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REPORT TITLE: Molecular Mechanisms of Nitroarene Degradation

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Sincerely,

David T. Gibson
Edwin B. Green Professor of Biocatalysis and Microbiology

Final Progress Report

(4) Statement of the Problem

The major objective of this research project was to elucidate the molecular mechanisms used by bacteria to degrade nitroaromatic environmental pollutants.

The focus of the project was to characterize the genes and proteins involved in the degradation of nitrobenzene and 2-nitrotoluene, two nitroarene compounds that are common environmental pollutants. The initial reactions used by *Comamonas* sp. strain JS765 and *Pseudomonas* sp. strain JS42 to initiate the degradation of nitrobenzene and 2-nitrotoluene, respectively, are shown in Fig. 1. Preliminary characterizations of the nitroarene degradation pathways present in these strains have been reported (1, 3, 7-10).

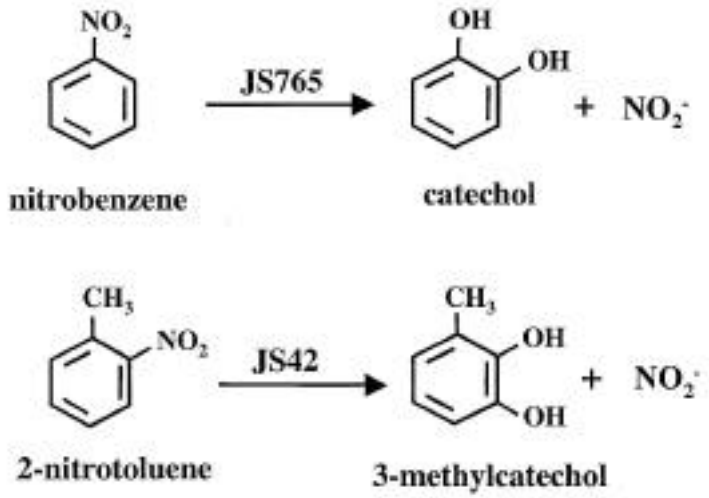


Fig. 1. Initial reactions in the degradation of nitrobenzene and 2-nitrotoluene.

(5) Summary of Results

a) Purification and characterization of the reductase component of the 2-nitrotoluene dioxygenase (2NTDO) system from *Pseudomonas* JS42.

The enzymes catalyzing the initial oxidations of nitrobenzene and 2-nitrotoluene belong to a family of multicomponent non-heme iron dioxygenases (2). Each system consists of a reductase which transfers electrons from NADH to a Rieske [2Fe-2S] ferredoxin which in turn passes the electrons to the dioxygenase which catalyzes the reaction. The genes the 2-nitrotoluene dioxygenase (2NTDO) system have been cloned and sequenced (8).

An expression plasmid carrying the gene encoding $Reductase_{2NT}$ was generated and the *E. coli* strain carrying the plasmid [JM109(DE3)(pDTG871)] was grown in a 10 L fermentor. The reductase was isolated from crude cell extracts by a three-step purification procedure utilizing Q-sepharose, Phenyl-sepharose and hydroxyapatite chromatography. The final product was greater than 99% pure. The purified reductase was orange-brown in color and the native molecular weight (35,000) showed that the active enzyme was a monomer. The N-terminal sequence of the recombinant reductase was determined and was in agreement with the deduced amino acid sequence.

The flavin nucleotide in $Reductase_{2NT}$ was identified as FAD by reverse-phase HPLC and the molar ratio of FAD to protein was determined as one. The reductase contained two moles of iron and two moles of

acid-labile sulfur per mole of protein. The UV-visible absorption spectrum of the reductase was determined and absorbance maxima at 271, 344, 397 and 459 nm were detected. The electron paramagnetic resonance spectrum of the purified protein was determined at 70ºK under partially and fully reduced conditions with dithionite. The spectra obtained at 20, 10 and 2ºK were identical. The spectrum of the partially reduced reductase showed absorbance maxima at g=2.003 and g=1.94, indicating the formation of a flavin semiquinone and a reduced [2Fe-2S] center, respectively. Characteristics of $Reductase_{2NT}$ are shown in Table 1. We are currently preparing a manuscript describing the purification and characterization of all three components of 2-nitrotoluene dioxygenase.

Table 1. Physical properties of $Reductase_{2NT}$

Properties	$Reductase_{2NT}$
Native Molecular Mass (kDa) [1]	29.37
Subunit molecular mass (kDa)	36.73[2], (35.303)[3]
Subunit structure	monomer
Iron (g-atoms per mol)	1.86
Acid labile sulfur (g-atoms per mol)	2.01
Flavin, (mol per mol)	1.10
Absorption spectra, λ_{max} (nm)	320, 417, 460, 438 (shoulder)
Extinction coefficients, $mM^{-1}cm^{-1}$ (λ_{max}, nm)	20.35 (320), 15.44 (417), 15.63 (460)
EPR (reduced), g_Z, g_Y, g_X	2.00, 1.94

[1] Gel filtration using Sephacryl 300 (Pharmacia)
[2] SDS-PAGE
[3] From deduced amino acid sequence

b) Crystallization of the oxygenase component of the 2-nitrotoluene dioxygenase (2NTDO) system.

Crystallization requires large amounts of purified $Oxygenase_{2NT}$. $Oxygenase_{2NT}$ was purified from cell extracts of *E. coli* JM109(DE3)(pDTG800), which expresses the cloned dioxygense genes *ntdAaAbAcAd*, by modification of a previously reported procedure (5). This procedure routinely yields approximately 200 mg of purified $Oxygenase_{2NT}$ for crystallization studies.

Crystals were generated using the "hanging drop" method. In this method 2µl of protein (20-40 mg/ml) is mixed with 2µl of the crystallizing solution on a siliconized glass cover slip. The cover slip containing the drop is then inverted and sealed over a reservoir containing 1ml of the crystallization solution. The concentration of the drop is then driven towards the concentration of the reservoir solution by vapor diffusion. The protein becomes supersaturated and crystals (or precipitation) form as the drop goes to equilibrium with the reservoir solution. Afer screening over 360 different crystallization conditions with differences in buffer composition (MES, Tris, phosphate, citrate, acetate, MOPS), precipitant (ammonium sulfate, ammonium citrate, ammonium phosphate, NaCl, KSCN, PEG 400, PEG 8000, MPD), pH, protein concentration and various other

additives (including dioxane, cacodylate, cobalt, copper, nickel and zinc), crystal forms that diffract to 5.0 Å and 3.8 Å were obtained. Preliminary analysis of data collected at Argonne National Laboratory demonstrates an $\alpha_3\beta_3$ mushroom-shaped protein similar to the observed structure of naphthalene dioxygenase (4). However, better diffracting crystals are still needed to complete the structure of the protein.

Fig. 1. Crystals of 2-nitrotoluene dioxygenase. This crystal forms diffracts to 3.8Å.

c) Identification of the genes encoding the nitrobenzene dioxygenase (NBDO) system of *Comamonas* strain JS765

The genes encoding the NBDO system were cloned and sequenced from *Comamonas* sp. JS765 as follows. A 500-bp fragment of the gene encoding the α-subunit of the NBDO oxygenase component was provided by Dr. Glenn Johnson and Dr. Jim C. Spain. This DNA fragment was used as a probe to identify the genes encoding the NBDO system. Colony hybridization experiments with recombinant *E. coli* clones containing a partial JS765 genomic *Eco*R1 library yielded a plasmid containing a 8881-bp *Eco*R1 fragment. Sequence analysis of the *Eco*R1 fragment revealed eight open reading frames (ORFs). The amino acid sequences of the predicted proteins from four of the ORFs showed high homology to the amino acid sequences of proteins from other known three component dioxygenase systems, in particular the 2-nitrotoluene 2,3-dioxygenase (2NTDO) from *Pseudomonas* sp. strain JS42. Based on amino acid sequence analysis, four of the proteins were designated Reductase_{NBZ}, Ferredoxin_{NBZ}, $\text{Oxygenase}\alpha_{NBZ}$, and $\text{Oxygenase}\beta_{NBZ}$ with gene designations *nbzAaAbAcAd*. The reductase and ferredoxin components are identical to the analogous proteins in the 2NTDO system and the $\text{Oxygenase}\alpha_{NBZ}$ and $\text{Oxygenase}\beta_{NBZ}$ components show 95% identity. ORF1 shares 99% amino acid sequence identity to ORF2 in the 2NTDO system. The remaining 3 ORFs have been tentatively identified as a regulatory protein (*nbzR*), a methyl-accepting chemotaxis protein and a transposase. The cloning and sequence analysis was published in: Lessner, D.J., G.R. Johnson, R.E. Parales, J.C. Spain, and D.T. Gibson (2000) Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. 68:634-641.

d) Purification and properties of nitrobenzene dioxygenase.

The NBDO system consists of a reductase, which is an iron-sulfur flavoprotein that transfers electrons to a Rieske [2Fe-2S] ferredoxin. The latter reduces the oxygenase which catalyzes the oxygenolytic elimination of nitrite from nitrobenzene with the concomitant formation of catechol. The analogous enzyme system in *Pseudomonas* JS42 oxidizes 2-nitrotoluene to 3-methylcatechol and nitrite.

E. coli DH5α(pDTG927), which carries the genes encoding nitrobenzene dioxygenase, was grown on L-broth in a 10 liter fermentor for 7 h. Cell extracts were prepared and nitrobenzene dioxygenase was purified to approximately 95% purity (Fig. 2) by a two step purification (Q-sepharose followed by Butyl-sepharose). The absorption spectrum (Fig. 3) and electron spin resonance spectrum (Fig. 4) are characteristic of Rieske non-heme iron dioxygenases. The purified enzyme was also used to analyze the substrate specificity of nitrobenzene dioxygenase (see below).

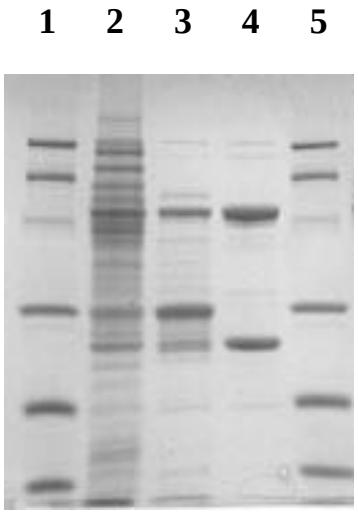


Fig. 2. SDS-PAGE analysis of samples taken during the purification of NBDO. Lane 1 and 5: MW markers, lane 2: crude cell extract, lane 3: Q-sepharose, lane 4: butyl-sepharose.

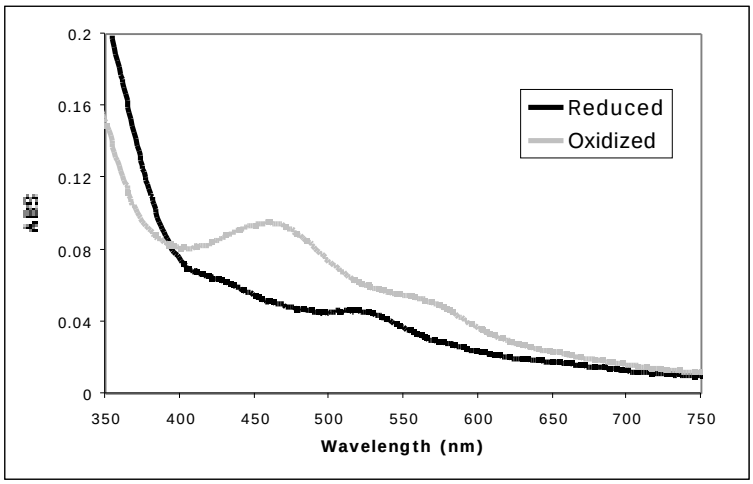


Fig. 3. Absorption spectra of NBDO (0.4 mg of protein in 0.7 ml total volume). Oxidized NBDO shows peaks at 330 nm, 460 nm, and 560 nm (shoulder). These peaks were greatly reduced and a new peak appeared at 520 nm upon reduction with NADH in the presence of catalytic amounts (15 μg each) of reductase$_{2NT}$ and ferredoxin$_{2NT}$.

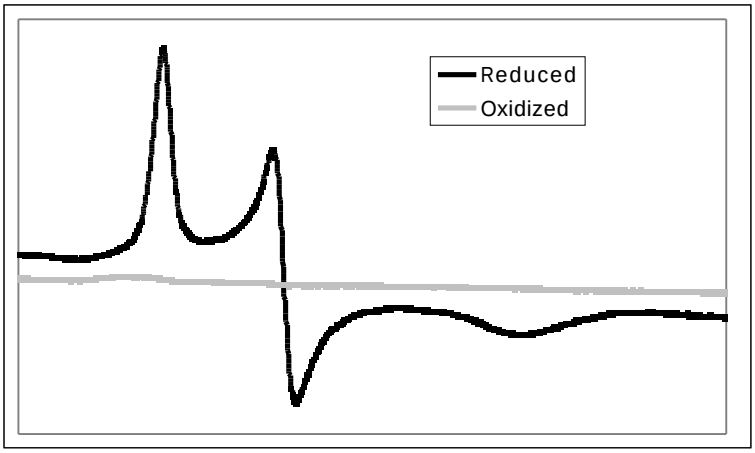


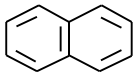
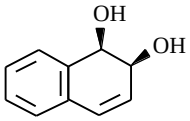
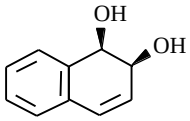
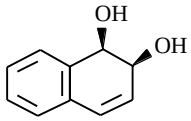
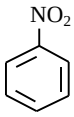
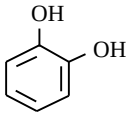
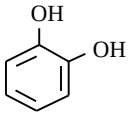
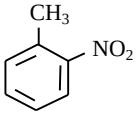
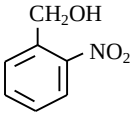
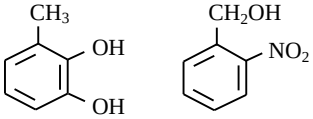
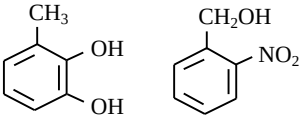
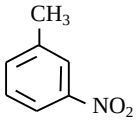
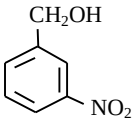
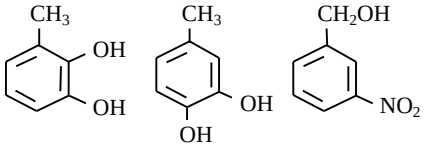
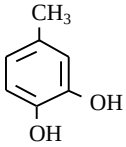
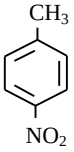
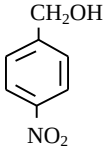
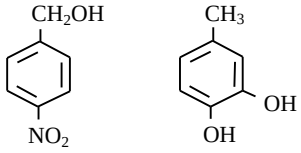
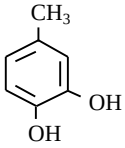
Fig. 4. EPR spectra of NBDO (1.3 mg of protein). Oxidized protein was EPR silent, but following reduction with sodium dithionite three signals appeared (g_x = 1.75, g_y = 1.92, and g_z = 2.02) which indicate the presence of a Rieske-type [2Fe-2S] redox center.

e) Substrate specificity of nitrobenzene dioxygenase.

Initial experiments were conducted with a recombinant *E. coli* strain that expresses nitrobenzene dioxygenase (NBDO) activity, *E. coli* DH5α(pDTG927). This strain was grown at 30˚C in Luria-Bertani medium supplemented with 0.7% glycerol. Cultures were shaken until the turbidity reached an A_{600} of 0.65-0.80. At this time isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was added to induce synthesis of the nitrobenzene dioxygenase system. Induced cells were used in biotransformation experiments with ten aromatic substrates. Experiments were conducted in triplicate in 125 ml baffled shaken flasks containing cells (final A_{600}, 2.0) and 20 ml of phosphate buffer. The flasks were shaken at 220 rpm and 26˚C. Samples were taken at intervals for analysis by high performance liquid chromatography (HPLC). For comparison, analogous experiments were conducted with recombinant *E. coli* strains expressing 2-nitrotoluene dioxygenase and naphthalene dioxygenase activities. Additional experiments were undertaken with the purified nitrobenzene dioxygenase and electron transfer components form the 2-nitrotoluene dioxygenase system.

Results of these studies are shown in Table 2 and were published in: Lessner, D.J., G.R. Johnson, R.E. Parales, J.C. Spain, and D.T. Gibson (2000) Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. 68:634-641.

Table 2. Substrate specificity of naphthalene, 2-nitrotoluene and nitrobenzene dioxygenases.

Substrate	NDO	2NTDO	NBDO
 Naphthalene	 >99% (+)-*cis*-(1*R*,2*S*)	 70% (+)-*cis*-(1*R*,2*S*)	 65% (+)-*cis*-(1*R*,2*S*)
 Nitrobenzene	None Detected		
 2-Nitrotoluene		 90% 10%	 55% 45%
 3-Nitrotoluene		 59% 27% 14%	
 4-Nitrotoluene		 76% 24%	

f) Regulation of nitrobenzene dioxygenase activity in *Comamonas* sp. strain JS765 and 2-nitrotoluene dioxygenase in *Pseudomonas* sp. strain JS42.

The genes for the nitrobenzene dioxygenase (NBDO) system and the 2-nitrotoluene dioxygenase (2NTDO) system have been cloned and sequenced (6, 8) and the gene organization is shown in Fig. 5. The genes encoding the reductase (Rd) and ferredoxin (Fd) components of both systems are identical. The α (Oxy_{alpha}) and β (Oxy_{beta}) genes encoding the respective dioxygenase components show 95% nucleotide sequence identity. The NBDO and 2NTDO gene clusters have an identical transcriptional regulatory nucleotide sequence (Reg) upstream of Rd. These regulatory proteins (NbzR from JS765 and NtdR from JS42) are 61% identical to NahR from *Pseudomonas putida* G7 (11). In order to determine if the *nbz* (JS765) and *ntd* (JS42) genes are cotranscribed and thus regulated as an operon we used reverse transcriptase and the polymerase chain reaction (RT-PCR) to generate the messenger RNA transcripts 1 and 2 shown in Fig. 5. Polyacrylamide gel

electrophoresis showed that at least one transcript is made that encompasses the *nbz* or *ntd* genes which indicates that they are cotranscribed as an operon.

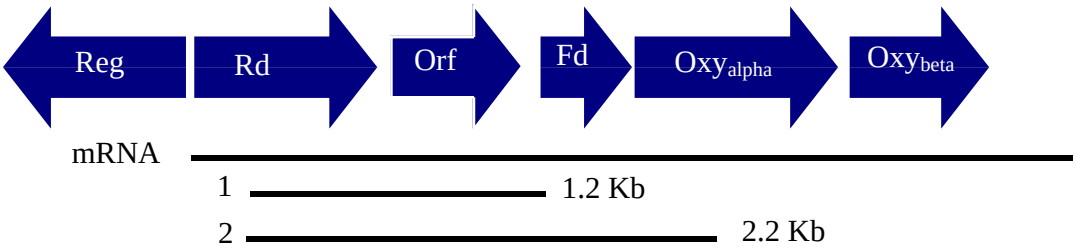


Fig. 5. Gene organization of the 2NTDO and NBZDO gene clusters and results of RT-PCR experiments.

Primer extension analysis was used to map the transcriptional start sites of the *nbz* and *ntd* operons. The results shown in Figure 6 show that the start site for both operons is the same and matches with the start sites for the aligned *nah* (naphthalene) and sal (salicylate) operons. These results also demonstrate that the -10 and-35 promoter recognition sequences are identical for the *nah*, *nbz*, and *ntd* promoters. No differences were seen in the start sites when JS765 and JS42 were grown in the presence of nitrobenzene, 2-nitrotoluene or salicylic acid.

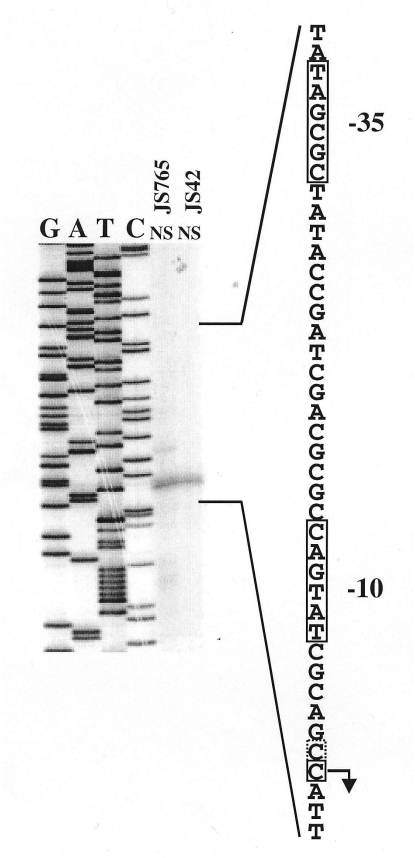


Fig. 6. Primer extension analysis to determine the transcriptional start sites of the *nbz* and *ntd* operons.

Assays of β-galactosidase activity from a chromosomal *nbzAa-lacZ* fusion in JS765 and JS42 showed that the expression of NBDO and 2NTDO is inducible by several nitroaromatic substrates as well as salicylate and anthranilate (Fig. 7). In each case, the level of 2NTDO activity was higher than the expression of NBDO in JS765. In order to identify possible inducer compounds, a chromosomal *nbzAa-lacZ* fusion was constructed in *E. coli* with NbzR/NtdR expressed from a plasmid. The results showed an increase in β-galactosidase activity

in the presence of salicylate and anthranilate but no induction by nitrobenzene or nitrotoluene. Catechol and 3-methylcatechol, the products of nitrobenzene and 2-nitrotoluene oxidation, respectively, did not induce β-galactosidase activity (Fig. 7).

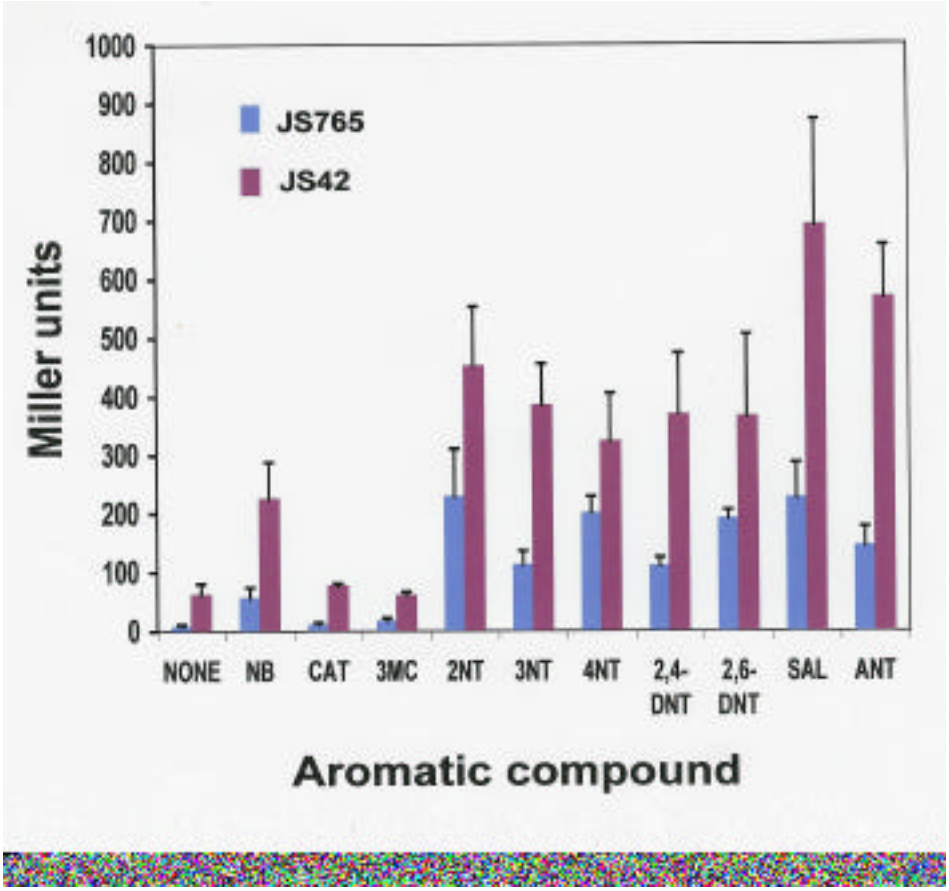


Fig. 7. Comparison of β-galactosidase activity of a *nbzAa-lacZ* fusion in JS765 and JS42.

Reverse genetics was used to generate a chromosomal mutation in the *ntdR* gene in JS42 in order to characterize the role of this regulatory gene. A kanamycin resistance cassette was inserted into the cloned *ntdR* gene, and this disrupted gene was introduced into the JS42 chromosome by homologous recombination. The presence of the disrupted gene was verified by Southern hybridization and PRC analyses. The JS42 *ntd::km* mutant is no longer capable of growth with 2-nitrotoluene and the *ntd* genes are not inducible in this strain. These results indicate that *ndtR* plays an essential role in the expression of the 2-nitrotoluene dioxygenase genes.

We are currently preparing a mauscript describing results of the regulation of the *ntd* and *nbz* operons.

6. Publications and Technical reports

(a) Papers published in peer-reviewed journals

Gibson, D.T. and R.E. Parales. 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr. Opin. Biotechnol. 11:236-243.

Lessner, D.J., G.R. Johnson, R.E. Parales, J.C. Spain, and D.T. Gibson. 2002. Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. 68:634-641.

(b) Papers published in non-peer-reviewed journals or in conference proceedings

None

(c) Papers presented at meetings, but not published in conference proceedings

Lessner, D.J. and D.T. Gibson. 2000. Cloning and characterization of the genes encoding nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. K-58, p. 430. In Abstracts of the 100th General Meeting of the American Society for Microbiology 2000, American Society for Microbiology, Washington, D.C.

Lessner, D.J., J.V. Parales, and D.T. Gibson. 2000. Purification and characterization of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. p. 54. In Abstracts of the 9th Biocatalysis and Bioprocessing Conference 2000, Iowa City, IA.

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7. Scientific personnel

Gibson, D.T., 3%
Parales, J.V., 100%
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8. Inventions

None

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13. ABSTRACT (Maximum 200 words)

The initial reaction in the degradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42 is catalyzed by 2-nitrotoluene dioxygenase (2NTDO). Electrons from NADH are transferred to 2NTDO via a reductase and a Rieske [2Fe-2S] ferredoxin. The reductase was purified to homogeneity and shown to be an iron-sulfur flavoprotein. 2NTDO was purified and crystallized. The crystals diffracted to 3.8Å on a synchrotron beamline. Preliminary analysis indicated that 2NTDO has similar structural features to naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain 9816. Better resolution is required for detailed structural studies. The genes encoding the nitrobenzene dioxygenase (NBDO) system in *Comamonas* sp. strain JS765 were cloned and sequenced. Four open reading frames (*nbzAaAbAcAd*) were identified as genes encoding the reductase, ferredoxin and α and β subunits of the NBDO system. NBDO was purified from IPTG-induced cells of *E. coli* DH5α(pDTG927) expressing *nbzAcAd*. The enzyme is a Rieske non-heme iron dioxygenase closely related to 2NTDO and NDO. The genes encoding NBDO in JS765 and 2NTDO in JS42 are cotranscribed as operons under the control of identical transcriptional regulatory proteins (NbzR and NtdR). The transcriptional start site for both operons are identical and several nitroaromatic compounds as well as salicylate and anthranilate can induce enzyme activity.

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