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# Biotransformation of the Fungicide Chlorothalonil by Bacterial Glutathione *S*-Transferase

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**Abstract** A gene responsible for the chlorothalonil-biotransformation was cloned from the chromosomal DNA of *Ochrobactrum anthropi* SH35B, an isolated bacterium strain from soil. We determined the nucleotide sequences and found an open reading frame for glutathione *S*-transferase (GST). The drug-hypersensitive *Escherichia coli* KAM3 cells transformed with a plasmid carrying the GST gene can grow in the presence of chlorothalonil. The GST of *O. anthropi* SH35B was expressed in *E. coli* and purified by affinity chromatography. The fungicide chlorothalonil was rapidly transformed by the purified GST in the presence of glutathione. No significant difference in the chlorothalonil-biotransformation effect was observed among the thiol compounds (cysteine, reduced glutathione, and β-mercaptoethanol). Thus, the result reported here is the first evidence on the chlorothalonil-biotransformation by conjugation with the cellular free thiol groups, especially glutathione, catalyzed by the bacterial GST.

**Key words:** *Ochrobactrum anthropi* SH35B, glutathione *S*-transferase, chlorothalonil

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. Chlorothalonil, however, is considered a potential pollutant due to its high application rate, persistence, and toxicity to human and other species. 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, is often considered as an environmentally favorable method [14, 17, 23]. To date, however, there have been no unambiguous reports about the bioremediation of soil contaminated by chlorothalonil and its metabolites.

We isolated a bacterium strain, *Ochrobactrum anthropi* SH35B, capable of efficiently dissipating the fungicide chlorothalonil. A gene responsible for the chlorothalonil-biotransformation was cloned from the chromosomal DNA of *O. anthropi* SH35B using the *Escherichia coli* KAM3 [21], which lacks major multidrug efflux pumps (*ΔacrB*) as host cells. It was determined to be an open reading frame (ORF) for glutathione *S*-transferase (GST) by the nucleotide sequence.

The syntheses of a metal-binding protein, metallothionein and heat shock proteins, as well as glutathione and glutathione *S*-transferase, in response to heavy metals, heat shocks, carbon starvation, and various xenobiotics have been observed in most of the species examined. The syntheses of such stress-responsible proteins are very rapidly induced to protect cells against the toxicity caused by stress, thereby allowing organisms to survive in harmful environmental conditions [2, 10–12, 18, 29]. Glutathione *S*-transferase (GST) catalyzes the conjugation of the sulfur atom of glutathione to a large variety of electrophilic compounds of both endobiotic and xenobiotic origin, resulting in detoxification [9, 32]. Therefore, we hypothesized that the bacterial GST may be involved in the detoxification of the fungicide chlorothalonil in soil.

The objective of the current study was to evaluate the possibility of the biological treatment for the biotransformation of chlorothalonil. In this paper, we report the cloning of a gene responsible for the chlorothalonil-biotransformation from the isolated strain, capable of efficiently dissipating the chlorothalonil.

## Materials and Methods

### Materials

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

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sulfoxide (DMSO). It was then added to the medium at the concentrations indicated. For the purification of the GST of *O. anthropi* SH35B, GST Bind Kits were purchased from Novagen (Madison, U.S.A.). 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.* [7], were used to determine 16S rDNA of the SH35B: (Forward) 5'-AGAGTTTGATCCTGGCTCAG-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°C, annealing of 1 min at 55°C, and extension of 2 min at 72°C. A final extension step consisting of 5 min at 72°C was included. Amplified 16S rDNA was purified from an agarose gel and then sequenced by dideoxy chain termination methods [27]. 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 [1]. 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 II(+). Competent cells of *E. coli* KAM3 (*ΔacrB*) were transformed with the ligated recombinant plasmids and then spread onto M9 minimal medium agar plates [26] containing 1% glucose and 0.37 mM chlorothalonil. The plates were incubated at 37°C 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 [27]. The nucleotide sequences reported here have been deposited in the GenBank database with the accession number AY378173.

### Protein Expression and Purification

In order to construct an expression plasmid for the GST of *O. anthropi* SH35B, PCR was carried out using two synthetic oligonucleotides based on the ORF to generate a unique *Eco*RI and *Bam*HI restriction enzyme site: (Forward) 5'-CGGAATTCATCGAAACTGATGGGAG-3', and (Reverse) 5'-GCGGATCCTTAGTTCAGGCCTTC-3'. The PCR product was digested by *Eco*RI-*Bam*HI restriction enzymes and then ligated into pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which had been digested with *Eco*RI and *Bam*HI. The resulting plasmid was designated as pTOaGST. *E. coli* JM105 cells [25] harboring pTOaGST were grown overnight at 37°C in Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin. After overnight culture, 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 IPTG and incubated for an additional 6 h. After harvesting, cells were resuspended in 100 mM potassium phosphate buffer (pH 6.5), and disrupted by sonication. Unbroken cells were removed by centrifugation at 100,000 ×*g* for 10 min, and the supernatant was taken for purification. The purification of GST was carried out according to the recommendations of the manufacturer (Novagen, Madison, U.S.A.). The crude enzymes and each fraction through the purification procedures were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with Coomassie brilliant blue R-250 (CBB; Sigma-Aldrich Corp., St. Louis, U.S.A.). The GST activity was measured as described previously [31] and protein content was determined by the Bradford method [3].

### Effect of the GST Gene on the Cell Growth

To investigate the effect of the GST on cell growth, *E. coli* KAM3 cells [21] harboring pTOaGST were grown on M9 minimal medium agar plates containing 1% glucose, 0.2 mM IPTG, and 0.37 mM chlorothalonil, and then incubated at 37°C for 3 days.

### The Biotransformation of Chlorothalonil by GST

To investigate the biotransformation of chlorothalonil by the purified GST, 0.5 ml of reaction buffer (100 mM phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence and absence of the purified GST (1 μg/ml) and 5 mM reduced glutathione. The reaction was performed for 30 min at 25°C, and stopped by the addition of 20 μl of 20% trichloroacetic acid. The remained chlorothalonil was then extracted with the same volume of hexane. The extract was evaporated and then dissolved in acetonitrile prior to analysis. To detect the chlorothalonil, high performance liquid chromatography (HPLC) analysis was performed using a Waters μBondapak $C_{18}$ column (3.9×150 mm; Waters, Milford, U.S.A.) with an eluent of water:acetonitrile (4:6) for 10 min at a flow rate of 1 ml/min. The HPLC effluent was detected in series by UV monitoring at 235 nm.

To investigate the effect of the biotransformation of chlorothalonil by thiol compounds, several thiol compounds were added to a reaction buffer containing 0.37 mM chlorothalonil. The reaction and analysis condition was the same as described above.

## RESULTS AND DISCUSSION

### Cloning of a Gene Responsible for the Chlorothalonil-Biotransformation

We isolated a bacterium strain, *O. anthropi* SH35B, capable of growing on the plate containing chlorothalonil as the only source for carbon. The isolated strain showed the ability of biotransforming efficiently the fungicide chlorothalonil [16]. 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 [21], which lacks major multidrug efflux pumps (*ΔacrB*) 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 [8]. 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 [8], 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.* [8]. The putative GST of *Brucella suis* 1330 [24] showed the highest sequence similarity throughout the entire sequence: 82% identity and 90% similarity. The GST of *Pseudomonas pseudoalcaligenes* [13] 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* SH35B (data not shown). The GST of *E. coli* showed 38% identity and 54% similarity.

### Purification and Characterization of the *O. anthropi* SH35B GST

Bacterial GSTs are present in very low amounts [30], resulting in a limit to the 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. In order to overproduce, we subcloned the gene encoding the GST into an IPTG inducible expression vector pTrc99A as described in Materials and Methods. To overproduce the GST, 1 mM IPTG was added to the culture broth at $A_{600}$ of 0.6 and incubated for an additional 6 h. Overproduction and purified enzymes were monitored by CBB staining of SDS-PAGE gels (Fig. 1). We observed an increase in the intensity of the band corresponding to about 22 kDa, which corresponds to the molecular mass calculated from its gene (Fig. 1, lane 1), but no band was detected in the control cells, *E. coli* JM105/pTrc99A (data not shown). Thus, we concluded that the 22 kDa band represents the overproduced GST. The overproduced GST was purified using the GST Bind Kits (Novagen, Madison, U.S.A.). The purified GST band had an apparent molecular mass near 22 kDa similar to the crude enzyme fraction (Fig. 1, lane 2). Table 1 summarizes the purification of the GST from the SH35B. The specific activity of the purified protein increased 16-fold compared with the crude enzyme. We obtained 0.12 mg of purified protein from about 1.2 mg of crude extract.

We investigated the physiological properties of the purified GST. The optimum temperature and pH were

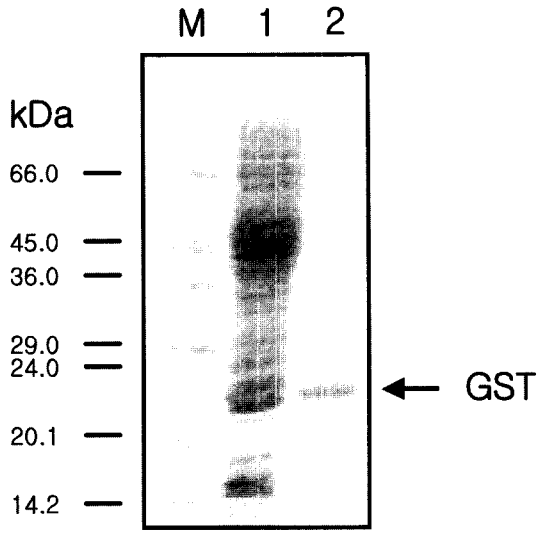


**Fig. 1.** Expression and purification of GST from *Ochrobactrum anthropi* SH35B.
*Escherichia coli* JM105/pTOaGST cells were induced with 1 mM IPTG for 6 h and harvested. Crude enzymes were prepared from the cells. GST was purified as described in Materials and Methods. M, standard protein marker;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

**Table 1.** Purification of glutathione *S*-transferase.

| Fraction | Protein (mg) | Specific activity (μmol/min/mg protein) | Purification rate (fold) |
|---|---|---|---|
| Crude enzyme | 1.19 | 0.8 | 1 |
| Eluate | 0.12 | 12.8 | 16 |

The data is the average of the triplicate experiments.

determined by incubating the enzyme at different temperatures and pH values according to the standard assay [31]. The maximum activity was observed at 25°C and the optimum pH was 7.4 (data not shown). Thermal stability was also investigated by incubating the enzyme at 25°C. The GST kept over 50% of its activity up to 3 h at pH 7.4 and 25°C (data not shown).

### Enhancement of Cell Growth by the GST

The isolated strain is able to grow on plates containing chlorothalonil, and has a high level of glutathione content and GST activity compared with other strains, *E. coli* and *Bacillus subtilis* [16]. It has been known that GST catalyzes the conjugation of the sulfur atom of glutathione to a large variety of electrophilic compounds of both endobiotic and xenobiotic origin, resulting in detoxification [9, 32]. Therefore, it was presumed that the GST may be involved in the detoxification of the fungicide chlorothalonil. To investigate this possibility, *E. coli* KAM3 cells, which are not able to grow on M9 minimal medium agar plates containing 1% glucose, 0.2 mM IPTG, and 0.37 mM chlorothalonil, were transformed with the plasmid pTOaGST and incubated for 3 days at 37°C. As shown in Fig. 2, we observed normal growth on the agar plate with *E. coli* KAM3 cells transformed with pTOaGST, but not control cells with the vector plasmid. Thus, the drug-hypersensitive *E. coli* KAM3 cells transformed with the GST gene showed elevated levels of resistance to chlorothalonil, suggesting that the GST may be involved in the detoxification of the fungicide chlorothalonil.

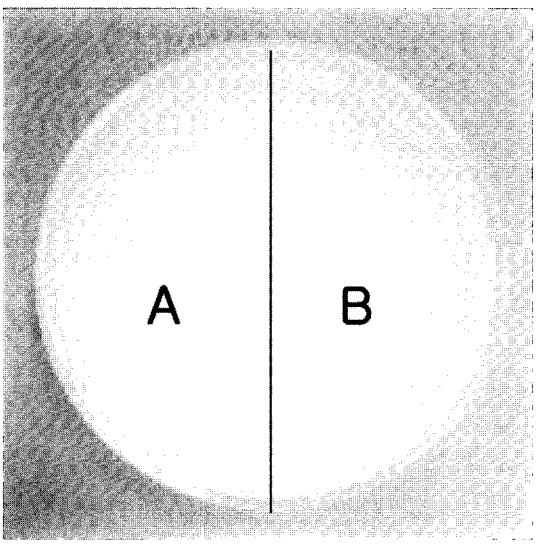


**Fig. 2.** Effect of the GST gene on the cell growth.
Cells were grown on M9 minimal medium agar plates containing 1% glucose, 0.2 mM IPTG, and 0.37 mM chlorothalonil, and then incubated at 37°C for 3 days. A, *Escherichia coli* KAM3/pTrc99A; B, *E. coli* KAM3/pTOaGST.

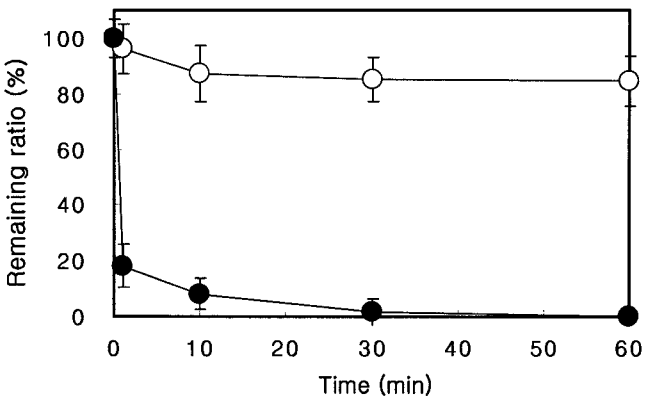


**Fig. 3.** The biotransformation of chlorothalonil by GST in the presence of glutathione.
0.5 ml of reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence of the purified GST (1 μg/ml) and 5 mM glutathione. The reaction was performed at 25°C, 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.

### The Biotransformation of Chlorothalonil by the GST

In plants, the GST activity is an important factor in determining the resistance to various 2-chloroacetanilide herbicides [28]. The conjugation with glutathione, to displace chlorine by the thiol group of glutathione, has been recognized as a major detoxification pathway in plants. As based on the report and our results of Fig. 2, we hypothesized that chlorothalonil is detoxified via the mechanism of glutathione *S*-conjugate formation catalyzed by the intracellular GST. In order to test our hypothesis, the biotransformation of chlorothalonil by the purified GST was investigated in the presence of glutathione *in vitro*. As shown in Fig. 3, the biotransformation of chlorothalonil by GST was observed in the presence of glutathione. The chlorothalonil content in the reaction mixture rapidly decreased (Fig. 3). Then, the chlorothalonil was not detected after 30 min of incubation (Fig. 3). An additional experiment was performed to determine whether protein or glutathione affected the dissipation of chlorothalonil (Fig. 4). In a reaction mixture containing only glutathione, some extent of chlorothalonil-dissipation was observed due to the nonenzymatic conjugation of the thiol group of glutathione [15]. We also investigated the effect of the interaction (adsorption) between chlorothalonil and protein. Proteins tested here (native GST, denatured GST, and bovine serum albumin) did not significantly affect the dissipation of chlorothalonil. There was also no significant difference among the tested proteins (Fig. 4). Thus, the chlorothalonil mainly dissipated with the conjugation of glutathione catalyzed by the GST (biotransformation).

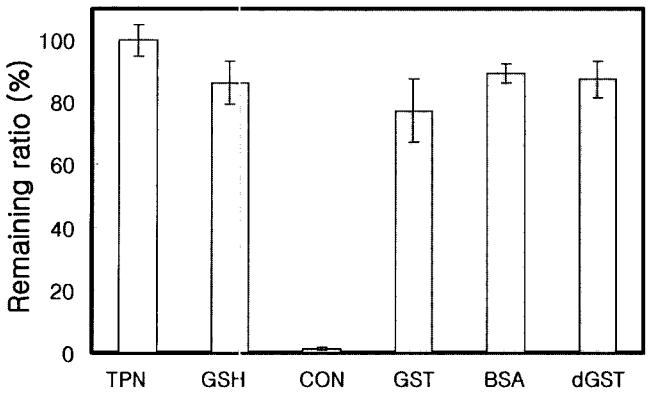


**Fig. 4.** Effect of the chlorothalonil-biotransformation by several compounds.
0.5 ml of reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence of the indicated ingredient. Denatured protein was prepared by the addition of 20 µl of 20% trichloroacetic acid before reaction. The reaction was performed for 30 min at 25°C. The other conditions were the same as described in Fig. 3. TPN, chlorothalonil alone; GSH, 5 mM glutathione; CON, 5 mM glutathione and the native GST (1 µg/ml); GST, native GST (1 µg/ml); BSA, native bovine serum albumin (1 µg/ml); dGST, denatured GST (1 µg/ml).

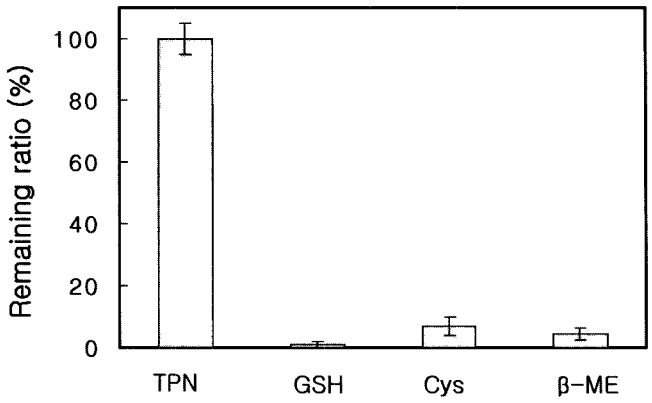


**Fig. 5.** Effect of thiol compouds on the chlorothalonil-biotransformation.
Thiol compounds were added to a reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil, and then the reaction was performed in the presence of GST (1 µg/ml) for 30 min at 25°C. The other conditions were the same as described in Fig. 3. TPN, chlorothalonil alone; GSH, 5 mM glutathione; Cys, 5 mM L-cysteine; β-ME, 5 mM β-mercaptoethanol.

### The Effect of Thiol Compounds on the Chlorothalonil-Biotransformation

As described above, the isolated strain had a higher level of glutathione content compared with other strains, *E. coli* and *Bacillus subtilis* [16]. Glutathione is an especially abundant nonproteinous thiol compound found in most aerobic organisms and has many physiological functions in cells [19, 20].

In order to test our hypothesis that cellular free thiol groups, mainly glutathione, are a major detoxification factor of chlorothalonil, the biotransformation rate of chlorothalonil caused by several thiol compounds was investigated. Several thiol compounds (cysteine, reduced glutathione, and β-mercaptoethanol) were added to a reaction buffer (100 mM phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil. Figure 5 shows the positive effect of the thiol compounds on the biotransformation of chlorothalonil. There was no significant difference in the effect of the chlorothalonil-biotransformation among the tested thiol compounds, which is consistent with our recent results obtained in yeasts [31]. We also observed some extent of the chlorothalonil-dissipation in the absence of the GST by thiol compounds such as glutathione (data not shown). As described above, it was supposed to be due to the nonenzymatic conjugation of the thiol group [15].

The results presented in this paper suggest that chlorothalonil is detoxified by conjugation with the cellular free thiol groups, mainly glutathione, catalyzed by the bacterial GST. The structure of glutathione *S*-conjugates, however, formed by the bacterial GST have not been elucidated. It is also suggested that a glutathione *S*-conjugate of chlorothalonil will be an intermediate in the degradation of chlorothalonil *in vivo* since the isolated strain is able to grow on agar plates containing chlorothalonil as the only carbon source. In order to study these issues in more detail, it is necessary to confirm the conjugate structures and to elucidate the pathway of the chlorothalonil metabolism.

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