Supporting Information

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SI Methods

Replacement of *pcpD* with a Kanamycin Resistance Cassette. A 448-bp fragment including the 3′ end of *pcpB*, the short intergenic region, and the first 4 bp of *pcpD* was amplified from genomic DNA using the primers DKO1F (CCGgaattcATTATCGCGACCAGCTCG) and DKO1B (GCTGAGTTGAAGGATCAGATTCATCGCACGGGTCTCCT). The forward primer added an EcoRI site (lowercase) to allow cloning into pUC19, and the reverse primer added a 20-bp sequence complementary to the kanamycin resistance gene from pACYC177 (underlined). A second fragment of 427 bp including the last 6 bp of *pcpD*, the intergenic region and a 5′ fragment from *pcpR* was amplified from genomic DNA using primers DKO2f (GATGCTCGATGAGTTTTTCTAAATCTGAATCGACCGCTCG) and DKO2b (ACTAggatccTCGAAGACCATCAGATGG). The forward primer added a 22-bp sequence complementary to the kanamycin cassette fragment (underlined), and the reverse primer added a BamHI (lowercase) restriction site to allow cloning into pUC19. The kanamycin resistance gene was amplified from the plasmid pACYC177 using the primers pKanF (ATCTGATCCTTCAACTCAGC) and pKanB (TTAGAAAAACTCATCGAGCATC). The amplified fragments were gel-purified and assembled by PCR using the primers DKO1f and DKO2b. Fragments (0.05 pmol each) were mixed, and the reaction was initiated by 5 cycles without primers followed by 25 cycles with primers. In each cycle, annealing was carried out at 58 °C for 30 s followed by extension at 72 °C for 2 min. The assembled fragment was digested with EcoRI and BamHI and ligated into pUC19 digested with the same enzymes to give the recombination plasmid pUC19_DKOcassette.

Sphingobium chlorophenolicum L1 cells were made competent for transformation by growth in 1/4 strength tryptic soy broth (1/4 TSB) to an OD_{600} of 0.6. The cells were harvested by centrifugation at 2,500 × *g* and 4 °C for 20 min and then washed three times with ice cold 10% glycerol. The cells were resuspended in 3 mL of 10% glycerol (0.6% of the original volume of the culture) and incubated with 5 μg of pUC19_DKOcassette. Electroporation was carried out three times in a 1-mm gap cuvette at EC1 settings (Micropulser; BioRad). The transformed cells were incubated in 1 mL of 1/4 TSB for 5 h at 30 °C, after which 10 μg/mL kanamycin was added, and the cells were grown for an additional 15 h. The transformed cells were then plated on 1/4 TSB agar plates supplemented with 7.5 μg/mL kanamycin. Colonies formed after 7 d were analyzed by PCR and DNA sequencing from the genomic DNA to verify the correct deletion of *pcpD* and the insertion of the kanamycin cassette in its place.

Cloning of *pcpD*. *pcpD* from *Sphingomonas* sp. RA2 was amplified by PCR using PfuUltra II Fusion HS DNA Polymerase (Agilent). The following primers used to amplify *pcpD* were obtained from Integrated DNA Technologies: (*i*) forward primer 5′-ACTAGAAGACTAgatcCATGACAAACCCCGTTTCG-3′ (underlining indicates a BbsI recognition site and lowercase indicates a restriction site compatible with BamHI); and (*ii*) reverse primer 5′-ATAGTTTAGCGGCCGCTCAGATGTCCAGCACCAG-3′ (underlining indicates a NotI site). The amplified DNA was digested with BbsI and NotI (New England BioLabs) according to the manufacturer's recommendations. The digested PCR product was purified using a QIAquick Gel extraction kit (Qiagen). The pET28 vector (Novagen) containing an ORF for a His_{10}-tagged Smt3 protein [small ubiquitin-related modifier (SUMO)] from *Saccharomyces cerevisiae* (1) was digested with BamHI and NotI. The cleavage products were separated on an agarose gel (1%), and the linearized vector was further purified using a QIAquick Gel extraction kit. The PCR fragment was ligated into the linearized vector downstream of and in-frame with the sequence encoding the His_{10}-tagged Smt3 protein using T4 DNA Ligase (New England BioLabs). The resulting plasmids were introduced into competent *E. coli* DH5α cells. After selection and growth of individual clones on LB containing kanamycin (50 mg/L), plasmids were purified using a QIAprep Spin Miniprep kit (Qiagen), and the sequence of the inserted *pcp*D was verified by DNA sequencing.

Expression and Purification of PcpD. The plasmid pET28-His_{10}-Smt3-pcpDRA2 was introduced into *E. coli* BL21(DE3) cells, and the protein was expressed according to the protocol of Jaganaman et al. (2) for purification of phthalate dioxygenase reductase from *Burkholderia cepacia*. Briefly, an overnight culture in Luria Broth was used to inoculate 1 L of Terrific broth medium containing 50 mg/L kanamycin. The culture was grown at 30 °C with shaking at 250 rpm until the OD_{600} reached 1.2. At that time, the culture was supplemented with 2 mM cysteine, 0.2 mg/mL ferrous sulfate, 0.2 mg/mL ferric citrate, and 0.2 mg/mL ferric ammonium citrate and transferred to 16 °C. After 20 min, expression of *pcpD* was induced by addition of 50 μM isopropyl-β-D-1-thiogalactoside (IPTG). After 18 h, the cells were harvested by centrifugation at 5,000 × *g* for 20 min at 4 °C.

The pellet from 0.5 L of cells was resuspended in 60 mL buffer B (50 mM sodium phosphate, pH 7.6, containing 500 mM NaCl and 0.2 mM DTT) supplemented with 20 mM imidazole, 1 mM phenylmethanesulfonylfluoride, and protease inhibitor mixture (Sigma; P8849; 1:500 dilution). The cells were lysed by two passages through a French press at 12,000 psi. Following centrifugation at 12,000 × *g* for 30 min at 4 °C to remove the cellular debris, nucleic acids were precipitated by the drop-wise addition of protamine sulfate to a final concentration of 0.3%. Following another centrifugation at 12,000 × *g* for 30 min, the clarified lysate was loaded on an Ni^{2+}-column (5 mL HisTrap HP from GE Healthcare). The column was washed with 10 column volumes of 50 and 100 mM imidazole in buffer B and eluted using a linear gradient of 100–500 mM imidazole in buffer B over 20 column volumes. Fractions containing PcpD were pooled and treated with Ulp1 (SUMO protease 1) to remove the Smt3 tag using the protocol described in Malakhov et al. (1). After Ulp1 treatment, the protein was reloaded onto a new Ni^{2+}-column to remove the His_{10}-Smt3 tag and any undigested protein. PcpD in the flow-through was concentrated, applied to a Superdex 200 16/60 gel filtration column (GE Healthcare) and eluted with buffer B. Fractions containing PcpD as determined by absorption spectra and SDS/PAGE were pooled, buffer exchanged to 50 mM sodium phosphate, pH 7.6, containing 150 mM NaCl, concentrated, flash frozen, and stored at −80 °C.

Preparation of PcpD Lacking the 2Fe-2S Domain. The 5′ region of *pcpD* from *Sphingomonas* sp. RA2 that encodes the flavin mononucleotide (FMN) and NADH binding domains (but lacks the sequence encoding the 2Fe-2S domain after Ala229) was amplified by PCR using PfuUltra II Fusion HS DNA Polymerase (Agilent). The following primers used to amplify the pcpD-FeS gene were obtained from Integrated DNA Technologies: (*i*) forward primer 5′-CCCATGGTGACAAACCCCGTTTCGACAATCG-3′ (underlining indicates an NcoI restriction site); and (*ii*) reverse primer 5′-ATAGTTTAGCGGCCGCTTACAAGGCCGCGCCGAAATG-3′ (underlining indicates an NotI site). The amplified DNA was digested with NcoI and NotI (New England BioLabs) according to the manufacturer's recommendations. The digested PCR product was purified using a QIAquick Gel extraction

kit (Qiagen) and cloned into a modified pET20b vector (Novagen) in which the *pelB* sequence had been replaced with a His_6-tag. The plasmid pET20His_6-[pcpD-FeS] was introduced into BL21 (DE3) cells. Growth of cells and induction of PcpD-FeS was performed as described above for production of PcpD, omitting the supplementation with cysteine and iron. PcpD-FeS was purified by nickel affinity chromatography and gel filtration as described for PcpD above.

1. Malakhov MP, et al. (2004) SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. *J Struct Funct Genomics* 5(1-2): 75–86.

2. Jaganaman S, Pinto A, Tarasev M, Ballou DP (2007) High levels of expression of the iron-sulfur proteins phthalate dioxygenase and phthalate dioxygenase reductase in Escherichia coli. *Protein Expr Purif* 52(2):273–279.

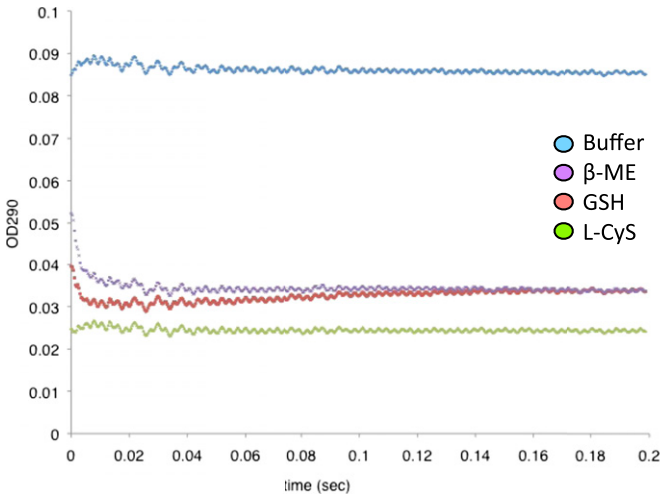

Fig. S1. Reaction of thiols with tetrachlorobenzoquinone (TCBQ). TCBQ was mixed in a Hi-Tech SF-61DX stopped-flow instrument with glutathione (GSH), L-cysteine (L-CyS), or β-mercaptoethanol (β-ME) in 50 mM potassium phosphate, pH 7.0, to yield final concentrations of 25 μM TCBQ and 250 μM thiol. Depletion of TCBQ was monitored at 290 nm. The traces shown represent the averages of four to five reactions.

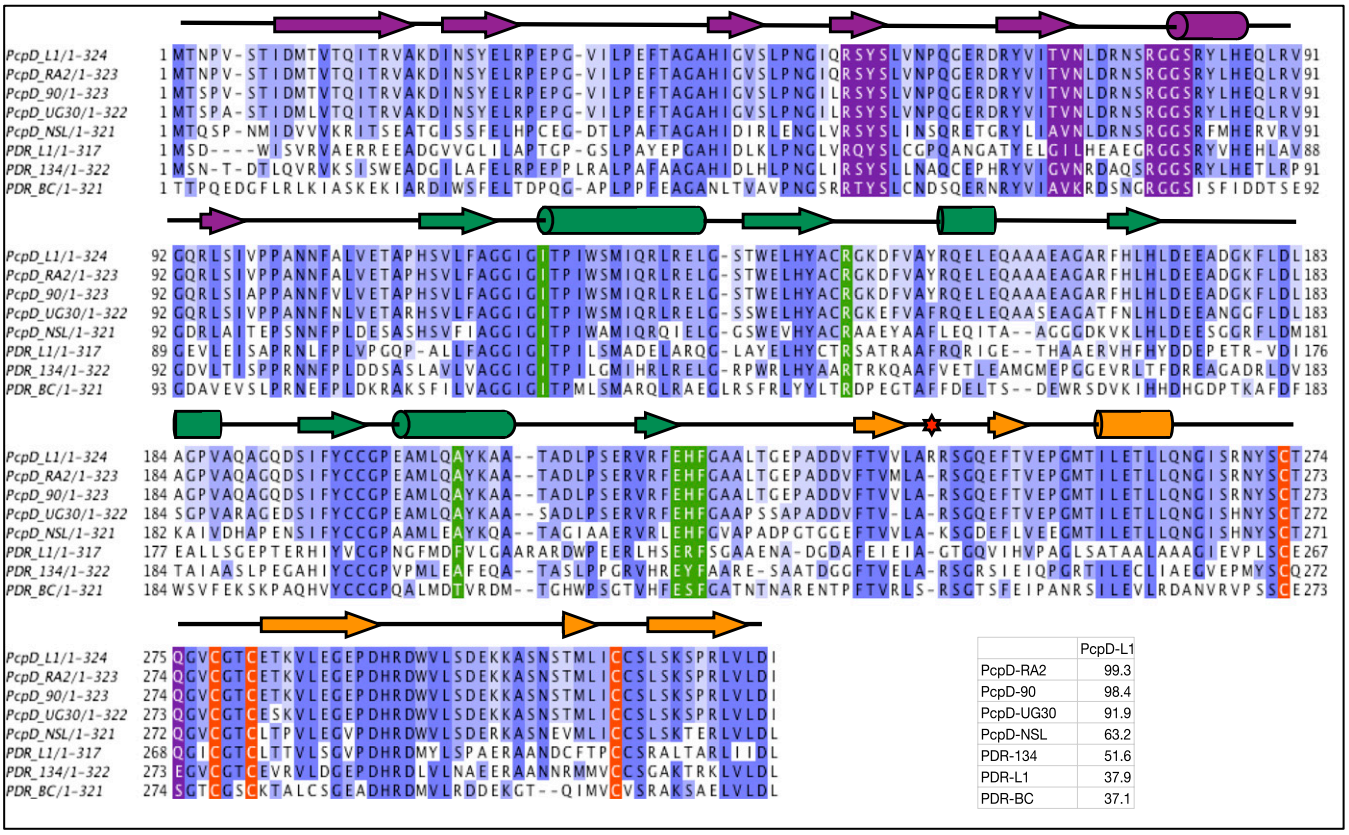

Fig. S2. Multiple sequence alignment of PcpD enzymes and related phthalate dioxygenase reductases. PcpD-L1, PcpD from *S. chlorophenolicum* L1; PcpD-RA2, PcpD from *S. chlorophenolicum* sp. RA2; PcpD-90, PcpD from *S. chlorophenolicum* ATCC 33790; PcpD-UG30, PcpD from *Sphingomonas*. sp UG30; PcpD-NSL, PcpD from *Novosphingobium lentum*; PDR-134, phthalate dioxygenase reductase from *Cupriavidus necator* JMP 134; PDR-L1, phthalate dioxygenase reductase from *S. chlorophenolicum L1*; and PDR-BC, PDR from *B. cepacia*. (Accession numbers for the previously unsequenced proteins from *S. chlorophenolicum* sp. RA2, *S. chlorophenolicum* ATCC 33790, and *N. lentum* are JX514946, JX514945, and JX514944, respectively.) Proteins were aligned using T-coffee (1) and viewed with Jalview (2). Secondary structure elements were derived from the structure of PDR-BC (PDB 2PIA) and colored according to the three domains of the protein: purple, FMN binding domain; green, NADH binding domain; and orange, 2Fe-2S domain. Individual residues that interact with NADH, FMN, and the iron sulfur cluster are colored according to the domains. The red star indicates the inserted arginine in PcpD-L1. *Inset* table shows the percent identity between PcpD-L1 and the other enzymes.

1. Di Tommaso P, et al. (2011) T-Coffee: A web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res* 39(Web Server issue):W13–W17.
2. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ (2009) Jalview version 2: A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25(9):1189–1191.

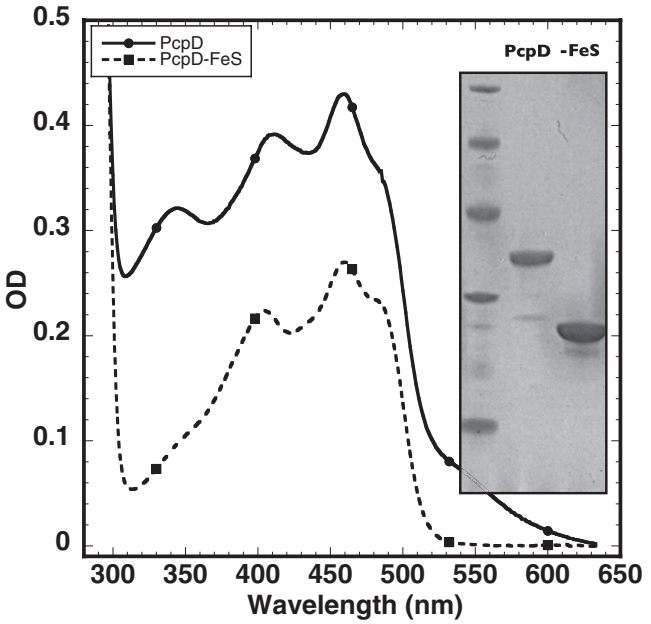

Fig. S3. Spectra of full-length PcpD and PcpD-FeS (25 μM). *Inset* is an SDS/PAGE of the purified enzymes: lane 1, molecular mass markers; lane 2, purified PcpD; lane 3, PcpD-FeS.

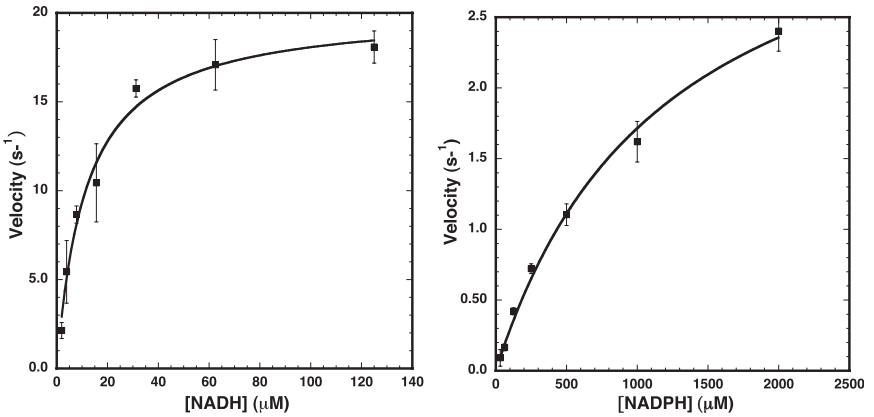

Fig. S4. PcpD uses NADH more efficiently than NADPH. Cytochrome *c* (90 μM) was mixed with 5 nM PcpD for the NADH reaction and 100 nM PcpD for the NADPH reaction. Reactions were started by the addition of the relevant nicotinamide cofactor, and the change in absorption at 550 was recorded. Data were converted to s^{-1} and plotted against NAD(P)H concentration. A fit of the data to the Michaelis-Menten equation gave K_m of 11.5 μM, k_{cat} of 20.1 s^{-1}, and k_{cat}/K_M of 1.74×10^6 $M^{-1}{\cdot}s^{-1}$ for NADH and K_M of 1.21 mM, k_{cat} of 3.7 s^{-1}, and a k_{cat}/K_M of 3.1×10^3 $M^{-1}{\cdot}s^{-1}$ for NADPH.

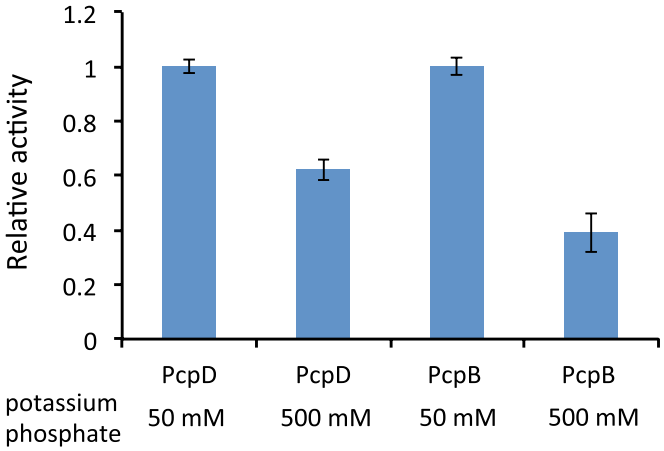

Fig. S5. The effect of salt on the activity of PcpB and PcpD. PcpB (0.75 μM) was assayed with PCP and NADPH (100 μM each), and the disappearance of NADPH was monitored at 340 nm. PcpD (1.25 nM) was assayed with cytochrome *c* (90 μM) and NADH (0.1 mM), and the reduction of cytochrome *c* was monitored at 550 nm.