Pathway and Evolutionary Implications of Diphenylamine Biodegradation by Burkholderia sp. Strain JS667

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Diphenylamine (DPA) is a common contaminant at munitions-contaminated sites as well as at aniline manufacturing sites. Little is known about the biodegradation of the compound, and bacteria able to use DPA as the growth substrate have not been reported. Burkholderia sp. strain JS667 and Ralstonia sp. strain JS668 were isolated by selective enrichment from DPA-contaminated sediment. The isolates grew aerobically with DPA as the sole carbon, nitrogen, and energy source. During induction of DPA degradation, stoichiometric amounts of aniline accumulated and then disappeared, which suggested that aniline is on the DPA degradation pathway. Genes encoding the enzymes that catalyze the initial steps in DPA degradation were cloned from the genomic DNA of strain JS667. The Escherichia coli clone catalyzed stoichiometric transformation of DPA to aniline and catechol. Transposon mutagenesis, the sequence similarity of putative open reading frames to those of well-characterized dioxygenases, and 18O₂ experiments support the conclusion that the initial reaction in DPA degradation is catalyzed by a multicomponent ring-hydroxylating dioxygenase. DPA is converted to aniline and catechol via dioxygenation at the L2 position of the aromatic ring and spontaneous rearomatization. Aniline and catechol are further biodegraded by the well-established aniline degradation pathway. Genes that encode the complete aniline degradation pathway were found 12 kb downstream of the genes that encode the initial dioxygenase. Expression of the relevant dioxygenases was confirmed by reverse transcription-PCR analysis. Both the sequence similarity and the gene organization suggest that the DPA degradation pathway evolved recently by the recruitment of two gene clusters that encode the DPA dioxygenase and aniline degradation pathway.

We have isolated aerobic bacteria able to use DPA as the growth substrate through selective enrichment with samples from DPA-contaminated sites. Here we describe the degradation pathway of DPA and the genes that encode the enzymes involved. The understanding of the DPA degradation pathway will provide the basis to predict and enhance DPA biodegradation at contaminated sites.

(A preliminary report of this work was presented previously at the 107th General Meeting of the American Society for Microbiology [40].)

MATERIALS AND METHODS

Isolation and growth of DPA degraders. Samples from the surface of the sediment in a DPA-contaminated stream at the DuPont Repauno plant in Gibbstown, NJ, were suspended in nitrogen-free minimal medium (BLK) (6) containing DPA (100 μM). The culture was incubated under aerobic conditions at room temperature. When DPA disappeared from the culture, portions (10%, vol/vol) were transferred into BLK containing DPA crystals (0.85 g/liter). After several serial transfers, samples were spread on BLK agar (1.8%) plates containing DPA (500 μM). Individual colonies were transferred into 5 ml of BLK containing DPA (500 μM) as the carbon and nitrogen source. DPA concentrations in the culture fluids were measured by high-performance liquid chromatography (HPLC) at appropriate intervals. Isolated DPA degraders were routinely grown in BLK liquid medium or agar plates containing DPA (1 mM).

Analytical methods. DPA and its degradation intermediates were separated by paired ion chromatography on a Merck Chromolith RP18e column (4.6 mm by 100 mm) with a Varian HPLC system equipped with photodiode array detector. The mobile phase consisted of part A (5 mM paired ion chromatography low-UV reagent A [Waters, MA] in 30% methanol–70% water) and part B (70% methanol–30% water). The flow rate was 3 ml/min. The mobile phase was changed from 100% part A to 100% part B over a 2-min period and then held at 100% part B for 2 min. DPA, aniline, and catechol were monitored at 280, 230, and 275 nm, respectively. Alternatively, catechol was analyzed on a Phenomenex Synergi Polar-RP column (4 μm; 2.0 mm by 150 mm) with an isotropic mobile phase composed of 10% methanol–90% water (23). The flow rate was 1.5 ml/min. Aniline, catechol, and DPA were also analyzed by gas chromatography mass
spectrum (GC/MS) (23). The compounds were separated on an Equity-1701 capillary column (30 m by 0.25 mm; 0.25-μm film thickness; Supelco, PA). Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The chromatography program was as follows: initial column temperature of 55°C for 1 min, temperature increase of 20°C/min to 280°C, and isothermal for 5 min. Protein was measured with a Pierce (Rockford, IL) bicinchoninic acid protein assay reagent kit.

**Respirometry.** Cells grown on DPA were harvested by centrifugation, washed with potassium phosphate buffer (pH 7.2; 20 mM), and suspended in the same buffer. Oxygen uptake was measured polarographically at 25°C with a Clark-type oxygen electrode connected to a YSI model 5300 biological oxygen monitor (29).

**Enzyme essays.** Cells were harvested by centrifugation, washed with potassium phosphate buffer (pH 7.2; 20 mM), and broken by two passages through a French press. Succinate-grown cells served as negative controls.

**Plasmids.**

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**PCR primers**

- **DPADO-F**: PCR amplification of 218-bp fragment of *dpaAa*; ATACGAAGTGCTACGTGCC
- **DPADO-R**: PCR amplification of 218-bp fragment of *dpaAa*; TCTCATTGATCCAGCCACGC
- **ANDO-F**: PCR amplification of 205-bp of *tdnAa*; ACATCTTTCAACCTGGCACTGA
- **ANDO-R**: PCR amplification of 205-bp of *tdnAa*; CATTCTCGGAATTGGCTGCT
- **CatDO-F**: PCR amplification of 164-bp fragment of *tdnC*; GGGTGTTGATGCTG
- **CatDO-R**: PCR amplification of 164-bp fragment of *tdnC*; ACAGGATCAGCGAATCT
- **Primer5F-R**: PCR amplification of 370-bp fragment of *tdnQ*; GGCGTGATGCTG
- **Primer4F-R**: PCR amplification of 370-bp fragment of *tdnQ*; AAAGGTTGTTCCATCT
- **TdnQ-F**: PCR amplification of 5-kbp fragment of *tdnQ*: ATTGGACAGGATGT
- **TdnR-R**: PCR amplification of 5-kbp fragment of *tdnQ*: TCCATACGGTGATT

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**Bacterial identification.** Total DNA from DPA-grown cells was randomly sheared by vortexing. DNA sequences (600 bp) were compared with the sequences in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) using BLAST.

**Gene library construction and screening.** A recombinant fosmid library of DNA from *Burkholderia* sp. strain JS667 was created according to the manufacturer’s directions (CopyControl fosmid library production kit; Epicentre Biotechnologies, WI). Total DNA from DPA-grown cells was randomly sheared by vortexing. DNA fragments were ligated into the fosmid vector pCC1FOS. Fosmids harboring 40-kb DNA fragments were transfected into *Escherichia coli* strain EPI300. Approximately 2,000 clones of the *E. coli* recombinant library were spread on LB agar plates containing chloramphenicol (12.5 μg/ml). For preliminary screening an ether solution of catechol (0.1%) was sprayed onto colonies on plates to screen for meta-cleavage of catechol (21). Several presumptive catechol dioxygenase clones were selected for further characterization based on the formation of a faint yellow color. For confirmation of phenotypes the clones were grown in LB containing chloramphenicol (12.5 μg/ml) and fosmid induction solution (Epicentre), harvested by centrifugation, washed, and suspended in phosphate buffer (20 mM; pH 7.2). Yellow color formation from catechol was tested visually and the ability to transform DPA was monitored by HPLC analysis of the culture fluid. The clone designated pJS702 had the ability to transform either catechol or DPA (Table 1). Clone pJS701 transformed DPA but not catechol.

**Generation of transposon mutants.** Fosmid pJS702 was purified from recombinant *E. coli* strain EPI300 using the FosmidMAX DNA purification kit (Epicentre Biotechnologies, WI) and then randomly mutated in vitro with a modified mini-Tn5 transposon carrying the kanamycin resistance cassette (Ez-Tn5<KAN-2>) according to the manufacturer’s directions (Ez-Tn5<KAN-2> insertion kit; Epicentre Biotechnologies, WI). The resulting fosmids were then reintroduced into *E. coli* strain EPI300 by electroporation. Transposon insertion mutants were selected by growth on LB medium supplemented with chloramphenicol (20 μg/ml) and kanamycin (25 μg/ml). The kanamycin-resistant transposon mutants were then screened as above for the ability to transform DPA or catechol. Clones pJS702 and pJS7022 are transposon insertion mutant of pJS702 (Table 1).

**DNA sequencing and sequence analysis.** The fosmids that lost the ability to transform DPA or catechol due to transposon insertion were sequenced using Ez-Tn5<KAN-2> specific outward-reading primers by the Nevada Genomics Center (Reno, NV). The flanking regions were sequenced by primer walking (19). The resulting fosmids were then reintroduced into *E. coli* strain EPI300 by electroporation. Transposon insertion mutants were selected by growth on LB medium supplemented with chloramphenicol (20 μg/ml) and kanamycin (25 μg/ml). The kanamycin-resistant transposon mutants were then screened as above for the ability to transform DPA or catechol. Clones pJS702 and pJS7022 are transposon insertion mutant of pJS702 (Table 1).

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sequence alignments were done using ClustalW (46) and phylogenetic analysis was performed by using the neighbor-joining algorithm found in BioEdit. Phylogenetic trees were drawn using the TreeView program (33).

**Biotransformation of substrates by fosmid clones.** Cells were incubated in 50 ml of LB (12.5 µg/ml chloramphenicol) at 25°C. When the optical density at 600 nm (OD<sub>600</sub>) reached 0.9, fosmid induction solution (Epicentre) was added to the cultures and they were incubated for another 4 h. Cells were harvested by centrifugation, washed twice with sterile BLK medium, and suspended to an OD<sub>600</sub> of 6 in BLK medium. The cell suspensions (1 ml) were transferred to individual test tubes. The reactions were initiated by the addition of substrates and suspensions were incubated at 25°C with shaking at 250 rpm. At appropriate intervals samples were mixed with equal volumes of acidified acetone (pH 1.5), centrifuged at 16,100 × g for 1 min, and analyzed by HPLC. Cloned carbazole dioxygenase from *Pseudomonas* sp. strain CA10 in intact cells of *E. coli* was tested for transformation of DPA analogues as above. Cells were grown and induced as described previously (37).

**18O<sub>2</sub> incorporation.** Cells of *E. coli* EPI300 pJS7021 (DPA- catechol-) were prepared as indicated above. The cell suspensions were transferred to a 50-ml round-bottom flask and incubated with DPA (400 µM) in the presence of 18O<sub>2</sub> as previously described (43). After 2 h, the metabolites were extracted from the culture fluid and analyzed by GC/MS.

**Total RNA extraction and RT-PCR.** Total RNA was isolated from DPA- or succinate-grown cells at mid-exponential phase (SV total RNA isolation system; Promega, WI). cDNA was synthesized from total RNA (340 ng) with High Capacity cDNA reverse transcription kits; Applied Biosystems, CA). Samples of the reverse transcription (RT) reaction mixtures (1 µl) were subjected to PCR amplification by the primer pairs DPADO-F and DPADO-R, ANDO-F and ANDO-R, and CatDO-F and CatDO-R (Table 1). The 30 cycles of amplification were carried out as follows: 95°C for 1 min, 56.5°C for 30 s, and 72°C for 30 s, after initial denaturation at 95°C for 10 min. The predicted sizes of the PCR products were 218, 205, and 164 bp, respectively.

**Results**

**Isolation of DPA degraders.** From the DPA enrichment culture, we isolated *Burkholderia* sp. strain JS667 and *Ralstonia* sp. strain JS668, which are able to use DPA as the sole carbon, nitrogen, and energy source. Both strains can utilize aniline, anthrancate, and catechol but not carbazole as growth substrates. DPA was completely biodegraded (Fig. 1) and no UV-absorbing products were detected in the culture fluid by HPLC. The growth was slow (about 15-h doubling time). HPLC analysis indicated that no UV-absorbing intermediates of DPA accumulated in actively growing cultures. A typical DPA degradation rate was 39 nmol DPA/mg of protein/min during exponential growth (Fig. 1). The growth yield was 0.23 mg of total protein per mg of DPA, which is similar to those of the dibeno-p-dioxin degrader *Sphingomonas* sp. strain RW1 (52) and the biphenyl degrader *Burkholderia xenovorans* LB400 (34). The capabilities of JS667 and JS668 to grow on DPA were relatively stable. After seven transfers in 0.75% (wt/vol) tryptic soy broth, small portions of JS667 (20.8%) and JS668 (5.5%) lost the ability to degrade DPA.

**DPA degradation kinetics during induction.** When succinate-grown cells of JS667 were transferred to medium containing DPA, substantial amounts of aniline accumulated and then disappeared (Fig. 2). Transient accumulation of aniline suggested strongly that it is an intermediate of DPA degradation. The lack of an induction period before DPA disappearance and aniline accumulation in the first phase indicate that the initial enzymes of the pathway are at least partially constitutive, whereas enzymes that catalyze aniline degradation are inducible.

Small amounts of an unknown intermediate were also detected by HPLC. GC/MS analysis of the unknown compound extracted from the culture fluids after growth of JS667 on 1 mM DPA revealed a characteristic mass fragment [M<sup>+</sup>] at m/z 185 with major fragment ions at m/z 168, 156, and 139, which are similar to those of 3- and 4-hydroxydiphenylamines (36). The unknown compound was tentatively identified as 2-hydroxydiphenylamine based on the difference in HPLC retention time and UV spectrum from those of 3- and 4-hydroxydiphenylamines. The compound might be generated non-enzymatically by dehydration of a dihydrodiol intermediate as a consequence of its structural instability (41) or enzymatically by a monooxygenase mechanism (15). Similar results were obtained when the above experiments were repeated with strain JS668, which suggests that the two strains employ a similar DPA degradation pathway (data not shown). Further
TABLE 2. Oxygen uptake by DPA-grown versus succinate-grown cells

<table>
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<th>Substrate</th>
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<tr>
<td></td>
<td>DPA</td>
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<tr>
<td>DPA</td>
<td>81 ± 21</td>
</tr>
<tr>
<td>Aniline</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>Catechol</td>
<td>351 ± 5</td>
</tr>
<tr>
<td>2-Hydroxy-DPA</td>
<td>48 ± 12</td>
</tr>
<tr>
<td>3-Hydroxy-DPA</td>
<td>63 ± 9</td>
</tr>
<tr>
<td>4-Hydroxy-DPA</td>
<td>15 ± 2</td>
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*a Reaction mixtures contained substrate (20 μM), cells (0.14 mg of protein), and air-saturated phosphate buffer (20 mM, pH 7.2) to a final volume of 1.85 ml. Data represent means of at least two experiments. 2-Nitrodiphenylamine, 4-nitrodiphenylamine, N-nitrosodiphenylamine, biphenyl, toluene, and phenol did not stimulate oxygen uptake.

studies to determine the DPA biodegradation pathway were carried out with strain JS667.

Oxygen uptake rates. Catechol, DPA, monohydroxylated DPA isomers, and aniline stimulated immediate and rapid oxygen uptake by DPA-grown cells (Table 2). The results suggest that enzymes involved in DPA degradation are induced during growth on DPA and that some of the above compounds are on the degradation pathway. Slight stimulation of oxygen uptake by DPA in succinate-grown cells supports the observation that the initial enzymes of the pathway have moderate constitutive activities. The oxidation of aniline and catechol required 3.1 ± 0.28 and 1.85 ± 0.13 mol of O₂ per mol of substrate, respectively. The result is consistent with conversion of aniline to catechol by a dioxygenase (30).

Enzyme assays. Catechol was oxidized by a catechol 2,3-dioxygenase detected in extracts prepared from cells of JS667 grown on DPA. The specific activity for catechol 2,3-dioxygenase was 75.5 ± 0.2 nmol/min/mg of protein when the cell extracts were incubated for 10 min at 40°C before the assay. Catechol 1,2-dioxygenase and 2,3-dihydroxybiphenyl-1,2-dioxygenase activities were not detectable in the cell extracts.

Cloning and in silico analysis of the genes involved in DPA degradation. In order to determine the initial reaction in the DPA degradation pathway, a fosmid library of total genomic DNA from strain JS667 was constructed and transposon mutagenesis was carried out with pJS702 (DPA⁺ catechol⁺). Some clones lost the ability to transform DPA or catechol and the regions flanking the insertions were sequenced via primer walking outward from the transposon. When the transposon was inserted in open reading frame 4 (ORF4) or ORF7 the clones lost the ability to transform DPA (Fig. 3A). Transposon insertion in ORF29 abolished the catechol dioxygenase activity.

The remainder of the genes that encode the DPA degradation pathway were identified by primer walking and PCR amplification (Fig. 3A). The nucleotide sequences of ORFs 1 to 31, which encode DPA and catechol dioxygenases, were identified by primer walking in pJS702. ORF31 was identical to the regulator (tdnR) in aniline dioxygenase (24) and was located at the end of pJS702. The nucleotide sequences of ORFs 32 to 36 were obtained by PCR amplification from the genomic DNA of JS667. Primers were designed based on ORF31 and the conserved region in the glutamine synthetase-like gene (tdnQ) of aniline dioxygenase (Table 1) (48).

The assembled sequence formed a 30,579-bp segment of DNA. The segment contained 35 complete ORFs and one partial ORF (ORF36) (Fig. 3A). Variability of G+C content and nucleotide sequence similarities reveal the patchwork-like structure of the DNA segment. The abrupt increase in G+C content downstream of ORF18 suggests that the DNA fragment was assembled from at least two different origins. The presence of several transposon remnants (ORFs 11 to 12 and 15 to 18) is further support for the hypothesis. On the other hand, the nucleotide sequences in ORFs 12 to 14 (2.4 kb) and in ORFs 22 to 29 (6.8 kb) were 94% and 90% identical to the homologous sequences in R. eutropha JMP134 and those of the hypothetical catechol degradative operon of Burkholderia sp. strain 383, respectively. ORFs 15, 18 to 21, and 30 to 36 were approximately 90% identical to nucleotide sequences in the genes that encode aniline degradation in Delftia tsuruhatensis AD9 (24) and Pseudomonas putida UCC22 (11). It appears that ORFs 15 and 18 were divided by the insertion of ORFs 16 and 17, insertion elements first reported in Burkholderia multivorans (32). The results suggest that the gene cluster that encodes the DPA degradation pathway was assembled by horizontal gene transfer.

The deduced amino acid sequence of ORF4 showed 67% identity to CarAaI, the terminal oxygenase component of carbazole 1,9a-dioxygenase from Sphingomonas sp. strain KA1 (Table 3). The consensus sequence for the binding of a [2Fe-2S] cluster in Rieske-type iron-sulfur proteins (35) and the mononuclear iron-binding residues (18) were conserved in ORF4 in the pattern CX₉₋₁₈CX₂₋₃H from the 70th amino acid and GX₉AXHX₉H from the 179th amino acid, respectively. The deduced amino acid sequences indicate that ORF7 and ORF8 are related to the ferredoxin reductase from Phenylolobacterium zucutenum HLK1 (45% identity) and the ferredoxin from Sphingomonas wittichii RW1 (49% identity), respectively. Conserved motifs for FAD-binding and NADH-binding sites (4) are found in ORF7. ORF8 contains four cysteine residues in the pattern CX₉CX₂CX₉₋₁₃C from the 40th amino acid, typical of putidaredoxin-type [2Fe-2S] cluster ligands (3). ORF4, ORF7, and ORF8 have been therefore designated dpaAa, dpaAd, and dpaAc, respectively.

Biotransformation of DPA by the fosmid clones. In order to determine the initial reaction in the DPA degradation pathway, cells of E. coli EPI300 harboring pJS7021 or pJS702 were tested for the ability to transform DPA. When DPA was transformed by E. coli EPI300 pJS7021 (DPA⁺ catechol⁺), stoichiometric amounts of catechol and aniline accumulated (Fig. 4). Similar results (not shown) were obtained with pJS701, which has the same phenotype as pJS701. When the experiment was repeated with E. coli EPI300 pJS702 (DPA⁺ catechol⁻), only aniline accumulated (data not shown). The results clearly indicate that DPA is initially transformed to catechol and aniline, and then catechol is degraded without accumulation in pJS702. The results are consistent with our failure to detect catechol in the medium of DPA-degrading cultures.

Incorporation of 18O₂ into catechol. The reaction mechanism of DPA dioxygenase was rigorously determined by measuring ¹⁸O₂/¹⁶O₂ incorporation. Cells of E. coli EPI300 pJS7021 (DPA⁺ catechol⁻) were incubated as above with DPA in an atmosphere that contained ¹⁸O₂ (56%) and ¹⁶O₂.
At the end of the incubation period, the catechol that accumulated was a mixture of $^{18}$O-$^{18}$O (52%) and $^{16}$O-$^{16}$O (48%) with no detectable $^{16}$O-$^{18}$O catechol (Fig. 5). The results clearly indicate that the initial enzyme functions as a dioxygenase by the incorporation of one molecule of oxygen into DPA to form an unstable intermediate that is converted to catechol and aniline.

**Substrate specificities.** The substrate specificity of the DPA dioxygenase encoded on pJS701 (DPA$^+/H_11001$ catechol$^+/H_11002$) was compared with that of carbazole dioxygenase from *Pseudomonas* sp. strain CA10. The lack of transformation of DPA by carbazole dioxygenase and the preference of DPA dioxygenase for DPA indicates that DPA dioxygenase is specialized for DPA (Table 4). In a separate experiment, cells expressing DPA dioxygenase transformed 3-hydroxydiphenylamine and 4-hydroxydiphenylamine at 27% and 30% of the rate of DPA transformation, respectively, which accounts for the stimulation of oxygen uptake by the two compounds (Table 2). The products of the transformation were not identified.

**RT-PCR amplification.** The results from RT-PCR clearly indicated that DPA, aniline, and catechol dioxygenases are expressed in JS667 during growth on DPA (Fig. 6). The am-
amplification of dpaAa in succinate-grown cells of JS667 further supports the moderate constitutive expression of DPA dioxygenase. The tdnC encoding catechol 2,3-dioxygenase was not transcribed in succinate-grown cells of JS667, which does not correspond to the moderate constitutive activities of catechol dioxygenase (Fig. 2 and Table 2). The presence of an additional catechol 2,3-dioxygenase encoded outside the region studied here would explain the observation (45). Negative results on PCR

### DISCUSSION

Based on the stoichiometric aniline production and by analogy with related pathways, there are two possible DPA degra-
dation pathways (Fig. 7). Attack by a dioxygenase at the 1,2 position would lead to the spontaneous conversion of the resulting compound to aniline and catechol (Fig. 7A). The initial reaction would be analogous to those catalyzed by aniline and diphenyl ether-, dibenzofuran-, and carbazole dioxygenases (2, 12, 37, 39). Alternatively, DPA dioxygenase could initially catalyze attack at the 2,3 position of the aromatic ring. The resulting cis-dihydrodiol intermediate would be converted to aniline and 2-hydroxymuconate via dehydrogenation, meta ring cleavage, and hydrolysis (Fig. 7B). The previous observation (41) that a modified biphenyl dioxygenase transforms DPA to the corresponding 2,3-dihydrodiol is consistent with pathway B. A binding model (13) suggests how biphenyl dioxygenase might acquire the ability to oxidize DPA at the 2,3-position. The results with DPA dioxygenase (Fig. 4) indicate clearly, however, that DPA is initially converted to aniline and catechol (pathway A), which are then completely degraded via the common aniline degradation pathway (Fig. 7C).

Detection of hydroxydiphenylamine in culture supernatants and stimulation of oxygen uptake in DPA-grown cells by hydroxydiphenylamine and 2-hydroxymuconate via dehydrogenation, meta ring cleavage, and hydrolysis (Fig. 7B). The previous observation (41) that a modified biphenyl dioxygenase transforms DPA to the corresponding 2,3-dihydrodiol is consistent with pathway B. A binding model (13) suggests how biphenyl dioxygenase might acquire the ability to oxidize DPA at the 2,3-position. The results with DPA dioxygenase (Fig. 4) indicate clearly, however, that DPA is initially converted to aniline and catechol (pathway A), which are then completely degraded via the common aniline degradation pathway (Fig. 7C).
droxydiphenylamine could be interpreted as evidence for sequential monooxygenase reactions or decomposition of a dihydrodiol intermediate. The $^{18}$O$_2$ experiment results, however, preclude the possibility that sequential monooxygenations play a significant role in the degradation pathway. 2-Hydroxydiphenylamine appears to be a minor side product. The role and extent of participation of the hydroxydiphenylamines are currently under investigation.

The DPA dioxygenase system, comprising a terminal dioxygenase component ($dpaAa$), ferredoxin reductase ($dpaAd$), and ferredoxin ($dpaAc$), is not readily classified by the previous classification systems for Rieske nonheme iron oxygenases that hydroxylate aromatic rings (22, 28). $dpaAa$ is phylogenetically related to $carAa$ of carbazole 1,9α-dioxygenases that catalyze attack at the angular position of carbazole (Fig. 8). The oxygenase components listed in Fig. 8 have a single subunit and they form a clade distinct from the multicomponent dioxygenases composed of large ($α$) and small ($β$) oxygenase subunits, including aromatic acid dioxygenases, benzenoid dioxygenases, and naphthalene dioxygenases (28). The ferredoxin and ferredoxin reductase components of the electron transfer system of DPA dioxygenase ($dpaAdAc$) are closely related to those of type IV dioxygenases, including dioxin dioxygenase (Sphingomonas sp. strain RW1), biphe-

![Diagram](http://aem.asm.org/)

**FIG. 6.** RT-PCR amplifications of the genes that encode DPA (A), aniline (B), and catechol (C) dioxygenases from Burkholderia sp. strain JS667 grown on DPA (1) or succinate (2). RT-PCRs without reverse transcriptase served as negative controls (3). M lanes contain the molecular size marker (Quick-Load 100-bp DNA ladder; New England Biolabs, Ipswich, MA).

**FIG. 7.** Alternative DPA degradation pathways (A and B) and the subsequent aniline/catechol degradation pathway (C). (Pathways A and C are supported by results of this study.) The X indicates that the hydrolytic branch in the meta-cleavage pathway is not predicted in JS667.
3-aminophenyl-2,3-diol to anthranilate and 2-hydroxypenta-2,4-dienoate. The hydrolysis is not necessary in pathway A (Fig. 7). The differences raise questions about how the two dioxygenase systems divergently evolved from a common ancestor. The presence of three vestigial genes (ORFs 3, 9, and 10) and transposon remnants suggests relatively recent evolution of the DPA dioxygenase system in JS667.

ORF5 and ORF6 showed substantial amino acid identities to CarBa (57%) and CarBb (54%), the two subunits of the meta-cleavage enzyme from Sphingomonas sp. strain KA1. CarB has about 20 times higher activity for 2,3-dihydroxybiphenyl than for catechol (38). The size of the product of ORF6, however, is about one-third the size of CarBb (catalytic subunit) from carbazole degraders. Approximately 170 amino acids in the N-terminal region of CarBb, including the active site histidine residues, are missing in ORF6 (44). This observation explains the absence of 2,3-dihydroxybiphenyl-1,2-dioxygenase activity in extracts prepared from cells of JS667. The truncation of the meta-cleavage enzyme in JS667 might help to avoid misrouting of the metabolites produced from DPA or structurally similar compounds. The inactivation of an extraneous meta-cleavage gene was previously proposed as a step in the evolution of chlorobenzene-degrading bacteria, where it seems to prevent misrouting of chlorocatechol (27, 51). The presence of the truncated CarBb that functions in carbazole degradation, but not in DPA degradation, provides strong evidence for the origin of the gene cluster that encodes DPA dioxygenase. The argument is analogous to the truncated salicylate hydroxylase that revealed the evolutionary origins of the nitroarene dioxygenase in 2,4-dinitrotoluene-degrading bacteria (10, 19).

Constitutive expression of DPA dioxygenase in JS667 is reminiscent of the expression of carbazole dioxygenases (26). In that system two different promoters located upstream of the gene that encodes the terminal dioxygenase component cause constitutive and inducible expression of the car operon. The regulatory protein (CarR) negatively controls the inducible expression. 2-Hydroxy-6-oxo-6-(2-aminophenyl)hexa-2,4-dienoate, an intermediate in carbazole degradation, functions as the inducer of the expression of the car operon (26). The inducer is not an intermediate in DPA biodegradation. The presence of a carR-like gene (ORF2) and two different putative promoter sequences 204 and 323 bp upstream of dpaAa suggests a similar regulation pattern in JS667. The promoter sequences, however, seem unrelated to those of the car operon, perhaps due to the different evolutionary history of the two dioxygenase systems. ORF1 belongs to the AraC/XylS family of transcriptional regulators that function as transcriptional activators in catabolic operons (47). We are currently investigating how the two different regulatory proteins are involved in the expression of DPA dioxygenase.

Genes that encode the aniline degradation pathway in JS667 are compactly organized without extraneous elements (Fig. 3C and Table 3), which suggests that evolution of the aniline degradative operon preceded recruitment of the gene cluster.
that encodes DPA dioxygenase. Gene duplication (tdnCD1 and tdnCD2) and unknown ORFs between the duplications are present in the other aniline degraders, strains AD9 and UCC22, but not in JS667. The extraneous elements are considered evolutionary remnants resulting from the recombination of two different gene clusters that encode aniline dioxygenase and the meta pathway enzymes (11). Highly identical unknown ORFs 15 and 18 to 21 among the aniline degraders suggest that similar evolutionary processes have been occurring during the development of the aniline degradative operon and that some of the unknown ORFs perform an important function. On the other hand, the organization of genes that encode the meta-cleavage pathway in JS667 is identical to that of the hypothetical phenol degradative operon in Burkholderia sp. strain 383, the phylogenetically closest relative to JS667 in the database. The only difference is that the gene (tdnF) that encodes 2-hydroxyxymucronic semialdehyde hydrolase is absent in JS667, which suggests that catechol is degraded via the 4-oxoacrotarone branch in JS667 (Fig. 7C) (1, 16).

The recruitment of the genes encoding DPA dioxygenase would be sufficient to allow the relatively common aniline-degrading bacteria to grow with DPA as a sole source of carbon, nitrogen, and energy. The hypothesis is supported by several lines of evidence: the significant differences in G+C content between the gene clusters that encode DPA dioxygenase and the aniline degradation pathway, several transposon remnants (ORFs 11, 12, and 15 to 18) between the gene clusters, and the relatively well-organized aniline degradative operon without superfluous genetic material compared to the genes that encode DPA dioxygenase. The immature organization seems to contradict the expectation that the genes encoding the degradation pathways of naturally occurring compounds are often linked in operons without superfluous genetic material. It seems reasonable to speculate that the system was assembled recently in response to contamination by DPA. Such a scenario was strongly supported for assembly of a chlorobenzene degradation pathway in response to chlorobenzene contamination (27, 49). We are currently isolating new DPA degraders to determine their distribution in the environment and the organization of the genes that encode DPA degradation, which will provide additional insight about the evolution of the DPA degradation pathway.

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