The Broad Substrate Chlorobenzene Dioxygenase and cis-Chlorobenzene Dihydrodiol Dehydrogenase of Pseudomonas sp. Strain P51 Are Linked Evolutionarily to the Enzymes for Benzene and Toluene Degradation*

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The chlorobenzene degradation pathway of Pseudomonas sp. strain P51 is an evolutionary novelty. The first enzymes of the pathway, the chlorobenzene dioxygenase and the cis-chlorobenzene dihydrodiol dehydrogenase, are encoded on a plasmid-located transposon Tn5280. Chlorobenzene dioxygenase is a four-protein complex, formed by the gene products of tcbAA for the large subunit of the terminal oxygenase, tcbAB for the small subunit, tcbAC for the ferredoxin, and tcbAD for the NADH reductase. Directly downstream of tcbAD is the gene for the cis-chlorobenzene dihydrodiol dehydrogenase, tcbB. Homology comparisons indicated that these genes and gene products are most closely related to those for toluene (todC1C2BAD) and benzene degradation (bedC1C2BA and bnzABCD) and distantly to those for biphenyl, naphthalene, and benzoate degradation. Similar to the tod-encoded enzymes, chlorobenzene dioxygenase and cis-chlorobenzene dihydrodiol dehydrogenase were capable of oxidizing 1,2-dichlorobenzene, toluene, naphthalene, and biphenyl, but not benzoate, to the corresponding dihydrodiol and dihydroxy intermediates. These data strongly suggest that the chlorobenzene dioxygenase and dehydrogenase originated from a toluene or benzene degradation pathway, probably by horizontal gene transfer. This evolutionary event left its traces as short gene fragments directly outside the tcbAB coding regions.

Bacteria that are able to use mono- or bicyclic aromatic compounds as their sole source of carbon and energy under aerobic growth conditions are present ubiquitously in the environment (1, 2). Considering the potential use and importance of such bacteria to help to remove many man-made polluting compounds, it is necessary to study the genetic and biochemical variations found among these types of bacteria and to investigate their evolutionary development (3). Only then can the limitations of existing metabolic pathways be understood and concepts be developed to select or engineer novel pathways (4, 5). Of special interest will be to obtain degradation of highly recalcitrant chlorinated aromatic compounds, such as chlorinated benzenes and biphenyls (6).

A very important enzyme complex in the aerobic degradation of many aromatic compounds is the multicomponent aromatic ring dioxygenase (7, 8). Aromatic ring dioxygenases, such as benzoate dioxygenase (9), toluate dioxygenase (10), naphthalene (11), biphenyl, toluene (12), or benzene dioxygenases (13), are enzyme complexes with three- or four protein subunits. This complex catalyzes a redox reaction in which molecular oxygen is incorporated in the aromatic ring at the expense of the oxidation of NADH (7, 8, 12). The resulting intermediate is a dihydrodiol derivative of the aromatic ring structure. A dihydrodiol dehydrogenase then catalyzes the (formal) oxidation of the dihydrodiol to the dihydroxy derivative and regenerates the reduced NADH. The components of the aromatic ring dioxygenase consist of different electron transport proteins (a ferredoxin and a reductase, or a combined ferredoxin-NADH reductase) and the terminal oxygenase (also called hydroxylase component or iron sulfur protein), which is thought to determine the substrate specificity of the enzyme and to carry out the substrate activation (7, 8). Despite their structural similarities, remarkable differences in substrate spectrum are found among the different aromatic ring dioxygenases (12, 14, 15).

We have focused on bacteria-degrading chlorinated benzenes, in particular Pseudomonas sp. strain P51. Upon growth on chlorobenzenes, strain P51 induces enzyme activities which catalyze the conversion of chlorobenzenes to chlorocatechols (16). The genes for these enzymes, the tcbAB genes, were cloned previously from a catabolic plasmid present in strain P51 and were proposed to encode an aromatic ring dioxygenase and a dihydrodiol dehydrogenase, similar to the enzymes of other aromatic pathways (16). Strain P51 also contains the genes for a so-called chlorocatechol oxidative pathway, which in strain P51 consists of an operon of four genes, tcbCDEF (17), and a regulatory gene tcbR (18). Interestingly, both gene clusters in strain P51 are located on a transmissible plasmid pP51. Furthermore, the tcbAB genes itself are part of a transposable element, Tn5280 (19). We therefore strongly believe that the chlorobenzene pathway is an evolutionary novelty in bacteria, formed by a novel combination of two existing gene clusters, perhaps through horizontal gene transfer.

To test this idea further, we wanted to characterize the genes for the chlorobenzene dioxygenase and for the cis-chlorobenzene dihydrodiol dehydrogenase of Pseudomonas sp. strain P51 in detail. We wanted to analyze further the capability of the enzymes to convert several different aromatic compounds. This would make it possible to compare the enzymes both genetically and biochemically with related enzymes from other aro-

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The abbreviations used are: Tn, transposon; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IS, inser-

tion element; bp, base pair(s); kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
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motic degradation pathways. The data in the paper indicate clearly that the chlorobenzene dioxygenase and the cis-chlorobenzene dihydrodiol dehydrogenase resemble most the toluene/benzene-type enzymes. Furthermore, evidence is presented to show the relics of the horizontal gene transfer events which may have lead to the imprecise excision of a genetic element containing the genes for an aromatic ring dioxygenase and dihydrodiol dehydrogenase from a toad-like operon.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions—Pseudomonas sp.** Strain P51 has been described previously (16). *Escherichia coli* strains DH5α and TG1 were used routinely for plasmid cloning and single-stranded M13 phase preparation, respectively (20). *E. coli* BL21 (DE3) was used for T7 RNA polymerase-directed expression of genes and gene fragments cloned into plasmid pET2c (21). We used the plasmids pUC18 and pUC19 (obtained from Boehringer Mannheim, Mannheim, Federal Republic of Germany) as cloning vectors for strain P51-derived DNA fragments. In general, *E. coli* strains were grown on LB medium at 37 °C (20), supplemented with the appropriate antibiotics. For expression of active chlorobenzene dioxygenase and cis-chlorobenzene dihydrodiol dehydrogenase, however, the *E. coli* strains were cultivated at 25 °C.

**DNA Techniques and Sequence Analysis—All DNA techniques, such as plasmid DNA isolation, transformations, or DNA-enzyme digestions, were carried out according to established procedures described elsewhere (20).** DNA sequence analysis was performed on both strands of the DNA by sequencing overlapping fragments cloned in M13mp18, as described (17). The source of DNA fragments containing the chlorobenzene dioxygenase and dihydrodiol dehydrogenase genes of strain P51 was plasmid pTCB60 (16). Restriction enzymes and other DNA-modifying enzymes were purchased from Life Technologies Europe (Paisley, UK), Appligene (Illkirch, France), or Boehringer Mannheim (Mannheim, FRG). Reagents for the polymerase chain reaction were obtained from Life Technologies Inc. (Cergy Pontoise, France).

**Expression Clones—To obtain overexpression of the individual components of the dioxygenase system and the dehydrogenase, we constructed a number of translational fusions with the start codon present on pET2c (Fig. 1) (21).** Hereto, artifical NcoI sites were created on the DNA to be cloned by applying PCR amplification in the presence of a mutagenic primer. For clones starting with the tcbAb gene, we amplified a 200-bp DNA fragment ranging from the start of tcbAb until the first SpI site downstream. The PCR fragment was then cleaved with NcoI and SpAI and ligated with a 2-kb SpAI-SclI fragment of plasmid pTCB71 (16) and, with pET8c, cut with NcoI and EcoRV. After transformation, this resulted in plasmid pTCB113. The sequence of the PCR-amplified part of this plasmid was determined and found to be identical with that of the wild-type strain P51. The plasmid pTCB113 was then used to transform strain DH5α (pETCB144). The cultures were inoculated from a single colony in 200 ml of LB medium with 50 μg/ml ampicillin and grown at 37 °C for about 36 h. The optical density of the cultures had then reached an A550 of 3.5. The cell culture was then centrifuged at 4000 rpm for 10 min at 20 °C, and cells were resuspended in 50 ml of M9 minimal salts medium (20). This was repeated once more and, after a final centrifugation step, the cells were resuspended in 10 ml of M9 medium and stored briefly on ice until use. A series of glass-stoppered tubes with a volume of 15 ml was prepared with the following components: 2.5 ml M9 medium, 50 μM glucose, 50 μl of a methanol solution containing the aromatic substrates, and 0.5 ml of the cell suspension. The final A550 of the cells in the assay was between 0.7 and 1.0. The tubes were incubated on a rotary shaking platform at a temperature of 30 °C. We tested the following aromatic substrates at a final concentration in the assay of 0.5 mM: 1,2-dichlorobenzene, toluene, biphenyl, naphthalene (all previously dissolved in methanol), and benzene. For each time point of the assay, two tubes were incubated. Samples of 1.0 ml were then taken from the tubes and centrifuged for 1 min at 13,000 rpm to remove the cells. The supernatant was transferred to a fresh tube and analyzed for the presence of dihydrodiol intermediates by HPLC (see below).

**cis-Chlorobenzene dihydrodiol dehydrogenase activity was tested in *E. coli* DH5α (pTCB149).** Cultures were grown on 50 ml of LB medium at 37 °C to an A550 of 1.0, after which the cells were harvested and a cell extract was prepared as described previously (16). The reaction mixture for dihydrodiol dehydrogenase activity contained 0.65 ml of 20 mM sodium phosphate buffer, pH 7.5, 25 μl of 20 mM NAD+ solution, 50 μl of the cell extract, and 50 μl of dihydrodiol substrate. As substrates we used the supernatants of the whole cell incubations after 2 h (see above). The assay mixture was incubated at 37 °C, and absorbance in A340 was measured on a spectrophotometer. When no more changes in A340 were observed, the assay mixture was analyzed on HPLC to check for the disappearance of the dihydrodiol and the presence of the dihydroxy compound.

**HPLC and GC–MS Analysis—** Analysis of dihydrodiols and dihydroxy compounds was performed on a Waters 625 LC HPLC system equipped with a photodiode array detector. Separation was carried out on a C18 reversed phase column (Nova-Pak 300 mm, 6 mm, 4 μm). Two running solutions were used which contained: A, 10 mM H3PO4 in H2O at a pH of 3.0, and B, 90% methanol and 10% of solution A. Elution from the column was performed by running a gradient as follows: 0–2 min, 40% of buffer B and 60% of buffer A; 2–30 min, linear increase to 70% of buffer B; 30–35 min, 70% of buffer B and decrease of buffer A to 30%; 35–40 min, 70% of buffer B and 30% of buffer A. Flow-rate through the system was 0.5 ml/min at a pressure of 3500 ps.i. Generally, an amount of 200 μl of the samples was injected. Under these conditions we observed the following retention times: 3,4-dichloro-1,2-dihydroxycyclohexa-3,5-diene (3,4-dichlorobenzene dihydrodiol), 10.8 min; 3,4-dichlorocatechol, 29.5 min; 1,2-dihydroxy-3-methylcyclohexa-3,5-diene (toluene dihydrodiol), 5.6 min; 1,2-dihydroxybenzotriphenyl ether, 14.8 min; 1,2-dihydroxy benzoic acid (naphthalene dihydrodiol), 13.4 min; 1,2-dihydroxy-3-phenylcyclohexa-3,5-diene (biphenyl dihydrodiol), 21.5 min; 2,3-dihydroxybiphenyl, 35.5 min. Authentic standards compounds which were tested to us and could be tested were 2,3-dihydroxybiphenyl, 3,4-dichlorocatechol, and 3-methylcatechol.

µl of dihydrodiol intermediates were extracted from supernatants of the whole cell incubations after 2 h with an equal volume of ethyl acetate and dried with sodium sulfate. Samples were derivatized with BSTFA and subjected to GC–MS analysis as described elsewhere (22).
RESULTS

Sequence Determination of the tcbAB Genes—We determined the nucleotide sequence of the region containing the tcbAB genes of Pseudomonas sp. strain P51 on both strands of the DNA. The tcbAB genes are located on a stretch of 5,402 base pairs which lay between IS1066 and IS1067 (19) (Figs. 1 and 2). The region showed the presence of five large unidirectional ORFs, encoding the different subunits of the chlorobenzene dioxygenase and the dihydrodiol dehydrogenase. Sequence homologies with other known dioxygenases allowed the assignment of putative protein functions to each of the ORFs (Table I). We propose to designate the genes as follows: tcbAa, coding for the large subunit of the terminal oxygenase; tcbAb, encoding the small terminal oxygenase subunit; tcbAc, the ferredoxin; tcbAd, the NADH reductase; and tcbB, the dihydrodiol dehydrogenase. Except for a small 109-bp gap between tcbAa and tcbAb and 8 bp between tcbAb and tcbAc, the ORFs were contiguous on the DNA. Downstream of tcbB, another ORF was found, which showed homology to catechol 2,3-dioxygenases, such as todE (23). This ORF, however, appeared to be interrupted by IS1067, causing a premature ending (Fig. 2).

Homologies with Other Bacterial Aromatic Ring Dioxygenases and Dihydrodiol Dehydrogenases—The amino acid sequences predicted for the TcbAa, -Ab, -Ac, -Ad, and TcbB proteins were compared with those from other bacterial aromatic ring dioxygenases by using the GCG programs FASTA, DISTANCES, and PILEUP (24, 25). The alignments and distance calculations showed for almost every individual component of the dioxygenases and for the dihydrodiol dehydrogenase a clustering in four different families (Fig. 3 and Table II). One family is formed by the dioxygenases which are composed of two components (and three protein subunits), i.e. benzoate dioxygenase of Acinetobacter calcoaceticus (9) and toluate dioxygenase of Pseudomonas putida (10). A second family contains the three-component dioxygenases of naphthalene metabolism, such as those encoded by the nah (26), ndo (27), pah (28), or dox (29) genes. The third family is composed of the dioxygenases for biphenyl and chlorobiphenyl conversion in Gram-negative bacteria (30–32), and a fourth one of the benzene (13, 33), toluene (23), and chlorobenzene dioxygenases. Two recently published sequences for a biphenyl dioxygenase from two Gram-positive microorganisms aligned more closely with the toluene/benzene family than with the biphenyl family itself (34, 35) (Fig. 3). The only exception in the alignments was the clustering of the reductase components. In this case, the positions of the reductases from P. paucimobilis KKS102, Rhodococcus sp. RHA1, and R. globulus P6 appeared to be intermediate (Table II). In general, the reductases seem to have diverged substantially more than the other components of the dioxygenases and the dihydrodiol dehydrogenase.

It is interesting that to a large extent the gene organization within the clusters has been conserved as well, but clearly differs between them. For example, the benzene/toluene family has the gene order: large subunit of the terminal oxygenase, small subunit, ferredoxin, reductase, and, in three cases, dehydrogenase. The group of the Gram-positive biphenyl dioxygenases, which appeared to be the cluster closest to that of the benzene/toluene dioxygenases, has an identical organization of the genes encoding the core dioxygenase, but differs in genes located downstream. The family of the Gram-negative biphenyl dioxygenases has a genetic organization comparable to that of the benzene/toluene family, but in two cases contain an extra
ORF between the genes for the terminal oxygenase and that for the ferredoxin. Also in these cases, the upstream regions lack homology between each other or with the corresponding regions of the benzene/toluene family. The biphenyl dioxygenase of strain KKS102 lacks the extra ORF, but also lacks the gene for the reductase at this position (Fig. 3). A stronger difference was found with the family of the naphthalene dioxygenases. Here the gene order is reductase, ferredoxin, large subunit of the terminal oxygenase. In the case of the benzoate and toluate dioxygenases, the largest difference in gene order with the others is found in the presence of a gene for the combined ferredoxin and reductase function (i.e. benC and xylZ) (8, 9).

Expression of the tcb-encoded Gene Products—We cloned all open reading frames from the tcbAB cluster in the expression vector pET8c under transcriptional control of the T7 promoter in E. coli BL21(DE3), to test if the gene products would have the size as predicted from the amino acid sequence. Upon induction in E. coli, we could detect all predicted protein bands and deletion derivatives on SDS-PAGE (Fig. 4). Interestingly, in some cases, read-through from one ORF into the other occurred only sparsely. For example, the tcbB gene could not be visibly expressed in clones containing tcbAa upstream of tcbAb. Only by using plasmids in which part of tcbAa was deleted, such as pTCB147, we found detectable expression of tcbAb. On the other hand, clones starting with tcbAc would also express downstream ORFs when present, such as in plasmid pTCB116 and pTCB120. Most protein bands observed on SDS-PAGE which were attributed to expression from a tcbA gene were of the size expected from computer predictions (Table I). The exception was TcbB, which migrated at a smaller apparent molecular mass than predicted (22 kDa instead of 33.1 kDa).

Chlorobenzene Dioxygenase and Dihydrodiol Dehydrogenase Activity in E. coli—The functionality of the tcbAB gene products was then tested by measurement of their enzymatic activity in E. coli. We cloned the complete DNA fragment with the tcbAaAbAcAd genes starting at the ATG codon of the tcbAa gene in pET8c. To our surprise, we could not detect any measurable activity of the chlorobenzene dioxygenase with this plasmid (see above) and by the formation of inclusion bodies. The genes were then removed from pET8c and cloned in pUC19 under control of the lac promoter (pTCB144). E. coli (pTCB144) showed the typical formation of blue-green colonies when grown on LB agar, due to the formation of indigo. This color became more pronounced when the colonies were incubated at 25 °C.

Whole cells of E. coli (pTCB144) were incubated with different aromatic substrates in minimal medium in order to produce the cis-dihydrodiols. E. coli (pTCB144) cells rapidly produced one single metabolite as detected by HPLC, when incubated with 1,2-dichlorobenzene, toluene, biphenyl, or naphthalene (Fig. 5). No conversion of benzoate was detected with these substrates. The UV spectra of the intermediates of toluene, naphthalene, and biphenyl incubation on HPLC were in agreement with 1,2-dichlorobenzene, toluene, although with a \( A_{max} \) of 272 nm. Further information on the identity of the four intermediates was obtained by GC-MS analysis of the BSTFA-derivatized form (Fig. 6). All
four mass spectra gave a similar fragmentation pattern with the molecular ions showing at m/z 324, 270, 392, and 306 for the products of dichlorobenzene (Fig. 6A), toluene (Fig. 6B), biphenyl (Fig. 6C), and naphthalene (Fig. 6D), respectively. The usually dominant (M + 15) ion (loss of one of the methyl groups of the trimethylsilyl moiety) is absent from all the mass spectra, but loss of OSi(CH$_3$)$_3$ (molecular mass of 89) is apparent in all of them. The ions at m/z 191 ([(CH$_3$)$_3$OSiOCH$_2$(CH$_3$)$_3$]$^+$), 147 ([(CH$_3$)$_3$OSiOSi(CH$_3$)$_3$]$^+$), and 73 ([(CH$_3$)$_3$Si]$^+$) dominate the four spectra. In the case of the product of dichlorobenzene, the ion at m/z 289 is formed by the loss of chlorine (mass of 35) from the molecular ion. Following the line of evidence for the formation of cis-dihydrodiols from aromatic compounds by toluene and naphthalene dioxygenase (36–38), for example, all our results are in agreement with the proposed cis-dihydrodiol structures which would be formed during conversion of toluene, 1,2-dichlorobenzene, biphenyl, and naphthalene by the chlorobenzene dioxygenase.

The dihydrodiols were then incubated with cell extracts of E. coli (pTCB149), which expresses the dihydrodiol dehydrogenase, and analyzed by HPLC. In the case of 1,2-dichlorobenzene, we found that the dihydrodiol was converted to one single product. This product cochromatographed with authentic 1,2-dichlorocatechol, and the UV spectra of the two compounds were identical. Biphenyl dihydrodiol was enzymatically converted to a compound with an identical retention time and UV spectrum as 2,3-dihydroxybiphenyl and, similarly, toluene dihydrodiol to a compound with identity to 3-methylcatechol. In the case of naphthalene 1,2-dihydrodiol, the presumed product 1,2-dihydroxynaphthalene could not be detected, as it became autooxidized quickly as described earlier (39, 40).

**DISCUSSION**

Chlorobenzene Dioxygenase Belongs to the Toluene and Benzene Dioxygenase Subclass—Pseudomonas sp. strain P51 has the ability to use chlorinated benzenes as sole carbon and energy source. The enzyme catalyzing the initial dioxygenation of the aromatic ring structure was presumed to be a three-component aromatic ring dioxygenase, like those found in other aerobic bacteria. Here we have shown that the genes for the chlorobenzene dioxygenase are contiguous on the DNA and indeed code for four protein subunits, two of which make up the terminal oxygenase, one the ferredoxin, and the last one the NADH reductase. Following the genes for the dioxygenase is a gene coding for a dihydrodiol dehydrogenase. All genes were shown to be functional by using expression studies and enzyme activity assays. Both biochemical and genetic evidence indicate that the chlorobenzene dioxygenase belongs to a subclass of aromatic ring dioxygenase enzymes to which the toluene and benzene dioxygenases also belong.

Our studies with the Tcb dioxygenase showed that it is not specific for catalyzing the conversion of 1,2-dichlorobenzene only, but capable of converting toluene, naphthalene, and biphenyl. Benzene was not converted by the Tcb dioxygenase, which is like other characterized three-component aromatic ring dioxygenases (8, 12). The outcome of the whole cell inco-
Uncorrected, pairwise distances calculated for the PILEUP alignments of the deduced amino acid sequences of the terminal oxygenase large subunits (A) and of the reductase components (B) of the aromatic ring dioxygenases

Denominator in the calculation was the length of the shorter sequence without gaps (24). Circles show a graphic representation with a circle diameter proportional to the calculated distances. Circle filling indicates an observed clustering in PILEUP at a distance higher than 0.8. Cross-hatched circle, benzoate/toluate family; checkered circle, naphthalene family; solid circle, biphenyl family; diagonally lined circle, toluene/benzone family.

Table II

Uncorrected, pairwise distances calculated for the PILEUP alignments of the deduced amino acid sequences of the terminal oxygenase large subunits (A) and of the reductase components (B) of the aromatic ring dioxygenases

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Fig. 4. SDS-PAGE of the cell extracts from *E. coli* BL21(DE3) strains containing the different plasmids with tcbAB genes. Lanes: 1, pET8c; 2, pTCB147 (tcbAb, tcbAc, and deletion of tcbAa); 3, pTCB120 (tcbAc, tcbAd, and tcbAd); 4, pTCB117 (tcbAc and a deletion of tcbAd); 5, pTCB116 (tcbAc, tcbAd, and a deletion of tcbB); 6, pTCB115 (tcbAc, tcbAb, tcbAd, and small region of tcbAd); 7, pTCB114 (tcbAc and tcbAb with a frameshift mutation); 8, pTCB113 (tcbAc). Symbols: Aa, gene product of tcbAa; Aa/H9004, product of the interrupted tcbAa gene; Ab, product of tcbAb; Ac, product of tcbAc; Ad, product of tcbAd; Ad, product of the interrupted tcbAd; B, product of tcbB; Bb, product of the interrupted tcbB gene. Migration of the molecular mass standards is indicated in kilodaltons on the right side.

Fig. 5. Formation of dihydrodiol intermediates by washed whole cells of *E. coli* (pTCB146). Shown are mean values of peak areas of the intermediates as measured on HPLC from two independent incubations. Formation of the dihydrodiols excreted in the supernatant was measured as an increase in absorbance at the wavelength of the respective absorption maximum, i.e. 252 nm for naphthalene dihydrodiol, 265 nm for toluene dihydrodiol, 272 nm for 1,2-dichlorobenzene dihydrodiol, and 303 nm for biphenyl dihydrodiol.
dehydrogenase converts all of these cis-dihydrodiols to dihydroxy intermediates (i.e., 3,4-dichlorocatechol, 3-methylcatechol, 2,3-dihydroxybiphenyl, and 1,2-dihydroxynaphthalene). These results strongly suggest that the formation of dihydrodiols and dihydroxy compounds by the Tcb dioxygenase and TcbB dihydrodiol dehydrogenase proceed as expected from the general line for dioxygenation (7, 8, 12).

It is not so clear which subunit of the aromatic ring dioxygenases determines the substrate specificity of the enzyme and why the toluene/benzene subclass enzymes have such a wide substrate spectrum. The remarkable potential of the Tod enzyme to catalyze incorporation of oxygen into a wide range of aromatic substrates has been well studied and explored (12, 41). For instance, the Tod dioxygenase oxidizes biphenyl, the main substrate of the group of bph-encoded enzymes. On the other hand, biphenyl dioxygenase from *P. pseudoalcaligenes* KF707 does not oxidize toluene (14, 42). This limitation supposedly arose in the small subunit of Bph dioxygenase, because when hybrid enzymes between Tod and Bph were constructed, some were found (e.g. BphA1TodC2AB) that gained both the Bph and Tod substrate range. The Bph-dioxygenases were mostly studied for their capability to convert (poly-) chlorinated biphenyls. For example, differences in polychlorinated biphenyl-congener specificity were found between the Bph dioxygenase from *Pseudomonas* sp. strain LB400 and *P. pseudoalcaligenes* KF707, which in this case were attributed to changes in the large subunit of the terminal oxygenase (15). It will be interesting to study in more detail whether the Tcb dioxygenase has acquired any new substrate specificities which enable it to convert higher chlorinated aromatic compounds more efficiently.

**Gene Rearrangements in the Evolutionary Divergence of Aromatic Ring Dioxygenases**—The large pool of genetic data on aromatic ring dioxygenase systems from different aerobic bacteria makes it possible to speculate about the different events which have taken place in the course of the evolutionary development of these microorganisms (43). The accumulation of small events (e.g. mutations) has likely led to the divergence of the different individual genes and gene products, as shown in Fig. 3. However, more striking larger genetic changes have also occurred. Rearrangements on the DNA have caused differences in gene order of the aromatic ring dioxygenase. This becomes obvious when we compare the gene order of the toluene/benzene family and the biphenyl family on one side, and that of the naphthalene family on the other (Fig. 3). In the naphthalene family, the genes for the reductase and ferredoxin have inverted their position with respect to the genes encoding the terminal oxygenase. The gene for the reductase may also be at a different position, as in the *bph* gene cluster of *P. paucimobilis* strain KKS102 (31, 44). Similarly, rearrangements have caused differences in the organization of genes located downstream of the aromatic ring dioxygenase, like the ones encoding the dihydrodiol dehydrogenase or the *meta*-cleavage enzymes (Fig. 3). These DNA rearrangements must have had their effects on gene expression and on enzyme synthesis, perhaps due...
to improper signals on the DNA or changed stability and structure of the RNA. For instance, how is it achieved that the right molar proportions of all components of the dioxygenase are synthesized? The benzene dioxygenase of P. putida ML2, which is transcribed from one single gene cluster with operonic organization, apparently has intracellular molar proportions of 1:0.45:0.8 of ISP-α subunit/ferrodoxin/reductase (45). Regulation of the right molar amounts may become less obvious when the reductase gene is not directly transcriptionally coupled. This may reflect the idea that the aromatic ring dioxygenase is a kinetic enzyme complex with a rather loose association between oxygenase component, ferrodoxin, and reductase (45). For the tcbAB genes it was interesting to notice that we could not see expression of tcbAB in E. coli under control of the T7 RNA polymerase from plasmid constructs on which tcbAA was still present, although expression was clearly forced upon the system in this case.

A major gene rearrangement has probably taken place in Pseudomonas sp. strain P51. In this microorganism the genes for the aromatic ring dioxygenase and the dihydrodiol dehydrogenase were most likely transposed from their original position upstream of the todAB genes originally present, although expression was clearly forced upon the system in this case.

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