Saturation Mutagenesis of 2,4-DNT Dioxygenase of *Burkholderia* sp. Strain DNT for Enhanced Dinitrotoluene Degradation

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Abstract: 2,4-Dinitrotoluene (2,4-DNT) and 2,6-DNT are priority pollutants, and 2,4-DNT dioxygenase of *Burkholderia* sp. strain DNT (DDO) catalyzes the initial oxidation of 2,4-DNT to form 4-methyl-5-nitrocatechol and nitrite but has significantly less activity on other dinitrotoluenes and nitrotoluenes (NT). Hence, oxidation of 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT were enhanced here by performing saturation mutagenesis on codon I204 of the α subunit (DntAc) of DDO and by using a membrane agar plate assay to detect catechol formation. Rates of degradation were quantified both by the formation of nitrite and by the formation of the intermediates with high performance liquid chromatography. The degradation of both 2,3-DNT and 2,5-DNT were achieved for the first time (no detectable activity with the wild-type enzyme) using whole *Escherichia coli* TG1 cells expressing DDO variants DntAc I204L and I204Y (0.70 ± 0.03 and 0.22 ± 0.02 nmol/min/mg protein for 2,5-DNT transformation, respectively). DDO DntAc variant I204L also transformed both 2,6-DNT and 2,4-DNT 2-fold faster than wild-type DDO (0.8 ± 0.6 nmol/min/mg protein and 4.7 ± 0.5 nmol/min/mg protein, respectively). Moreover, the activities of DDO for 2NT and 4NT were also enhanced 3.5-fold and 8-fold, respectively. Further, DntAc variant I204Y was also discovered with comparable rate enhancements for the substrates 2,4-DNT, 2,6-DNT, and 2NT but not 4NT. Sequencing information obtained during this study indicated that the 2,4-DNT dioxygenases of *Burkholderia* sp. strain DNT and *B. cepacia* R34 are more closely related than originally reported. This is the first report of engineering an enzyme for enhanced degradation of nitroaromatic compounds and the first report of degrading 2,5-DNT. © 2005 Wiley Periodicals, Inc.

Keywords: dinitrotoluene; 2,4-DNT dioxygenase; saturation mutagenesis

INTRODUCTION

2,4,6-Trinitrotoluene (TNT) is the most common explosive and the production intermediates 2,6-dinitrotoluene (2,6-DNT) and 2,4-DNT are found in the soil and ground water at TNT facilities (Johnson et al., 2002; Nishino et al., 2000). 2,6-DNT and 2,4-DNT are also used for the production of polyurethane foams as a precursor of toluene diisocyanate (Nishino et al., 1999), and both are listed as priority pollutants by the U.S. Environmental Protection Agency (EPA) (Nishino et al., 1999). Wastes containing 2,4-DNT are regulated as hazardous if the concentration is over 0.13 ppm (Suen and Spain, 1993), and both 2,4-DNT and 2,6-DNT are carcinogenic to rats and mice (EPA/600/8-88/032, 1988) whereas 2,3-DNT and 2,5-DNT are suspected human carcinogens (1994). 2,3-DNT, 2,4-DNT, 2,5-DNT, and 2,6-DNT cause systemic intoxication (Gosselin et al., 1976). 2-Nitrotoluene (2NT) and 4NT are also listed as high production chemicals by the EPA, and there is potential for human exposure (Dunnick et al., 2003). 2NT and 4NT may cause cancer in mammalian species (Dunnick et al., 2003) and are suspected of causing anemia in chronic exposures (Clayton and Clayton, 1991).

Burkholderia sp. strain DNT was isolated from water samples from Waconda Bay near the Volunteer Army Ammunition Plant in Chattanooga, Tenn. (Spanggord et al., 1991), and *B. cepacia* R34 was found from surface water at the Radford Army Ammunition plant in West Virginia (Nishino et al., 2000). These strains use 2,4-DNT as a sole carbon and energy source (Johnson et al., 2002; Nishino et al., 2000). Catabolism of 2,4-DNT is initiated by both 2,4-DNT dioxygenases (DDO and R34 DDO), and the 2,4-DNT oxidation pathway was reported previously (Johnson et al., 2002; Suen and Spain, 1993) with the first product 4-methyl-5-nitrocatechol (4M5NC) (Fig. 1). Though, 2,6-DNT is not a sole carbon and energy source of either *Burkholderia* sp. strain DNT or *B. cepacia* R34, DDO and R34 DDO oxidize 2,6-DNT to 3M4NC (Nishino et al., 2000) (Fig. 1).

DDO is very similar to R34 DDO since its α and β subunits have 93% and 99.5% identity, respectively. As shown in this report, even though DDO and R34 DDO are more similar at the DNA level than first thought, DDO transforms 4NT to

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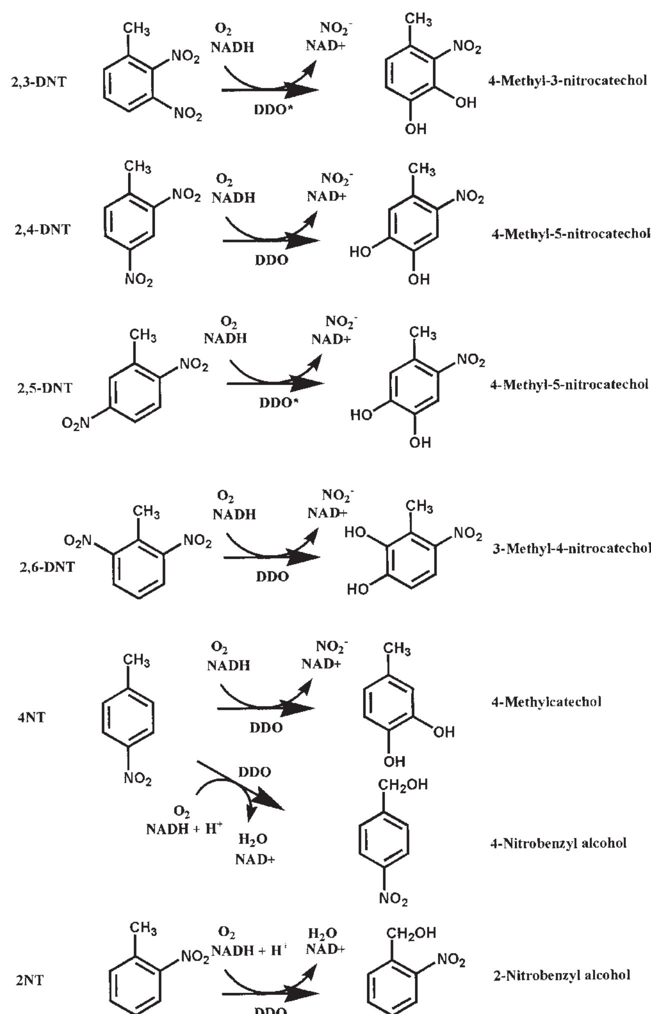

Figure 1. Oxidation of 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 4NT, and 2NT by DDO DntAc variants I204L and I204Y. The new oxidation pathways of 2,3-DNT and 2,5-DNT by DDO enzyme variants are marked with asterisks (*).

both 4-methylcatechol and 4-nitrobenzyl alcohol, but R34 DDO transforms 4NT to 4-nitrobenzyl alcohol (Table I), so the enzymes do not have the same activity.

Although there are many physiological roles for oxygenases, they can be grouped broadly into two applications: biodegradation and biosynthesis (van Beilen et al., 2003). Directed evolution and saturation mutagenesis have been used to enhance the behavior of the oxygenases for these applications; for example, the activity of toluene *ortho*-monooxygenase of *B. cepacia* G4 was enhanced for chlorinated ethenes and naphthalene degradation (Canada et al., 2002) as well as for indigoid production (Rui et al., 2005), and the regiospecific oxidation of toluene may be controlled completely (Fishman et al., 2005). The large subunit of biphenyl dioxygenase (BDO) from *Pseudomonas pseudoalcaligenes* KF707 and *B. cepacia* LB400 have also been shuffled to enhance the degradation of polychlorinated biphenyls, benzene, and toluene (Kumamaru et al., 1998), and random mutagenesis and saturation mutagenesis were

Table I. Initial product formation rate (nmol/min/mg protein) for the substrates 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT by *E. coli* TG1 expressing wild-type R34 DDO, wild-type DDO, and saturation mutagenesis variants DDO DntAc I204L and I204Y. Rates were determined both from nitrite released and from products detected with HPLC (300 μ*M* substrate concentrations except 4NT which was 150 μ*M*).

		W/T R34 DDO		W/T DDO		I204L DDO				I204Y DDO			
Substrate	Product	Nitrite	HPLC	Nitrite	HPLC	Nitrite	Rel.[a]	HPLC	Rel.[a]	Nitrite	Rel.[a]	HPLC	Rel.[a]
2,3-DNT	4M3NC[b]	N/A	—	N/A	—	1.0 ± 0.2	—	—	∞	0.31 ± 0.05	—	—	∞
2,4-DNT	4M5NC	1.1 ± 0.2	1.7 ± 0.3	2.3 ± 0.7	2.2 ± 0.2	4.7 ± 0.5	2.0	5.2 ± 1.4	2.2	4.7 ± 0.9	2.2	3.4 ± 0.1	1.5
2,5-DNT	4M5NC	N/A	0	N/A	0	0.70 ± 0.03	∞	0.2 ± 0.1	∞	0.22 ± 0.02	∞	0.10 ± 0.08	∞
2,6-DNT	3M4NC	0.7 ± 0.3	0.07 ± 0.02	0.4 ± 0.2	0.09 ± 0.07	0.8 ± 0.6	2.0	0.20 ± 0.04	2.2	1.0 ± 0.4	2.5	0.25 ± 0.02	2.8
2NT	2-nitrobenzyl alc.[c]	N/A	0.0130 ± 0.0004	N/A	0.04 ± 0.02	N/A	—	0.14 ± 0.04	3.5	N/A	—	0.13 ± 0.01	3.3
4NT	4-methylcatechol	N/A	0	0.10 ± 0.06	0.09 ± 0.06	0.76 ± 0.06	7.6	0.5 ± 0.2	5.6	N/A	—	0	0
	4-nitrobenzyl alc.	N/A	0.1 ± 0.2	N/A	0.051 ± 0.008	N/A	—	0.060 ± 0.004	1.2	N/A	—	0.02 ± 0.01	0.4

N/A: nitrite should not be formed.
[a]Relative to wild-type DDO.
[b]The product 4M3NC was detected by using GC-MS from variants DntAc DDO I204L and I204Y but not from wild-types.
[c]alc.: alcohol.

used to improve the activity of toluene dioxygenase by increasing the acceptability for 4-picoline which is a poor substrate (Sakamoto et al., 2001).

DDO is a three-component enzyme system; electrons are transferred from NADH through a flavoprotein reductase (DntAa), an iron-sulfur [2Fe-2S] ferredoxin (DntAb), and an iron-sulfur oxygenase which consists of a large (α) subunit (DntAc) and a small (β) subunit (DntAd) (Parales et al., 1998b; Suen et al., 1996). Naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4 (NDO) is also a three-component enzyme system similar to DDO (80% identity for the alpha subunit). The X-ray crystal structure of NDO and the active site residues have been determined (Kauppi et al., 1998) which enabled the amino acids located near the active site to be studied for their roles in controlling the regioselectivity and enantioselectivity of the enzyme (Parales, 2003). For example, F352 in the α subunit of NDO (NahAc) significantly affects the regio- and enantioselectivity of the oxidation of naphthalene, biphenyl, and phenanthrene (Parales et al., 2000a,b). By replacing valine at this position to phenylalanine (V350F) in the related enzyme R34 DDO, we changed the substrate specificity from 2,4-DNT to substituted *o*- and *m*-phenols to create new synthetic routes to nitrohydroquinone, methylhydroquinone, and methoxyhydroquinone for green chemistry (Keenan et al., 2004).

Yu et al. (2001) identified A206 position in the α subunit of NDO (which is analogous to I204 in DntAc of DDO, Fig. 5) as important in determining regioselectivity with phenanthrene since variant A206I formed 57% phenanthrene *cis*-3,4-dihydrodiol compared to 90% for wild-type NDO. Further, mutation at the analogous residue M220A in the α subunit (TodC1) of a hybrid toluene dioxygenase from *Pseudomonas putida* F1 and *Burkholderia sp.* strain PS12 restored dehalogenase activity for 1,2,4,5-tetrachlorobenzene (Beil et al., 1998). Hence, codon I204 of DDO DntAc has been shown to affect dioxygenase activity for related enzymes so it was mutated here with the goal of increasing the nitrotoluene substrate range of DDO to include 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT. Two DDO DntAc variants with enhanced activity were found, I204L and I204Y, and the degradation products were identified. For comparison, the wild-type R34 DDO reactions were also investigated.

EXPERIMENTAL METHODS

Bacterial Strains and Growth Conditions

Escherichia coli TG1 (Sambrook et al., 1989) was used to express DDO from pBS(Kan)DNT (Fig. 2) or R34 DDO from pBS(Kan)R34 (Keenan et al., 2004) under the control of a *lac* promoter. Strains were cultured as described previously (Keenan et al., 2004), and exponentially-grown cells were resuspended in sodium phosphate buffer (pH 6.5) at an OD of 5–15.

B. cepacia R34 was used with broad-host-range plasmid pVLT31 (de Lorenzo et al., 1993) and pVLT31-I204L which

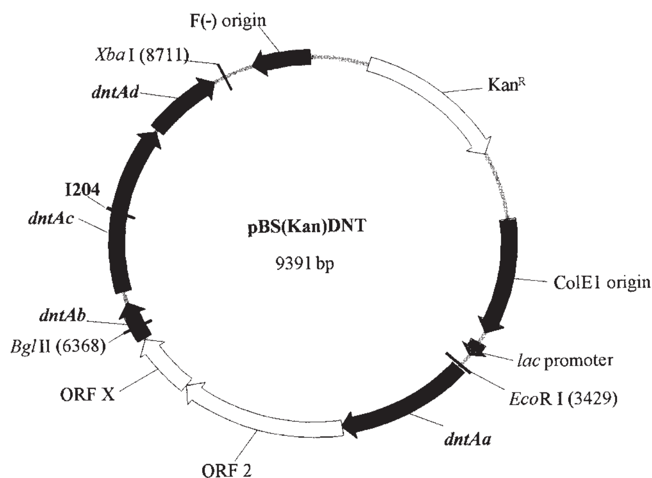

Figure 2. Plasmid map of pBS(Kan)DNT for the constitutive expression of DDO (wild-type and variants) from *dntAaAbAcAd* and the relevant restriction enzymes used for cloning (*Bgl*II, *Xba*I, and *Eco*RI), the kanamycin resistance gene, and the constitutive *lac* promoter are shown.

contains the DDO DntAc I204L mutation (described below). Cells were grown from single colonies in Luria-Bertani (LB) medium containing tetracycline (100 µg/mL) to maintain the plasmid at 30°C and 250 rpm and incubated with 1 m*M* of isopropyl-β-D-thiogalactopyranoside (IPTG) after 24 h. After the culture reached an OD of 2.0, the cells were washed with 100 m*M* sodium phosphate buffer (pH 6.5), resuspended in an equal volume of buffer, and transferred to MSB medium (Spain and Nishino, 1987) (final OD 0.1) containing 1 m*M* of IPTG, tetracycline (100 µg/mL), and 0.3 m*M* of 2,5-DNT as a sole carbon source.

Chemicals

2,3-DNT, 2,4-DNT, 2,6-DNT, 2NT, 4NT, and 4-methylcatechol were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). 2,5-DNT was obtained from Accustandards Inc. (New Haven, Conn). 2-Nitrobenzyl alcohol and 4-nitrobenzyl alcohol were purchased from Fisher Scientific Co. (Fairlawn, NJ), and 3M4NC and 4M5NC were provided by G. R. Johnson of Tyndall Air Force Base.

Construction of pBS(Kan)DNT

The expression vector pBS(Kan)DNT (Fig. 2) was constructed for expression of DDO from the *dntAaAbAcAd* locus (5.3 kb from pJS48 (Suen et al., 1996)) using the *lac* promoter. The *dnt* locus was cloned into pBS(Kan) (Canada et al., 2002) using a polymerase chain reaction (PCR) with a mixture of *Taq* (Promega, Madison, WI) and *Pfu* (Stratagene, La Jolla, CA) polymerases (1:1) that consisted of an initial hot-start at 96°C for 3 min, after which the polymerases was added, followed by 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 7 min (the final elongation was 72°C for 7 min). The forward primer BSIIR34EcoF (Table II) generated the *Eco*RI site, and the reverse primer

Table II. PCR primers used for construction of pBS(Kan)DNT, saturation mutagenesis of positions I204 DntAc for DDO, and sequence analysis of both the wild-type DDO locus (*dntAaAbAcAd*) and the DntAc DDO variants at codon I204.

Purpose	Primer	Sequence
Plasmid construction	BSIIR34EcoF	5′-GCATGG**GAATTC**CAACTGAAAAAAGAGCTTGCATGG-3′
	pBSIIDNTXbaR	5′-CCACTC**TCTAGA**ACTGAAATCCAGGGTGTTGCACTTCAC-3′
Saturation mutagenesis	DNTAcBgl2F	5′-ATGAGCGAGAACTGGATCG-3′
	DNTDNTI204F	5′-GCGGAAAACTTTGTAGGTGACNNNTACCAC-3′
	DNTR34V204R	5′-GATGCGTGCGTCCAACCAATGTGGTANNNGTCACCTAC-3′
	DNTAcXbaR	5′-AGGGTTTTCCCAGTCACG-3′
Sequence analysis	T3MO 107F outer	5′GACCATGATTACGCCAAGCGCGC3′
	ContigAa	5′-CATCGTTTTCAATGAACTGTCCG-3′
	pBSKanDntdntAb	5′-GACCGAGTGTACGGTGTCGTCAAGG-3′
	DNTctgB	5′-AAATCCGGAAATCACCCC-3′
	DNTctgC	5′-TTGTGTGCGGTTACCACG-3′
	DNTctgD	5′-GACCACCAAGTATGGCAGTG-3′
	DNTctgE	5′-GATTTGGGTTTCGGCAAG-3′
	DNTctgF	5′-ACTTCGCTCCACTTCCGA-3′

Restriction site underlined and bold.

pBSIIDNTXbaR (Table II) generated an *Xba*I site; after double digestion, the PCR fragment was cloned into the multiple cloning site of pBS(Kan). The resulting plasmid was electroporated into *E. coli* TG1 competent cells using a Bio-Rad GenePulser/Pulse Controller (Hercules, CA) at 15 kV/cm, 25 μF, and 200 Ω. The pink color produced by DDO expressed from pBS(Kan)DNT in *E. coli* TG1 colonies on agar plates, and the indigo produced in broth cultures indicated correct construction of the plasmid which was confirmed by DNA sequencing.

Saturation Mutagenesis at I204

Saturation mutagenesis was performed at codon I204 of DDO in DntAc (GenBank accession no. U62430). All possible 64 codons were created at the target position by replacing the codon with NNN via overlap-extension PCR. Two degenerate primers, DNTDNTI204F (Table II) and DNTR34V204R (Table II), were designed to randomize position 204 in DntAc. The two additional primers for cloning, DNTAcBgl2F (Table II) and DNTAcXbaR (Table II), were chosen to utilize the two unique restriction sites, *Bgl*II and *Xba*I, that are upstream and downstream, respectively, from position I204 (Fig. 2). pBS(Kan)DNT (200 ng) was used as the template in the initial PCR reaction, and *Vent* DNA polymerase (New England Biolabs, Inc., Beverly, MA) was used in the PCR to minimize random point mutations. A 1,059 bp DNA degenerate fragment was amplified using primers DNTAcBgl2F and DNTR34V204R, and a 1,498 bp DNA degenerate fragment was amplified using DNTDNTI204F and DNTAcXbaR. After purifying from a 0.6% agarose gel, the two PCR fragments were combined at a 1:1 ratio as templates to obtain the full-length PCR product by using primers DNTAcBgl2F and DNTAcXbaR. PCR (MJ Research minicycler™, Watertown, MA) was performed at 96°C for 2 min, 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 2 min 30 seconds, with a final extension of 72°C for 7 min. The resulting randomized PCR product (2,557 bp) was cloned into pBS(Kan)DNT after double digestion of both vector and insert with *Bgl*II and *Xba*I, replacing the wild-type region. The resulting plasmid library was electroporated into *E. coli* TG1 competent cells as described above.

Construction pVLT31-I204L

The 5.3 kb *Eco*RI and *Xba*I fragment from pBS(Kan)DNT with variant DntAc I204L was cloned into the same sites of the broad-host-range vector pVLT31 (de Lorenzo et al., 1993) which contains the tetracycline-resistance gene. The resulting plasmid was conjugated into *B. cepacia* R34 using *E. coli* S17-1(λpir) (de Lorenzo et al., 1993) as a host for biparental mating (Yee et al., 1998) with selection on MSB containing tetracycline (10 μg/mL). The resulting strain was *B. cepacia* R34-I204L with DDO variant I204L under control of *tac* promoter. The vector pVLT31 plasmid was also conjugated into wild-type *B. cepacia* R34 as a negative control.

Molecular Techniques and Protein Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (Sambrook et al., 1989), and plasmid DNA was isolated using a Midi or Mini Kit (Qiagen, Inc., Chatsworth, CA) and digested by the restriction enzymes from New England Biolabs, Inc.(Beverly, MA). DNA fragments were isolated from agarose gels using the GeneClean III Kit (Bio 101, Vista, CA). Ligation reactions were performed at 16°C for 20 hours (Sambrook et al., 1989) with a 3 to 1 molar ratio

(insert:vector). T4 DNA ligase and 5X T4 DNA ligase buffer were from Invitrogen (Carlsbad, CA).

Colony Screening and Nitrite Detection

A nylon membrane agar colony screen was used with 300 μ*M* of 2,6-DNT (Keenan et al., 2004). The dark brown color surrounding colonies indicated catechol was formed intracellularly which was secreted and auto-oxidized to quinones and semiquinones which have red brown color.

Nitrite released from DNT degradation using whole cells was detected spectrophotometrically (Keenan et al., 2004). Exponentially-grown cells resuspended in sodium phosphate buffer were incubated with 300 μ*M* of 2,3-DNT, 2,4-DNT, 2,5-DNT, or 2,6-DNT or 150 μ*M* of 4NT at a cell density of OD 5-10 in sodium phosphate buffer (100 m*M*, pH 6.5) for 5 to 60 min. For 2,4-DNT and 2,6-DNT degradation, the supernatants changed from colorless to yellow due to the accumulation of 4M5NC (Spanggord et al., 1991) and 3M4NC (Nishino et al., 2000), respectively. The nitrite concentration was determined using a linear calibration curve (0.72 to 145 μ*M*). Two to three replicates of both wild-type enzymes and the enzyme variants were analyzed.

Product Formation Rates by HPLC

Reverse-phase, high performance liquid chromatography (HPLC) was used to identify the products and to determine the product formation rates from 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT. Samples (10 mL) of exponentially-grown, washed cells (OD 5 to 15) were contained in sealed 60 mL serum vials were incubated with 300 μ*M* of 2,4-DNT, 2,5-DNT, or 2,6-DNT or 150 μ*M* of 2NT or 4NT. 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT stock solutions were diluted in acetonitrile. The samples were incubated at 37°C on a shaker at 300 rpm for 5–60 min. 750 μL samples were taken at 5, 15, 30, 45, and 60 min using a 3 mL syringe to determine the rates of formation. Samples were centrifuged in a microcentrifuge for 5 min at 16,000 × g. Supernatants (20 μL) were collected and analyzed using HPLC with a Chromolith^{TM} Performance RP-18e column (Merck KGaA, 4.6 × 100 mm) for 4M5NC and 3M4NC products and a Zorbax SB-C8 column (Agilent Technologies, 5 μm, 4.6 × 250 mm) with a Waters Corporation (Milford, MA) 515 solvent delivery system. Compounds were detected by a photodiode array detector (Waters 996). A gradient elution was used with H_2O (0.1% formic acid) and acetonitrile (95:5 0–1.5 min at 1 mL/min, 95:5 at 3.5 min at 3 mL/min, 85:15 at 5 min at 3 mL/min, 85:15 at 8 min at 1 mL/min, 60:40 at 9 min at 1 mL/min, 60:40 at 12 min at 1 mL/min, and 95:5 at 15 min at 1 mL/min) as the mobile phase for the 4M5NC and 3M4NC products. For 2-nitrobenzyl alcohol, 4-methylcatechol, and 4-nitrobenzyl alcohol, a gradient elution was used with H_2O (0.1% formic acid) and acetonitrile (70:30 0–8 min, 40:60 at 15 min and 70:30 at 20 min) as the mobile phase at the flow rate of 1 mL/min. The compounds were identified by comparison of retention time and UV-visible spectra to those of authentic standards as well as by co-elution with the standards. Two to three replicates of both wild-type enzymes and the enzyme variants were analyzed.

Product Identification via Gas Chromatography-mass Spectrometry Analysis

4M3NC from 2,3-DNT oxidation was identified by gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5970B GC-MS instrument equipped with a HP-1 column (12 m × 0.2 mm, 0.33 μ*M* thickness), and the ionization voltage was 70 eV. The initial column temperature was 120°C for 2 min which was increased at 10°C/min to 270°C followed by an isothermal operation for 6 min. The injector and detector temperatures were 270°C and 275°C. Samples were prepared in a similar manner to the samples for HPLC and were incubated for 1 h with 300 μ*M* of a 2,3-DNT stock solution dissolved in acetonitrile. Samples were centrifuged in a microcentrifuge for 5 min at 16,000 × g, and supernatants were extracted with ethyl acetate. Reaction mixtures were acidified to pH 2.5 by adding hydrochloric acid prior to extracting the product. The solvent was evaporated under nitrogen, and the residue was dissolved in 40 μL of ethyl acetate. Trimethylsilyl derivatives were prepared with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (Alltech Associates Inc., Deerfield, Ill.) by adding 40 μL of BSTFA into the samples, and the samples were incubated for 30 min at room temperature. 4M3NC was identified by GC-MS retention time (Rt) and characteristic mass fragments (note that the molecular weight includes 144 from trimethylsilyl derivitization): Rt 8.4 min and molecular ion [M+ (% relative intensity)] at m/z 313 (16) with major fragment ions at m/z 298 (58), 281 (12), and 73 (100) which were confirmed by comparison to the GC-MS data of Lessner et al. (2002). Two to three replicates of both wild-type enzymes and the enzyme variants were analyzed.

DNA Sequencing and Homology Modeling

The primers shown in Table II were used to sequence the complete *dnt* locus (Keenan et al., 2004); all sequencing was performed three times. The wild-type DDO α subunit (DntAc) was modeled using SWISS-MODEL Server (Guex et al., 1999; Guex and Peitsch, 1997; Peitsch, 1995; Schwede et al., 2003) based on the polymer chain A NahAc template (Protein Data Bank 1O7G (Karlsson et al., 2003)) of *Pseudomonas* sp. NCIB 9816-4 NDO. The DDO DntAc I204L variant was modeled from the generated wild-type DDO model using the DeepView program (Swiss-Pdb Viewer) (Guex et al., 1999; Guex and Peitsch, 1997; Peitsch, 1995; Schwede et al., 2003). The DeepView program performed the amino acid substitutions isosterically for the variant DDO alpha subunits based on residue interactions, steric hindrance, and energy minimization. Figure 3 was prepared using PyMOL (DeLano Scientific LLC, http://pymol.sourceforge.net/).

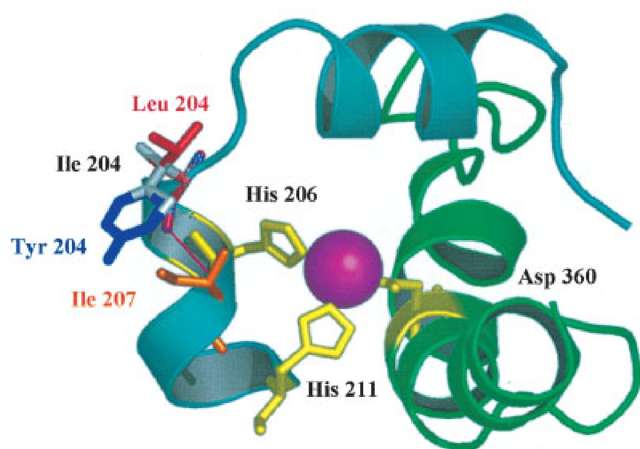

Figure 3. Active site of the DDO α subunit (DntAc three-dimensional structure model) showing Ile 204 of the wild-type DDO (white), the variant DntAc I204L (red), and variant DntAc I204Y (blue). Residues His 206, His 211, and Asp 360 (yellow) coordinately bind with the mononuclear iron (pink sphere). Portions of the two ribbons of DDO which form the substrate channel are shown in light blue (terminating at Lys 190 and His 211) and green (terminating at Ala 343 and Ser 376). The orange residue (Ile 207) participates in the new hydrogen bonds (pink) with the mutated residues. Only a portion of an alpha subunit three-dimensional structure model is shown from the catalytic oxygenase component that consists of three alpha and three beta subunits ($\alpha_3\beta_3$).

RESULTS

2,6-DNT Screening

DDO DntAc variants I204L and I204Y were identified from saturation mutagenesis of I204 by screening 300 colonies from the library since it was reported by Rui et al. (2004) that there is a 99% probability that all 64 codons will be sampled if 292 colonies from the single site of random mutagenesis are screened. Whereas wild-type DDO produces yellow/brown oxidized products with 2,6-DNT, DDO DntAc variants I204L and I204Y were found to enhance the production of yellow/brown metabolites on the nylon membrane. DNA sequencing indicated there was only one codon changed for both variants.

Nitrite Detection From Oxidation of Nitroaromatics

The reactions of wild-type DDO, wild-type R34 DDO, and the DDO DntAc variants I204L and I204Y for the nitro compounds used in this work are summarized in Figure 1. Previously, nitrite has been detected from the degradation of 2,4-DNT and 2,6-DNT using wild-type DDO and R34 DDO (Johnson et al., 2002; Nishino et al., 2000; Parales et al., 1998a; Spanggord et al., 1991).

The I204L and I204Y DDO DntAc variants oxidized 2,6-DNT 2-fold and 2.5-fold faster than wild-type DDO, respectively (Table I). For 2,3-DNT and 2,5-DNT degradation, activity was achieved for the first time with the I204L and I204Y DntAc variants (Table I) since there was no detectable activity for either of the wild-type enzymes.

Since 2,4-DNT is the natural substrate of DDO (Nishino et al., 2000), its rate of oxidation was also checked via nitrite release for the variants. The initial rate of 2,4-DNT oxidation by wild-type DDO was 2-fold faster than wild-type R34 DDO, and the rate of 2,4-DNT oxidation by the DDO DntAc variants I204L and I204Y were 2-fold and 2.2-fold faster than wild-type DDO (Table I). Hence, two new enzyme variants were discovered that have elevated nitrite release for 2,3-DNT, 2,4-DNT, 2,5-DNT, and 2,6-DNT. For 4NT oxidation, the initial rate of DDO DntAc I204L variant was 7.6-fold faster than wild-type DDO (Table I).

HPLC and GC-MS Analysis of the 2,3-DNT, 2,4-DNT, 2,5-DNT, and 2,6-DNT Transformations

HPLC analysis was used to obtain the oxidation rates of the variant DntAc and wild-type enzymes. As expected 2,4-DNT was transformed to 4M5NC by wild-type DDO (Nishino et al., 2000; Spanggord et al., 1991), wild-type R34 DDO (Johnson et al., 2002), and the two new DDO DntAc variants I204L and I204Y. 2,6-DNT was transformed to 3M4NC by both wild-type DDO and R34 DDO (Nishino et al., 2000) and the I204L and I204Y DDO DntAc variants (Fig. 1). As with the nitrite analysis, the initial rates of 2,4-DNT oxidation by DDO was 1.3 fold faster than R34 DDO, and the 2,4-DNT degradation rate of the I204L and I204Y DntAc variants were 2.2 and 1.5 fold faster than wild-type DDO (Table I). For 2,6-DNT oxidation, the initial rates by R34 DDO was similar to the rate of DDO, and the 2,6-DNT degradation rates of the DntAc I204L and I204Y variants were 2.2 and 2.8 fold faster than wild-type DDO.

For 2,5-DNT degradation, 4M5NC (Fig. 1) was detected from the DDO DntAc I204L and I204Y variants in contrast to wild-type DDO and R34 DDO which had no detectable activity (Fig. 4A and B). For 2,3-DNT oxidation, the 4M3NC product was detected from the DDO DntAc I204L and I204Y variants by GC-MS in contrast to wild-type DDO which had no detectable activity. The GC-MS fragmentation chromatogram of 4M3NC is shown in Figure 4C. Therefore, for all the DNT substrates, the DntAc I204L variant had elevated activity.

HPLC Analysis of 2NT and 4NT Transformations

As seen previously for wild-type DDO (Parales et al., 1998a; Suen et al., 1996), 2NT was transformed to 2-nitrobenzyl alcohol by wild-type DDO and R34 DDO. Both the I204L and I204Y DntAc DDO variants (Fig. 1) were also found to form 2-nitrobenzyl alcohol. The 2NT transformation rate of wild-type DDO was 3 fold faster than wild-type R34 DDO, and the 2NT transformation rates of the I204L and I204Y DntAc variants were 3.5 and 3.25 fold faster than wild-type DDO (Table I).

In agreement with earlier studies with wild-type DDO (Parales et al., 1998a; Suen et al., 1996), 4-methylcatechol and 4-nitrobenzyl alcohol were detected from 4NT oxidation by both wild-type DDO and by the I204L DntAc variant

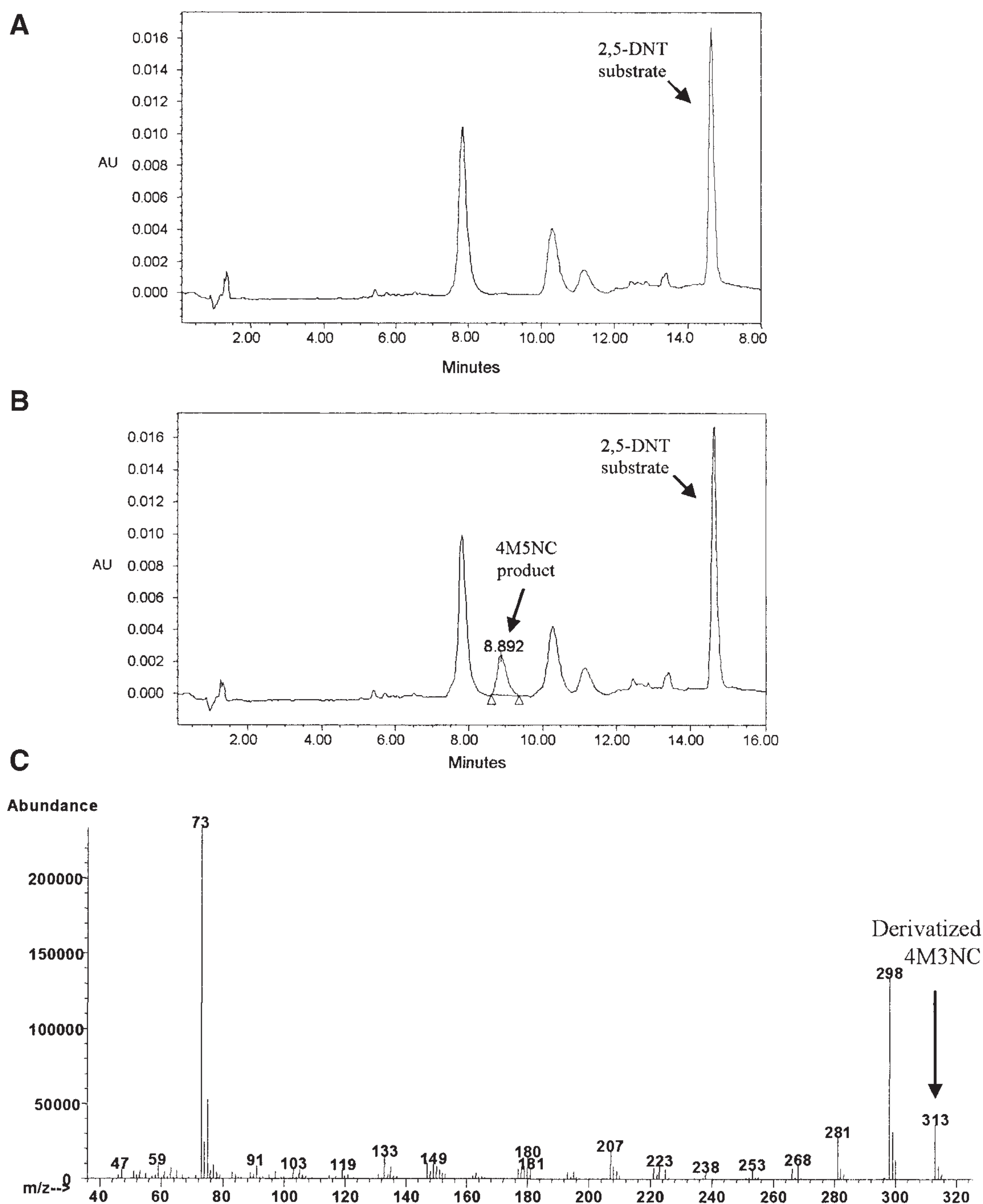

Figure 4. HPLC chromatograms of 2,5 DNT degradation (300 μ*M*) using wild-type DDO (**A**) and the I204L variant (**B**) after 60 min. (**C**) GC-MS fragmentation of the product 4M3NC from the degradation of 2,3-DNT.

(Fig. 1). Only 4-nitrobenzyl alcohol was detected from 4NT oxidation by wild-type R34 DDO and the I204Y DntAc variant. The 4-methylcatechol formation rate of I204L DntAc variant was 5.6 faster than wild-type DDO which was corroborated by the nitrite assay (7.6 fold, Table I). The 4-nitrobenzyl alcohol formation rate of wild-type R34 DDO was 2 fold faster than wild-type DDO, and the I204L DntAc variant had a similar rate of 4NT oxidation compared to wild-type DDO (Table I). The I204Y DntAc variant was a down variant for 4NT, but the DntAc I204L variant had elevated activity (3-6 fold) for both 2NT and 4NT.

Expression of DDO and Growth on 2,5-DNT

SDS-PAGE was used to determine the expression level of the enzymes (Sakamoto et al., 2001). The DDO DntAc I204L and I204Y variants had the same expression level as the pBS(Kan)DNT wild-type enzyme based on identification of the subunits DntAc (49 kDa) and DntAd (23 kDa); hence, the changes in rate and regiospecificity were due to changes in catalytic activity, not expression levels. Upon cloning DDO DntAc variant I204L into *B. cepacia* R34 using the broad-host-range vector pVLT31, we were unable to detect an

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         1                                                    50
DntAc    MSYQN..LVS EAGLTQKHLI YGDKELFQHE LKTIFARNWL FLTHDSLIPS
NahAc    MNYNNKILVS ESGLSQKHLI HGDEELFQHE LKTIFARNWL FLTHDSLIPA

         51                                                  100
DntAc    PGDYVKAKMG VDEVIVSRQN DGSVRAFLNV CRHRGKTIVD AEAGNAKGFV
NahAc    PGDYVTAKMG IDEVIVSRQN DGSIRAFLNV CRHRGKTLVS VEAGNAKGFV

         101                                                 150
DntAc    CGYHGWGYGS NGELQSVPFE KELYGDAIKK KCLGLKEVPR IESFHGFIYG
NahAc    CSYHGWGFGS NGELQSVPFE KDLYGESLNK KCLGLKEVAR VESFHGFIYG

         151                                                 200
DntAc    CFDAEAPPLI DYLGDVAWYL EPTFKHSGGL ELVGPPAKVV VKGNWKVFAE
NahAc    CFDQEAPPLM DYLGDAAWYL EPMFKHSGGL ELVGPPGKVV IKANWKAPAE

         201   I204                                          250
DntAc    NFVGDIYHIG WTHASTLRAG QAIFAPLAGN AMLPPEGTGL QATTKYGSGI
NahAc    NFVGDAYHVG WTHASSLRSG ESIFSSLAGN AALPPEGAGL QMTSKYGSGM

         251                                                 300
DntAc    GVSLDAYSGV QSADLVPEMM AFGGAKQEKL AKEIGDVRAR IYRSQVNGTV
NahAc    GVLWDGYSGV HSADLVPELM AFGGAKQERL NKEIGDVRAR IYRSHLNCTV

         301                                                 350
DntAc    FPNNCFLTGA GVFKVFNPID ENTTEAWTYA IVEKDMPEDL KRRLADAAQR
NahAc    FPNNSMLTCS GVFKVWNPID ANTTEVWTYA IVEKDMPEDL KRRLADSVQR

         351                                                 400
DntAc    STGPAGYWES DDNDNMVLS. QNAKKYQSSN SDLIADLGFG KDVYGDECYP
NahAc    TFGPAGFWES DDNDNMETAS QNGKKYQSRD SDLLSNLGFG EDVYGDAVYP

         401                                                 450
DntAc    GVVSKSAFSE TNHRGFYRAY QAHISSSNWA EFENTSRNWH TELTKTTDR
NahAc    GVVGKSAIGE TSYRGFYRAY QAHVSSSNWA EFEHASSTWH TELTKTTDR
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Figure 5. Alpha subunit amino acid sequence alignment of DDO (DntAc) and NDO (NahAc). Residue A206 of NDO is analogous to residue I204 of DDO as in bold and underlined. Amino acids that are different between DDO and NDO are highlighted in grey.

increase in cell number or mass using colonies, total protein, and absorbance (results not shown).

DntAc Modeling

To determine the impact of the amino acid substitutions on the active site structure, the program DeepView-Swiss-PdbViewer was used to make the model of DntAc DDO (451 amino acids) using the α subunit of NDO as a template. There is relatively high identity between DntAc DDO and the α subunit of NDO template, and the correct folds were generated as judged by the positions of the mono nuclear iron coordinating residues in DDO (H206, H211, and D360) compared to NDO: the distances between the respective C_{α} of the iron binding residues were less than 0.07 Å for all three residues from both enzymes. As shown in Figure 3, both mutations I204L and I204Y in DntAc may introduce a hydrogen bond (I204 in wild-type DDO does not have a hydrogen bond); the carbonyl group of L204 has a probable hydrogen bond with the amino group of I207 (distance 3.17 Å), and the carbonyl group of Y204 has another probable hydrogen bond with the amino group of I207 (distance 3.17 Å).

DNA Sequencing

We sequenced the entire wild-type *dntAaAbAcAd* locus from our cloning plasmid pBS(Kan)DNT as well as the entire *dntAaAbAcAd* locus from the original plasmid that was used to determine the DNA sequence from *Burkholderia* sp. strain DNT, pJS48 (Suen et al., 1996). The DDO DNA sequences from pBS(Kan)DNT and pJS48 are identical, so there were no changes in the DNA sequence due to cloning into pBS(Kan). However, nine sequencing errors were identified in the published DNA sequence of DDO, GenBank accession no. U62430 (Suen et al., 1996) that had a significant impact on the protein sequence. One nucleotide was found to be incorrectly inserted (at base pair position 873 of *dntAa* in GenBank accession no. U62430) and four nucleotides changes were found in the reductase gene *dntAa* (base pair

positions 875, 876, 878, and 879 of *dntAa* in GenBank accession no. U62430 should be changed from **TGCAT** to **GCCTA**). Removing the wrong nucleotide and correcting the four nucleotides resulted in 55 amino acid changes. One incorrectly inserted and one incorrectly deleted nucleotide were also found in the ferredoxin gene *dntAb* (between codons P15 and L31 of DntAb), and another two nucleotides changes were found in the *dntAd* gene (codons 42 and 43 of DntAd should be changed from GC**G C**AC to GC**C G**AC**)**. Each of the changes was verified by sequencing three times, and the corrected DNA sequences for *dntAa* (accession number AY936476), *dntAb* (accession number AY524770), and *dntAd* (accession number AY524771) have been deposited in GenBank. The 14 amino acids changes in DntAb (R16E, R18D, R19V, D20I, R21G, H22I, Q23N, Y24I, R25V, R26G, Q27K, G28E, D29I, and C30A) and the one amino acid change in DntAd (D43H) cause DDO to match the equivalent residues in both R34 DDO and NDO. With these DNA changes, the corrected DDO sequence now has 99.7% identity to the analogous DntAa of R34 DDO, 100% identity to the analogous DntAb of R34 DDO, and has 99.5% identity to the analogous DntAd of R34 DDO; hence, these enzymes are more evolutionarily-related than first indicated (DntAc is 93% identical). This corroborates the similar activities seen with these two enzymes for cresols, nitrophenols, and methoxyphenols (Keenan et al. 2004, unpublished).

DISCUSSION

These results show clearly that saturation mutagenesis of DDO may be used to create DntAc I204L and I204Y variants with enhanced 2,4-DNT and 2,6-DNT degradation, and that these variants are the first DDO that can transform 2,3-DNT to 4M3NC and 2,5-DNT to 4M5NC in contrast to wild-types DDO and R34 DDO which have no activity on these substrates. To our knowledge, this is the first degradation of 2,5-DNT using a specific enzyme. Variants DntAc I204L and I204Y have increased activity towards 2NT producing 2-nitrobenzyl alcohol, and I204L DntAc variant also has enhanced activity towards 4NT producing 4-methylcatechol and 4-nitrobenzyl alcohol, whereas the I204Y DntAc variant lost this activity (Table I). It is interesting that the I204Y mutation changes the mechanism from dioxygenation to monooxygenation with 4NT: both wild-type DDO and the I204L variant produce both 4-methylcatechol and 4-nitrobenzyl alcohol from 4NT oxidation whereas the I204Y variant produces only 4-nitrobenzyl alcohol. Similarly, the I204Y variant had elevated monooxygenation of 2NT (Table I). We have previously mutated positions I204, S349, and T350 in the DDO alpha subunit and found that the most important for DNT oxidation was position I204 (unpublished).

For 2,5-DNT oxidation, it was found that 4M5NC was the transformation product which is the same product as that derived from 2,4-DNT, the original sole carbon and energy source substrate of *Burkholderia* sp. strain DNT and *B. cepacia* R34. Hence, it is possible that after cloning the DDO DntAc variant I204L or I204Y into *Burkholderia* sp. strain DNT or *B. cepacia* R34, a strain may be created allowing growth on 2,5-DNT since the rest of the pathway is the same as the 2,4-DNT degradation pathway (Johnson et al., 2002; Nishino et al., 2000). However, cloning the DDO DntAc I204L variant into *B. cepacia* R34 using the broad-host-range vector pVLT31 did not result in growth on 2,5-DNT. Perhaps the problem lies in the toxicity of this compound and that it is very difficult to show growth on 2,4-DNT itself. However, this strain degrades 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT which might be useful for contaminated sites that contain mixtures of these compounds.

Although non-conserved loops are a major factor in model accuracy (Guex et al., 1999), the NDO NahAc template was used (78% amino acid identity to DDO DntAc) to yield a homology model which deviates by less than 0.07 Å. The model in Figure 3 shows the altered residues of DDO position I204 lie in a presumed substrate channel. It is interesting that although isoleucine and leucine have the same molecular weight and hydrophobicity, the leucine substitution caused the variant to have significant activity for 2,3-DNT and 2,5-DNT and enhanced activity for 2,4-DNT, 2,6-DNT, 2NT, and 4NT; hence, this is one of the first reports that shows a change in enzyme activity due to an amino acid substitution that does not alter the residue size and hydrophobicity. Beil et al. (1998) reported that the substitution of M220A at the analogous position in the α subunit of the toluene dioxygenase from *P. putida* F1 facilitates tetrachlorobenzene to access the active site; hence, substitution of a smaller and more hydrophobic residue was beneficial. Similarly, when smaller and more hydrophobic amino acids were substituted in the α subunit of BDO from *B. cepacia* LB400 (T335A/F336I/I341T, T335A/F336L/I341T, and T335A/F336L/N338T), there was enhanced activity for polychlorinated biphenyl degradation (Mondello et al., 1997).

Both the DntAc mutations I204L and I204Y in DDO may introduce a hydrogen bond which suggests that hydrogen bonding plays a role for enhancing in the activity for 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT since the hydrogen bond might make the substrate channel more rigid and might affect the substrate binding affinity as seen for ribulose biphosphate carboxylase/oxygenase (Bainbridge et al., 1998). It was reported that D137N variant of hypoxanthine phosphoribosyltransferases displays 40-fold reduction in k_{cat}/K_m for nucleotide formation with hypoxanthine as a substrate, and from the crystal structure, they showed that the Asn 137 side chain of the D137N variant does not form the same hydrogen bonds with the substrate as the wild-type enzyme (Canyuk et al., 2001; Xu and Grubmeyer, 1998). Disruption of hydrogen bonds may cause the important amino acids in the catalytic site to lose their catalytic activity (Bainbridge et al., 1998). Hence, forming hydrogen bonds seems to affect the activity of DDO. Clearly, further structural study would be required for more detailed analysis such as the substrate docking.

NITROAROMATIC NOMENCLATURE

DNT	dinitrotoluene
TNT	2,4,6-trinitrotoluene
NT	nitrotoluene
DDO	2,4-DNT dioxygenase of *Burkholderia* sp. strain DNT
R34 DDO	2,4-DNT dioxygenase of *Burkholderia cepacia* R34
DntAa	flavoprotein reductase
DntAb	iron-sulfur ferredoxin
DntAc	large (α) subunit of the iron-sulfur oxygenase
DntAd	small (β) subunit of the iron-sulfur oxygenase
4M5NC	4-methyl-5-nitrocatechol
3M4NC	3-methyl-4-nitrocatechol
4M3NC	4-methyl-3-nitrocatechol
NDO	naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4
BDO	biphenyl dioxygenase

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References

Anonymous. 1988. Health Effects Assessment for 2,4- and 2,6-Dinitrotoluene. U.S. Environmental Protection Agency EPA/600/8-88/032.

Anonymous. 1994. Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices for 1994–1995, Cincinnati, OH.

Bainbridge G, Anralojc PJ, Madgwick PJ, Pitts JE, Parry MAJ. 1998. Effect of Mutation of Lysine-128 of the Large Subunit of Ribulose Bisphosphate Carboxylase/Oxygenase from *Anacystis nidulans*. Biochem J 336:387–393.

Beil S, Mason JR, Timmis KN, Pieper DH. 1998. Identification of Chlorobenzene Dioxygenase Sequence Elements Involved in Dechlorination of 1,2,4,5-Tetrachlorobenzene. J Bacteriol 180(21):5520–5528.

Canada KA, Iwashita S, Shim H, Wood TK. 2002. Directed Evolution of Toluene *ortho*-Monooxygenase for Enhanced 1-Naphthol Synthesis and Chlorinated Ethene Degradation. J Bacteriol 184:344–349.

Canyuk B, Focia PJ, Eakin AE. 2001. The Role for an Invariant Aspartic Acid in Hypoxanthine Phosphoribosyltransferases Is Examined Using Saturation Mutagenesis, Functional Analysis, and X-ray Crystallography. Biochemistry 40:2754–2765.

Clayton GD, Clayton FE, editors. 1991. Patty's Industrial Hygiene and Toxicology. 4th editon. New York: John Wiley Sons.

de Lorenzo V, Eltis L, Kessler B, Timmis KN. 1993. Analysis of *Pseudomonas* Gene Products Using *lacI*^{q}/*Ptrp*-lac Plasmids and Transposons that Confer Conditional Phenotypes. Gene 123:17–24.

Dunnick JK, Burka LT, Mahler J, Sills R. 2003. Carcinogenic Potential of *o*-Nitrotoluene and *p*-Nitrotoluene. Toxicology 183:221–234.

Fishman A, Tao Y, Rui L, Wood TK. 2005. Controlling the Regiospecific Oxidation of Aromatics via Active Site Engineering of Toluene para-Monooxygenase of *Ralstonia pickettii* PKO1. J Biol Chem 280(1):506–514.

Gosselin RE, Hodge HC, Smith RP, Gleason MN. 1976. Clinical Toxicology of Commercial Products. Baltimore: Williams and Wilkins.

Guex N, Peitsch MC. 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18:2714–2723.

Guex N, Diemand A, Peitsch MC. 1999. Protein modeling for all. Trends Biochem Sci 24(9):364–367.

Johnson GR, Jain RK, Spain JC. 2002. Origins of the 2,4-Dinitrotoluene Pathway. J Bacteriol 184(15):4219–4232.

Karlsson A, Parales JV, Parales RE, Gibson DT, Eklund H, Ramaswamy S. 2003. Crystal Structure of Naphthalene Dioxygenase: Side-on Binding of Dioxygen to Iron. Science 299:1039–1042.

Kauppi B, Lee K, Carredano E, Parales RE, Gibson DT, Eklund H, Ramaswamy S. 1998. Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. Structure 6:571–586.

Keenan BG, Leungsakul T, Smets BF, Wood TK. 2004. Saturation mutagenesis of *Burkholderia cepacia* R34 2,4-DNT dioxygenase for synthesizing nitrohydroquinone, methylhydroquinone, and methoxyhydroquinone. Appl Environ Microbiol 70:3222–3231.

Kumamaru T, Suenaga H, Mitsuoka M, Watanabe T, Furukawa K. 1998. Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. Nat Biotechnol 16:663–666.

Lessner DJ, Johnson GR, Parales RE, Spain JC, Gibson DT. 2002. Molecular Characterization and Substrate Specificity of Nitrobenzene Dioxygenase from *Comamonas* sp. Strain JS765. Appl Environ Microbiol 68(2):634–641.

Mondello FJ, Turcich MP, Lobos JH, Erickson BD. 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl Environ Microbiol 63:3096–3103.

Nishino SF, Spain JC, Lenke H, Knackmuss H-J. 1999. Mineralization of 2,4- and 2,6-dinitrotoluene in soil slurries. Environmental Science and Technology 33:1060–1064.

Nishino SF, Paoli GC, Spain JC. 2000. Aerobic Degradation of Dinitrotoluenes and Pathway for Bacterial Degradation of 2,6-Dinitrotoluene. Appl Environ Microbiol 66(5):2139–2147.

Parales RE. 2003. The role of active-site residues in naphthalene dioxygenase. J Ind Microbiol Biotechnol 30:271–278.

Parales JV, Parales RE, Resnick SM, Gibson DT. 1998a. Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 is determined by the c-terminal region of the α subunit of the oxygenase component. J Bacteriol 180(5):1194–1199.

Parales RE, Emig MD, Lynch NA, Gibson DT. 1998b. Substrate Specificities of Hybrid Naphthalene and 2,4-Dinitrotoluene Dioxygenase Enzyme Systems. J Bacteriol 180(9):2337–2344.

Parales RE, Lee K, Resnick SM, Jiang H, Lessner DJ, Gibson DT. 2000a. Substrate Specificity of Naphthalene Dioxygenase: Effect of Specific Amino Acids at the Active Site of the Enzyme. J Bacteriol 182(6):1641–1649.

Parales RE, Resnick SM, Yu C-L, Boyd DR, Sharma ND, Gibson DT. 2000b. Regioselectivity and enantioselectivity of naphthalene dioxygenase during arene *cis*-dihydroxylation: control by phenylalanine 352 in the α subunit. J Bacteriol 182(19):5495–5504.

Peitsch MC. 1995. Protein modelling by e-mail. Bio/Technology 13:658–660.

Rui L, Kwon YM, Fishman A, Reardon KF, Wood TK. 2004. Saturation Mutagenesis of Toluene *ortho*-Monooxygenase of *Burkholderia cepacia* G4 for Enhanced 1-Naphthol Synthesis and Chloroform Degradation. Appl Environ Microbiol 70(6):3246–3252.

Rui L, Reardon KF, Wood TK. 2005. Protein Engineering of Toluene *ortho*-Monooxygenase of *Burkholderia cepacia* G4 for Regiospecific Hydroxylation of Indole to Form Various Indigoid Compounds. Appl Microbiol Environ 66:422–429.

Sakamoto T, Joern JM, Arisawa A, Arnold FH. 2001. Laboratory Evolution of Toluene Dioxygenase To Accept 4-Picoline as a Substrate. Appl Microbiol Biotechnol 67(9):3882–3887.

Sambrook J, Fritsch EF, Maniatis T, editors. 1989. Molecular Cloning:A Laboratory Manual. 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schwede T, Kopp J, Guex N, Peitsch MC. 2003. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385.

Spain JC, Nishino SF. 1987. Degradation of 1,4-Dichlorobenzene by a *Pseudomonas* sp. Appl Environ Microbiol 53(5):1010–1019.

Spanggord RJ, Spain JC, Nishino SF, Mortelmans KE. 1991. Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl Environ Microbiol 57(11):3200–3205.

Suen W-C, Spain JC. 1993. Cloning and characterization of *Pseudomonas* sp. strain DNT genes for 2,4-dinitrotoluene degradation. J Bacteriol 175(6):1831–1837.

Suen W-C, Haigler BE, Spain JC. 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J Bacteriol 178(16):4926–4934.
van Beilen JB, Duetz WA, Schmid A, Witholt B. 2003. Practical issues in the application of oxygenases. Trends in Biotechnology 21(4):170–177.
Xu Y, Grubmeyer C. 1998. Catalysis in Human Hypoxanthine-Guanine Phosphoribosyltransferase: Asp 137 Acts as a General Acid/Base. Biochemistry 37:4114–4124.
Yee DC, Maynard JA, Wood TK. 1998. Rhizoremediation of Trichloroethylene by a Recombinant, Root-Colonizing *Pseudomonas fluorescens* Strain Expressing Toluene *ortho*-Monooxygenase Constitutively. Appl Environ Microbiol 64(1):112–118.
Yu C-L, Parales RE, Gibson DT. 2001. Multiple mutations at the active site of naphthalene dioxygenase affect regioselectivity and enantioselectivity. Journal of Industrial Microbiology & Biotechnology 27:94–103.