Gene Cloning and Molecular Characterization of a Two-Enzyme System Catalyzing the Oxidative Detoxification of β-Endosulfan

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The gram-positive bacterium Mycobacterium sp. strain ESD is able to use the cyclodiene insecticide endosulfan as a source of sulfur for growth. This activity is dependent on the absence of sulfate or sulfur in the growth medium. A cosmid library of strain ESD DNA was constructed in a Mycobacterium-<em>Escherichia coli</em> shuttle vector and screened for endosulfan-degrading activity in <em>Mycobacterium smegmatis</em>, a species that does not degrade endosulfan. Using this method, we identified a single cosmid that conferred sulfur-dependent endosulfan-degrading activity on the host strain. An open reading frame (esd) was identified within this cosmid that, when expressed behind a constitutive promoter in a mycobacterial expression vector, conferred sulfite- and sulfate-independent β-endosulfan degradation activity on the recombinant strain. The translation product of this gene (Esd) had up to 50% sequence identity with an unusual family of monoxygenase enzymes that use reduced flavins, provided by a separate flavin reductase enzyme, as cosubstrates. An additional partial open reading frame was located upstream of the Esd gene that had sequence homology to the same monoxygenase family. A flavin reductase gene, identified in the <em>M. smegmatis</em> genome, was cloned, expressed, and used to provide reduced flavin mononucleotide for Esd in enzyme assays. Thin-layer chromatography and gas chromatography analyses of the enzyme assay mixtures revealed the disappearance of β-endosulfan and the appearance of the endosulfan metabolites, endosulfan monoaldehyde and endosulfan hydroxyether. This suggests that Esd catalyzes the oxygenation of β-endosulfan to endosulfan monoaldehyde and endosulfan hydroxyether. Esd did not degrade either α-endosulfan or the metabolite of endosulfan, endosulfan sulfate.

Organochlorine pesticides generally are mineralized very slowly or not at all in the environment. This recalcitrance has led to the banning of the use of most of these compounds throughout the world. An exception to this is the insecticide endosulfan, which has the same primary action and target site as other organochlorines (3). However, it has a relatively reactive cyclic sulfite diester group (27) and, as a consequence, has chemical and physical properties significantly different from those of other organochlorine insecticides that affect both its environmental fate and its biological fate. In particular, the environmental persistence of endosulfan is lower than that of other chemicals of the same class, although it is higher than that of many other insecticides. Since deregistration of most organochlorine insecticides in many countries, the continued availability of endosulfan has been important because of its use in integrated pest management and resistance management strategies. However, wastewater residue problems associated with the use of endosulfan have led to interest in postapplication detoxification of this insecticide.

Endosulfan can be detoxified in a single enzymatic reaction, making it an ideal candidate for bioremediation (24). Detoxification is thought to occur by removal of the sulfur moiety, and the resultant hexachloro product poses little environmental threat (6, 10). Two pathways for single-step detoxification of endosulfan by cultured soil microbial populations have been described. In the first pathway endosulfan is hydrolyzed to endosulfan diol. Because endosulfan is particularly susceptible to abiotic hydrolysis and forms endosulfan diol at pH values of 7.0 and above (18), it is difficult to estimate the contribution of enzymatic hydrolysis rather than chemical hydrolysis to this pathway of degradation. This is especially problematic because often the growth of microbes increases the alkalinity of the medium in which they are grown (2, 18, 20). This uncertainty needs to be resolved before isolation of the enzymes responsible for this reaction is attempted. The second pathway of metabolism involves the formation of endosulfan monoaldehyde (24) and has been found in the soil bacterium <em>Mycobacterium</em> sp. strain ESD and described (25). The formation of endosulfan monoaldehyde occurs only enzymatically, and therefore this pathway has been the focus of endosulfan bioremediation studies in our laboratory.

The commercial endosulfan insecticide is a mixture of two diastereoisomers, α-endosulfan and β-endosulfan, at a ratio of 7:3. <em>Mycobacterium</em> sp. strain ESD metabolizes the isomers to endosulfan sulfate and endosulfan monoaldehyde (24). However, the α isomer is predominantly oxidized to endosulfan sulfate, and the β isomer is predominantly converted to endosulfan monoaldehyde (24). <em>Mycobacterium</em> sp. strain ESD was isolated by providing endosulfan as the sole source of sulfur in successive enrichment cultures initially inoculated with endosulfan-contaminated soil. The formation of both endosulfan sulfate and endosulfan monoaldehyde by <em>Mycobacterium</em> sp. strain ESD occurs as a response to sulfur starvation and does not occur in the presence of relatively low levels of inorganic sulfate or sulfite. In this report we describe the cloning, molecular characterization, and expression of a monoxygenase capable of degrading β-endosulfan from <em>Mycobacterium</em> sp. strain ESD. <em>Mycobacterium-<em>Escherichia coli</em> shuttle vectors allowed isolation of the enzyme by selection of an
active enzyme in a related Mycobacterium species, Mycobacterium
smegeticus, which is normally unable to metabolize endosulfan.
Reduced flavin was provided to this enzyme in cell-free enzyme assay mixtures by expression of a flavin reductase
gene isolated from the M. smegeticus gene isolated from the

**Table 1. Bacterial strains and DNA vectors used in this study**

<table>
<thead>
<tr>
<th>Strain or DNA vector</th>
<th>Description or genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mycobacterium</strong> sp. strain <strong>ESD</strong></td>
<td>Endosulfan degrading</td>
<td>25</td>
</tr>
<tr>
<td><strong>M. smegeticus</strong> mc2</td>
<td>Not endosulfan degrading</td>
<td>H. Billman-Jacobe, New England Biolabs</td>
</tr>
<tr>
<td>E. coli strain TG1</td>
<td>F′ traD6 lacI ΔlacZΔM15 proAΔ R (r (mcerB) thi Δlac-proAB) strain capable of growth in minimal media</td>
<td></td>
</tr>
<tr>
<td>E. coli strain EP1305</td>
<td>F′ ΔlacC-mrr mcrA recA13 supE44 ara-14 galK2 lacY1 rpsL20</td>
<td>Epicenter Technologies</td>
</tr>
<tr>
<td>pGEM</td>
<td>Mycobacterium-E. coli shuttle cosmid vector, Ap′</td>
<td>Promega</td>
</tr>
<tr>
<td>pMV216</td>
<td>Mycobacterium-E. coli shuttle expression vector, Kn′</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET14b</td>
<td>E. coli expression vector, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pYUB172</td>
<td>Approximately 50-kb Mycobacterium sp. strain ESD DNA</td>
<td>This study</td>
</tr>
<tr>
<td>pYUB415-Apa3</td>
<td>Fragment from pYUB415 that confers endosulfan-degrading activity on M. smegeticus</td>
<td>This study</td>
</tr>
<tr>
<td>pYUB4145-Apa3</td>
<td>3-kb ApaI fragment from pYUB415 that confers endosulfan-degrading activity on M. smegeticus</td>
<td>This study</td>
</tr>
<tr>
<td>pMV216-esd</td>
<td>ESD cosmid vector in pMV216</td>
<td>This study</td>
</tr>
<tr>
<td>pET14b-esd</td>
<td>ESD expression construct in pET14b</td>
<td>This study</td>
</tr>
<tr>
<td>pET14b-MaFR</td>
<td>MaFR expression construct in pET14b</td>
<td>This study</td>
</tr>
</tbody>
</table>


**Materials and Methods**

**Media and reagents.** Sulfur-free medium (SFM) (pH 6.9) was prepared by the method of Sutherland et al. (24). Endosulfan or endosulfan sulfate (Sigma-Aldrich, Sydney, New South Wales, Australia) was added to bacterial cultures at a concentration of 50 μM when it was required. Modified Luria broth (LB) contained 10 g of tryptone, 5 g of yeast extract, and 0.5 g of NaCl in 1 liter of 100 mM potassium phosphate buffer (pH 6.9). LB (23) and LB containing 0.05% sodium citrate, and 0.5% Sarkosyl. Glass beads (diameter, 450 to 600 μm; Sigma) were added, and the cells and beads were mixed vigorously with a vortex mixer for 10 min. After the beads were settled by a brief centrifugal pulse, the supernatant was removed and extracted with phenol-chloroform several times. The DNA was precipitated with ethanol, resuspended in Tris-EDTA, and stored at −20°C.

**Construction of cosmid library.** A Mycobacterium sp. strain ESD genomic library was constructed in the Mycobacterium-E. coli cosmid shuttle vector pYUB415 as follows. Genomic DNA of Mycobacterium sp. strain ESD was partially digested with SacI/3Al restriction endonuclease and then separated by gel electrophoresis in a 1% low-melting-point agarose gel (23). An agarose gel slice containing 30- to 45-kb DNA fragments was melted, and the DNA was ligated in the gel to BamHI-digested, calf intestinal alkaline phosphatase-treated pYUB415 (23). After ligation, the GELase agarose gel-digesting enzyme preparation (Epicenter Technologies, Madison, Wis.) was added according to the manufacturer’s instructions. The ligated DNA was packaged into MaxPlax packaging extracts (Epicenter Technologies) and used to infect E. coli strain EP1305 cells freshly prepared according to the manufacturer’s instructions (Epicenter Technologies). Strains containing pYUB415 derivatives were grown in the presence of ampicillin (E. coli) or hygromycin (M. smegeticus).

**Screening of cosmid clones and subsequent subclones.** Cosmid DNA was isolated from 2-ml cultures of individual library clones by using the alkaline lysis procedure of Sambrook et al. (23), purified by using the QIAquick system (Qiagen, Victoria, Australia), and then electroporated into freshly prepared M. smegeticus mc2 competent cells (prepared as described by Jacobs et al. [13]). The M. smegeticus strain used to screen the Mycobacterium sp. strain ESD cosmid library does not have detectable endosulfan-metabolizing activity (25) and can grow in SFM supplemented with either 50 μM magnesium sulfate or sodium sulfate. DNA (5 to 50 pg of 1 μl of distilled H2O) was incubated with 50 μl of prepared cells for 1 min on ice and then transferred to a vial with a 0.2-cm gap (Bio-Rad, Sydney, New South Wales, Australia). This preparation was exposed to one electrical pulse consisting of 2.500 V and 25 μF with the resistance set at 1,000 Ω. Electroporated cells were incubated for 3 h in LB and then plated onto LB agar containing hygromycin and incubated at 28°C for 4 days. Colonies were washed with SFM and used to inoculate SFM containing hygromycin and β-endosulfan. After 7 days of incubation at 28°C and 180 rpm, the culture was analyzed for endosulfan degradation by thin-layer chromatography (TLC) (24).

**Construction of esd expression constructs.** To create a mycobacterial esd expression construct, the esd gene was amplified by PCR from the ApaI DNA fragment from Mycobacterium sp. strain ESD containing the Esd gene by using the primers provided by the manufacturer. The PCR product was cloned into pGEM T Easy vector (Promega, Madison, Wis.) by following the manufacturer’s instructions to create pGEM- esd. This plasmid was then digested with PstI and HindIII and cloned into similarly digested pMV261 (Table 1) to produce pMV261-esd.
To create an E. coli expression construct, the esp gene was amplified by PCR using pYUB415-Apa3 as the template and CCATATGACCCGACAGCTA CACCTC forward and CAGATCTTTATCCGACCCGGTGCGCCA reverse primers (NdeI and BamHI sites, respectively, are underlined, and the initiation codon is indicated in boldface type). PCR was performed by using Taq polymerase (Life Technologies, Rockville, Md.) and buffer provided by the manufacturer supplemented with 1.5 mM MgCl₂. After incubation for 5 min at 95°C, 30 cycles of amplification (95°C for 30 s, 65°C for 30 s, and 72°C for 30 s) were performed, followed by a 5-min extension step at 72°C. The PCR product was cloned into the pGEM T Easy vector (Promega) by following the manufacturer’s instructions. The PCR product was digested with NdeI and BamHI and cloned into NdeI-BamHI-digested pET14b (Novagen, Madison, Wis.), which contains a N-terminal His tag to aid recombinant protein purification, to produce pET14b-esd.

Construction of a flavin reductase expression plasmid. The flavin reductase gene sequence of M. smegmatis was identified by using the BLAST program to identify sequences in the unannotated M. smegmatis genome sequence (obtained from The Institute for Genomic Research website [http://www.tigr.org]) homologous to the daaD gene sequence of Rhodococcus erythropolis strain D-1 (accession number AB051429 [19]). This sequence extends from position 137818 to position 138306 of the M. smegmatis genome sequence (obtained from TIGR-1772 [5073]). An open reading frame (ORF) (95°C for 30 s) were increased at a rate of 20°C/minute at 25°C. The enzyme was oxidized per minute at 25°C (E_{max} = 6.220 M⁻¹ cm⁻¹).

Nonrecombinant M. smegmatis cell extracts were prepared by growing 400-ml cultures to the stationary phase, harvesting the cells by centrifugation, and then washing the cells in 50 ml of 50 mM HEPES buffer (pH 6.9) and resuspending the cells in the same buffer. Cells were lysed by sonication, and the cell debris was removed by centrifugation at 16,000 × g for 5 min. The supernatants were stored at −20°C until they were used.

**GC analysis.** Endosulfan sulfate (50 μg) was added to enzyme assay mixtures at the conclusion of the incubation time as an internal standard, and then each reaction mixture was extracted with 2 ml of ethyl acetate. The organic phase was passed through a 6-cm MgSO₄ column in a Pasteur pipette stoppered with glass wool to remove any residual water, gently evaporated under a dry nitrogen stream, and then dissolved in 200 μl of dichloromethane prior to GC analysis.

**RESULTS**

Isolation of a gene encoding an endosulfan-degrading protein. A gene encoding a protein capable of degrading β-endosulfan was isolated after we screened 370 clones from the Mycobacterium sp. strain ESD cosmid library in M. smegmatis mc2, a strain that does not metabolize the insecticide. A single cosmid (pYUB-172) was identified that conferred β-endosulfan-degrading activity on M. smegmatis. When M. smegmatis containing pYUB-172 was grown in SFM with β-endosulfan as the sole source of sulfur, all the insecticide disappeared and the metabolites endosulfan monoaldehyde and endosulfan hydrosyether were produced. When α-endosulfan was provided as the sole source of sulfur, no degradation was apparent and no metabolites were observed. In Mycobacterium sp. strain ESD the endosulfan-degrading activity was absent when the bacterium was grown in the presence of a low concentration of sulfate (20 μM) (25). When sulfate was provided to M. smegmatis containing pYUB-172 as an additional source of sulfur in the medium, the recombinant did not degrade endosulfan, demonstrating that sulfur regulation is maintained on the cosmid. E. coli TG1 containing pYUB-172 did not degrade endosulfan irrespective of the presence of an alternative sulfur source.

DNA fragments generated by digestion of pYUB-172 with various restriction nucleases were cloned into pYUB415 and screened for endosulfan-degrading activity in M. smegmatis. Fragments with activity were further subcloned and screened in the same manner until the DNA fragment containing activity was reduced to a 3.0-kb ApaI DNA fragment (pYUB415-Apa3).
Analysis of a single ORF within the *ApaI* fragment and homology of its product to other proteins. DNA sequence analysis of the 3.0-kb *ApaI* pYUB415-Apa3 fragment revealed a 1,347-bp ORF (esd), which encoded a 448-amino-acid protein (Esd, with a predicted molecular mass of 49.8 kDa) with a ribosome binding site (GGGAG) 9 bp upstream from the initiation codon (data not shown). As expected for sulfur-regulated proteins, Esd contained a low number of sulfur-containing amino acids compared to the number of sulfur-containing amino acids contained by non-sulfur-regulated proteins. The protein contained only a single methionine (frequency of sulfur-containing amino acids in the enzyme, 0.22%). The deduced amino acid sequence of the ORF was compared with the amino acid sequences of other proteins in the SwissProt and SpTrEMBL databases and was found to have significant similarity to the sequences of several other proteins (Fig. 1). The highest level of identity (50%) was to the *tdsA* product, a thermophilic flavomonooxygenase of *Paenibacillus* sp. strain A11-2 that catalyzes the conversion of dibenzothiophene 5,5-dioxide to 2-(2'-hydroxyphenyl)benzene sulfonate (12). The ORF product also had significant similarity (46% identity) to the product of *dszA* (formerly *ssoA*), the *tdsA* homologue in *Rhodococcus* sp. strain IGTS8 (4), and 38% identity to component A of a nitritetriacetate monooxygenase of *Chelatobacter heintzii* ATCC 29600 (16). The four proteins had similar lengths (448 to 453 amino acids), and similarities occurred throughout the proteins (Fig. 1). The regions conserved in the other proteins were also conserved in Esd, and no major insertion or deletion differences specific to Esd were identified.

The ORF was amplified by PCR and cloned behind the constitutive promoter of the mycobacterial expression vector pMV261. The resulting construct (designated pMV261::esd) transferred the β-endosulfan-degrading phenotype to *M. smegmatis* without the sulfate regulation observed with pYUB415-Apa3, demonstrating that sulfate did not have a direct effect on enzymatic activity. When α-endosulfan or endosulfan sulfate was included in the medium rather than β-endosulfan, no disappearance of substrate was observed and no endosulfan metabolites were detected, suggesting that the enzyme was specific for the β isomer of endosulfan.

Sequence analysis of the 3.0-kb *ApaI* fragment of pYUB415-Apa3 did not reveal any ORF in the 400-bp region downstream from *esd* whose product had significant sequence homology to any protein in the SwissProt or SpTrEMBL database. Upstream of *esd* was a region for which it was difficult to generate sequence data, presumably due to an extensive secondary structure. This region contained a 53-bp palindromic sequence that contained a pair of mismatched base pairs midway through the sequence. An additional ORF (870 bp) was located upstream of *esd* and extended into the end of the 3.0-kb fragment. The amino acid sequence of the product of this ORF had 24% identity to the amino acid sequence of the *dszC* product of *R. erythropolis* extending from the amino acid at position 64 to the end. The *dszC* gene is located in an operon with *dszA* and encodes an enzyme that oxidizes dibenzothiophene to dibenzothiophene 5,5′-dioxide (4). The *dszC* homologue of *Mycobacterium* sp. strain ESD extended into the region that we were unable to sequence immediately upstream of *esd*.

Effect of flavin reductase gene expression on endosulfan degradation in enzyme assays. Initially, we did not detect β-endosulfan-degrading activity in cell-free enzyme assay mixtures containing *E. coli*-expressed Esd (data not shown). It has been shown that the activity of DszA of *Rhodococcus* was enhanced by the presence of either endogenous or heterologous flavin reductase (9, 11, 12, 17, 19, 28). As sequence analysis suggested that Esd was homologous to DszA, we isolated and overexpressed a flavin reductase gene from *M. smegmatis* to supply flavin reductase in Esd enzyme assays.

An ORF (MsFR) in the *M. smegmatis* genome sequence contained a 487-bp region whose translated product had 61% identity to the protein sequence encoded by *dszD*, a flavin reductase gene from *R. erythropolis*. In contrast to the low frequency of sulfur-containing amino acids in Esd, MsFR contained three cysteine and three methionine residues (frequency of sulfur-containing amino acids, 3.8%). DszD is a flavin reductase enzyme that is part of a two-component flavin-diffusible monooxygenase family of enzymes. Galán et al. (9) compared the amino acid sequences of the flavin reductase components of this family and identified several conserved residues. MsFR contains the first of these residues, a serine residue located before a pair of proline residues in the N terminus of the protein. However, the highly conserved GDH motif located in the C terminus of members of this family is a GDS motif in MsFR. This motif was speculated to be involved in NAD(P)H binding (9). The relevance of this is not known.

We amplified the MsFR gene using PCR, inserted the amplificon into pET14b (pET14b-MsFR), and overexpressed the protein (MsFR) in *E. coli*. Purification with a nickel column resulted in a yield of approximately 0.5 mg of MsFR or 0.9 mg of Esd from 100-ml overexpressing cultures with approximately 95 and 90% purity, respectively. The purified protein expressed utilized FMN and NADH as an electron acceptor and an electron donor, respectively (Table 2). FMN was a better electron acceptor for MsFR than riboflavin and FAD were, but the latter two compounds could support some enzyme activity. Methylene blue and tetrahydrobiopterin were not utilized as electron acceptors, and NADPH could not replace NADH as the electron donor.

When MsFR was included in assay mixtures containing Esd overexpressed from pET14b-esd in *E. coli* along with NADH, FMN, or FAD and a solubilizing agent (Table 3), β-endosulfan disappeared and the metabolites, endosulfan monoaldehyde and endosulfan hydroxyether, were detected. Endosulfan is very hydrophobic, and initial assays demonstrated that in the absence of added detergent, BSA, or *M. smegmatis* cell extract dependent on both the age of the culture and was not available to the enzyme (data not shown). Activity was greatly enhanced in the presence of *M. smegmatis* cell extract compared to the activity in the presence of BSA or Triton X-100 (Table 3). Furthermore, the enhancing activity of the *M. smegmatis* cell extract depended on both the age of the culture from which it was prepared (an extract from a stationary-phase culture had a significantly greater enhancing effect than an extract prepared from log-phase cells [Table 3]) and the presence of 10 mM MgCl2. TLC analysis indicated that the relative proportions of the two metabolites, endosulfan monoaldehyde and endosulfan hydroxyether, were similar in the presence of added detergent, BSA, and *M. smegmatis* cell ex-
tract. When either α-endosulfan or endosulfan sulfate was included in the assay mixture rather than β-endosulfan, no metabolites were observed.

**Pathway analysis.** The metabolites endosulfan monoaldehyde (recovered by TLC on alumina) and endosulfan hydroxyether (a gift from I. Kennedy) were included in enzyme assay mixtures as substrates (under the conditions described in Materials and Methods and in the presence of 0.08% Triton X-100)
The complete reaction mixture contained 0.4 nmol of Esd (20 μg), 20 mM FMN, 4 mM NADH, and 500 μM β-endosulfan in 1 ml of 50 mM HEPES buffer (pH 6.9).

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**TABLE 2. Substrate specificity of the flavin reductase protein, MsFR**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Activity (μmol·min⁻¹·μg⁻¹ of MsFR⁻¹)</th>
<th>Coupled reaction with Esd</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>FMN</td>
<td>463.2 ± 39.9</td>
<td>Yes</td>
</tr>
<tr>
<td>Riboflavin</td>
<td></td>
<td>92.6 ± 23.1</td>
<td>NRc</td>
</tr>
<tr>
<td>FAD</td>
<td></td>
<td>58.0 ± 14.7</td>
<td>Yes</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>0</td>
<td>NDd</td>
<td></td>
</tr>
<tr>
<td>Tetrazydrobioprotein</td>
<td>0</td>
<td>NDd</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>FMN</td>
<td>0.63 ± 0.21</td>
<td>NDc</td>
</tr>
<tr>
<td>NADH</td>
<td>FMN, no MsFR</td>
<td>0.84 ± 0.12</td>
<td>NDc</td>
</tr>
</tbody>
</table>

* Electron acceptors were included at the following concentrations: 10 to 100 μM FMN; 10 to 100 μM riboflavin; 10 to 100 μM FAD; 50 μM methylene blue; and 50 μM tetrazydrobioprotein. The reaction was initiated by adding 0.5 mg of electron donor (NADH or NADPH) as described in Materials and Methods.

**DISCUSSION**

We exploited the reactive nature of the cyclic sulfite diester group of endosulfan to isolate a strain of *Mycobacterium* (strain ESD) that degrades the insecticide in order to access the sulfur as a nutrient source (24, 25). From this strain we isolated *esd*, a gene that encodes an enzyme capable of degrading β-endosulfan when it is provided with reduced flavin. A sequence comparison revealed that *esd* was most similar to the *tdsA* gene of *Paenibacillus* sp. strain A11-2 (12) and its homologue, *dszA* of *Rhodococcus* sp. strain IGTS8 (4). The products of these genes are members of the two-component flavin-sensitive monooxygenase family (TC-FDM) of enzymes that require reduced FMN supplied by an NAD(P)H-dependent flavin reductase. This family differs from other monooxygenase families as the enzymes do not contain bound flavin but rather use the compound as a cosubstrate. The flavin reductase enzymes that provide reduced FMN are interchangeable, and physical contact with the monooxygenase is not required for activity (9, 11, 28).

Our early attempts to isolate a gene-enzyme system with endosulfan-degrading activity from *Mycobacterium* sp. strain ESD by screening a cosmid library in *E. coli* were unsuccessful (data not shown). We now suspect that this was because, at least in part, degradation involves both monooxygenase and flavin reductase components and that the *E. coli* host cells did not have appropriate flavin reductase activity to support the activity of recombinant cells containing the monooxygenase enzyme. Isolation of the monooxygenase component was successful after a *Mycobacterium* sp. strain ESD genomic DNA cosmid library was screened in *M. smegmatis* as an organism that did not have endosulfan-degrading activity but did possess appropriate flavin reductase activity. When it was found that the *esd* product had sequence similarity to other monooxygenases that require a separate flavin reductase, we searched the *M. smegmatis* genome for sequences with homology to the sequences of genes encoding enzymes that are potentially capable of providing reduced flavin to this type of enzyme. A flavin reductase gene was identified by analysis of the *M. smegmatis* genome sequence, and this sequence was used as a template for active flavin reductase (MsFR). Recombinant MsFR could reduce FMN, FAD, and riboflavin with NADH as the electron donor, but only reduced FMN and reduced FAD could be utilized by the monooxygenase as electron donors in the degradation of endosulfan (Table 2).

The specific activity of MsFR is similar to the specific activities of other flavin reductase enzymes belonging to the TC-FDM family that reduce flavin as a substrate rather than as a prosthetic group (7, 8, 9, 14, 19, 21, 26). The substrate specificities of these enzymes vary; some of them act on FAD and riboflavin better than on FMN (8, 21), some of them have similar activities with the three flavins (9, 26), and some of them, like MsFR, have higher activity with FMN (19). Similarly, some of the flavin reductases utilize NADPH in preference to NADH (7), while others are inert with NADPH and exhibit activity with NADH (8, 9, 14, 19, 26).

An additional problem with the initial screening in an *E. coli* host may have been at the level of regulation of gene expression. Expression of the monooxygenase component is strongly regulated by sulfur levels in the parent strain, and based on similar studies (4, 22), this regulatory mechanism is unlikely to be recognized in the *E. coli* host strain. Genes encoding other monooxygenases of the TC-FDM family are also controlled by sulfur-regulated promoters not recognized in *E. coli* (4, 22). Like *esd*, these related genes are efficiently expressed under the control of alternate promoters to produce active protein. The proteins encoded by these genes are involved in the desulfuration of methanesulfonates and other alkanesulfonates that are part of the response of the bacterium to sulfur limitation (for a review see reference 15). This involves expression of a set of 7 to 14 proteins, which allows the bacterium to utilize organosulfur-containing compounds in the absence of more bioavailable sulfur sources, such as sulfate and sulfite. Sulfur regulation is retained by the 3-kb *ApaI* fragment that confers

**TABLE 3. Activity of the endosulfan-degrading gene product, Esd, expressed in E. coli**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Solubilizing factor added</th>
<th>Amt of β-endosulfan metabolized (μmol·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completea</td>
<td>BSAb</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>0.08% Triton X-100</td>
<td>4.1 ± 1.4</td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em> stationary-phase cell extractb</td>
<td>18.4 ± 6.9</td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em> log-phase cell extractb</td>
<td>0.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>NDd</td>
<td></td>
</tr>
<tr>
<td>No Esd protein</td>
<td><em>M. smegmatis</em> stationary-phase cell extractb</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>No MsFR protein</td>
<td><em>M. smegmatis</em> stationary-phase cell extractb</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>No NADH</td>
<td><em>M. smegmatis</em> stationary-phase cell extractb</td>
<td>2.2 ± 0.3</td>
</tr>
</tbody>
</table>

* The complete reaction mixture contained 0.4 nmol of Esd (20 μg), 0.5 nmol of MsFR (12 μg), 50 μM FMN, 4 mM NADH, and 500 μM β-endosulfan in 1 ml of 50 mM HEPES buffer (pH 6.9).

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* The amount added was 7.5 mg of protein per ml.

* ND, none detected.
endosulfan-degrading activity on *M. smegmatis* but was absent when *esd* was expressed behind a constitutive mycobacterial promoter, suggesting that the regulation is cis to *esd*, most likely in the 53-bp palindromic region upstream of the gene that we have had difficulty sequencing. Expression of the *dszA* gene from *R. erythropolis* IGTS8 is also strongly repressed by sulfate, as well as by sulfur-containing amino acids. It has been proposed that two possible regions at bases −263 to −244 and −93 to −38 immediately upstream from the initiator codon (positions 1 to 3) are responsible for this regulation (17). A potential hairpin structure was also located between bases −75 and −57.

We were surprised to find that Esd was a member of a monooxygenase family and that catalysis required reduced flavin, as it has previously been proposed that the mechanism for endosulfan metabolism by *Mycobacterium* sp. strain ESD is most likely to be base-catalyzed hydrolysis, like the hydrolysis that occurs under alkaline conditions to produce endosulfan diol (24). Esd appears to perform oxygenation on β-endosulfan to produce endosulfan monoaldehyde and endosulfan hydroxyether. As neither metabolite acts as a substrate for the other, it appears that the reactions occur in tandem. We propose that the steric requirements of the enzyme allow access of both methylene groups of β-endosulfan to possible oxidation. If only one of these methylene groups reacts, then the subsequent proton rearrangement releases endosulfan monoaldehyde from the active site. However, if both methylene groups are oxidized, then the organic moiety cyclizes to a hemiacetal (that is, endosulfan hydroxyether) as shown in Fig. 2. The *esd* gene does not confer the ability to degrade the α isomer of endosulfan on *M. smegmatis*, and presumably there is another gene in the *Mycobacterium* sp. strain ESD genome that is responsible for the observed β-endosulfan-degrading activity of this strain (24). Although α-endosulfan and β-endosulfan are diastereoisomers with distinct physical and chemical properties, they are both degraded to endosulfan monoaldehyde in *Mycobacterium* sp. strain ESD (24), and we were surprised to find that the *esd* product was apparently inert with the α isomer.
The activity of the purified protein expressed by \textit{E. coli} was significantly enhanced when \textit{M. smegmatis} cell extracts were included in the cell-free assay mixture, although the products of the reaction remained the same. Endosulfan is very hydrophobic (Kow log \text{p} \text{H} = 4.8; solubility in water, 0.32 mg \cdot \text{liter}^{-1}) and partitioned rapidly out of the aqueous phase to the glass surfaces of our reaction vessels when it was added to enzyme assay mixtures at concentrations that were required for detection (which were significantly higher than the solubility limit of the insecticide). Detergent, BSA, or mycobacterial cell extract was an essential component of the reaction mixture and increased the apparent solubility of the insecticide and allowed degradative activity to be detected. In the absence of these supplements the endosulfan partitioned to the glass surface of the reaction vessel and degradative activity was not detected. Many soil bacteria produce biosurfactants to enhance degradation of hydrophobic compounds (5). The \textit{M. smegmatis} extracts increase the apparent solubility and therefore the bioavailability of endosulfan to the soluble \textit{esd}-encoded enzyme in our system. The enhanced degradation in the presence of a natural biosurfactant suggests that the Esd activity in our enzyme assays is limited by endosulfan solubility rather than the activity of the enzyme. The bioavailability of the enzyme and the ability of the enzyme to degrade endosulfan at concentrations present in aqueous solutions are currently being investigated.

The genes encoding DszA in \textit{R. erythropolis} and TdsA in \textit{Paenibacillus sp.} strain A11-2, two of the three proteins with significant sequence similarity to Esd, are located in operon-like structures within the genomes of the two organisms (4, 12). These operon-like structures each encode two other enzymes, DszB and DszC and TdsB and TdsC, which form pathways that convert dibenzothiophene to 2-hydroxyphenyl and sulfite. The organization of these genes suggests that they are coordinately regulated by a single promoter. Regulation of this region of the \textit{dsz} gene cluster by sulfur has been mentioned previously. Interestingly, the flavin reductase enzyme that is presumed to provide electrons to the DszA and DszC enzymes of this cluster is located elsewhere in the genome (11). This is also the case with other TC-FDM systems (see reference 15 for a review). The other enzyme with significant sequence similarity to Esd, the nitrilotriacetae monooxygenase of \textit{C. heintzii}, is found in association with a flavin reductase, but the genes encoding these enzymes are not coordinately regulated. These genes are located close together in the genome but are orientated divergently, with a 307-bp intergenic region separating them (16). We did not find any evidence that there is an operon-like structure surrounding the \textit{esd} gene of \textit{Mycoabacterium sp.} strain ESD; the 3.0-kb fragment from \textit{Mycobacterium sp.} strain ESD that had endosulfan-degrading activity contained only the single complete gene, and this gene was expressed under sulfur-limited conditions, indicating that the fragment contained a promoter. No ORF was identified in the 400-bp region downstream from \textit{esd}. We also did not find evidence that there is a proximal flavin reductase, although our search was limited to the 3-kb \textit{ApaI} fragment containing \textit{esd}. We did, however, identify an incomplete ORF upstream of \textit{esd}, orientated in the same direction as \textit{esd}, with sequence homology to \textit{dszC} of \textit{R. erythropolis}. This ORF is interesting in light of the fact that \textit{Mycobacterium sp.} strain ESD could metabolize both \(\alpha\)- and \(\beta\)-endosulfans to endosulfan monoaodehyde but \textit{esd} is specific for the \(\beta\) isomer of endosulfan. Possibly more significant is the finding that the product of the ORF was most similar to DszC, an enzyme that oxidizes dibenzothiophene to dibenzothiophene 5,5'-dioxide. This reaction is very similar to the oxidation of endosulfan to endosulfan sulfate observed in \textit{Mycobacterium} sp. strain ESD. It is thought that this oxidation is catalyzed by a non-cytochrome P450 monooxygenase as it is not inhibited by the cytochrome P450 inhibitor piperonyl butoxide (24). We are currently investigating whether this gene is involved in either \(\alpha\)-endosulfan metabolism or oxidation of either isomer to endosulfan sulfate.

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**REFERENCES**