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Protein Engineering of the 4-Methyl-5-Nitrocatechol Monooxygenase from *Burkholderia* sp. Strain DNT for Enhanced Degradation of Nitroaromatics

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4-Methyl-5-nitrocatechol (4M5NC) monooxygenase (DntB) from *Burkholderia* sp. strain DNT catalyzes the second step of 2,4-dinitrotoluene degradation by converting 4M5NC to 2-hydroxy-5-methylquinone with the concomitant removal of the nitro group. DntB is a flavoprotein that has a very narrow substrate range. Here, error-prone PCR was used to create variant DntB M22L/L380I, which accepts the two new substrates 4-nitrophenol (4NP) and 3-methyl-4-nitrophenol (3M4NP). At 300 μM of 4NP, the initial rate of the variant expressing M22L/L380I enzyme (39 ± 6 nmol/min/mg protein) was 10-fold higher than that of the wild-type enzyme (4 ± 2 nmol/min/mg protein). The values of k_{cat}/K_m of the purified wild-type DntB enzyme and purified variant M22L/L380I were 40 and 450 (s^{-1} M^{-1}), respectively, which corroborates that the variant M22L/L380I enzyme has 11-fold-higher efficiency than the wild-type enzyme for 4NP degradation. In addition, the variant M22L/L380I enzyme has fourfold-higher activity toward 3M4NP; at 300 μM, the initial nitrite release rate of M22L/L380I enzyme was 17 ± 4 nmol/min/mg protein, while that of the wild-type enzyme was 4.4 ± 0.7 nmol/min/mg protein. Saturation mutagenesis was also used to further investigate the role of the individual amino acid residues at positions M22, L380, and M22/L380 simultaneously. Mutagenesis at the individual positions M22L and L380I did not show appreciable enhancement in 4NP activity, which suggested that these two sites should be mutated together; simultaneous saturation mutagenesis led to the identification of the variant M22S/L380V, with 20% enhanced degradation of 4NP compared to the variant M22L/L380I. This is the first report of protein engineering for nitrite removal by a flavoprotein.

Nitroaromatic compounds are widely used as dyes, plasticizers, explosives, solvents, and pesticides (26) and are prevalent pollutants (31). 4-Nitrophenol (4NP) is a priority environmental pollutant found in industrial effluents and formed by the hydrolysis of parathion (15); 4NP exposure leads to human health problems (14). 3-Methyl-4-nitrophenol (3M4NP) is a toxic major breakdown product of fenitrothion that is used as an agricultural insecticide (1). 2,4-Dinitrotoluene (2,4-DNT) is the major intermediate from 2,4,6-trinitrotoluene production (27), is listed as a priority pollutant by the U.S. Environmental Protection Agency (27), and is an animal carcinogen (6).

Burkholderia sp. strain DNT was isolated from Waconda Bay near the Volunteer Army Ammunition Plant in Chattanooga, Tenn., and uses 2,4-DNT as a sole carbon and energy source (27). This strain initiates the degradation of 2,4-DNT using 2,4-DNT dioxygenase, which removes one nitro group to form 4-methyl-5-nitrocatechol (4M5NC) (21, 27). 4M5NC monooxygenase catalyzes the next step in the 2,4-DNT degradation pathway by converting 4M5NC to 2-hydroxy-5-methylquinone (Fig. 1) (11, 27).

4M5NC monooxygenase, encoded by *dntB*, uses flavin adenine dinucleotide (FAD) as a cofactor and NADPH as an electron donor (11). 4M5NC monooxygenase has a narrow substrate range; only 4M5NC and 4-nitrocatechol (4NC) serve as good substrates (11). The basic biochemistry and molecular genetics for the 4M5NC monooxygenase were previously described (11, 12, 29); however, its catalytic mechanism and substrate binding sites have not been discerned, since there is no crystal structure (existing crystal structures of flavoproteins match poorly).

Error-prone PCR (epPCR) and DNA shuffling are powerful mutagenesis methods used to rapidly generate variant gene products (3) that can be screened for desired phenotypes. Several investigations have described the use of epPCR for the mutagenesis of genes for hydroxylating oxygenases. For example, the substrate preference of the toluene dioxygenase from *Pseudomonas putida* was altered to accept 4-picolin as a substrate (23), alkane oxidation was enhanced in cytochrome P450 monooxygenase BM-3 from *Bacillus megaterium* (9), and the substrate preferences for 2-hydroxybiphenyl 3-monooxygenase from *Pseudomonas azelaica* HBP1 were expanded to include guaiacol, 2-hydroxybiphenyl (19), indole, and indole derivatives (20). epPCR was also used to tune the flavoenzyme vanillyl-alcohol oxidase from *Penicillium simplicissimum* (30); beneficial single-point mutations resulted in 40-fold-enhanced activity toward creosol. The crystal structure revealed that all the beneficial

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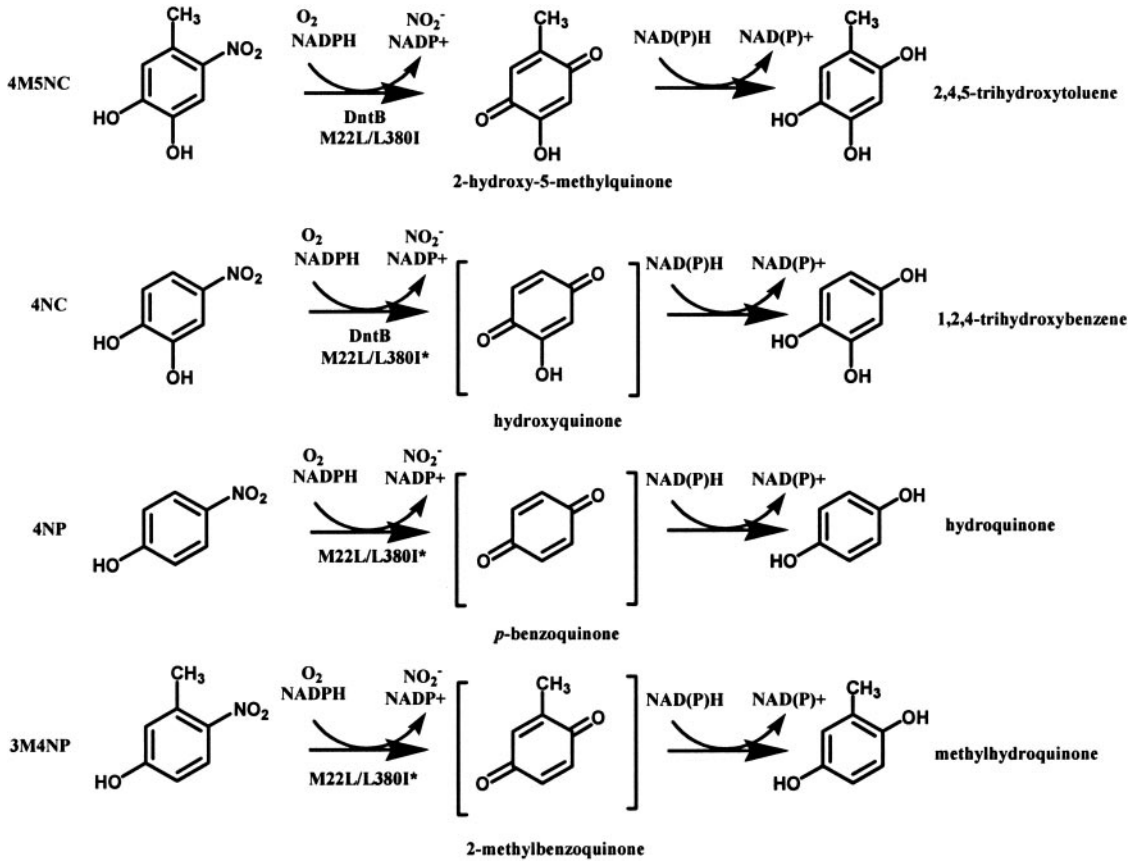

FIG. 1. Oxidation of 4M5NC, 4NC, 4NP, and 3M4NP by wild-type DntB and variant M22L/L380I. The proposed oxidation pathways of 4NC, 4NP, and 3M4NP by the DntB M22L/L380I variant are marked with asterisks. Oxidation of 4M5NC by wild-type DntB is based on Haigler et al. (11, 12). The retention time and maximum absorbance wavelength used in the HPLC analysis were 14.93 min and 245.3/353.1 nm for 4M5NC, 8.31 min and 264.4 nm for 2,4,5-trihydroxytoluene or 2-hydroxy-5-methylquinone, 12.0 min and 242.9/345.9 nm for 4NC, 4.6 min and 253.6 nm for 1,2,4-trihydroxybenzene, 15.4 min and 225.1/315.8 nm for 4NP, 3.8 min and 221.6/287.1 nm for hydroquinone, 19.1 min and 312.2 nm for 3M4NP, and 6.08 min and 288 nm for methylhydroquinone, respectively.

mutations were distant from sites for substrate interaction, which illustrates how the residues controlling catalytic activity are difficult to rationally predict (30).

DNA shuffling (28) is another method of directed evolution that has been used successfully to obtain enzymes that might improve the efficiency of biodegradative pathways. For example, toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 was evolved to increase the oxidation of both naphthalene and chlorinated ethenes (4), naphthalene dioxygenase from *Ralstonia* sp. strain U2 was evolved to recognize nitroaromatics (e.g., 4-amino-2-nitrotoluene) as substrates (16), and biphenyl dioxygenases from *B. cepacia* LB400 and *Pseudomonas pseudoalcaligenes* KF707 were evolved to extend the substrate specificity for polychlorinated biphenyls (2). DNA shuffling was also used recently for an extradiol ring-cleaving dioxygenase of *Pseudomonas* sp. strain C18, which is downstream in the naphthalene degradation pathway, to create a variant that had 770-fold-higher activity for cleaving 3,4-dihydroxybiphenyls (10).

Except for the aforementioned evolution of the extradiol ring-cleaving dioxygenase, most protein engineering has focused on the first enzyme in degradation pathways, but this is not a complete solution to improve the degradation pathway, since the other enzymes in the pathway might be inhibited by metabolites (10). After engineering the first enzyme in the 2,4-DNT pathway for degrading DNT (17), we report here the use of protein engineering for the evolution of the second step in the 2,4-DNT pathway, catalyzed by the flavoprotein DntB, for increasing its substrate range to include 4NP and 3M4NP, and we show that positions M22 and L380 of DntB are important for monooxygenase activity and substrate preference. Variants M22L/L380I and M22S/L380V were also identified as beneficial enzymes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* TG1 (24) was used to express the 4M5NC monooxygenase from pBS(Kan)DntB DNT under the control of a *lac* promoter. The strain was cultured from single colonies in 25 ml of Luria-Bertani (LB) medium (24) with 100 μg/ml kanamycin to maintain the plasmid and was incubated overnight at 37°C at 250 rpm. One milliliter of the overnight culture was used to inoculate 50 ml of LB with 100 μg/ml kanamycin and 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were grown exponentially to a turbidity at 600 nm of 1.5 to 2. Cultures harvested during the exponential phase were used for product identification and rate determinations. The cells were centrifuged at 13,000 × *g* for 7 min at 25°C in a Beckman (Palo Alto, CA) J2-HS centrifuge. The collected cells were resuspended in 100 mM sodium phosphate buffer (pH 6.5) at a turbidity of 3 to 5.

Chemicals. 1,2,4-Trihydroxybenzene, 2-chloro-4-nitrophenol (2C4NP), and guaiacol were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). FAD

TABLE 1. PCR primers used for constructing pBS(Kan)DntB DNT, for saturation mutagenesis of positions M22 and L380 of DntB, and for sequence analysis of the wild-type *dntB* and its variants

Purpose	Primer	Sequence[a]
Plasmid construction	DntB DNT front	5′-GCCAGGGTTTCGGCCCTA**GAATTC**CGTCATCCC-3′
	DntB DNT rear	5′-CGGCGGGCG**TCTAGA**GTGCTCCGCTCGGC-3′
Error-prone PCR	DntAa NotI Front	5′-AGTGAGCGCAAGCGGCCGCATGTGAGTTAGCTCACTC-3′
	pBSKan Rev	5′-GGGCCTCTTCGCTATTACGCCAGC-3′
Saturation mutagenesis	dntB DNT M22 front	5′-CCTCATCGTGGGCGGGAGC**NNS**GTCGGACTGTCC-3′
	dntB DNT M22 rear	5′-GAACAACGCCGTGGACAGTCCGAC**SNN**GCTCCCGCCC −3′
	dntB DNT L380 front	5′-CCTTCAGCCGCTACATCCGCCGG**NNS**GCACCCGAGTTCC −3′
	dntB DNT L380 rear	5′-GCTCGTCCAGGAACTCGGGTGC**SNN**CCGGCGGATGTAGC-3′
	DntBDNT M+L front	5′ GTCAGCGCCGGCTTCACTACCGACACCGCCGTCG 3′
	DntBDNT M+L rear	5′ CCGGACGAGTTCGACGGCGGTGTCGGTAGTGAAGC 3′
Sequence analysis	TpMO107FO	5′-GACCATGATTACGCCAAGCGCGC-3′
	DNT DNTB2	5′-GTCTCTCGCCGGTGGGGAAACGG-3′
	DNT DNTB3 new	5′-GCTTCACTACCGACACCGCCGTCG-3′
	DNTAcXba Rev	5′-AGGGTTTTCCCAGTCACG −3′
	SEQ PET Front upstream	5′-CATACCCACGCCGAAACAAG-3′

[a] Restriction and mutation sites are underlined and boldface. S indicates nucleotide C or G.

and NADPH were obtained from Alexis Biochemicals (San Diego, CA). 4NC, 4NP, 3M4NP, hydroquinone, *p*-benzoquinone, 2-methylbenzoquinone, and methylhydroquinone were obtained from Fisher Scientific Co. (Fairlawn, NJ). 4M5NC was synthesized previously by Spanggord et al. (27).

Construction of pBS(Kan)DntB DNT. The recombinant plasmid pBS(Kan)DntB DNT was constructed for inducible expression of 4M5NC monooxygenase from *dntB* (1.65 kb from pJS59) using the *lac* promoter. *dntB* was cloned into pBS (Kan) (4) using PCR with *Pfu* (Stratagene, La Jolla, CA) polymerase that consisted of an initial hot start at 96°C for 2 min, after which the polymerase was added, followed by 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 2 min (the final elongation was 72°C for 7 min). All the primers used in this work are listed in Table 1. The forward primer DntB DNT front generated the EcoRI site, and the reverse primer DntB DNT rear generated an XbaI site; after double digestion, the PCR fragment was cloned into the multiple cloning site of pBS(Kan). Prior to ligation of the insert and vector fragments, Antarctic phosphatase (New England Biolabs, Inc., Beverly, MA) was used to treat the vector fragment to avoid self-ligation and decrease the vector background. The resulting plasmid was electroporated into *E. coli* TG1 competent cells using a Bio-Rad (Hercules, CA) GenePulser/Pulse Controller at 15 kV/cm, 25 μF, and 200 Ω. To verify correct plasmid construction, several random colonies were used for plasmid isolation and restriction digests (EcoRI, XbaI, and RsrII), and the *dntB* insert was sequenced.

Error-prone PCR of *dntB*. epPCR (3) was performed to introduce random mutations throughout *dntB* (GenBank accession no. U68411). The two primers for cloning, DntAa NotI Front and pBSKan Rev (Table 1), were designed to utilize the two unique restriction sites, BglII and XbaI, that are upstream and downstream, respectively, from the *dntB* gene. For the low-fidelity reaction, the mixture (100 μl) contained pBS(Kan)DntB DNT template (100 ng), primers DntAa NotI Front and pBSKan Rev (30 pmol each), 0.2 mM (each) of dATP and dGTP, 1 mM (each) of dCTP and dTTP, 2.5 mM of $MgCl_2$, 0.7 mM of $MnCl_2$, and 5 U of *Taq* DNA polymerase (New England Biolabs, Inc., Beverly, MA). PCR (MJ Research [Watertown, MA] minicycler) was performed at 96°C for 2 min, with 30 cycles of 94°C for 1 min, 70°C for 1 min, and 72°C for 2 min, and a final extension of 72°C for 7 min. The resulting randomized PCR product (2,137 bp) was cloned into pBS(Kan) after double digestion of both vector and insert with EcoRI and XbaI and after Antarctic phosphatase treatment of the vector. The resulting plasmid library was transferred into *E. coli* TG1 competent cells using the electroporation method described above. The error rate was found to be 0.5 to 1.3% based on sequencing 450 bp from three random colonies.

Saturation mutagenesis at M22 and L380 of DntB. Saturation mutagenesis was performed at codons M22 and L380 of DntB. Thirty-two codons for all 20 amino acids were created at the target position by replacing the codon with NNS via overlap extension PCR (5).

The degenerate primers dntB DNT M22 front and dntB DNT M22 rear were designed to randomize position M22 in DntB. The two additional primers for cloning, DntAa NotI Front and pBSKan Rev, were chosen to utilize the two unique restriction sites, BglII and XbaI, that are upstream and downstream, respectively, from position M22. pBS(Kan)DntB DNT (100 ng) was used as the template in the initial PCR, and *Pfu* DNA polymerase was used in the PCR to minimize random point mutations. A 373-bp degenerate DNA fragment was amplified using primers DntAa NotI Front and dntB DNT M22 rear, and a 1,828-bp degenerate DNA fragment was amplified using dntB DNT M22 front and pBSKan Rev. After being purified 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 DntAa NotI Front and pBSKan Rev. PCR was performed at 96°C for 2 min, with 30 cycles of 94°C for 45 seconds, 80°C for 45 seconds, and 72°C for 2 min, and a final extension of 72°C for 7 min. The resulting randomized PCR product (2,137 bp) was cloned into pBS(Kan) after double digestion of both vector and insert with BglII and XbaI and after Antarctic phosphatase treatment of the vector. The resulting plasmid library was transferred into competent *E. coli* TG1 cells as described above.

A similar approach was used for saturation mutagenesis at L380 of DntB. The degenerate primers dntB DNT L380 front and dntB DNT L380 rear were designed to mutagenize position L380 in DntB. A 1,445-bp degenerate DNA fragment was amplified using primers DntAa NotI Front and dntB DNT L380 rear, and a 739-bp degenerate DNA fragment was amplified using dntB DNT L380 front and pBSKan Rev.

Saturation mutagenesis was also performed simultaneously at M22 and L380 of DntB. To combine the fragments containing the randomized positions M22 and L380, two internal primers, DntBDNT M+L front and DntBDNT M+L rear, were designed that allowed the individual saturation mutagenesis PCR products of both positions (1,111 bp for M22 and 1,069 bp for L380) to be combined due to overlap of 44 bases; these two primers replaced pBSKan Rev and DntAa NotI Front, which were used for saturation mutagenesis for M22 and L380, respectively. The two additional primers for cloning, DntAa NotI Front and pBSKan Rev, were chosen to utilize the two unique restriction sites, BglII and XbaI, that are upstream and downstream, respectively, from positions M22 and L380.

His-tagged protein purification. To purify the 4M5NC monooxygenases (the wild type and its variant M22L/L380I) using a polyhistidine tag, genes encoding both 4M5NC monooxygenases were amplified from the pBS(Kan) vectors by PCR using primers DntB DNT pET front and DntB DNT pET rear (Table 1) under the same thermal amplification profile used for pBS(Kan)DntB DNT. The PCR products were cleaved with NdeI and HindIII and inserted into pET28b(+) (Novagen, Madison, WI) to generate pET-DntB and pET-M22L/L380I, which yielded a 6-amino-acid His tag, as well as a thrombin cleavage site at the N termini of the 4M5NC monooxygenases (in total, 20 amino acids, NH_3^+-MGS SHHHHHHSSGLVPRGSH, were added to the complete wild-type amino acid sequence). Both genes were sequenced using primer SEQ PET Front upstream (Table 1) to confirm that there was no amino acid change in the recombinant plasmids at the N termini of the 4M5NC monooxygenases. To overexpress the

4M5NC monooxygenases, pET-DntB and pET-M22L/L380I were transformed into *E. coli* BL21(DE3) (Novagen, Madison, WI) by electroporation.

Both wild-type 4M5NC monooxygenase and its variant M22L/L380I with a His tag were purified using an Ni-nitrilotriacetic acid column (Invitrogen, Carlsbad, CA). The strains were initially inoculated into 25 ml of LB medium containing kanamycin (100 μg/ml) from single colonies and cultured overnight at 37°C and 250 rpm. Eight milliliters was then inoculated into 1 liter of fresh LB medium with the same antibiotic and incubated at 37°C. IPTG (4 mM) was added at a cell optical density at 600 nm (OD_{600}) of ~0.4 to induce the T7 promoter, and the cultures were incubated at 37°C for 6 h. Cells (1 liter) were harvested by centrifugation at 10,000 × *g* and 4°C for 10 min and resuspended in 10 ml of the purification buffer (50 mM NaH_2PO_4, pH 8.0, 0.5 M NaCl, 60 μM of FAD). Cell lysis was performed by sonication using the Braunsonic 1510 sonicator at 20 cycles for 10 s at 10 W with 10-s intervals (B. Braun Medical Inc., Bethlehem, PA), and the cells were centrifuged at 20,000 × *g* and 4°C for 1 h to get rid of the cell debris. The supernatants containing the soluble 4M5NC monooxygenases were loaded into a 10-ml Ni-nitrilotriacetic acid column for purification. His-tagged enzymes were eluted with the purification buffer with imidazole (250 mM) and dialyzed against TE buffer (100 mM Tris-Cl, 0.2 mM EDTA, 60 μM of FAD) overnight and then dialyzed against storage buffer (10 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, 60 μM of FAD, and 20% glycerol, pH 7.5). FAD was added (3.5× molar excess based on 1 mol FAD/mol protein) to all the buffers to avoid losing FAD during protein purification (18).

The concentrations of the purified enzymes were evaluated by comparing standard protein samples (Precision plus protein standards; Bio-Rad Laboratories, Hercules, CA) of known concentration using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24). To corroborate these values, a method based on spectrophotometric measurement of the purified enzymes in 6 M guanidine at 280 nm was used (8). The concentrations of His-DntB and His-M22L/L380I were 0.25 μg/μl and 1 μg/μl, respectively, which were used for the calculation of the specific activities of enzymes.

Molecular techniques. Plasmid DNA was isolated using a Midi or Mini Kit (QIAGEN, Inc., Valencia, CA) and digested by the restriction enzymes from New England Biolabs, Inc. (Beverly, MA). DNA fragments were isolated from agarose gels using a QIAquick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). Ligation reactions were performed at 16°C for 20 h (24) with a 3-to-1 molar ratio (insert-vector). T4 DNA ligase and 10× T4 DNA ligase buffer were from New England Biolabs, Inc. (Beverly, MA).

Colony screening and nitrite detection. A nylon membrane agar colony-screening method (17) was used with whole cells expressing the evolved enzymes with 500 μM of 4NC, 500 μM of 4NP, 1 mM of 3M4NP, 1 mM of 2C4NP, or 1 mM of guaiacol. The screened plates were incubated overnight at 37°C.

Monooxygenase activity was measured indirectly from the nitrite that accumulated due to substrate hydroxylation (Fig. 1). Nitrite concentrations were determined by combining reaction supernatants with sulfanilamide and *N*-(1-naphthyl)ethyldiamine and then measuring the colored complex that formed spectrophotometrically (7). Purified enzymes (100 μg) were added to 5 ml of sodium phosphate buffer (100 mM; pH 6.5) and were incubated with 300 μM of 4M5NC, 4NC, 4NP, or 3M4NP with 300 μM of NADPH for 5 to 60 min at 250 rpm at 37°C. The sulfanilamide and *N*-(1-naphthyl)ethyldiamine reagents were added in equal volumes of 100 μl to 1 ml of the sample. The formation of azo dye was detected at 543 nm after 10 min of incubation at room temperature. The nitrite concentration was determined using a linear calibration curve (0.72 to 145 μM). At least three time points from the linear parts of the nitrite release rate curves were used for the calculation of specific initial rates. Two replicates of both wild-type enzyme and the enzyme variants were analyzed. To obtain the apparent V_{max}, K_m, and k_{cat} values for 4NP oxidation by both purified wild-type DntB and the M22L/L380I variant at 300 μM of NADPH, 4NP was used at 50, 100, 200, 300, 500, and 1,000 μM. The steady-state kinetic parameters (apparent V_{max} and K_m) were determined based on a Michaelis-Menten kinetic equation plot.

Variants M22L, L380I, M22L/L380M, and M22S/L380V were evaluated using nitrite released from the oxidation of 4NP by whole cells; cells were incubated with 500 μM of 4NP at a cell density (OD_{600}) of 3.0 to 5.0 in sodium phosphate buffer (100 mM, pH 6.5) for 5 to 60 min at 250 rpm at 37°C. Rates from whole cells were determined with a protein concentration of 0.22 mg protein/ml/OD_{600} unit (16). Two or three replicates of both wild-type enzymes and the enzyme variants were analyzed.

Product identification by HPLC. Reverse-phase high-performance liquid chromatography (HPLC) was used to identify the products from 4NC, 4NP, and 3M4NP. Samples were prepared in the same way as the samples for nitrite assay. The samples were incubated for 60 min at 250 rpm at 37°C. Samples (20 μl) were collected and analyzed using HPLC with a Zorbax SB-C8 column (Agilent Technologies; 5 μm, 4.6 by 250 mm) and a Waters Corporation (Milford, MA) 515 solvent delivery system. Compounds were detected by a photodiode array detector (Waters 996) and injected by an autosampler (Waters 717 plus). A gradient elution was used with H_2O (0.1% formic acid) and acetonitrile (75:25 from 0 to 3 min, 50:50 at 18 min, and 75:25 at 25 min) as the mobile phase at 1.5 ml/min. The compounds were identified by comparison of the retention time and UV-visible spectra to those of authentic standards, as well as by coelution with the standards. Two or three replicates of both the wild-type enzyme and the enzyme variant were analyzed.

DNA sequencing. A dye terminator chain-sequencing technique based on the dideoxy method of sequencing DNA (25) was used with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Wellesley, MA). A PE Biosystems ABI 373 DNA sequencer (Perkin-Elmer) was used to determine the nucleotide sequence of the *dntB* gene for both wild-type DntB and its variants. Vector NTI software (InfoxMax, Inc., Bethesda, MD) was used to analyze the sequencing data. The primers shown in Table 1 were used to sequence the complete *dntB* gene; all sequencing was performed at least two times.

Nucleotide sequence accession number. The corrected DNA sequences for *dntB* have been deposited in GenBank (accession number DQ298257).

RESULTS

Protein engineering of DntB. To expand the substrate specificity of 4M5NC monooxygenase, epPCR was performed, and 3,000 colonies were screened for activity on 4NP. The 4M5NC monooxygenase variant M22L/L380I was identified, since the colony expressing this enzyme turned dark brown after overnight incubation at 37°C on the 4NP screening plate whereas the wild-type colony turned only light brown. The variant M22L/L380I was the only colony found that turned dark brown on the 4NP screening plate. The negative control *E. coli* TG1/pBS(Kan) had no color, so background *E. coli* oxygenases have no activity on this substrate. The dark-brown color arises from the intracellular dihydroxylated product (in this case, hydroquinone) (Fig. 1), which auto-oxidizes to quinones upon secretion (17). DNA sequencing indicated there were two amino acid codon changes, M22L and L380I, in *dntB*.

After the variant M22L/L380I was identified, saturation mutagenesis at M22 and L380 of DntB individually was performed to see if better enzymes could be obtained. Variants M22L and M22S and variants L380V, L380I, and L380M were identified after 300 colonies were screened to ensure that all possible variants were checked (22) on the 4NP screening plate from each library of saturation mutagenesis at M22 and L380, respectively. Colonies expressing these variants were darker brown than the wild type, but not as brown as cells expressing variant M22L/L380I, which indicated that although both positions M22L and L380I affect enzyme activity, both mutations in these residues are required to obtain activity significantly greater than that of wild-type DntB.

To try all possible amino acids at both positions M22 and L380 of DntB, simultaneous saturation mutagenesis was performed. A saturation mutagenesis library of 650 variant colonies was screened to ensure that all of the possible variants were checked at both positions (22). DntB variants M22L/L380M and M22S/L380V were identified, since the colonies from which they were obtained both had a dark-brown color as intense as that of the DntB variant M22L/L380I on the 4NP screening plates. For the 3M4NP screening plates, the same trend in color formation was seen, although there was lighter color. There was no difference in the color formed between

TABLE 2. Initial rates of nitrite release for substrates 4M5NC, 4NC, 4NP, and 3M4NP by purified wild-type DntB DNT and purified M22L/L380I

Substrate (300 μM)	Rate (nmol/min/mg protein)		Relative[a]
	Wild-type DntB	M22L/L380I	
4M5NC	41 ± 4	58 ± 2	1.4
4NC	31 ± 3	49 ± 1	1.6
4NP	4 ± 2	39 ± 6	9.8
3M4NP	4.4 ± 0.7	17 ± 4	3.9

[a] Relative to wild-type DntB DNT.

colonies expressing wild-type DntB and its variants using the 4NC, 2C4NP, and guaiacol screening plates.

HPLC identification of the 4NC, 4NP, and 3M4NP transformations. HPLC analysis was used to identify the oxidation products of purified wild-type DntB and its variant, M22L/L380I. 1,2,4-Trihydroxybenzene was detected from 4NC oxidation by both wild-type DntB and variant M22L/L380I. Hydroquinone and methylhydroquinone were detected from oxidation of 4NP and 3M4NP, respectively, by the purified M22L/L380I enzyme; hence, activity toward these substrates was achieved for the first time with the purified variant M22L/L380I, since no oxidation products were detected for both reactions with purified wild-type DntB. Hydroquinone and methylhydroquinone were also detected on the time scale of these reactions from the intermediates *p*-benzoquinone and 2-methylbenzoquinone, respectively, with or without any enzyme in the reaction mixtures, which indicates that *p*-benzoquinone and 2-methylbenzoquinone are not stable under these conditions.

Nitrite detection from oxidation of nitroaromatics. Nitrite is released from the degradation of 4M5NC by DntB (11), and 4NC was reported to be the only other compound that could serve as a substrate for the wild-type enzyme (11). In this work, we confirmed that nitrite is derived from the degradation of 4M5NC and 4NC using purified wild-type DntB. More importantly, we found that variant M22L/L380I also generates nitrite from the degradation of the two new substrates 4NP and 3M4NP (Fig. 1). The purified variant M22L/L380I oxidized 300 μM 4NC 1.6-fold faster than purified wild-type DntB (Table 2). For 4NP and 3M4NP oxidation, the purified M22L/L380I enzyme oxidized 4NP and 3M4NP 9.8- and 3.9-fold faster, respectively, than purified wild-type DntB at 300 μM (Table 2).

To compare the M22L/L380I variant with the wild-type enzyme, apparent V_{max}, K_m, and k_{cat} values for 4NP oxidation were determined (Table 3). For the purified wild-type DntB enzyme, the apparent V_{max}, K_m, and k_{cat} values were 14 nmol/min/mg protein, 350 μM, and 0.014 s^{-1}, respectively. For the purified variant M22L/L380I enzyme, the apparent V_{max}, K_m, and k_{cat} values were 44 nmol/min/mg protein, 100 μM, and 0.045 s^{-1}, respectively. Therefore, the k_{cat}/K_m values of the purified wild-type DntB enzyme and purified variant M22L/L380I were 40 and 450 (s^{-1} M^{-1}), respectively, which shows that the M22L/L380I variant has 11-fold-higher efficiency than the wild-type enzyme for 4NP degradation. It was found that DntB was inhibited at concentrations of 4NP higher than 1 mM.

TABLE 3. Kinetic values for the purified wild-type DntB and M22L/L380I enzymes toward 4NP[a]

Enzyme	Apparent V_{max} (nmol/min/mg protein)	Apparent K_m (μM)	Apparent k_{cat} (s^{-1})	Apparent k_{cat}/K_m (s^{-1} M^{-1})
Wild-type DntB	14 ± 3	350 ± 75	0.014 ± 0.004	40 ± 14
M22L/L380I	44 ± 2	100 ± 5	0.045 ± 0.003	450 ± 38

[a] At 300 μM of NADPH. The rates were determined from nitrite release at 50, 100, 200, 300, 500, and 1,000 μM 4NP concentrations based on a Michaelis-Menten model.

TABLE 4. Initial rates of nitrite release for the substrate 4NP[a]

Strain	Codon change	Rate (nmol/min/mg protein)	Relative[b]
Wild-type DntB		0.2 ± 0.1	1.0
M22L	ATG to TTG	0.1 ± 0.1	0.5
L380I	CTC to ATC	0.2 ± 0.2	1.0
M22L/L380I	ATG to TTG/CTC to ATC	0.5 ± 0.4	2.5
M22L/L380M	ATG to TTG/CTC to ATG	0.1 ± 0.1	0.5
M22S/L380V	ATG to TCG/CTC to GTG	0.6 ± 0.6	3.0

[a] At 500 μM by *E. coli* TG1 expressing wild-type DntB DNT and its variants M22L, L380I, M22L/L380I, M22L/L380M, and M22S/L380V.
[b] Relative to wild-type DntB.

Since 4M5NC is the natural substrate of DntB (29), its rate of oxidation was also checked via nitrite release for both purified wild-type DntB enzyme and its variant, M22L/L380I. The initial rate of 4M5NC oxidation by the purified variant M22L/L380I enzyme was 1.4-fold higher than that of the wild-type DntB enzyme at 300 μM substrate concentration (Table 2).

The initial nitrite release rates of whole cells expressing the variants M22L, L380I, M22L/L380M, and M22S/L380V were also determined to compare them to the activities of variant M22L/L380I and wild-type DntB using 500 μM of the substrate (Table 4). Variants M22L, L380I, and M22L/L380M had 5-, 2.5-, and 5-fold-lower rates of oxidation of 4NP than the variant M22L/L380I, and variant M22S/L380V had about 20% greater initial rate of nitrite release from the oxidation of 4NP than the variant M22L/L380I. Hence, a new enzyme variant, M22L/L380I, was discovered that has elevated nitrite release for 4NP and 3M4NP, and simultaneous saturation mutagenesis created an even better variant (M22S/L380V). To ensure that the differences in whole-cell activities were not due to differences in expression, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed, which indicated that the variants M22L, L380I, M22L/L380I, M22L/L380M, and M22S/L380V all had the same expression level as wild-type DntB (data not shown).

DNA sequencing. We sequenced the entire wild-type *dntB* gene from our cloning plasmid pBS(Kan)DntB, as well as the entire *dntB* gene from the original plasmid (pJS59) that was used to determine the DNA sequence from *Burkholderia* sp. strain DNT. Plasmid pJS59 is derived from pJS53 (29). The *dntB* DNA sequences from pBS(Kan)DntB and pJS59 are identical, so there were no changes in the DNA sequence due to cloning into pBS(Kan). However, two sequencing errors were

identified in the published DNA sequence of *dntB*, GenBank accession number U68411 (12), that had an impact on the protein sequence. The two nucleotide changes at base pair positions 934 and 935 of *dntB* in U68411 should be changed from ***CG***C to ***GC***C, which results in an amino acid change in DntB from arginine to alanine (R312A). These changes were verified by sequencing at least five times.

DISCUSSION

In this study, we used epPCR mutagenesis to identify amino acid residues in the 4M5NC monooxygenase from strain DNT that contribute to catalytic selectivity by increasing activity for the substrates 4NP, 3M4NP, 4NC, and 4M5NC. Previously, it was reported that hydroxyl groups *meta* and *para* to the nitro group are critical to the catalytic reaction of wild-type DntB (12). However, the engineered DntB enzymes (unlike wild-type DntB) are able to degrade 4NP and 3M4NP, which lack the hydroxyl group at the *meta* position to the nitro group (Fig. 1); hence, the *meta* hydroxyl may be made dispensable through engineering. The hydroxyl group *para* to the nitro group is critical for enzyme activity, since this position is essential for quinone formation after removal of the nitro group (12). In contrast, the methyl group in substrates 4M5NC and 3M4NP is not essential for activity, since the wild-type enzyme has activity toward both 4M5NC and 4NC and the M22L/L380I variant has activity toward both 3M4NP and 4NP.

From HPLC product identification, hydroquinone and methylhydroquinone were detected from 4NP and 3M4NP oxidation, respectively, by variant M22L/L380I but not from wild-type DntB. However, released nitrite was detected from the 4NP and 3M4NP oxidation by both the variant M22L/L380I and wild-type DntB enzymes. This discrepancy might arise because of the instability and very small quantities of hydroquinone and methylhydroquinone formed from wild-type DntB compared to that of variant M22L/L380I. The proposed pathways for 4NC, 4NP, and 3M4NP degradation by variant M22L/L380I are shown in Fig. 1. It was reported previously that 4NC was a substrate of DntB, but the reaction has not been characterized (12). Here, we postulate that hydroquinone, *p*-benzoquinone, and 2-methylbenzoquinone are the intermediates in the conversion of 4NC to 1,2,4-trihydroxytoluene, 4NP to hydroquinone, and 3M4NP to methylhydroquinone, respectively (Fig. 1). These deductions are based on direct observation of 1,2,4-trihydroxytoluene, hydroquinone, and methylhydroquinone in the reactions with the purified enzymes with 4NC, 4NP, and 3M4NP and also on the fact that *p*-benzoquinone and 2-methylbenzoquinone are reduced to hydroquinone and methylhydroquinone, respectively, in the presence of NADPH with or without the enzymes. Also, similar reactions were seen for 2NP and 4NP oxidation by *Moraxella* sp. (26, 31); however, the accumulation of hydroxyquinone, *p*-benzoquinone, and 2-methylbenzoquinone was not detected.

Our results show that to improve the activity of DntB, both mutations M22L and L380I were necessary, since the individual mutations at M22L and L380I did not bring appreciable improvement in 4NP oxidation, but mutating the two residues simultaneously resulted in a 10-fold enhancement in activity (Table 2). By determining the crystal structure, perhaps structure-function relationships could be deduced for these two residues; however, it is just as likely that these beneficial mutations are far from the active site (30). This new variant, M22L/L380I, not only exhibits a wider range of oxygenation capabilities, it also maintains the original activity with small improvements for 4M5NC (1.4-fold) and 4NC (1.6-fold) degradation (Table 2). Our previous work using saturation mutagenesis had already enhanced 2,4-DNT dioxygenase activity (the first step in the 2,4-DNT degradation pathway) to create the DntAc variant I204L of strain DNT; this evolved enzyme degrades 2,4-DNT to 4M5NC twofold faster than wild-type 2,4-DNT dioxygenase, as well as having novel activity on 2,3-DNT and 2,5-DNT (17). *Burkholderia* sp. strain DNT accumulates 4M5NC during growth on 2,4-DNT (the culture turns yellow [unpublished results]), suggesting that the DntB step is rate limiting in this pathway. Therefore, adding variant M22L/L380I, which has 1.4-fold-faster 4M5NC degradation, to the original *Burkholderia* sp. strain DNT may help to improve the overall rate of 2,4-DNT degradation, and further screening may yield beneficial mutants for 4M5NC oxidation.

In this work, we focused on studying 4M5NC monooxygenase from *Burkholderia* sp. strain DNT; however, there is another 4M5NC monooxygenase that was discovered previously from *Burkholderia cepacia* R34, which also oxidizes 4M5NC (13). Though these two enzymes have similar enzyme activities, they have significant differences in protein sequence, since they share only 50% of their amino acid sequences (13); hence, it may be interesting to perform mutagenesis on this enzyme as well.

Though the wild-type 4M5NC monooxygenase has a limited substrate range for bioremediation, we showed in this work that this substrate limitation could be overcome by protein engineering. The M22L/L380I variant now accepts 4NP and 3M4NP as substrates, which may be useful for bioremediation, since 4NP and 3M4NP are priority pollutants.

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