Supporting Information

Evolution of a New Bacterial Pathway for 4-Nitrotoluene Degradation

Kou-San Ju[#] and Rebecca E. Parales

Department of Microbiology, College of Biological Sciences, 1 Shields Ave, University of California, Davis, CA 95616

Corresponding author: Rebecca E. Parales
Department of Microbiology
226 Briggs Hall
1 Shields Ave.
University of California,
Davis, CA 95616
Phone: (530)-754-5233
FAX: (530)-752-9014
email: reparales@ucdavis.edu

[#]**Current address:** Institute for Genomic Biology, University of Illinois, Urbana, IL 61801

SI Results

Oxidation of other substrates by variant dioxygenases. To determine if the amino acid substitutions altered activity with other substrates, the activities of the evolved and constructed enzymes expressed in *E. coli* (Table S1) were measured with naphthalene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene (naphthalene *cis*-dihydrodiol) was produced from naphthalene by 2NTDO and all of the variants, except the S242N and L238V S242N mutants. 2,6-Dinitrotoluene was oxidized to 3-methyl-4-nitrocatechol and activity remained low for all of the enzymes (12 ± 3 to 26 ± 18 nmoles NO_2^- mg protein^{-1}) except the L238V mutant, which produced slightly more nitrite (77 ± 15 nmoles NO_2^- mg protein^{-1}). Although 2,4-dinitrotoluene is not a substrate for wild-type 2NTDO, the L238V and M248I single mutants, and the L238V M248I and S242N M248I double mutants were able to produce small amounts of 4-methyl-3-nitrocatechol or 4-methyl-5-nitrocatechol (Table S2).

Growth of *Acidovorax* strains on acetaldehyde and propionaldehyde. Acetaldehyde and propionaldehyde are predicted intermediates of 2NT and 4NT degradation, respectively. No growth on acetaldehyde or propionaldehyde was observed with JS42, the 4NT$^+$, or the complemented JS42Ac strains when incubated on plates for up to 10-days at either room temperature or 30° C. The same strains formed colonies 1-2 mm in diameter on MSB plates containing 10 mM succinate after 10 days in the presence of acetaldehyde or propionaldehyde, indicating that the inability to grow on the aldehydes was not due to toxic effects. It is possible that the appropriate genes for propionaldehyde degradation were not induced under these conditions. In contrast, when induced with ethanolamine or 1,2-propanediol the positive control strain *S. enterica* LT2 grew on plates with acetaldehyde or propionaldehyde provided as vapor.

Growth of *Pseudomonas putida* mt-2 on methylbenzoates. *P. putida* mt-2 grows on both 3- and 4-methylbenzoate, generating 3- and 4-methylcatechol, respectively (Murray *et al.*, 1972). As in JS42, the 4-methycatechol is converted to pyruvate and propionaldehyde. Strain mt-2 was grown on 2 mM 3- or 4-methybenzoate to examine for differences in cell yields. In contrast to results with JS42 (Table 3), growth of strain mt-2 on 3- and 4-methybenzoate resulted the same cell yield (135 μg protein ml^{-1}), indicating that acetaldehyde and propionaldehyde are used with equal efficiency; neither compound was detected in the culture headspace. These results suggest that the difference in cell yields during growth on 3- versus 4-methycatechol is specific to JS42 and not a general phenomenon among strains that grow on 4-methylcatechol using the *meta*-cleavage pathway.

Characterization of the aldehyde dehydrogenase. The difference in cell yield when the *Acidovorax* strains are grown on 2NT versus 4NT could be a result of the inability of the acetaldehyde dehydrogenase (CtdQ) to acylate both acetaldehyde and propionaldehyde to form acetyl-CoA and propionyl-CoA, respectively. CoA-ester formation by CtdQ was compared to that of XylQ (from *Pseudomonas putida* mt-2), and DmpF (from *Pseudomonas* sp. strain CF600). In 30 min reactions, extracts of *E. coli* expressing CtdQ produced 79 μM acetyl-CoA and 83 μM propionyl-CoA from 10 mM acetaldehyde or propionaldehyde, respectively, whereas extracts containing XylQ produced 116 μM acetyl-CoA and 83 μM propionyl-CoA, and extracts expressing DmpF produced 250 μM acetyl-CoA and 269 μM propionyl-CoA. Therefore, approximately equivalent amounts of acetyl-CoA and propionyl-CoA were generated from

acetaldehyde and propionaldehyde by extracts expressing the three individual dehydrogenases, demonstrating no significant difference in substrate specificity for the two aldehyde substrates.

We verified that *ctdQ* was expressed in JS42-KSJ-11 by performing RT-PCR experiments. A product of appropriate size was obtained using RNA purified from 4NT-grown cells as well as from cells grown on rich medium (data not shown). No products were formed in control reactions lacking reverse transcriptase. We verified that no mutations were present in *ctdQ* in the evolved strain by PCR-amplifying *ctdQ* from JS42-KSJ11 and sequencing the complete gene.

Construction of a propionaldehyde salvage pathway. The inability of JS42 to utilize the propionaldehyde produced from 4-methycatechol although the strain is capable of robust growth on propionate (doubling time similar to that on succinate) suggested that the conversion of propionaldehyde to propionyl-CoA could be the bottleneck. Since the acetaldehyde dehydrogenase CtdQ had similar activities with acetaldehyde and propionaldehyde, and the 2-methylcitrate pathway appears to be present in JS42 based on the genome sequence (see below), JS42 should not only be able to convert propionaldehyde into propionyl-CoA, but metabolize propionyl-CoA to succinate and pyruvate, both of which are growth substrates for JS42.

Several strategies were attempted to engineer the evolved strains to salvage the accumulated propionaldehyde when grown on 4NT. In an attempt to increase conversion of propionaldehyde to propionyl-CoA, plasmids containing the genes encoding the 4-hydroxy-2-ketovalerate aldolase and acylating acetaldehyde dehydrogenase from JS42 (*ctdQK*) and *Pseudomonas putida* CF600 (*dmpFG*) were constructed for overexpression in JS42 (pKSJ216, and pKSJ215, respectively; Table S1). The aldolase and dehydrogenase genes were both overexpressed, as it

was previously shown that the two polypeptides function together in an enzyme complex, and both are required for maximal activity (Manjasetty *et al.*, 2003; Powlowski *et al.*, 1993). In addition, a plasmid allowing the expression of *pduL* (phosphotransacylase) and *pduW* (propionate kinase) from *Salmonella enterica* LT2 was constructed (pKSJ243) to provide a pathway for the conversion of propionyl-CoA to propionate in JS42. The encoded enzymes are used by strain LT2 during growth on 1,2-propanediol, converting propionyl-CoA into propionyl-phosphate (PduL), and then propionyl-phosphate into propionate with the production of one ATP (Liu *et al.*, 2007). The genes were also combined to allow the co-expression of *dmpFG-pduLW* (pKSJ244) or *ctdQK-pduLW* (pKSJ245) (Table S1).

The five constructed plasmids were introduced into JS42 and JS42-KSJ11 by conjugative matings, and the resulting strains were tested for growth on 2NT and 4NT (Table S4). JS42-KSJ11 was selected for analysis as it demonstrated the most robust growth on 4NT (Table 3). Plasmid pBBR1MCS2 was also introduced as a negative control. Introduction of the plasmids resulted in decreased growth rates of JS42 on 2NT, but cell yields were unchanged (Table S4). Introduction of pBBR1MCS2 into JS42-KSJ11 resulted in slightly slower growth on 2NT and 2.5-fold slower growth on 4NT. Plasmids pKSJ215 (*dmpFG*) and pKSJ216 (*ctdQK*) further decreased the growth rates; overexpression of these genes may have depleted CoA pools and resulted in slowed growth. These results indicate that CtdQ is not the limiting step in the pathway. Expression in JS42-KSJ11 of *pduLW* alone (pKSJ243) or in the presence of *dmpFG* (pKSJ244) or *ctdQK* (pKSJ245) increased the growth rates on 2NT and 4NT, and also increased the cell yields on 4NT. The presence of pKSJ244 or pKSJ245 also increased the cell yields on 2NT (Table S4). These results indicate that the presence of PduL and PduW was beneficial for growth on both 2NT and 4NT. The reason for this is not completely clear but if entry of

propionyl-CoA into the methylcitrate pathway is a limiting step, PduLW could decrease the build up of propionyl-CoA, releasing needed CoA and allowing cells to generate needed energy in the form of ATP. Unfortunately, none of the engineered strains appeared to be fully capable of utilizing propionaldehyde, and propionaldehyde was detected in the headspace of all cultures growing on 4NT.

Propionate metabolism in *Acidovorax* sp. strain JS42. Inspection of the genome sequence of JS42 revealed that it contains all of the necessary genes for the metabolism of propionate by use of the 2-methylcitrate cycle. Propionyl-CoA synthetase, which catalyzes the formation of propionyl-CoA from propionate, is encoded by *prpE* (Ajs_2016; accession YP_986268). This protein shares 63% and 63% amino acid sequence identity, respectively, to the products of *prpE* from *S. enterica* LT2 (AAO70081) (Horswill and Escalante-Semerena, 1997, 1999) and *Ralstonia eutropha* (YP_726919) (Bramer and Steinbuchel, 2001). This gene is in a cluster with the other genes of the 2-methylcitrate cycle. 2-Methylcitrate synthase, which catalyzes the formation of 2-methylcitrate from oxaloacetate and propionyl-CoA, is encoded by *prpC* (Ajs_1635; accession YP_985903). This protein shares 76% and 80% amino acid sequence identity to PrpC of *S. enterica* LT2 (AAC44815) (Horswill and Escalante-Semerena, 1997) and *Ralstonia eutropha,* respectively (AAL03989) (Bramer and Steinbuchel, 2001).

Similar to other bacteria such as *Shewanella oneidensis*, *Vibrio cholera, Burkholderia sacchari*, and *Ralstonia eutropha* CH34, the gene for *prpD*, which encodes 2-methylcitrate dehydratase (Grimek and Escalante-Semerena, 2004), appears to be absent in JS42. Instead, 2-methylcitrate appears to be converted to *trans*-2-methylaconitate by an aconitase-like protein, which in JS42 is

designated *acnD* (Ajs_1634; accession YP_985902). This protein shares 75% sequence identity with AcnD from *S. oeidensis* MR-1 (AAN53428) (Grimek and Escalante-Semerena, 2004). Next, 2-methylaconitate isomerase, encoded by *prpF* in JS42 (Ajs_1633; accession YP_985901), converts *trans*-2-methylaconitate into *cis*-2-methylaconitate. This enzyme shares 73% sequence identity with PrpF of *S. oeidensis* MR-1 (AAN53427) (Garvey *et al.*, 2007; Grimek and Escalante-Semerena, 2004). Aconitase re-hydrates *cis*-2-methylaconitate to form 2-methylisocitrate (*acnB* gene of JS42, Ajs_2787; accession YP_987004). The *acnB* gene is located distal to *acnD* and the *prp* genes in JS42. The encoded enzyme is 74% identical in sequence to AcnB of *E. coli* MG1655 (AAC73229) (Brock *et al.*, 2002).

Finally, 2-methylisocitrate lyase, encoded by *prpB* (Ajs_1632; accession YP_985900), is predicted convert 2-methylisocitrate to succinate and pyruvate. The encoded enzyme is 42% identical in sequence to PrpB from *S. enterica* LT2 (AAC44814) (Horswill and Escalante-Semerena, 1997) and 42% identical to PrpB from *R. eutropha* (AAL03988) (Bramer and Steinbuchel, 2001).

SI Experimental procedures

DNA manipulations. Standard methods were used to manipulate plasmids and DNA fragments (Maniatis *et al.*, 1982). Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs (Beverly, Mass). Plasmids were purified using commercially available plasmid purification kits or as previously described (Sambrook *et al.*, 1989). DNA fragments were purified with a QIAquick Gel Extraction kit (Qiagen). A Purgene DNA purification kit (Gentra Systems, Minneapolis, Minn.) was used to isolate genomic DNA.

Oligonucleotides were synthesized by MWG-Biotech (Greensboro, N.C.) or Invitrogen (Carlsbad, Calif.). Fluorescent automated DNA sequencing was carried out at the University of California, Davis, sequencing facility with an Applied Biosystems 3730 automated sequencer.

E. coli strains were transformed with plasmid DNA by standard procedures (Maniatis *et al.*, 1982). *E. coli* S17-1 was used to introduce plasmids into *Acidovorax* strains by conjugative matings on TY plates at 30° C. After 48 hours, cells were scraped from plates, resuspended in 10 ml of M9 medium (Miller, 1975), and plated onto MSB plates containing 10 mM succinate, vitamins, and the appropriate antibiotics. Exconjugants were purified by repeated single colony isolation. The presence of plasmids was confirmed by plasmid purification and diagnostic restriction digests.

Identification of mutations and construction of dioxygenase expression clones. The *ntdAcAd* genes from the $4NT^+$ strains were PCR-amplified from purified genomic DNA using Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) and the primer pair NtdAc_F1 (5'-GGCCTCG**AGGAGG**GACATATGAGTTACCAAAACTTAGTGAGTGAAGC-3'; XbaI and NdeI restriction sites underlined; synthetic Shine-Delgarno sequence in bold) and NtdAd_R1 (5'-GCTGGCATGCGAGCTCACAGGAAGACCAACAGGTTGTGGGTC-3'; SphI and SacI sites underlined). The resulting ~2 kb DNA fragments were sequenced to identify mutations. Plasmids pKSJ90, pKSJ91, and pKSJ92 (Table S1) were generated by digesting the PCR-amplified *ntdAcAd* genes from the $4NT^+$ strains with KpnI and MfeI, and subcloning the resulting 774-bp fragments into pDTG850.

Site-directed mutagenesis of the *ntdAc* gene by single overlap-extension PCR (Horton *et al.*, 1989) was used to create missense mutations that resulted in L238V and S242N amino acid substitutions in the catalytic subunit of 2NTDO. Primers for mutagenesis included NtdAc_F1, NtdAc_R1 (5'- GGCGTCTAGAATTAGCGATCAGTTGTCTTGGTG-3'; XbaI site underlined), L238V-F (5'-CCGAAGGCGCGGGC**G**TGCAAATGACCAGCAAG-3'), L238V-R (5'-CTTGCTGGTCATTTGCA**C**GCCCGCGCCTTCGG-3'), S242N-F (5'-GCTTGCAAATGACCAA**A**CAAGTATGGCAGTGG-3'), and S242N-R (5'-CCACTGCCATACTTG**T**TGGTCATTTGCAAGC-3'). Mutagenic bases are in bold. The 1.2-kb DNA fragments from the second round of PCR were digested with KpnI and MfeI, and the resulting 774-bp fragments were subcloned into pDTG850 and verified by sequencing. Plasmids pDTG850 and pKSJ92 served as templates for mutagenesis in the construction of pKSJ119, pKSJ120, and pKSJ121. Plasmid pKSJ119 was then used as a template to construct pKSJ122.

For expression in *Acidovorax* strains, plasmid pKSJ44 was constructed by subcloning the 4.7-kb SacI-fragment containing the *ntdAaAbAcAd* gene cluster from pDTG800 into pBBR1MCS5. The 2.4-kb AvrII-MfeI fragments from pKSJ90, pKSJ91, and pKSJ92 were subsequently ligated with pKSJ44, creating pKSJ93, pKSJ94, pKSJ95, respectively.

Construction of acylating aldehyde dehydrogenase expression clones. The *dmpFG* genes from *Pseudomonas* sp. strain CF600 were PCR-amplified from plasmid pVI316Δ using primer pair DmpFQ-F1 (GTTCTAGACATATGAACCAGAAACTCAAAGTCGCGATC; XbaI and NdeI sites underlined) and DmpFQ-R1 (CGTAAGCTTCCTAGGTCATGCGCGGTTCTCCTTGTGGGCC; HindIII and AvrII sites

underlined). The resulting ~ 2-kb product was purified, digested with XbaI and HindIII, and ligated with pUC18 to generate pKSJ211. For expression in JS42, *dmpFG* was excised from plasmid pKSJ211 by digestion with KpnI and HindIII, purified, ligated with pBBR1MCS2 to generate pKSJ215. The *ctdQ* and *xylQ* genes from *Acidovorax* sp. JS42 and *P. putida* mt-2, respectively, were PCR-amplified from purified genomic DNA using primer pairs ctdQ-F1 (GTTTCTAGATGACCCAAAAAATCAAGTGC; XbaI site underlined) and ctdQ-R1 (GGTAAGCTTCAGGCAGCTTGCAAGGTGAGCTTG; HindIII site underlined), and xylQ-F1(GTTTCTAGAATGAACAAGAAACTGAAAGTAGCG; XbaI site underlined) and xylQ-R1 (GGTAAGCTTCAGGCGTTCAGCAGCGACTGCGC; HindIII site underlined), respectively. The resulting ~ 1-kb products were purified, digested with XbaI and HindIII, and ligated to pUC18 to generate pKSJ191 and pKSJ197, respectively. The *ctdK* gene was PCR-amplified from purified JS42 genomic DNA using primer pair CtdK-F2 (GTTCTAGAGCTAGC**AGGAGG**GACATATGACACAAAAGATCACCCTGCACG; XbaI, NheI, and NdeI sites underlined; synthetic Shine-Delgarno sequence in bold) and CtdK-R1 (GGTAAGCTTCAGGCGTTGACGGTCAGGCCGCGTG; HindIII site underlined). The resulting ~ 1-kb product was purified, digested with XbaI and HindII, and ligated with pUC18 to generate pKSJ144. A *ctdQK* expression clone was constructed by single-overlap extension PCR using primer pairs CtdQK-F1 (CGTTCTAGA**AGGAGG**GACATATGACCCAAAAAATCAAGTGCGCCCTG; XbaI and NdeI sites underlined, synthetic Shine-Delgarno sequence in bold)) and CtdQK-SOE-R1 (GCAGGGTGATCTTTTGTGTCATATGTC**CCTCCT**GTGCGCTTCAGGCAGCT; synthetic Shine-Delgarno sequence in bold), and CtdQK-SOE-F1

(AGCTGCCTGAAGCGCAC**AGGAGG**GACATATGACACAAAAGATCACCCTGC) and CtdQK-R1 (CGTAAGCTTCCTAGGTCAGGCGTTGACGGTCAGGCCGCGTG; HindIII and AvrII sites underlined), with pKSJ144 and purified genomic DNA as templates. The ~ 2.0-kb product from the second PCR reaction was purified, digested with XbaI and HindII, and ligated with pUC18 to generate pKSJ212. For expression in JS42, *ctdQK* was excised from plasmid pKSJ212 by digestion with KpnI and HindIII, purified, ligated with pBBR1MCS2 to generate pKSJ216. The *ctdQ* gene from strain JS42-KSJ11 was amplified by PCR using primers ctdJ-F1 (5'-CGTTCTAGAGGAGGGACATATGACCATGACCCCTGCCTTGATCGAACAAC-3') and ctdQ-R1 (see above).

Construction of expression clones for propionaldehyde metabolism. The *pduL* and *pduW* genes (Genbank AF026270) from *Salmonella enterica* serovar Typhimurium strain LT2 were PCR-amplified from purified genomic DNA using primer pairs PduL-F1 (CGTTCTAGACCT**AGGAGG**GACATATGGATAAAGAGCTTCTGCAATCAACG; XbaI, AvrII, and NdeI sites underlined, synthetic Shine-Delgarno sequence in bold) and PduL-R1 (CGTAAGCTTCCTAGGTCATCGCGGGCCTACCAGCCGGGC; HindIII and NheI sites underlined), and PduW-F1 (CGTTCTAGAGCTAGC**AGGAGG**GACACATGGCCATTAACGCCGGTAGCTCA; XbaI and NheI sites underlined, synthetic Shine-Delgarno sequence in bold (Chen *et al.*, 1994)) and PduW-R1 (CGTAAGCTTTCAGGCTGGTACACAAAGCCCTTC; HindIII site underlined). The resulting 633-bp and 1.2-kb products were purified, digested with XbaI and HindIII, and ligated to similarly digested pUC18 to generate pKSJ209 and pKSJ210, respectively. A *pduLW* expression clone was constructed by single-overlap extension PCR (Horton *et al.*, 1989) using

primers PduL-F1 with PduLW-SOE-R1 (CCGGCGTTAATGGCCATGTGTC**CCTCCT**GCTAGCTCATCGCGGGCCTACC; NheI site underlined, synthetic Shine-Delgarno sequence in bold), and PduLW-SOE-F1 (GGTAGGCCCGCGATGAGCTAGC**AGGAGG**GACACATGGCCATTAACGCCGG; NheI site underlined, synthetic Shine-Delgarno sequence in bold) with PduW-R1. The ~ 1.9-kb product from the second PCR reaction was purified, digested with XbaI and HindIII, and ligated with pUC18 to generate pKSJ242. For expression in JS42, *pduL*, *pduW*, and *pduLW* were excised from plasmids pKSJ209, 210, and 242 by digestion with KpnI and HindIII, purified, ligated with pBBR1MCS2 to generate pKSJ213, pKSJ214, and pKSJ243, respectively. The ~ 1.9-kb DNA fragment containing *pduLW* was excised from pKSJ242 by digestion with AvrII and HindIII, purified, and ligated to with similarly digested pKSJ215 and pKSJ216, to generate plasmids pKSJ244 and pKSJ245, respectively.

RT-PCR experiments. RT-PCR experiments to verify the *ctdQ* was expressed in *Acidovorax* sp. strain JS42-KSJ11 were carried out using the One-Step RT-PCT kit (Qiagen) with primers ctdQ-F1 and ctdQ-R1.

Growth assays. To test growth on acetaldehyde and propionaldehyde, JS42 and the $4NT^{+}$ strains were streaked on MSB agar containing vitamins. *Salmonella enterica* LT2 was streaked on MSB agar containing vitamins, 150 nM cyanocobalamin, and 0.02% 1,2-propanediol or ethanolamine. Plates were incubated at room temperature or 30° C in a 5.5-L metal tin containing an open test tube taped to the interior, with 10 mL of acetaldehyde or

propionaldehyde as the sole carbon source for growth. Aldehyde was replenished every three days.

To test growth in liquid media, strains were inoculated into glass screw-capped culture tubes containing 5-ml of MSB containing vitamins and yeast extract (0.05%), with 2 mM nitrobenzene, 2NT, or 4NT for JS42 strains, and with 2 mM 3-methylbenzoate or 4-methylbenzoate for *Pseudomonas putida* mt-2. Gentamicin or kanamycin was included when appropriate. Overnight cultures were used to inoculate tubes to an initial OD_{660} of 0.04 to 0.05. Tubes were sealed with butyl stoppers and incubated at 30° C on a roller drum at 75 RPM. Culture turbidity and total protein content was monitored over time.

Headspace analysis. Propionaldehyde production was examined by sampling the atmosphere of the cultures with a gas-tight syringe and analyzing 100-μl of headspace by GC-MS (Ju and Parales, 2006), by isothermal separation at 36° C. Under these conditions, the peak observed at 2.6 min ([m/z] 58) was identified as propionaldehyde by comparing to the standard.

Preparation of crude-cell extracts. JS42-KSJ11 was grown in 2.8-L Fernbach flasks containing 800 ml of MSB containing 10 mM succinate, 1 mM 4NT, vitamins, and 0.05% (wt/vol) yeast extract at 30° C. At an OD_{660} of 1.0, cultures were harvested by centrifugation and 10 grams of cells (wet weight) was resuspended in 20 ml of 50 mM phosphate buffer (pH 7.3) with 1 mg of lysozyme and 2 μg each of DNaseI and RNase (Roche), and incubated at 30° C for 30 minutes with periodic mixing. Cell suspensions were passed twice through a chilled French pressure cell, maintaining an internal cell pressure of 20,000 lb/in^2. Residual cells, membranes,

and debris were removed by centrifugation at 14,476 RCF for 20 min at 4° C. The resulting cell extracts were immediately used for enzyme assays, as described below.

Stoichiometry of pyruvate and propionaldehyde formation. Five ml of JS42-KSJ11 crude cell extracts were added into glass screw-capped culture tubes sealed with butyl stoppers. 4-Methylcatechol was added to a final concentration of 3 mM and incubation was at 28° C on a roller drum at 75 RPM. Propionaldehyde formation was monitored by GC-MS as described above. When levels of propionaldehyde reached a maximum (~2.5 h), reactions were quenched by adding 100 μl of 6 N HCl and 10 ml of 0.1% of 2,4-diphenylhydrazine dissolved in 2N HCl (Dagley and Gibson, 1965). Samples were derivatized overnight at room temperature. Precipitated material was recovered by centrifugation (10,000 RPM for 10 min) and washing with 20 ml of 2 N HCl. After the fourth wash the 2,4-dinitrohydrazones were extracted three times with an equal volume of ethyl acetate, concentrated by rotary evaporation, and evaporated to dryness.

The dried products were redissolved in 100 μl of acetonitrile, and 1-μl volumes spotted onto Kieselgel 60 F_{254} TLC plates, along with standards of the dinitrophenylhydrazones of pyruvate and propionaldehyde. Matching R_F values to authentic materials run on the same chromatogram was used to identify analytes from the reaction mixtures. The following solvent systems were used: *A*. ethyl acetate - isopropanol - acetic acid (10:2:1) (Shigeno and Nakahara, 1991); *B*. benzene - tetrahydrofuran - acetic acid (15:9:1) (Byrne, 1965); *C*. diethyl ether - *n*-hexane (1:3) (Dagley and Gibson, 1965). The R_F values of 2,4-dinitrophenylhydrazones in solvents *A*, *B*, and *C* for pyruvate were in *A*, 0.6; in *B*, 0.54 and no migration in *C*; for propionaldehyde in *A*, 0.77;

in *B*, 0.74; and in *C*, 0.35. To determine yields, reaction samples were spotted with known amounts of reference compounds, and quantified by densitometry using a FluoroChem 8900 digital imaging system (Alpha Innotech).

Analysis of CoA-ester formation. *E. coli* DH5α containing plasmids pUC18, pKSJ191, pKSJ197, or pVI303Δ (Table S1) were grown in 800-ml LB medium containing 200 μg ml^{-1} ampicillin in 2.8 L Fernbach flasks at 37° C. After 12 hours, cultures were harvested by centrifugation and resuspended in 15 ml of 50 mM Na^+/K^+-phosphate buffer (pH 7.5) with 1 mM dithiothreitol. Crude cell extracts were prepared as described above, and dialyzed against 1 L of 50 mM Na^+/K^+-phosphate buffer (pH 7.5) at 4° C for 6 hours, and against a second liter of the same buffer overnight. The formation of CoA-esters was tested as previously described (Powlowski *et al.*, 1993), using glass screw-cap culture tubes containing 5-ml of 50 mM Na^+/K^+-phosphate buffer with 50 μmol acetaldehyde or propionaldehyde, 7.2 μmol NAD^+, and 1.6 μmol coenzyme A. Reactions were initiated by the addition of 5 mg of crude cell extract, immediately stoppering the reaction vial, and incubating at 30° C in a shaking water bath (100 RPM). After 30 min, reactions were adjusted to pH 5 by the addition of 3 N HCl and placed on ice to stop the reaction. Samples were filtered to remove precipitates and kept on ice prior to analysis.

High-pressure liquid chromatography was used to separate acetyl-CoA and propionyl-CoA from reaction mixtures. A Beckman Coulter System Gold 125 high-performance liquid chromatography system with a model 168 diode array multiwavelength detector and a Zorbax SB-C18 column (4.6 x 250 mm, 5 μm particles; Agilent Technologies) was operated with a mobile phase consisting of 50 mM Na^+/K^+-phosphate buffer (pH 5.3):acetonitrile (95:5) and a

flow rate of 0.8-ml min^{-1}. CoA-esters were detected at 254 nm. Under these conditions, acetyl-CoA and propionyl-CoA had retention times of 13.4 and 36.6 min, respectively. Authentic acetyl-CoA and propionyl-CoA were used to generate standard curves for quantification.

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TABLE S1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
DH5α	Cloning host; *thi*	Invitrogen
S17-1	Host for plasmid mobilization; *thi*	(Simon *et al.*, 1983)
Salmonella enterica serovar Typhimurium		
LT2	Wild type	J.R. Roth
Acidovorax sp.		
JS42	Wild-type strain. Degrades 2NT and nitrobenzene	(Haigler *et al.*, 1994)
JS42Ac	*ntdAc* mutant of JS42, $2NT^-$, $nitrobenzene^-$, Km^R	(Ju *et al.*, 2009)
JS42-KSJ9	$4NT^+$ derivative of JS42	This study
JS42-KSJ10	$4NT^+$ derivative of JS42	This study
JS42-KSJ11	$4NT^+$ derivative of JS42	This study
Pseudomonas sp.		
9816/11	Derivative of NCIB 9816; naphthalene *cis*-dihydrodiol dehydrogenase mutant	(Klecka and Gibson, 1979)
Pseudomonas putida		
mt-2	Wild-type strain. Degrades toluene, *m*-, *p*-xylene, 3-, 4- methylbenzoate	(Murray *et al.*, 1972; Williams and Murray, 1974; Worsey and Williams, 1975)
Plasmids		
pBBR1MCS2	Broad host-range expression vector, Km^R	(Kovach *et al.*, 1995)
pBBR1MCS5	Broad host-range vector, Gm^R	(Kovach *et al.*, 1995)
pDTG800	pUC18 containing *ntdAaAbAcAd* from JS42, encodes 2NTDO, Amp^R	(Parales *et al.*, 1996)
pDTG850	pUC13 containing *ntdAaAbAcAd* from JS42, Amp^R	(Parales *et al.*, 1998)
pKSJ44	pBBR1MCS5 containing *ntdAaAbAcAd* from pDTG800, Gm^R	This study
pKSJ90	Derivative of pDTG850 containing the *ntdAc* KpnI-MfeI gene fragment from JS42-KSJ9, encodes 2NTDO M248I, Amp^R	This study
pKSJ91	Derivative of pDTG850 containing the *ntdAc* KpnI-MfeI gene fragment from JS42-KSJ10, encodes 2NTDO S242N M248I, Amp^R	This study

pKSJ92	Derivative of pDTG850 containing the *ntdAc* KpnI-MfeI gene fragment from JS42-KSJ11, encodes 2NTDO L238V M248I, Amp^R	This study
pKSJ93	Derivative of pKSJ44 containing the *ntdAc* KpnI-MfeI gene fragment from pKSJ90, Gm^R	This study
pKSJ94	Derivative of pKSJ44 containing the *ntdAc* KpnI-MfeI gene fragment from pKSJ91, Gm^R	This study
pKSJ95	Derivative of pKSJ44 containing the *ntdAc* KpnI-MfeI gene fragment from pKSJ92, Gm^R	This study
pKSJ119	Derivative of pDTG850, encodes 2NTDO L238V, Amp^R	This study
pKSJ120	Derivative of pDTG850, encodes 2NTDO S242N, Amp^R	This study
pKSJ121	Derivative of pKSJ92, encodes 2NTDO L238V S242N M248I, Amp^R	This study
pKSJ122	Derivative of pKSJ119, encodes 2NTDO L238V S242N, Amp^R	This study
pKSJ144	pUC18 containing *ctdK* from JS42, encodes 4-hydroxy-2-ketovalerate aldolase, Amp^R	This study
pKSJ191	pUC18 containing *ctdQ* from JS42, encodes acylating acetaldehyde dehydrogenase, Amp^R	This study
pKSJ197	pUC18 containing *xylQ* from mt-2, encodes acylating acetaldehyde dehydrogenase, Amp^R	This study
pKSJ209	pUC18 containing *pduL* from LT2, encodes phosphotransacylase, Amp^R	This study
pKSJ210	pUC18 containing *pduW* from LT2, encodes phosphtransacylase, Amp^R	This study
pKSJ211	pUC18 containing *dmpFG* from *Pseudomonas* sp. strain CF600, encodes acylating acetaldehyde dehydrogenase and 4-hydroxy-2-ketovalerate aldolase, Amp^R	This study
pKSJ212	pUC18 containing *ctdQK* from JS42; encodes acylating acetaldehyde dehydrogenase and 4-hydroxy-2-ketovalerate aldolase, Amp^R	This study
pKSJ213	pBBR1MCS2 containing *pduL* from LT2, Km^R	This study
pKSJ214	pBBR1MCS2 containing *pduW* from LT2, Km^R	This study
pKSJ215	pBBR1MCS2 containing *dmpFG* from *Pseudomonas* sp. strain CF600, Km^R	This study
pKSJ216	pBBR1MCS2 containing *ctdQK* from JS42, Km^R	This study
pKSJ242	pUC18 containing *pduLW* from LT2, Amp^R	This study
pKSJ243	pBBR1MCS2 containing *pduLW*, Km^R	This study
pKSJ244	pBBR1MCS2 containing *dmpFQ-pduLW*, Km^R	This study
pKSJ245	pBBR1MCS2 containing *ctdQK-pduLW*, Km^R	This study
pUC18	General purpose expression vector, Amp^R	(Norrander *et al.*, 1983)

pVI303Δ	Expression clone for *dmpDEFGH* from *Pseudomonas* sp. strain CF600, Amp^R	(Shingler *et al.*, 1992)
pVI316Δ	Expression clone for *dmpFG* from *Pseudomonas* sp. strain CF600, Amp^R	(Powlowski *et al.*, 1993; Shingler *et al.*, 1992)

Amp^R, ampicillin resistant; Km^R, kanamycin resistant; Gm^R, gentamicin resistant.

Table S2. Product formation from 2,4-dinitrotoluene by wild-type and mutant dioxygenases expressed in *E. coli*.

Enzyme[a]	Source Strain[b]	Products (%) produced from 24DNT[c]	
		4M3N CAT	4M5N CAT
2NTDO	JS42	---	---
L238V	n/a	14	86
M248I	JS42-KSJ9	40	60
L238V M248I	JS42-KSJ11	---	100
S242N M248I	JS42-KSJ10	---	100

[a] No products were detected with the S242N, L238V S242N, or L238V S242N M248 variants.

[b] n/a, not applicable; mutant enzyme was generated by site-directed mutagenesis.

[c] N =3; standard deviations were < 3%; 24DNT, 2,4-dinitrotoluene; 4M3NC, 4-methyl-3-nitrocatechol; 4M5NC, 4-methyl-5-nitrocatechol; ---, none detected.

Table S3. Similarity of gene products from the catechol degradation gene cluster of JS42 to selected homologs.

Gene Product	Source Strain	Function	Length (AA)	% Identity	Alignment Range		Accession no.
					JS42 Peptide	Homolog	
CtdR1	*Acidovorax* sp. strain JS42	LysR-type regulator	306				YP_984543
CdoR	*Comamonas* sp. strain JS765		306	100	1-306	1-306	AAC79916
CtdR2	*Acidovorax* sp. strain JS42		301	67	1-297	1-297	YP_984546
CbnR	*Ralstonia eutropha* NH9		294	33	1-294	1-293	BAA74529
CtdT1	*Acidovorax* sp. strain JS42	Ferredoxin	123				YP_984544
CdoT	*Comamonas* sp. strain JS765		123	100	1-123	1-123	AAC79917
CbzT	*Pseudomonas putida* GJ31		119	71	3-113	2-112	AAD05249
TdnD1	*Pseudomonas putida* UCC22		119	67	11-119	11-118	BAB62044
CtdE1	*Acidovorax* sp. strain JS42	Catechol 2,3-dioxgenase	314				YP_984545
CdoE	*Comamonas* sp. strain JS765		314	100	1-314	1-314	AAC79918
TdnC	*Pseudomonas putida* UCC22		314	83	1-314	1-314	CAA42452
XylE	*Pseudomonas putida* mt-2		307	44	2-314	4-307	P06622
ORF1	*Acidovorax* sp. strain JS42	Conserved hypothetical	293				
CdoX1	*Comamonas* sp. strain JS765		188	98	1-175	1-175	AAG17128
ORF1	*Pseudomonas putida* UCC22		386	92	1-255	1-254	BAB62047
CtdR2	*Acidovorax* sp. strain JS42	LysR-type regulator	301				YP_984546
CdoR2	*Comamonas* sp. strain JS765		301	66	1-298	1-298	AAG17132
ClcR	*Pseudomonas* sp. B13		294	36	1-289	1-288	CAE92862

CtdT2	*Acidovorax* sp. strain JS42	Ferredoxin	111				YP_984547
TdnD2	*Pseudomonas putida* UCC22		121	83	1-111	1-111	BAB62049
CbzT	*Pseudomonas putida* GJ31		119	48	3-108	8-114	AAD05249
PhlG	*Pseudomonas putida* H		101	40	4-98	6-100	CAA56746
CtdE2	*Acidovorax* sp. strain JS42	Catechol 2,3-dioxygenase	303				YP_984548
TdnC2	*Pseudomonas putida* UCC22		303	93	1-303	1-308	BAB62050
NahH	*Pseudomonas fluorescens* PC20		307	53	5-303	4-307	AAW81680
NahH	*Pseudomonas putida* G7		307	52	5-303	4-307	YP_534833
XylE	*Pseudomonas putida* mt-2		307	44	4-307	2-314	P06622
ORF2	*Acidovorax* sp. strain JS42	Hypothetical protein	143				YP_984549
ORF4	*Pseudomonas putida* UCC22		143	99	1-143	1-143	BAB62051
ORFX2	*Delftia* sp. AN3		143	69	5-142	6-143	ABI20715
CtdG	*Acidovorax* sp. strain JS42	2-Hydroxymuconate semialdehyde dehydrogenase	486				YP_984550
TdnE	*Pseudomonas putida* UCC22		486	98	1-486	1-486	BAB62052
CbzG	*Pseudomonas putida* GJ31486		496	77	1-486	1-486	AAX38583
CdoG	*Comamonas* sp. strain JS765		486	72	1-486	1-486	AAG17134
XylG	*Pseudomonas putida* mt-2		486	63	1-486	1-486	CAC86805
CtdF	*Acidovorax* sp. strain JS42	2-Hydroxymuconate semialdehyde hydrolase	286				YP_984551
TdnF	*Pseudomonas putida* UCC22		288	97	1-286	1-286	BAB62053
CdoFa	*Comamonas* sp. strain JS765		175	84	7-152	13-157	AAG17130
CdoFb	*Comamonas* sp. strain JS765		125	77	157-279	1-123	AAG17131

DmpD	*Pseudomonas putida* CF600		283	73	1-280	1-279	CAA36993
XylF	*Pseudomonas putida* mt2		281	64	1-280	1-278	CAC86804
CtdJ	*Acidovorax* sp. strain JS42	2-Oxopent-4-dienoate hydratase	262				YP_984552
TdnG	*Pseudomonas putida* UCC22		262	94	1-262	1-262	BAB62054
CdoH	*Comamonas* sp. strain JS765		262	90	1-262	1-262	AAG17135
XylJ	*Pseudomonas putida* mt-2		222	70	3-223	1-222	CAC86803
CtdQ	*Acidovorax* sp. strain JS42	Acetaldehyde dehydrogenase	302				YP_984553
TdnI	*Pseudomonas putida* UCC22		302	99	1-302	1-302	BAB62056
CdoI	*Comamonas* sp. strain JS765		302	95	1-302	1-302	AAG17136
XylQ	*Pseudomonas putida* mt-2		312	56	1-293	1-310	CAC86802
CtdK	*Acidovorax* sp. strain JS42	4-Hydroxy-2-oxovalerate aldolase	342				YP_984554
TdnJ	*Pseudomonas putida* UCC22		343	99	1-342	1-343	BAB62057
CdoJ	*Comamonas* sp. strain JS765		347	93	1-271	1-271	AAG17137
XylK	*Pseudomonas putida* mt-2		345	54	3-336	7-340	CAC86801
CtdI	*Acidovorax* sp. strain JS42	4-Oxalocrotonate decarboxylase	262				YP_984555
TdnK	*Pseudomonas putida* UCC22		262	95	1-262	1-262	BAB62058
CdoK	*Comamonas* sp. strain JS765		262	90	1-262	1-262	AAG17138
XylI	*Pseudomonas putida* mt-2		264	59	3-261	5-263	CAC86800
ORF3	*Acidovorax* sp. strain JS42	Putative exported protein	327				YP_984556
CdoL	*Comamonas* sp. strain JS765		249	95	1-249	1-249	AAG17139
ORFZ	*Delftia* sp. AN3		325	72	1-325	1-323	ABI20722

CtdH	*Acidovorax* sp. strain JS42	4-Oxalocrotonate isomerase	63				YP_984557
TdnL	*Pseudomonas putida* UCC22		63	87	1-63	1-63	BAB62059
CnbG	*Comamonas* sp. strain CNB-1		63	77	1-63	1-63	YP_001967692
XylH	*Pseudomonas putida* mt-2		63	42	1-63	1-63	CAC86799

Table S4. Growth of JS42 and JS42-KSJ11 carrying cloned components for a constructed propionaldehyde salvage pathway.

Strain	Plasmid-Encoded Genes	Doubling Time[a] (h)		Cell Yield[b] (μg Protein ml^{-1})	
		2NT	4NT	2NT	4NT
JS42 (pBBR1MCS2)	none	3.8	---[c]	112	---[c]
JS42 (pKSJ215)	*dmpFG*	6.7	---	113	---
JS42 (pKSJ216)	*ctdQK*	6.8	---	112	---
JS42 (pKSJ243)	*pduLW*	5.3	---	113	---
JS42 (pKSJ244)	*dmpFG-pduLW*	6.2	---	114	---
JS42 (pKSJ245)	*ctdQK-pduLW*	6.4	---	114	---
JS42-KSJ11 (pBBR1MCS2)	none	6.5	7.8	128	63
JS42-KSJ11 (pKSJ215)	*dmpFG*	7.8	8.4	129	63
JS42-KSJ11 (pKSJ216)	*ctdQK*	14.1	8.4	128	63
JS42-KSJ11 (pKSJ243)	*pduLW*	5.3	7.6	127	77
JS42-KSJ11 (pKSJ244)	*dmpFG-pduLW*	6.0	6.9	130	78
JS42-KSJ11 (pKSJ245)	*ctdQK-pduLW*	5.3	7.6	144	81

[a] N = 3; standard deviations were 7% or less
[b] N = 3; standard deviations were 5% or less
[c] ---, no growth observed

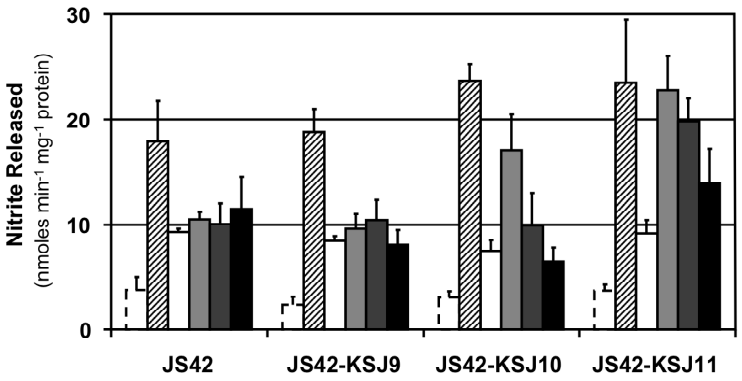

Fig. S1. Nitroarene dioxygenase activity in JS42 and $4NT^{+}$ strains grown in MSB containing succinate in the absence of inducer (white, dashed border), or in the presence of 100 μM salicylate (diagonal lines), nitrobenzene (white, solid border), 2NT (light grey), 3NT (dark grey), or 4NT (black) as inducers. One drop of nitrobenzene (~ 3μL) was provided as substrate and the amount of nitrite was measured and normalized to the total protein content in each culture. Error bars indicate standard deviations (N = 3).

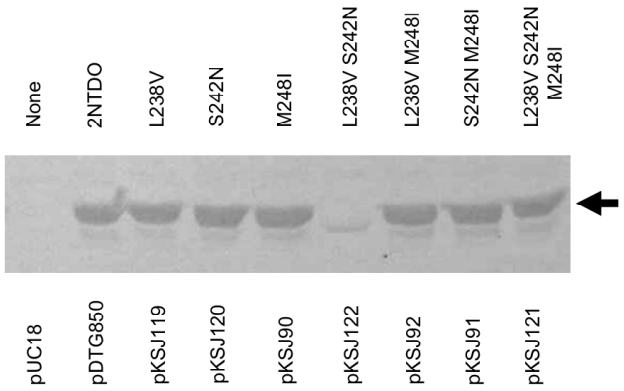

Fig. S2. Western blot analysis of the recombinant wild-type and mutant 2NTDO α subunits (indicated by the arrow) using a polyclonal antibody for naphthalene dioxygenase.

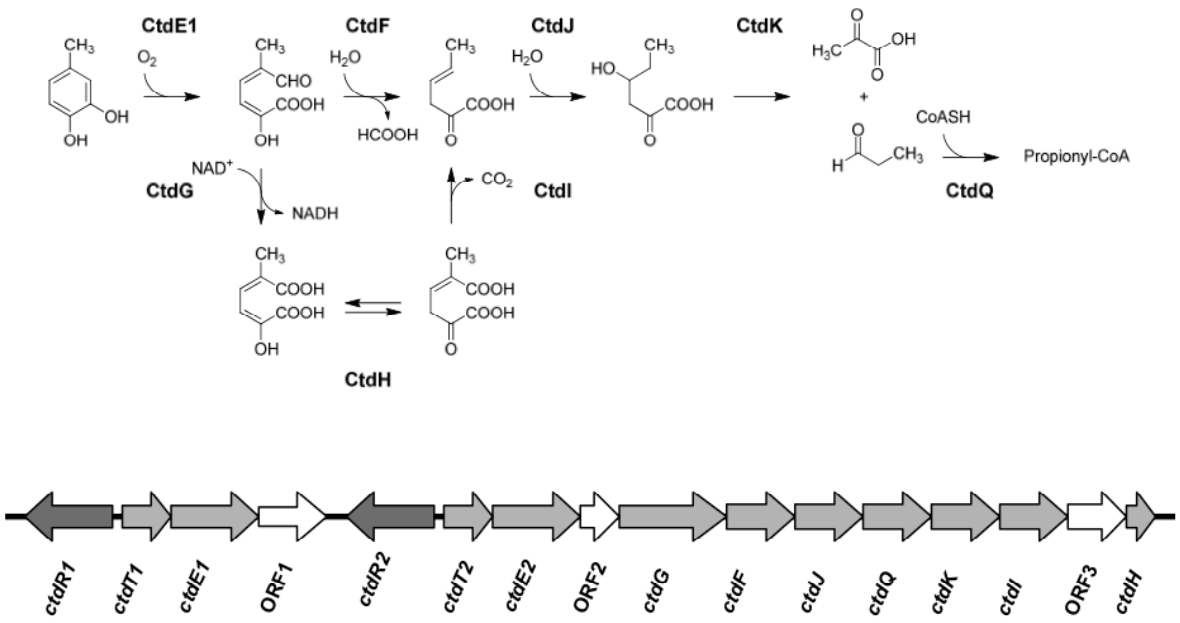

Fig. S3. The *meta* ring-cleavage pathway in *Acidovorax* sp. JS42, and physical map of the region of the genome encoding putative catechol degradation (*ctd*) genes in JS42. The pathway shown is for 4-methylcatechol degradation; the same pathway is used for catechol and 3-methylcatechol degradation with slight variations in the intermediates formed. The catechol degradation (Ctd) enzymes were identified based on the analysis of the JS42 genome sequence: CtdE1/ CtdE2, catechol 2,3-dioxygenase; CtdF, 2-hydroxymuconate semialdehyde hydrolase; CtdG, 2-hydroxymuconate semialdehyde dehydrogenase; CtdH, 4-oxalocrotonate isomerase; CtdI, 4-oxalocrotonate decarboxylase; CtdJ, 2-oxopent-4-dienoate hydratase; CtdK, 4-hydroxy-2-oxovalerate aldolase; CtdQ, acetaldehyde dehydrogenase (acylating).

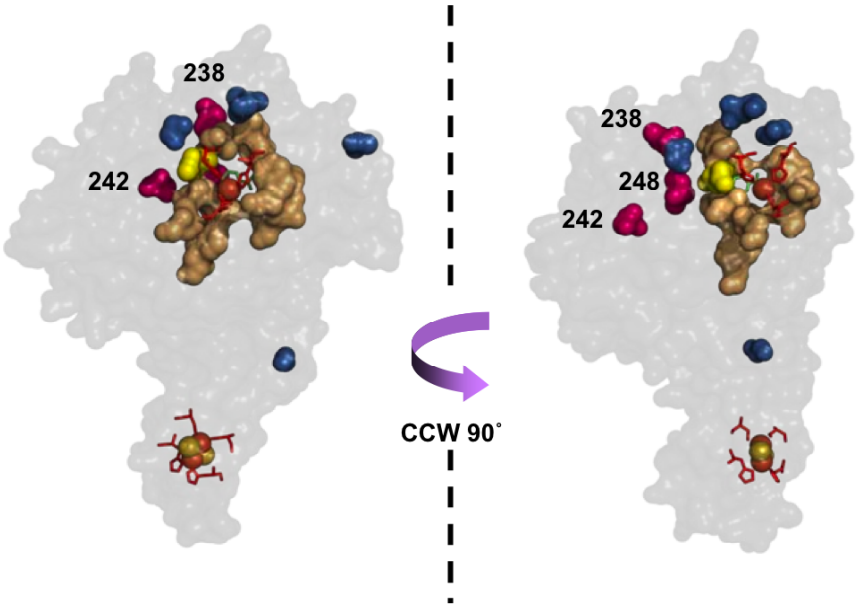

Fig. S4. Homology model of a single α-subunit of 2NTDO. A) Front-view. B) Side-view (90° clockwise-rotation). The mononuclear iron (orange) and Rieske-center (orange and yellow) are shown as spheres with their coordinating amino acids displayed in red. Nitrobenzene in the active site of the dioxygenase, is green, and aspartate 203, which is critical for electron transfer from the Rieske center to the active site iron (Friemann *et al.*, 2005; Parales *et al.*, 1999), is yellow. Residues that were changed in the dioxygenases from the $4NT^{+}$ strains are pink and are labeled with the amino acid number. For comparison, amino acids shown to affect dioxygenase activity in naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4, nitrobenzene dioxygenase, or 2NTDO (Ju and Parales, 2006; Lee *et al.*, 2005; Parales *et al.*, 1999; Parales *et al.*, 2000a; Parales *et al.*, 2000b; Yu *et al.*, 2001) are tan, and positions affecting substrate specificity in engineered variant of naphthalene dioxygenase from *Ralstonia* sp. strain U2 (Keenan *et al.*, 2005) are blue. The homology model was generated with the SWISS-MODEL server (Arnold *et al.*, 2006) using the NBDO NbzAc crystal structure (PDB ID: 2BMQ, chain as the template (Friemann *et al.*, 2005). The root mean square deviation of the modeled 2NTDO was 0.06 Å using 445 C_{α} atoms of NbzAc and NtdAc. Molecular representations were created using PyMol (DeLano Scientific).