A GENETIC AND BIOCHEMICAL INVESTIGATION OF BACTERIAL DEGRADATION OF THE INSECT REPELLENT *N*,*N*-DIETHYL-*M*-TOLUAMIDE (DEET)

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2009

A GENETIC AND BIOCHEMICAL INVESTIGATION OF BACTERIAL DEGRADATION OF THE INSECT REPELLENT *N*,*N*-DIETHYL-*M*-TOLUAMIDE (DEET)

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Cornell University 2009

DEET (*N,N*-diethyl-*m*-toluamide) is the active compound most commonly found in insect repellent products. It has been frequently detected as a micropollutant of waters around the world. This dissertation examines the degradation of DEET by bacteria isolated from activated sewage sludge. In particular, we looked at the biochemical and genetic aspects of the degradation of DEET by *Pseudomonas putida* DTB. We also investigated the ability of *Arthrobacter protophormiae* DE1 to degrade diethylamine, a by-product of DEET metabolism by strain DTB.

The metabolites produced during growth of strain DTB using DEET as a carbon source were analyzed. The results indicate that DTB degrades DEET by first cleaving the amide bond in DEET, producing 3-methylbenzoate and diethylamine. It then further metabolizes 3-methylbenzoate through 3-methylcatechol, which undergoes *meta* cleavage to produce Krebs cycle intermediates. The gene responsible for the first step in this pathway was identified by screening a fosmid library constructed in *Escherichia coli*. This gene, *dth*A, encodes a serine hydrolase with similarity to members of the α/β hydrolase family of proteins.

DthA was expressed as a histidine-tagged protein in *E. coli*, purified by metal affinity chromatography, and characterized. It has a broad substrate range towards aromatic and aliphatic esters and amides and an optimum temperature of 53 ºC. *In*

silico analysis and site-directed mutagenesis showed that DthA contains a catalytic triad composed of residues Ser166, Asp292, and His320, which are essential for activity. The results also indicate that Tyr84, Trp167, and Trp218 form an oxyanion hole important for stabilizing a reaction intermediate. Met170 and Trp214 seem important in substrate binding, as well.

A. protophormiae DE1 was able to grow using diethylamine as sole carbon and nitrogen source. When grown together with strain DTB, the coculture could use DEET as a sole source of carbon and nitrogen, which neither strain could do alone. Analysis of the metabolites produced by strain DE1 indicates that it metabolizes diethylamine by subsequent removal of the ethyl groups. We have identified *dea*A, a gene encoding an amine oxidase implicated in diethylamine degradation, by screening a fosmid library of DE1 genomic DNA constructed in *E.coli*.

BIOGRAPHICAL SKETCH

Giomar Rivera-Cancel grew up in and near Caguas, Puerto Rico. Her interest in science and nature led her to attend the NASA SHARP PLUS Program at Cornell University the summer after her junior year in high school, where she had her first research experience. She went to college at the University of Puerto Rico at Río Piedras, Puerto Rico, where she received her B.S. in Biology in 2003. While in college, she enjoyed the opportunity to do undergraduate research, first as an intern in the laboratory of Dr. Lily Young at Rutgers University in New Brunswick, New Jersey and then with Dr. Gary Toranzos Soria at Río Piedras. Encouraged by these two wonderful experiences, she chose to return to Cornell University for her Ph.D., where she joined the laboratory of Dr. Anthony Hay. She was awarded a State University of New York (SUNY) Fellowship to support her first two years of study and the Provost's Diversity Fellowship to fund her last semester at Cornell.

Giomar has been an eager supporter of increasing the participation of women and minorities in science. In 2007 and 2008, she volunteered in Expanding Your Horizons, a math and science conference for 7^{th} to 9^{th} grade girls held at Cornell, where she offered a workshop on bacterial batteries in 2008. She also went on a recruitment trip to Puerto Rico in 2008 to offer orientation to prospective students on the internship opportunities available for minorities and the graduate programs of study offered at Cornell. She hopes that throughout her life she can be a role model and a mentor for young women and minorities interested in science.

To Abner, thank you for being my rock and for always

knowing how to make me smile.

A mi Madre, a ti te debo lo que soy hoy. Gracias por encaminarme por el buen rumbo.

ACKNOWLEDGMENTS

I want to thank my advisor, Dr. Anthony Hay, for guiding me and believing in me, even at times when I did not believe in myself. I also have to acknowledge my committee members Dr. Beth Ahner and Dr. David Wilson for contributing to my project through their advice and insightful suggestions. I thank the students, postdocs, faculty, and administrative staff at the Department of Microbiology for helping me in my work whenever they could. Thanks to Dr. Holger Sondermann for giving me the opportunity to learn about protein crystallography in his lab and to Nabanita De for her assistance. I appreciate the financial support provided to me by Cornell University through the SUNY and Provost's Diversity fellowships.

I am forever grateful to my labmates (Bryan Campbell, Mitchell Thompson, Kevin George, Jeffrey Peacock, Daniela Bocioaga, Karl Rueggeberg, Amy Risen, and Gabriela Hidalgo) for their wonderful companionship, for always cheering me up, and for keeping me sane amidst the frustrations of research. Thank you to former lab members (Andreas Toba, Abigail Porter, Jeanne Kagle, Robert Murdoch, and Lauren Junker) for sharing their wisdom with me. Thank you to my family, for always loving and supporting me. Thank you to my friends, they have been my family away from home. Most of all, I want to thank my fiancé, Abner, without whom this journey would have been meaningless. His constant support was the one thing that kept me going through the good and the bad times.

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CHAPTER 1

INTRODUCTION

PHARMACEUTICAL AND PERSONAL CARE PRODUCTS (PPCPS)

PPCPs are bioactive chemicals used for medicinal or personal care purposes (28). These include: prescription and over-the-counter drugs, veterinary drugs, fragrances, lotions, cosmetics, sun-screen products, detergents and their degradation products, disinfectants, and insect repellents, among others. They are emerging pollutants, mainly of aquatic environments, that are often found in the ng/l to μg/l range.

In the past, this group of chemicals had received little attention. This was in part because analytical methods allowing their detection at low concentrations were not available. The number of existing PPCPs has increased exponentially as a result of technological and medical advances over the past few decades, and so has the number of these compounds in the environment (17). This has lead to an acknowledgement of their importance as environmental pollutants.

Thanks to the development of new detection methods, many studies have been published reporting the presence of PPCPs in surface and ground waters, water sediments, waste water treatment plants, and drinking water (5, 7, 30, 45, 49, 58, 63). Kolpin *et al*. (30) did a survey of organic contaminants in U.S. streams during the years 1999 and 2000. The PPCPs caffeine (stimulant), cotinine (nicotine metabolite), triclosan (antimicrobial disinfectant), 4-nonylphenol (nonionic detergent metabolite), *N*,*N*-diethyl-*m*-toluamide (insect repellent), trimethoprim (antibiotic), and acetaminophen (antipyretic) were among the most frequently detected contaminants. Daughton and Ternes (17) also did an extensive study of PPCPs in the environment finding synthetic hormones (17α-ethynylestradiol), anti-inflammatories (ibuprofen,

diclofenac), lipid regulators (clofibric acid), antiepileptics (carbamazepine), and fragrances (galaxolide).

Although PPCPs are detected at low concentrations in the environment, they are still a cause of concern. For most PPCPs, the long-term ecological and human health effects of exposure to low concentrations are unknown. The effect of multiple pharmaceuticals that act on the same biochemical target could be considerable (17) and chemicals with unrelated mechanisms could have unpredictable synergistic effects (1, 4). In addition, a chemical that may pose a low risk to one species can be much more harmful to a nontarget one such as the antimicrobial triclosan, which has been shown to be toxic to algae at environmentally relevant concentrations (40). For this reasons, research efforts should be concentrated on understanding the environmental fate of these compounds, as well as the physicochemical and biological processes that could be enhanced in order to facilitate the removal of PPCPs before they get into the natural waters, which often serve as drinking water supplies to humans.

BACTERIAL DEGRADATION OF PPCPS

A number of examples of bacterial degradation of PPCPs have been published (14, 25, 38, 42, 44). A quick glance at the PPCPs identified in environmental samples reveals that the majority of them have one or more aromatic rings. This provides an opportunity for biological transformation by microbes with pathways for the degradation of aromatic compounds like toluene, xylene, and polycyclic aromatic hydrocarbons. One example of this is ibuprofen degradation by *Sphingomonas* sp. Ibu-2, an organism isolated from activated sewage sludge that is capable of utilizing this compound as a sole source of carbon and energy (38). Ibuprofen degradation by Ibu-2 starts by removing the propionic acid moiety of ibuprofen and hydroxylating the ring at the 1 and 2 positions, forming isobutylcatechol (figure 1.1). This substituted

catechol is then metabolized via *meta*-cleavage (38), as suggested by the accumulation of this compound when an inhibitor of the enzyme catechol 2,3-dioxygenase was added.

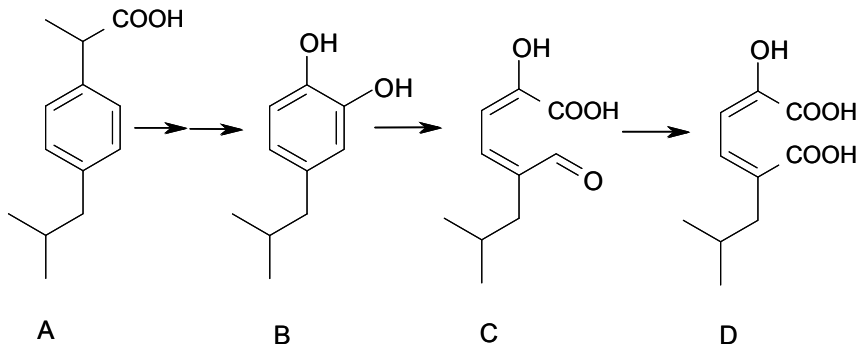

Figure 1.1. Degradation of ibuprofen (A) by *Sphingomonas* sp. Ibu-2. The metabolites in this pathway are: (B) isobutylcatechol, (C) 5-formyl-2-hydroxy-7-methylocta-2,4-dienoic acid, and (D) 2-hydroxy-5-isobutylhexa-2,4-dienedioic acid. Adapted from Murdoch *et al.* (38).

In some cases, microbial transformation of PCPPs can also lead to accumulation of metabolites with higher toxicity than the parent compounds. Such is the case of the nonionic surfactants nonylphenol polyethoxylates (NPEOs) and octylphenol polyethoxylates (OPEOs), also known as alkylphenol polyethoxylates (APEOs). Microbial degradation of APEOs under aerobic conditions leads to removal of all but the last one or two ethoxy moieties proximal to the aromatic ring (23). The resultant alkylphenol mono- and di- ethoxylates are weakly estrogenic (58) and are refractory under aerobic conditions, but are converted to alkylphenols by anaerobic microorganisms (23). Alkylphenols are 10-20 times more estrogenic than the mono or diethoxylates and are much more toxic than the long chain APEOs. Nonylphenol and octylphenol have been repeatedly detected in sewage treatment plant effluents and sludges (26, 50).

Despite the large amounts of alkylphenols that survive the wastewater treatment processes, there exist microorganisms that can degrade them. Three of these

organisms, *Sphingomonas* sp. TTNP3(14), *Sphingomonas xenophaga* Bayram (22), and *Sphingomonas* sp. PWE1 (42), accomplish the removal of the side chain of these alkylphenols via a type II ipso substitution mechanism (15).

Porter *et al*. (42) were able to identify a flavin monooxygenase from *Sphingomonas* sp. PWE1 that could hydroxylate the octylphenol ring on the same carbon that the alkyl substituent is on (figure 1.2). The product of this reaction then undergoes type II *ipso* substitution, resulting in the formation of either hydroquinone and a carbocation corresponding to the alkyl side chain, or octyloxyphenol (42). Other metabolites detected during octylphenol degradation by PWE1 were 1,2,4-trihydroxybenzene, 2,4,4-trimethyl-2-pentanol (resulting from the S_n1 reaction of the carbocation with water), and 2,4,4-trimethyl-1-pentene (product of an E1 elimination reaction of the carbocation) (42).

There are still many more examples of microorganisms that can metabolize and grow on PPCPs but whose degradation pathways have not been elucidated in detail. These are reviewed elsewhere (28).

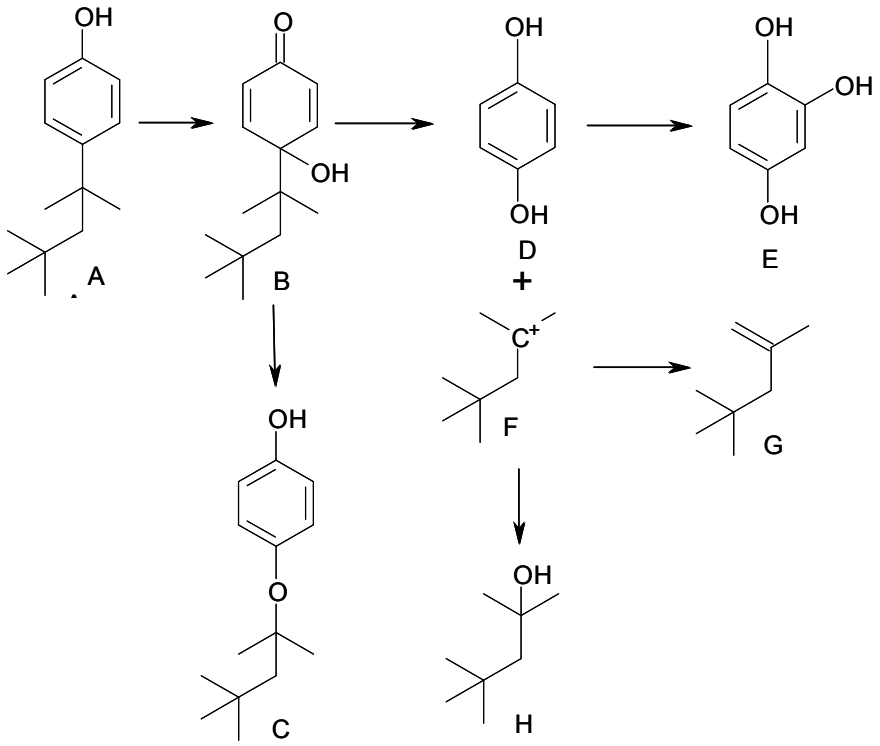

Figure 1.2. Degradation of octylphenol (A) by *Sphingomonas* sp. PWE1. The metabolites in this pathway are: (B) *ipso* hydroxylation intermediate, (C) octyloxyphenol, (D) hydroquinone (E) 1,2,4-trihydroxybenzene, (F) alkyl side chain carbocation, (G) 2,4,4-trimethyl-1-pentene, and (H) 2,4,4-trimethyl-2-pentanol. Adapted from Porter *et al.* (42).

DEET: HISTORY, TOXICITY, AND ENVIRONMENTAL FATE

DEET (figure 1.3) is one of many PPCPs that have just recently begun to receive attention as emerging environmental pollutants. The discovery of DEET arose from a study aiming to correlate structure with the repellent activity of synthetic compounds (33, 41). DEET was developed in 1946 for use by U.S. Army personnel in insect infested areas and commercially marketed since 1965 (54). It is thought that this compound protects from biting insects by inhibiting their attraction to substances given off by the host, such as lactic acid which is present in human sweat (20). Ditzen *et al* (19) have suggested that DEET also acts by blocking insects' odorant receptor

neurons that respond to a compound emitted in human breath, 1-octen-3-ol. However, another study by Syed and Leal (53) refutes this hypothesis by proposing that there is an olfactory receptor neuron for DEET, separate from that for 1-octen-3-ol, and that the repellency effect is a result of the insects smelling DEET and avoiding it.

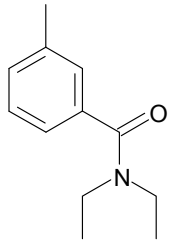

Figure 1.3. Structure of *N,N*-diethyl-*m*-toluamide (DEET).

Commercial formulations of DEET contain between 4 and 100% of the compound and usually come in an ethanol base. DEET-containing products are primarily intended for application to skin and clothes. Dermal absorption is less than 20% in humans and is higher when DEET is diluted in ethanol than when it is undiluted (48). The absorbed DEET is primarily excreted in the urine in several metabolized forms (48). Studies in rats have identified two major pathways for the metabolism of DEET. One pathway involves the oxidation of the aromatic methyl group and the other involves a single dealkylation of the *N*-ethyl group, yielding *N,N*-diethyl-*m*-hydroxymethylbenzamide and *N*-ethyl-*m*-toluamide, respectively (51). These metabolites were also detected in a study with human liver microsomes and human cytochrome P450 isoforms expressed in baculovirus-infected insect cells (55).

There have been several reports of adverse health effects, in particular neurological effects, related to DEET overexposure (9, 11, 18). The mechanism of toxicity in these cases is unknown. Some studies suggest that exposure to the pesticide permethrin in combination with DEET might produce synergistic toxicity (2, 3). It has

also been suggested that DEET might enhance the effect of the cholinesterase inhibitor pyridostigmine bromide, when used concurrently (2, 3, 10, 34). It is possible that DEET might be enhancing the toxicity of these compounds by increasing their absorption or availability in the body. Another possibility is that it might be competing for the same detoxification systems that act on these other chemicals.

Since DEET is classified as a residential-use product, it has not been considered to have potential environmental effects. For this reason, ecotoxicological studies on this compound have not been as extensive as for other pesticides (16). Yet, studies indicating a widespread distribution of DEET call for a more thorough risk assessment (45). DEET has been detected throughout the North Sea at concentrations of up to 1.1 ng/l (59) and the coast of northern Norway (downstream of an untreated sewage plume) at up to 13 ng/l (58). It also has been found in groundwater within a leachate plume downgradient of a municipal landfill (7) at a maximum concentration of 13 µg/l. A study by Kolpin *et al*. (30) listed DEET as one of the most frequently detected organic compounds in U.S. streams suspected to receive wastewater from livestock production and urbanized areas (30). The maximum concentration detected in this study was 1.1 µg/l. Although DEET is not generally used on livestock, use of DEET on horses and by humans involved in livestock production might explain its detection downstream of agricultural settings. Given that DEET is applied to the skin and clothing, it is also reasonable to assume that it is entering sewer systems after rinsing from the skin and clothing during washing.

Wastewater treatment processes, however, seem not to remove DEET completely. A study by Knepper (29) revealed that DEET was degraded to some extent in wastewater treatment plants in Germany, but only after a period of adaptation and with influent concentrations over 1 µg/l. Failure to remove DEET and other PPCPs during the wastewater treatment process can lead to contamination of drinking

water supplies. There is research showing that commonly used drinking water purification methods fail to remove DEET. A study investigating the removal of an array of endocrine-disruptors and PPCPs from simulated drinking water (60) found that DEET was poorly removed when chemical coagulation, adsorption to powder activated carbon, and oxidation with chlorine where used.

The frequent detection of DEET in riverine environments in the U.S. reported by Kolpin (30) *et al.* and Sandstrom (45) *et al.* might be a consequence of limited degradation by microbes in wastewater treatment plants. There is little information about the capacity for microorganisms to degrade DEET, although several fungi have been reported to partially degrade DEET (44). Chapter 2 discusses in detail the biodegradation of DEET via 3-methylbenzoic acid and diethylamine by *Pseudomonas* sp. DTB, the only microorganism that, to date, has been reported to grow on DEET.

AROMATIC ACID DEGRADATION

Aromatic acids, such as 3-methylbenzoic acid, are often intermediates in the degradation of aromatic compounds (27). In the aerobic oxidation of aromatic acids, the aromatic ring is destabilized by the addition of hydroxyl groups (61). The pathway encoded by the Tol plasmid of *Pseudomonas putida* mt-2 (62) is a well known example of this. In this pathway, benzoate, 3-methylbenzoate, and 4-methylbenzoate are metabolized by dioxygenation followed by dehydrogenation to produce catechol, 3-methylcatechol, and 4-methylcatechol, respectively (62). The ring in these catechols is then cleaved by the action of an extradiol catechol dioxygenase in what is known as *meta*-cleavage (figure 1.4) (62). Other pathways utilize intradiol (*ortho*) cleavage to break the catechol ring (figure 1.4) (56, 61). The ring fission products are then converted into central metabolites in bacteria: pyruvate and acetaldehyde for the *meta*

fission product (39) and succinyl-CoA and acetyl-CoA for the *ortho* fission product (24).

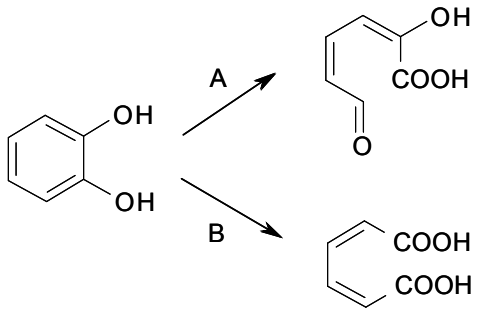

Figure 1.4. (A) Extradiol (*meta*) and (B) intradiol (*ortho*) cleavage of catechol by catechol dioxygenases.

ALKYLAMINES: ABIOTIC AND BIOTIC TRANSFORMATIONS

Conversion to toxic nitrosamines

As was seen for NPEOS and OPEOS, when pollutants are only partially degraded, the remaining metabolites could be less or more toxic than the parent compound. A great number of PPCPs have amine or amide groups (17) that could potentially be metabolized into alkylamines. Toxic nitrosamines can be formed by the abiotic or biotic nitrosation of secondary alkylamines and their presence has been detected in sewage sludge from municipal water treatment plants (37). Nitrosamines act as DNA-alkylating agents and are carcinogenic in animals, their effect being dependent on the specific nitrosamine, the type of tissue, and the species (32). *N*-diethylnitrosamine, for instance, is a potent liver carcinogen in rodents, snakes, birds, mollusks, and monkeys (32, 46). Nitrosamines can be produced from secondary amines during water treatment by their reaction with chloramines, a common disinfection reagent. They can also be formed endogenously by the reaction of secondary aliphatic and aromatic amines with nitrite in the acidic environment of the stomach (43). It has been demonstrated that this reaction is also catalyzed by the

enzymatic action of intestinal bacteria at neutral pH (52). Enzymatic catalysis is not the only way in which microorganisms can influence the formation of nitrosamines in the human body and in other environments; they could also promote the formation of these compounds by lowering the pH and by reducing nitrate to nitrite or oxidizing ammonia to nitrite (6).

Bacterial metabolism of alkylamines

As will be discussed in chapter 2, degradation of DEET by bacteria can produce diethylamine as an end product. The metabolism of secondary aliphatic amines such as diethylamine has not been well characterized, however, that of methylamines by methylotrophic bacteria has been studied more in detail. The pathways employed by methylotrophic bacteria that use trimethylamine as a sole carbon source are highly modular and many different enzymes with the same functionalities are used (figure 1.5). Degradation starts with oxidation of trimethylamine, generating dimethylamine and formaldehyde (12). This can be accomplished by a trimethylamine dehydrogenase, as for *Hyphomicrobium* (35). It can also be done in two steps by utilizing a trimethylamine monooxygenase that generates trimethylamine-*N*-oxide, followed by a trimethylamine-*N*-oxide demethylase. This is the case in *Pseudomonas aminovorans* and *Bacillus* PM6 (8). The dimethylamine produced by either mechanism is then oxidized using a dimethylamine monooxygenase (8). An exception to the rule is *Hyphomicrobium* X, which utilizes a dimethylamine dehydrogenase when growing anaerobically on dimethylamine (36). The oxidation of methylamine is even more varied, with three enzyme systems available: methylamine oxidase, methylamine dehydrogenase, and *N*-methylglutamate synthase. Methylamine oxidase is a copper-quinoprotein observed in *Arthrobacter* P1 that produces hydrogen peroxide (31, 57). *Pseudomonas* AM1 uses methylamine dehydrogenase, which needs the cofactor pyrroloquinoline quinine (PQQ) as an

electron acceptor (8). In *Hyphomicrobium* species, *N*-methylglutamate synthase is used to produce N-methylglutamate and ammonia from methylamine and glutamate (35, 47). The formaldehyde produced by these reactions is then fed to the RuMP cycle or the serine cycle, depending on the organism (8).

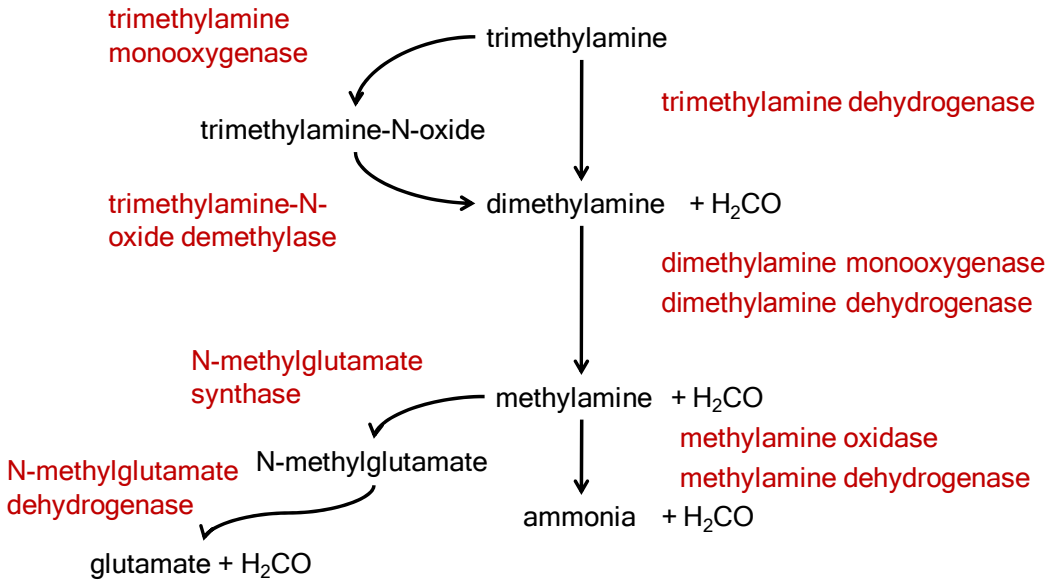

Figure 1.5. Pathways for the utilization of methylamines by methylotrophic bacteria. The enzymes used for each step are in red.

Some of the enzymes described above have broad substrate specificity. The trimethylamine dehydrogenase from bacterium 4B6, isolated by Colby and Zatman (13), can also utilize secondary amines with *N*-methyl and/or *N*-ethyl groups (12). The dimethylamine monooxygenase from *Pseudomonas aminovorans* was shown to also metabolize diethylamine at 40% of the rate with dimethylamine (21). Similarly, dimethylamine dehydrogenase from *Hyphomicrobium* X metabolizes diethylamine at 78% of the rate with dimethylamine (36).

FINAL REMARKS

Even though biotic degradation has been observed for some PPCPs, the mechanism by which this happens and the species involved are unknown for many of them. The following chapters describe the basic research into the metabolism of one PPCP: DEET. In chapter 2, the biochemical pathway of DEET degradation by *Pseudomonas putida* DTB is explained. In chapter 3, the biochemical characteristics of DEET hydrolase, the enzyme involved in the first step of DEET degradation, are presented. The final chapter investigates the degradation of the diethylamine moiety of DEET by a strain of *Arthrobacter protophormiae*.

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CHAPTER 2

BACTERIAL DEGRADATION OF N,N-DIETHYL-*M*-TOLUAMIDE (DEET): CLONING AND HETEROLOGOUS EXPRESSION OF DEET HYDROLASE

ABSTRACT

Pseudomonas putida DTB grew aerobically with DEET (*N,N*-diethyl-*m*-toluamide) 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, *dth*A, was heterologously expressed and shown to encode the ability to hydrolyze DEET into 3-methylbenzoate and diethylamine.

INTRODUCTION

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 (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.

Applied and Environmental Microbiology (2007) vol.73, p. 3105–3108, doi:10.1128/AEM.02765-06

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 as 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 the 16S rRNA gene from *Pseudomonas putida* KT2440 over 1419 nucleotides.

PATHWAY OF DEET DEGRADATION BY *PSEUDOMONAS PUTIDA* DTB

To determine the degradation pathway of DEET, DTB was inoculated into minimal salts medium (MSM) (4) amended with 2.6 mM DEET. Growth was monitored by measuring attenuance (D) at 600 nm. The culture was sampled over a 98 hour period after inoculation. Samples were diluted with one volume of methanol and centrifuged at 21,000 x 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 (Figure 2.1). The same was observed when cell-free extracts were incubated with DEET. A compound with the same retention time as 3-methylcatechol was also detected in cell-free 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 in *P. putida* mt-2 (18).

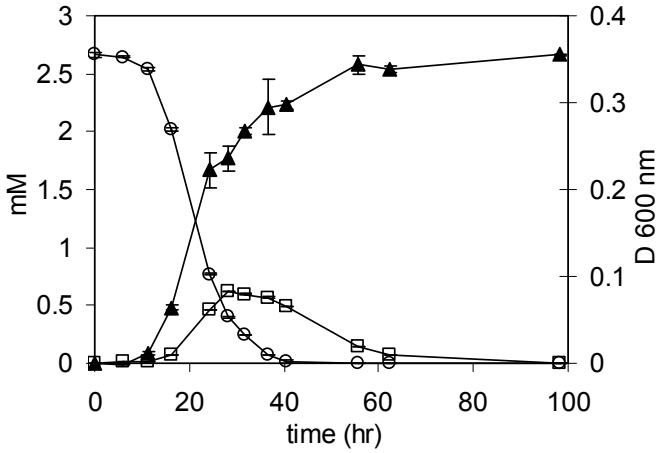

Figure 2.1. Growth of *P. putida* DTB on DEET and transient accumulation of 3-methylbenzoate. Symbols: ○, DEET concentration; □, 3-methylbenzoate concentration; ▲, D 600 nm. One data set typical of growth under these conditions is shown. The error bars indicate one standard deviation from three technical replicates.

It was observed that DTB was unable to grow on DEET without an additional source of nitrogen in the media, an indication that it could not further metabolize the diethylamine produced from the initial break down 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 hours, 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 (GC-MS) as described in Sacher *et al*. (11). This analysis revealed the accumulation of 2.74 ± 0.18 mM diethylamine in the 66 hour-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.

The metabolites detected in culture supernatants and in cell-free 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 figure 2.2. 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. Neither does it involve oxidation of the aromatic methyl group as has been observed in rats and human liver microsomes (14, 17).

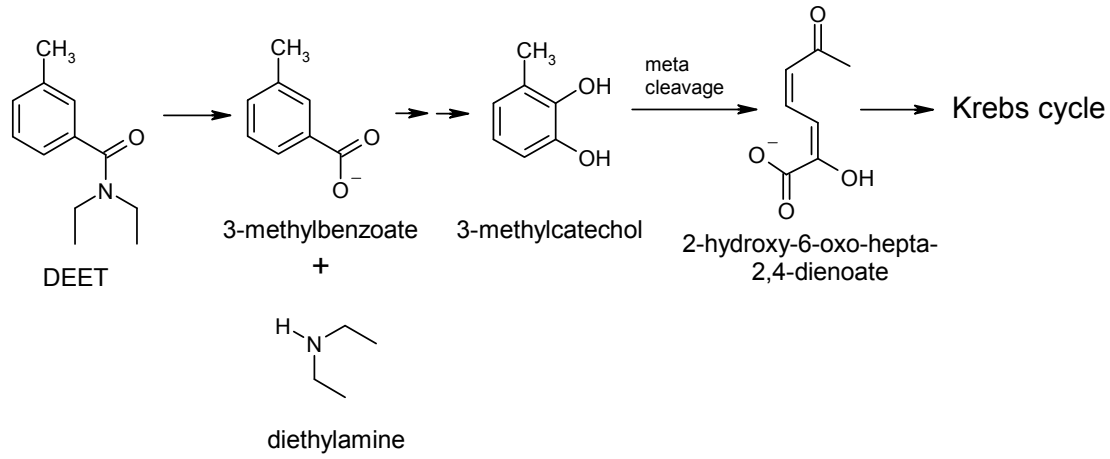

Figure 2.2. Proposed pathway for the degradation of DEET by *P. putida* DTB.

IDENTIFICATION OF DEET HYDROLASE

A fosmid library from DTB genomic DNA was constructed in *E. coli* using the CopyControl Fosmid Library Production Kit from Epicentre (Madison, WI) following 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 (Epicentre) on 96-well plates. After a 48 hour incubation period, the plates were centrifuged at 3,000 rpm for 20 minutes and the supernatants were screened for the presence of diethylamine using a colorimetric assay for the detection of secondary amines based on the method of Schär *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 (Epicentre) to randomly insert a Tn*5* <R6Kγ*ori*/KAN-2> transposon. The process yielded 13 mutants that had lost the ability to metabolize DEET out of a total of 192. The insertion site of four mutants

that lost activity was determined by bidirectional sequencing using primers KAN-2 FP-1 and R6KAN-2 RP-1 (Epicentre). 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 (orf) of 1.9 kb, which we named *dth*A (for DEET hydrolase). 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 *dth*A. PCR revealed that this orf was also in the 5 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 *dth*A 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 re-designed to include KpnI restriction sites and used to amplify *dth*A. The PCR product was gel purified with 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-free extracts from *E. coli* JM109 with and without either pGEM-*dth*A or pGEM-*dth*A::kan to hydrolyze DEET. Cell-free 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-free extract (15 mg protein/ml) and 15 mg of DEET in 30 mM phosphate buffer for a total volume of 1 ml and a final DEET molarity of 78.4 mM. Reactions were carried out at 30°C for 30 minutes and stopped by adding 250 µl of trichloroacetic acid 15% (w/v). Samples were centrifuged at 21,000 x g for 30 minutes 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-*dth*A were able to hydrolyze DEET, producing 64 ± 1 nmoles 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 *dth*A 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 *dth*A with these four sequences was performed using Clustal X version 1.83 (15) and is shown in figure 2.3. 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

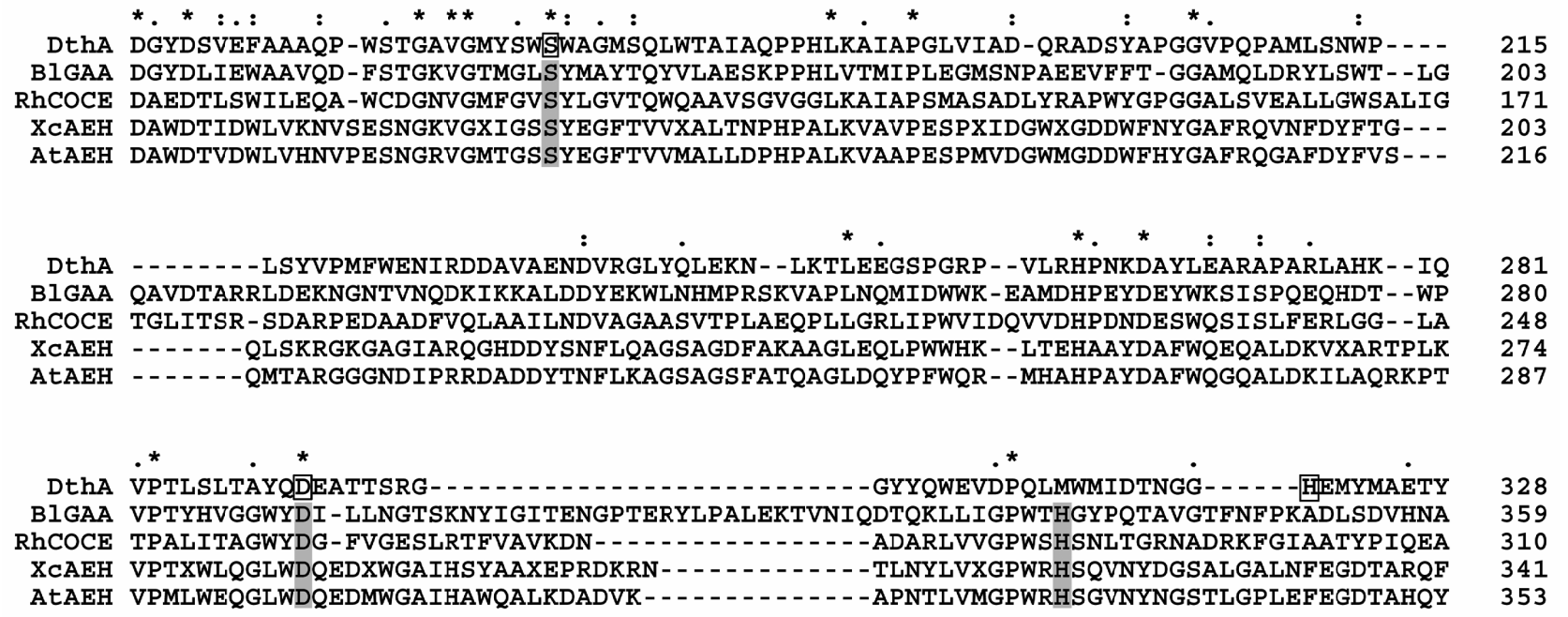

Figure 2.3. Partial multiple sequence alignment of DthA with glutaryl 7-aminocephalosporanic acid acylase from *Brevibacillus laterosporus* J1 (BlGAA), cocaine esterase from *Rhodococcus* sp. MB1 (RhCOCE), and amino acid ester hydrolases from *Xanthomonas citri* (XcAEH) and *Acetobacter turbidans* (AtAEH). Identical amino acids, conservative substitutions, and semiconservative substitutions are indicated by stars, stacked double dots, and single dots, respectively. Highlighted in grey are the catalytic triad residues of BlGAA, RhCOCE, XcAEH, and AtAEH. The putative catalytic residues of DthA are shown boxed.

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 in cell-free extracts of *E. coli* pGEM-*dth*A. The extracts were incubated in phosphate buffer with the following inhibitors: aprotinin (15.3 mM), PMSF (5 mM), and AEBSF (2 mM) for 15 minutes at 30°C. Then, DEET hydrolysis activity was 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 only decreased activity by 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 similar mechanism of inhibition to PMSF, completely inhibited DEET hydrolysis by DthA. It is not clear why AEBSF completely inhibited activity in DthA and the other hydrolases 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 (*dth*A) and a partial sequence of the 16S rRNA gene have been deposited in GenBank under accession numbers EF123069 and EF123070, respectively.

This work was partially supported by a State University of New York (SUNY) fellowship awarded to G. R. C.

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CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF DEET HYDROLASE FROM *PSEUDOMONAS PUTIDA* DTB

ABSTRACT

The enzyme DEET hydrolase (DthA) from *Pseudomonas putida* DTB was purified and characterized. In addition to hydrolyzing DEET, as the first step in the metabolic pathway for this compound, DthA also hydrolyzes a variety of esters and amides. Native DthA is a tetrameric enzyme with an Mr of 334 KDa as determined by gel filtration. Its activity was optimal at 53 ºC and at pH 8.0. A homology model of the tertiary structure of DthA was used to guide mutational analysis of DthA. Site directed mutagenesis demonstrated that DthA is a serine hydrolase containing a Ser-His-Asp catalytic triad and oxyanion-hole residues. Finally, DthA crystals were obtained by the vapor diffusion method which diffracted to 2.66 Å.

INTRODUCTION

The widely used insect repellent DEET has been introduced into the environment fairly recently (18). Despite being around for only 62 years at low concentrations in surface waters (10), we were able to isolate a bacterium that grows on DEET as an only carbon source and identify the enzyme (DthA) responsible for the hydrolysis of this compound (14).

In our previous work, we presented a Clustal X alignment of DthA with homologous proteins from the α/β hydrolase fold family (14). α/β hydrolases contain a conserved catalytic triad consisting of a nucleophile, an acid residue, and a histidine. They also have an oxyanion hole, a group of residues that help stabilize the covalent substrate-enzyme intermediate formed during the reaction. The alignment suggested

that S166 and D292 are the nucleophile and acid residue, respectively. The conserved histidine residue could not be identified from this alignment; however, alignments produced using PSI-BLAST suggested this to be H320.

The first part of the present work describes the purification, biochemical properties, and substrate range of DthA. The second part describes our efforts to confirm the *in silico* predictions about important active site residues using site directed mutagenesis.

METHODS

Chemicals

All compounds used for substrate specificity studies were purchased from Acros, with the exception of toluanilide and cocaine, which were purchased from Sigma. Cocaine was obtained as a 1 mg/ml standard in acetonitrile, which we evaporated under nitrogen gas and resuspended to 2 mg/ml in pure water and stored at -20 °C, to avoid hydrolysis. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Gold Biotechnology.

Cloning into expression vector

The *dth*A gene was amplified from *Pseudomonas putida* DTB using the forward primer 5'-TACATATGACTCAAAATCATAATAATCCGG-3' with a 5' *Nde*I restriction site and the reverse primer 5'-ATGGTACCATCAATGATGATGATGATGATGACAGCCCGTTAGCGGGTC-3' containing a 5' *Kpn*I site and a hexa-histidine tag. The 1.8 kb fragment obtained was TA cloned into the vector pGEM (Promega) to produce pGdthA and transformed into *Escherichia coli* JM109. The *dthA* fragment was digested from pGdthA using *Nde*I and *Kpn*I, gel purified, and ligated to *Nde*I/*Kpn*I-digested pRSET-A (Invitrogen). The

resulting vector, pRdthA, was transformed into JM109, from which it was purified and then transformed into *E. coli* BL21(DE3)pLysS.

Site-directed mutagenesis

DthA variants were created by PCR amplification of the complete sequence of pRdthA with mutagenic primers. One unit of high fidelity Phusion polymerase (Finnzymes) and 15 pg of template were utilized per 50 µl reaction. Codon substitutions were carried out using the 5'-phosphorylated primers listed in table 3.1 (substitutions are underlined). Self-ligation of the PCR products was carried out with T4 DNA ligase (Promega) and transformed into JM109. Transformants were sequenced to check for the appropriate substitutions and absence of frameshift mutations. Plasmids were isolated from transformants containing the correct substitutions and transformed into BL21(DE3)pLysS.

Table 3.1. Mutagenic primers used in this study

Mutation	Primer Sequence
S166A	F: 5'-TGTACAGCTGGGCCTGGGCCGG-3' R: 5'-TGCCGACCGCCCCTGTGCTC-3'
D292A	F: 5'-GCCTACCAGGCCGAAGCAACGA-3' R: 5'-GGTGAGAGAGAGAGTGGGCAC-3'
H320A	F: 5'-ACCAATGGCGGGGCCGAGATGTATAT-3' R: 5'- GTCGATCATCCACATCAATTGCGG -3'
Y84F	F: 5'-TTCAGCGGCTTTGCCCCAAGCG -3' R: 5'-ATGCAGCAATGTCGGAAAAGGCCC-3'
W167A	F: 5'-CAGCTGGTCCGCGGCCGGGATG-3' R: 5'- TACATGCCGACCGCCCCTG -3'
Y218F	F: 5'- GCCGTTGAGCTTTGTTCCGATGT-3' R: 5'- CAGTTCGAAAGCATCGCCGGCTG-3'
W214A/V	F: 5'- GATGCTTTCGAACGYGCCGTTGAGC -3' R: 5'- GCCGGCTGCGGGACGC-3'
W214E	F: 5'-GATGCTTTCGAACGAGCCGTTGAGC-3' R: 5'- GCCGGCTGCGGGACGC-3'
M170A/Q	F: 5'- CTGGGCCGGGSMGTCGCAGCTC -3' R: 5'- GACCAGCTGTACATGCCGACCGCCCC-3'

F, forward primer
R, reverse primer

Purification of histidine-tagged DthA

For DthA overexpression, *E. coli* BL21(DE3)pLysS containing pRdthA or mutant derivatives was grown on 100 ml Terrific Broth to an OD_{600} of ~0.5 and induced with 1 mM IPTG overnight at room temperature. Cells were harvested by centrifugation and resuspended in 25 mM Tris/100 mM NaCl/20 mM imidazole. Cell extracts were prepared by sonication and subsequent centrifugation at 18,000 rpm and 4 °C for 20 min. The cleared lysate was applied to a Ni-NTA column (Qiagen). The column was washed twice with 25 mM Tris/100 mM NaCl/40 mM imidazole and the protein was eluted with 25 mM Tris/100 mM NaCl/300 mM imidazole. The purified enzyme was stored at -20 ºC in 40% glycerol.

Wild type DthA used for crystallization was purified as described above with the following exceptions: 1) the volume of growth medium used was 8 l, 2) induction of the cultures with IPTG was done at 18 ºC, 3) protein was passed through a desalting column and a gel filtration column after eluting from the Ni-NTA column, and 4) the final protein preparation was concentrated using an Amicon ultracentrifugation filter device with a 30,000 molecular weight cutoff (Millipore) and stored at -70 ºC.

Molecular mass determination

The molecular mass of the native enzyme was determined by size-exclusion chromatography with a HiLoad 16/60 Superdex 200 (GE Healthcare) FPLC column. The buffer used for equilibration and elution was composed of 25 mM Tris and 100 mM NaCl (pH 7.6). The column was calibrated with apoferritin (M_r = 443,000), alcohol dehydrogenase (M_r = 150,000), bovine serum albumin (M_r = 66,000), and ovalbumin (M_r = 44,000) (Sigma).

Kinetic assays

Initial DEET hydrolysis rates were measured spectrophotometrically at 30 °C on a Synergy HT spectrophotometer using KC4 software version 3.4 (Bio-Tek) by following the change in absorbance at 215 nm every 5 s for 100 s. The difference between the molar extinction coefficients of substrate and product at 215 nm was 7,300 $M^{-1}cm^{-1}$. The assay was started by adding 1 µl of enzyme solution to 999 µl of pre-warmed 30 mM potassium phosphate buffer pH 7.6 with DEET in a quartz cuvette. The concentration of enzyme used ranged from 0.5 µg/ml to 22.5 µg/ml. Initial rates for the wild type enzyme were determined by performing this assay in triplicate with the following DEET concentrations: 2, 5, 10, 25, 50, and 60 µM. For the initial rates of the mutants, the assay was performed in triplicate with 50 µM DEET. The errors reported are the standard error of three measurements.

Enzyme assays

The standard activity assay was performed in a microfuge tube by incubating 45 µg of enzyme with 5 mM of each substrate for 5 to 15 minutes (depending on the level of activity with the specific substrate) at 30 °C in 1 ml of 30 mM potassium phosphate buffer pH 7.6. The buffer was pre-warmed to the temperature of the assay. Assays were stopped by adding 100 µl of 15% (w/v) trichloroacetic acid. All assays were performed at least in duplicate. Negative controls without enzyme were used in all the assays.

The effect of pH on DEET hydrolysis activity was determined by performing the assay described above at different pHs. The buffers used were: 50 mM citrate/sodium phosphate pH 5, 50 mM sodium phosphate pH 6 to 7.5, 50 mM Tris pH 8 to 9. After stopping the reactions, samples were filtered and analyzed by high-performance liquid chromatography (HPLC) (12).

The effect of temperature on activity was investigated by performing the standard activity assay with DEET at different temperatures between 22 °C and 65 °C and analyzed by HPLC as described for the pH experiments

Substrate specificity towards aromatic compounds (except for cocaine) was assayed by performing the standard activity assay at 30 °C and pH 7.6. The samples were extracted with an equal volume of ethyl acetate after stopping the reaction. The extracts were dried with sodium sulfate and analyzed by gas chromatography-mass spectrometry (GC-MS) as described elsewhere (12).

For the methyl-alkanoates, the assays were conducted in crimp-sealed vials. Each substrate was added at 10 mM and 5 mM n-nonane was used as an internal standard. After stopping the reaction by injecting trichloroacetic acid through the seal, 250 µl of the headspace was analyzed by GC-MS as described above.

The activity with cocaine was measured using the standard activity assay with the following modifications: the substrate concentration was 1.6 mM, the final volume of the assay was 100 µl, and 225 µg of enzyme were used. Because nonenzymatic hydrolysis of cocaine was observed when trichloroacetic acid was added, the assays were stopped by boiling for 5 minutes. The samples were analyzed by HPLC by quantifying the benzoate produced. The mobile phase used was 80% sodium phosphate buffer (25 mM, pH 7) and 20% methanol.

Modeling of DthA structure

A model of the tertiary structure of DthA was made using the HHpred and Modeller programs on the Bioinformatics Toolkit platform of the Max Planck Institute for Developmental Biology (http://toolkit.tuebingen.mpg.de) (15). The model was validated using PROCHECK (11). An alignment of the structures of the cocaine esterase from *Rhodococcus* sp. MB1 and the α-amino acid ester hydrolases from Acetobacter turbidans and Xanthomonas citri, previously identified as DthA

homologues (14), with the DthA model was generated using Swiss-pdb viewer (7) and displayed using ESPript (6).

Crystallization.

DthA was crystallized by the hanging-drop vapor diffusion technique by mixing 0.8 µl of protein solution (8-12 mg/ml) with 0.8 µl of reservoir solution containing 0.1 M sodium malonate and PEG 3350 4-10%, pH 5.0-5.5. The crystals were grown at 20 ºC.

RESULTS

Purification of DthA

DthA was heterologously expressed in *E. coli* BL21(DE3)pLys as a C-terminally histidine-tagged protein. An N-tagged version of DthA was also expressed but was inactive. C-tagged DthA was purified by nickel affinity chromatography. The migration of the purified protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (figure 3.1) is in agreement with the 70 kDa molecular mass predicted from the sequence. The purified enzyme was very stable, retaining most of its activity for at least 5 months when stored in 40% glycerol at -20°C.

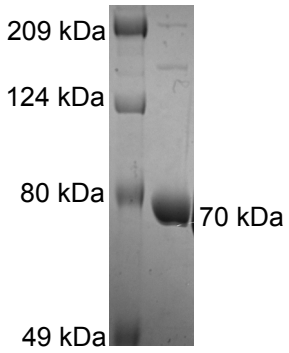


Figure 3.1. SDS-PAGE of purified DthA. Gel was stained with Coomassie Brilliant Blue after electrophoresis. The left lane contains molecular mass standards and right lane contains purified DthA. Molecular masses (deduced from the sequence for DthA) are indicated in kilodaltons.

Molecular mass determination

DthA eluted at approximately at 334 kDa on a size exclusion chromatography column (figure 3.2). This is between the expected molecular mass for a tetramer or pentamer of DthA, 280 kDa and 350 kDa respectively, suggesting that the enzyme might exist in solution in one of these conformations.

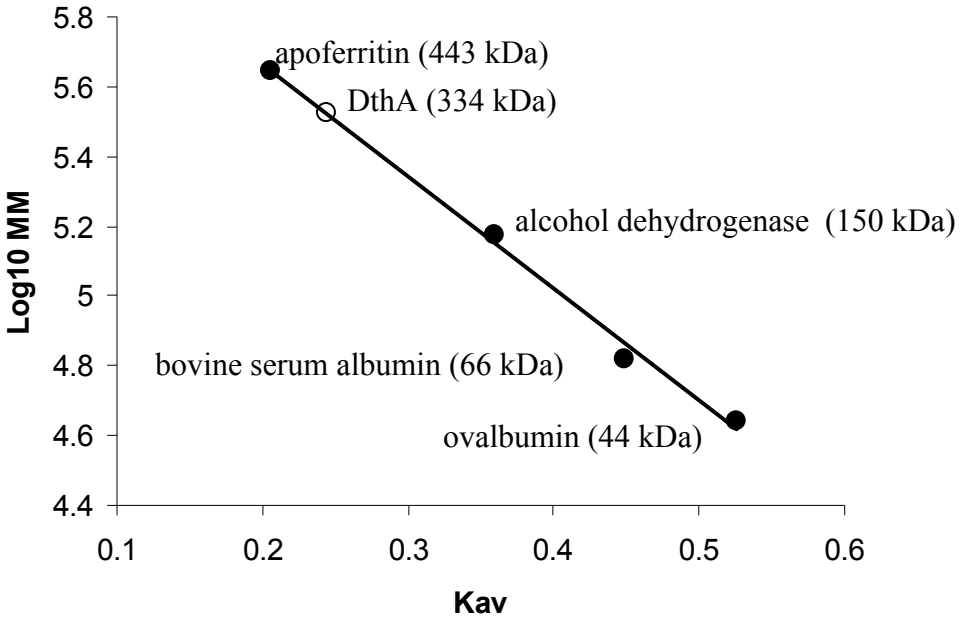


Figure 3.2. Molecular mass estimation of native DthA. The partition coefficients of molecular mass standards (●) and DthA (○) were plotted against the logarithm of the molecular weight.

Determination of kinetic parameters with DEET

The kinetic parameters for wild type DthA were determined under initial rate conditions as described in the methods using 0.5 µg of protein. The K_m is 6.52 (± 1.27) µM and the V_{max} is 1.83 (± 0.10) x 10^{-2} µmoles/s and k_{cat} is 3.04 (± 0.16) x 10^3 s^{-1} (figue 3.3).

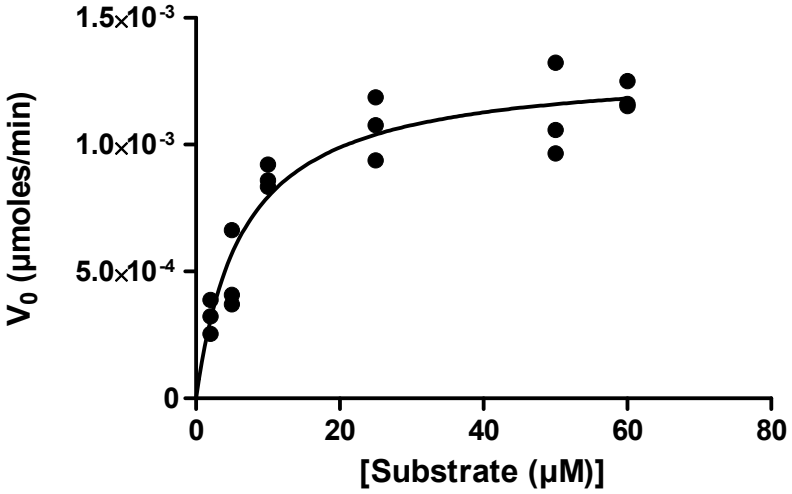
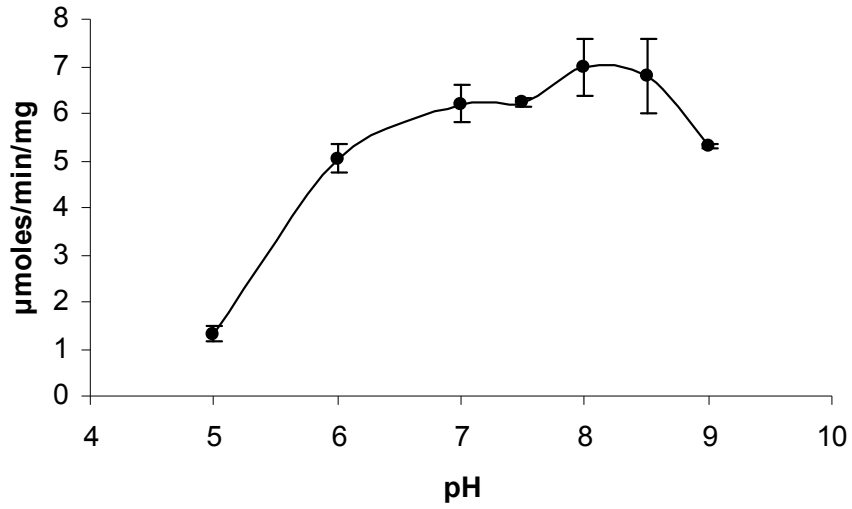


Figure 3.3. Kinetic analysis of DEET hydrolysis by DthA. Kinetic assays were performed by measuring the rate of decrease in absorbance at 215 nm as a result of DEET hydrolysis. K_m, V_{max}, and k_{cat} were calculated by least squares fitting the data to the Michaelis-Menten equation using GraphPad Prism (GraphPad software).

Determination of optimal pH and temperature

DthA has a broad pH range of activity, with its optimum around pH 8 (figure 3.4 A). It was active over a surprisingly high temperature range, between 40 and 65 °C, with its optimum temperature around 53 °C (figure 3.4 B).

(A)



(B)

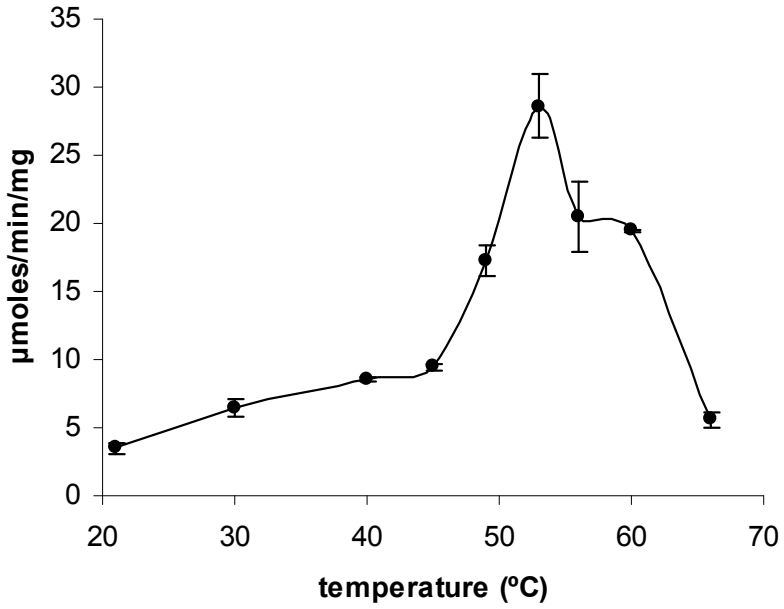


Figure 3.4. Effect of pH (A) and temperature (B) on DthA activity.

Substrate specificity

When the substrate specificity of DthA towards structurally related analogs was examined, we found it could hydrolyze a number of aromatic esters in addition to amides (fig. 3.5). In fact, it was almost four times more active on methylbenzoate than it was on DEET. It was also active against nonaromatic esters. The level of activity on methylbutyrate and methylhexanoate was similar to that on DEET and slightly lower for the branched chain methylisobutyrate. No transformation products could be detected when benzamide, hippuric acid, or *m*-methylhippuric acid were supplied as substrates (data not shown).

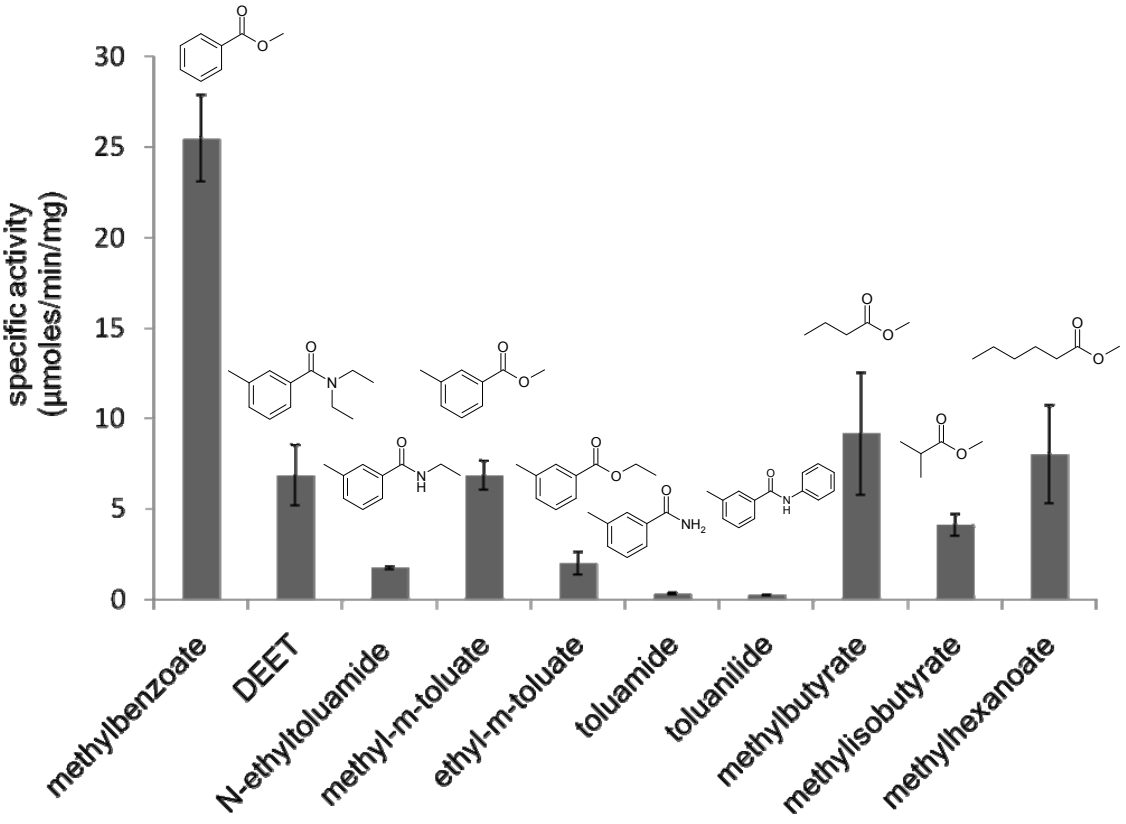


Figure 3.5. Substrate specificity of DthA. Hydrolysis of aromatic esters and amides was assayed using GC-MS by measuring the appearance of the hydrolysis product benzoic acid or 3-methylbenzoic acid. For the methyl-alkanoates, hydrolysis was assayed by measuring the disappearance of the substrate using GC-MS as well.

In previous work (14), we found homology between DthA and the cocaine esterase from *Rhodococcus* sp. MB1 (24% sequence identity using BLAST (Altschul, 1997 #67)). Therefore, we wanted to know if DthA was also active on cocaine. Only trace activity, 0.011 ± 0.002 μmoles/min/mg, was observed with this substrate.

Model of DthA

In order to better identify the residues important for activity and substrate binding, we created a model of DthA based on homology to the *Rhodococcus* sp. MB1 cocaine esterase (RhCOCE) and the α-amino acid ester hydrolases from *Acetobacter turbidans* (AtAEH) and *Xanthomonas citri* (XcAEH) (Figure 3.6). The overall average Procheck G-factor of the resulting model was -0.32 and more than 95 % of the residues were found in the most favored or additional allowed regions. The DEET molecule was docked manually into this model using Swiss-pdb viewer (7). Also, a structural alignment of the sequences of DthA and its homologues (Figure 3.7) was built with Swiss-pdb viewer and displayed with ESPript (6).

The overall predicted structure of DthA (Figure 3.6 A) suggests that DthA consists of three domains: an N-terminal α/β-hydrolase domain, a large helical cap insertion, and a C-terminal ß-strand domain. The same domain arrangement is observed in the homologous structures. A closer look into the cleft formed by the interfaces of the three domains reveals the active site (Figure 3.6 B). The active site contains a catalytic triad formed by S166, D292, and H320 whose positions in the model are in agreement with the canonical arrangement of these residues in α/β-hydrolases (13)

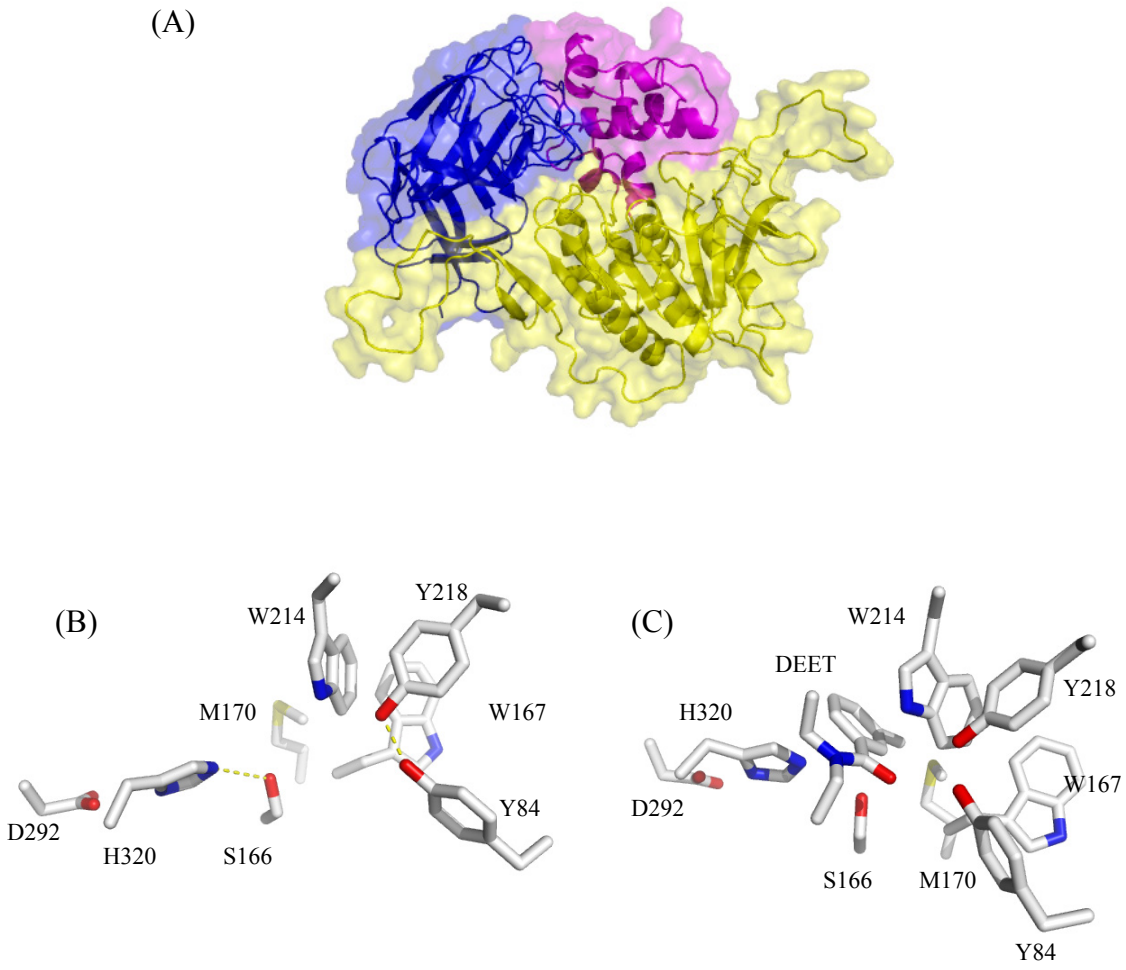


Figure 3.6. Model of DthA tertiary structure. (A) Ribbon diagram of DthA monomer. Domains are displayed in the following colors: yellow, N-terminal α/β-hydrolase domain; magenta, helical cap domain; blue, C-terminal ß-strand domain. (B) Active site residues. (C) DEET molecule docked into active site. Images were prepared with PyMOL (5).

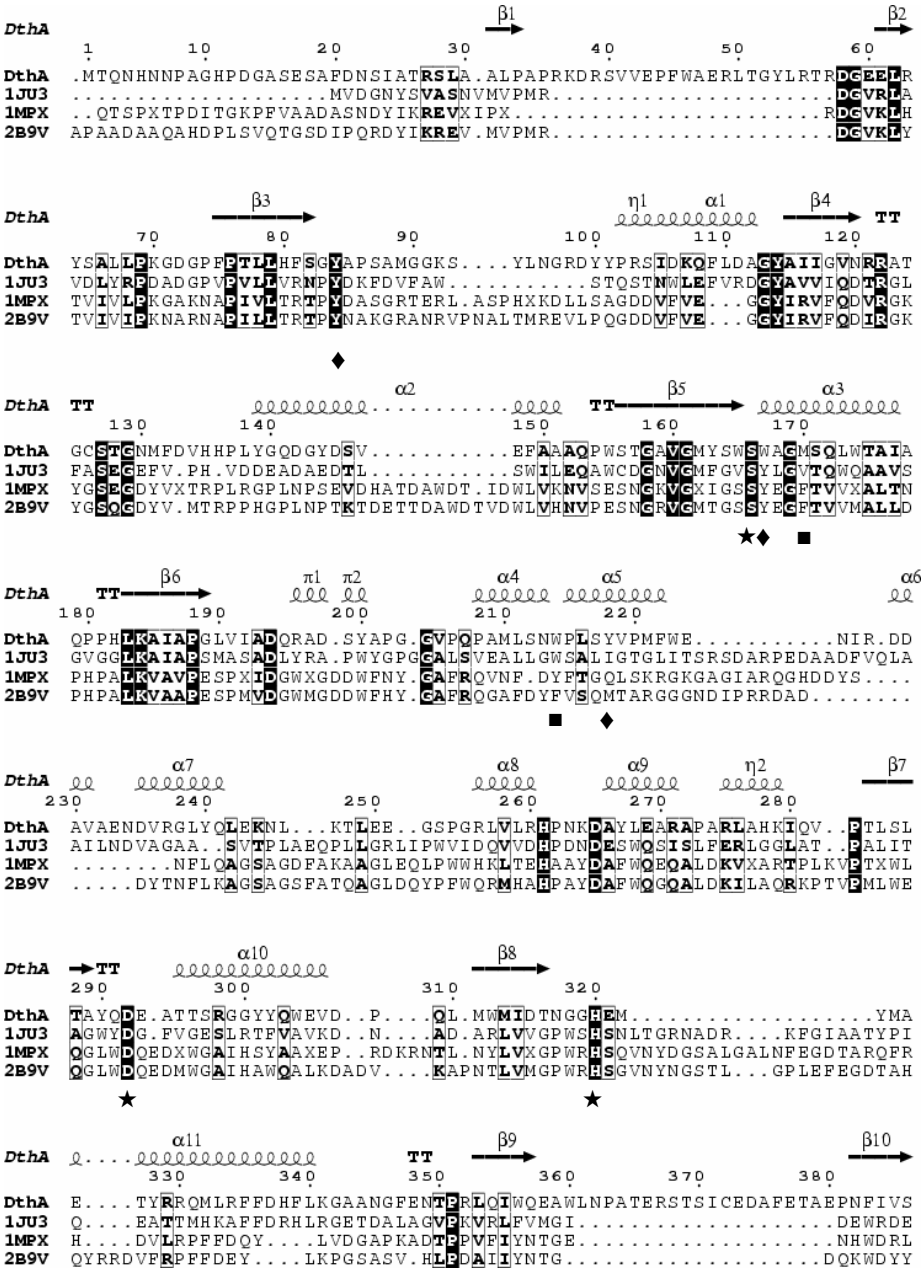
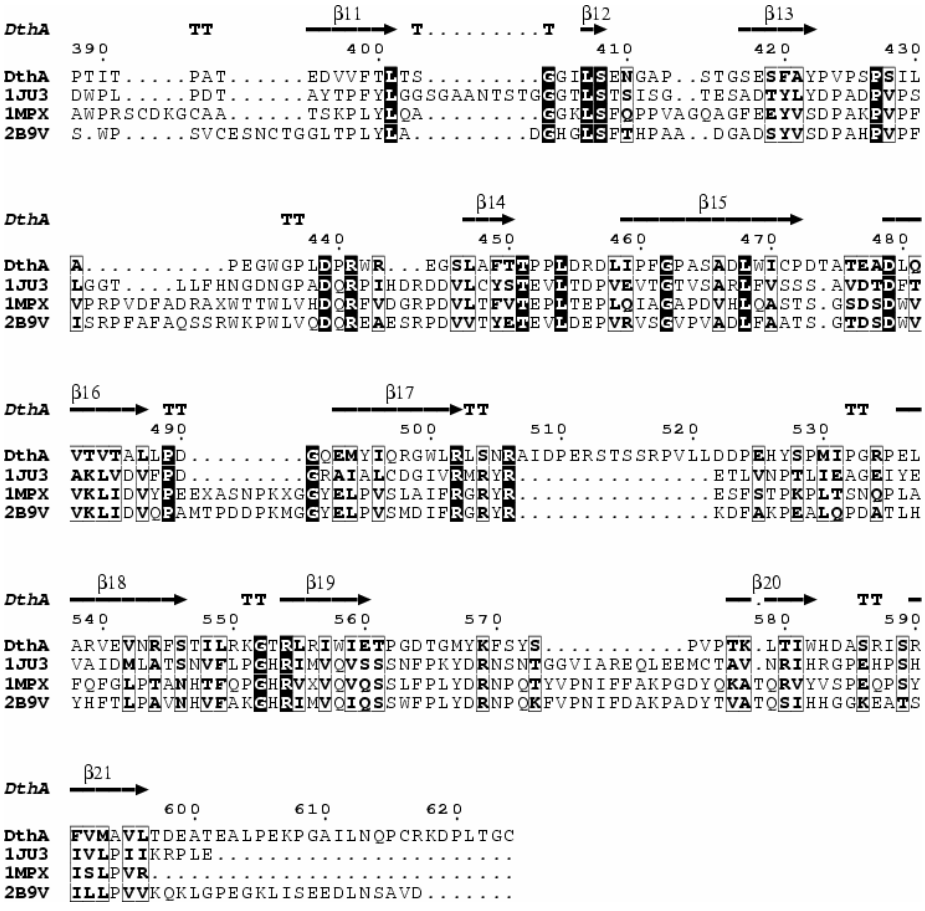


Figure 3.7. Structural alignment of DthA and homologues. The sequences of the homologous proteins are indicated by the PDB accession codes of the structures used in the alignment: 1JU3, *Rhodococcus* sp. MB1 cocaine esterase; 1MPX, *Xanthomonas citri* α-amino acid ester hydrolase; 2B9V, *Acetobacter turbidans* α-amino acid ester hydrolase. The secondary structure derived from the DthA model is shown on top of the alignment with squiggles for helices, arrows for beta strands, and "TT" for turns. The numbers on top of the alignment correspond to the residues in DthA. Strictly conserved residues are highlighted in black and partially conserved residues are boxed. Catalytic triad residues are identified with stars (★), oxyanion-hole residues with diamonds (♦), and other residues important for activity with squares (■).

Figure 3.7. (Continued)



Mutational analysis

Previously we reported that residues S166, D292, and H320 are likely constitute the catalytic triad based on sequence alignment data (14). In addition, we observed that serine protease inhibitors were able to inhibit DthA's activity, further supporting the role of a serine residue as the nucleophile. In order to confirm that DthA is indeed a serine hydrolase and that these three residues formed the catalytic triad, they were mutated to alanine and the activity of the mutated proteins was assayed (table 3.2). As expected, no hydrolysis activity was observed for S166A, D292A, and H320A with our assay. We also wanted to confirm the importance of two putative oxyanion-hole residues identified through sequence alignments, Y84 and W167, and one identified in the model of the structure, Y218. The oxyanion hole helps to stabilize the transtition state that occurs during hydrolysis by donating hydrogen bonds to the carbonyl hydrogen, which acquires a negative charge during this step (13). Y84 and Y218 were mutated to phenylalanine residues in order to assess the importance of the hydroxyl groups in forming hydrogen bonds with the substrate. This resulted in a more than 2-fold and 3-fold decrease in activity for Y84F and Y214F, respectively. The mutation of W167 to alanine brought a 10-fold decrease in the initial activity rate. W214 and M170, two residues that are near the active site in the structural model, were also mutated. In the model, the side chain of W214 appears to stack with the aromatic moiety of DEET. For this reason we decided to mutate W214 to residues with side chains with a variety of sizes and charges. Mutations to alanine, valine or glutamate were all inactive. When M170, which is located on one side of the DEET molecule, was mutated to a polar (glutamine) or a small and non-polar (alanine) residue, the enzyme was inactive too.

Table 3.2. Effect of mutations on DthA activity.

DthA mutant	Initial reaction rate (μmoles/min/mg)[a]
WT	2.23 (± 0.37)
Y84F	0.831 (± 0.196)
Y218F	0.659 (± 0.069)
W167A	0.214 (± 0.057)
S166A	nd[b]
D292A	nd[b]
H320A	nd[b]
W214A/E/V	nd[b]
M170A/Q	nd[b]

[a] (± standard deviation)
[b] hydrolysis not detected (limit of detection = 0.002 μmoles/min/mg)

DthA crystallization.

DthA crystallized as rods (figure 3.8 A) under the conditions described in the methods section. The crystals appeared in 2 to 5 days. Figure 3.8 B shows the diffraction pattern of a crystal that diffracted to 2.66 Å. This crystal belonged to the space group P222 and had unit cell dimensions of a=140 Å, b=200 Å, c=100 Å; α=β=γ=90° (figure 3.8 B).

We attempted to solve the phase data obtained from this crystal by molecular replacement by using the crystal structures of the DthA homologues (1JU3, 1MPX, and 2B9V), but this was unsuccessful. Using the homology model built in this work didn't produce a solution either. A Matthews coefficient of 2.5 $Å^3$/Da and a solvent content of 50.8% was calculated for this crystal. From the Matthews coefficient, however, it is expected that there are 4 molecules of the DthA monomer per asymmetric unit.

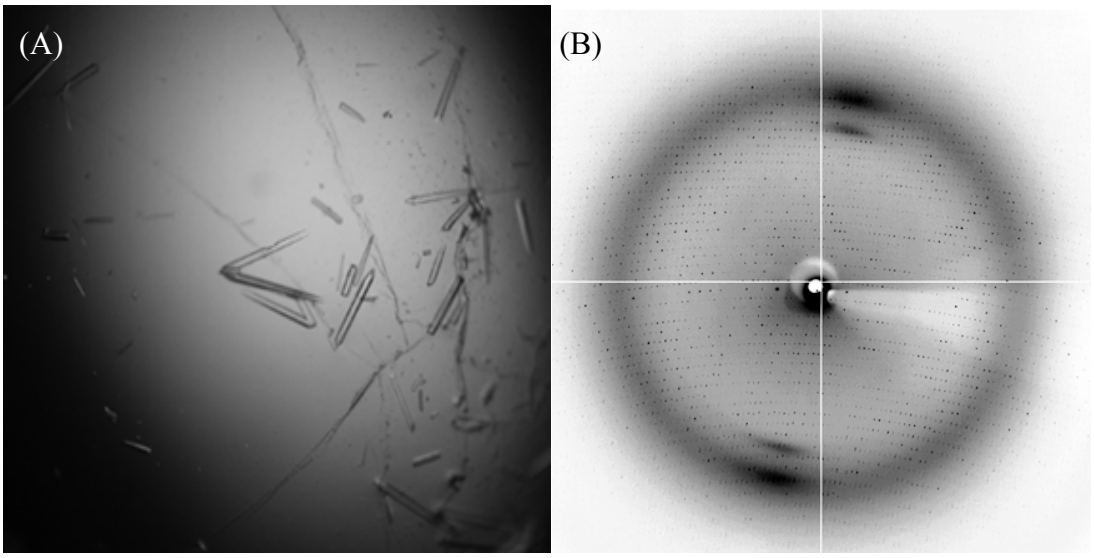


Figure 3.8. (A) Photomicrograph of DthA crystals. (B) Diffraction pattern from a DthA crystal.

DISCUSSION

DthA is an enzyme from *Pseudomonas putida* DTB that was identified due to its ability to hydrolyze DEET. To our knowledge, this is the only enzyme with this activity that has been described. Sequence analysis has shown that DthA has sequence similarity with α/β-hydrolases (*Rhodococcus* sp. MB1 cocaine esterase and *Acetobacter turbidans* and *Xanthomonas citri* α-amino acid ester hydrolases) (14). As seen in the model in figure 3.6 A, the α/β-hydrolase domain is on the N-terminus and follows the classical structure of proteins from the α/β-hydrolase family (13). It is composed of a mostly parallel beta sheet formed by ten strands and flanked on each side by α-helices. This domain contributes the three catalytic residues: Ser166, Asp292, and His320. Trp167 and Tyr84 are also in the α/β-hydrolase domain and they form the oxyanion hole, the former through the backbone amide and the latter through the sidechain hydroxyl group. The N-terminal domain is interrupted by a helical cap domain composed of 5 helices (residues 195-272). This insertion is very common in

this family of enzymes (13). In DthA, the cap domain contributes Tyr218, whose sidechain hydroxyl also seems to form part of the oxyanion-hole. The C-terminal domain is composed exclusively of ß-strands and adopts a jellyroll topology, as seen in RhCOCE, AtAEH, and XcAEH.

The results of mutating the residues mentioned above confirm their importance in DthA activity. All the catalytic triad mutants were devoid of activity. The mutants of the residues expected to be interacting with the aromatic ring of DEET, W214 and M170, were also inactive. Docking the DEET molecule into the model suggests that W214 is important in substrate binding, as it is likely to form pi-stacking interactions with one face of the ring in aromatic substrates. Such pi-stacking interactions with the substrate are also observed in RhCOCE (17). On the other hand, the oxyanion-hole mutants were active, albeit less than half of the wild type activity in all cases. The dramatic decrease in activity in the W167A mutant was unexpected, since this residue is thought to be involved in hydrogen bonding through the main chain amide in other homologous enzymes. In fact, in BlGCA and RhCOCE, mutating the equivalent residue, tyrosine in both cases, to alanine only reduced the activity slightly (17, 19). It is possible that W167 is playing other roles in DthA, in addition to forming the oxyanion hole. This could include helping to form the nucleophile elbow (13), a tight turn in which the catalytic serine sits so that it is accessible to the substrate. Interactions between W167 and other residues could be important in forming this nucleophile elbow. It is also possible that other interactions may account for this big decrease in activity. For example, in AtAEH, mutating the equivalent tyrosine to alanine had the indirect effect of changing the orientation of another oxyanion-hole residue, resulting in an enzyme with altered kinetic properties (3).

From size exclusion chromatography alone, it is not clear whether DthA exist as a tetramer or a pentamer in solution. However, analysis of structurally related

hydrolases revealed that AtAEH(3) and XcAEH(2) exist as tetramers. In these proteins, the four monomers are arranged in a spherical shape with all the active sites facing the inside of the tetramer, forming a water-filled central cavity (2, 3). When we consider these findings in light of the Matthews coefficient calculated from the X-ray diffraction data, we are inclined to think that the oligomeric state of DthA in solution is indeed tetrameric. It is not clear why DthA appeared to migrate at an apparent weight of 334 kDa and not closer to the 280 kDa expected. This may be because of the additional bulk resulting from the formation of the predicted central cavity.

These results contrast with those reported for RhCOCE (4) and another homologue of DthA, the glutaryl 7-amino cephalosporanic acid acylase from *Bacillus laterosporus* J1 (BlGCA) (1), which appear to be monomeric. In addition, in XcAEH and AtAEH there is an N-terminal arm before the first ß-sheet that makes contacts with other monomers and plays a role in tetramer formation. Interestingly, the first 19 residues of DthA before ß-strand 1, are missing in the monomeric RhCOCE (figure 3.7).

The optimal pH and temperature for DthA were higher than the homologous XcAEH (8), which are 6.4 and 35°C, respectively. Its optimum pH is also higher than that reported for the AtAEH (16), which is pH 6. However, these biochemical characteristics were more similar to those of BlGCA, which has an optimum pH of 8 and optimum temperature of 40°C (1). Kinetic studies with RhCOCE also reveal a maximum rate of hydrolysis at pH 9 (17).

DthA has a broad substrate specificity, being able to hydrolyze a number of other amides and esters in addition to DEET, including aliphatic compounds (figure 3.5). The enzyme showed preference for tertiary amides such as DEET over secondary (e.g. *N*-ethyltoluamide, toluanilide) and primary (e.g. toluamide) amides. A plausible explanation for this could be that the secondary and primary amides could be forming

a hydrogen bond with the τ-N in H320, preventing it from forming a productive interaction with S166 that favors deprotonation, a step that facilitates the nucleophilic attack to the substrate by S166. In the case of the aromatic ester substrates, DthA favored shorter O-alkyl substituents and non-substituted aromatic rings. As for aliphatic substrates, there was a preference for straight-chain compounds. Despite its homology to RhCOCE, only trace activity was observed with cocaine. Perhaps this is due to steric clashes with the bulky ecgonine attached to the ester group in the cocaine molecule. This is supported by the low activity observed with toluanilide, which also has a bulky group attached to the amide nitrogen. Of the compounds tested, DthA was most active towards methylbenzoate, a common component in flower scents (9) that is also used in the fragrance industry. The high activity with methylbenzoate, 25.5 (± 2.4) μmoles/min/mg, suggests that perhaps DthA might have evolved for the metabolism of this or other related compounds.

In conclusion, we have shown that DthA is an α/β hydrolase with a catalytic triad formed by Ser166, Asp292, and His320. It has broad substrate specificity towards hydrophobic substrates and a high optimum temperature. The results of site directed mutagenesis reflects a similar pattern to that seen for other α/β hydrolases concerning residues that are important for catalysis, although we were able to identify some previously unidentified regions that were important in substrate binding. Final conclusions about the role of particular residues based on results from the mutational analysis or the homology model, however, must be made with care until a structure of this enzyme is available. We are, therefore, working on determining the crystal structure of DthA using multi-wavelength anomalous dispersion and selenomethionine crystals.

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CHAPTER 4

ISOLATION OF AN *ARTHROBACTER PROTOPHORMIAE* STRAIN CAPABLE OF GROWTH ON DIETHYLAMINE AS SOLE CARBON AND NITROGEN SOURCE FROM N,N-DIETHYL-*M*-TOLUAMIDE (DEET) DEGRADING CONSORTIUM

ABSTRACT

The present work describes the isolation of *Arthrobacter protophormiae* DE1, which was able to use diethylamine as a sole carbon and nitrogen source. When grown in coculture with *Pseudomonas putida* DTB, both organisms grew on DEET as sole source of carbon and nitrogen. Cell free extracts of diethylamine grown DE1 rapidly liberated acetaldehyde from diethylamine, yielding ethylamine, which was also degraded, although at a much lower rate. A chromosomal library of DE1 was constructed in *Escherichia coli* and screened for its ability to degrade diethylamine. Twenty fosmids were identified that encoded the ability to degrade diethylamine and one was then subject to *in vitro* Tn5 mutagenesis. The site of Tn5 insertion was identified in 9 mutants. Six of these contained insertions either inside or upstream of a putative amine oxidase that appears to be responsible for diethylamine utilization in DE1.

INTRODUCTION

Previously, we characterized the degradation of DEET by *P. putida* DTB (16). That bacterium grows at the expense of the 3-methylbenzoic acid moiety of DEET, but leaves the diethylamine moiety of DEET untouched, requiring the presence of an alternate source of nitrogen in the media when grown in pure culture (16). The consortium from which DTB was isolated, however, could grow on DEET in nitrogen-

free media. This indicated that other organisms in that consortium were degrading either DEET itself or at least the liberated diethylamine as a sole source of nitrogen. Since no isolates were obtained from agarose plates containing DEET as a sole source of both carbon and nitrogen, we reasoned that another organism was likely responsible for liberating nitrogen for growth from diethylamine. We therefore set about to isolate diethylamine degrading bacteria from the consortium. In as much as secondary amines like diethylamine can form toxic nitrosamines in the environment (9), it was of interest to us to know the mechanism of diethylamine degradation by microorganism associated with the consortium from which strain DTB was isolated. Here, we report on the characterization of diethylamine degradation by one of these isolates, a member of the genus *Arthrobacter*.

METHODS

Enrichment and isolation

A DEET enrichment was set up as previously described (16). After several passages on minimal salts medium (MSM) (8) with 2.6 mM DEET, an aliquot was inoculated into nitrogen-free medium (NFM; 10 mM K_2HPO_4, 3 mM NaH_2PO_4, 10 μM $FeCl_3$, 100 μM $CaCl_2$, and 1 mM $MgSO_4$ added after autoclaving) with 4.8 mM diethylamine. Once the liquid culture got visibly turbid, aliquots were plated on NFM plates with 4.8 mM diethylamine and incubated at room temperature for a week. Morphologically distinct colonies were re-streaked on fresh plates and incubated as before, and subsequently inoculated into liquid NFM diethylamine.

Despite being isolated from an NFM diethylamine-grown consortium, growth of several of the colonies isolated from NFM diethylamine agar plates was variable in NFM diethylamine liquid medium, therefore the diethylamine degradation rate of

selected colonies were initially determined in MSM with 5% LB and 4.8 mM diethylamine using the colorimetric assay for the detection of secondary amines (16).

Isolate identification

Colony PCR was performed on one isolate that grew very robustly on NFM diethylamine plates, designated as strain DE1, using the 16S rRNA gene primers 27F and 1492R (11). The PCR product was sequenced using the same primers.

Cultivation conditions

DE1 and DTB were grown on MSM or NFM with diethylamine (4.8 mM), ethylamine (5.4 mM), or DEET (2.6 mM) at room temperature with shaking. For cocultivation studies, DTB was grown on NFM amended with $(NH_4)_2SO_4$ (10 mM) to reduce the possibility that differences in the medium composition, other than the presence or absence of ammonia, were responsible for growth. Growth was monitored turbidometrically by determining the optical density at 600 nm.

Washed cell assays with diethylamine exposed and non-exposed cells

DE1 was grown on MSM with 10% LB media with or without diethylamine (4.8 mM). Washed cell suspensions were prepared in 30 mM potassium phosphate buffer pH 7.6 according to standard protocols (8). The cell suspensions were amended with 9.6 mM diethylamine and 2 sets of 1 ml samples were taken at 0.5, 1, 2, 4, and 8 hours. The samples were spun down and the supernatants were frozen at -20°C until derivatized. One set of samples was derivatized using the method developed by Sacher *et al*. (17) for the detection of primary and secondary amines. The other set was derivatized using the method for acetaldehyde detection by de Andrade *et al*. (5).

Assays with cell-free extracts

Cells from an overnight culture of DE1 grown on LB were washed once with 25 mM sodium phosphate buffer pH 7.0 and resuspended in 1 ml of the same buffer. The suspension was sonicated and centrifuged (14) The cleared lysate was used for the

assays. The assays were set up in triplicate by mixing 50 µl of cell-free extract with 450 µl of potassium phosphate buffer pH 7.6 and either diethylamine (9.6 mM), ethylamine (10.7 mM) or acetaldehyde (17.9 mM). The assay with acetaldehyde also contained NAD (0.4 mM). NAD was not added to the assays with diethylamine or ethylamine in an attempt to minimize acetaldehyde dehydrogenase activity, as NAD is a cofactor for this enzyme, so that the production of acetaldehyde was detectable. The assays were incubated at room temperature along with controls without enzyme. The assay was stopped by adding 3 volumes of acetaldehyde derivatization reagent (5), which contains 46% acetonitrile. Protein content of the cell-free extracts was measured using the Bradford assay (3).

Identification of gene responsible for diethylamine utilization

A fosmid library of strain's DE1 genomic DNA was constructed in *E. coli* EPI 300 using the CopyControl Fosmid Library Production Kit from Epicentre according to the manufacturer's protocol. The library was incubated in MSM with 5% LB, diethylamine, and 12.5 µg/ml in order to identify those fosmids that encoded the ability to remove diethylamine from culture. Diethylamine in culture supernatants was screened for as previously described (16).

The clones that were positive for diethylamine removal were confirmed by repeating the assay with those clones only and two negative controls. The fosmid from one of the clones was subjected to *in vitro* Tn5 transposon mutagenesis as described previously (16). The mutants were grown on LB with chloramphenicol (12.5 µg/ml) and kanamycin (50 µg/ml). The cultures were centrifuged and the spent medium was discarded. The pellets were resuspended in MSM with 5% LB, 9.6 mM diethylamine, kanamycin (50 µg/ml), and induction solution (Epicentre). After incubation at room temperature for 3 days, the clones were spun down and the supernatants were screened

for the presence of diethylamine (16). The mutants that lost the ability to use diethylamine were sequenced. The sequence was analyzed using BLAST (2).

RESULTS

Selection and Identification of Arthrobacter protophormiae

A number of isolates that formed robust colonies on NFM diethylamine plates were selected for analysis. Strain DE1 degraded more diethylamine than other isolates that also formed robust colonies on NFM diethylamine plates (data not shown). This was determined by comparing the amounts of diethylamine remaining on the supernatants of three day old cultures grown on MSM with 5% LB and 4.8 mM diethylamine using the colorimetric assay for the detection of secondary amines (16). The 16S rRNA sequence of strain DE1 was analyzed using BLAST (2) and found to be 99% identical to that of *Arthrobacter protophormiae*.

Growth on diethylamine and ethylamine

DE1 grew well on NFM plates with diethylamine, first appearing as off-white colonies that turned yellow as time passed. However, growth on liquid was somewhat unpredictable and required vigorous aeration. Figure 4.1 shows representative growth curve of DE1 growing on NFM with diethylamine. Growth on ethylamine, on the other hand, was poor and gave rise to small flocs. The optical density of the ethylamine cultures did not pass 0.12 in any of the attempts to grow DE1 with ethylamine on NFM or MSM.

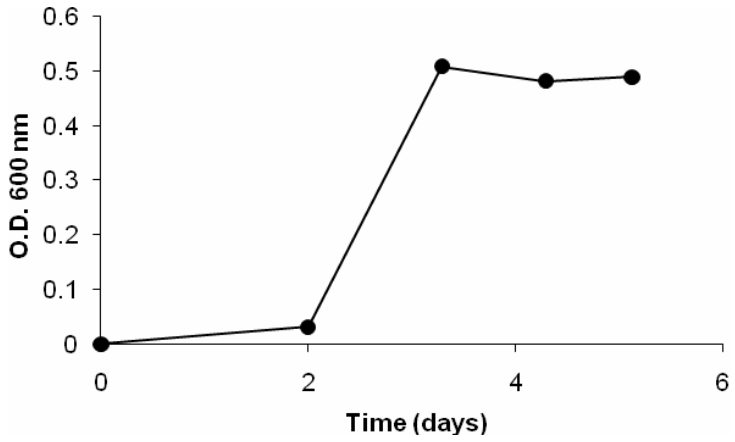

Figure 4.1. Growth of DE1 on diethylamine in media without nitrogen (NFM).

Coculture of Arthrobacter protophormiae DE1 and Pseudomonas putida DTB using DEET as a sole source of carbon and nitrogen

Although DE1 grew well on diethylamine it was unable to grow on NFM DEET as either a carbon or nitrogen source (figure 4.2). Strain DTB on the other hand, which we previously described (10) was able to grow on DEET when ammonia was present in the growth media, but could not use it as a nitrogen source. When both strains were inoculated together, however, they were able to grow on DEET as a sole source of carbon and nitrogen (figure 4.2). Interestingly, this coculture experienced a significant lag before growth began that was not observed when DTB grew alone on NFM DEET supplemented with ammonia (figure 4.2). The supernatant in the coculture also turned yellow at the late-exponential phase (data not shown), suggesting the accumulation of an aromatic ring fission product, which is consistent with growth at the expense of the aromatic ring of DEET.

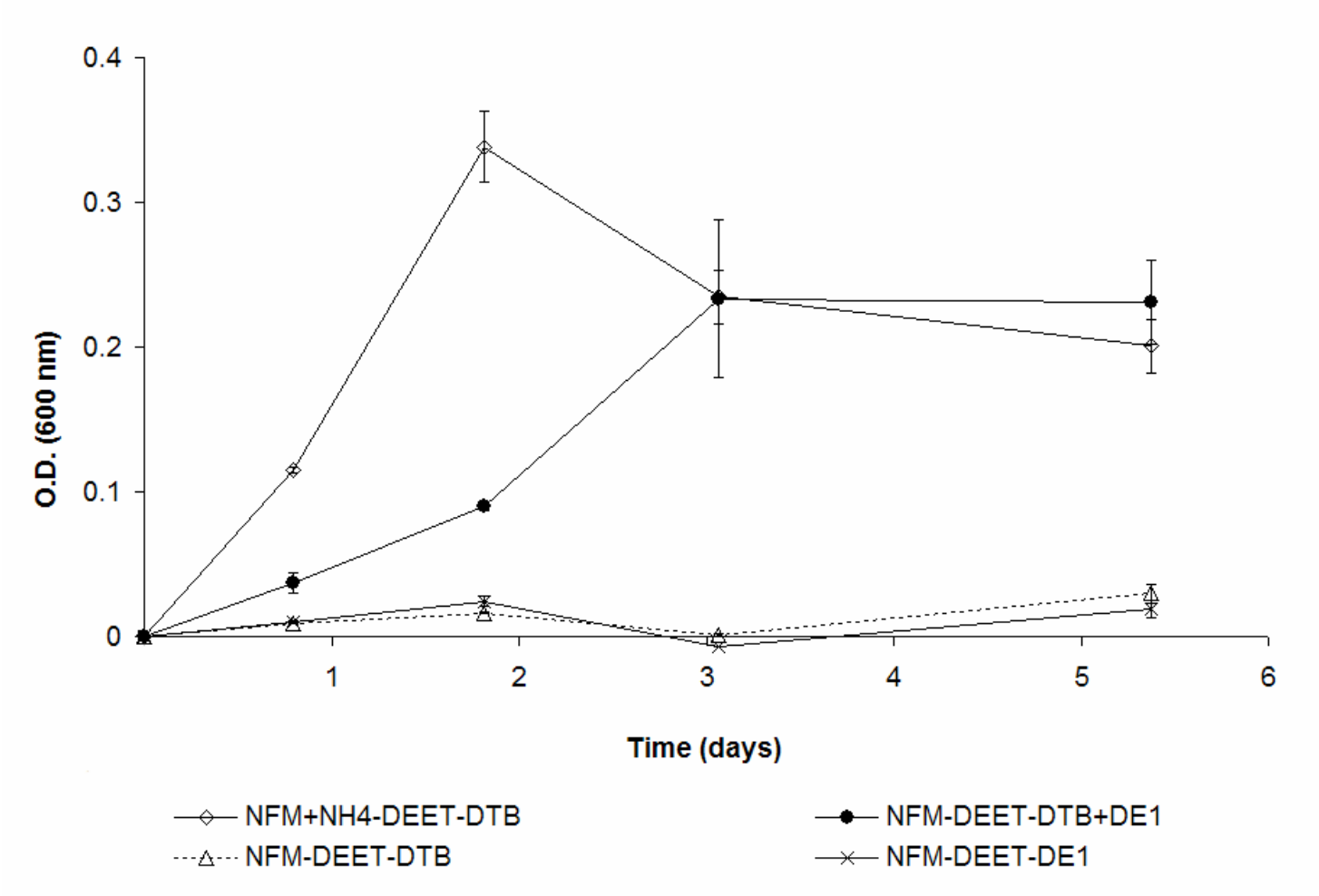

Figure 4.2. Growth of *Pseudomonas putida* DTB on DEET as sole source of carbon (open diamonds), as a source of carbon and nitrogen in coculture with *Arthrobacter protophormiae* DE1 (circles). Cultures of DTB (open triangles and dotted line) and DE1 (crosses) on nitrogen free media with DEET were also monitored for growth.

Diethylamine metabolism in washed cell suspensions

Washed cell suspensions of DE1 effected the rapid disappearance of diethylamine accompanied by a conconmitant increase in ethylamine which was then slowly degraded (figure 4.3). The rate of diethylamine utilization was faster in the resting cell suspensions that were grown in the presence of diethylamine (1.2 μmoles/hr/O.D. unit) than those that were not (0.15 μmoles/hr/O.D. unit). Also, no net disappearance of ethylamine was observed for the non-exposed cells, while in the exposed cells the average rate of ethylamine disappearance after the first hour of incubation was 0.025 μmoles/hr/O.D. unit. No acetaldehyde was detected during the assay for either the exposed or the non-exposed cells.

Diethylamine metabolism in cell free extracts

Given that whole cells rapidly metabolized acetaldehyde and the known dependence of this reaction on NAD in Arthrobacter P1 (12), we sought to detect acetaldehyde in cell free extracts where NAD could not be regenerated. The average rate of acetaldehyde formation from diethylamine in cell free extracts was added as a substrate was 0.44 μmoles/min/mg. No acetaldehyde formation above the control's level was detected from ethylamine.

Acetaldehyde utilization was also measured in the cell-free extracts. NAD was added to this reaction as it is required as a cofactor by acetaldehyde dehydrogenase (12). The rate of acetaldehyde consumption was 0.10 μmoles/min/mg.

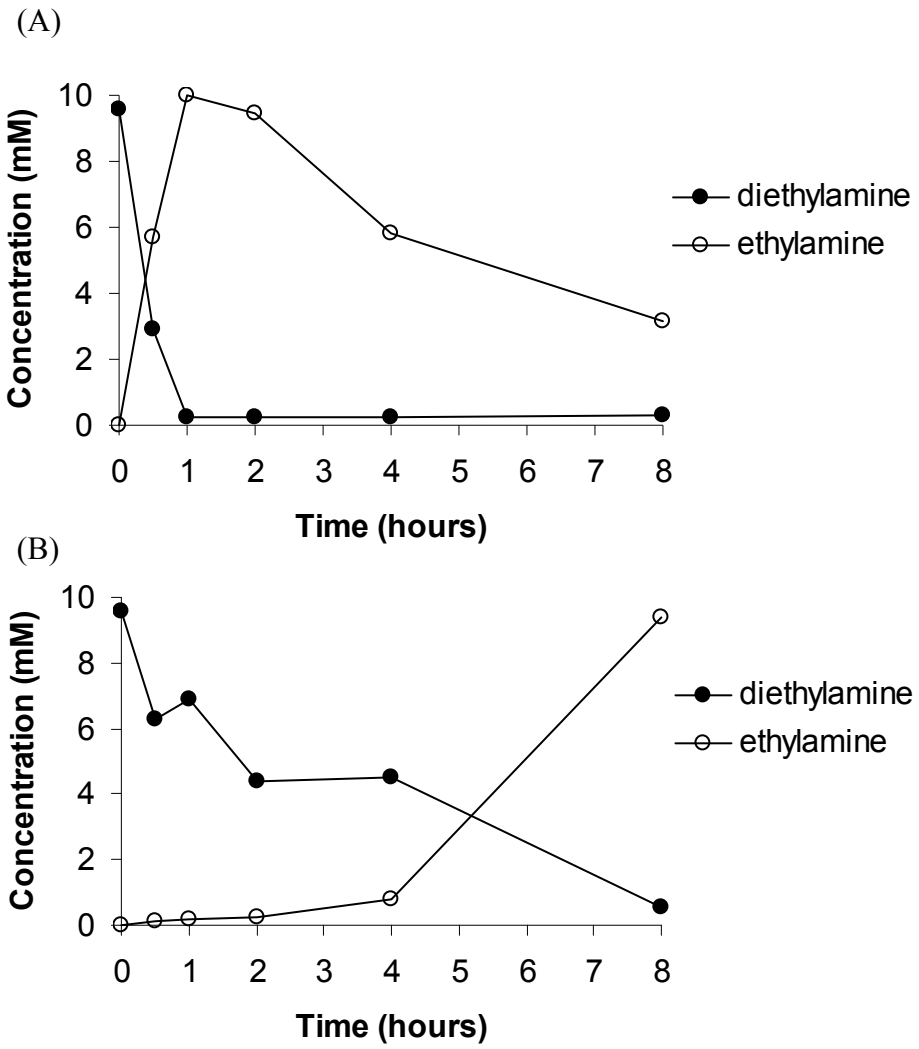

Figure 4.3. Metabolism of diethylamine by washed cell suspensions from diethylamine (A) exposed (B) non-exposed cultures.

Identification of a putative amine oxidase and an <u>eutQ</u> homologue

Screening the DE1 fosmid library yielded 20 clones that were able to consume the diethylamine in the media out of a total of 864. Transposon mutagenesis with one positive clone, 2f2, yielded 15 mutants that lost this activity out of 284, of which 9 were sequenced. The sequence around the insertion sites in 6 of these mutants contained two open reading frames (orfs) (figure 4.4). Two of the transposon

insertions were in orf 1, two were in orf 2, and two were in between these orfs. The other 3 mutants had insertions inside the vector.

Orf 1 was 417 nucleotides long and was predicted to start with the unconventional start codon TTG. It corresponds to a putative protein of unknown function, DUF861 (99% similarity), from *Arthrobacter aurescens* TC1. The last 74 amino acids contain a cupin domain. This domain is found in proteins with very diverse functionalities, including enzymes, regulatory proteins, and non-enzymatic storage proteins from plant seeds (7). Two rounds of PSI-BLAST with the amino acids comprising the cupin domain showed that this protein has similarity to EutQ, a protein of unknown function from the ethanolamine utilization operon in *Salmonella typhimurium* (10).

Orf 2 had similarity to a putative flavin-containing amine oxidase from *Thauera* sp. MZ1T (36% identity, 57% similarity), as well as to other putative amine oxidases. It also shows similarity to two well studied amine oxidases, MaoN from *Aspergillus niger* (30% identity, 40% similarity) (18, 19) and human monoamine oxidase B (21% identity, 43% similarity) (1). Given its apparent involvement in diethylamine degradation we have given orf 2 the name *deaA*

TCTCGGATTAAGGTGCATAGTGATTGGAGTCACCAAGTTCGTCGGAGGGCAACAGTG
GCATCCGGCAAGACAAGAGATATCGATTTGACAACCTGGAGGAAAGCGTGAAGAAGT
CAAGTATCGGAGAGACGGAGTACGAGCCGTTCATCGTCGGAGATCGGCAACTCGGCG
AGGTGCATTGGTTGCGTCAGGACGCCGCCGAAAGCGGAGTAGTGCTGGCCGGACTGT
GGAAGGCAGAAGATGCCACAACGCCTAGGGAATTCGATTACCCATTCGGCTACGAAG
AGACAATCCAGGTGCTTGAGGGCTCGGTGACAATC*GACTTCCCCAAC*TCACCGAG
CGTGACGCTGCGCGCCGGCGATCTCGCTTCGTTCACAAAGGGAACCACCTCTGTCTG
GCACGTCCAGATGCCATTCAAGAAGTTCTTCGTCTGCTCGTGATTCGGTCTGGCGCC
GGCCACAATTCGATGTTCCTCTAACGAGAGAACGAATC*GCTCGACATAAGCAGCGG
AATCCGGAATGGTTTCCGGCGCCGTGTTATTGGGCGGCGACTCACCTCTGCTCCAGC
CGAAGCTGAAGGTCACGGAAAAAGTGTGCAACGA*CCCCCGACCTTTGGGCGTACCA
GTGGCAATAAATCACCCTAAGTCGAGCACGACAACCTGATAGTTACCCACTCTTAAA
GATATTCCTGATAAGGATTGATATGACTCCCAATTCGTATGACGCGATCGTCATTGG
TGGCGGGTTCGCCGGAGTGACGGCTGCCCGAGAACTAACGAACGCTGGTCGTTCCGT
TCTTCTCCTCGAAGCGCGCGATCGGCTCGGAGGCCGTACTCATTACGAGCATGCTGC
AAGTCTTGGACGTGAAGTAGAAATGGGCGGCACTTGGGTCCACTGGCTCCAACCGCA
CGTGTGGGCTGAACTCACACGCTATGGTAGCGAACTCATCGAGAGCATTGGGGCTGC
GGCCCCGGAAGAAGTGGTCTACCGTACCCAGGGAGAGCTGAAACGCGTTGATATTCA
AGCTGCATGGCCGAACATGGTCGATGCGATGCTTAAATTCTATGGGAACTATGCCGA
CGTGGCTTTCCCGCGACCATACGAGCCGTTGTATGCCAGGGAGGAACTCAAGAGATT
CGACGGGATGTCCATTCA*GGATCGCATCGACCAACTTGAGGATCTGACCACCGAAC
AAAGGGATATCCTCAACGCCTACTACTCAC*TGTGTTGTAATGCCCCGGTAACTGGC
GGGGGTTTGGCGACAATGGTCAGATGGTTCGCGCTTTCGTGTAAGAGTGTCAGCGCA
ACGTTCGACATGCTCGCCCGATTCAAAATCGCTGGCGGCACCAAGAGCCTGATCGAT
AAAATGATCGCCGACAGTTCGGCAGTCGTTCGCCTGGACACGCCGGTCGCGTCTATC
CACAGCAACGAGGAGGGCTCCACTGTGACACTCAGCAGCGGTGAAACGGTTCGGGCG
TCCGCTGTAGTCGTTGCATTGCCCATCGCCGTGCTCGGCGACGTAGCGTTCGAGCCA
CCGTTGCTGCAAACCAAGCTCCCGGCCATTTCAACTGGACAGCTGTGCGAAGGACTC
AAGTTCTGGGTCCGGATTAAGACTCGTGATGCGAAGCCGATGTTCGCAATGGCACCG
GATAACGAGGTTCTCAACTATGCTCACACAGAAGAAATTTACGATGACGGCCAGTTG
CTGGTGGTCTTCGGTATCGATGGCTCCAAGATCGATGACCCGAACTCGATTGACGAA
ATCGACCCACACATCAAGCGCCTGTTGGGCGATCACGTCGAAGTCATCGCAGTGCAT
GGGAAATACTGGCGCGACGATGAATTCGCAAAGGGCGGCTGGTCAGTCTACCGTCCG
GATCAGCTGACCAACGACTTGGAAGCATTGCAAAGTCCGCAACCACCAGTGTTCTTC
GCCACCGGCGATATTCGCCAATGGGTTGGAATGGATATATCGATGGGCGCCATTTGA
AACCGGGATCACTG

Figure 4.4. Nucleotide sequence of region responsible for diethylamine utilization in DE1. The two operons are boxed and the sites of the transposon insertions in 2F2 are marked by asterisks. The region where the second operon is truncated in *Arthrobacter aurescens* TC1 is highlighted in gray (see discussion).

DISCUSSION

Arthrobacteria are nutritionally versatile bacteria, in particular with regard to nitrogen metabolism. Several strains of Arthrobacter have been isolated based on their ability to use nitrogenous compounds, such as atrazine (20) and nicotine (4), as a sole source of carbon and nitrogen. Also, analysis of the genome of *Arthrobacter aurescens* TC1, an atrazine degrader, revealed the presence of 14 genes encoding amine oxidases (13). In that respect, it was not a surprise that we obtained an *Arthrobacter* species by selecting for growth on diethylamine as a sole source of carbon and nitrogen.

We chose *Arthrobacter protophormiae* DE1 for further study because of its superior diethylamine degradation rate when compared to other isolates (data not shown). DE1 experienced a two day lag period when grown on diethylamine (figure 4.1), suggesting that one or more of the genes responsible for diethylamine utilization is inducible. Further support for this came from our observation that extracts from cells exposed to diethylamine during growth degraded diethylamine almost eight times faster than extracts from non-exposed cells (figure 4.3).

Ethylamine was also degraded by DE1, but at a much slower than diethylamine, as seen in the experiments with washed cell suspensions (figure 4.3). This might explain why we did not detect acetaldehyde accumulation from ethylamine in cell-free extract, as the endogenous level of NAD remaining in the extract was likely sufficient to allow oxidation of the small amount of acetaldehyde expected to be liberated from ethylamine.

When *A. protophormiae* DE1 was cocultured with *P. putida* DTB, both were able to grow on DEET as a sole source of carbon and nitrogen (figure 4.2). DE1 cannot cleave the amide bond in DEET, but once DTB hydrolyzes this bond, the diethylamine moiety became available for metabolism by DE1. DTB, which cannot

metabolize diethylamine, can only grow on DEET in nitrogen free media when DE1 is present. DTB failed to grow in NFM when provided with ethylamine as its only carbon and nitrogen source, achieving an optical density of only 0.007 even after prolonged incubation. Therefore, DTB must be obtaining its nitrogen by using the ammonia released by DE1's metabolism of ethylamine. The fact that DE1 was able to slowly degrade ethylamine supports this, even though we did not actually detect the release of ammonia from ethylamine.

Based on what is known about metabolism of methylamines in *Arthrobacter* P1, which uses a monooxygenase to metabolize dimethylamine (6), we initially suspected that DE1 could be employing a monooxygenase for diethylamine degradation too. Neither of the orfs identified in the transposon mutagenesis, however, suggested the involvement of a P1-like monoxygenase. Rather, *deaA* which is necessary for diethylamine utilization is similar to flavin-containing amine oxidases; enzymes whose functions are also consistent with the activity we have observed.

Although the exact function of orf 1 is unknown, the two orfs are likely to be in the same operon because mutations in orf 1and the intergenic region also eliminate activity. It is not clear if the loss of activity caused by these insertions was due to polar effects or if the protein encoded by orf 1 affects the activity of the putative amine oxidase. Analyzing the differences in the activity of mutants in orf 1 and *dea*A might help clarify its importance, although heterologous expression of each orf indivually will be required to better understand the requirements for diethylamine degradation.

The region around these two orfs in *A. aurescens* TC1 is populated by genes related to amine and acetaldehyde utilization. In TC1 there is an acetaldehyde dehydrogenase, a putative copper-containing tyramine oxidase, and an acetate CoA-ligase just downstream of the *dea*A homolog. Interestingly, *dea*A is interrupted by a

transposon insertion in TC1. There is also a protein from the major facilitator superfamily (MFS). The MFS proteins are permeases that transport small solutes (15). The MFS-like putative protein could be involved in transport of aliphatic amines or amino acids across the cell membrane, although experimental evidence is needed to test that hypothesis.

Upstream of the orf 1 homolog in TC1, there are also a few genes involved in the synthesis of vitamin B12. Vitamin B12 is a cofactor of ethanolamine lyase (10) in *Salmonella typhimurium*. A gene coding for an ethanolamine lyase, however, was not found near this area in *A. aurescens* TC1.

In summary, we have shown *Arthrobacter protophormiae* DE1 can utilize diethylamine as a sole source of carbon and nitrogen. This ability allows a coculture of *Pseudomonas putida* DTB and *Arthrobacter protophormiae* DE1 to grow on DEET as a source of carbon and nitrogen. We have also presented evidence that diethylamine can be metabolized to ethylamine by *E. coli* heterologously expressing a gene from *A. protophormiae* DE1 (*dea*A) which encodes a putative flavin-containing amine oxidase. DUF861, a gene of unknown function, seems to share an operon with *dea*A and is also required for amine oxidase activity in *E. coli* although the role of the protein it encodes is unknown. Further work is needed to determine if orf 1 and *dea*A are responsible for the diethylamine degradation activity detected in strain DE1 and if they also play a role in ethylamine degradation.

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CHAPTER 5

CONCLUSIONS

SUMMARY OF FINDINGS ABOUT THE BACTERIAL PATHWAY FOR DEET DEGRADATION

Much information about the presence of PCPPs in the environment has emerged in the last decade (2-4). Nonetheless, there is still much left to know about biological transformation of these compounds in the environment. Answering the who and the how of the biotic transformation of PPCPs is important, not only to know whether or not a particular compound can be removed, but to devise better ways to remove them during the wastewater treatment. This knowledge is also valuable in that it allows us to know if more toxic compounds can be produced as the result of these transformations, as is the case for alkylphenol polyethoxylates (see chapter 1), and consequently degraded. The objective of the present work has been to answer the who and the how of the degradation of a particular PPCP: DEET.

Figure 5.1 summarizes the findings of this study regarding the degradation of DEET by bacteria. Chapter 2 describes the isolation of *Pseudomonas putida* DTB, which is responsible for initiating the metabolism of DEET as described in the top portion of the pathway in figure 5.1. This is the first organism to be reported that can grow on DEET. It accomplishes this by cleaving the amide bond in DEET by means of a DEET hydrolase, the products of this reaction being diethylamine and 3-methylbenzoate. The later is metabolized through the *meta*-cleavage pathway and used as a source of carbon and energy by *P. putida* DTB. The DEET hydrolase, DthA, is a serine hydrolase whose characterization is described in chapter 3 and its proposed mechanism is described in figure 5.2.

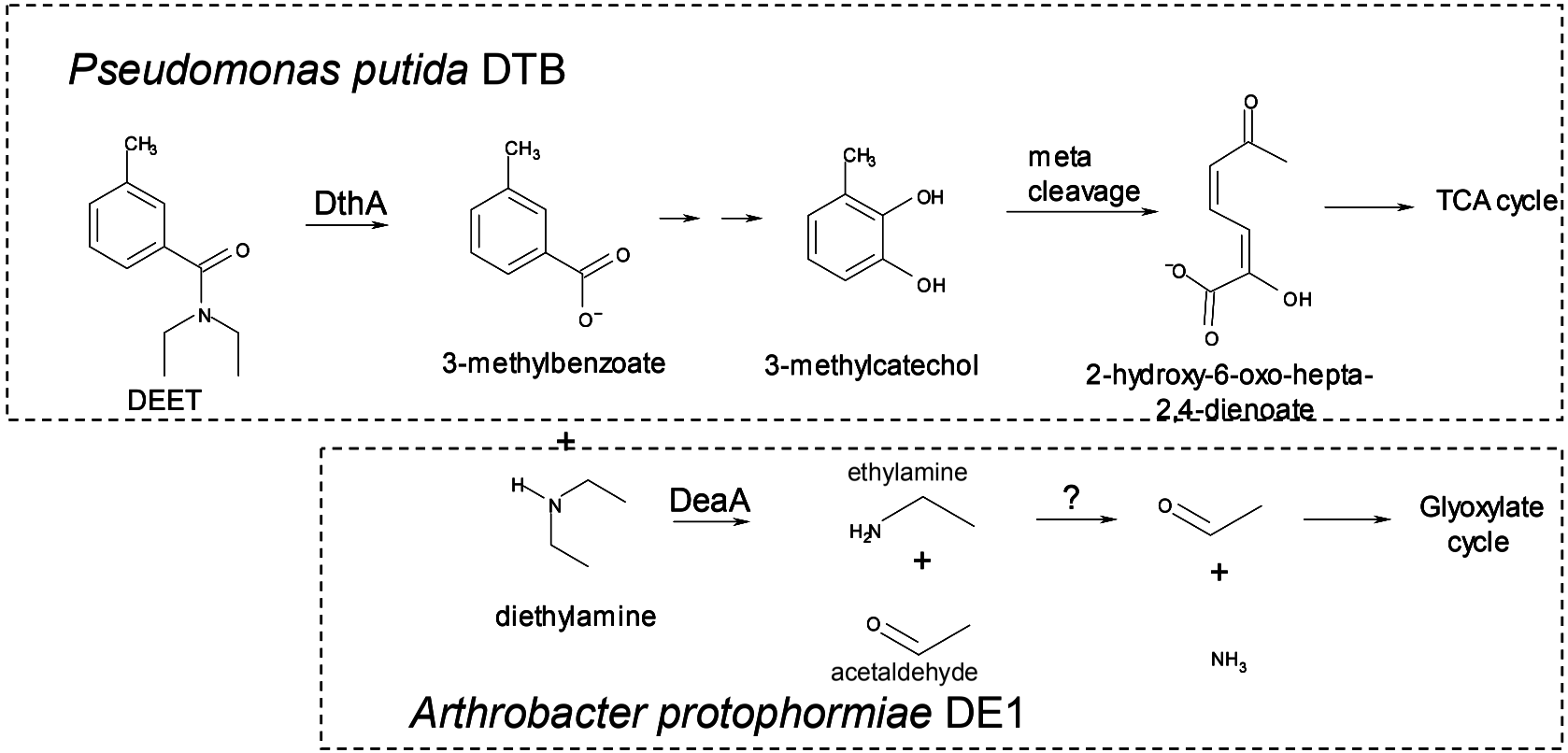

Figure 5.1. DEET metabolism by *P. putida* DTB and *A. protophormiae* DE1. The figure shows the proposed mechanism of DEET degradation by strains DTB (top panel) and DE1 (bottom panel) in coculture. The metabolism of DEET by DTB starts with hydrolysis of DEET by the enzyme DthA, followed by metabolism of 3-methylbenzoate. The metabolism of diethylamine by DE1 consists of subsequent removal of ethyl groups, producing acetaldehyde and NH_3. An amine oxidase (DeaA) is thought to be responsible for the removal of the first ethyl group. It is not clear yet if DeaA also removes the ethyl group from ethylamine.

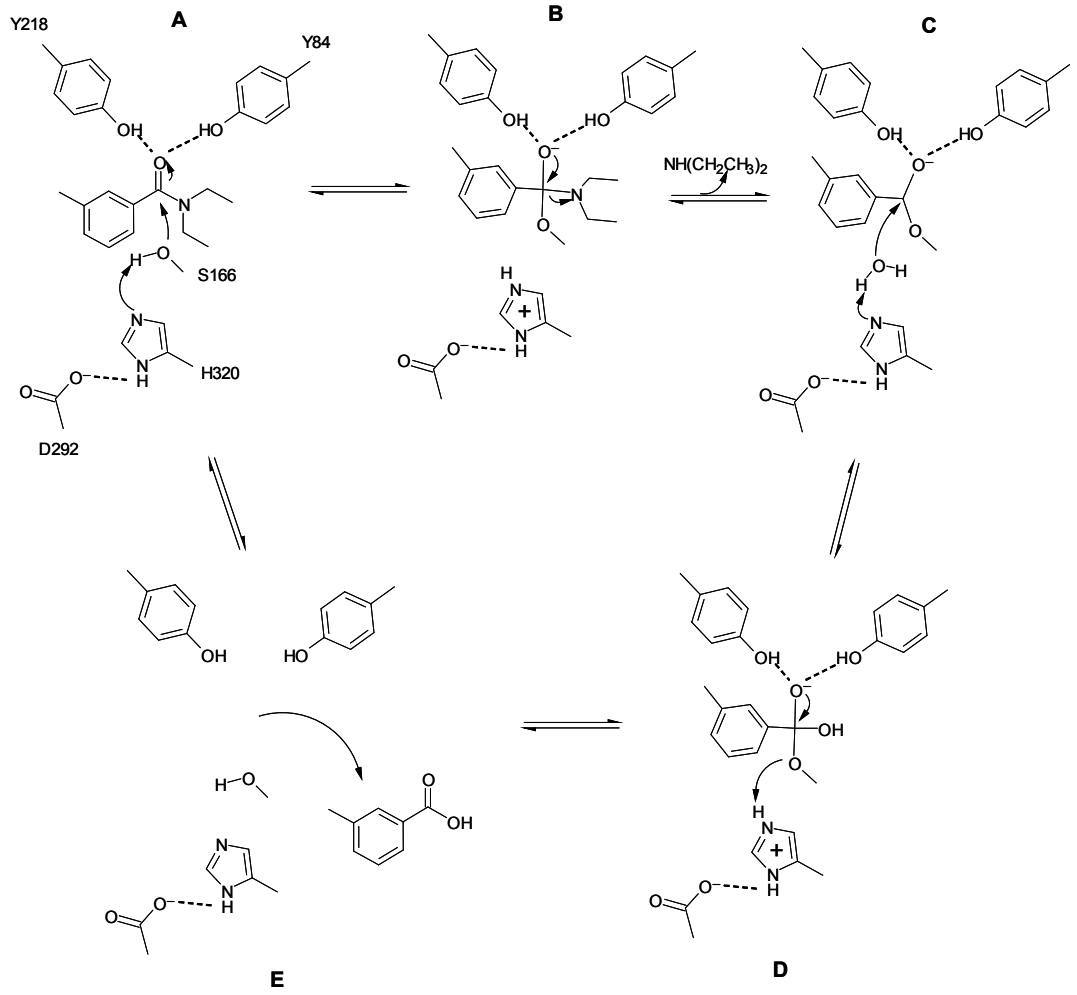

Figure 5.2. Proposed mechanism of DEET hydrolysis by DthA. For clarity, only some of the residues that were found to be important for activity in this study are shown in this figure. When the substrate binds, hydrolysis starts with the nucleophilic attack of S166 to the carbonyl carbon of the substrate (A). Meanwhile, H320 accepts a hydrogen atom from S166 and D292 helps relieve the positive charge acquired by H320 (A and B). A covalent tetrahedral intermediate is formed between the substrate and S166 (B), which is stabilized by hydrogen bonding between the carbonyl oxygen of the substrate and the oxyanion hole residues (Y218 and Y84 are shown). The tetrahedral intermediate collapses, releasing diethylamine. In C, water acts as the nucleophile in the same manner as S166 did before, forming a second tetrahedral intermediate (D). This intermediate collapses and the enzyme is regenerated (E).

This study provides an example of how the transformation of some PCPPs can produce toxic metabolites, as one of the intermediates, diethylamine, can be converted to a carcinogenic nitrosamine by undergoing one of several reactions discussed in chapter 1. As diethylamine is not further degraded by strain DTB, one objective of this study was to find other microorganisms in the consortium from which DTB was isolated that could degrade diethylamine. The bottom portion of figure 5.1 shows the mechanism of degradation of diethylamine by *Arthrobacter protophormiae* DE1. The isolation of *A. protophormiae* DE1 and details about the proposed degradation pathway are described in chapter 4. As a result of this study, two contiguous genes were found that appear to be involved in diethylamine degradation in strain DE1. One of these genes, *dea*A, is similar to amine oxidases, thus, it is likely responsible for diethylamine oxidation.

This work has reported, for the first time, the complete degradation of DEET by a consortium of microorganisms. It also has revealed many of the biochemical and genetic aspects related to this process. There are, however, several questions of interest that remain to be answered. The following section, "Future Directions", presents some of these questions.

FUTURE DIRECTIONS:

Genetic location and origin of the gene encoding the DEET hydrolase in DTB

With the interest of finding out in what genetic context *dth*A was located, the sequence surrounding *dth*A was determined. The sequence 2.6 kilobases upstream and 3.7 kilobases downstream of *dth*A was submitted to ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) for identification of open reading frames (orfs). Among the orfs that were identified in this manner, none were found that encoded enzymatic activities related to DEET degradation. BLAST analysis (1) of

these orfs revealed three putative transposases upstream of *dth*A (figure 5.3). Downstream of *dth*A, several genes encoding regulatory proteins were found, followed by a gene encoding a putative transposase. Of the putative regulatory genes, two are similar to σ^{54}-dependent transcriptional regulators (orfs 6 and 7 in figure 5.3). The other two are similar to a TonB-dependent receptor (orf 5) and a sensor kinase (orf 8).

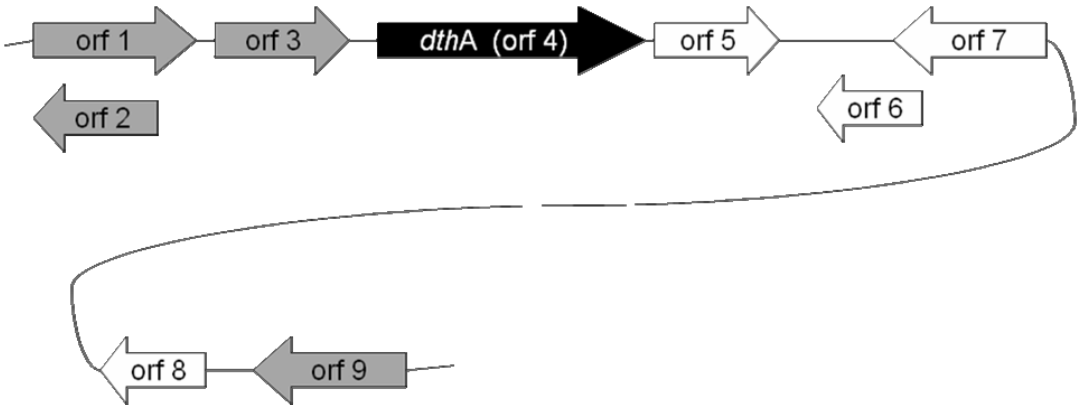

Figure 5.3. Genomic context of *dth*A. The total sequence length is 8.2 kilobases. Orfs are indicated by arrows and shading corresponds to function. Putative transposases are shaded grey, *dth*A is in black, and putative regulatory proteins are in white. The positions of these orfs relative to the total sequence presented are as follows: orf 1, 601-1854; orf 2, 1764-970; orf 3, 1874-2398; *dth*A, 2633-4507; orf 5, 4524-4877; orf 6, 5472-5095; orf 7, 6106-5411; orf 8, 6507-6094; orf 9, 7265-7684.

The presence of these transposases around *dth*A suggests that DTB might have acquired this gene by horizontal gene transfer. Also supporting this hypothesis is the fact that BLAST analysis did not reveal any matches between *dth*A and any gene from other pseudomonads. The GC content of *dth*A, however, does not support or disprove this hypothesis. *dth*A has a GC content of 58%, which is in close agreement with the

62% GC content of the *Pseudomonas putida* KT2440 genome (5). Thus, the answer to how DTB obtained *dth*A remains a topic for further study.

At this moment, the molecular target of the regulatory genes found downstream of *dth*A has not been determined. It does not appear that these genes regulate *dth*A as the expression of *dth*A seems to be constitutive. Evidence for this is that when normalized cell free extracts of DTB cells grown on DEET or on acetate were incubated with DEET, they had similar rates of DEET metabolism (0.032 μmoles/min/mg for DEET grown extracts and 0.021 μmoles/min/mg for acetate grown extracts). Further study is necessary to determine if these downstream genes have any role regulating other genes of the DEET degradation pathway.

The genetic location of *dth*A is another aspect of interest. DTB could not be cured of the ability to degrade DEET after many transfers in minimal salts medium with acetate as sole carbon source. This suggests that *dth*A might be located in the chromosome. This assertion, however, needs to be corroborated by additional experimentation.

Substrate specificity of the amine oxidase (DeaA) from DE1

The results of the experiments with washed cell suspensions and cell free extracts from strain DE1, presented in chapter 4, reveal that the metabolism of diethylamine is much faster than that of ethylamine. This raises several questions. First, what is the gene responsible for ethylamine metabolism? Is it *dth*A? Since *dth*A was found by screening only for metabolism of diethylamine, it is unknown yet if its product can metabolize ethylamine, albeit at a lower rate than diethylamine. Second, what are the determinants for DeaA's preference of secondary amines over primary ones? *dea*A and the orf upstream of it have been recently cloned into the pGEM-T Easy vector, each one by itself and together, and transformed into *Escherichia coli*. This will allow us to do experiments to gain more insight into these questions.

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