Escherichia coli* numbering). PCR products were cloned into a TA cloning vector (Invitrogen Co., USA) and the sequences were determined. Sequences were processed using a

model 373 Stretch PE Biosystems automated sequencer (PE Biosystems Co., USA). The sequences determined in this study and those retrieved from databases were aligned using Clustal W, version 1.8. Alignments were refined by visual inspection. A neighbor-joining tree was constructed using the njplot software in Clustal W, version 1.8.

*Growth conditions*

Growth curve experiments were performed using 100-ml Erlenmeyer flasks with 20 ml MM containing 0.2 mM DF or 0.1 mM DD dissolved in 20 µl of ethanol as the sole carbon source. Flasks with MM containing ethanol without DF or DD were used as controls. After incubation at 30 °C?and 200 rpm, the cultures were removed at 6-hrs intervals and the cell density was measured using a spectrophotometer ($OD_{600}$). Spectrophotometry was performed using a Hewlett-Packard 8452A (Hewlett-Packard Co., USA). To analyze the spectrum of the aromatic compounds that could be metabolized as the sole carbon source for growth, cells were grown for one week in liquid MM medium in the presence of various aromatic compounds at a 0.1 mM final concentration. Growth was expressed in terms of the increase in turbidity.

*Preparation of resting cells, identification of metabolites, and analytical procedures*

Cells were grown in 100-ml Erlenmeyer flasks with MM supplemented with 0.2 mM DF as the sole carbon source. The cells were grown to the exponential growth phase (OD 600 of 2.0), washed twice with 50 mM sodium phosphate buffer (pH 7.5), and resuspended in 5 ml of the same buffer to adjust the OD600 to 1.0. DF, DD, and CDD dissolved in ethanol were added at suitable concentrations (see Results) to cell suspensions in 100-ml Erlenmeyer flasks with Teflon seal screw caps. After incubation with shaking at 200 rpm and 30 °C for a suitable duration (see Results), the entire culture was transferred to 100-ml separatory funnels and extracted with the same volume (5 ml) of ethyl acetate. One milliliter of the extract that was evaporated to dryness with a gentle stream of nitrogen gas was derivatized with bis- (trimethylsilyl)-acetamide (BSA, Tokyo Kasei Co. Ltd.,Tokyo). Control experiments were performed with heat-inactivated cells (121 °C, 20min). Each set of experiments was performed in triplicate. The samples were analyzed by gas chromatography-mass spectrometry (GC-MS) (Hewlett-Packard Co., USA ; model HP5890, HP5971A) employing a coiled capillary glass column (0.25 mm inner diameter, 30 m long) packed with methylsilicon DB5. Helium was used as the carrier gas at a flow rate of 0.8 ml/min. The GC column temperature on the GC was increased from 80 °C to 280 °C at a rate of 10 °C /min. Relative percentages of degradation were calculated based on the integrated total ion peak area.

*Chemicals*

DF and DD were purchased from Wako Chemical Co.(Tokyo). 1-CDD, 2,3-CDD, 2,7-CDD, 2,8-CDD, 1,2,3-CDD and 2,3,7-CDD were purchased from Cambridge Isotope Laboratories, Inc. (MA, USA)

**Results and Discussion**

A dibenzo-*p*-dioxin-degrading bacterial strain, *Rhodococcus opacus* SAO101, was isolated from forest soil samples collected from the subtropical islands of Japan by enrichment of a mineral salt medium containing dibenzofuran as the sole carbon and energy source. The isolated bacterium could utilize dibenzo-*p*-dioxin as the sole carbon and energy source, and also many monocyclic aromatic compounds, such as toluene, phenol, and chlorobenzene, as well as bicyclic aromatic compounds, such as biphenyl, naphthalene, and dibenzothiophene. Furthermore, strain SAO101 has a high co-oxidative potential for chlorinated dibenzo-*p*-dioxin. Metabolite analysis of dibenzo-*p*-dioxin degradation by

strain SAO101 revealed the formation of dihydrodiol, 2,2',3'-trihydroxybiphenyl ether, and a *meta*-cleavage compound of 2,2',3'-trihydroxybiphenyl ether, and the accumulation of dihydroxy compounds. On the basis of these results, the metabolic pathway of dibenzo-*p*-dioxin was proposed.

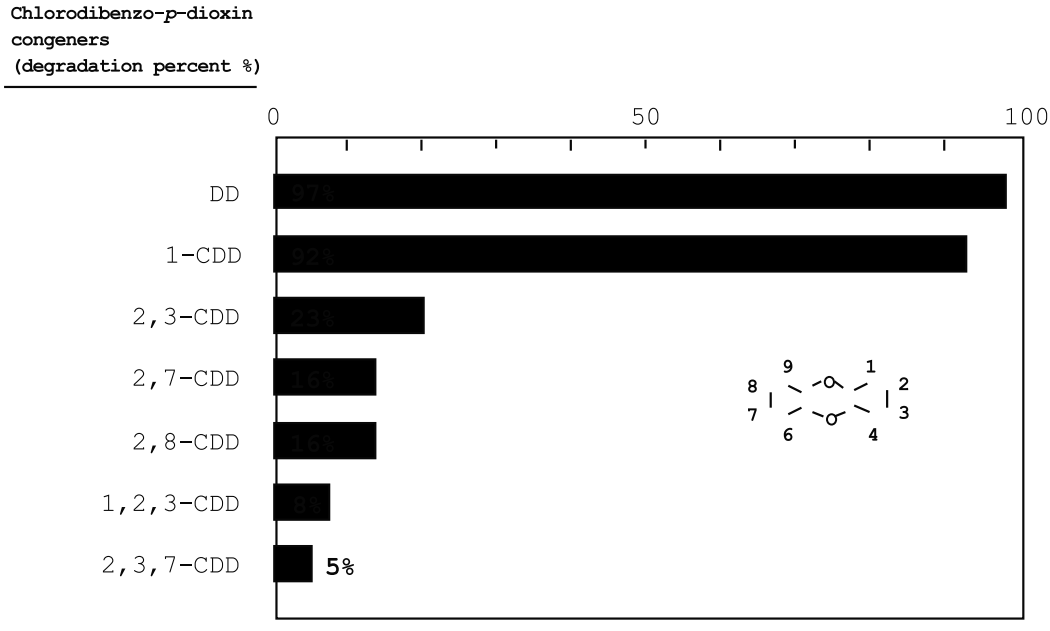


**Figure 1.** Degradation of chlorinated DD congeners by resting cells of *R. opacus* SAO101. DF-grown cells of each strain were washed, then incubated with CDD at 30 °C for 7 d. The concentration of CDD was 1 ppm.

**Acknowledgments**

We greatly appreciate the excellent technical assistance of Mrs. Tomoko Kimura. We thank Mr. Kazuo Miyaji for chemical analysis.

**References**

1. Cerniglia,C.E., Morgan,J.C., and Gibson,D.T. (1979) Biochem. J., 180, 175-185.
2. Fortnagel,P., Harms,H., Wittich,R.-M., Krohn,S., Meyer,H., Sinnwell, V., Wilkes,H., and Francke,W. (1990) Appl. Environ. Microbiol. 56, 1148-1156.
3. Harms,H., Wittich,R.-M., Sinnwell,V., Meyer,H., Fortnagel,P., and Francke,W. (1990) Appl. Environ. Microbiol.56,1157-1159.
4. Klecka,G.M. and Gibson,D.T. (1979) *J. Biochem.* 180,639-645.
5. Klecka,G.M. and Gibson,D.T. (1980) Appl. Environ. Microbiol.39, 288-296.
6. Monna,L., Omori,T., and Kodama,T. (1993) Appl.Environ.Microbiol. 59,285-289.

7. Strubel, V., Rast, H. G., Fietz, W., Knackmuss, H.-J., and Engesser, K. H. (1989) FEMS Microbiol. 58, 233-238.
8. Wittich,R.-M., Wilkes,H., Sinnwell,V., Francke,W., and Fortnagel,P. (1992) Appl. Environ. Microbiol. 58,1005-1010.
9. Becher,D., Specht,M., Francke,W., and Schauer,F. (2000) Appl. Environ. Microbiol.66, 4528-4531.
10. Wilkes,H., Wittich,R.-M., Timmis,K.N., Fortnagel,P., and Francke,W. (1996). Appl. Environ. Microbiol.62,367-371.
11. Kimura, N., and Urushigawa, Y. (2001) J. Biosci. Bioengi. 92, 138 – 143.