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Bacterial Degradation of *N*,*N*-Diethyl-*m*-Toluamide (DEET): Cloning and Heterologous Expression of DEET Hydrolase▽

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Pseudomonas putida **DTB grew aerobically with *N*,*N*-diethyl-*m*-toluamide (DEET) as a sole carbon source, initially breaking it down into 3-methylbenzoate and diethylamine. The former was further metabolized via 3-methylcatechol and *meta* ring cleavage. A gene from DTB, *dthA*, was heterologously expressed and shown to encode the ability to hydrolyze DEET into 3-methylbenzoate and diethylamine.**

N,*N*-diethyl-*m*-toluamide (DEET) is the active ingredient in most topical insect repellent products. Approximately 30% of the U.S. population use DEET-containing products, and domestic usage of DEET is estimated to be 1800 tonnes annually (16). It has been frequently detected in U.S. streams (in 74% of the streams surveyed) in the low parts-per-billion levels (5).

There is very little information about the microbial metabolism of DEET. Only partial degradation by the fungi *Cunninghamella elegans* and *Mucor ramannianus* R-56, via N oxidation and N deethylation, has been shown previously (13). Here we report the isolation of a bacterium capable of utilizing DEET as a sole carbon and energy source. We also describe the identification and heterologous expression of a gene from this bacterium encoding a DEET hydrolase. To our knowledge, this is the first report of a microorganism able to use DEET as a sole source of carbon and energy.

Chemicals. DEET (98%), 3-methylbenzoate (99%), 3-methylcatechol (99%), diethylamine (>99%), benzenesulfonyl chloride (99%), acetaldehyde (99.5%), and glacial acetic acid were purchased from Acros Organics (Morris Plains, NJ). Phenylmethylsulfonyl fluoride (PMSF) and aprotinin were obtained from Sigma (St. Louis, MO) and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) from Fluka (Buchs, Switzerland). Methanol and sodium nitroprusside were purchased from Fisher Scientific (Pittsburgh, PA).

Isolation of *Pseudomonas putida* DTB. Activated sludge from a municipal wastewater treatment plant in Ithaca, NY, was enriched with DEET (2.6 mM) according to standard protocols (4). A bacterial strain was isolated in pure culture and designated DTB. A fragment of the 16S rRNA gene from strain DTB was amplified by PCR and sequenced using the universal primers 27F and 1492R (6). The sequence of this fragment was compared with those deposited in the GenBank database using BLAST (1) and was found to be 100% identical to that of the 16S rRNA gene from *Pseudomonas putida* KT2440 over 1,419 nucleotides.

Pathway of DEET degradation by *P. putida* DTB. To determine the DEET degradation pathway, DTB was inoculated into minimal salts medium (MSM) (4) amended with 2.6 mM DEET. Growth was monitored by measuring attenuance at 600 nm. The culture was sampled over a 98-h period after inoculation. Samples were diluted with 1 volume of methanol and centrifuged at 21,000 × *g* for 10 min. The supernatants were analyzed by high-performance liquid chromatography (HPLC) (8) by monitoring absorbance at 220 nm and compared with DEET, 3-methylbenzoate, and 3-methylcatechol standards. The mobile phase consisted of 60% methanol and 40% 40 mM acetic acid.

HPLC analysis of culture supernatants showed the disappearance of DEET to be concomitant with the transient appearance of 3-methylbenzoate (Fig. 1). The same was observed when cell extracts were incubated with DEET. A compound with the same retention time as 3-methylcatechol was also detected in cell extracts incubated with DEET (data not shown). Cells of DTB produced a yellow color with maximum absorbance at 378 nm when incubated with either DEET or 3-methylcatechol (data not shown). This color disappeared upon acidification and reappeared upon neutralization, which is diagnostic of 2-hydroxy-6-oxo-hepta-2,4-dienoate, the *meta* cleavage product of 3-methylcatechol. These observations suggest that the 3-methylbenzoate produced from DEET hydrolysis was further metabolized through the *meta* cleavage pathway, as has been described for *P. putida* mt-2 (18).

It was observed that DTB was unable to grow on DEET without an additional source of nitrogen in the medium, an indication that it could not further metabolize the diethylamine produced from the initial breakdown of DEET. To confirm this, DTB was grown in triplicate in MSM with 2.6 mM DEET in screw cap bottles with shaking. After 66 h, cultures were centrifuged and the supernatant was derivatized with benzenesulfonyl chloride by the method of Sacher et al. (11), with the exception that chloroform was used instead of dichloromethane. The derivatized samples were then analyzed via gas chromatography-mass spectrometry as described by Sacher et al. (11). This analysis revealed the accumulation of 2.74 ± 0.18 mM diethylamine in the 66-h-old cultures from which 2.6 mM of DEET had been depleted, whereas no diethylamine was detected in uninoculated controls. This suggests a stoichiometric release of diethylamine which could not be metabolized further and explains why DEET could not be used as a nitrogen source.

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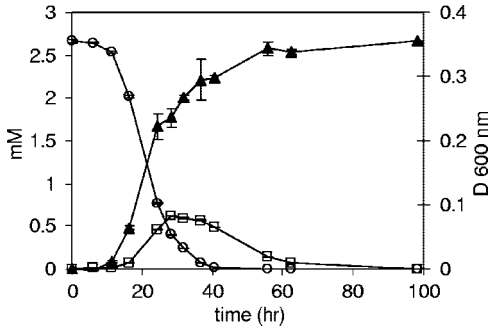

FIG. 1. Growth of *P. putida* DTB on DEET and transient accumulation of 3-methylbenzoate. Symbols: ○, DEET concentration; □, 3-methylbenzoate concentration; ▲, attenuance (D) at 600 nm. One data set typical of growth under these conditions is shown. The error bars indicate one standard deviation from three technical replicates.

The metabolites detected in culture supernatants and in cell extracts incubated with DEET, combined with the appearance of the yellow color diagnostic of a *meta* cleavage product, strongly suggest that DEET degradation follows the path outlined in Fig. 2. Biotransformation by DTB starts with hydrolysis of the amide bond in DEET, producing 3-methylbenzoate and diethylamine. Then, 3-methylbenzoate is converted into 3-methylcatechol, which undergoes ring cleavage in an extradiol manner and is further metabolized into compounds that enter the Krebs cycle. Unlike fungal metabolism by *Cunninghamella elegans* and *Mucor ramannianus* R-56 (13), this pathway does not involve N oxidation or N deethylation, nor does it involve oxidation of the aromatic methyl group as has been observed in rats and human liver microsomes (14, 17).

Identification of DEET hydrolase. A fosmid library from DTB genomic DNA was constructed in *Escherichia coli* by using the CopyControl fosmid library production kit from Epicenter (Madison, WI) according to the manufacturer's instructions. The library was screened for diethylamine production from DEET by growing the clones on MSM with 5% LB, 5.2 mM DEET, 12.5 μg/ml chloramphenicol, and CopyControl induction solution (Epicenter) on 96-well plates. After a 48-h incubation period, the plates were centrifuged at 3,000 rpm for 20 min and the supernatants were screened for the presence of diethylamine by using a colorimetric assay for the detection of secondary amines which was based on the method of Schar et al. (12) but scaled down for use in 96-well plates. Appearance of a purple color indicated the presence of diethylamine. The library yielded six positive clones out of a total of 928.

The fosmid from one positive clone, 7d2, was isolated and subjected to transposon mutagenesis using the EZ-Tn5 insertion kit (Epicenter) to randomly insert a Tn*5* ⟨R6K*γori*/KAN-2⟩ transposon. The process yielded 13 mutants that had lost the ability to metabolize DEET out of a total of 192. The insertion sites of four mutants that lost activity were determined by bidirectional sequencing using primers KAN-2 FP-1 and R6KAN-2 RP-1 (Epicenter). These sequences were assembled using the DNAStar software package (DNAStar Inc., Madison, WI) and the resulting contig was submitted to Orf Finder at the NCBI website (http://www.ncbi.nlm.nih.gov/projects/gorf/). The search yielded a predicted open reading frame of 1.9 kb, which we named *dthA* (for *D*E*E*T *h*ydrolase). Forward and reverse primers (IsF [5′-TGGTGACAGTTACCGCCTAAAGCA-3′] and GspR [5′-CCAAATGTTCTGACCCACGGACAA-3′], respectively) were designed and synthesized (Integrated DNA Technologies, Coralville, IA) to amplify a 2,285-base-pair region including *dthA*. PCR revealed that this open reading frame was also in the five other fosmid library clones that exhibited DEET hydrolysis activity in the original screen (data not shown).

Subcloning of DEET hydrolysis gene in *Escherichia coli*. To demonstrate that *dthA* indeed conferred the ability to cleave the amide bond in DEET, this gene and a transposon-interrupted copy of it were separately subcloned in *E. coli*. Primers IsF and GspR were redesigned to include KpnI restriction sites and used to amplify *dthA*. The PCR product was gel purified with the Zymoclean Gel DNA recovery kit (Zymo Research, Orange, CA) and cloned into the pGEM-T Easy cloning vector (Promega, Madison, WI). Chemically competent *E. coli* JM109 cells were transformed with the ligation product.

We tested the ability of cell extracts from *E. coli* JM109 with and without either pGEM-*dthA* or pGEM-*dthA*::kan to hydrolyze DEET. Cell extracts were prepared by washing LB-grown cells with 30 mM potassium phosphate buffer and resuspending in sonication buffer (100 mM Tris, 1 μM dithiothreitol, pH 8). The cells were disrupted by sonication (8), and the cleared lysate was stored in 50% glycerol at −20°C. Protein concentration was determined with the Bio-Rad (Hercules, CA) protein assay by the method of Bradford (3) using bovine serum albumin as a standard. The DEET hydrolysis assay system contained 50 μl of cell extract (15 mg protein/ml) and 15 mg of

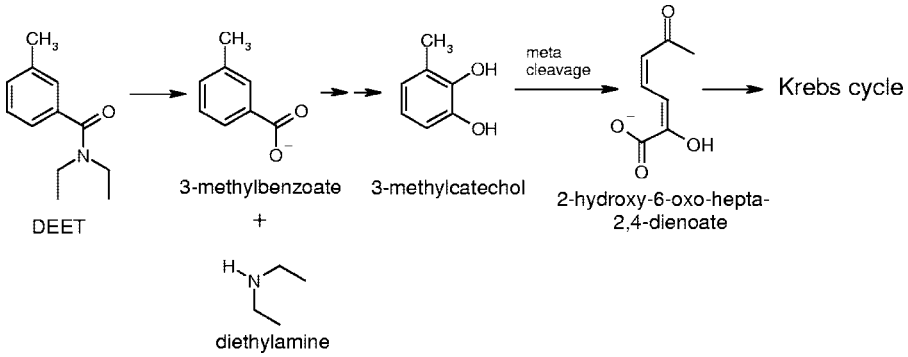

FIG. 2. Proposed pathway for the degradation of DEET by *P. putida* DTB.

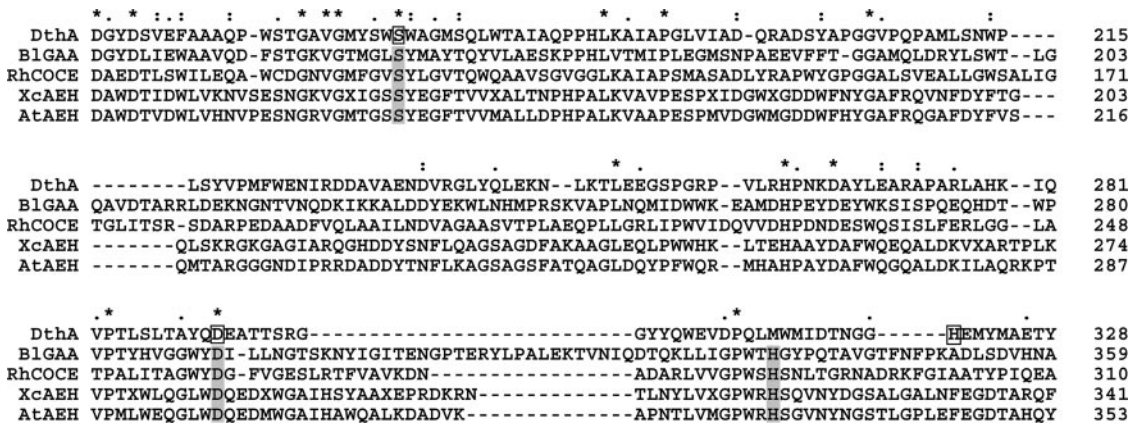

FIG. 3. Partial multiple sequence alignment of DthA with glutaryl 7-aminocephalosporanic acid acylase from *Brevibacillus laterosporus* J1 (BlGAA), cocaine esterase from *Rhodococcus* sp. strain MB1 (RhCOCE), and amino acid ester hydrolases from *Xanthomonas citri* (XcAEH) and *Acetobacter turbidans* (AtAEH). Identical amino acids, conservative substitutions, and semiconservative substitutions are indicated by asterisks, double dots, and single dots, respectively. Highlighted in gray are the catalytic triad residues of BlGAA, RhCOCE, XcAEH, and AtAEH. The putative catalytic residues of DthA are boxed.

DEET in 30 mM phosphate buffer for a total volume of 1 ml and a final DEET concentration of 78.4 mM. Reactions were carried out at 30°C for 30 min and stopped by adding 250 μl of trichloroacetic acid (15%, wt/vol). Samples were centrifuged at 21,000 × *g* for 30 min to separate the precipitated protein. The supernatants were diluted 1:5 in methanol and analyzed by HPLC as described for the analysis of culture supernatants. Only extracts from *Escherichia coli* JM109 pGEM-*dthA* were able to hydrolyze DEET, producing 64 ± 1 nmol of 3-methylbenzoate/min/mg protein, demonstrating that this gene confers the ability to cleave the amide bond in DEET.

Relatedness of DthA to other proteins. The nucleotide sequence of *dthA* was analyzed using BLAST (1) and found to be weakly related to a putative x-prolyl dipeptidyl peptidase from *Delftia acidovorans* SPH-1 (36% identity). Blast and PSI-BLAST (1) analysis also yielded similarity to four proteins of known function belonging to the α/β hydrolase fold family of enzymes. This diverse family includes proteases, esterases, and lipases, among other functions (9). An alignment of the deduced amino acid sequence of *dthA* with these four sequences was performed using Clustal X version 1.83 (15) and is shown in Fig. 3. The results suggested the presence of conserved residues in DthA which are characteristic of α/β hydrolases and which form part of a Ser-His-Asp catalytic triad in previously characterized proteins (9). Residues S166 and D292 from DthA aligned with the serine and aspartic acid residues that have been identified, respectively, as the nucleophile and the acidic residues that form part of the catalytic triad in the homologous proteins (2, 7, 10, 19). No histidine residues from DthA aligned with the catalytic histidine residues in the Clustal X alignment. However, four iterations of PSI-BLAST produced alignments where H320 from DthA aligned with the catalytic histidine of its homologues (data not shown).

To investigate the involvement of a nucleophilic serine in catalysis, the effect of three serine protease inhibitors was investigated with cell extracts of *E. coli* pGEM-*dthA*. The extracts were incubated in phosphate buffer with the inhibitors aprotinin (15.3 mM), PMSF (5 mM), and AEBSF (2 mM) for 15 min at 30°C. DEET hydrolysis activity was then assayed as described above and compared to that of a control without inhibitors.

Aprotinin (15.3 mM) did not have a significant effect on DEET hydrolysis. PMSF, which acts by sulfonylating the serine residue in the active site of serine hydrolases, decreased activity by only 36 ± 9%. Interestingly, PMSF has also been reported to cause weak or no inhibition of other hydrolases that share homology with DthA (2, 10). AEBSF, however, which has a mechanism of inhibition similar to that of PMSF, completely inhibited DEET hydrolysis by DthA. It is not clear why AEBSF completely inhibited activity in DthA whereas PMSF did not.

Although the inhibition by AEBSF is consistent with the presence of a serine nucleophile in the active site and the in silico results are suggestive of a Ser-His-Asp catalytic triad, a more in-depth investigation of the catalytic residues in DthA using site-directed mutagenesis is needed in order to confirm the role of these residues in DEET hydrolysis.

Conclusions. In summary, we have isolated a bacterium, *P. putida* DTB, that metabolizes DEET by hydrolyzing the amide bond to produce 3-methylbenzoate and diethylamine. This observed enzymatic activity contrasts with dealkylation of the *N*-ethyl group, or oxidation of either the nitrogen or the methyl group, as has been observed in eukaryotic organisms (13, 14, 17). A DEET hydrolase responsible for this activity was cloned from DTB, expressed in *E. coli*, and found to be related to members of the α/β hydrolase fold family of enzymes.

Nucleotide sequence accession numbers. The full sequence of the DEET hydrolase gene (*dthA*) and a partial sequence of the 16S rRNA gene have been deposited in GenBank under accession numbers EF123069 and EF123070, respectively.

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