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Degradation of Benzene by *Pseudomonas veronii* 1YdBTEX2 and 1YB2 Is Catalyzed by Enzymes Encoded in Distinct Catabolism Gene Clusters

Daiana de Lima-Morales,* Diego Chaves-Moreno, Melissa L. Wos-Oxley, Ruy Jáuregui,* Ramiro Vilchez-Vargas,* Dietmar H. Pieper

Microbial Interactions and Processes Research Group, Helmholtz Centre for Infection Research, Braunschweig, Germany

Pseudomonas veronii 1YdBTEX2, a benzene and toluene degrader, and *Pseudomonas veronii* 1YB2, a benzene degrader, have previously been shown to be key players in a benzene-contaminated site. These strains harbor unique catabolic pathways for the degradation of benzene comprising a gene cluster encoding an isopropylbenzene dioxygenase where genes encoding downstream enzymes were interrupted by stop codons. Extradiol dioxygenases were recruited from gene clusters comprising genes encoding a 2-hydroxymuconic semialdehyde dehydrogenase necessary for benzene degradation but typically absent from isopropylbenzene dioxygenase-encoding gene clusters. The benzene dihydrodiol dehydrogenase-encoding gene was not clustered with any other aromatic degradation genes, and the encoded protein was only distantly related to dehydrogenases of aromatic degradation pathways. The involvement of the different gene clusters in the degradation pathways was suggested by real-time quantitative reverse transcription PCR.

Members of the *Pseudomonas* genus are highly versatile and known to colonize diverse habitats such as humans, animals, plants, water, and soil (1), and *Pseudomonas veronii* strains have been implicated in human pathogenicity (2), heavy metal biosorption (3), and dibenzo-*p*-dioxin, chloroaromatic, and alkyl methyl ketone degradation (4, 5). *P. veronii* strains were also pointed out as key catabolic players in a BTEX (benzene, toluene, ethylbenzene, xylenes)-contaminated area where benzene had been the major contaminant (6). Two strains, termed *P. veronii* 1YB2 and *P. veronii* 1YdBTEX2, were subjected to a detailed analysis, as they contained catabolism gene variants shown to be highly abundant at the site (6). Interestingly, both strains differed in their substrate specificities, and whereas *P. veronii* 1YdBTEX2 grows on benzene and toluene, strain 1YB2 grows on benzene only. The difference in substrate specificity was due to small sequence differences in the α-subunit of the initial multicomponent Rieske nonheme iron oxygenase responsible for the activation of the aromatic ring (7).

Even though benzene is the most simple aromatic compound, the biodegradation pathways used by bacteria to degrade this compound have been documented in detail in only a few cases (8). Typically, the degradation of benzene is supposed to occur in analogy to the pathways observed for toluene degradation and to proceed via either initial dioxygenation (9) or two subsequent initial monooxygenations (10). In the case of initial dioxygenation, enzymes involved in the degradation of benzene and toluene are expected to show the highest similarities to enzymes termed benzene/toluene dioxygenases (11, 12). However, *P. veronii* strains 1YdBTEX2 and1YB2 harbor dioxygenases most closely related to isopropylbenzene dioxygenases (7). In the well-characterized isopropylbenzene-degradative pathways of *Pseudomonas putida* RE204 and *Pseudomonas fluorescens* IP01 (13, 14), the genes coding for the oxygenase subunits are organized in operons together with genes encoding a *cis*-dihydrodiol dehydrogenase and an extradiol dioxygenase of subfamily I.3.A (15). However, *P. veronii* 1YB2 and 1YdBTEX2 do not express a subfamily I.3.A extradiol dioxygenase (EXDO K2) during growth on benzene but recruit a

subfamily I.2.A extradiol dioxygenase (catechol 2,3-dioxygenase [C23O]) (EXDO A) with high similarity to the archetype TOL plasmid-encoded enzyme (6, 16). To our knowledge, such a recruitment has not been reported previously for the degradation of benzene or toluene via a dioxygenolytic route. Since *P. veronii* 1YdBTEX2 and 1YB2 were indicated to be key catabolic players with unique pathway arrangements, we sequenced the genomes of both strains to elucidate the organization of the benzene and/or toluene catabolic pathways.

MATERIALS AND METHODS

Strains and culture conditions. *P. veronii* 1YB2 and *P. veronii* 1YdBTEX2 were cultured in mineral medium (17) containing 50 mM phosphate buffer (pH 7.4) supplemented with trace elements and growth substrates at concentrations of 2 mM. Benzene or toluene was supplied via the gas phase (2 to 5 mM each) (16), and cultures were incubated at room temperature without agitation.

Genome sequencing and assembly. Genomes were assembled as previously described (18). Briefly, both genomes were sequenced on an Illumina GAII genome analyzer generating 110-bp paired ends. Approxi-

mately 10 million short sequence reads were obtained for each genome. Whole-genome shotgun sequencing data were assembled by using Velvet (19) and Edena (20). Both data sets were then merged by using Minimus (21). In order to further diminish the number of gaps, scaffolds were constructed by using SSPACE (22), and comparisons between contigs and scaffolds were made by using MAUVE (23). Contigs were also compared to the *P. fluorescens* SBW25 genome (24). These strategies resulted in a possible assembly for a number of contigs. In order to confirm this information and to close gaps between contigs, primers framing these gaps were designed, and PCR products were sequenced by Sanger sequencing.

Phylogenetic comparisons. Nucleotide sequences of 16S rRNA genes and the DNA gyrase subunit B gene (*gyrB*) were aligned by using MUSCLE 3.7 (25). Phylogenetic trees were constructed with MEGA5 (26) by using the neighbor-joining (N-J) algorithm (27) with Jukes-Cantor correction and pairwise deletion of gaps and missing data. A total of 100 bootstrap replications were done to test for branch robustness.

The complete set of protein and coding sequences from *P. veronii* 1YdBTEX2 and 1YB2; *P. fluorescens* SBW25, A506, Pf0-1, and F113; *P. protegens* CHA0 and Pf-5; *P. brassicacearum* NFM421; and *P. aeruginosa* PAO1 were obtained from the NCBI ftp website (http://www.ncbi.nlm.nih.gov/Ftp). Comparisons between pairs of strains were performed independently at the protein and coding sequence levels through an in-house Perl script detecting the reciprocal best hit (RBH). For every RBH pair (proteins and coding sequences), the identity score was calculated considering the percent identity and the percent coverage of the query (alignment length × % identity/query length) (28, 29). If a sequence reported no RBH, the identity was scored as zero. Two data matrices containing the identity score for all protein or coding sequence comparisons among the 10 genomes were then generated (59,527 proteins). Similarity matrices were generated by using the Bray-Curtis coefficient (30), and dendrograms were created by group-average agglomerative hierarchical clustering. All statistical routines were carried out by using PRIMER 6 (v.6.1.6; Primer-E, Plymouth Marine Laboratory, United Kingdom) (31).

Partial purification and identification of benzene dihydrodiol dehydrogenase. Cells of *P. veronii* 1YdBTEX2 and *P. veronii* 1YB2 (100 ml of culture) were harvested and disrupted with a French press (Aminco, Silver Spring, MD). Benzene dihydrodiol dehydrogenase was partially purified by using a fast protein liquid chromatography system (Amersham Biosciences, Freiburg, Germany). Cell extracts (1 ml) containing 1.4 to 2.0 mg of protein were applied directly to the column, and proteins were eluted with a linear gradient of 0 to 0.5 M NaCl over 33 ml at a flow rate of 0.5 ml/min. Fraction volumes were 0.5 ml. The activity of benzene dihydrodiol dehydrogenase was determined spectrophotometrically by measuring the 3,5-cyclohexadiene-1,2-diol (benzene dihydrodiol) (0.1 mM)-dependent reduction of NAD$^+$ (0.1 mM) in Tris-HCl (100 mM) (pH 7.5) at 340 nm ($\varepsilon_{NADH} = 6{,}220$ M$^{-1} \cdot$ cm^{-1}). Protein concentrations were determined by the Bradford method (32).

Aliquots of active fractions were subjected to SDS-PAGE on a Bio-Rad Miniprotean II instrument as described previously (33), and major protein bands with a molecular mass of ~50 kDa obtained from *P. veronii* 1YdBTEX2 were analyzed by N-terminal amino acid sequencing performed with an Applied Biosystems model 494 A Procise HT sequencer, as previously described (34). Proteins from *P. veronii* 1YdBTEX2 and 1YB2 annotated by the RAST server (35) were searched for the presence of the determined N-terminal sequence.

Real-time quantitative PCR. Primers annealing inside the genes of interest and targeting genes encoding the α-subunit of the isopropylbenzene dioxygenase (ipbAfw [GCTGGTTCAATGATGACTTCTC] and ipbArev [GATGAAAGACCAGACTTCGAC]), benzene dihydrodiol dehydrogenase (DHfw [GTGATTTTCTGCTCGAAGCTG] and DHrev [ATCCCAGGTCATCGCCGTAC]), EXDO A (ExdoA-1fw [GTTGACAAATTCTCCGTGGT] and ExdoA-1rev [TCTGCATACAACTCGAAGTGA]), and EXDO A2 (ExdoA-2fw [GCGTGTACTCGATATGGACA] and ExdoA-2rev [TGGGTCAGCTGATACAGACA]) were designed. After PCR, fragments were cloned into the pGEM-T Easy vector (Promega). Vectors

with inserts were transformed into *Escherichia coli* JM109 cells, and positive clones were confirmed by PCR. The appropriate plasmids were purified by using the QIAprep Spin Miniprep kit (Qiagen, Germany).

For RNA preparation, strain 1YdBTEX2 was grown with benzene or toluene, strain 1YB2 was grown with benzene as the carbon source (inducing conditions), and both strains were grown on glucose (2 g/liter) as a control (noninducing conditions). RNA extractions were performed as previously described (36), and cDNA was prepared from 800 ng of RNA by using 0.3 μg of random primers and 400 U of Superscript III reverse transcriptase (RT) (Invitrogen, Life Technologies, Germany), according to the instructions of the providers.

The primers described above were used for the amplification of cDNA. Real-time PCRs were performed as previously described (37), using a LightCycler 480 real-time PCR system programmed for a hold at 95°C for 10 min and completion of 50 cycles of 94°C for 15 s, 56°C for 30 s, and 72°C for 30 s. Results were analyzed by using LightCycler 480 software (Roche Applied Science). Standard curves were generated, and a melting step was added to confirm amplification specificity.

Nucleotide sequence accession numbers. The genome sequences of *P. veronii* 1YdBTEX2 and 1YB2 have been deposited in GenBank under accession numbers AOUH00000000 (18) and JGYI00000000, respectively.

RESULTS

General characteristics and phylogenetic analyses of *P. veronii* 1YdBTEX2 and 1YB2. The *Pseudomonas* strain YdBTEX2 and 1YB2 genomes were sequenced, and ~9.5 million and 3.2 million reads were assembled, giving 63 and 115 contigs, respectively (18). The genomes contained 5,981 and 6,884 candidate protein-encoding genes, respectively, where >5,100 of the encoded proteins are shared between the strains (nucleotide sequence identity of ≥80%) (see Table S1 in the supplemental material for comparison with finished genomes of strains of the *P. fluorescens* group).

Comparison of the 16S rRNA gene (Fig. 1A) and a 798-base sequence stretch of the DNA gyrase subunit B-encoding gene *gyrB* (Fig. 1B) with the respective genes of *P. veronii* CIP104663^T, *P. fluorescens* IAM12022^T, and other genome-sequenced *P. fluorescens* group organisms (24, 38–42) as well as comparison of the similarity of total predicted proteins (see Fig. S1 in the supplemental material) support the notion that both 1YB2 and 1YdBTEX2 belong to the *P. veronii* species.

Genetic analysis of determinants of the benzene catabolic pathway. In previous studies, some of the isopropylbenzene dioxygenase-encoding genes (*ipb*) (7) and the genes encoding an EXDO A protein involved in the degradation of benzene by both strains (6, 16) were identified. Genome sequencing indicated the presence of a complete isopropylbenzene catabolism operon in both strains (GenBank accession numbers NZ_AOUH01000041 and NZ_JGYI01000039) (Fig. 2A), with a gene organization similar to the one found for plasmid pRE4 of *P. putida* RE204 (accession number AF006691) (14). The presence of putative transposases flanking the *ipb* operons in both 1YB2 and 1YdBTEX2 indicates that they were probably acquired via horizontal gene transfer. Curiously, the activity of the putatively encoded isopropylcatechol 2,3-dioxygenase (EXDO K2) has never been observed in any strain (16). In accordance with the absence of any activity, the extradiol dioxygenase-encoding gene of 1YB2 showed the presence of an internal stop codon probably resulting in a nonfunctional gene product. In 1YdBTEX2, the extradiol dioxygenase-encoding gene was obviously functional; however, sequence analysis revealed the presence of stop codons in both isopropylbenzene dihydrodiol dehydrogenase-encoding genes (*ipbB* genes)
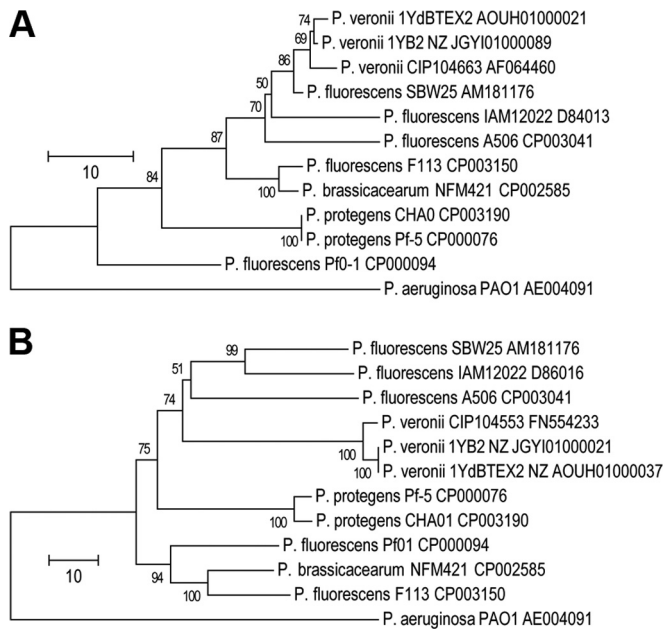
FIG 1 Phylogenetic relationships among *Pseudomonas fluorescens* group organisms. Phylogenetic trees are based on 1,513- to 1,515-base 16S rRNA gene fragments (A) and 798-base DNA gyrase subunit B gene (*gyrB*) fragments (B) of *P. aeruginosa* PAO1; *P. veronii* 1YdBTEX2, 1YB2, and CIP104663[T]; *P. fluorescens* A506, SBW25, F113, Pf0-1, and IAM12022[T]; *P. brassicacearum* NFM421; and *P. protegens* CHA01 and Pf-5. Phylogenetic trees were constructed with MEGA5 (26) using the N-J algorithm (27) with Jukes-Cantor correction and pairwise deletion of gaps and missing data. Bootstrap values of >50% from 100 neighbor-joining trees are indicated to the left of the nodes. The bar indicates nucleotide differences per site.

upstream of the extradiol dioxygenase-encoding gene, probably resulting in nonfunctional gene products in both strains.

Sequence comparisons show that in *P. veronii* 1YdBTEX2, EXDO A is encoded by a salicylate catabolism gene cluster (GenBank accession number NZ_AOUH01000059), which is recruited for benzene degradation. The presence of a complete salicylate catabolism gene cluster (*nahGTHINLOMKJ*) (Fig. 2) allows the strain to grow on this substrate. Typically, salicylate catabolism gene clusters are localized in the proximity of naphthalene catabolism gene clusters, as is the case in *P. putida* G7 (43), where both the *nahAaAbAcAdBCDEF* genes encoding enzymes for the transformation of naphthalene to salicylate and the genes responsible for salicylate oxidation are localized on plasmid NAH7 (Fig. 2B). However, there was no indication of the presence of a naphthalene operon in *P. veronii* 1YdBTEX2. In contrast, in *P. veronii* 1YB2, the EXDO A-encoding gene is localized near a truncated naphthalene gene cluster (Fig. 2B) composed of only the *nahAaAbBF* genes where the naphthalene dihydrodiol dehydrogenase-encoding gene (*nahB*) is interrupted (GenBank accession number NZ_JGYI01000001), which explains the failure of the strain to grow on naphthalene. The absence of a *nahG* gene encoding salicylate hydroxylase explains the failure of strain 1YB2 to grow on salicylate.

P. veronii 1YdBTEX2 harbors a second extradiol dioxygenase (EXDO A2), which exhibits 84% sequence identity with EXDO A and which is localized in a gene cluster comprising only genes for downstream processing of catechol ring cleavage products (GenBank accession number NZ_AOUH01000042) (Fig. 2). The

absence of any gene encoding enzymes for the formation of catechol may indicate the function of this operon in the processing of catechol derived from different sources by gene products encoded elsewhere on the genome.

Identification of the benzene dihydrodiol dehydrogenase recruited for benzene degradation. Evidently, no functional dihydrodiol dehydrogenase was observed in any of the above-mentioned gene clusters, and screening of the genomes did not reveal any open reading frame that could encode an enzyme with high similarity to those transforming isopropylbenzene dihydrodiol, naphthalene dihydrodiol, or benzene dihydrodiol in either strain. However, benzene-grown cells of *P. veronii* 1YdBTEX2 and 1YB2 exhibited benzene dihydrodiol dehydrogenase activities of 590 and 860 U/g protein, respectively. The benzene dihydrodiol dehydrogenases of *P. veronii* 1YdBTEX2 and 1YB2 were partially purified from benzene-grown cells by anion-exchange chromatography, and activities of both cell extracts eluted at an NaCl concentration of 0.38 ± 0.01 M. In each case, 32 to 40% of the applied activity was recovered, reaching specific activities of 4,800 U/g protein.

Proteins in the active fractions were subjected to N-terminal sequencing. Only one open reading frame of each genome (GenBank accession number WP_046487322 for 1YB2 and accession number WP_017849553 for 1YdBTEX2) matched the observed MQRFQNKVVLVTGAA N-terminal sequence. Exploring the gene organization, the benzene dihydrodiol dehydrogenase-encoding genes in both strains are localized in identical gene clusters, devoid of other genes encoding aromatic degradation enzymes (accession numbers NZ_AOUH01000056 and NZ_JGYI01000072). Importantly, the benzene dihydrodiol dehydrogenases of both 1YdBTEX2 and 1YB2 are only distantly related to previously described dehydrogenases involved in aromatic degradation pathways (Fig. 3).

Expression of genes of the benzene catabolic pathway. According to the data presented above, benzene degradation in both strains is most likely catalyzed by proteins of at least three catabolism gene clusters (see also Fig. S2 in the supplemental material). In addition, 1YdBTEX2 harbors two distinct extradiol dioxygenases of the EXDO A subfamily (EXDO A and EXDO A2). To analyze whether both dioxygenases were recruited and to determine whether all three gene clusters described above were transcribed in response to benzene and/or toluene, transcript levels of key genes of the above-mentioned gene clusters were quantified. This analysis comprised the quantification of expression of genes encoding the α-subunit of isopropylbenzene dioxygenase (*ipbAa*), the novel benzene dihydrodiol dehydrogenase, and EXDO A and EXDO A2 (1YdBTEX2 only) during growth on benzene (*P. veronii* 1YB2) and on benzene and toluene (*P. veronii* 1YdBTEX2) compared to transcript levels observed under noninducing conditions (growth on glucose).

The novel type of benzene dihydrodiol dehydrogenase was expressed constitutively in both strains with high transcript levels of 1.5×10^5 to 3.1×10^5 copies/ng cDNA (Fig. 4). EXDO A was also expressed constitutively but at levels of 4.6×10^3 to 10.3×10^3 copies/ng cDNA in the case of *P. veronii* 1YdBTEX2 and at a higher level of 5.9×10^3 to 20×10^3 copies/ng cDNA in the case of *P. veronii* 1YB2. In contrast, IpbAa was inducible in both strains, with transcript levels being increased by roughly 2 orders of magnitude after growth on benzene or toluene (1.5×10^5 to 2.5×10^5 copies/ng cDNA) compared to levels in glucose-grown cells
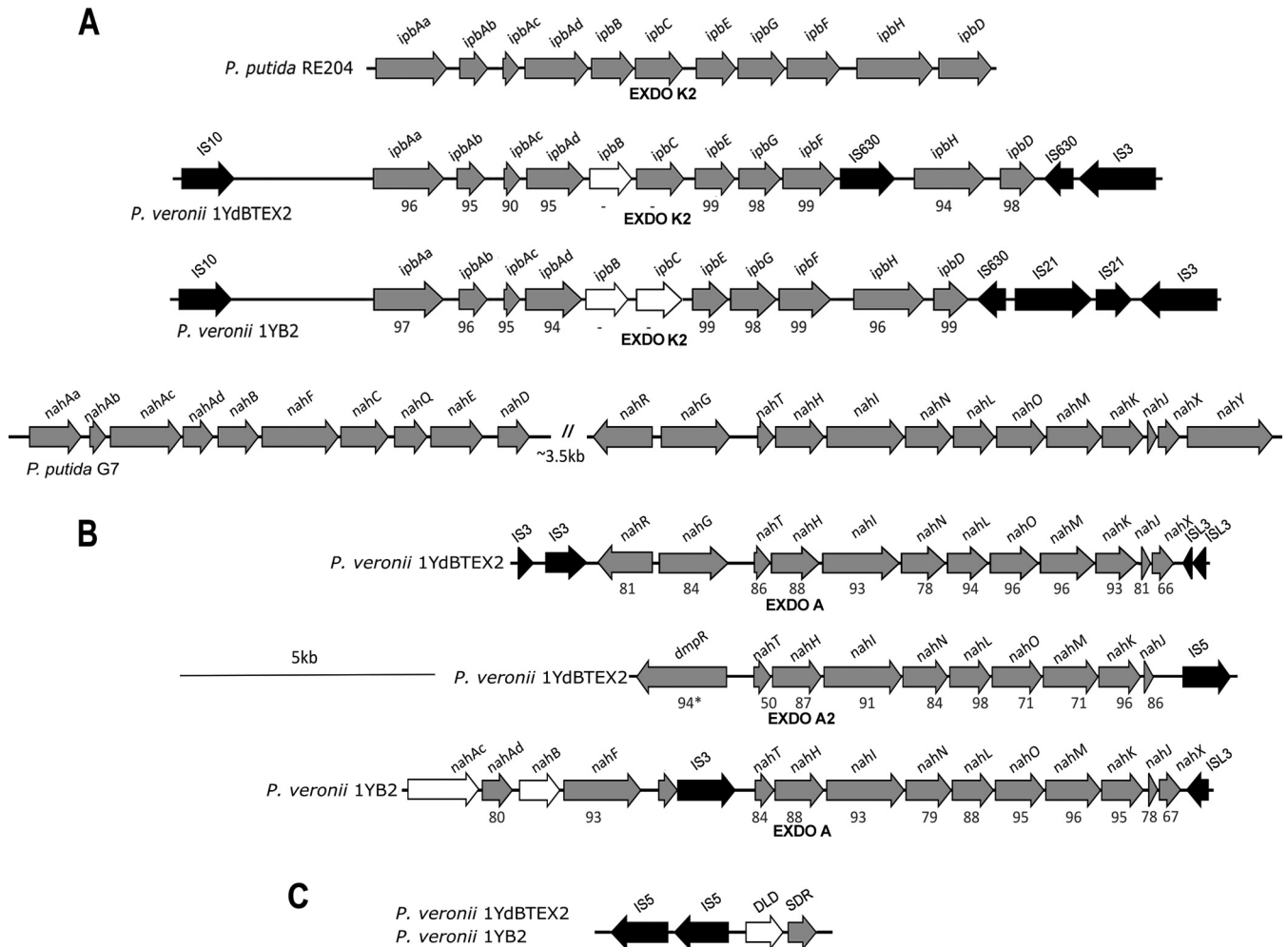
FIG 2 Organization of gene clusters involved in benzene degradation by *P. veronii* 1YdBTEX2 and 1YB2. Gene clusters of *P. putida* RE204 and *P. putida* G7 were used for comparison. (A) Isopropylbenzene gene clusters (*ipb*) of *P. putida* RE204 (14) (GenBank accession number AF006691; locus tags AAC03436 to AAC03446), *P. veronii* 1YdBTEX2 (accession number NZ_AOUH01000041; locus tags H736_RS0125995 to H736_RS0126080), and *P. veronii* 1YB2 (accession number NZ_JGYI01000039; locus tags Y055_RS13435 to Y055_RS13520). (B) Upper and lower naphthalene catabolism gene clusters (*nah*) located on plasmid Nah7 of *P. putida* G7 (accession number NC_007926; locus tags NAH7_34 to NAH7_56) (43), a gene cluster in *P. veronii* 1YdBTEX2 encoding enzymes for salicylate degradation (accession number NZ_AOUH01000059), a gene cluster in *P. veronii* 1YdBTEX2 harboring a second EXDO A (EXDO A2)-encoding gene (accession number NZ_AOUH01000042; locus tags H736_RS0126300 to H736_RS0126240), and the truncated *nah* gene cluster in *P. veronii* 1YB2 (accession number NZ_JGYI01000001; locus tags Y055_RS00010 to Y055_RS00080). (C) Gene encoding benzene dihydrodiol dehydrogenase and its genetic environment (accession numbers NZ_AOUH01000056 [locus tags H736_RS0127880 to H736_RS0127895] and NZ_JGYI01000072 [locus tags Y055_RS18330 to Y055_RS18315]). White arrows indicate interrupted genes, and black arrows indicate mobile genetic elements. SDR indicates the gene encoding benzene dihydrodiol dehydrogenase of the short-chain dehydrogenase/reductase family, and DLD indicates the gene encoding a truncated putative dihydrolipoamide dehydrogenase. Putative mobile genetic elements were annotated by using TnpPred (52), and putative families of transposases are indicated. The percent sequence identity between the encoded proteins of *P. putida* RE204 and *P. veronii* 1YdBTEX2 or 1YB2 (A) and between the encoded proteins of *P. putida* G7 and *P. putida* 1YdBTEX2 or 1YB2 (B) are given below the gene clusters.

$(5.3 \times 10^2$ to 8.2×10^2 copies/ng cDNA). Similarly, EXDO A2 (present only in 1YdBTEX2) was inducible, with transcript levels increasing from 5.3×10^2 copies/ng cDNA to 2.9×10^4 and 1.2×10^5 copies/ng cDNA after growth on benzene and toluene, respectively (Fig. 4).

DISCUSSION

Considering that *P. veronii* 1YdBTEX2 and 1YB2 were isolated from a benzene-contaminated site, it was not surprising that they were able to degrade a variety of aromatic hydrocarbons. The use of at least three different catabolism gene clusters for benzene degradation, including a novel benzene dihydrodiol dehydroge-

nase, was upon first view unexpected, as such a mosaic kind of pathway is more typically observed for other genera such as *Sphingomonas* (44). However, this highlights the plasticity of the *Pseudomonas* genome in performing more efficient degradation in the face of dramatic environmental challenges.

The inactivation of a gene encoding an extradiol dioxygenase as observed for the EXDO K2-encoding gene in the isopropylbenzene catabolism gene cluster of 1YB2 was previously reported for other gene clusters. The degradation of 1,2,4-trichlorobenzene by *Ralstonia* sp. strain PS12 is initiated by a Rieske nonheme iron oxygenase with high similarity to isopropylbenzene dioxygenases (45). However, the further degradation of central chlorocatechols
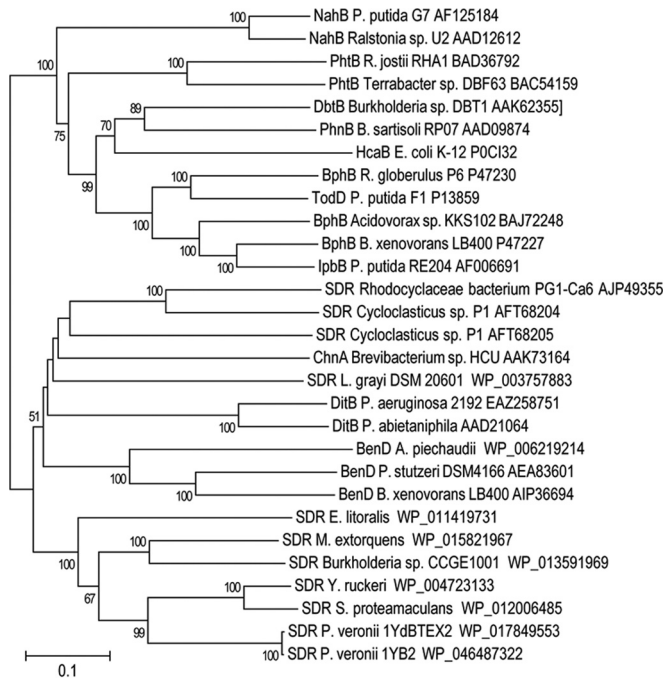
FIG 3 Evolutionary relationships among members of the short-chain dehydrogenase/reductase enzyme family. The evolutionary histories were inferred by using the neighbor-joining method and the *p*-distance model. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Phylogenetic analyses were conducted with MEGA5 (26). Bootstrap values of >50% from 100 neighbor-joining trees are indicated to the left of the nodes. The bar indicates amino acid differences per site. *R. jostii*, *Rhodococcus jostii*; *B. sartisoli*, *Burkholderia sartisoli*; *R. globerulus*, *Rhodococcus globerulus*; *B. xenovorans*, *Burkholderia xenovorans*; *L. grayi*, *Listeria grayi*; *P. abietaniphila*, *Pseudomonas abietaniphila*; *A. piechaudii*, *Achromobacter piechaudii*; *P. stutzeri*, *Pseudomonas stutzeri*; *E. litoralis*, *Erythrobacter litoralis*; *M. extorquens*, *Methylobacterium extorquens*; *Y. ruckeri*, *Yersinia ruckeri*; *S. proteamaculans*, *Serratia proteamaculans*.

is achieved by an intradiol cleavage chlorocatechol pathway, whereas extradiol dioxygenases are typically inactivated during chlorocatechol turnover (46) and, thus, have no function. To avoid the energy-costing synthesis of an enzyme without function, the respective gene is inactivated in strain PS12 (47). Similarly, it may be speculated that inactivation of the EXDO K2-encoding gene avoids the unnecessary synthesis of the respective gene products, which in turn may be of a selective advantage.

The degradation of benzene via an extradiol cleavage pathway requires a 2-hydroxymuconic semialdehyde dehydrogenase (48); however, the corresponding genes are absent from gene clusters encoding enzymes for biphenyl/isopropylbenzene/toluene degradation via a dioxygenolytic route, and only a 2-hydroxymuconic semialdehyde hydrolase (encoded by *ipbD*) (Fig. 2) is typically encoded (9, 14). Thus, a 2-hydroxymuconic semialdehyde dehydrogenase has to be recruited from elsewhere. In *P. veronii* 1YdBTEX2, a 2-hydroxymuconic semialdehyde dehydrogenase-encoding gene (*nahI*) (Fig. 2) was localized in a salicylate catabolic cluster together with a gene encoding an EXDO A-type extradiol dioxygenase (Fig. 2). In *P. veronii* 1YB2, EXDO A- and 2-hydroxymuconic semialdehyde dehydrogenase-encoding genes were localized in a truncated naphthalene gene cluster (Fig. 2). Thus, recruitment of a gene cluster encoding an extradiol dioxygenase and a 2-hydroxymuconic semialdehyde dehydrogenase would be sufficient to allow growth on benzene and make expression of the extradiol dioxygenase encoded in the isopropylbenzene dioxygenase gene cluster dispensable. However, the reasons why dihydrodiol dehydrogenase encoded by this gene cluster is also inactivated and why such an activity is recruited from elsewhere in both strains are not so obvious. Interestingly, in *Pseudomonas putida* ML2, one of the few bacterial strains isolated based on its ability to grow on benzene (49) and characterized in detail regarding enzymes involved in benzene degradation, the dehydrogenation of *cis*-benzene dihydrodiol is catalyzed by BedD, a *cis*-benzene dihydrodiol dehydrogenase with high similarity to glycerol dehydrogenases (50), whereas genes encoding enzymes related to toluene *cis*-dihydrodiol dehydrogenases of the short-chain dehydrogenase
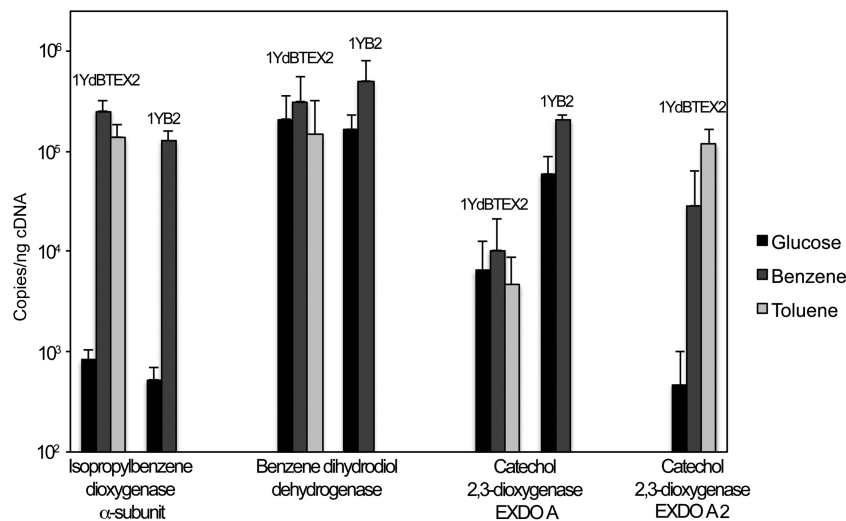

FIG 4 Expression levels of catabolism genes involved in benzene or toluene degradation by *P. veronii* 1YdBTEX2 and 1YB2. Accumulation of transcripts of catabolism genes during growth on benzene for both *P. veronii* 1YdBTEX2 and 1YB2 and on toluene only for *P. veronii* 1YdBTEX2 was measured by real-time quantitative reverse transcription-PCR. Glucose was used as a noninducing negative control.

family were absent. Curiously, degradation of intermediate catechol proceeds via an intradiol cleavage pathway (see Fig. S2 in the supplemental material) (8), which belongs to the core genomes of most *Pseudomonas* strains (51). Similarly to the situation for strain ML2, dehydrogenation of intermediate *cis*-benzene dihydrodiol in 1YB2 and 1YdBTEX2 is catalyzed by enzymes that are not closely related to toluene *cis*-dihydrodiol dehydrogenases. Thus, in *P. putida* ML2 as well as *P. veronii* 1YB2 and 1YdBTEX2, degradation of benzene, except for the initial dioxygenation step, is not catalyzed by enzymes similar to those involved in toluene degradation via a dioxygenolytic pathway. Whether this is due to the poor activity of enzymes of the toluene dioxygenase pathway remains to be elucidated.

ACKNOWLEDGMENTS

FUNDING INFORMATION

REFERENCES

1. **Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW.** 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol Rev **35:** 652–680. http://dx.doi.org/10.1111/j.1574-6976.2011.00269.x.
2. **Cheuk W, Woo PC, Yuen KY, Yu PH, Chan JK.** 2000. Intestinal inflammatory pseudotumour with regional lymph node involvement: identification of a new bacterium as the aetiological agent. J Pathol **192:** 289–292. http://dx.doi.org/10.1002/1096-9896(2000)9999:9999<::AID-PATH767>3.0.CO;2-F.
3. **Vullo DL, Ceretti HM, Daniel MA, Ramirez SA, Zalts A.** 2008. Cadmium, zinc and copper biosorption mediated by *Pseudomonas veronii* 2E. Bioresour Technol **99:**5574–5581. http://dx.doi.org/10.1016/j.biortech.2007.10.060.
4. **Hong HB, Nam IH, Murugesan K, Kim YM, Chang YS.** 2004. Biodegradation of dibenzo-p-dioxin, dibenzofuran, and chlorodibenzo-p-dioxins by *Pseudomonas veronii* PH-03. Biodegradation **15:**303–313. http://dx.doi.org/10.1023/B:BIOD.0000042185.04905.0d.
5. **Onaca C, Kieninger M, Engesser KH, Altenbuchner J.** 2007. Degradation of alkyl methyl ketones by *Pseudomonas veronii* MEK700. J Bacteriol **189:**3759–3767. http://dx.doi.org/10.1128/JB.01279-06.
6. **Junca H, Pieper DH.** 2004. Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. Environ Microbiol **6:**95–110.
7. **Witzig R, Junca H, Hecht HJ, Pieper DH.** 2006. Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. Appl Environ Microbiol **72:**3504–3514. http://dx.doi.org/10.1128/AEM.72.5.3504-3514.2006.
8. **Tan H-M, Fong KP-Y.** 1993. Molecular analysis of the plasmid-borne *bed* gene cluster from *Pseudomonas putida* ML2 and cloning of the *cis*-benzene dihydrodiol dehydroxygenase gene. Can J Microbiol **39:**357–362. http://dx.doi.org/10.1139/m93-052.
9. **Zylstra GJ, McCombie WR, Gibson DT, Finette BA.** 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. Appl Environ Microbiol **54:**1498–1503.
10. **Shields MS, Montgomery SO, Chapman PJ, Cuskey SM, Pritchard PH.** 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium g4. Appl Environ Microbiol **55:**1624–1629.
11. **Duarte M, Jauregui R, Vilchez-Vargas R, Junca H, Pieper DH.** 2014. AromaDeg, a novel database for phylogenomics of aerobic bacterial degradation of aromatics. Database **2014:**bau118. http://dx.doi.org/10.1093/database/bau118.
12. **Gibson DT, Parales RE.** 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr Opin Biotechnol **11:**236–243. http://dx.doi.org/10.1016/S0958-1669(00)00090-2.
13. **Aoki H, Kimura T, Habe H, Yamane H, Kodama T, Omori T.** 1996. Cloning, nucleotide sequence, and characterization of the genes encoding enzymes involved in the degradation of cumene to 2-hydroxy-6-oxo-7-methylocta-2,4-dienoic acid in *Pseudomonas fluorescens* IP01. J Ferment Bioeng **81:**187–196. http://dx.doi.org/10.1016/0922-338X(96)82207-0.
14. **Eaton RW, Selifonova OV, Gedney RM.** 1998. Isopropylbenzene catabolic pathway in *Pseudomonas putida* RE204: nucleotide sequence analysis of the *ipb* operon and neighboring DNA from pRE4. Biodegradation **9:**119–132. http://dx.doi.org/10.1023/A:1008386221961.
15. **Eltis LD, Bolin JT.** 1996. Evolutionary relationships among extradiol dioxygenases. J Bacteriol **178:**5930–5937.
16. **Junca H, Plumeier I, Hecht HJ, Pieper DH.** 2004. Difference in kinetic behaviour of catechol 2,3-dioxygenase variants from a polluted environment. Microbiology **150:**4181–4187. http://dx.doi.org/10.1099/mic.0.27451-0.
17. **Dorn E, Knackmuss H-J.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. Biochem J **174:**85–94.
18. **de Lima-Morales D, Chaves-Moreno D, Jarek M, Vilchez-Vargas R, Jauregui R, Pieper DH.** 2013. Draft genome sequence of *Pseudomonas veronii* strain 1YdBTEX2. Genome Announc **1**(3):e00258-13. http://dx.doi.org/10.1128/genomeA.00258-13.
19. **Zerbino DR, Birney E.** 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res **18:**821–829. http://dx.doi.org/10.1101/gr.074492.107.
20. **Hernandez D, Francois P, Farinelli L, Osteras M, Schrenzel J.** 2008. De novo bacterial genome sequencing: millions of very short reads assembled on a desktop computer. Genome Res **18:**802–809. http://dx.doi.org/10.1101/gr.072033.107.
21. **Sommer DD, Delcher AL, Salzberg SL, Pop M.** 2007. Minimus: a fast, lightweight genome assembler. BMC Bioinformatics **8:**64. http://dx.doi.org/10.1186/1471-2105-8-64.
22. **Boetzer M, Henkel CV, Jansen HJ, Butler D, Pirovano W.** 2011. Scaffolding pre-assembled contigs using SSPACE. Bioinformatics **27:**578–579. http://dx.doi.org/10.1093/bioinformatics/btq683.
23. **Darling AC, Mau B, Blattner FR, Perna NT.** 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res **14:**1394–1403. http://dx.doi.org/10.1101/gr.2289704.
24. **Silby MW, Cerdeno-Tarraga AM, Vernikos GS, Giddens SR, Jackson RW, Preston GM, Zhang XX, Moon CD, Gehrig SM, Godfrey SA, Knight CG, Malone JG, Robinson Z, Spiers AJ, Harris S, Challis GL, Yaxley AM, Harris D, Seeger K, Murphy L, Rutter S, Squares R, Quail MA, Saunders E, Mavromatis K, Brettin TS, Bentley SD, Hothersall J, Stephens E, Thomas CM, Parkhill J, Levy SB, Rainey PB, Thomson NR.** 2009. Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. Genome Biol **10:**R51. http://dx.doi.org/10.1186/gb-2009-10-5-r51.
25. **Edgar RC.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113. http://dx.doi.org/10.1186/1471-2105-5-113.
26. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28:**2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
27. **Saitou N, Nei M.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406–425.
28. **Moreno-Hagelsieb G, Latimer K.** 2008. Choosing BLAST options for better detection of orthologs as reciprocal best hits. Bioinformatics **24:** 319–324. http://dx.doi.org/10.1093/bioinformatics/btm585.
29. **Sierra R, Rodriguez RL, Chaves D, Pinzon A, Grajales A, Rojas A, Mutis G, Cardenas M, Burbano D, Jimenez P, Bernal A, Restrepo S.** 2010. Discovery of *Phytophthora infestans* genes expressed in planta through mining of cDNA libraries. PLoS One **5:**e9847. http://dx.doi.org/10.1371/journal.pone.0009847.
30. **Bray JR, Curtis JT.** 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr **27:**325–349. http://dx.doi.org/10.2307/1942268.
31. **Clarke KR, Warwick RM.** 2001. Change in marine communities: an approach to statistical analysis and interpretation, 2nd ed. Primer-E, Plymouth, United Kingdom.

32. **Bradford MM.** 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.

33. **Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685. http://dx.doi.org/10.1038/227680a0.

34. **Heim S, Ferrer M, Heuer H, Regenhardt D, Nimtz M, Timmis KN.** 2003. Proteome reference map of *Pseudomonas putida* strain KT2440 for genome expression profiling: distinct responses of KT2440 and *Pseudomonas aeruginosa* strain PAO1 to iron deprivation and a new form of superoxide dismutase. Environ Microbiol **5:**1257–1269. http://dx.doi.org/10.1111/j.1462-2920.2003.00465.x.

35. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics **9:**75. http://dx.doi.org/10.1186/1471-2164-9-75.

36. **Vilchez-Vargas R, Geffers R, Suarez-Diez M, Conte I, Waliczek A, Kaser VS, Kralova M, Junca H, Pieper DH.** 2013. Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system. Environ Microbiol **15:**1016–1039. http://dx.doi.org/10.1111/j.1462-2920.2012.02752.x.

37. **Camarinha-Silva A, Wos-Oxley ML, Jauregui R, Becker K, Pieper DH.** 2012. Validating T-RFLP as a sensitive and high-throughput approach to assess bacterial diversity patterns in human anterior nares. FEMS Microbiol Ecol **79:**98–108. http://dx.doi.org/10.1111/j.1574-6941.2011.01197.x.

38. **Jousset A, Schuldes J, Keel C, Maurhofer M, Daniel R, Scheu S, Thuermer A.** 2014. Full-genome sequence of the plant growth-promoting bacterium *Pseudomonas protegens* CHA0. Genome Announc **2**(2)**:**e00322-14. http://dx.doi.org/10.1128/genomeA.00322-14.

39. **Loper JE, Hassan KA, Mavrodi DV, Davis EW, II, Lim CK, Shaffer BT, Elbourne LD, Stockwell VO, Hartney SL, Breakwell K, Henkels MD, Tetu SG, Rangel LI, Kidarsa TA, Wilson NL, van de Mortel JE, Song C, Blumhagen R, Radune D, Hostetler JB, Brinkac LM, Durkin AS, Kluepfel DA, Wechter WP, Anderson AJ, Kim YC, Pierson LS, III, Pierson EA, Lindow SE, Kobayashi DY, Raaijmakers JM, Weller DM, Thomashow LS, Allen AE, Paulsen IT.** 2012. Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. PLoS Genet **8:**e1002784. http://dx.doi.org/10.1371/journal.pgen.1002784.

40. **Ortet P, Barakat M, Lalaouna D, Fochesato S, Barbe V, Vacherie B, Santaella C, Heulin T, Achouak W.** 2011. Complete genome sequence of a beneficial plant root-associated bacterium, *Pseudomonas brassicacearum*. J Bacteriol **193:**3146. http://dx.doi.org/10.1128/JB.00411-11.

41. **Paulsen IT, Press CM, Ravel J, Kobayashi DY, Myers GS, Mavrodi DV, DeBoy RT, Seshadri R, Ren Q, Madupu R, Dodson RJ, Durkin AS, Brinkac LM, Daugherty SC, Sullivan SA, Rosovitz MJ, Gwinn ML, Zhou L, Schneider DJ, Cartinhour SW, Nelson WC, Weidman J, Watkins K, Tran K, Khouri H, Pierson EA, Pierson LS, III, Thomashow LS, Loper JE.** 2005. Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. Nat Biotechnol **23:**873–878. http://dx.doi.org/10.1038/nbt1110.

42. **Redondo-Nieto M, Barret M, Morrisey JP, Germaine K, Martinez-Granero F, Barahona E, Navazo A, Sanchez-Contreras M, Moynihan JA, Giddens SR, Coppoolse ER, Muriel C, Stiekema WJ, Rainey PB, Dowling D, O'Gara F, Martin M, Rivilla R.** 2012. Genome sequence of the biocontrol strain *Pseudomonas fluorescens* F113. J Bacteriol **194:**1273–1274. http://dx.doi.org/10.1128/JB.06601-11.

43. **Yen KM, Gunsalus IC.** 1982. Plasmid gene organization: naphthalene/salicylate oxidation. Proc Natl Acad Sci U S A **79:**874–878. http://dx.doi.org/10.1073/pnas.79.3.874.

44. **Armengaud J, Timmis KN, Wittich RM.** 1999. A functional 4-hydroxysalicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-*p*-dioxin pathway genes in *Sphingomonas* sp. strain RW1. J Bacteriol **181:**3452–3461.

45. **Beil S, Happe B, Timmis KN, Pieper DH.** 1997. Genetic and biochemical characterization of the broad-spectrum chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12: dechlorination of 1,2,4,5-tetrachlorobenzene. Eur J Biochem **247:**190–199. http://dx.doi.org/10.1111/j.1432-1033.1997.00190.x.

46. **Bartels I, Knackmuss H-J, Reineke W.** 1984. Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl Environ Microbiol **47:**500–505.

47. **Beil S, Timmis KN, Pieper DH.** 1999. Genetic and biochemical analyses of the *tec* operon suggest a route for evolution of chlorobenzene degradation genes. J Bacteriol **181:**341–346.

48. **Harayama S, Mermod N, Rekik M, Lehrbach PR, Timmis KN.** 1987. Roles of the divergent branches of the *meta*-cleavage pathway in the degradation of benzoate and substituted benzoates. J Bacteriol **169:**558–564.

49. **Tan H-M, Mason JR.** 1990. Cloning and expression of the plasmid-encoded benzene dioxygenase genes from *Pseudomonas putida* ML2. FEMS Microbiol Lett **72:**259–264. http://dx.doi.org/10.1111/j.1574-6968.1990.tb03899.x.

50. **Fong KPY, Goh CBH, Tan HM.** 1996. Characterization and expression of the plasmid-borne *bedD* gene from *Pseudomonas putida* ML2, which codes for a NAD(+)-dependent *cis*-benzene dihydrodiol dehydrogenase. J Bacteriol **178:**5592–5601.

51. **Perez-Pantoja D, Donoso R, Junca H, Gonzalez B, Pieper DH.** 2009. Phylogenomics of aerobic bacterial degradation of aromatics, p 1356–1397. *In* Timmis KN (ed), Handbook of hydrocarbon and lipid microbiology. Springer-Verlag, Berlin, Germany.

52. **Riadi G, Medina-Moenne C, Holmes DS.** 2012. TnpPred: a Web service for the robust prediction of prokaryotic transposases. Comp Funct Genomics **2012:**678761. http://dx.doi.org/10.1155/2012/678761.