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Selection for Growth on 3-Nitrotoluene by 2-Nitrotoluene-Utilizing *Acidovorax* sp. Strain JS42 Identifies Nitroarene Dioxygenases with Altered Specificities

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***Acidovorax* sp. strain JS42 uses 2-nitrotoluene as a sole source of carbon and energy. The first enzyme of the degradation pathway, 2-nitrotoluene 2,3-dioxygenase, adds both atoms of molecular oxygen to 2-nitrotoluene, forming nitrite and 3-methylcatechol. All three mononitrotoluene isomers serve as substrates for 2-nitrotoluene dioxygenase, but strain JS42 is unable to grow on 3- or 4-nitrotoluene. Using both long- and short-term selections, we obtained spontaneous mutants of strain JS42 that grew on 3-nitrotoluene. All of the strains obtained by short-term selection had mutations in the gene encoding the α subunit of 2-nitrotoluene dioxygenase that changed isoleucine 204 at the active site to valine. Those strains obtained by long-term selections had mutations that changed the same residue to valine, alanine, or threonine or changed the alanine at position 405, which is just outside the active site, to glycine. All of these changes altered the regiospecificity of the enzymes with 3-nitrotoluene such that 4-methylcatechol was the primary product rather than 3-methylcatechol. Kinetic analyses indicated that the evolved enzymes had enhanced affinities for 3-nitrotoluene and were more catalytically efficient with 3-nitrotoluene than the wild-type enzyme. In contrast, the corresponding amino acid substitutions in the closely related enzyme nitrobenzene 1,2-dioxygenase were detrimental to enzyme activity. When cloned genes encoding the evolved dioxygenases were introduced into a JS42 mutant lacking a functional dioxygenase, the strains acquired the ability to grow on 3-nitrotoluene but with significantly longer doubling times than the evolved strains, suggesting that additional beneficial mutations occurred elsewhere in the genome.**

Nitroaromatic compounds are toxic and stable in the environment and are important components in the manufacture of dyes, polymers, explosives, and pesticides (1). The synthesis and widespread use of these products have caused environmental contamination of soil and groundwater (2, 3). In addition, nitroaromatic compounds tend to undergo reduction, resulting in the formation of mutagenic aromatic amines (4). Due to the stability, toxicity, and mutagenicity of these chemicals, the U.S. Environmental Protection Agency has designated several nitroaromatic compounds as priority pollutants (5).

Although the majority of nitroarene compounds are anthropogenic, a number of microorganisms have developed the ability to utilize compounds of this chemical class as sources of carbon, nitrogen, and energy (6). Bacterial strains capable of growth on nitrobenzene (7–9), mononitrotoluenes (10–16), and dinitrotoluenes (17–19) have been isolated from various contaminated environments. For example, *Acidovorax* sp. strain JS42 (formerly *Pseudomonas* sp. strain JS42) uses nitrobenzene (NB) and 2-nitrotoluene (2NT) as sole sources of carbon, nitrogen, and energy (15, 20), while *Comamonas* sp. strain JS765 is capable of growth on NB and 3-nitrotoluene (3NT) (9, 21). The first enzymes of each degradation pathway, 2-nitrotoluene 2,3-dioxygenase (2NTDO) (in strain JS42) and nitrobenzene 1,2-dioxygenase (NBDO) (in strain JS765), are homologous multicomponent enzyme systems that add both atoms of molecular oxygen to nitroarene substrates, forming nitrite and (methyl)catechol. Both enzyme systems are comprised of identical iron-sulfur flavoprotein reductase and Rieske iron-sulfur ferredoxin components that transfer electrons from NAD(P)H to the catalytic oxygenase components, which are 95% identical in amino acid sequence (22). Key amino acid differences at the active sites of 2NTDO and NBDO confer different substrate preferences and result in the formation of different products (21, 23). Although both enzymes can oxidize NB and all three mononitrotoluene isomers to (methyl)catechols that serve as growth substrates for both strains, *Acidovorax* sp. strain JS42 is unable to grow on either 3NT or 4-nitrotoluene (4NT) (15), and *Comamonas* sp. strain JS765 is unable to grow on 2NT or 4NT (9).

In this study, we used *Acidovorax* sp. JS42 as a model system to study how enzymes and pathways change when the organism is challenged with new growth substrates. Sequence analyses suggested that the genes encoding 2NTDO in strain JS42 evolved and diverged under selection from genes encoding a naphthalene dioxygenase such as that from *Ralstonia* sp. strain U2 (24). As a way

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to understand how enzymes and pathways change over time, we placed JS42 cells under selection, requiring them to use 3NT as a sole source of carbon and energy. Here we describe the isolation and characterization of evolved variants of JS42 capable of growth on 3NT and analysis of their evolved dioxygenases.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are shown in Table 1. The rich media used were LB (25) for *Escherichia coli* strains and tryptone-yeast extract (TY) medium (1% tryptone, 0.5% yeast extract; Difco, Sparks, MD) for *Acidovorax* strains. Rich media were solidified with 1.6% (wt/vol) agar (Fisher Scientific, Pittsburg, PA). Minimal MSB medium (26) was supplemented with 10 mM glucose and 1 mM thiamine for the growth of *E. coli* or 10 mM sodium succinate and 1% (vol/vol) Balch's vitamins (27) lacking *p*-aminobenzoate and thiamine for growth of *Acidovorax* strains. For plates, MSB medium was solidified with 1.8% (wt/vol) Noble agar (Difco). NB (99%), 2NT (99%), and 4NT (99%) were obtained from Acros Organics (Morris Plains, NJ), and 3NT (99%) was obtained from Avocado (Heysham, Lancashire, United Kingdom). Growth rates of *Acidovorax* strains on nitroaromatic compounds were determined by using screw-cap tubes containing 5 ml MSB medium; 1 mM 2NT, 3NT, 4NT, or NB; and 0.01% yeast extract. Tubes were incubated at 30°C in a TC-7 roller drum (New Brunswick) at 70 rpm, and turbidity was monitored as the optical density at 660 nm (OD_{660}). When needed, gentamicin and ampicillin were provided at 10 and 150 μg/ml, respectively. For purification of reductase, *Escherichia coli* BL21(DE3)(pDTG871) was grown as described previously (22). For purification of ferredoxin and oxygenase components, DH5α(pKMM1), VJS415(pKMM30), and DH5α(pKMM31) through DH5α(pKMM35) were grown overnight at 30°C in 4 liters of TB Dry broth (Mo Bio Laboratories, Inc., Carlsbad, CA) containing 50 μg/ml kanamycin. DH5α(pDTG904) (28) was used for partial purification of catechol 2,3-dioxygenase (CDO); the strain was grown in 1 liter of LB supplemented with 100 μg/ml ampicillin at 30°C in order to obtain cell extracts.

Selection of $3NT^+$ mutants of *Acidovorax* sp. strain JS42. Variants of *Acidovorax* sp. JS42 that grew on 3NT were selected in two ways. For short-term selections, *Acidovorax* sp. JS42 cultures that were grown overnight in MSB medium containing succinate were harvested, washed, resuspended, and plated onto MSB plates with 3NT vapor provided from a small tube fixed to the lid of the petri plate. Incubation at 30°C yielded 3NT-positive ($3NT^+$) colonies within 6 to 10 days. Independent colonies were purified for further study (strains JTP8 and JTP17) (Table 1). For long-term selections, serial transfers of liquid cultures with 3NT vapor provided as the sole carbon and energy source were carried out weekly for 1 month. After plating onto MSB plates with 3NT in the vapor phase in a closed container, isolated colonies were serially transferred onto solid medium for 5 months under the same conditions. Five strains were selected for further analysis (KSJ1, KSJ2, KSJ3, KSJ4, and KSJ8) (Table 1).

DNA manipulations. The manipulation of plasmids and DNA fragments was conducted according to standard methods (29). Restriction endonucleases and DNA modification enzymes were obtained from New England BioLabs (Beverly, MA). Plasmids were purified by using commercial kits (Fermentas, Glen Burnie, MD, and Qiagen, Valencia, CA). DNA fragments were purified from gel slices with a QIAquick gel extraction kit (Qiagen), and a Puregene DNA purification kit (Gentra Systems, Minneapolis, MN) was used to isolate genomic DNA. DNA sequencing of PCR products and cloned fragments was carried out at the University of California, Davis, sequencing facility with an Applied Biosystems 3730 automated sequencer.

Cloning of mutant *ntdAc* genes from evolved strains. The *ntdAcAd* genes from the $3NT^+$ strains were PCR amplified from purified genomic DNA by using primer pair NtdAc_F1 and NtdAd_R1 (Table 2). The resulting ~2-kb DNA fragments were cloned and sequenced to identify mutations. After the location of each mutation was identified, PCRs using genomic DNA from $3NT^+$ mutants as the template and primers nitroAc and U791F (Table 2) were used to amplify the fragments carrying the mutations. These fragments were cut with MfeI and KpnI and ligated with similarly digested pDTG850 and pKSJ44. The presence of the cloned fragments was confirmed by PCR and sequencing. For expression of NBDO in *Acidovorax* sp. JS42Ac, pDTG927 was digested with SacI, and the resulting 4.6-kb DNA fragment was ligated with pBBR1MCS5 to create pKSJ42.

To obtain oxygenase proteins for purification, wild-type and mutant oxygenase genes were cloned into vector pK18 (30) and expressed in *E. coli* DH5α or the tryptophanase mutant strain VJS415 (31) to prevent the formation of indigo by NBDO. Plasmid pKMM30, which was used for the production of recombinant wild-type NBDO oxygenase, was constructed by digesting pDTG927 with SacI and inserting the resulting fragment carrying *nbzAaAbAcAd* into pK18. The expression clone pKMM31, which was used for the production of recombinant wild-type 2NTDO oxygenase, was similarly constructed by digesting pDTG850 with SacI. Plasmids pKMM32 to pKMM35, which were constructed by digesting pJTP200, -300, -400, and -700, respectively, with MfeI and SpeI and using the resulting *ntdAc* fragments to replace the *ntdAc* fragment of pKMM31, were used for purification of mutant 2NTDO proteins (I204V, I204T, A405G, and I204A, respectively).

Site-directed mutagenesis. To evaluate the contribution of the A226V mutation to the activity of the dioxygenase, site-directed mutagenesis was used to construct a clone carrying only the A226V mutation. Standard PCR using wild-type JS42 genomic DNA as the template and primers NitroAc and ntdAcC677T-R (Table 2) generated a DNA fragment carrying the 5′ end of the *ntdAc* gene including the mutated A226 codon. A second PCR using JS42 DNA as the template and primers U791F and ntdAcC677T-F (Table 2) generated a 3′ *ntdAc* gene fragment including the mutated A226 codon. Primerless PCR with the two PCR fragments allowed the extension of complementary strands to form a complete *ntdAc* gene. The resulting PCR fragment was then digested with MfeI and KpnI and cloned into pDTG850 and pKSJ44. The resulting plasmids were verified by sequencing and were named pJTP1900 and pJTP2000, respectively.

NBDO variants with G204A, G204I, G204T, and G204V substitutions and a 2NTDO variant with an I204G substitution were generated by using the Infusion kit according to the manufacturer's instructions (Clontech, Mountain View, CA), using the BsrG1-MluI fragment from pDTG800 or pDTG927 and the primers listed in Table 2. The resulting plasmids (pJP500 to pJP504) (Table 1) were screened by restriction digestion to identify the introduced sites (Table 2) and verified by sequence analysis. Strains of *E. coli* DH5α carrying the plasmids were used for nitrite assays and biotransformations (described below) to characterize the activity and substrate specificity of the mutant enzymes. The SacI fragment from each clone was ligated into similarly digested pBBR1MCS5, generating pJP510 to pJP514 (Table 1) for introduction into *Acidovorax* sp. strain JS42Ac to test for complementation by growth on nitroaromatic substrates.

Biotransformations of nitroaromatic substrates. Briefly, *E. coli* DH5α strains carrying cloned genes encoding wild-type or mutant 2NTDO or NBDO enzymes were grown at 30°C in MSB medium containing glucose, thiamine, and ampicillin to an OD_{660} of 0.8 to ~1.0. Cultures were harvested and resuspended in MSB medium containing 10 mM glucose to an OD_{660} of ~2.0. Cell suspensions were transferred into 300-ml flasks in 25-ml aliquots and exposed to 1 mg/ml 2NT or 3NT for 6 h (30°C at 225 rpm). The products were extracted from the clarified supernatant with ethyl acetate and analyzed by gas chromatography-mass spectrometry (GC-MS), as described previously (32, 33).

***In vivo* specific activity of dioxygenases.** The activities of 2NTDO, NBDO, and their variants in *E. coli* were determined by using the cell suspensions (OD_{660} of ~2.0) described above. Specific activities were determined by measuring the rate of nitrite formed (sampling at 5-min intervals over 30 min or 15-min intervals over 90 min, depending on the activity of each cell suspension) at 30°C with shaking (200 rpm) during exposure to 1 mM 2NT, 3NT, or NB (27, 33). Total cell protein levels were

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
DH5α	Cloning host; *thi*	Invitrogen
S17-1	Host for plasmid mobilization, *thi*	63
BL21(DE3)	Expression host	Invitrogen
VJS415	Expression host K-12 strain W3110; Δ(*trpEA*)*2 tna2*	31
Acidovorax sp.		
JS42	Wild-type 2-nitrotoluene-degrading strain	15
JS42Ac	*ntdAc*::Km mutant of JS42; Km^r	41
JTP8	$3NT^+$; 2NTDO-I204V(A610G)	This study
JTP17	$3NT^+$; 2NTDO-I204V(A610G)	This study
KSJ1	$3NT^+$; 2NTDO-I204T(T611C)	This study
KSJ2	$3NT^+$; 2NTDO-A405G(C1214G)	This study
KSJ3	$3NT^+$; 2NTDO-I204T A226V(T611C,C667T)	This study
KSJ4	$3NT^+$; 2NTDO-I204V(A610G)	This study
KSJ8	$3NT^+$; 2NTDO-I204A(A610G,T611C)	This study
Plasmids		
pBBR1MCS5	Broad-host-range vector; Gm^r	64
pDTG800	pUC18 containing *ntdAaAbAcAd*; Ap^r	65
pDTG850	pUC13 containing *ntdAaAbAcAd*; Ap^r	40
pDTG871	pT7-7 containing *ntdAa*; Ap^r	22
pDTG904	pK19 containing *cdoE* from *Comamonas* JS765; Ap^r	28
pDTG927	pUC19 containing *nbzAaAbAcAd* from *Comamonas* sp. JS765; Ap^r	21
pFL880	pT7-7 containing *ntdAb*; Ap^r	This study
pJPK13	Cloning vector; Km^r	66
pJTP200	pDTG850 containing the 2NTDO-I204V mutation; Ap^r	This study
pJTP300	pDTG850 containing the 2NTDO-I204T mutation; Ap^r	This study
pJTP400	pDTG850 containing the 2NTDO-A405G mutation; Ap^r	This study
pJTP600	pDTG850 containing 2NTDO-I204T A226V mutations; Ap^r	This study
pJTP700	pDTG850 containing the 2NTDO-I204A mutation; Ap^r	This study
pJTP1900	pDTG850 containing the 2NTDO-A226V mutation; Ap^r	This study
pJTP610	pKSJ44 containing 2NTDO-I204T A226V mutations; Gm^r	This study
pJTP710	pKSJ44 containing the 2NTDO-I204A mutation; Gm^r	This study
pJTP1300	pKSJ44 containing the 2NTDO-I204T mutation; Gm^r	This study
pJTP1400	pKSJ44 containing the 2NTDO-A405G mutation; Gm^r	This study
pJTP1800	pKSJ44 containing the 2NTDO-I204V mutation; Gm^r	This study
pJTP2000	pKSJ44 containing the 2NTDO-A226V mutation; Gm^r	This study
pJVP500	pDTG850 containing the 2NTDO-I204G mutation; Ap^r	This study
pJVP501	pDTG927 containing the NBDO-G204A mutation; Gm^r	This study
pJVP502	pDTG927 containing NBDO-G204I mutation; Gm^r	This study
pJVP503	pDTG927 containing the NBDO-G204T mutation; Gm^r	This study
pJVP504	pDTG927 containing the NBDO-G204V mutation; Gm^r	This study
pJVP510	pKSJ44 containing the 2NTDO-I204G mutation; Gm^r	This study
pJVP511	pKSJ42 containing the NBDO-G204A mutation; Gm^r	This study
pJVP512	pKSJ42 containing the NBDO-G204I mutation; Gm^r	This study
pJVP513	pKSJ42 containing the NBDO-G204T mutation; Gm^r	This study
pJVP514	pKSJ42 containing the NBDO-G204V mutation; Gm^r	This study
pK18	Cloning vector; Km^r	30
pKMM1	pJPK13 containing *nbzAb*; Km^r	This study
pKMM30	pK18 containing *nbzAaAbAcAd*; Km^r	This study
pKMM31	pK18 containing *ntdAaAbAbAd*; Km^r	This study
pKMM32	pKMM31 containing the 2NTDOα I204TV mutation; Km^r	This study
pKMM33	pKMM31 containing the 2NTDOα I204TT mutation; Km^r	This study
pKMM34	pKMM31 containing the 2NTDOα A405G mutation; Km^r	This study
pKMM35	pKMM31 containing the 2NTDOα I204A mutation; Km^r	This study
pKSJ42	pBBR1MCS5 containing *nbzAaAbAcAd* from pDTG927; Gm^r	This study
pKSJ44	pBBR1MCS5 containing *ntdAaAbAcAd* from pDTG800; Gm^r	41

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance.

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)[a]	Use or description
NitroAc	CCACCCAACCCAATCACTACC	2NTDO-A226V
ntdAcC677T-R	GCTTAGCGTTGCC**CAC**AAGAGAACTAAATACCG	2NTDO-A226V
ntdAcC677T-F	TATTTAGTTCTCTT**GTG**GGCAACGCTAAGC	2NTDO-A226V
U791F	TTCACCGCATCATTCAGTTGG	2NTDO-A226V
NtdAc_F1	GGCCTCG***AGGAG***GGACATATGAGTTACCAAAACTTAGTGAGTGAAGC	Subcloning mutant dioxygenase genes; XbaI, NdeI; synthetic RBS
NtdAd_R1	GCTGGCATGCGAGCTCACAGGAAGACCAACAGGTTGTGGGTC	Subcloning mutant dioxygenase genes; SphI, SacI
pDTG800 For	CACCCGCGAAGACCTGTACAAGCGCGAATTGGAGCGCCTG	2NTDO-I204G; BsrGI
pDTG800 Rev	CGTTTCCATTTGTCTTCACGCGTTGCATAGAAGACGTCAAC	2NTDO-I204G; MluI
I204G For	AACTTTGTAGGTGAC**GGT**TACCACGTTGGTTGGACGCACG	2NTDO-I204G; BstEII
I204G Rev	CGTGCGTCCAACCAACGTGGTA**ACC**GTCACCTACAAAGTT	2NTDO-I204G; BstEII
pDTG927 For	CACCCGCGAAGACCTGTACAAGCGCGAATTGGAGCGCCTG	NBDO-G204 mutants; BsrGI
pDTG927 Rev	CGTTTCCATTTGTCTTCACGCGTTGCATAGAAGACGTCAAC	NBDO-G204 mutants; MluI
G204I For	AACTTTGTAGGTGAT**ATC**TACCACGTTGGTTGGACGCACG	NBDO-G204I; EcoRV
G204I Rev	CGTGCGTCCAACCAACGTGGTA**GAT**ATCACCTACAAAGTT	NBDO-G204I; EcoRV
G204T For	AACTTTGTAGGTGAC**ACG**TACCACGTTGGTTGGACGCACG	NBDO-G204T; AflIII
G204T Rev	CGTGCGTCCAACCAACGTGGTA**CGT**GTCACCTACAAAGTT	NBDO-G204T; AflIII
G204V For	AACTTTGTAGGTGAC**GTG**TACCACGTTGGTTGGACGCACG	NBDO-G204V; AflIII
G204V Rev	CGTGCGTCCAACCAACGTGGTA**CAC**GTCACCTACAAAGTT	NBDO-G204V; AflIII
G204AForNEW	AACTTTGTAGGTGAC**GCG**TACCACGTTGGTTGGACGCACG	NBDO-G204A; MluI
G204ARevNEW	CGTGCGTCCAACCAACGTGGTA**CGC**GTCACCTACAAAGTT	NBDO-G204A; MluI
ntdAbF	ATCATATGTCCGAGAACTGGATTGATGCC	*ntdAb* cloning; NdeI
ntdAbR	GATAAGCTTAGTCCAGCTTGAGCATCACGCG	*ntdAb* cloning; HindIII

[a] Changed codons are shown in boldface type. The indicated restriction sites are underlined in sequences. The ribosome binding site (RBS) is shown in italic type.

determined by resuspending cell pellets in an equal volume of 100 mM NaOH, boiling for 10 min, and measuring the protein concentration, as previously described, with bovine serum albumin as the standard (33, 34).

Preparation of cell extracts and protein purification. Proteins in this study were purified by using modifications (detailed below) of previously described methods (22). All purification procedures were performed at 4°C by using an automated fast protein liquid chromatography system (Bio-Rad Laboratories, Hercules, CA), except for the partial purification of catechol 2,3-dioxygenase (CDO), in which cell extracts were heat treated at 65°C for 15 min, followed by removal of denatured proteins by centrifugation at 145,000 × *g* for 2 h at 4°C. Cell extracts were prepared by allowing frozen cell suspensions to thaw on ice. DNase I was added to a final concentration of 0.01 mg/ml, and lysozyme was added to a final concentration of 1 mg/ml. Cell suspensions were passed through a chilled French pressure cell, maintaining an internal cell pressure of 20,000 lb/in^2. Cell debris and membranes were removed by centrifugation at 145,000 × *g* for 2 h at 4°C. We found that removal of salts by dialysis improved protein binding to the resin, so prior to loading of columns, cell extracts were placed into Fisherbrand regenerated cellulose dialysis tubing (Fisher Scientific) and dialyzed at 4°C with stirring in 2 liters of MEGD (pH 6.8) (50 mM morpholineethanesulfonic acid [MES] [pH 6.8], 5% ethanol, 5% glycerol, and 1 mM dithiothreitol [DTT]). After 4 h of stirring, buffer was replaced with 2 liters of fresh MEGD (pH 6.8), and dialysis was allowed to continue overnight. Precipitates were removed by centrifugation at 17,000 × *g* for 30 min at 4°C, and the resulting extracts were immediately used for protein purification. Chromatography columns and column resins used for protein purification were obtained from GE Healthcare.

The reductase was purified essentially as described previously (22), except that the extract from *E. coli* BL21(DE3)(pDTG871) was dialyzed as described above, and the buffer used throughout purification was MEGD (pH 6.8). The ferredoxin was purified essentially as described previously (22), except that the dialyzed cell extract from DH5α(pKMM1) was used as the source of the protein. To construct pKMM1, *ntdAb* was amplified from pDTG850 by using primers ntdAbF and ntdAbR (Table 2) and the fragment was cloned into pT7-7, generating pFL880. pKMM1 was generated by digesting pFL880 with XbaI and EcoRI and inserting the resulting fragment into pJPK13.

A revised protocol for purification of NBDO and 2NTDO oxygenase components was developed, as a butyl-Sepharose resin with the appropriate properties is no longer commercially available. The dialyzed cell extract from either VJS415(pKMM30) or DH5α(pKMM31-pKMM35) was fractionated on a Q-Sepharose FF column as described previously (22), and the concentrated fraction with oxygenase activity was brought to 0.8 M ammonium sulfate. After 30 min, the precipitate was removed by centrifugation at 17,000 × *g* for 30 min. The supernatant was applied onto an XK26/40 column containing 200 ml (bed volume) of phenyl-Sepharose. Oxygenase remained bound even after salt had been removed from the column. Bound oxygenase was eluted at a flow rate of 0.5 ml/min with MEGD buffer (pH 10). Fractions exhibiting oxygenase activity were pooled and concentrated, and the buffer was exchanged to 1 mM KPO_4 (pH 6.8) by ultrafiltration with a YM100 membrane. The final polishing step using hydroxyapatite was described previously (22). The purified reductase, ferredoxin, and each individual oxygenase component resulted in either single bands for reductase and ferredoxin or two bands for the oxygenases, as previously reported (22). Since the reductase (36.2 kDa) and ferredoxin (11.5 kDa) components are identical in the 2NTDO and NBDO systems (21), the purified proteins could be used for assaying both 2NTDO and NBDO activities, and differences in activities and substrate specificities could be attributed solely to the oxygenase components.

Protein determinations. Protein concentrations were determined by the method of Bradford (34), with bovine serum albumin as the standard, or by using a Nanodrop 1000 spectrophotometer (Thermo Scientific), using previously determined extinction coefficients for NBDO, 2NTDO, reductase, and ferredoxin (22).

Enzyme assays. During protein purification, enzyme activities were determined by measuring the amount of nitrite released from the substrate, as previously described (35). Reaction mixtures contained 200 μl of 50 mM MES (pH 6.8), 0.4 mM fresh NADH, 0.2 mM ferrous ammonium sulfate (FAS), 1 mM substrate (NB, 2NT, or 3NT), and purified NBDO or 2NTDO components.

Kinetic analysis. Kinetic parameters of wild-type and mutant oxygenases were determined spectrophotometrically by examining their initial velocities using a coupled enzyme assay in which excess CDO was included, in addition to the components required for nitrite release from

TABLE 3 Calculated extinction coefficients

Enzyme	% product formed from 3NT			Extinction coefficient (M^{-1} cm^{-1})
	3MC	4MC	3NBZA	
2NTDO	72	25	4	21,600[a]
2NTDO-I204A	2	97	1	28,000[a]
2NTDO-I204V	13	83	5	27,000[a]
2NTDO-I204T	5	93	2	27,700[a]
2NTDO-A405G	17	83	0	26,600[a]
NBDO	0	100	0	28,100[b]

[a] Extinction coefficients calculated for product formation from 3NT were based on the product ratios in Fig. 2, which are shown here in numerical format to make the calculations clear. The extinction coefficients for 3-methylcatechol (3MC) and 4-methylcatechol (4MC) are 19,400 M^{-1} cm^{-1} and 28,100 M^{-1} cm^{-1}, respectively (39). In order to take into account both products (3-methylcatechol and 4-methylcatechol), which have overlapping absorbances at 382 and 388 nm, respectively, a weighted extinction coefficient was calculated as follows: (19,400 × 0.17) + (28,100 × 0.83) = 26,621. 3NBZA, 3-nitrobenzyl alcohol.
[b] See reference 39.

TABLE 4 Growth of evolved 3NT$^+$ *Acidovorax* strains[a]

Strain	Dioxygenase present	Doubling time (h) on substrate[b]			Final A_{660} on substrate		
		2NT	3NT	NB	2NT	3NT	NB
JS42	Wild-type 2NTDO	4.0	—	5.4	0.25	0.06	0.23
KSJ8[c]	2NTDO-I204A	—	8.9	—	0.10	0.21	0.12
KSJ1[c]	2NTDO-I204T	5.8	7.6	—	0.15	0.21	0.12
KSJ3[c]	2NTDO-I204T A226V	6.0	7.0	—	0.16	0.22	0.12
KSJ2[c]	2NTDO-A405G	4.1	10.3	3.6	0.27	0.20	0.23
JTP8[d]	2NTDO-I204V	6.1	9.4	61[e]	0.20	0.19	0.13
JTP17[d]	2NTDO-I204V	4.4	5.9	5.6	0.22	0.23	0.29
KSJ4[c]	2NTDO-I204V	5.2	7.4	98[e]	0.21	0.22	0.14

[a] Substrates were provided at 1 mM, as this concentration gave the fastest doubling times. None of the strains grew on 4NT ($n \geq 3$; standard deviations were 15% or lower).
[b] —, no growth observed beyond that of the no-carbon control (final A_{660} of ≤0.12).
[c] Strains obtained in long-term selections.
[d] Strains obtained in short-term selections.
[e] Cultures turned yellow, indicating accumulation of the catechol ring cleavage product 2-hydroxymuconic semialdehyde, but good growth was observed on plates with NB supplied in a vapor form (see Table S1 in the supplemental material).

nitroaromatic substrates. Similar coupled assays were used previously to quantify initial dioxygenase activity (36). This CDO from *Comamonas* sp. strain JS765 was previously shown to be active with all three catechols produced from nitroaromatic substrates by 2NTDO, NBDO, and their derivatives (37). Activity was assayed at room temperature as previously described (37), by monitoring absorbance changes for 0.5-s intervals over 1 min to monitor the formation of the ring cleavage products of catechol (375 nm), 3-methylcatechol (382 nm), and 4-methylcatechol (388 nm) using an Agilent 8453 UV-visible (UV-Vis) spectrophotometer (Agilent Technologies, Santa Clara, CA). Reactions were carried out with 50 mM air-saturated MES (pH 7.0) in a total volume of 1 ml. Reaction mixtures contained 0.7 mg partially purified CDO, 0.4 mM fresh NADH, 0.2 mM FAS, various concentrations (0.1 to 100 μM) of the substrate (NB, 2NT, or 3NT), 0.3 μM reductase, 2 μM ferredoxin, and 1 μM oxygenase. The substrate concentrations could be varied sufficiently within their solubility ranges to allow determination of reliable apparent k_{cat} and K_m values. Under these conditions, minor background rates (without a substrate) were subtracted from the rates obtained for assay mixtures containing all components, including the substrate. The number of concentrations tested per substrate ranged from 8 to 10. The molar absorption coefficients of the ring cleavage products of catechol, 3-methylcatechol, and 4-methylcatechol, which were previously determined to be 33,000 (at 375 nm), 19,400 (at 382 nm), and 28,100 (at 388 nm), respectively (38, 39), were used to calculate the amount of product formed per second. Some of the dioxygenases produced mixtures of 3-methylcatechol, 4-methylcatechol, and 3-nitrobenzyl alcohol from the substrate 3NT. Alcohol formation is not observed in this assay, and since the 3- and 4-methylcatechol absorbances overlap, a weighted extinction coefficient was calculated based on the ratio of products formed. The weighted extinction coefficients were calculated for this study based on previously determined extinction coefficients (39) and the product ratios determined in this study. The tabulated product ratios for 3NT and weighted extinction coefficients are provided in Table 3. The apparent K_m and k_{cat} values were determined by nonlinear fitting of the Michaelis-Menten model using the GraphPad Prism program.

RESULTS

Each 3NT$^+$ mutant contained one or more mutations in *ntdAc*. Using short- and long-term selections, we isolated 14 variants of JS42 that grew on 3NT, 6 from short-term direct-plating experiments and 8 from long-term liquid enrichments. Previous studies showed that the rate of product formation by 2NTDO from 3NT was significantly lower than that from 2NT (23). Therefore, we hypothesized that mutations in 2NTDO that improved activity with 3NT may have occurred. The *ntdAc* gene encodes the α subunit of 2NTDO, which controls substrate specificity (23, 40), so the *ntdAc* and *ntdAd* genes (encoding the 2NTDO α and β subunits) from each 3NT$^+$ variant were PCR amplified and sequenced. The *ntdAc* gene from each evolved strain was found to have one or more missense mutations that encoded different amino acids at positions 204, 226, and 405 in the α subunit of 2NTDO. No mutations were found in *ntdAd*. The six independent 3NT$^+$ strains recovered from the short-term selections each contained an I204V substitution (Table 1 [only the two strains shown were further characterized]). The eight 3NT$^+$ strains recovered from long-term selection were more varied (Table 1), containing substitutions in the α subunit that included I204V (4/8 strains; only 1 was studied further), I204T (1/8), I204A (1/8), I204T A226V (1/8), and A405G (1/8).

Growth of evolved strains on nitroarene substrates. Growth of three of the evolved strains carrying the 2NTDO-I204V substitution (two from short-term and one from long-term selection) and each strain carrying the I204T, I204T A226V, I204A, and A405G substitutions was monitored in liquid cultures containing 1 mM 2NT, 3NT, 4NT, or NB. The three independent mutants carrying the 2NTDO-I204V allele did not have the same doubling times on various substrates, suggesting that additional mutations that affect growth on nitroarene compounds may be present elsewhere in their genomes (Table 4). Several of the 3NT$^+$ strains either were unable to grow or grew more slowly on NB and/or 2NT than wild-type strain JS42, suggesting that the improved ability to grow on 3NT came at the expense of efficient growth on 2NT and NB. Because some of the final cell yields in liquid culture were very low (Table 4), we confirmed these results by testing for growth on plates with substrates provided in a vapor form (see Table S1 in the supplemental material). We found that although strains JTP8 and KSJ4 grew very poorly on NB in liquid medium, accumulating a yellow intermediate (presumably the catechol ring cleavage product 2-hydroxymuconic semialdehyde), these strains grew quite well on solid medium in the presence of NB vapor (see Table S1 in the supplemental material). None of the mutant

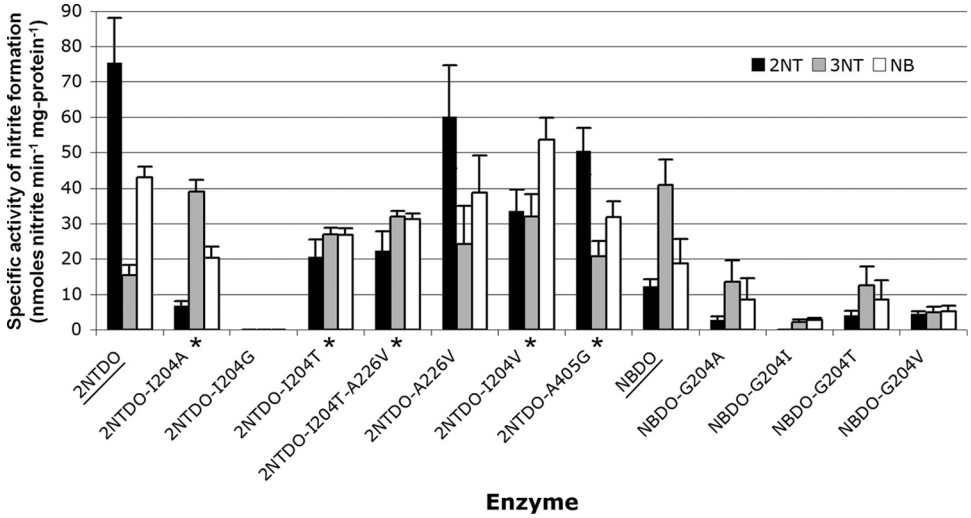

FIG 1 Specific activities of nitrite formation by wild-type and mutant dioxygenases in *E. coli* monitored with whole-cell nitrite assays. 2NT, 3NT, and NB were provided as the substrates. Wild-type enzymes are underlined; enzymes indicated by asterisks were obtained from evolved JS42 strains, and the other mutant enzymes were generated by site-directed mutagenesis. Values are averages of data from at least 3 independent experiments, and error bars represent standard deviations.

strains acquired the ability to grow on 4NT on plates or in liquid (data not shown).

Mutations in *ntdAc* increase the activity of 2NTDO with 3NT. To test the effect of the *ntdAc* mutations on 2NTDO activity, PCR products carrying mutant *ntdAc* gene fragments were cloned into pDTG850 (40), which harbors all of the *ntd* structural genes (*ntdAa*, encoding reductase; *ntdAb*, encoding ferredoxin; and *ntdAc* and *ntdAd*, encoding the α and β subunits of 2NTDO, respectively). To determine the contribution of the A226V mutation to the enzyme activity of the I204T A226V mutant, an additional construct encoding only the A226V substitution was created by site-directed mutagenesis. Each of these plasmids (Table 1) was introduced into *E. coli* DH5α, and *in vivo* specific activities were determined with 2NT, 3NT, and NB as the substrates. Compared to wild-type 2NTDO, all of the enzymes from the evolved strains (Fig. 1, asterisks) had increased specific activities with 3NT; some were comparable to that of wild-type NBDO, the enzyme from *Comamonas* sp. JS765, a strain capable of growth on 3NT (21). In contrast, most of the evolved enzymes had reduced activities with 2NT and NB (Fig. 1). On its own, the A226V substitution did not have a significant effect on activity with any of the tested substrates (Fig. 1).

The corresponding amino acid substitutions in NBDO are detrimental to enzyme activity. The results presented above indicated that the residue at position 204 is critical for the activity of 2NTDO with 3NT. NBDO, an enzyme that is very efficient at 3NT oxidation (21), has a glycine at position 204, and since we did not obtain a mutant enzyme with such a change in our selections, we introduced a glycine into 2NTDO to determine if this substitution would be beneficial. Surprisingly, the 2NTDO-I204G mutant enzyme was severely impaired (Fig. 1); nitrite formation from all substrates was below the limit of detection (~2.0 nmol min^{-1} mg · $protein^{-1}$). We also tested the effects of the corresponding substitutions at position 204 in the NBDO context. Unlike 2NTDO, NBDO did not tolerate substitutions at position 204: all of the constructed enzymes had significantly reduced activities with all substrates compared to wild-type NBDO (Fig. 1).

Amino acid substitutions at positions 204 and 405 alter the regiospecificity of the enzymes. The ratios of products formed from 2NT and 3NT by wild-type and mutant dioxygenases were measured by GC-MS analysis of ethyl acetate extracts of *E. coli* culture supernatants after incubation with substrates for 6 h (Fig. 2A and B, respectively). The preferred location of dioxygenation of 3NT shifted from the 2,3- to the 3,4-positions of the aromatic ring for all of the evolved 2NTDO enzymes (Fig. 2, asterisks), which is similar to the reaction catalyzed by NBDO. The dioxygenases with I204A and I204T substitutions showed a decreased ability to oxidize the aromatic ring of 2NT, resulting in the formation of significantly more 2-nitrobenzyl alcohol (Fig. 2). The ability of the enzymes to efficiently catalyze dioxygenation of the aromatic ring is an important activity because (methyl)catechols support the growth of JS42, while nitrobenzyl alcohols do not (15). This change in specificity therefore likely contributed to the lower final cell densities of the corresponding strains on 2NT (Table 4). In contrast to results with the 2NTDO-I204A and 2NTDO-I204T enzymes, those carrying I204V and A405G substitutions converted 2NT to a ratio of 3-methylcatechol to 2-nitrobenzyl alcohol similar to that of wild-type 2NTDO; the corresponding strains carrying these mutations retained the ability to grow quite well on 2NT (Table 4). To rule out the possibility that the reduced growth was due to toxicity of the accumulating nitrobenzyl alcohols, we grew wild-type JS42 on 2NT in the presence of up to 500 mM 2-nitrobenzyl alcohol but observed no differences in doubling times or final cell yields (data not shown). These results indicate that toxicity was not the cause of the reduced growth rates, and it is likely that the reduced availability of catabolizable methylcatechol products from 2NT contributed to the low overall growth yields of the strains carrying these mutations (Table 4).

The constructed 2NTDO-I204G enzyme also produced more nitrobenzyl alcohols relative to catechols than did wild-type 2NTDO from both 2NT and 3NT, and most of the constructed NBDO variants had altered regiospecificities with both substrates (Fig. 2). These results confirm the importance of the residue at position 204 for proper positioning of nitrotoluenes for oxidation

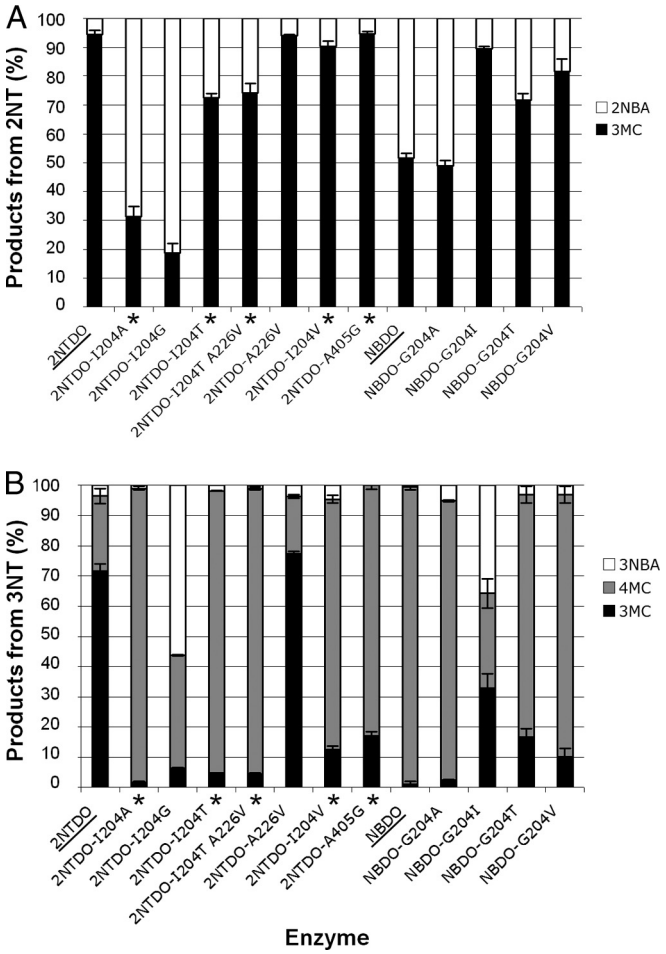

FIG 2 Oxidation products formed from 2NT and 3NT in biotransformation reactions using *E. coli* expressing wild-type and mutant forms of 2NTDO and NBDO. (A) Products formed from 2NT. 3MC, 3-methylcatechol; 2NBA, 2-nitrobenzyl alcohol. (B) Products formed from 3NT. 4MC, 4-methylcatechol. Wild-type enzymes are underlined; enzymes indicated by asterisks were obtained from evolved JS42 strains, and the other mutant enzymes were generated by site-directed mutagenesis. Values are averages of data from at least 3 independent experiments, and error bars represent standard deviations.

of the aromatic ring. The single mutation at position 226, which was constructed by site-directed mutagenesis, did not affect the regiospecificity of the enzyme with either substrate (Fig. 2), further indicating that this residue does not contribute to the specificity of nitrotoluene oxidation.

Mutations in *ntdAc* are sufficient to allow growth of JS42 on 3NT. To test whether the *ntdAc* mutations are sufficient to allow growth on 3NT, each mutant *ntdAc* gene was cloned into pKSJ44 (Table 1), which is a broad-host-range plasmid containing the complete *ntd* gene cluster. Plasmids containing wild-type or mutant *ntdAc* alleles were introduced into JS42Ac, a mutant strain that does not produce a functional 2NTDO (41). As expected, the introduction of pKSJ44 complemented the mutation in JS42Ac and allowed growth on NB and 2NT (Table 5). The additional copies of the wild-type *ntd* gene cluster on the multicopy plasmid were not sufficient, however, to allow growth on 3NT. In contrast, the introduction of pKSJ42, which carries a wild-type *nbz* operon encoding NBDO, allowed JS42Ac to grow on NB and 3NT but not on 2NT (Table 5). This result was expected because NBDO catalyzes efficient oxidation of NB and 3NT (Fig. 1) (21). Introduction of the plasmids encoding the evolved 2NTDOs with substitutions at positions 204 and 405 conferred the ability to grow on 3NT but with much longer doubling times than those of the evolved strains (Table 4). These results suggest that the evolved strains carry other, as-yet-unidentified mutations outside the dioxygenase gene cluster that improve 3NT degradation. In contrast to the enzyme with both the I204T and A226V substitutions, the enzyme carrying the constructed A226V substitution did not support growth of JS42Ac on 3NT (Table 5).

As seen with the evolved strains (Table 4), several of the complemented JS42Ac strains were unable to grow on 2NT and/or NB (Table 5), and none of the strains grew on 4NT (data not shown). Only the strains carrying the I204V and A405G substitutions had near-wild-type doubling times on 2NT, and the I204V-carrying strain stopped growing at a very low density. Analysis of growth of the complemented *ntdAc* mutant on plates suggested that additional mutations were required to allow growth on 2NT and NB, because growth was seen only when a large inoculum was plated, and single colonies arose randomly over a 2-week period (see Table S2 and Fig. S1 in the supplemental material). These findings may explain some of the variability in the liquid cultures that we observed, and they provide further evidence that mutations in

TABLE 5 Growth of complemented *Acidovorax* sp. JS42Ac

Strain	Dioxygenase present[a]	Doubling time (h) on substrate[b]			Final A_{660} on substrate		
		2NT	3NT	NB	2NT	3NT	NB
JS42Ac(pBBR1MCS5)	None	—	—	—	0.07	0.12	0.07
JS42Ac(pKSJ44)	Wild-type 2NTDO	5.1	—	7.1	0.27	0.09	0.16
JS42Ac(pJTP710)	2NTDO-I204A	—	43	—	0.05	0.16	0.12
JS42Ac(pJTP1300)	2NTDO-I204T	—	25	—	0.10	0.19	0.12
JS42Ac(pJTP610)	2NTDO-I204T A226V	—	38	8.3	0.08	0.15	0.17
JS42Ac(pJTP2000)	2NTDO-A226V	13	—	—	0.22	0.07	0.12
JS42Ac(pJTP1800)	2NTDO-I204V	5.8	38	7.4	0.12	0.15	0.21
JS42Ac(pJTP1400)	2NTDO-A405G	5.6	41	19	0.24	0.14	0.21
JS42Ac(pKSJ42)	Wild-type NBDO	—	23	6.3	0.07	0.19	0.24

[a] Growth of JS42Ac expressing 2NTDO-I204G was not tested, as no nitrite formation was detected with this enzyme (Fig. 1). JS42Ac expressing the genes encoding NBDO-G204I, NBDO-G204A, NBDO-G204T, and NBDO-G204V did not grow on 2NT or 3NT.

[b] Substrates were provided at 1 mM, as this concentration gave the fastest doubling times ($n \geq 3$; standard deviations were 15% or lower). —, no growth observed beyond that of the no-carbon control (final A_{660} of ≤0.12).

TABLE 6 Apparent K_m values of (methyl)catechol formation by the wild-type and evolved enzymes for the indicated substrates

Enzyme	Avg apparent K_m (μM) for substrate ± SD[a]		
	2NT	3NT	NB
2NTDO	3.8 ± 0.2	40 ± 6.4	4.7 ± 0.3
I204A	19 ± 4.2	9.2 ± 0.7	47 ± 5.4
I204T	5.7 ± 0.5	5.4 ± 0.1	9.9 ± 0.7
I204V	3.0 ± 0.1	3.9 ± 0.1	4.8 ± 0.1
A405G	1.2 ± 0.2	7.8 ± 0.2	6.2 ± 0.7
NBDO	13.7 ± 2.7	4.2 ± 0.4	4.5 ± 0.2

[a] Values are expressed as averages of data from three replicates ± standard deviations.

TABLE 7 Apparent k_{cat} values of (methyl)catechol formation by the wild-type and evolved enzymes for the indicated substrates

Enzyme	Avg apparent k_{cat} (s^{-1}) for substrate[a]		
	2NT	3NT	NB
2NTDO	0.34	0.15	0.27
I204A	0.12	0.27	0.19
I204T	0.21	0.21	0.22
I204V	0.25	0.26	0.25
A405G	0.19	0.13	0.23
NBDO	0.16	0.27	0.30

[a] Values are expressed as averages of data from three replicates. Standard deviations were <10%.

2NTDO that result in improved growth on 3NT are detrimental for growth on 2NT and NB. As expected based on their low activities (Fig. 1), none of the mutant NBDO enzymes with the corresponding site-directed mutations allowed growth on 2NT, 3NT, or NB (data not shown).

Steady-state kinetic analysis of the evolved dioxygenases. By using a coupled assay mixture containing catechol 2,3-dioxygenase (CDO), 2NTDO and NBDO exhibited Michaelis-Menten kinetics for the dependence of the initial rate of (methyl)catechol cleavage product formation on the concentration of the indicated substrate (2NT, 3NT, or NB). Based on the determined apparent K_m values (Table 6), the affinity of wild-type 2NTDO for the three substrates was in the order 2NT > NB > 3NT, while wild-type NBDO had the following affinity order: 3NT > NB > 2NT. Thus, 2NTDO and NBDO differ significantly in their preferences for these selected nitroaromatic substrates. It should be noted that the coupled CDO assay used to calculate kinetic parameters monitors only the physiologically relevant reaction catalyzed by 2NTDO; the enzyme and some of its variants also produce various amounts of nitrobenzyl alcohols (Fig. 2), which are not substrates for CDO and are not metabolized further by *Acidovorax* sp. strain JS42. The small amounts of nitrobenzyl alcohols produced from 2NT or 3NT by most of the enzymes (Fig. 2) should have little effect on the obtained values; however, the kinetic parameters for the I204A and I204T forms of 2NTDO with 2NT obtained from an assay that takes into account both catechol and alcohol formation might differ significantly from those obtained here.

Based on the measurement of (methyl)catechol formation from nitroarene substrates, the evolved 2NTDO proteins all had lower apparent K_m values for 3NT (3.9 to 9.2 μM) than did wild-type 2NTDO (40 μM). These values are approaching the apparent K_m of NBDO for 3NT (4.2 μM). In contrast, the affinities of the evolved 2NTDO proteins for 2NT and NB were variable. In comparison to wild-type 2NTDO, 2NTDO-A405G and 2NTDO-I204V both had increased affinities for 2NT, while 2NTDO-I204A and 2NTDO-I204T had decreased affinities for 2NT and NB (Table 6).

Compared to wild-type 2NTDO, apparent k_{cat} values for methylcatechol formation from 3NT were slightly higher for the mutant enzymes with substitutions at position 204 and slightly lower for the A405G mutant (Table 7). However, all of the evolved enzymes had higher catalytic efficiencies with 3NT than did wild-type 2NTDO (Table 8). The largest improvement was seen with the I204V variant, which had a 18-fold increase in the catalytic efficiency of methylcatechol formation with 3NT as the substrate compared to 2NTDO, which was comparable to that of NBDO (Table 8). Interestingly, this substitution was identified in multiple independently evolved strains (Table 1). In contrast, the enzyme with the A405G substitution showed the smallest overall improvement with 3NT but was the most versatile of all of the evolved enzymes, with improved catalytic efficiencies for methylcatechol formation with both 2NT and 3NT compared to wild-type 2NTDO (Table 8). In general, improved catalytic efficiencies of the evolved enzymes are consistent with improvements in growth of the complemented strain with the three substrates (compare Tables 5 and 8); discrepancies can likely be attributed to additional and as-yet-unidentified mutations elsewhere in the genomes.

DISCUSSION

Based on the crystal structure of NBDO, which is 95% identical in amino acid sequence to 2NTDO (21), the residue at position 204 is in the active site of the enzyme (42) (see Fig. S2 in the supplemental material). Substitutions at the corresponding residue are known to affect the specificity of related enzymes, including naphthalene, toluene, and 2,4-dinitrotoluene dioxygenases (43–45). In NBDO, G204 interacts with the methyl group of 3NT when it is bound at the active site of the enzyme (42). The NBDO crystal structure suggests that an isoleucine at position 204 (the residue present in 2NTDO) would result in an active-site pocket that is unable to accommodate or efficiently oxidize 3NT due to steric hindrance (42). The steric hindrance of 3NT by the isoleucine residue at position 204 may be the cause of the substantial difference in affinity in 2NTDO; all of the evolved proteins had a smaller residue at position 204, which may have allowed the accommodation of the 3NT methyl group in the substrate pocket. Since NBDO has a glycine at position 204, and we did not obtain an evolved 2NTDO with a glycine in our selections, possibly because it would require at least two transversions to generate a Gly codon, we generated such an enzyme by site-directed mutagenesis. However, further decreasing the size of the residue at this position

TABLE 8 k_{cat}/K_m values of (methyl)catechol formation by the wild-type and evolved enzymes for the indicated substrates

Enzyme	k_{cat}/K_m (M^{-1} s^{-1})		
	2NT	3NT	NB
2NTDO	9.0×10^4	3.7×10^3	5.8×10^4
I204A	6.5×10^3	2.9×10^4	4.0×10^3
I204T	3.7×10^4	3.9×10^4	2.2×10^4
I204V	8.3×10^4	6.8×10^4	5.3×10^4
A405G	1.6×10^5	1.7×10^4	3.7×10^4
NBDO	1.2×10^4	6.5×10^4	6.7×10^4

resulted in an enzyme with very poor activity and increased relative production of dead-end nitrobenzylalcohols (Fig. 1 and 2). The residue at position 405, which is conserved in NBDO and 2NTDO, is not located in the active site (42) (see Fig. S2 in the supplemental material), and the corresponding residue has not been noted previously to be important in determining substrate specificity in any Rieske dioxygenases. Several studies have found that amino acid substitutions that are not in the immediate vicinity of the bound substrate can still have a distinct effect on the structure of the active site (46–48). The introduction of a glycine at position 405 must somehow provide enough compensatory changes to the substrate pocket to allow 3NT to productively bind in the presence of the isoleucine at position 204.

Other than the JS42 variants obtained in this selection, there are only a few bacterial isolates that have been documented to use 3NT as the sole carbon and energy source: *Comamonas* sp. strain JS765 (21), three *Diaphorobacter* sp. strains (11), *Rhodococcus* sp. strain ZWL3NT (10), and *Micrococcus* sp. strain SMN-1 (14). All of these strains appear to have a similar oxidative pathway for the degradation of nitroarene substrates that involves the release of nitrite; however, while JS765 converts 3NT to 4-methylcatechol, *Rhodococcus* sp. strain ZWL3NT was reported to generate 3-methylcatechol from 3NT (10), and the methylcatechol isomer(s) formed from 3NT by *Diaphorobacter* and *Micrococcus* strains has not been identified. Strain JS765 is capable of growth on NB and 3NT but not on 2NT or 4NT (9, 21), whereas *Rhodococcus* sp. strain ZWL3NT was reported to grow on 2NT and 3NT but not on 4NT (10), and *Micrococcus* sp. strain SMN-1 and *Diaphorobacter* sp. strain DS2 grew on 2NT, 3NT, and NB and less well on 4NT (11, 14). Only the enzymes from *Comamonas* sp. strain JS765 and *Diaphorobacter* sp. strain DS2 have been examined in detail. Both initial dioxygenases from these strains preferentially oxidize 3NT > NB > 2NT > 4NT (21, 49). Interestingly, 3NTDO from *Diaphorobacter* sp. strain DS2 has an isoleucine at position 204 and a glycine at position 405 (50). Modeling based on the structure of NBDO indicated that 3NTDO from *Diaphorobacter* has a larger active site than does NBDO (50). It is possible that the glycine at position 405 plays a role in determining the larger size of the active site.

Since JS42 is capable of growing on both 3- and 4-methylcatechols as sole carbon sources, we were surprised to find that all of the mutant dioxygenases that allowed growth on 3NT had altered regiospecificities that resulted in the formation of predominantly 4-methylcatechol from 3NT. These enzymes therefore acquired specificities similar to that of NBDO from *Comamonas* sp. strain JS765, which forms only 4-methylcatechol from 3NT (21). Since strain JS765 is capable of growth on 3NT, this specificity change suggests the possibility of an inherent requirement for metabolism through 4-methylcatechol. The crystal structure of NBDO with 3NT bound in the active site shows 3NT positioned specifically for oxidation at carbons 3 and 4 of the aromatic ring (42), so it is possible that the change in specificity of the evolved 2NTDOs is simply due to the positioning of 3NT in the active site of the enzyme. Constraints in the active site may preclude efficient positioning of 3NT for oxidation at the 2,3-position.

An alternative explanation for the change in regiospecificity could be more efficient growth using the oxalocrotonate branch of the *meta* pathway, which accommodates catechol and 4-methylcatechol, rather than the hydrolytic branch, through which 3-methylcatechol is metabolized (51). Adding to the complexity of the *meta* pathway, the JS42 genome encodes two different putative catechol 2,3-dioxygenases, CtdE1 and CtdE2 (52); however, we can rule out the possibility that differences in the activity or specificity of these enzymes contribute to preferred metabolism via 4-methylcatechol. Previous studies showed that CtdE1 is essential for the growth of the strain on 2NT (41, 53) and that CDO from *Comamonas* sp. strain JS765, which is identical in sequence to CtdE1, had similar activities with 3-methylcatechol and 4-methylcatechol (28). We confirmed this finding with CtdE1 from strain JS42 and showed that the inactivation of *ctdE2* had no effect on the ability of JS42 to grow on 2NT (data not shown). Another possibility could involve differences in the regulation of the genes encoding downstream steps in the pathway. The genes encoding both branches of the *meta* pathway are clustered in what appears to be several transcriptional units at a single locus in strain JS42 (52), and at this time, we cannot rule out a role for differential expression, as we are just now beginning to unravel the mechanism of gene regulation.

We recently reported the isolation of three variants of strain JS42 that were selected for growth on 4NT (52). Each variant had mutations that resulted in one or more amino acid substitutions in the α subunit of 2NTDO, but none of the substitutions was the same as any of those found in the $3NT^+$ variants. All three evolved enzymes shared a single amino acid substitution (M248I) near but not within the active site of 2NTDO, and two of the enzymes had a second amino acid substitution (L238V or S242N) nearby (see Fig. S2 in the supplemental material). All three mutant enzymes had improved activity with 4NT, and when introduced into JS42Ac, all three mutant enzymes permitted growth on both 2NT and 4NT (52). In contrast, none of the enzymes had improved activity with 3NT, and the evolved strains did not acquire the ability to grow on 3NT (52). Taken together with the results presented here, it appears that subtle changes in different regions of 2NTDO within and surrounding the active site have significant effects on enzyme activity and regiospecificity with various mononitrotoluene isomers.

Bacteria are known for the ability to adapt to the presence of nonnative carbon and energy sources (54, 55). Moreover, bacteria exposed to toxic synthetic chemicals in the environment evolve to take advantage of these potential sources of carbon and energy by developing new catabolic pathways (19, 56–59). This and similar studies (52, 60–62) highlight the rapid evolution of bacteria to utilize toxic human-made carbon and energy sources in laboratory time scales, allowing evolution to be studied in real time rather than being reconstructed from history.

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