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13. ABSTRACT *(Maximum 200 words)*

Funded studies focused on the biodegradation of three nitroaromatic compounds, nitrobenzene, 2-nitrotoluene, and 2,4-dinitrotoluene, by bacterial strains isolated in Dr. Jim C. Spain's laboratory, Tyndall AFB. Genes and enzymes required for the degradation of these compounds were characterized. Several genes involved in the degradation of nitrobenzene and phenol from *Comamonas* sp. strain JS765 were cloned and sequenced. A gene cluster from *Pseudomonas* sp. strain JS42 encoding 2-nitrotoluene dioxygenase was cloned and sequenced and was found to be related to gene clusters from *Burkholderia* sp. strain DNT and *Pseudomonas* sp. strain NCIB 9816-4 which encode 2,4-dinitrotoluene and naphthalene dioxygenases, respectively. The *ntd* gene cluster cloned in *E. coli* proved useful in studies that demonstrated that the C-terminal half of the alpha subunit of the oxygenase component of 2-nitrotoluene dioxygenase is responsible for determining the substrate specificity of the enzyme. Construction and analysis of hybrid dioxygenases demonstrated that the oxygenase beta subunits of naphthalene, 2-nitrotoluene and 2,4-dinitrotoluene dioxygenases do not play a role in determining substrate specificity.

14. SUBJECT TERMS
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OBJECTIVES: The major objective of this research project was to determine the mechanisms used by bacteria to degrade nitroaromatic compounds.

During the grant period, the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. strain JS42 were cloned and sequenced. Also, genes required for the degradation of nitrobenzene from *Comamonas* sp. strain JS765 were cloned, sequenced, and their functions characterized. A major focus of the research was the characterization of the factors responsible for the substrate specificities of 2-nitrotoluene and 2,4-dinitrotoluene dioxygenases.

1.) Cloning of the 2-nitrotoluene dioxygenase (2NTDO) genes from *Pseudomonas* sp. strain JS42.

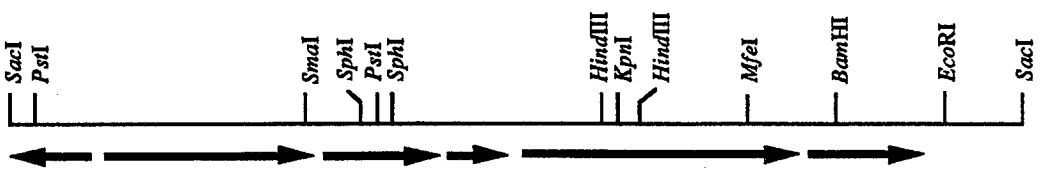
Summary. The ability of the *E. coli* strain carrying the cloned 2-nitrotoluene dioxygenase gene cluster to oxidize 2NT to 3-methylcatechol with concomitant release of nitrite indicates that all of the genes necessary to encode 2NTDO activity were successfully cloned. The genes are located on the 4912-bp *Sac*I DNA fragment from *Pseudomonas* sp. JS42. Sequence analyses of the cloned 2NTDO genes revealed that at the molecular level, $Ferredoxin_{2NT}$, $Reductase_{2NT}$, $ISP\alpha_{2NT}$ and $ISP\beta_{2NT}$ have high amino acid sequence identity to analogous polypeptides from three-component dioxygenase systems in general, and have extremely high amino acid sequence identity to their counterparts from the naphthalene dioxygenase (NDO) system. This high amino acid identity between 2NTDO and NDO suggests that they share a common evolutionary background. Results of this study were published (31).

Cloning of the *ntd* genes. Southern hybridization experiments demonstrated that a probe made from pDTG141(46) containing the NDO genes *nahAaAbAcAd* from *Pseudomonas* sp. NCIB 9816-4 strongly hybridized to a 5kb-*Sac*I fragment of JS42 chromosomal DNA. A

genomic library was constructed using *Sac*I-digested pUC18 (50) and purified *Sac*I-digested JS42 chromosomal DNA of approximately 4-6 kb in size. Competent *Escherichia coli* DH5α (GIBCO-BRL Laboratories, Gaithersburg, MD) cells were transformed with this gene library and plated onto Luria agar (5) supplemented with 200 μg/ml ampicillin. Ampicillin resistant colonies demonstrating strong hybridization to the pDTG141 probe were then screened for 2NTDO activity by monitoring nitrite release in the presence of 2NT (1). One recombinant, designated DH5α(pDTG800) showed strong hybridization to the pDTG141 probe and nitrite release in the presence of 2NT. Whole cell biotransformation assays with DH5α(pDTG800) demonstrated that this recombinant strain was capable of catalyzing the oxidation of 2NT to 3-methylcatechol, indicating that all the genes required for 2NTDO activity were present on the pDTG800 plasmid.

Sequence analysis of pDTG 800. DNA sequence analysis of pDTG800 revealed that it consisted of a 4912-bp *Sac*I DNA fragment inserted into the *Sac*I site of pUC18. Five ORFs exhibiting homology to polypeptides from several multicomponent dioxygenases were identified. Based on sequence homology, predicted polypeptides from four of the ORFs were designated Reductase$_{2NT}$, Ferredoxin$_{2NT}$, ISPα $_{2NT}$ (iron sulfur protein, large subunit) and ISPβ$_{2NT}$ (iron-sulfur protein, small subunit) with gene designations *ntdAa*, *ntdAb*, *ntdAc*, and *ntdAd* respectively (*ntd* for nitrotoluene degradation genes). Gene locations and calculated MWs of the predicted polypeptides are shown in Fig. 1.

Fig. 1 ***ntd*** **Gene Cluster from** ***Pseudomonas*** **sp. JS42**



Gene	*ntdR*	*ntdAa*	ORF2	*ntdAb*	*ntdAc*	*ntdAd*
Coding Region		487-1473	1519-2079	2120-2434	2523-3866	3881-4465
Protein	Regulatory?	Reductase $_{2NT}$	?	Ferredoxin $_{2NT}$	ISPα $_{2NT}$	ISPβ $_{2NT}$
Predicted MW		35,303	21,259	11,481	49,490	23,091

As shown in Table 1, the amino acid sequences of the predicted polypeptides from the 2NTDO genes have high amino acid sequence identity to analogous polypeptides from other three component dioxygenase systems in general, and have extremely high amino acid sequence identity to those from NDO. The amino acid sequence of $Reductase_{2NT}$ was compared to the NDO and TDO reductase sequences and was found to contain conserved cysteines thought to coordinate [2Fe-2S] clusters in chloroplast-type ferredoxins (27, 30). This "plant-type" [2Fe-2S] center is not generally found in reductases from three component dioxygenase systems and was previously thought to be unique to the reductase component of the NDO system (27). The N-terminal region of $Ferredoxin_{2NT}$ was compared to the ferredoxins from NDO and TDO and found to contain the Rieske iron-sulfur motif, $CXHX_{15-17}CX_2H$, characteristic of Rieske iron-sulfur proteins (4, 27, 39). These conserved cysteines and histidines are the proposed sites for the coordination of [2Fe-2S] clusters and are characteristic of ISPα subunits and some ferredoxins from three-component dioxygenase systems. Comparison of the amino acid sequence of $ISP\alpha_{2NT}$ with the amino acid sequence of the ISPα polypeptides of NDO and TDO showed that the N-terminus of $ISP\alpha_{2NT}$ also contained the Rieske iron-sulfur motif. As shown in Table 1, the high amino acid sequence identity between the polypeptides from 2NTDO and NDO clearly show that 2NTDO is related to NDO. The predicted amino acid sequence of ORF2 has high amino acid sequence identity to ISPα subunits and contains the characteristic Rieske iron-sulfur motif for ISPα subunits but appears to encode a polypeptide that would be uncharacteristically small (21,259 MW) for an ISPα subunit. $Reductase_{2NT}$, $Ferredoxin_{2NT}$, $ISP\alpha_{2NT}$ and $ISP\beta_{2NT}$ have highest amino acid sequence identity to the DNDTO system polypeptides from *Burkholderia* sp. strain DNT (47). The polypeptide most similar to ORF2 was

a similar, but full size ORF from the 2,4-dinitrotoluene dioxygenase (*dnt*) gene cluster from *Burkholderia* sp strain DNT.

Table 1. **Amino acid identities with 2NTDO polypeptides**

Dioxygenase enzyme system	Reductase	Ferredoxin	ISPα	ISPβ
2,4-Dinitrotoluene (*Burkholderia* sp. DNT)	87%	79%	88%	92%
Naphthalene (*Pseudomonas* sp. NCIB 9816-4)	67%	72%	84%	76%
Trichlorobenzene (*Pseudomonas* sp. P51)	23%	35%	34%	25%
Biphenyl (*Pseudomonas* sp. LB400)	19%	40%	32%	30%
Toluene (*Pseudomonas putida* F1)	19%	36%	34%	25%

Cloning and sequencing of regulatory genes for nitroaromatic gene expression. A partial open reading frame with homology to *nahR*, was located upstream of the *ntd* genes from *Pseudomonas* sp. strain JS42. The *nahR* gene encodes the activator of naphthalene degradation genes in *Pseudomonas putida* G7. A clone bank of JS42 DNA was generated and a clone carrying the complete *ntdR* gene was identified by colony hybridization.

The *ntdR* gene was sequenced. An inspection of the sequence upstream of the 2,4-dinitrotoluene dioxygenase (*dnt*) genes from *Burkholderia* sp. strain DNT revealed the presence of a gene similar to *ntdR*. The complete *dntR* gene was subcloned from a large clone supplied by J. Spain, Tyndall AFB, and the sequence was completed. The deduced amino acid sequences of the two putative regulatory proteins are 97% identical (8 amino acid differences). These genes are predicted to be involved in expression of the *dnt* and *ntd* structural genes, although this has not yet been demonstrated.

Conclusions. A gene cluster from *Pseudomonas* sp. strain JS42 encoding 2-nitrotoluene dioxygenase was cloned and sequenced and was found to be related to gene clusters from *Burkholderia* sp strain DNT and *Pseudomonas* sp. strain NCIB 9816-4 which encode 2,4-dinitrotoluene and naphthalene dioxygenases, respectively. A possible regulatory gene was located upstream of the *ntd* and *dnt* gene clusters. The *ntd* gene cluster cloned in *E. coli* proved useful in substrate specificity studies (see below).

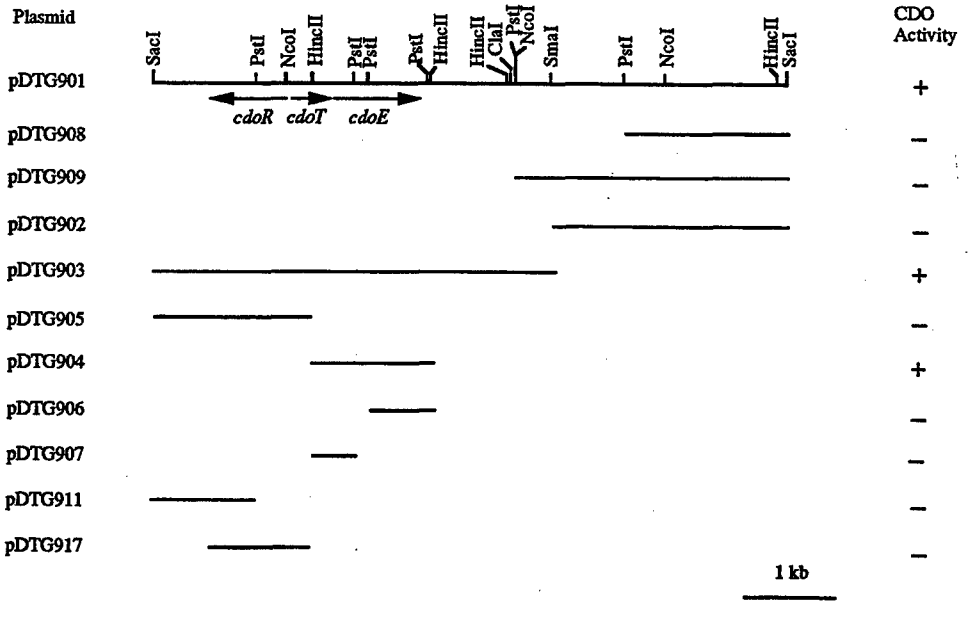
2. Cloning of nitrobenzene degradation genes from *Comamonas* sp. strain JS765.

Summary. The organism isolated in Dr. Jim C. Spain's laboratory degrades nitrobenzene by an initial dioxygenation reaction that eliminates nitrite to form catechol. A gene that encodes catechol 2,3-dioxygenase from *Comamonas* sp. JS765 was cloned and sequenced. Catechol 2,3-dioxygenase carries out the second step in the degradation of nitrobenzene. The catechol 2,3-dioxygenase gene is located in a cluster with a plant type ferredoxin gene and a LysR regulatory gene. This work has been published (35). By further sequencing of the original clone, genes which appear to encode a multicomponent phenol hydroxylase were also identified. Growth studies with JS765 had previously indicated that phenol was not a growth substrate. JS765 was

shown to mutate to a phenol$^+$ strain at high frequency under selection with phenol as sole carbon source. The basis for this mutation is not yet known and studies are ongoing. This work was presented at the Annual Meeting of the American Society for Microbiology (34).

Gene localization and subcloning. A cosmid library was generated by ligating a partial *Sau*3AI digest of JS765 genomic DNA with *Bam*HI-digested pHC79 cosmid. Of 490 colonies screened on catechol spray plates, one turned yellow. Cosmid DNA was isolated from this recombinant and designated pDTG900. Restriction mapping indicated that approximately 34 kb of DNA was inserted in the 6.4 kb vector. Digestion with *Sac*I revealed the presence of 5 fragments (17.0, 7.2, 6.7, 6.2 and 2.7 kb) which were subcloned into *Sac*I-digested pK19. The subclone carrying the 7.2-kb *Sac*I fragment was shown to have catechol 2,3-dioxygenase activity, and was designated pDTG901. The restriction map of pDTG901 is shown in Figure 2. Construction of deletion clones and subclones and subsequent analyses for catechol 2,3-dioxygenase activity localized the gene to the 1.3-kb *Hin*cII fragment on pDTG904 (Figure 2).

Fig. 2 Restriction Map and Subclones to Locate the Catechol 2,3-Dioxygenase Gene from JS765



Sequence analysis. Both strands of the 1.3-kb *Hinc*II fragment of pDTG904 were completely sequenced using a combination of subclones (pDTG904, pDTG906, pDTG907) and oligonucleotide primers. Portions of pDTG901, pDTG905, pDTG911, and pDTG917 were sequenced in order to complete the sequence of the two upstream open reading frames (ORFs) in the gene cluster. The G+C content of the sequenced region was 64%, typical of *Comamonas* sp. genes which range from 61-68% G+C (6, 48).

Nucleotide sequence analysis revealed three ORFs which were designated *cdoRTE* . The *cdoR* gene encodes a 306 amino acid protein. Homology searches with the amino acid sequence deduced from *cdoR* suggested that the 33,639 molecular weight protein is a member of the LysR family of transcriptional regulators. Pairwise comparisons revealed the highest amino acid identities (34-36%) with activators of the *ortho* or modified-*ortho* cleavage pathway genes (Table 2). These regulators include ClcR from plasmid pAC27 (3), CatR from *Pseudomonas putida* PRS2000 (41), TfdR from *Ralstonia eutropha* JMP134 (25), and TcbR from *Pseudomonas* sp. P51 (49). Residues 18-37 of the CdoR form a helix-turn-helix motif very similar to the LysR consensus sequence (43). The *cdoR* gene is divergently transcribed from the *cdoT* gene, and putative sigma-70 type promoters for the *cdoR* and *cdoT* genes were identified. These observations suggest that CdoR regulates transcription of *cdoTE*, although this has not yet been tested.

The *cdoT* gene encodes a protein with a calculated molecular weight of 12,983. Analysis of the amino acid sequence deduced from *cdoT* revealed strong homology to plant-type ferredoxins, especially the XylT-type ferredoxins which are associated with aromatic compound degradation. Homologous proteins include TbuW from the toluene/benzene monooxygenase

gene cluster of *R. pickettii* PKO1 (22), XylT from the TOL plasmid (12), NahT from the NAH7 plasmid (12, 51), and DmpQ, PhhQ, and PhlG from the phenol hydroxylase gene clusters of *Pseudomonas* sp.CF600 (45), *P. putida* P35X (28) , and *P. putida* H (14), respectively. Conserved cysteines characteristic of plant-type ferredoxins (12) were identified in the deduced amino acid sequence. These amino acids are potential ligands for plant-type [2Fe-2S] clusters.

Each of the plant-type ferredoxin genes mentioned above is located directly upstream of a catechol 2,3-dioxygenase gene. Only *xylT* has been characterized in detail. Mutations in *xylT* from the TOL plasmid prevented growth on *para*-substituted aromatic compounds. The associated catechol 2,3-dioxygenase (XylE) was shown to be sensitive to 4-methylcatechol, and *in vivo* studies demonstrated that XylT was able to reactivate XylE that had been inactivated by 4-methylcatechol (37). In *Pseudomonas* sp. CF600, DmpQ was shown to be required for growth with *para*-substituted phenols (38), and based on this, DmpQ is predicted to play a role similar to that of XylT: modulation of catechol 2,3-dioxygenase (in this case DmpB) activity. By analogy, it is possible that CdoT may expand the substrate range of CdoE by modifying its activity in the presence of suicide substrates.

A 314 amino acid protein with a molecular weight of 35,020 is encoded by *cdoE*. The deduced amino acid sequence had high homology with several catechol 2,3-dioxygenases , especially those of the 1.2.C subfamily of extradiol dioxygenases (7). The most similar protein is TdnC (84% amino acid identity) from the aniline and toluidine degrading *P. putida* UCC2 (42). CDOII from the TOL plasmid pWW15 (21), and TbuE from *R. pickettii* PKO1 are (22) also very similar. The deduced amino acid sequence was found to contain conserved residues involved in metal binding, catalysis, and dimerization that were identified in crystallization studies of 2,3-dihydroxybiphenyl 1,2-dioxygenses (11, 44).

Catechol 2,3-dioxygenase activity in cell extracts. Extracts of recombinant strains were tested for the ability to convert catechol to 2-hydroxymuconic semialdehyde (HMS). The extract from JM109(pDTG900) had very low activity (Table 2). This result was not surprising, since the cosmid does not carry a strong promoter for expression and the copy number of the cosmid is low. Activities in extracts of DH5α carrying pDTG901 or pDTG904 were significantly higher. As seen with the catechol 2,3-dioxygenase from nitrobenzene-induced JS765 (29), heat treatment of the DH5α(pDTG901) extract (60°C for 10 min) did not significantly reduce enzyme activity (Table 2).

Table 2. Catechol 2,3-dioxygenase activity in cell extracts

Extract source	Catechol 2,3-dioxygenase specific activity (μmol/min/mg of protein)
JS765[a]	3.04
DH5α(pDTG900)	0.005
DH5α(pDTG901)	0.55 (0.47)[b]
DH5α(pDTG904)	0.26

[a] Data from (29).
[b] Activity after incubation of crude cell extract at 60°C for 10 min.

In contrast to results with JS765 (29), the yellow color produced from catechol by DH5α(pDTG901) extracts did not fade with time, even when catechol was provided at a reduced concentration (100 μM) or when NAD (500 μM) was added. This result suggests that the HMS hydrolase and HMS dehydrogenase are not encoded on the DNA fragment.

Substrate range of catechol 2,3-dioxygenase. The substrate range of catechol 2,3-dioxygenase was investigated by measuring O_2 uptake by cell extracts with various substituted catechols. No O_2 uptake by extracts of JM109(pDTG900) was detected. Although the relative rates were not absolutely proportional, the pattern of substrate oxidation by extracts of DH5α carrying either pDTG901 or pDTG904 was identical to that of the enzyme from nitrobenzene-induced JS765. High O_2 uptake rates with catechol, 3-methylcatechol, and 4-methylcatechol indicated that these compounds were good substrates for the enzyme (Table 3). The initial O_2 consumption rate with 3-chlorocatechol was high, but rapidly decreased, suggesting that the enzyme is inactivated in the presence of this substrate. No O_2 uptake was observed with 4-nitrocatechol or protocatechuate, suggesting that these compounds do not serve as substrates. This substrate range is similar to that of the catechol 2,3-dioxygenase from *R. pickettii* PKO1 (22), an observation made by Nishino and Spain (29) based on studies of the enzyme present in extracts of nitrobenzene-induced JS765. These results provide further evidence that CdoE and TbuE belong to the same subfamily.

Table 3. Oxygen uptake by cells extracts

Assay substrate	O_2 uptake (μmol/min/mg of protein)		
	DH5α(pDTG901)[a]	DH5α(pDTG904)[a]	JS765[b]
Catechol	2.07	0.10	0.35
3-Chlorocatechol	1.22	0.07	0.27
3-Methylcatechol	1.11	0.07	0.26
4-Methylcatechol	0.84	0.05	0.25
4-Nitrocatechol	<0.01	<0.01	<0.01
Protocatechuate	<0.01	<0.01	<0.01

[a] Substrate concentration, 1 mM

[b] Data from (29); Substrate concentration, 100 μM.

Isolation and characterization of phenol$^+$ variants of JS765. When JS765 was plated on phenol auxanographic plates, colonies began appearing after 4 days of incubation and continued to accumulate for several days. Phenol$^+$ variants of JS765 appeared with a frequency of about 3 in 10^5 and the phenol$^+$ phenotype appeared to be stable. Similar experiments with toluene did not result in any growth.

Isolated colonies from phenol auxanographic plates grew in liquid culture containing 3 mM phenol as sole carbon source with doubling times of 3.3 to 4.2 h. One isolate grew with all three cresol isomers and 3,4-dimethylphenol, but no growth was seen with other dimethylphenol or nitrophenol isomers. This substrate range is similar to that of the phenol-degrading strain *Pseudomonas* sp. CF600 (45). In CF600, phenol is converted to catechol by a multicomponent phenol hydroxylase. Catechol is then degraded via a *meta* pathway (38).

Genes encoding phenol hydroxylase were identified upstream of the catechol 2,3-dioxygenase gene from JS765. Five of the six expected genes for a multicomponent hydroxylase were sequenced and the deduced amino acid sequences were very similar to those for phenol hydroxylases from *Pseudomonas* sp. strain CF600 (45), *P. putida* P35X (28), and *P. putida* H (14) as well as to the toluene/benzene-2-monooxygenase from *P. pickettii* PKO1 (2), and the toluene-3-monooxygenase from *Pseudomonas* sp. strain JS150 (19). Sequence comparisons indicate that the JS765 phenol hydroxylase components are more similar in amino acid sequence to the components of toluene/benzene-2-monooxygenase from *Pseudomonas* sp. JS150 than the phenol hydroxylase components of CF600. Taken together, these results suggest that the genes encode a phenol hydroxylase.

Conclusions. An interesting and unique gene cluster has been identified from JS765. The gene organization (*xylT*-type ferredoxin gene followed by catechol 2,3-dioxygenase gene) is also conserved in the *cdo*, *xyl*, *nah*, *tbu*, *dmp*, *phh*, and *phl* gene clusters. However, only in the *cdo* gene cluster described here is a regulatory gene located directly upstream of and divergently transcribed from the ferredoxin gene. The regulatory protein encoded by *cdoR* is most like those of *ortho*-cleavage pathways, and the coinducers associated with these pathways have been shown to be the *ortho* cleavage products *cis,cis*-muconate (36) or 2-chloro-*cis,cis*-muconate (26, 49), compounds which would not be formed by a *meta* cleavage pathway such as the one described here. It will be interesting to identify the inducer associated with the *cdo* gene cluster. The nitrobenzene dioxygenase genes do not appear to be present on the clone under study. However, upstream of the *cdo* gene cluster is located a series of genes that appear to encode a multicomponent phenol hydroxylase. It appears that in JS765, these genes are not expressed as the strain does not grow with phenol, but spontaneous mutants are easily isolated. The basis for this genetic change has not yet been elucidated.

3. Construction and analysis of hybrid 2-nitrotoluene/2,4-dinitrotoluene dioxygenases.

Summary. 2-nitrotoluene, 2,4-dinitrotoluene, and naphthalene dioxygenases have unique but partially overlapping substrate specificities. By constructing hybrid 2NTDO and DNTDO large subunits, substrate specificity was shown to be determined primarily by the C-terminal half of the α subunit of the oxygenase. This study has been published (32).

Substrate specificities of 2NTDO, DNTDO and NDO. A comparison of the substrates oxidized by recombinant *E. coli* strains expressing 2NTDO, DNTDO and NDO was carried out. 2NTDO catalyzed the dihydroxylation of the aromatic nucleus of 2NT, 3-nitrotoluene (3NT), 4-

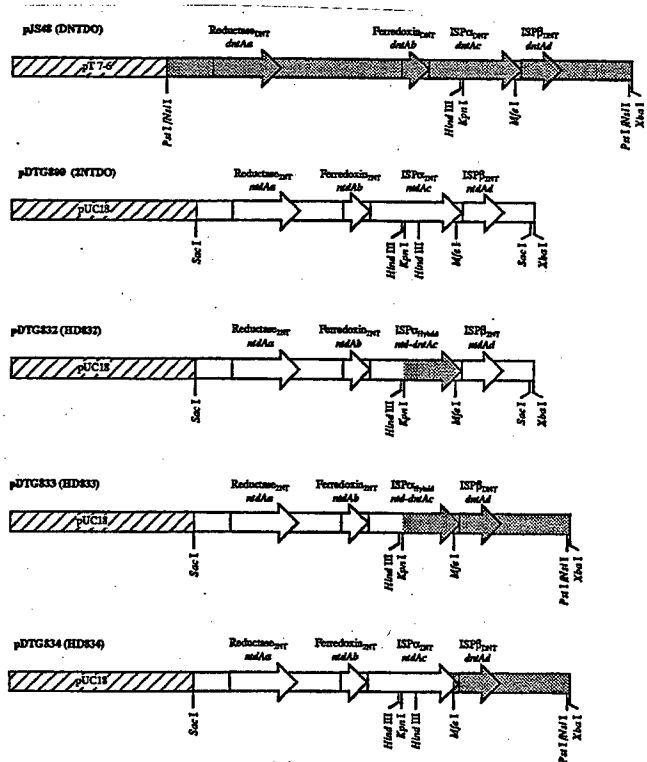
nitrotoluene (4NT) and nitrobenzene. Small amounts of 2-, 3- and 4-nitrobenzyl alcohol were also formed from 2NT, 3NT and 4NT, respectively. DNTDO formed the benzylic alcohol derivatives of all nitrotoluene isomers. In addition, a small amount of the dioxygenation product of 4NT was formed. Similarly, NDO only catalyzed the benzylic hydroxylation of 2NT, 3NT and 4NT. Neither DNTDO nor NDO formed oxidation products from nitrobenzene. All three dioxygenases catalyzed the dihydroxylation of naphthalene to form *cis*-naphthalene dihydrodiol. Of these three dioxygenases, only DNTDO catalyzed the dihydroxylation of the aromatic nucleus of 2,4-DNT. None of these products were detected in biotransformations with *E. coli* carrying the vector only.

Comparisons of the nucleotide and deduced amino acid sequences of 2NTDO, DNTDO and NDO. Individual components of the three dioxygenases 2NTDO, DNTDO and NDO were found to have high nucleotide and deduced amino acid sequence identities. Of particular interest are the high deduced amino acid sequence (88%) and nucleotide sequence identities (95%) between the α subunits from 2NTDO and DNTDO. Results described above indicate that DNTDO and NDO have similar substrate specificities and these differ significantly from that of 2NTDO. This observation allowed us to locate twelve positions in the $ISP\alpha_{2NT}$ deduced amino acid sequence that differ from the deduced amino acid sequences of both $ISP\alpha_{DNT}$ and $ISP\alpha_{NAP}$. Eleven of these amino acids are clustered in the C-terminal region of $ISP\alpha_{2NT}$. This region is downstream of conserved histidine and cysteine residues involved in binding the Rieske [2Fe-2S] center (4, 27, 39) and the amino acids thought to be involved in coordinating mononuclear iron at the active site of the enzyme (18, 20).

Construction of hybrid dioxygenases. Conserved *Kpn*I and *Mfe*I restriction sites flank the region of DNA encoding the portion of the C-terminal-coding regions of $ISP\alpha_{2NT}$ and

$ISP\alpha_{DNT}$ where the 11 amino acids of interest are clustered. The *KpnI/MfeI* DNA fragment was isolated from pJS48, which carries the genes encoding DNTDO (47), and was used to replace the analogous DNA fragment in pDTG800. The latter plasmid carries the genes encoding 2NTDO (31). The new plasmid was designated pDTG832. To determine if ISPα and ISPβ interactions were of importance in 2NTDO substrate specificity, the 1.6-kb *MfeI/XbaI* DNA fragments (containing the gene encoding $ISP\beta_{2NT}$) from both pDTG800 and pDTG832 were replaced by the analogous *MfeI/XbaI* DNA fragment from pJS48 (containing the gene encoding $ISP\beta_{DNT}$). The resulting plasmids were designated pDTG834 and pDTG833 respectively. Gene organization and partial restriction maps of pDTG800, pDTG832, pDTG833, pDTG834 and pJS48 are shown in Fig. 3. *E. coli* strains transformed with these plasmids were used in the biotransformation studies described below. The hybrid dioxygenase enzymes produced by DH5α carrying pDTG832, pDTG833 and pDTG834 were designated HD832, HD833, and HD834 respectively.

Fig. 3. Organization of DNTDO, 2NTDO and Constructed Hybrid Gene Clusters



Biotransformation of naphthalene by hybrid dioxygenases. The three dioxygenases, 2NTDO, DNTDO and NDO, oxidize naphthalene to *cis*-naphthalene dihydrodiol. Consequently, the biotransformation of naphthalene was used as a diagnostic tool to determine if active enzymes were expressed by the hybrid gene clusters. Biotransformation experiments demonstrated that all three hybrid dioxygenases oxidized naphthalene to *cis*-naphthalene dihydrodiol. Results from time course studies show that 2NTDO and HD833 produce *cis*-naphthalene dihydrodiol at a much higher rate (0.48 and 1.22 μg/min/mg total protein, respectively) than either HD832 or HD834 (0.07 μg/min/mg total protein each).

Substrate specificity of the hybrid dioxygenases. The ability of the hybrid dioxygenases to oxidize a variety of substrates was determined. The results show that HD832 did not catalyze the dihydroxylation of 2NT, 3NT, 4NT or nitrobenzene. The enzyme did however catalyze the oxidation of the methyl substituents of 2NT, 3NT and 4NT. In addition, HD832 gained the ability to catalyze the dihydroxylation of 2,4-DNT to 4-methyl-5-nitrocatechol. Thus, replacing the C-terminal region of $ISP\alpha_{2NT}$ with the corresponding region from $ISP\alpha_{DNT}$ resulted in a change in the substrate specificity and regiospecificity of 2NTDO. Replacement of the ISPβ subunit in HD832 or 2NTDO with the corresponding subunit from DNTDO (HD833 and HD834 respectively) resulted in no change in substrate specificity or regiospecificity. These results show that the ISPβ subunit does not play a role in determining substrate specificity or regiospecificity.

Enantiomeric purity of *cis*-naphthalene dihydrodiols formed by 2NTDO and the hybrid dioxygenases. The enantiomeric compositions of the *cis*-naphthalene dihydrodiols formed by 2NTDO, DNTDO, NDO and the hybrid dioxygenases were determined. The results

show that the enantiomeric composition of the *cis*-naphthalene dihydrodiol formed by 2NTDO is 70% (+) - (1*R*, 2*S*). In contrast, the enantiomeric compositions of the *cis*-naphthalene dihydrodiols formed by DNTDO and NDO were 96% and >99% (+) - (1*R*, 2*S*) respectively.

The enantiomeric composition of the *cis*-naphthalene dihydrodiol formed by the hybrid dioxygenase HD832 was 98% (+) - (1*R*, 2*S*). These results clearly indicate that C-terminal region of $ISP\alpha_{2NT}$ plays a major role in determining the enantiomeric compositions of the products formed by 2NTDO. The enantiomeric compositions of the *cis*-naphthalene dihydrodiols formed by HD833 and HD834 were also determined and found to be 96% and 70% (+) -(1*R*, 2*S*) respectively, indicating that the ISPβ subunit of the terminal oxygenase of 2NTDO does not influence the enantiomeric compositions of the products formed by 2NTDO.

Conclusions. This study characterized the overlapping but distinct substrate specificities of the three dioxygenases NDO, 2NTDO and DNTDO. Results indicate that the substrate specificities of 2NTDO and DNTDO are determined primarily by the C-terminal half of the oxygenase α subunit. These studies lay the groundwork for construction and analysis of site-directed mutations in 2NTDO that will identify specific amino acid residues responsible for determining the specificity of this enzyme.

4. The role of the small subunit in substrate specificity was explored by construction and analysis of hybrid dioxygenases.

Summary: These experiments demonstrated which small subunits were functional with the various large subunits and whether the introduction of a different small subunit affected substrate specificity of the enzymes. A two-plasmid system was used in which the reductase, ferredoxin and large (α) subunit genes were present on one expression plasmid and the small (β)

subunit gene was carried on a second compatible expression plasmid. A monoclonal antibody raised against the ISP_{NAP} was used to verify expression of the α subunits of NDO and DNTDO. This study has been published (33).

Construction of the two plasmid expression system. A new expression vector, pREP1, was constructed for use in this study. This plasmid, a derivative of pACYC184, carries a chloramphenicol resistance gene, a T7 promoter, a multiple cloning site, and is compatible with ColE1 plasmids. In this study, the genes encoding the reductases, ferredoxins and α subunits from the NDO and DNTDO systems were expressed from pREP1 derivatives (pDTG162 and pDTG953, respectively) in JM109(DE3). Genes encoding β subunits were coexpressed from compatible ColE1 plasmids. All genes were inducible either directly or indirectly with IPTG. The gene encoding the NDO β subunit, *nahAd*, was directly inducible as it is under the control of the *lac* promoter in pDTG824, a pUC18 derivative. All other genes were under the control of the T7 promoter in plasmids carried in JM109(DE3), a strain that has an IPTG-inducible T7 polymerase gene inserted in the chromosome. Six hybrid enzymes, two wild-type enzymes and control enzymes containing no β subunit were produced using the two-plasmid expression system.

Expression of cloned dioxygenase genes. Purified ISP_{NAP} was used to immunize mice, and from a library of hybridomas we were able to identify a monoclonal antibody that reacts with the large subunit of ISP_{NAP}. The monoclonal antibody specific for the α subunit of NDO reacted with the α subunits of NDO, DNTDO, and 2NTDO, but not TDO in crude cell extracts. Similar results were obtained when purified oxygenase components of NDO, 2NTDO and TDO were analyzed by Western blotting. Use of this antibody demonstrated that the α subunits of NDO and DNTDO were produced by all recombinant strains except control JM109(DE3) carrying the two

vectors only. Polyclonal antiserum raised against NDO reacted with the α subunits of NDO and DNTDO, and also with the β subunit of NDO. This polyclonal antiserum was used to verify the production of the β subunit of NDO. The polyclonal antibody also reacted with a nonspecific *E. coli* protein present in all crude cell extracts. Monoclonal antibody 301β, which was raised against the β subunit of TDO (24), was used to demonstrate the presence of the TDO β subunit in extracts. These results show that all oxygenase α and β subunits were present in *E. coli* extracts with the exception of the β subunits of 2NTDO and DNTDO. Antibodies for the detection of these two β subunits were not available.

Indigo formation. One rapid qualitative measure of dioxygenase activity is the conversion of indole to indigo. The ability to form indigo by *E. coli* strains expressing wild-type and hybrid dioxygenases was tested on agar plates. While NDO and TDO have been shown to efficiently convert indole to indigo (8, 52), DNTDO does so less efficiently (47), and 2NTDO only forms trace amounts. The formation of blue colonies by JM109(DE3)(pDTG162)(pDTG824), and JM109(DE3)(pDTG162)(pDTG951), which produce NDO-β_{2NT} and NDO-β_{DNT} respectively, was the first indication that these hybrid enzymes were active. None of the other hybrid enzyme producing strains turned blue. Control strains expressing wild-type NDO and DNTDO formed blue colonies. Strains that lacked a small subunit or contained only vectors remained white.

***cis*-Naphthalene dihydrodiol formation.** Naphthalene was used to test hybrid enzyme activity, since NDO, TDO, 2NTDO and DNTDO each catalyze the conversion of naphthalene to *cis*-naphthalene dihydrodiol. Specific activities of wild-type and hybrid enzymes with naphthalene as substrate are shown in Table 4. Since 2NTDO was produced from a single plasmid, its specific activity cannot be directly compared to the specific activities of the other

enzymes. These activities are consistently lower (5 to 10-fold) than activities in strains carrying single expression plasmids. One possible reason is that the four genes are no longer coordinately regulated from the same DNA fragment, and coupled translation of the α and β subunits cannot occur. A second possibility is that differences in plasmid copy number result in unequal amounts of α and β gene product formed. Amounts of *cis*-naphthalene dihydrodiol formed in 5 h biotransformations are shown in Table 5. NDO-β_{TDO}, DNTDO-β_{TDO} and DNTDO-β_{NDO} did not form detectable amounts of *cis*-naphthalene dihydrodiol even after prolonged incubation (24 h) as judged by HPLC or GC-MS analyses. *cis*-Naphthalene dihydrodiol was not formed by control strains that did not contain a β subunit gene.

Table 4. Comparison of enzyme activities and stereoselectivity of wild-type and hybrid dioxygenases with naphthalene as substrate

Dioxygenase	Specific activity[a] (μg min^{-1} mg^{-1})	Enantiomeric purity % (+)-1*R*,2*S*
Wild-type enzymes		
NDO	2.6	>99
2NTDO	ND[b]	70
DNTDO	0.38	96
Hybrid enzymes		
NDO-β_{2NT}	1.3	>99
NDO-β_{DNT}	0.37	>99
DNTDO-β_{2NT}	0.08	97

[a] μg *cis*-naphthalene dihydrodiol formed min^{-1} mg $protein^{-1}$ in whole cell biotransformations.
[b] ND, not determined

Table 5. Product formation[a] by wild-type and hybrid dioxygenases

Dioxygenase	*cis*-Naphthalene dihydrodiol from NAP (μg/ml)	2-Nitrobenzyl alcohol from 2NT[b] (μg/ml)	4-Methyl-5-nitrocatechol from DNT (μg/ml)
NDO	800	33.4	–[c]
NDO-ß$_{2NT}$	279	35.4	–
NDO-ß$_{DNT}$	113	8.7	–
NDO-ß$_{TOL}$	–	nd[d]	nd
NDO (no ß)	–	–	–
DNTDO	96.9	trace[e]	18.8
DNTDO-ß$_{2NT}$	32.3	–	4.9
DNTDO-ß$_{NAP}$	–	–	–
DNTDO-ß$_{TOL}$	–	nd	nd
DNTDO (no ß)	–	–	–

[a] Products formed from naphthalene (NAP), 2-nitrotoluene (2NT) and 2,4-dinitrotoluene (DNT) were detected and quantified by HPLC after 5 h biotransformations.

[b] No 3-methylcatechol was detected in any 2NT biotransformations although control DH5α(pDTG800) producing wild-type 2NTDO formed 3-methylcatechol and 2-nitrobenzyl alcohol in a 9:1 ratio.

[c] –, no products were detected by HPLC analysis of 5 h aqueous samples or by GC-MS after extraction and concentration of 24 h biotransformations.

[d] Not determined.

[e] Detected by GC-MS after extraction and concentration of 24 h biotransfomations. Not detected in aqueous samples by HPLC, where the limits of detection for *cis*-naphthalene dihydrodiol, 2-nitrobenzyl alcohol and 4-methyl-5-nitrocatechol were each approximately 2 μg/ml.

Stereochemistry of *cis*-naphthalene dihydrodiol formed by hybrid dioxygenases.

The stereochemistry of the *cis*-naphthalene dihydrodiol formed by hybrid dioxygenases was determined by chiral stationary-phase HPLC. NDO forms enantiomerically pure (+)-(1*R*,2*S*)-*cis*-naphthalene dihydrodiol from naphthalene (16, 17). DNTDO and 2NTDO form characteristic ratios of the (+) and (-) enantiomers of *cis*-naphthalene dihydrodiol (32, 47). Results with hybrid

enzymes indicated that the β subunit does not play a role in determining the enantiomeric purity of the *cis*-naphthalene dihydrodiol formed (Table 4).

Products formed from 2-nitrotoluene and 2,4-dinitrotoluene. While all of the wild-type oxygenases in this study are capable of forming 2-nitrobenzyl alcohol from 2-nitrotoluene (23, 40, 47), only 2NTDO forms 3-methylcatechol from this substrate (10). Two NDO hybrid enzymes (NDO-β_{2NT} and NDO-β_{DNT}) formed 2-nitrobenzyl alcohol from 2-nitrotoluene (Table 5), but formed no 3-methylcatechol as judged by HPLC and GC-MS analyses. No oxidation products were formed from 2-nitrotoluene by the other hybrid enzymes or by the control strains carrying no β subunit gene (Table 5). From these results, the β subunit does not appear to determine the position of attack (regiospecificity) on 2-nitrotoluene, since neither NDO-β_{2NT} nor DNTDO-β_{2NT} was capable of converting 2-nitrotoluene to 3-methylcatechol.

Of the wild-type oxygenases, only DNTDO can catalyze the conversion of 2,4-dinitrotoluene to 4-methyl-5-nitrocatechol. Of the hybrid enzymes, only DNTDO-β_{2NTDO} was capable of this conversion (Table 5). These results suggest that the α subunit confers the ability to oxidize 2,4-dinitrotoluene to 4-methyl-5-nitrocatechol, since DNTDO-β_{2NTDO} was capable carrying out this reaction and NDO-β_{DNTDO} was not.

Conclusions. This study demonstrates that the β subunits of NDO, 2NTDO and DNTDO do not play a role in determining substrate specificity. These results are in contrast to other reports with biphenyl, toluate and benzene dioxygenases that indicated that the β subunit might be controlling substrate specificity (9, 13, 15).

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PUBLICATIONS

1. Parales, J.V., A. Kumar, R.E. Parales, and D.T Gibson. 1996. **Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42.** Gene **181:**57-61.

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3. Gibson, D.T. 1997. **On learning to drive and being taken for a ride.** J. Ind. Microbiol. Biotechnol. **19:**312-323.

4. Parales, R.E., M.D. Emig, N.A. Lynch, and D.T Gibson. 1998. **Substrate specificity of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems.** J. Bacteriol. **180:**2337-2344.

5. Parales, J.V., R.E. Parales, S.M. Resnick, and D.T. 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.

6. Gibson, D.T., J.V. Parales, D. An, F. Lee, and M. Misumoto. **Purification and characterization of the three components of 2-nitrotoluene dioxygenase.** (In preparation).

PRESENTATIONS

1. David T. Gibson. Opening Lecture, **"The Carbon Cycle and the Biodegradation of Organic Pollutants."** Presented at the UIB-GBF-CSIC-TUB Symposium on Biodegradation of Organic Pollutants, Palma de Mallorca, Spain, June 29-July 3, 1996.

2. David T. Gibson. **"On learning to Drive and Being Taken for a Ride."** Closing presentation at the divisional symposium "Biocatalysis and Biodegradation of Aromatic Hydrocarbons: a Tribute to David T. Gibson" at the 96th General Meeting of the American Society for Microbiology, New Orleans Louisiana, May 19-23, 1996.

4. David T. Gibson. **"Fission of the Aromatic Nucleus at pH 7.0 and the Temperature of an English Summer's Day."** Presented at the Grinnell College Symposium on Microbes as Allies, Grinnell College, Grinnell, Iowa, April 9-11, 1996.

5. Rebecca E. Parales. **"Initial Reactions in the Oxidation of 2-Nitrotoluene."** Presented at the 96th General Meeting of the American Society for Microbiology, New Orleans Louisiana, May 19-23, 1996.

6. Rebecca E. Parales. **"Degradation of 2-Nitrotoluene by *Pseudomonas* sp. strain JS42."** Presented at the Department of Microbiology, Southern Illinois University, Carbondale, Illinois, April 12, 1996.

7. Juanito V. Parales presented a poster, **"Genetic Engineering Changes the Substrate Oxidation Profile of 2-Nitrotoluene 2,3-Dioxygenase"** at the 96th General Meeting of the American Society for Microbiology, New Orleans Louisiana, May 19-23, 1996. Co-authors contributing to this work were Dr. Rebecca E. Parales and Sol M. Resnick.

8. David T. Gibson. Procter & Gamble Award in Applied and Environmental Microbiology Lecture, **"Enzymatic Oxidation of Aromatic Hydrocarbons: 1861-1996"** at the 97th General Meeting of the American Society for Microbiology, Miami, Fla., May 4-8, 1997.

9. Rebecca E. Parales presented a poster, **"Construction and expression of hybrid dioxygenases: Comparison of oxidation products formed."** at the 97th General Meeting of the American Society for Microbiology, Miami, Fla., May 4-8, 1997. Matthew D. Emig was a contributing author.

10. Juanito V. Parales presented a poster, **"Purification and characterization of the ferredoxin component of 2-Nitrotoluene 2,3-Dioxygenase from *Pseudomonas* sp. JS42"** at the 97th General Meeting of the American Society for Microbiology, Miami, Fla., May 4-8, 1997.

11. Rebecca E. Parales presented a poster, **"Selection of spontaneous mutants of the nitrobenzene-degrading strain *Comamonas* sp. JS765 that grow with phenol and cresols."** at the 98th General Meeting of the American Society for Microbiology, Atlanta, Ga., May 17-21, 1998. David T. Gibson was a contributing author.

12. Rebecca E. Parales presented a seminar, **"Functional analyses of hybrid dioxygenases."** at the VI International Congress on Pseudomonas: Molecular Biology and Biotechnology, Madrid, Spain, September 4-8, 1997.