Journal of Industrial Microbiology & Biotechnology (2001) 27, 94–103

www.nature.com/jim

Multiple mutations at the active site of naphthalene dioxygenase affect regioselectivity and enantioselectivity

C-L Yu, RE Parales and DT Gibson

Department of Microbiology and the Center for Biocatalysis and Bioprocessing, University of Iowa, Iowa City, IA 52242, USA

The importance of five amino acids at the active site of the multicomponent naphthalene dioxygenase (NDO) system was determined by generating site-directed mutations in various combinations. The substrate specificities of the mutant enzymes were tested with the substrates indole, indoline, 2-nitrotoluene (2NT), naphthalene, biphenyl, and phenanthrene. Transformation of these substrates measured the ability of the mutant enzymes to catalyze dioxygenation, monooxygenation, and desaturation reactions. In addition, the position of oxidation and the enantiomeric composition of products were characterized. All enzymes with up to three amino acid substitutions were able to catalyze dioxygenation reactions. A subset of these enzymes could also catalyze the monooxygenation of 2NT and desaturation of indoline. Single amino acid substitutions at positions 352 and 206 had the most profound effects on product formation. Of the single mutations made, only changes at position 352 affected the stereochemistry of naphthalene *cis*-dihydrodiol formed from naphthalene, but in the presence of the F352I mutation, changes at positions 206 and 295 also affected enantioselectivity. Major shifts in regioselectivity with biphenyl and phenanthrene resulted with several of the singly, doubly, and triply mutated enzymes. A new product not formed by the wild-type enzyme, phenanthrene *cis*-9,10-dihydrodiol, was formed as a major product from phenanthrene by enzymes with two (A206I/F352I) or three amino acid substitutions (A206I/F352I/H295I). The results indicate that a variety of amino acid substitutions are tolerated at the active site of NDO. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 94–103.

Keywords: aromatic hydrocarbons; biodegradation; dioxygenase; naphthalene; site-directed mutagenesis

Introduction

Bacterial Rieske nonheme iron oxygenases [11] represent one of the primary groups of enzymes responsible for the initial step in the aerobic biodegradation of aromatic hydrocarbons and related compounds in nature. These enzymes consist of one or two components that transfer electrons from NAD(P)H to the catalytic component. One well-studied example, the naphthalene dioxygenase (NDO) system (EC 1.14.12.11) from *Pseudomonas* sp. NCIB 9816-4, is a multicomponent enzyme system that catalyzes the dioxygenation of naphthalene (Figure 1A). An iron–sulfur flavoprotein reductase and a Rieske [2Fe–2S] ferredoxin transfer electrons from NADH to the oxygenase component (NDO). The reduced form of NDO catalyzes the addition of both atoms of molecular oxygen to naphthalene to yield enantiomerically pure (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (naphthalene *cis*-dihydrodiol) [8,9,15,16]. NDO is an $\alpha_3\beta_3$ hexamer and is the first Rieske nonheme iron oxygenase for which the three-dimensional structure has been determined [23]. The α subunit of NDO contains a Rieske [2Fe–2S] center, which accepts electrons from the ferredoxin component, and transfers them to nonheme mononuclear iron at the active site [8].

NDO has a relaxed substrate specificity and catalyzes the dioxygenation of many two- and three-ring aromatic and hydroaromatic (benzocyclic) compounds to their respective *cis*-dihydrodiols. NDO also catalyzes a variety of other oxidation reactions, including monohydroxylation, desaturation, *O*- and *N*-dealkylation, and sulfoxidation [36]. The extremely broad substrate range and high regio- and enantioselectivities of NDO and related enzyme systems make these enzymes important not only in the degradation of environmental pollutants, but also in the biocatalytic formation of chiral synthons for the production of biologically active chemicals and pharmaceuticals [19].

A closely related enzyme system from *Pseudomonas* sp. strain JS42, 2-nitrotoluene 2,3-dioxygenase (2NTDO), is involved in the degradation of 2-nitrotoluene (2NT). 2NTDO initiates the degradation of 2NT by catalyzing dioxygenation of the aromatic ring of 2NT (Figure 1B). An unstable nitrohydrodiol is formed, which spontaneously breaks down to 3-methylcatechol and nitrite [1,17]. The α subunits of NDO and 2NTDO, which have been shown to control the substrate specificities of both enzyme systems [27,28], are 84% identical in amino acid sequence [26]. Despite their similarities, these two enzymes have key differences in substrate specificity. For example, while NDO forms enantiomerically pure (+)-naphthalene *cis*-dihydrodiol from naphthalene, 2NTDO forms a 7:3 mixture of the (+)- and (−)-enantiomers (Figure 1A). Both enzymes oxidize 2NT at the methyl group to form 2-nitrobenzyl alcohol, but 2NTDO preferentially oxidizes the aromatic ring of 2NT (Figure 1B) [27].

Investigations of cytochromes *P*450 have suggested that the different reaction types catalyzed by these enzymes are unlikely to be due to different mechanisms, but to positioning of the substrate in the active site [14]. According to the crystal structure of NDO, 17 amino acids line the substrate binding pocket [6,23]. Amino acid sequence alignments of NDO and 2NTDO identified five differences inside the substrate pocket that are likely to change the

Correspondence: Dr RE Parales, Department of Microbiology, 3-730 Bowen Science Building, University of Iowa, Iowa City, IA 52242, USA
Received 25 September 2000; accepted 29 June 2001

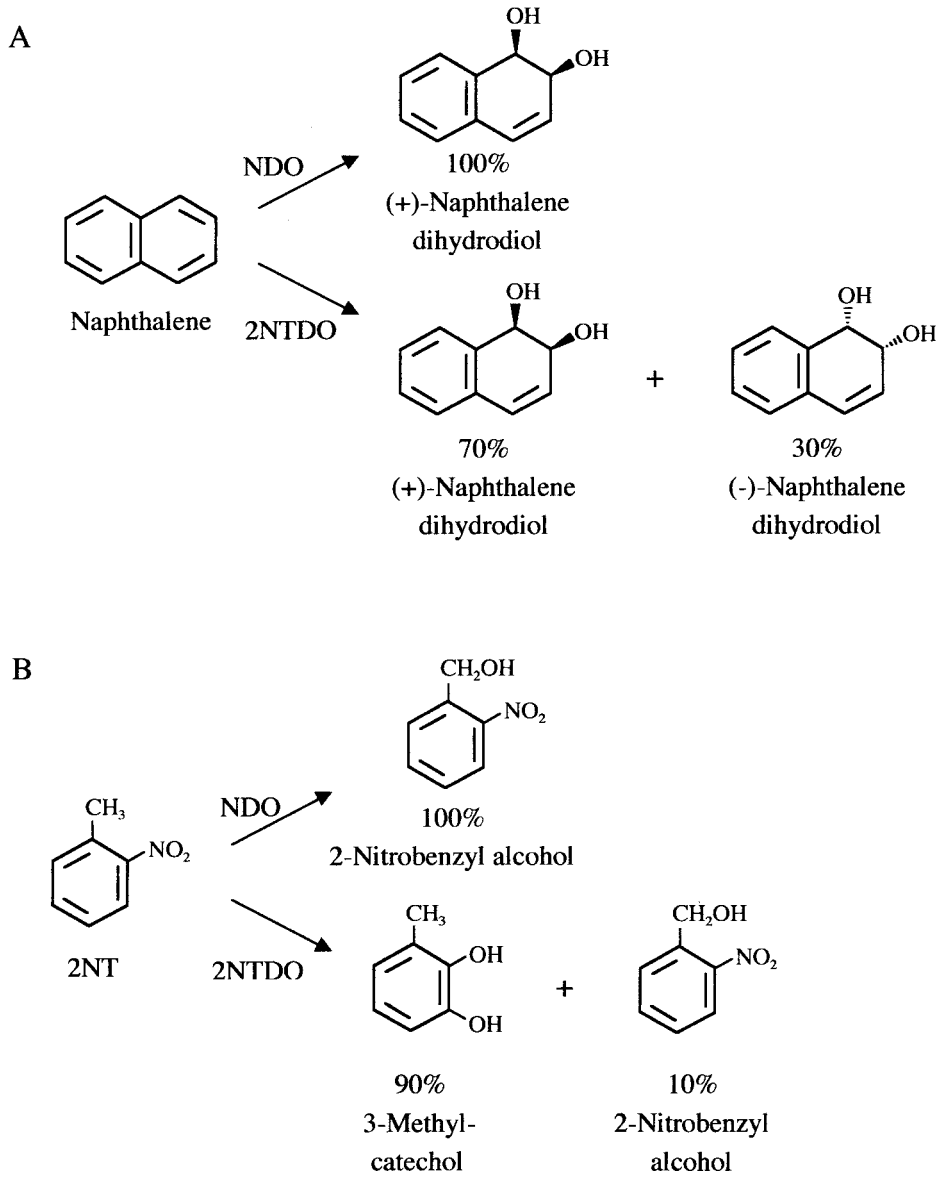

Figure 1 Reactions catalyzed by the NDO system and the 2NTDO system with naphthalene and 2NT as substrates [17,20,21,27,41]. Relative amounts of products are indicated when multiple products are formed.

topology of the substrate pocket and thereby the substrate specificity of these enzymes (Figure 2). These amino acids (NDO numbering), A206, L253, V260, H295, and F352, were predicted to confer the observed differences in substrate specificity of NDO and 2NTDO [5]. Previous results indicated that amino substitutions at phenylalanine 352 of NDO change the regio- and enantioselectivities of NDO [30,31].

In this study, site-directed mutagenesis was used to substitute amino acids in NDO with those at the corresponding positions in 2NTDO to form variants with the following changes in a variety of combinations: A206I, L253T, V260N, H295I, and F352I. Fourteen mutant NDO enzymes that contain between one and five mutations were expressed in *Escherichia coli* JM109(DE3). The substrate specificities of the mutant enzymes were tested with indole, indoline, 2NT, naphthalene, biphenyl, and phenanthrene as substrates. This series of substrates allowed us to assess the ability of the mutant enzymes to catalyze dioxygenation, monooxygenation, and desaturation reactions, and to characterize the regio- and enantioselectivities of dioxygenation.

Materials and methods

Bacterial strains and plasmids

E. coli DH5α (Life Technologies, Gaithersburg, MD) and JM109(DE3) (Promega, Madison, WI) were used for subcloning and gene expression experiments, respectively. Competent *E. coli* ES1301 and JM109 [44] were purchased from Promega, and were used in the site-directed mutagenesis procedure. Plasmid pDTG141 [40] carries the four genes encoding NDO (*nahAaAbAcAd*; sequences reported in Refs. [26,38]) from *Pseudomonas* sp. NCIB 9816-4 under control of the T7

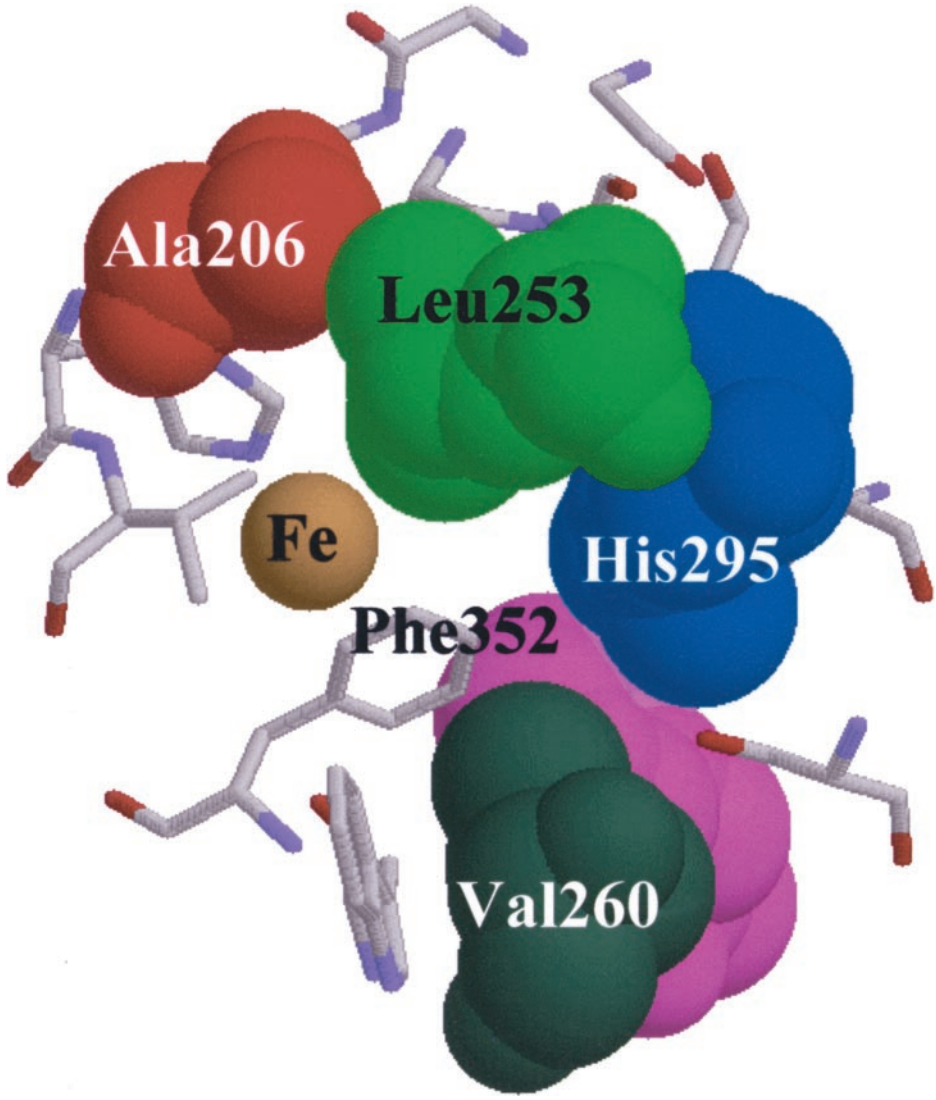

Figure 2 Structure of the active site of NDO showing mononuclear iron and surrounding amino acid residues. The residues that were changed in this study are indicated in red, A206; light green, L253; blue, H295; magenta, F352; and dark green, V260.

promoter of pT7-5 [42]. Plasmid pMASTER-1 [29] carries the *Kpn*I–*Xba*I fragment of pDTG141 containing *nahAcAd*, which encodes the large and small subunits of NDO on pALTER-1 (Promega).

Media and growth conditions

E. coli strains were grown at 30°C or 37°C in Luria–Bertani (LB) medium [7]. Antibiotics were added to the following concentrations as appropriate: 150 μg/ml ampicillin and 15 μg/ml tetracycline. JM109(DE3) strains carrying plasmids of interest were maintained on minimal medium plates (MSB) [39] containing 10 mM glucose, 0.1 mM thiamine, and ampicillin. For plates, MSB was solidified with 1.8% Noble Agar (Difco Laboratories). For small-scale biotransformations, JM109(DE3) containing pDTG141 or its mutant derivatives was cultured at 30°C in MSB medium containing glucose, thiamine, and ampicillin. Dioxygenase genes were induced for 2 h by addition of 100 μM isopropyl-β-D-thiogalactopyanoside when the culture turbidity at 660 nm reached approximately 0.8.

Molecular techniques

Plasmid DNA was isolated as described previously [24] or by using the QIAprep spin miniprep Kit (Qiagen, Chatsworth, CA). For sequencing, DNA was further purified with a Centricon-100 filter unit (Amicon, Beverly, MA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Promega DNA fragments were purified from gel slices with the QIAquick Gel Extraction Kit as specified by the manufacturer (Qiagen). Restriction digests, ligation reactions, transformation of *E. coli* strains, and agarose gel electrophoresis were performed by standard protocols [37].

Site-directed mutagenesis

Mutagenesis was carried out using the Altered Sites II Mutagenesis kit (Promega). Plasmid pMASTER-1 [29] was used as the template in the first round of mutagenesis. Single mutations V260N and F352I were previously constructed using pMASTER-1 [30,31] and, in some cases, these plasmids were used as templates for mutagenesis. To generate the multiple mutants,

further rounds of mutagenesis were carried out using the various derivatives of pMASTER-1. Each mutagenic oligonucleotide was designed with a silent mutation that altered the restriction pattern of the plasmid (Table 1) to facilitate screening for clones carrying the desired mutation. Phosphorylated oligonucleotides used for mutagenesis were synthesized by Genosys Biotechnologies (Midland, TX). The nucleotide sequences of both strands of the entire insertion in pMASTER-1 were determined for each mutation.

Fluorescent automated DNA sequencing was carried out at the University of Iowa DNA Facility with an Applied Biosystems 373A automated DNA sequencer. After verification of each mutation by restriction digestion and sequence analysis, the 1.5-kb *Kpn*I–*Xba*I fragments carrying each mutation were individually cloned into *Kpn*I–*Xba*I-digested pDTG141 to generate complete dioxygenase gene clusters under the control of the T7 promoter. After this subcloning step, the presence of each mutation was verified by restriction digestion and sequence analysis. The resulting plasmids were introduced into JM109(DE3) for gene expression and biotransformation studies.

Biotransformations

Induced cultures were prepared as described above and biotransformations were initiated by adding 20 mM glucose, 100 mM phosphate buffer (pH 7.2), and substrate (naphthalene, biphenyl, phenanthrene, or indole, 0.05% w/v; 2NT, 0.025% v/v). Flasks (reaction volume 100 ml) were incubated with shaking at 30°C for 12–18 h. The cells were then removed by centrifugation (8000×*g*, 10 min, 4°C) and the supernatant solutions were extracted three times with an equal volume of sodium hydroxide-washed ethyl acetate [34]. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated at 32°C under reduced pressure before analysis.

Analysis of biotransformation products

Thin-layer chromatography and gas chromatography mass spectrometry (GC-MS) were used to detect and identify the products as previously described [33,34]. Phenylboronic acid (PBA) derivatives of biphenyl and phenanthrene biotransformation products were analyzed by GC-MS as previously described [33]. Product identifications were based on comparisons to authentic chemicals. Naphthalene *cis*-dihydrodiol was purified for chiral stationary-phase high-performance liquid chromatography (chiral HPLC) analysis by preparative layer chromatography (PLC) [35]. The enantiomeric composition of the naphthalene *cis*-dihydrodiol was determined by chiral HPLC using a Chiralcel OJ column (Chiral Technologies, Exton, PA) as previously described [35]. Under these conditions, the (+)-and (−)-enantiomers of naphthalene *cis*-dihydrodiol eluted with retention times of 30 and 33 min, respectively. Phenanthrene *cis*-9,10-dihydrodiol, phenanthrene *cis*-3,4-dihydrodiol, and phenanthrene *cis*-1,2-dihydrodiol produced by the double mutant AF were separated by multiple-elution PLC (four times) with chloroform/acetone (9:1) (silica thickness, 2.0 mm).

Indigo formation

JM109(DE3) strains carrying mutated plasmids were grown overnight at 37°C on nitrocellulose filters placed on the surface of MSB agar plates containing glucose, thiamine, and ampicillin. Production of indigo was observed as colonies turned blue after exposure to indole or indoline vapor as previously described [29].

Product yields

Total yields (product dry weight) of naphthalene *cis*-dihydrodiol, biphenyl *cis*-dihydrodiols, and phenanthrene *cis*-dihydrodiols were determined after extraction, PLC purification, and concentration to dryness. The amounts of products formed after 18 h in 200-ml cultures containing 100 mg substrate by JM109(DE3)(pDTG141) expressing wild-type NDO were naphthalene *cis*-dihydrodiol, 67 mg; biphenyl *cis*-dihydrodiols, 21 mg; and phenanthrene *cis*-dihydrodiols, 36 mg. Yields of indigo were determined as described previously [10]. A 100-ml biotransformation sample containing cells of JM109(DE3)(pDTG141) expressing wild-type NDO yielded 11 mg of indigo after an 18-h incubation. Amounts of products formed by mutant NDO enzymes are reported as a percentage of the amount formed by the wild-type enzyme.

Chemicals

Naphthalene was purchased from Fisher Scientific (Pittsburgh, PA). Indole, indoline, indigo, phenanthrene, biphenyl, 2NT, 3-methylcatechol, and 2-nitrobenzyl alcohol were obtained from Aldrich Chemical (Milwaukee, WI). Naphthalene *cis*-1,2-dihydrodiol, biphenyl *cis*-2,3-dihydrodiol, biphenyl *cis*-3,4-dihydrodiol, phenanthrene *cis*-3,4-dihydrodiol, and phenanthrene *cis*-1,2-dihydrodiol were prepared biologically as previously described [12,21,22,31,32]. Synthetic phenanthrene *cis*-9,10-dihydrodiol was provided by Professor Derek Boyd (QU, Belfast, UK).

Gel electrophoresis and Western blot analyses

Cell pellets (from 1-ml cultures) were resuspended in 200 μl of sodium dodecyl sulfate polyacrylamide gel electrophoresis sample loading buffer [2] and boiled for 10 min. Proteins were separated on duplicate sodium dodecyl sulfate 12% polyacrylamide gels [2]. One gel was stained with Coomassie blue R-250 to verify that approximately equal amounts of protein were loaded in each lane. The second gel was subjected to Western blotting as described previously [18,25] with an antibody specific for the α subunit of NDO [28]. Antigens were visualized with alkaline

Table 1 Oligonucleotides used for site-directed mutagenesis

Mutation	Mutagenic oligonucleotide[a]	Restriction site change
A206I	5′-CTTTGTGGGAGAT**AT**ATACCACGTGGGT-3′	Remove *Nsi*I
L253T	5′-GCGGCATGGGTGT**CAC**GTGGGACGGATATTCA-3′	*Pml*I
H295I	5′-GATTTATCGCAGC**ATC**CTCAACTGCACCG-3′	*Sfa*NI

[a]Restriction site underlined; base changes in boldface.

Table 2 NDO mutants constructed

Mutant NDO designation	Mutations present in NDO α subunit	Reference
A206I	A206I	This study
L253T	L253T	This study
V260N	V260N	[30]
H295I	H295I	This study
F352I	F352I	[31]
AH	A206I, H295I	This study
VF	V260N, F352I	This study
AL	A206I, L253T	This study
AF	A206I, F352I	This study
AFL	A206I, F352I, L253T	This study
AHV	A206I, H295I, V260N	This study
AHF	A206I, H295I, F352I	This study
AHVF	A206I, H295I, V260N, F352I	This study
AHVFL	A206I, H295I, V260N, F352I, L253T	This study

phosphatase-conjugated goat antimouse immunoglobulin G (Pierce, Rockford, IL).

Results

Site-directed mutagenesis and initial analysis of the mutant NDO enzymes

Based on the crystal structure of NDO and amino acid sequence alignments with 2NTDO, it was predicted that a minimum of five amino acid differences at the active site of NDO result in the differences in the substrate specificities of the two related enzymes [5,6,23]. Single mutations at the five positions were constructed using pMASTER-1 and multiple mutations were generated using the newly generated derivatives of pMASTER-1. Five amino acids in the α subunit of NDO were changed (A206I, L253T, V260N, H295I, and F352I) in various combinations to generate the single and multiple mutants shown in Table 2. JM109(DE3) strains carrying pDTG141 mutant derivatives were grown on plates and exposed to indole to test activity with this substrate. All single mutants and the double mutants AF and AL formed indigo from indole (Table 3), although in varying amounts (Figure 3). With the exception of the AFL mutant, the other multiple mutants did not form visible amounts of indigo. These results indicate that all of the single mutants and at least three of the multiple mutants were active and could catalyze the dioxygenation of indole. Further studies demonstrated that several of the enzymes that did not form indigo from indole were capable of dioxygenating other substrates (see below).

Production of the mutant NDO α subunits

JM109(DE3)(pT7-5), JM109(DE3)(pDTG141), and the strains carrying the NDO mutants that did not form indigo were analyzed by gel electrophoresis and Western blot analysis to determine whether full-size α subunits were formed. Protein that corresponded in mobility to the single band observed for purified NDO was produced by strains carrying the wild type and all mutant constructs (Figure 4). No band was detected in cell lysates of JM109(DE3)(pT7-5). These results suggest that the inability to convert indole to indigo by the multiple mutants was not due to the absence of protein or the formation of truncated α subunits.

Desaturation and monooxygenation by mutant NDO enzymes

Desaturation and monooxygenation activities of the NDO multiple mutants were detected with indoline and 2NT as substrates, respectively. NDO catalyzes the desaturation of indoline to form indole, which is then dioxygenated to form indigo [13]. Indigo was formed from indoline by all single mutants, but only the double mutants AF, AL, and the triple mutant AFL produced indigo from this substrate. The other multiple mutants did not form detectable amounts of indigo from indoline.

Table 3 Activity of mutant NDO enzymes with indole, indoline, and 2NT

Mutant NDO designation[a]	Formation of indigo from indole[b]	Formation of indigo from indoline[b]	Major product from 2NT
Wild-type NDO	+++	+++	2-nitrobenzyl alcohol
A206I	++	++	2-nitrobenzyl alcohol
L253T	++	++	2-nitrobenzyl alcohol
V260N	+[c]	+	2-nitrobenzyl alcohol
H295I	++	++	2-nitrobenzyl alcohol
F352I	+[d]	+	2-nitrobenzyl alcohol
AH	−	−	N.D.[e]
VF	−	−	N.D.
AL	+	+	2-nitrobenzyl alcohol
AF	+	+	2-nitrobenzyl alcohol
AFL	+	+	2-nitrobenzyl alcohol
AHV	−	−	N.D.
AHF	−	−	N.D.
AHVF	−	−	N.D.
AHVFL	−	−	N.D.
Wild-type 2NTDO[f]	+	+	3-methylcatechol

[a]See Table 2.
[b]+++, Colonies dark blue; ++, colonies medium blue; +, colonies pale blue; −, colonies white (similar to negative control strain JM109(DE3)(pT7-5).
[c]Data reported in Ref. [30].
[d]Data reported in Ref. [31].
[e]None detected.
[f]Data reported in Refs. [17,27].

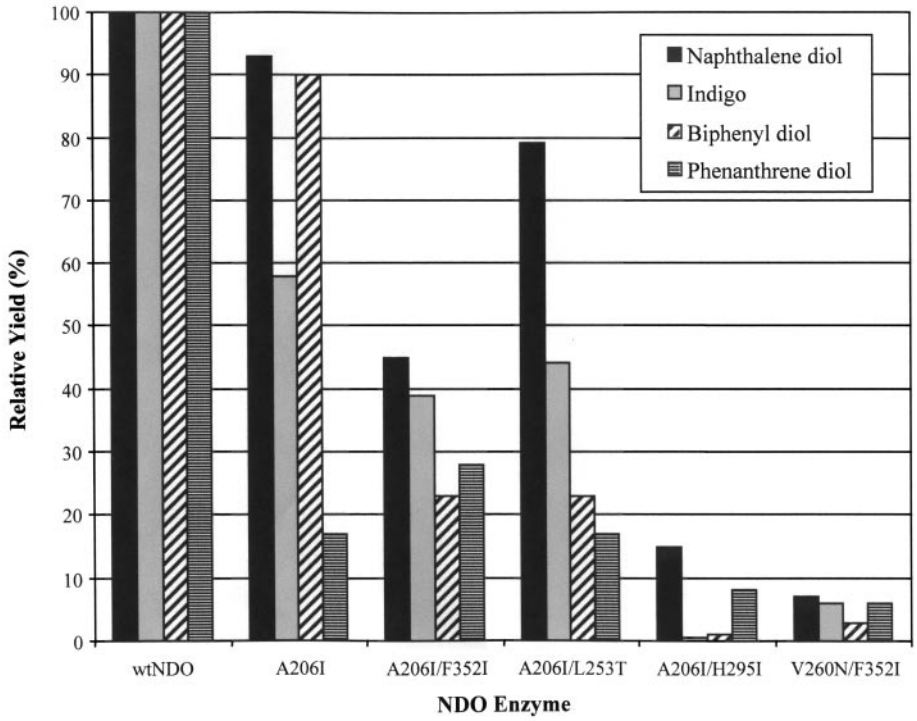

Figure 3 Yields of products formed from indole, naphthalene, biphenyl, and phenanthrene by NDO variants relative to amounts formed by wild-type NDO. Quantifications were carried out as described in Materials and Methods. In the case of biphenyl dihydrodiol and phenanthrene dihydrodiol, bars represent the sums of all isomers formed.

The wild-type NDO, all of the single mutants, and the AF, AL, and AFL mutants formed 2-nitrobenzyl alcohol from 2NT (Table 3). None of the mutant NDO enzymes formed 3-methylcatechol from 2NT, indicating that none of the amino acid substitutions conferred the ability to dioxygenate the aromatic ring. The results show that the same subset of multiply mutated enzymes was unable to catalyze dioxygenation of indole, desaturation of indoline, or monooxygenation of 2NT.

Regioselectivity of NDO mutants

With the exception of mutant AHVFL, which formed no detectable products with any of the substrates tested, all mutant NDO enzymes formed naphthalene *cis*-1,2-dihydrodiol from naphthalene. Thus, regioselectivity with naphthalene remained unchanged. The amount of product formed by the double mutants was determined and compared with that formed by the A206I mutant and the wild-type enzyme (Figure 3). The amount of product formed by the triple mutants and the quadruple mutant was even lower (data not shown).

When compared with wild-type NDO, the ratios of biphenyl *cis*-2,3- and biphenyl *cis*-3,4-dihydrodiols formed by single and multiple mutants differed significantly (Table 4). Our previous work indicated that the F352I mutant formed a ratio of biphenyl *cis*-2,3- and biphenyl *cis*-3,4-dihydrodiols (17:83), a result completely opposite to the ratio observed for wild type (87:13) [31]. The ratios of the dihydrodiols formed by the L253T mutant (62:38) and the AFL mutant (58:42) were also quite different from that of wild type. Many of the multiple mutants formed ratios of biphenyl *cis*-dihydrodiols quite similar to the wild type. The VF double mutant formed a very small amount of biphenyl *cis*-2,3-dihydrodiol and no 3,4-dihydrodiol was detected. The quadruple mutant AHVF produced only trace amounts of biphenyl *cis*-2,3-dihydrodiol. No product was detected from the AHVFL variant of NDO.

With phenanthrene as substrate, single mutants A206I and F352I formed 57% and 76% phenanthrene *cis*-3,4-dihydrodiol, respectively, compared to 90% for wild-type NDO (Table 4). The ratios of products formed by the other three single mutants varied slightly from that of the wild-type enzyme. The ratio of phenanthrene *cis*-3,4- to phenanthrene *cis*-1,2-dihydrodiols formed by the double mutants VF (76:24) and AH (50:50) were

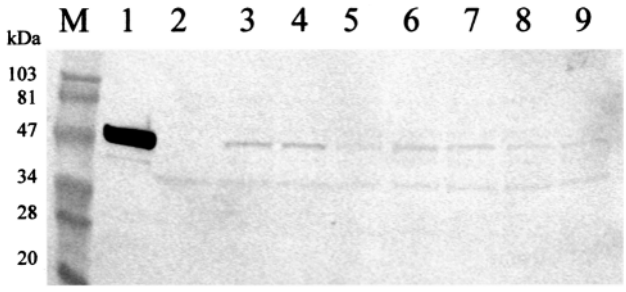

Figure 4 Western blot showing the α subunits formed by JM109(DE3) carrying pDTG141 derivatives with the mutations indicated. A monoclonal antibody specific for the α subunit of NDO was used [28]. Lanes: M, prestained low-range SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA); 1, purified wild-type NDO; 2, pT7-5 (negative control); 3, wild-type NDO (pDTG141); 4, AH; 5, VF; 6, AHV; 7, AHF; 8, AHVF; 9, AHVFL. See Table 2 for descriptions of mutant enzymes. The antibody also reacted nonspecifically with a protein (~35 kDa) present in all *E. coli* extracts.

Table 4 Regioselectivity of mutant NDO enzymes with biphenyl and phenanthrene[a]

Mutant NDO[b] designation	Products formed from biphenyl		Products formed from phenanthrene		
	Percentage of biphenyl *cis*-2, 3- dihydrodiol	Percentage of biphenyl *cis*-3, 4- dihydrodiol	Percentage of phenanthrene *cis*-3, 4- dihydrodiol	Percentage of phenanthrene *cis*-1, 2- dihydrodiol	Percentage of phenanthrene *cis*-9, 10- dihydrodiol
Wild-type NDO[c]	87	13	90	10	–[d]
A206I	95	5	57	43	–
L253T	62	38	>99	–	–
V260N[c]	93	7	94	6	–
H295I	87	13	83	17	–
F352I[e]	17	83	76	24	–
AH	92	8	50	50	–
VF	trace	–	76	24	–
AL	82	18	14	86	–
AF	79	21	45	23	32
AFL	58	42	10	84	6
AHV	>99	–	–	–	–
AHF	78	22	25	34	41
AHVF	trace	–	–	–	–
AHVFL	–	–	–	–	–
Wild-type 2NTDO	trace	–	trace	–	–

[a]Extracts of culture supernatants from *E. coli* strains producing wild-type and mutant NDO enzymes were derivatized with PBA and analyzed by GC-MS. Product distributions were determined from the GC-MS peak area integrations of total ion current chromatograms. The data shown are averages of at least two independent experiments and results typically varied less than ±1–2%.
[b]See Table 2.
[c]Data reported in Ref. [30].
[d]None detected.
[e]Data reported in Ref. [31].

similar to those seen with the single mutants F352I (75:25) and A206I (57:43), respectively. These results suggest that the changes at positions 352 and 206 are more important than those at positions 260 and 295 in determining regioselectivity with phenanthrene. Interesting results were obtained with mutants AL and AFL. The ratios of phenanthrene *cis*-3,4- to phenanthrene *cis*-1,2-dihydrodiols formed were completely opposite to that formed by the wild type (Table 4). This result is similar to that seen with a variant of NDO with an F352V mutation [30]. Significant changes were also found with double mutant AF and triple mutants AHF and AFL. Each of these proteins produced a third product, phenanthrene *cis*-9,10-dihydrodiol, that is not formed by wild-type NDO (Table 4). In an earlier study, a small amount of this compound was detected in biotransformations with a NDO variant with an F352L mutation [30]. Total amounts of products formed by some of the mutant enzymes with either biphenyl or phenanthrene as substrate were significantly lower than wild-type levels (Figure 3). However, no attempt was made to optimize product formation efficiencies in these studies. In biotransformations with the AHV, AHVF, and AHVFL multiple mutants, no products were detected from phenanthrene. Phenanthrene was also a very poor substrate for wild-type 2NTDO.

In a large-scale biotransformation, induced cells of JM109(DE3)(pDTG141-A206I-F352I) transformed 500 mg of phenanthrene to 54 mg of product. After separation by PLC, analysis showed that the product contained 32% phenanthrene *cis*-9,10-dihydrodiol, 45% phenanthrene *cis*-3,4-dihydrodiol, and 23% phenanthrene *cis*-1,2-dihydrodiol.

Enantioselectivity of NDO mutants

The stereochemistry of the naphthalene *cis*-dihydrodiol formed by the mutant enzymes was determined by chiral HPLC (Table 5). Previous results showed that the single mutant F352I formed about 6% (−)-naphthalene-*cis*-(1*S*,2*R*)-dihydrodiol [31]. The four other single mutants formed enantiomerically pure (+)-naphthalene *cis*-(1*R*,2*S*)-dihydrodiol (Table 5). All of the double and triple

Table 5 Enantioselectivity of mutant NDO enzymes with naphthalene[a]

Mutant NDO designation[b]	Percentage of (+)-naphthalene-(1*R*,2*S*)-dihydrodiol	Percentage of (−)-naphthalene-(1*S*,2*R*)- dihydrodiol
Wild-type NDO	>99	–[c]
A206I	>99	–
L253T	>99	–
V260N[d]	>99	–
H295I	>99	–
F352I[e]	93	7
AH	>99	–
VF	94	6
AL	>99	–
AF	70	30
AFL	70	30
AHV	>99	–
AHF	63	37
AHVF	N.D.[f]	N.D.
AHVFL	–	–
Wild-type 2NTDO[g]	70	30

[a]Enantiomeric composition was determined by chiral HPLC as described in Materials and Methods.
[b]See Table 2.
[c]None detected.
[d]Data reported in Ref. [30].
[e]Data reported in Ref. [31].
[f]Naphthalene *cis*-dihydrodiol was formed but in quantities too low to allow stereochemical analysis.
[g]Data reported in Ref. [27].

Table 6 Comparison of amino acids in related Rieske nonheme iron oxygenases

Enzyme (strain)[a]	GenBank accession number	Primary substrate	Amino acid at position[b]				
			206	253	260	295	352
NahAc (G7)	M83949	Naphthalene	Ala	Leu	Val	His	Phe
NahAc (9816-4)	U49496	Naphthalene	Ala	Leu	Val	His	Phe
NtdAc (JS42)	U49504	2NT	Ile	Thr	Asn	Ile	Ile
DntAc (DNT)	U62430	2,4-Dinitrotoluene	Ile	Ser	Val	Gln	Thr
TcbAa (P51)	U15298	Trichlorobenzene	Ala	Phe	Leu	Met	Phe
TecA1 (PS12)	U78099	Tetrachlorobenzene	Ala	Phe	Leu	Thr	Phe
TodC1 (F1)	J04996	Toluene	Met	Phe	Leu	Met	Phe
BedC1 (ML2)	L04642	Benzene	Met	Phe	Met	Met	Phe
BphA (LB400)	M86348	Biphenyl	Met	Trp	Ser	Val	Phe
BphA1 (KF707)	M83673	Biphenyl	Met	Trp	Met	Phe	Phe
CumA1 (IPO1)	D37828	Isopropyltoluene	Met	Trp	Leu	Val	Phe
TdnA1 (UCC22)	D85415	Aniline	Gly	Leu	Ile	Asn	–[c]
BenA (ADP1)	M76990	Benzoate	Gly	Phe	Leu	Cys	Phe
XylX (PaW1)	M647474	Toluate	Gly	Phe	Met	Cys	Phe
CmtAb (F1)	U24215	Isopropylbenzoate	Gly	Ala	Ile	Leu	Leu

[a]α Subunit of the enzyme and bacterial strain from which the enzyme originates.
[b]Position refers to numbering in NahAc (9816-4). Amino acid sequence alignments were carried out with the Pileup program (Wisconsin sequence analysis package; Genetics Computer Group, Madison, WI).
[c]Based on alignments, there is a gap at this position.

mutants that contained the F352I change formed significant amounts of the (−)-enantiomer of naphthalene *cis*-1,2-dihydrodiol. The AHVFL mutant formed no detectable products, and the amount of product formed by the AHVF mutant was too small to allow stereochemical analysis (Table 5). Compared with single mutant F352I, the relative amounts of the (−)-enantiomer formed by the double mutant AF increased from 6% to 30%. The triple mutant AHF formed the largest proportion of the (−)-enantiomer (37%). All other multiple mutants that did not contain the F352I change formed enantiomerically pure (+)-naphthalene *cis*-(1*R*,2*S*)-dihydrodiol. These results indicate that the amino acid at position 352 is critical in determining the enantioselectivity of the enzyme with naphthalene. However, in the context of the F352I mutation, secondary changes in the substrate pocket had a major influence on enantioselectivity. Results with the AF, AFL, and AHF mutants were very similar to results with wild-type 2NTDO (Table 5), indicating that only two amino acid substitutions are necessary to change the enantioselectivity of NDO to that of 2NTDO.

Discussion

In this study, 14 variants of NDO with between one and five amino acid substitutions near the active site were screened for their ability to catalyze *cis*-dihydroxylation, monooxygenation, and desaturation reactions. Several of the enzymes were able to catalyze all reaction types (Tables 3 and 4). In addition, the regio- and enantioselectivities of the enzymes were characterized. Previous work demonstrated that the F352I substitution in NDO resulted in a major change in the regioselectivity with biphenyl and phenanthrene [31]. It is interesting to note that all active multiply mutated NDO enzymes that carry the F352I substitution actually behaved more like the wild-type enzyme than the F352I mutant when biphenyl was provided as substrate (Table 4). This was not the case with phenanthrene as the substrate (Table 4). Of particular interest are the double and triple mutants AF, AL, AFL, and AHF. The AF and AHF enzymes produced as a major product phenanthrene *cis*-9,10-dihydrodiol, a compound not formed by either wild-type enzyme. The AL and AFL enzymes formed phenanthrene *cis*-1,2-dihydrodiol (the minor product formed by wild-type NDO) as the major product from phenanthrene (Table 4).

Specific amino acid substitutions were made in order to change the active site of NDO to be more similar to that of the related enzyme 2NTDO. We found that enzymes with four or five amino acid substitutions had little or no activity with any of the substrates tested. This result was somewhat surprising because the overall amino acid identities of the NDO and 2NTDO α subunits are quite similar. Apparently, the multiple amino acid substitutions had wider ranging effects on the structure of the enzyme that resulted in inactivation. In general, the amino acid substitutions at positions 295 and 260 had more detrimental effects on activity with all substrates tested than those at 352, 253, and 206 (Figure 3). By comparing the products formed by the two wild-type enzymes with those formed by the active NDO variants, we identified amino acids that were important in determining the differences in specificity. None of the NDO variants was capable of dihydroxylating the ring of 2NT, suggesting that other amino acids that may not interact directly with the substrate are important in determining whether enzymes are capable of catalyzing this reaction. However, we found that only two amino acid changes were required to change the enantioselectivity of NDO to that of 2NTDO with naphthalene as the substrate. In a previous study, we found that all assayed amino acid substitutions at position 352 of NDO resulted in the formation of detectable amounts of (−)-naphthalene *cis*-dihydrodiol [31]. No single amino acid substitutions that were assayed other than those at position 352 affected the stereochemistry of naphthalene *cis*-dihydrodiol [>99%(+)] (Table 5; Refs. [30,31]). However, in the context of an isoleucine at position 352, major differences in enantioselectivity were observed when further amino acid substitutions were introduced at positions 206 and 295, as seen with the double and triple mutants AF, AFL, and AHF (Table 5).

The amino acid at position 206 of NDO corresponds in amino acid sequence alignments with Met220 in the α subunit of toluene dioxygenase [45]. In the chlorobenzene dioxygenases TecA [3]

and TcbA [43], an alanine is present at this position. (Table 6). Unlike toluene dioxygenase, TecA is able to oxidize 1,2,4,5-tetrachlorobenzene. However, a single amino acid change (M220A) resulted in a toluene dioxygenase variant with activity towards 1,2,4,5-tetrachlorobenzene [4]. These results indicate that the amino acid at position 206 (NDO numbering) is important in determining substrate specificity in widely divergent (<35% amino acid identity) Rieske nonheme iron oxygenases, suggesting that the three-dimensional structures of the active sites of these enzymes are at least partially conserved.

Amino acids predicted to interact with substrates at positions 206, 253, 260, and 295 (NDO numbering) [5,6] are quite variable in dioxygenases whose primary substrates include aromatic hydrocarbons, chlorinated aromatics, amino- and nitroaromatic compounds, and aromatic acids (Table 6). This is consistent with the widely differing sizes, structures, and polarities of these substrates. Only the amino acid at position 352 is somewhat conserved (Table 6). This and previous data suggest that a wide range of amino acids can be tolerated near the active sites of Rieske nonheme iron oxygenases and these changes can have large effects on product formation from a variety of substrates. However, even with the crystal structure of NDO available, it is difficult at this time to predict the actual effects of specific amino acid substitutions at the active site of the enzyme.

Acknowledgements

This work was supported by Public Health Service grant GM29909 from the National Institute of General Medical Sciences. We thank Derek Boyd, the Queen's University of Belfast, Belfast, UK, for providing phenanthrene *cis*-9,10-dihydrodiol.

References

1 An D, DT Gibson and JC Spain. 1994. Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42. *J Bacteriol* 176: 7462–7467.
2 Ausubel FM, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith and K Struhl. 1993. Current Protocols in Molecular Biology. Wiley, New York.
3 Beil S, B Happe, KN Timmis and DH Pieper. 1997. Genetic and biochemical characterization of the broad spectrum chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12 — dechlorination of 1,2,4,5-tetrachlorobenzene. *Eur J Biochem* 247: 190–199.
4 Beil S, JR Mason, KN Timmis and DH Pieper. 1998. Identification of chlorobenzene dioxygenase sequence elements involved in dechlorination of 1,2,4,5-tetrachlorobenzene. *J Bacteriol* 180: 5520–5528.
5 Carredano E. 1999. PhD Thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden.
6 Carredano E, A Karlsson, B Kauppi, D Choudhury, RE Parales, JV Parales, K Lee, DT Gibson, H Eklund and S Ramaswamy. 2000. Substrate binding site of naphthalene 1,2-dioxygenase: functional implications of indole binding. *J Mol Biol* 296: 701–712.
7 Davis RW, D Botstein and JR Roth. 1980. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
8 Ensley BD and DT Gibson. 1983. Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. *J Bacteriol* 155: 505–511.
9 Ensley BD, DT Gibson and AL Laborde. 1982. Oxidation of naphthalene by a multicomponent enzyme system from *Pseudomonas* sp. strain NCIB 9816. *J Bacteriol* 149: 948–954.
10 Ensley BD, BJ Ratzkin, TD Osslund, MJ Simon, LP Wackett and DT Gibson. 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. *Science* 222: 167–169.
11 Gibson DT and RE Parales. 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* 11: 236–243.
12 Gibson DT, RL Roberts, MC Wells and VM Kobal. 1973. Oxidation of biphenyl by a *Beijerinckia* species. *Biochem Biophys Res Commun* 50: 211–219.
13 Gibson DT, SM Resnick, K Lee, JM Brand, DS Torok, LP Wackett, MJ Schocken and BE Haigler. 1995. Desaturation, dioxygenation and monooxygenation reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4. *J Bacteriol* 177: 2615–2621.
14 Guengerich FP. 1991. Reactions and significance of cytochrome P-450 enzymes. *J Biol Chem* 266: 10019–10022.
15 Haigler BE and DT Gibson. 1990. Purification and properties of ferredoxin$_{NAP}$, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. *J Bacteriol* 172: 465–468.
16 Haigler BE and DT Gibson. 1990. Purification and properties of NADH–ferredoxin$_{NAP}$ reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. *J Bacteriol* 172: 457–464.
17 Haigler BE, WH Wallace and JC Spain. 1994. Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42. *Appl Environ Microbiol* 60: 3466–3469.
18 Harlow E and D Lane. 1988. Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19 Hudlicky T, D Gonzalez and DT Gibson. 1999. Enzymatic dihydroxylation of aromatics in enantioselective synthesis: expanding asymmetric methodology. *Aldrichimica Acta* 32: 35–62.
20 Jeffrey AM, HJC Yeh, DM Jerina, TR Patel, JF Davey and DT Gibson. 1975. Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. *Biochemistry* 14: 575–583.
21 Jerina DM, JW Daly, AM Jeffrey and DT Gibson. 1971. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. *Arch Biochem Biophys* 142: 394–396.
22 Jerina DM, H Selander, H Yagi, MC Wells, JF Davey, V Mahadevan and DT Gibson. 1976. Dihydrodiols from anthracene and phenanthrene. *J Am Chem Soc* 98: 5988–5996.
23 Kauppi B, K Lee, E Carredano, RE Parales, DT Gibson, H Eklund and S Ramaswamy. 1998. Structure of an aromatic ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. *Structure* 6: 571–586.
24 Lee S-Y and S Rasheed. 1990. A simple procedure for maximum yield of high-quality plasmid DNA. *BioTechniques* 9: 676–679.
25 Lynch NA, H Jiang and DT Gibson. 1996. Rapid purification of the oxygenase component of toluene dioxygenase from a polyol-responsive monoclonal antibody. *Appl Environ Microbiol* 62: 2133–2137.
26 Parales JV, A Kumar, RE Parales and DT Gibson. 1996. Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42. *Gene* 181: 57–61.
27 Parales JV, RE Parales, SM Resnick and DT Gibson. 1998. 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: 1194–1199.
28 Parales RE, MD Emig, NA Lynch and DT Gibson. 1998. Substrate specificities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems. *J Bacteriol* 180: 2337–2344.
29 Parales RE, JV Parales and DT Gibson. 1999. Aspartate 205 in the catalytic domain of naphthalene dioxygenase is essential for activity. *J Bacteriol* 181: 1831–1837.
30 Parales RE, K Lee, SM Resnick, H Jiang, DJ Lessner and DT Gibson. 2000. Substrate specificity of naphthalene dioxygenase: effect of specific amino acids at the active site of the enzyme. *J Bacteriol* 182: 1641–1649.
31 Parales RE, SM Resnick, CL Yu, DR Boyd, ND Sharma and DT Gibson. 2000. Regioselectivity and enantioselectivity of naphthalene dioxygenase during arene *cis*-dihydroxylation: control by phenylalanine 352 in the α subunit. *J Bacteriol* 182: 5495–5504.
32 Resnick S. 1997. PhD Thesis. University of Iowa, Iowa City, IA.
33 Resnick SM and DT Gibson. 1996. Regio- and stereospecific oxidation of 9,10-dihydroanthracene and 9,10-dihydrophenanthrene by naphthalene dioxygenase: structure and absolute stereochemistry of metabolites. *Appl Environ Microbiol* 62: 3355–3359.
34 Resnick SM, DS Torok and DT Gibson. 1993. Oxidation of carbazole to 3-hydroxycarbazole by naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase. *FEMS Microbiol Lett* 113: 297–302.

35 Resnick SM, DS Torok, K Lee, JM Brand and DT Gibson. 1994. Regiospecific and stereoselective hydroxylation of 1-indanone and 2-indanone by naphthalene dioxygenase and toluene dioxygenase. *Appl Environ Microbiol* 60: 3323–3328.
36 Resnick SM, K Lee and DT Gibson. 1996. Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. *J Ind Microbiol* 17: 438–457.
37 Sambrook J, EF Fritch and T Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
38 Simon MJ, TD Osslund, R Saunders, BD Ensley, S Suggs, A Harcourt, W-C Suen, DL Cruden, DT Gibson and GJ Zylstra. 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. *Gene* 127: 31–37.
39 Stanier RY, NJ Palleroni and M Doudoroff. 1966. The aerobic pseudomonads; a taxonomic study. *J Gen Microbiol* 43: 159–271.
40 Suen W-C. 1991. PhD Thesis. University of Iowa, Iowa City, IA.
41 Suen W-C, BE Haigler and JC Spain. 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. *J Bacteriol* 178: 4926–4934.
42 Tabor S and CC Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* 82: 1074–1078.
43 Werlen C, H-P Kohler and JR van der Meer. 1996. The broad substrate chlorobenzene dioxygenase and *cis*-chlorobenzene dihydrodiol dehydrogenase of *Pseudomonas* sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation. *J Biol Chem* 271: 4009–4016.
44 Yanisch-Perron C, J Vieira and J Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33: 103–119.
45 Zylstra GJ and DT Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *E. coli*. *J Biol Chem* 264: 14940–14946.