Supplementary data

Biosynthesis of hydroxydiphenylacetylene by regiospecific monooxygenation

Heather R. Luckarift, Glenn R. Johnson and Jim C. Spain*
Air Force Research Laboratory, MLQL, 139 Barnes Drive, Suite # 2, Tyndall AFB, FL 32403-5323 Fax 850 283 6090; Tel: 850 283 6058; E-mail: jim.spain@tyndall.af.mil

Fig. S1; $^{13}$C and $^1$H NMR data for meta and para-hydroxydiphenylacetylene

The samples were dissolved in acetone–d$_6$. NMR spectra were recorded on a Varian Inova spectrometer equipped with a 5 mm indirect detection probe, operating at 500 MHz for $^1$H and at 125 MHz for $^{13}$C. Chemical shifts are reported in ppm relative to TMS. The $^1$H and $^{13}$C chemical shifts assignments presented below were based on the $^1$H-$^{13}$C one-bond and long-range correlations seen in the ghmbc (gradient heteronuclear multiple bond correlation) spectra. (Personal communication, Dr Ion Ghiviriga, University of Florida)
S2. Construction of expression plasmids pJS407 and pJS409

Plasmids pJS407 and pJS409 containing the genes for the toluene-2- and toluene-4-monoxygenases were derived from recombinant plasmid pRO2016 (Johnson and Olsen, 1997) (Fig. S2). The two oxygenase operons were subcloned into expression vector pSE380 (bla pMB1 oriColE1 pBR322) ptrc lac F' Invitrogen, Carlsbad, Calif, USA) using stepwise approaches. The native promoter regions of the operons were deleted by using polymerase chain reaction (PCR). Standard methods and conditions were followed for DNA manipulations and analysis and cell transformations (Ausbel et al., 1998).

**Fig. S2.** Restriction maps and summary of the derivation of pJS406 and pJS409. Arrows below pRO2016 map depict regions encoding the monoxygenases and their transcription direction. Asterisk indicates restriction sites added using PCR.

1. **pJS407** – The 3' end of the toluene-2-monoxygenase was subcloned into pSE380 as a 1.9-kb HindIII-NorI restriction fragment to yield pJS402 and then transferred to competent E. coli Top10F' (F' [lacIq, Tn10 (TetR)] mcrA Δ (mrr-hsdRMS-mcrBC) O80 lacZΔM15 ΔlacX74 deor recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (str8) endA1 nicG) by using electroporation. The 5' end of the operon was subcloned into pBlueScript KS+ (bla ColE1ori lacZ I strain origin) (Strategene, La Jolla, Calif, USA) by excising the 3.2-kb EcoRI-Xhol from pRO2016 and ligating to compatibly cleaved pBlueScript to form pJS401 which was transferred to E. coli Top10F'. The PCR was used to amplify a 550-bp portion of the toluene-2-monooxygenase operon extending from 33-bp upstream of the translational start site to point past the unique DraIII restriction recognition site within the operon. A BamHI restriction recognition site was engineered in the upstream primer to ease subsequent subcloning. PCR was done using pRO2016 as template DNA and oligonucleotides designated, tbmA+ (5' ATC GGA TCC GTG GCA AAC CCG ACC TCA ACA GC 3') and tnbmA (5' AAC TGC CCG ACG ATC CAC TCA G) as primers. The PCR product (PCR-1, Fig. S2) was treated with DraIII then ligated to the 4.0-kb NorI-BamHI fragment of the pJS401 DNA insert. The ligation product was subsequently treated with BamHI and NorI, ligated to compatibly-cleaved pBlueScript to form pJS402, and then transferred to competent E. coli Top10F'. Finally, to reform a functional operon encoding the toluene-2-monoxygenase, the 2.8-kb NorI-BamHI insert from pJS406 was excised and ligated to compatibly cleaved pJS402 to yield recombinant plasmid pJS407, which was transferred to competent E. coli Top10F'. Nucleotide sequence determinations were done to confirm that the intended construct was obtained.

2. **pJS409** – The 3' end of the toluene-4-monoxygenase operon was obtained as a 4.0-kb NorI-Xhol restriction fragment, subcloned into pBlueScript to form pJS403 and transferred to E. coli Top10F'. With the toluene-4-monoxygenase, the 5' end of the toluene-4-monoxygenase operon was obtained using PCR. Oligonucleotide primers were synthesized that annealed downstream from the NorI restriction site within the toluene-4-monoxygenase (tmbL- 5' AAT TAC CCC ATG ACG TAC TCA TCA G and an upstream region near the translation start site of the operon (tmbL+ 5' GCCGGA TCC AAA AAC ACT ACA GAC CCT ACC A 3'). Following the PCR using pRO2016 as template and tmbL+ and tmbL- as primers, the 0.8-kb product (PCR-2, Fig. S2) was gel purified, then treated with NorI. The NorI-cleaved PCR product was ligated to the 4.0-kb NorI-Xhol DNA insert from pJS403; treated with restriction enzymes BamHI and XhoI, and then ligated to compatibly cleaved pSE380 to yield recombinant plasmid pJS409, which was transferred to competent E. coli Top10F'. Again, nucleotide sequence determinations were done to confirm that the intended construct was obtained.