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Cloning and Characterization of Bacterial Gene Involved in Chlorothalonil-Biotransformation

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A gene responsible for the chlorothalonil-biotransformation was cloned from the chromosomal DNA of *Ochrobactrum anthropi* SH35B, capable of efficiently dissipating the chlorothalonil. The gene encoding glutathione S-transferase (GST) of *O. anthropi* SH35B was expressed in *Escherichia coli*, and the GST was subsequently purified by affinity chromatography. The fungicide chlorothalonil was rapidly transformed by the GST in the presence of glutathione. LC-MS analysis supported the formation of mono-, di- and tri-glutathione conjugates of chlorothalonil by the GST. The mono-glutathione conjugate was observed as an intermediate in the enzymatic reaction. The tri-glutathione conjugate has not been previously reported and seems to be the final metabolite in the biotransformation of chlorothalonil. It was here reported the glutathione-dependent biotransformation of chlorothalonil catalyzed by the bacterial GST.

Key words : chlorothalonil, glutathione S-transferase, biotransformation

Introduction

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad-spectrum chlorinated fungicide, which is highly efficient against the pathogens that infect mainly vegetables, fruits, and other crops[1]. Although chlorothalonil is not highly toxic to mammals, it is extremely toxic to fish[2] and is classified in the B2 group, which is considered as a probable human carcinogen, by the U.S. Environmental Protection Agency (U.S. EPA) due to the carcinogen hexachlorobenzene that is produced as a by-product during synthesis of the active ingredient[3]. To remove toxic organic compounds such as pesticides, both biological and chemical treatments have been suggested. A biological treatment of the toxic organic compounds (bioremediation), using microorganisms or enzymes produced from the microorganisms or plants, is often considered as an environmentally favorable method[4]. To date, however, there have been no unambiguous reports about the bioremediation of soil contaminated by chlorothalonil.

Ochrobactrum anthropi SH35B, capable of efficiently biotransforming the fungicide chlorothalonil, was isolated from soil. A gene responsible for the chlorothalonil-biotransformation was cloned from the strain[5]. The gene was determined to be an open reading frame (ORF) for the glutathione S-transferase (GST) by the nucleotide sequence. It has been known that the GST catalyzes the conjugation of the glutathione sulfur atom to a large variety of electrophilic compounds of both endobiotic and xenobiotic origin, resulting in detoxification[6,7]. Therefore, the GST might be involved in the detoxification of the fungicide chlorothalonil.

The experiment reported here was performed to investigate the mechanism of the biotransformation of chlorothalonil by *O. anthropi* SH35B. In this study, we report the glutathione-dependent biotransformation of chlorothalonil catalyzed by the bacterial GST, which was expressed in *Escherichia coli* and purified by affinity chromatography.

Materials and Methods

Chemicals

Chlorothalonil was purchased from Wako Pure Chemicals (Osaka, Japan) and was prepared by dissolving it in

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dimethyl sulfoxide (DMSO). All other reagents were of reagent grade and purchased from commercial sources.

Microorganism Isolation and Identification

The bacterial strain SH35B, which is able to grow on plates containing chlorothalonil as a single carbon source was isolated from soil of Goryeong, Gyeongsangbukdo, Korea. To identify the isolated strain culture morphology, biochemical reactions and 16S ribosomal DNA (rDNA) sequences were investigated. Two oligonucleotides, based on the report of Dunbar et al.[8], were used to determine 16S rDNA of the SH35B: (Forward) 5'-AGAGTTTGATCCTG GCTCAG-3' and (Reverse) 5'-TACCTTGTTACGACTT-3'. PCR was carried out using intact cells as a template. The thermal profile used was a 25-cycle consisting of denaturation of 1 min at 94℃, annealing of 1 min at 55℃, and extension of 2 min at 72℃. A final extension step consisting of 5 min at 72℃ was included. Amplified 16S rDNA was purified from an agarose gel and then sequenced by dideoxy-chain-termination methods. The isolated strain was identified as *O. anthropi* by culture morphology, biochemical reactions, and homology research based on 16S rDNA.

Cloning of a Gene Responsible for the Chlorothalonil-biotransformation

Chromosomal DNA was prepared from *O. anthropi* SH35B by the method of Berns and Thomas[9]. The DNA was partially digested with the restriction enzyme *Sau3*AI. DNA fragments of about 2.5 kbp were separated and then subcloned into the *Bam*HI site of pBluescript SK (+). Competent cells of *E. coli* KAM3 (Δ*acrB*) were transformed with the ligated recombinant plasmids and then spread onto M9 minimal medium agar plates containing 1% glucose and 0.37 mM chlorothalonil. The plates were incubated at 37℃ for 3 days. Candidate colonies were replica-plated, and plasmids were isolated from each of the candidates. Plasmid DNAs were used for restriction mapping and sequencing. The nucleotide sequence of the inserted DNA was determined by the dideoxy chain termination method. The nucleotide sequences reported here have been deposited in the GenBank database with the accession number AY378173.

Expression and Purification of *O. anthropi* SH35B GST

In order to construct an expression plasmid for the GST of *O. anthropi* SH35B, a polymerase chain reaction (PCR) was carried out using two synthetic oligonucleotides based on the ORF (GenBank accession number AY378173) to generate a unique *Eco*RI and *Bam*HI restriction enzyme site, respectively: (Forward) 5'-CGGAATTCATCGAAACTGA TGGGAG-3', and (Reverse) 5'-GCGGATCCTTAGTTCA GGCCTTC-3'. The PCR product was digested by *Eco*RI-*Bam*HI restriction enzyme and then ligated into pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which had been digested with *Eco*RI-*Bam*HI. The resulting plasmid was designated as pTOaGST. *E. coli* JM105 cells, transformed with pTOaGST, were grown overnight at 37℃ in Luria-Bertani medium (LB) containing 100 μg/ml of ampicillin. After overnight culture, the cells were diluted 50-fold into a fresh medium and grown to A_{600} of 0.6, at which point the GST expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 6 h. After harvesting, the cells were resuspended in 100 mM potassium phosphate buffer (pH 6.5), and disrupted by sonication. The unbroken cells were removed by centrifugation at 100,000 × g for 10 min, and the supernatant was taken for purification. The purification of the GST was carried out using GST Bind Kits according to the recommendations of the manufacturer (Novagen, Madison, WI). Protein purity was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining. The GST activity was measured as described previously[10] and the protein content was determined by the Bradford method[11].

Biotransformation of Chlorothalonil by the GST

To investigate the biotransformation of chlorothalonil by the purified GST, 0.5 ml of a buffer solution (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated at 25℃ in the presence and absence of the purified GST (1 μg/ml) and 5 mM reduced glutathione. At specific time, the enzymatic reaction was stopped by the addition of 60μl of 20% trichloroacetic acid. The mixture was extracted with the same volume of

hexane for the detection of the remained chlorothalonil and with the same volume of methanol for the metabolites that resulted from the enzymatic reaction. The extract was filtrated through a 0.45 μm membrane filter and analyzed by a high performance liquid chromatography (HPLC).

Analytical Methods

The content of the remaining chlorothalonil was determined using a Younglin-M930 HPLC (Younglin Co., Seoul, Korea) equipped with a Waters μBondapak C_{18} column (3.9 × 150 mm; Waters Co., Milford, MA). An elution of water : acetonitrile (2 : 3) was used at a flow rate of 1 ml per min for 10 min. The eluate was monitored at 254 nm using a M720 UV-visible absorbance detector (Younglin Co., Seoul, Korea).

In order to isolate and identify the metabolites that formed from the biotransformation of chlorothalonil, liquid chromatography / mass spectral (LC-MS) analyses were performed using an Agilent 1100 LC/MSD mass spectrometer equipped with an Agilent 1100 HPLC system and a diode-array detector (LC-DAD; Agilent Tech, Palo Alto, CA). The mass spectrometer was operated in the positive atmosphere pressure ionization electrospray (API-ES) mode with the capillary exit voltage at 100 V, the high energy dynode at 10 kV, and the multiplier at 2.1 kV. Full scans were acquired from m/z 50 to 1200 at 1.67 scans/sec. Algilent-Chemstation software was used to collect concurrent LC-DAD and positive API-ES data. Chlorothalonil metabolites were purified using a Zorbax C_{18} column (4.6 × 150 mm; i.d., 3.5 μm; Agilent Tech, Palo Alto, CA). For the isolation of chlorothalonil, the metabolite **A** and **B**, a linear gradient elution of 60% water with 1 % acetic acid: 40% methanol (v/v) to 100% methanol was used at a flow rate of 0.7 ml per min for 45 min. For the isolation of metabolite **C**, the mobile phase was only changed at a flow rate of 0.4 ml per min.

Results and Discussion

Cloning of a Gene Responsible for the Chlorothalonil-biotransformation

It was isolated a bacterium strain, *O. anthropi* SH35B, capable of growth on the plate containing chlorothalonil as the only source for carbon. The isolated strain showed the ability of biotransforming efficiently the fungicide chlorothalonil (manuscript in preparation). A gene responsible for the chlorothalonil-biotransformation (or resistance) was cloned from the chromosomal DNA of *O. anthropi* SH35B using a drug-hypersensitive *E. coli* KAM3[12], which lacks major multidrug efflux pumps ($\Delta acrB$) and is sensitive to chlorothalonil, as described in Materials and Methods. We obtained four candidate recombinant plasmids which enabled the KAM3 cells to grow in the presence of chlorothalonil. Restriction analysis revealed that these four plasmids contained the same DNA region (data not shown). One of them, pBOA3, was used for further analysis. The nucleotide sequence of inserted DNA revealed that it contained an open reading frame to be almost the same as the GST gene of *O. anthropi*, which has been registered in the GenBank database with the accession number Y17279[13]. We found, however, that there are some differences (70 different nucleotides at various positions) in the sequences between both GST genes (88% identity in the nucleotide sequence). The GST gene, 603 bp in length, specifies a putative 201-amino acid protein with a calculated molecular mass of about 22 kDa. The identity in the amino acid sequences between both GSTs was 94.5%. Only eleven amino acid residues were different. The Ser-11 residue, however, which has been known to be an important residue for catalysis by activation of the thiol group of glutathione[13], was conserved.

Comparison of the deduced primary structure of the GST with those of proteins present in the GenBank database indicated that the greatest similarity was with bacterial GSTs as suggested by Favaloro et al.[13]. The putative GST of *Brucella suis* 1330[14] showed the highest sequence similarity throughout the entire sequence: 82% identity and 90% similarity. The GST of *Pseudomonas pseudoalcaligenes*[15] showed 44% identity and 58% similarity. Many of the bacterial GSTs registered in the GenBank database also showed similar levels of identity and similarity to the GST of *O. anthropi* SH35 (data not shown). The GST of *E. coli* showed 38% identity and 54% similarity.

Purification and Characterization of *O. anthropi* SH35B GST

Bacterial GSTs are present in very low amounts[16], resulting in a limited study of the physical/chemical properties. Therefore, the first step is overproduction of the enzyme to study the properties of the *O. anthropi* SH35B GST. The GST was overexpressed and purified as described in Materials and Methods. An increase in the intensity of the band corresponding to about 22 kDa, which corresponds to the molecular mass calculated from its gene, was observed (Fig. 1, lane 1). The GST purified by the GST Bind Kits also appeared at the same molecular mass (Fig. 1, lane 2). The specific activity of the purified protein increased 16-fold compared with the crude enzyme. A total of 0.12 mg of purified protein from about 1.2 mg of crude extract was obtained.

Biotransformation of Chlorothalonil by the GST

In plants, the GST activity is an important indicator for determining the resistance to various 2-chloroacetanilide herbicides[17]. The conjugation with glutathione, to displace chlorine by the thiol group of glutathione, has been recognized as a major detoxification pathway in plants. Recently, it was also reported that the drug-hypersensitive *E. coli* KAM3 cells, into which the GST gene was transferred, showed elevated levels of resistance to chlorothalonil, suggesting that the GST may be involved in the detoxification of the fungicide chlorothalonil[5]. Based on the above results, it was hypothesized that chlorothalonil will be detoxified via the mechanism of the conjugation with glutathione catalyzed by the intracellular GST in *O. anthropi in vivo*. In order to verify our hypothesis, the biotransformation of chlorothalonil by the GST was investigated in the presence of glutathione *in vitro*. The chlorothalonil content in the reaction mixture rapidly decreased and a negligible amount of chlorothalonil was observed at 30 min after the reaction (Fig. 2). A control experiment was also performed to determine whether GST or glutathione affected the dissipation of chlorothalonil. To some extent, chlorothalonil dissipation (10-15%) was observed due to the nonenzymatic conjugation with the thiol group[18] and the interaction (adsorption) between the chlorothalonil and GST (active GST and denatured GST by boiling) in the absence of glutathione. However there was no significant difference in the chlorothalonil-biotransformation effect among thiol compounds tested (cysteine, reduced glutathione, and β-mercaptoethanol; data not shown). Thus, the chlorothalonil was mainly dissipated by a mechanism of the conjuga-

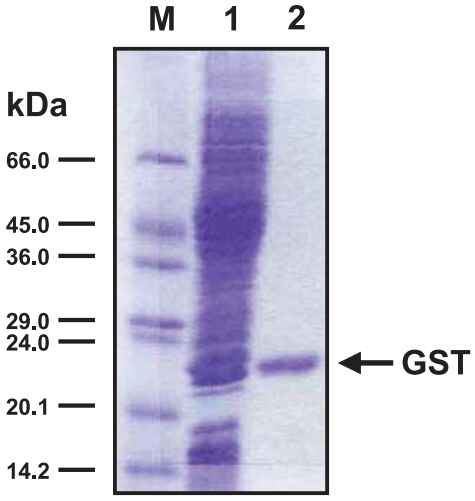

Fig. 1. Expression and purification of glutathione *S*-transferase (GST) from *Ochrobactrum anthropi* SH35B. *Escherichia coli* JM105/ pTOaGST cells were induced with 1 mM IPTG for 6 h and harvested. Crude enzymes were prepared from the cells. The GST was purified as described in Materials and Methods. M, standard protein marker; lane 1, crude enzymes; lane 2, purified GST.

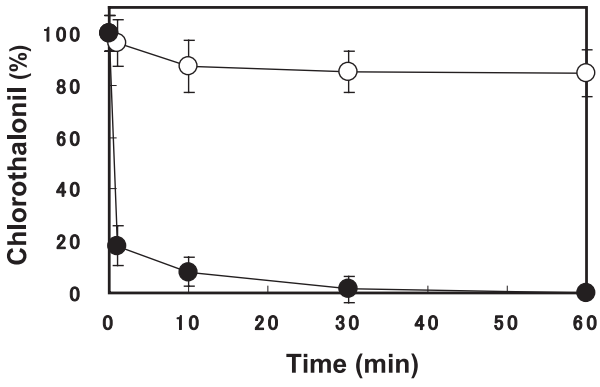

Fig. 2. The biotransformation of chlorothalonil by glutathione *S*-transferase (GST) in the presence of glutathione. 0.5 ml of reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence of the purified GST (1 μg/ml) and 5 mM glutathione. The reaction was performed at 25℃, and stopped by the addition of 20 μl of 20% trichloroacetic acid at the indicated times. The amount of chlorothalonil was analyzed by HPLC as described in Materials and Methods. ○-○, chlorothalonil alone; ●-●, in the presence of 5 mM glutathione and the purified GST.

tion with glutathione catalyzed by the GST.

Identification of Chlorothalonil Metabolites

HPLC analysis showed that chlorothalonil was metabolized to three metabolites (A, B, and C) by the GST in the presence of glutathione (Fig. 3). The formation of the metabolites was dependent upon the presence of glutathione, incubation time and the GST concentration (data not shown). After short incubation periods (< 30 sec), metabolites A and B were observed as major metabolites (Fig. 3 a). After 5 min of the reaction, a negligible content of metabolite A was detected and mainly metabolite B was formed (Fig. 3 b), suggesting that metabolite A is an intermediate in the biotransformation of chlorothalonil. After 30 min of the reaction, only metabolites B and C were detected as major products in the enzymatic reaction (Fig. 3 c). The content of metabolite C was apt to enhance with an increase of the reaction time, however, metabolite B did not completely convert to metabolite C under the current conditions, even though the reaction time had increased to 24 h (data not shown). The retention time of metabolite B was at about 6 min in Fig. 3 a and b, and about 14 min in Fig. 3 c due to using different HPLC condition as described in Materials and Methods. No other HPLC peak was detected.

It was proposed that metabolites A, B, and C are glutathione conjugates because they originated from the reaction catalyzed by the purified GST in the presence of glutathione. Considering the different elution times of the metabolites (Fig. 3), metabolites may differ in the number of glutathione conjugations, because hydrophathy changes with increasing conjugation. A compound with more conjugations generally elutes faster than a less conjugated compound due to increased hydrophilicity under the HPLC-separation conditions.

To clarify this hypothesis, a LC-MS analysis was performed as described in Materials and Methods. The metabolites were identified by comparing the electron impact mass fragmentation patterns with those of the expected reaction molecules. The mass spectrum of chlorothalonil showed sodiated molecules at *m/z* 289 $[M+Na]^+$ (Fig. 4 a). The mass spectrum of metabolite A consisted of molecules at *m/z* 536 [M-GS] and protonated molecules at *m/z* 538 $[M-GS+2H]^+$ (Fig. 4 b), which were consistent with the structure of the one-glutathione conjugated to chlorothalonil after the loss of chlorine. The mass spectrum of metabolite B consisted of molecules at *m/z* 807 [M-2GS] and protonated molecules at *m/z* 809 $[M-2GS+2H]^+$ (Fig. 4 c), suggesting that the metabolite B is the structure of the two-glutathione conjugated to chlorothalonil after the loss of two chlorines. The ion detected at *m/z* 831 was consistent with the sodiated molecules at *m/z* 807 (Fig. 4 c).

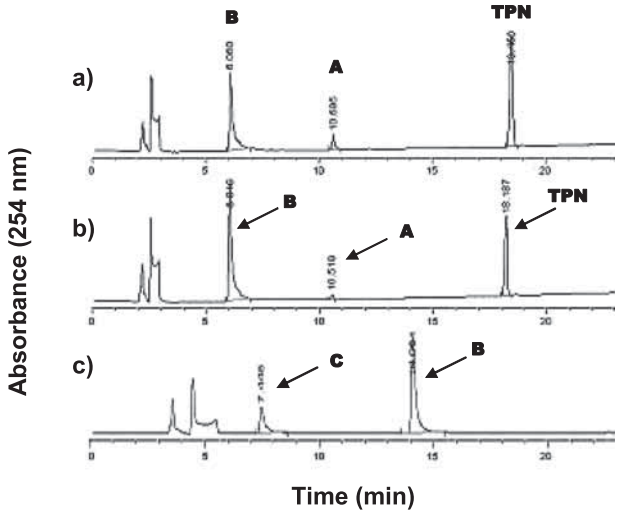

Fig. 3. HPLC elution profile of chlorothalonil (TPN), and metabolites A, B, and C formed from the enzymatic reaction of glutathione *S*-transferase. The metabolites A, B, and C were isolated as described in Materials and Methods. The enzymatic reaction was performed for 30 sec (a), for 5 min (b), and for 30 min (c).

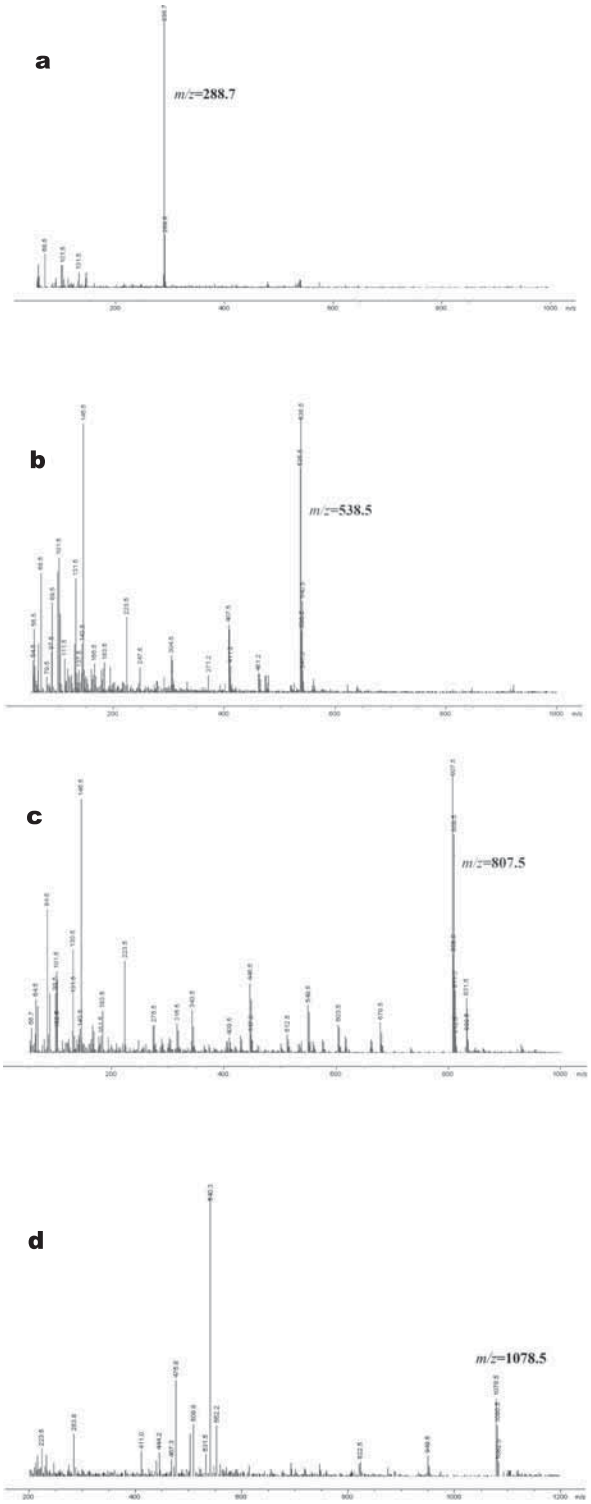

Fig. 4. Mass spectra of chlorothalonil (a) and its metabolites formed from the enzymatic reaction of glutathione *S*-transferase. b, Metabolite **A**; c, metabolite **B**; d, metabolite **C**.

The mass spectrum of metabolite C showed a peak at *m/z* 1078 corresponding to the structure of the tri-glutathione conjugate [M-3GS] and a peak at *m/z* 1080 corresponding to the protonated molecular ion $[M-3GS+2H]^+$ (Fig. 4 d). However, the major peak was detected at *m/z* 540 (Fig. 4 d), not at *m/z* 1078 or 1080 in the mass spectrum of the metabolite C. We attributed this to cleavage of the protonated molecule ion at *m/z* 1080. Considering the results from the HPLC elution and the mass spectrum profiles, metabolite C was proposed to be the structure of the three-glutathione conjugated to chlorothalonil. No other conceivable metabolites of chlorothalonil were detected.

Although identification of the reaction products in this study was tentative, it appeared reasonable. On the basis of the results obtained in this study, a reaction pathway for the glutathione-dependent biotransformation of chlorothalonil catalyzed by the GST is proposed (Fig. 5). In the enzymatic reaction, the one-glutathione conjugate was formed initially, but it seems to be a substrate for the GST, resulting in the substitution of a second chlorine atom to give the two-glutathione conjugate. The two-glutathione conjugate also seems to be a substrate for the enzymatic reaction to produce the three-glutathione conjugate, which appears to be the final metabolite in the glutathione-dependent biotransformation of chlorothalonil.

A similar mechanism has been discussed for the biotransformation of chlorothalonil in the rat liver cytosol and two metabolites, 4-(glutathion-*S*-yl)-2,5,6-trichloroisophtalonitril and 4,6-bis(glutathion-*S*-yl)-2,5-dichloroisophtalonitril, were reported[19]. Metabolite B reported here showed the same UV-spectrum profiles, absorption maxima at 264, 309 and 340 nm, as 4,6-bis(glutathion-*S*-yl)-2,5-dichloroisophtalonitril. Indeed, 4-(glutathion-*S*-yl)-2,5,6-trichloroisophtalonitril was also reported as an intermediate[19] such as the metabolite A reported in this study. Thus the structures of metabolites A and B correspond to 4-(glutathion-*S*-yl)-2,5,6-trichloroisophtalonitril and 4,6-bis(glutathion-*S*-yl)-2,5-dichloroisophtalonitril, respectively. To our knowledge, the tri-glutathione conjugate (metabolite C) has not been previously reported.

In general, the formation of glutathione conjugates is associated with the detoxification of xenobiotics[6,7]. The glutathione conjugates represent the biotransformation pathway for chlorothalonil in *O. anthropi*, resulting in the detoxification of chlorothalonil. Thus we believe the results

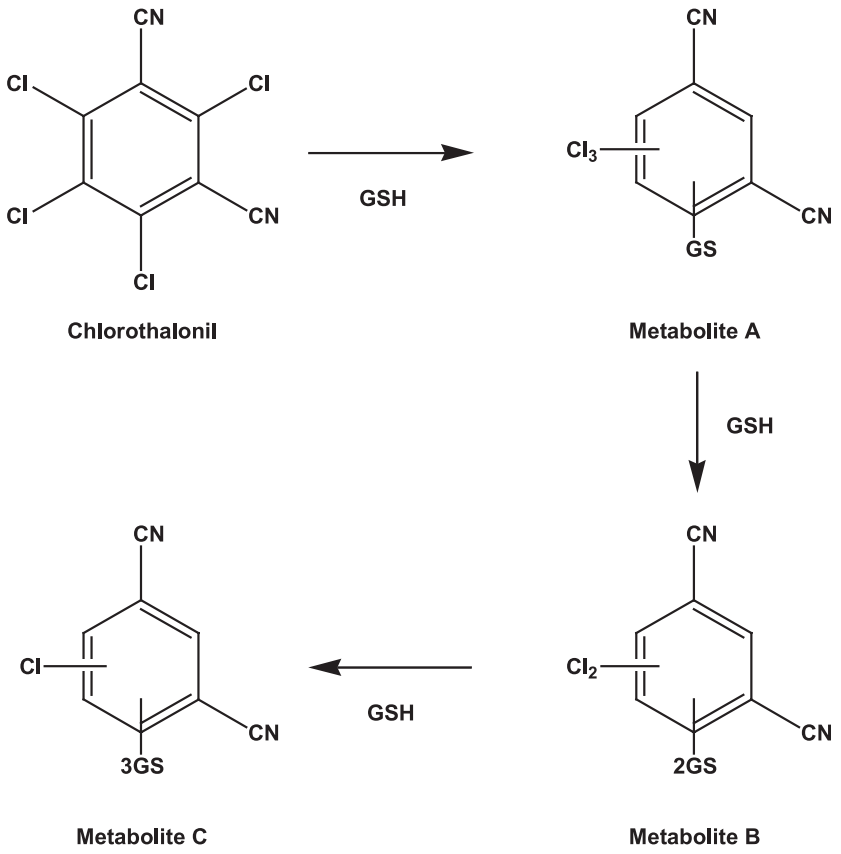

Fig. 5. Proposed pathway of glutathione-dependent biotransformation of chlorothalonil catalyzed by glutathione *S*-transferase. GSH indicates glutathione.

reported in this study are the first evidence on the glutathione-dependent biotransformation of chlorothalonil catalyzed by the bacterial GST. Several questions, however, are still unanswered: Which compound is the final metabolite in the biotransformation of chlorothalonil and how is the final glutathione conjugate excreted from the cells? It has been reported that glutathione conjugates formed in the liver are efficiently excreted in the bile due to a high-affinity transport for glutathione conjugates in the canalicular membrane[20]. The glutathione conjugate reported in this study is also likely excreted by a specific transporter after several enzymatic reactions such as γ-glutamyl transpeptidase *in vivo*[21]. To address these issues, it will be necessary to confirm a structure that results from the conjugation with glutathione in the bacterial medium.

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