THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 8, Issue of February 23, pp. 4009–4016, 1996

Printed in U.S.A.

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*

(Received for publication, September 14, 1995, and in revised form, December 5, 1995)

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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 Tn*5280*. 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 dicyclic 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 chlorosubstituted 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 *cis*-dihydrodiol derivative of the aromatic ring structure. A dihydrodiol dehydrogenase then catalyzes the (formal) oxidation of the dihydrodiol to a 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, Tn*5280*[1] (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 nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U15298.

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[1] The abbreviations used are: Tn, transposon; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IS, insertion element; bp, base pair(s); kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

matic 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 *tod*-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 phage preparation, respectively (20). *E. coli* BL21(DE3) was used for T7 RNA polymerase-directed expression of genes and gene fragments cloned in plasmid pET8c (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 elsewhere (17). The source of the DNA fragment 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.

Construction of tcb 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 pET8c (Fig. 1) (21). Hereto, artifical *Nco*I 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 *tcbAa* gene, we amplified a 200-bp DNA fragment ranging from the start of *tcbAa* until the first *Sph*I site downstream. The PCR fragment was then cleaved with *Nco*I and *Sph*I and ligated with a 2-kb *Sph*I-*Sca*I fragment of plasmid pTCB71 (16) and, with pET8c, cut with *Nco*I and *Eco*RV. 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 *tcbAa* gene. From plasmid pTCB113 we then constructed pTCB115 by exchanging the 1.2-kb *Mlu*I-*Sca*I fragment from pTCB113 with the 2.0-kb *Mlu*I-*Pst*I fragment, which contains *tcbAb*, *tcbAc*, and part of *tcbAd*. In plasmid pTCB114 the frame of the *tcbAb* gene was interrupted by cutting the DNA with *Aat*II, filling the ends by using Klenow enzyme, and religating. Plasmid pTCB147 was constructed from pTCB115 by cutting with *Sac*II, removing the 1.0-kb fragment, creating blunt ends with T4 DNA polymerase treatment, and religating. Clones starting with the *tcbAc* gene were made by introducing a *Nco*I site at the start codon of *tcbAc* similarly as described above. A small region of the DNA was amplified by PCR between the start codon of *tcbAc* and the first downstream *Sal*I site, then digested with *Nco*I and *Sal*I, and ligated with a 1.3-kb *Sal*I-*Eco*RI fragment containing the region of *tcbAc* and *tcbAd* and with pET8c, cut with *Nco*I and *Eco*RI. This resulted in plasmid pTCB117. A DNA fragment containing the complete *tcbAd* and *tcbB* sequence was then introduced in plasmid pTCB117 to form pTCB120. A deletion derivative was created which lacked the complete *tcbB* sequence by cutting this plasmid with *Bam*HI and *Sac*I, removing protruding ends, and religating (pTCB116). Finally, a clone containing only *tcbB* was made by cloning a 1.2-kb *Eco*RI-*Mro*I fragment of pTCB119 in pUC19 cut with *Eco*RI and *Sma*I. This plasmid was named pTCB149.

The pET8c-derived plasmids did not, unfortunately, express active Tcb enzymes in *E. coli* (see below). Therefore, we constructed a clone containing the complete *tcbAaAbAcAd* gene sequence in pUC19. Plasmid pTCB115 was hereto cut with *Mlu*I and *Bam*HI, and a 4.7-kb fragment was isolated and ligated with a 3.0-kb *Mlu*I-*Bam*HI fragment of pTCB60. After transformation, this resulted in plasmid pTCB130. We then removed the complete 4.2-kb *Xba*I-*Bam*HI insert of plasmid pTCB130 and ligated this fragment into pUC19, cut with *Xba*I and *Bam*HI. This resulted in plasmid pTCB144. Colonies of *E. coli* containing this plasmid turned blue green when growing on LB plates at 30 °C, which indicates synthesis of active dioxygenase in the cells (11).

Overproduction of Individual Components of the Dioxygenase and Dehydrogenase—To test if the observed ORFs could be translated to proteins of the predicted size, the pET8c-derived plasmids containing the various parts of the *tcb* genes were tested for expression in *E. coli* BL21(DE3). These cultures were grown on LB medium at 37 °C to an A_{550} of 0.5. We then induced T7-mediated gene expression by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM to the cell culture. Cultures were then allowed to grow for another 2 h, after which the cells were harvested from a 1-ml sample. The obtained cell pellet was resuspended in 50 μl of protein sample buffer according to Laemmli (50), and 5–10 μl were analyzed on 12.5% SDS-PAGE.

Analysis of Chlorobenzene Dioxygenase and Chlorobenzene Dihydrodiol Dehydrogenase Activity—Chlorobenzene dioxygenase activity was tested by analyzing the formation of dihydrodiol intermediates from aromatic substrates incubated with washed cell suspensions of *E. coli* DH5α (pTCB144). The cultures were inoculated from a single colony in 200 ml of LB medium with 50 μg/ml ampicillin and grown at 25 °C for about 36 h. The optical density of the cultures had then reached an A_{550} 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 mineral 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 were filled with the following reagents: 4.5 ml of M9 containing 1 mM glucose, 50 μl of a methanol solution containing the aromatic substrates, and 0.5 ml of the cell suspension. The final A_{550} 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 benzoate. 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 A_{550} 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 the change in A_{340} was measured on a spectrophotometer. When no more changes in A_{340} 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 nm, 4 μm). Two running solutions were used which contained: A, 10 mM H_3PO_4 in H_2O 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 and decrease of buffer A to 30%; 30–40 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 p.s.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; 3-methylcatechol, 14.8 min; 1,2-dihydroxy-1,2-dihydronaphthalene (naphthalene dihydrodiol), 13.4 min; 1,2-dihydroxy-3-phenylcyclohexa-3,5-diene (biphenyldihydrodiol), 21.5 min; 2,3-dihydroxybiphenyl, 35.5 min. Authentic standard compounds which were available to us and could be tested were 2,3-dihydroxybiphenyl, 3,4-dichlorocatechol, and 3-methylcatechol.

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).

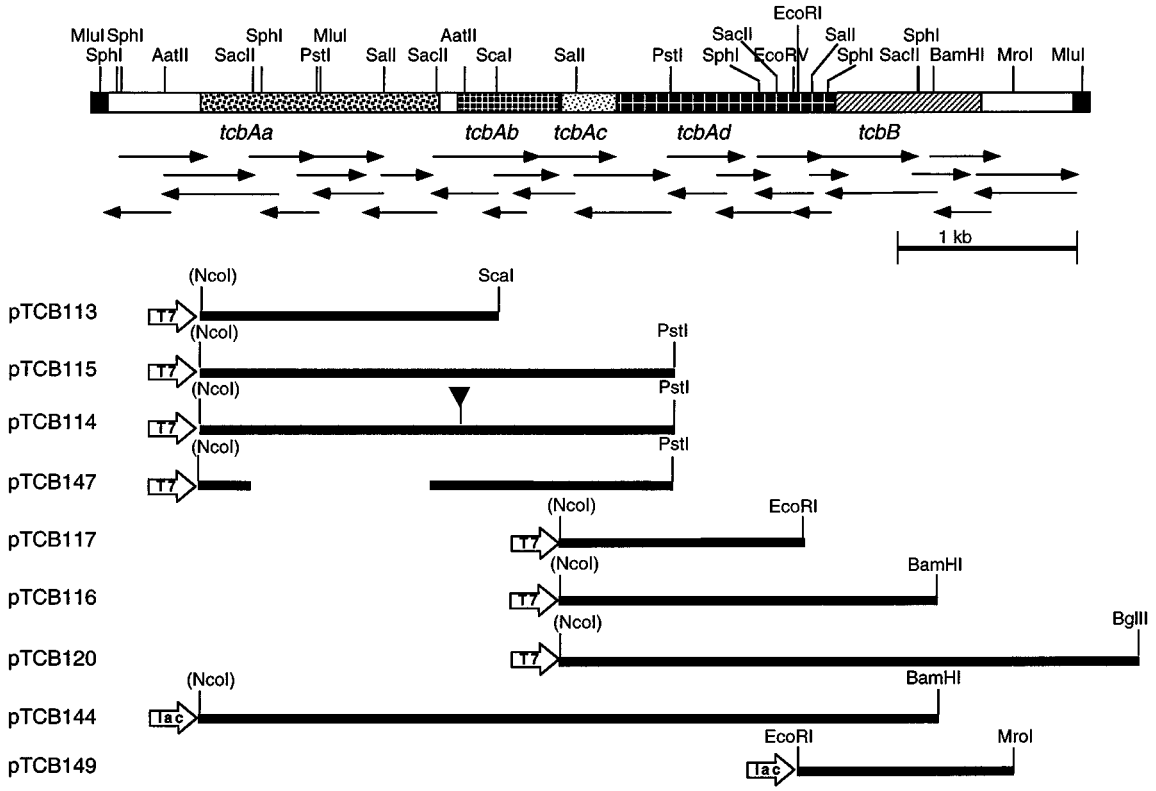

FIG. 1. **Physical map of the region of the *tcbAB* genes and of the different cloned DNA fragments described in this study.** The different hatchings in the physical map indicate the size and location of the ORFs derived from the DNA sequence analysis. The *black ends* on both sides of the physical map indicate the start of the insertion elements, IS*1066* (*left*) and IS*1067* (*right*). The *arrows* below show the size and location of the DNA sequences which were determined from the M13mp18-derived subclones. Relevant restriction sites are indicated on the DNA, but not all vector-located restriction sites are shown. Restriction sites within *brackets* point to the *Nco*I sites which were introduced by PCR to facilitate cloning. The *open arrows* in front of the cloned DNA fragments symbolize the different promoters of the used vector, *i.e. T7*, T7 promoter of pET8c; *lac*, *lac* promoter of pUC18.

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 IS*1066* and IS*1067* (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 IS*1067*, 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. globerulus* 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

TABLE I
Genes and gene products of the chlorobenzene dioxygenase and dihydrodiol dehydrogenase

Gene	ORF (nucleotide number in Fig. 2)	Calculated molecular mass	Observed molecular mass	Function
		kDa		
tcbAa	475–1827	54.1	55	Terminal oxygenase large subunit
tcbAb	1937–2500	22.9	24	Terminal oxygenase small subunit
tcbAc	2509–2832	12.9	14	Ferredoxin
tcbAd	2832–4064	49.3	48	Reductase
tcbB	4061–4888	33.1	22	Dehydrogenase

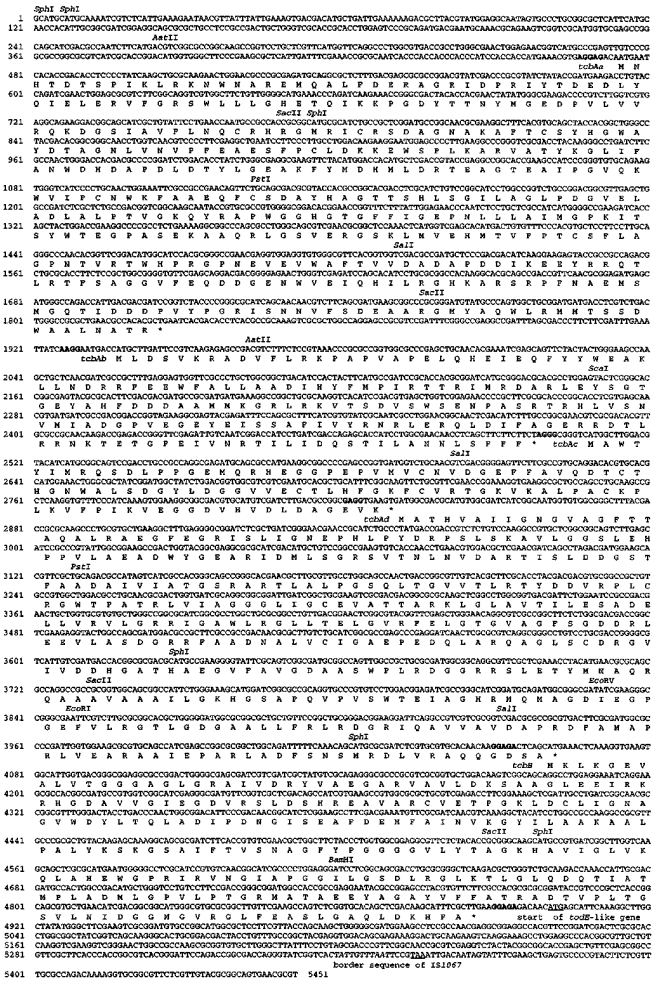

FIG. 2. **DNA sequence of the coding strand of the 5,451-bp *Sph*I-*Mlu*I fragment containing the *tcbAB* genes and the predicted amino acid translation of the ORFs encoding the individual protein subunits.** Relevant restriction sites are indicated, as well as DNA sequences which could function as ribosome binding sites (in *bold*). The putative start codon of the ORF with homology to the sequence of *todE* is shown downstream of *tcbB*. The sequence in *italics*, starting at position 5,338, indicates the border repeat sequence of IS*1067*. The stop codon, which is introduced by the insertion of IS*1067* in this ORF, is shown *underlined* at position 5,353.

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 is found with the family of the naphthalene dioxygenases. Here the gene order is reductase, ferredoxin, large subunit of the terminal oxygenase, small subunit, dehydrogenase. In the *dox*-encoded system, no (clustered) gene for a reductase was described (29), but this may similarly be located directly upstream. The naphthalene reductases (*i.e. nahAa* and *pahAa*) are also substantially shorter than those of the others (329 amino acids *versus* approximately 410) and biochemically different (7). In the case of the benzoate and toluate dioxygenase, 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 *tcbAb* 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 *tcb* 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 (not shown). We think that this may be caused by the unbalanced expression of the different ORFs when expressed from the T7 promoter (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 cells. The UV spectra of the intermediates of toluene, naphthalene, and biphenyl incubation on HPLC were in agreement with the λ_{max} values published previously for the corresponding dihydrodiols (36–38). For the product of 1,2-dichlorobenzene incubation, we observed a UV spectrum similar to that of toluene, although with a λ_{max} at 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

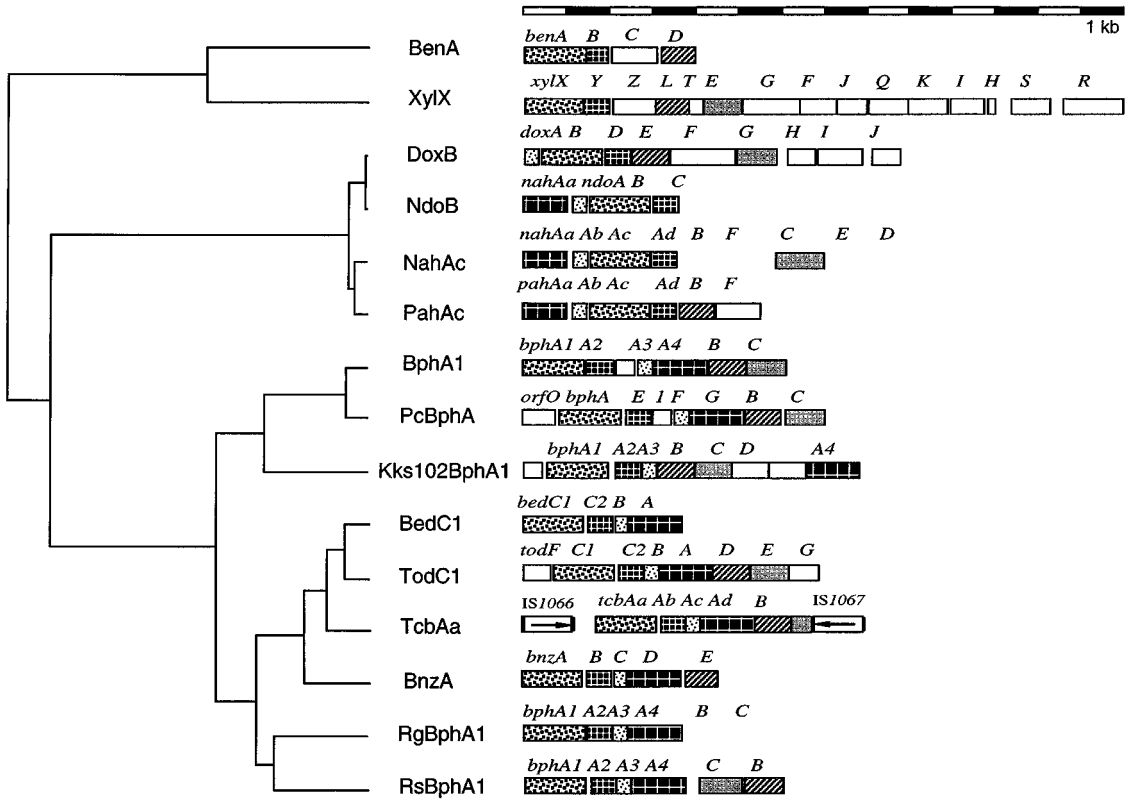

FIG. 3. **PILEUP clustering of an amino acid sequence alignment predicted from the gene sequence of a number of aromatic ring dioxygenases for the terminal oxygenase large subunits (at a gap creation penalty of 3.0 and a gap extension cost of 0.1) (24).** The *right part* of the figure shows the genetic organization of the aromatic ring dioxygenases and surrounding genes. *Horizontal bars* indicate the size and location of the ORFs on the DNA of these organisms. Similar *hatchings* and *shadings* represent homologous genes and derived gene products. Symbols: *checkered box*, large subunit of the terminal oxygenase (ISP_{large}); *waffled box*, small subunit of the terminal oxygenase (ISP_{small}); *dotted box*, ferredoxin; *white on black lined box*, reductase; *diagonally lined box*, dihydrodiol dehydrogenase; *shaded box*, *meta*-cleavage enzyme. The figure does not show the location of all genes within a particular gene cluster. *ben* genes, *Acinetobacter calcoaceticus* (9); *xyl*, *P. putida* mt2 (3, 10); *dox*, Ref. 29; *ndo*, *P. putida* NCIB9816 (26, 27); *nah*, *P. putida* G7 (26); *pah*, (28); *bph*, *P. pseudoalcaligenes* KF707 (30, 48); *PcBph*, *bph* genes of *Pseudomonas* sp. strain LB400 (32, 49); *Kks102Bph*, *bph* genes of *P. paucimobilis* strain KKS102 (31); *bed*, *P. putida* ML2 (33); *tod*, *P. putida* F1 (23, 46); *tcb*, *Pseudomonas* sp. strain P51; *bnz*, *P. putida* 136-R3 (13); *RgBph*, *bph* genes of *Rhodococcus globerulus* P6 (35); *RsBph*, *bph* genes of *Rhodococcus* sp. RHA1 (34).

four mass spectra gave a similar fragmentation pattern with the molecular ions showing at *m/z* 324, 270, 332, and 306 for the products of dichlorobenzene (Fig. 6*A*), toluene (Fig. 6*B*), biphenyl (Fig. 6*C*), and naphthalene (Fig. 6*D*), 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 ($[(CH_3)SiOCHOSi(CH_3)_3]^+$), 147 ($[(CH_3)_2SiOSi(CH_3)_3]^+$), and 73 ($[(CH_3)_3Si]^+$) 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 3,4-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. Benzoate 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 incu-

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

Denominator in the calculation was the length of the shorter sequence without gaps (24). Circles show a graphic representation with a circle diameter propertional 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/benzene family.

A

	BenA	XylX	DoxB	NdoB	NahAc	PahAc	BphA1	PcBphA1	BphA1 Kks102	BedC1	TodC1	TcbAa	BnzA	RgBphA1	RsBphA1
BenA	1.0000	0.7473	0.4044	0.4044	0.4022	0.4044	0.4130	0.4065	0.3922	0.4013	0.3978	0.3947	0.3764	0.3883	0.3991
XylX	⊕	1.0000	0.4200	0.4200	0.4156	0.4133	0.3956	0.3056	0.3802	0.4013	0.4000	0.3947	0.3786	0.3846	0.3890
DoxB	○	○	1.0000	0.9978	0.9733	0.9644	0.4733	0.4733	0.4711	0.5000	0.4978	0.5000	0.4811	0.5022	0.5089
NdoB	○	○	◍	1.0000	0.9733	0.9644	0.4733	0.4733	0.4711	0.5000	0.4978	0.5000	0.4811	0.5022	0.5089
NahAc	○	○	◍	◍	1.0000	0.9800	0.4778	0.4778	0.4689	0.5000	0.5000	0.5000	0.4833	0.5022	0.5089
PahAc	○	○	◍	◍	◍	1.0000	0.4756	0.4756	0.4689	0.4956	0.4956	0.4956	0.4811	0.5000	0.5067
BphA1	○	○	○	○	○	○	1.0000	0.9739	0.8431	0.7894	0.7933	0.7849	0.7261	0.7457	0.7804
PcBphA1	○	○	○	○	○	○	●	1.0000	0.8301	0.7827	0.7867	0.7805	0.7194	0.7413	0.7783
BphA1 Kks102	○	○	○	○	○	○	●	●	1.0000	0.7694	0.7644	0.7428	0.6971	0.7146	0.7473
BedC1	○	○	○	○	○	○	◯	◯	◯	1.0000	0.9733	0.9357	0.8976	0.8137	0.8559
TodC1	○	○	○	○	○	○	◯	◯	◯	▨	1.0000	0.9556	0.9243	0.8267	0.8689
TcbAa	○	○	○	○	○	○	◯	◯	◯	▨	▨	1.0000	0.8797	0.8160	0.8603
BnzA	○	○	○	○	○	○	◯	◯	◯	▨	▨	▨	1.0000	0.7572	0.7929
RgBphA1	○	○	○	○	○	○	◯	◯	◯	▨	▨	▨	◯	1.0000	0.8525
RsBphA1	○	○	○	○	○	○	◯	◯	◯	▨	▨	▨	◯	▨	1.0000

B

	NahAa	PahAa	BphA4	PcBphG	BphA4 Kks102	BedA	TodA	TcbAd	BnzD	RgBphA4	RsBphA4
NahAa	1.0000	0.9666	0.3070	0.3070	0.2371	0.3161	0.3131	0.3070	0.3131	0.2827	0.3070
PahAa	◍	1.0000	0.3009	0.3009	0.2401	0.3131	0.3100	0.3009	0.3100	0.2705	0.3040
BphA4	○	○	1.0000	0.9976	0.4792	0.6675	0.6724	0.6626	0.6553	0.6601	0.6650
PcBphG	○	○	●	1.0000	0.4817	0.6015	0.6724	0.6626	0.6553	0.6601	0.6650
BphA4 Kks102	○	○	◯	◯	1.0000	0.4720	0.4818	0.4720	0.4756	0.4574	0.4623
BedA	○	○	◯	◯	◯	1.0000	0.8856	0.8491	0.6415	0.6691	0.7129
TodA	○	○	◯	◯	○	▨	1.0000	0.8905	0.9780	0.6691	0.7178
TcbAd	○	○	◯	◯	○	▨	▨	1.0000	0.8707	0.6788	0.7129
BnzD	○	○	◯	◯	○	◯	▨	▨	1.0000	0.6512	0.7024
RgBphA4	○	○	◯	◯	○	◯	◯	◯	◯	1.0000	0.6973
RsBphA4	○	○	◯	◯	○	◯	◯	◯	◯	◯	1.0000

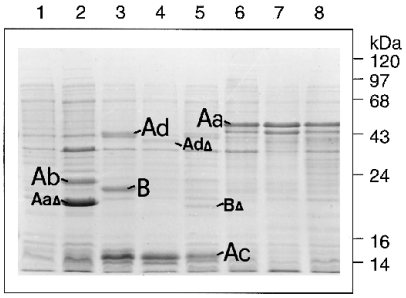

FIG. 4. **SDS-PAGE of the cell extracts from *E. coli* BL21(DE3) strains containing the different plasmids with *tcbAB* genes.** *Lanes: 1*, pET8c; *2*, pTCB147 (*tcbAb*, *tcbAc*, and deletion of *tcbAa*); *3*, pTCB120 (*tcbAc*, *tcbAd*, and *tcbB*); *4*, pTCB117 (*tcbAc* and a deletion of *tcbAd*);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

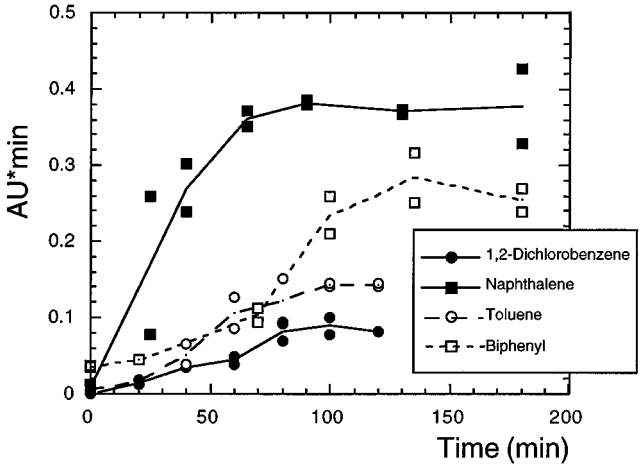

FIG. 5. **Formation of dihydrodiol intermediates by washed whole cells of *E. coli* (pTCB146).** Shown are mean values of peak areas of the intermediates as measured on HPLC from two independent incubations. Formation of the dihydrodiols excreted in the supernatant was measured as an increase in absorbance at the wavelength of the respective absorption maximum, *i.e.* 262 nm for naphthalene dihydrodiol, 265 nm for toluene dihydrodiol, 272 nm for 1,2-dichlorobenzene dihydrodiol, and 303 nm for biphenyl dihydrodiol.

bations suggests that naphthalene and biphenyl are converted even faster than 1,2-dichlorobenzene and toluene. However, we do not know in what way solubility of these compounds, different uptake, and excretion rates influence this outcome. We cannot exclude that other products, such as other stereoisomers or monohydroxylated rings, were formed in the incubations, since no attempts were made to determine a mass balance in the whole cell incubations. Furthermore, we did not determine the absolute stereoconfiguration of the products. HPLC analysis, however, suggested the formation of single intermediates. UV and mass spectrum of these compounds were in agreement with the structures of *cis*-dihydrodiols, as published previously (36–38). In the subsequent enzyme incubations, we obtained good evidence that the TcbB 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 biphenyls-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.

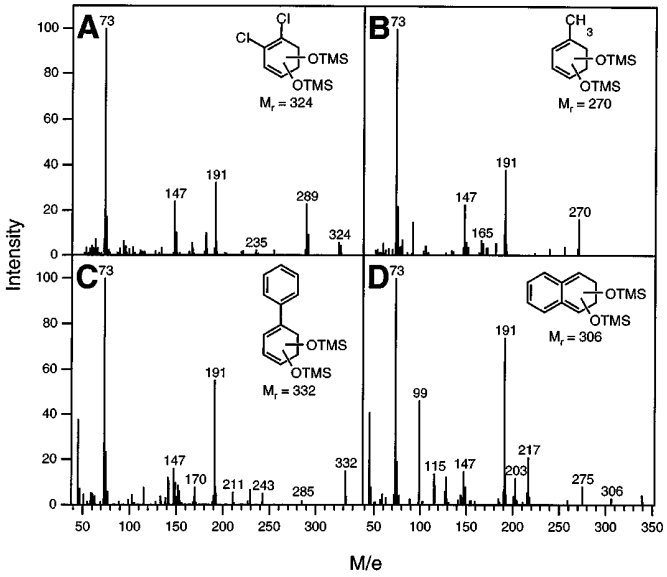

FIG. 6. **Electron ionization mass spectra of the trimethylsilyl derivatives of the products of whole cell incubations with *E. coli* (pTCB144) and the following substrates.** *A*, 1,2-dichlorobenzene; *B*, toluene; *C*, biphenyl; *D*, naphthalene. The structural formula of the proposed product has been drawn above the respective mass spectrum. The fragmentation pattern is discussed in the text.

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

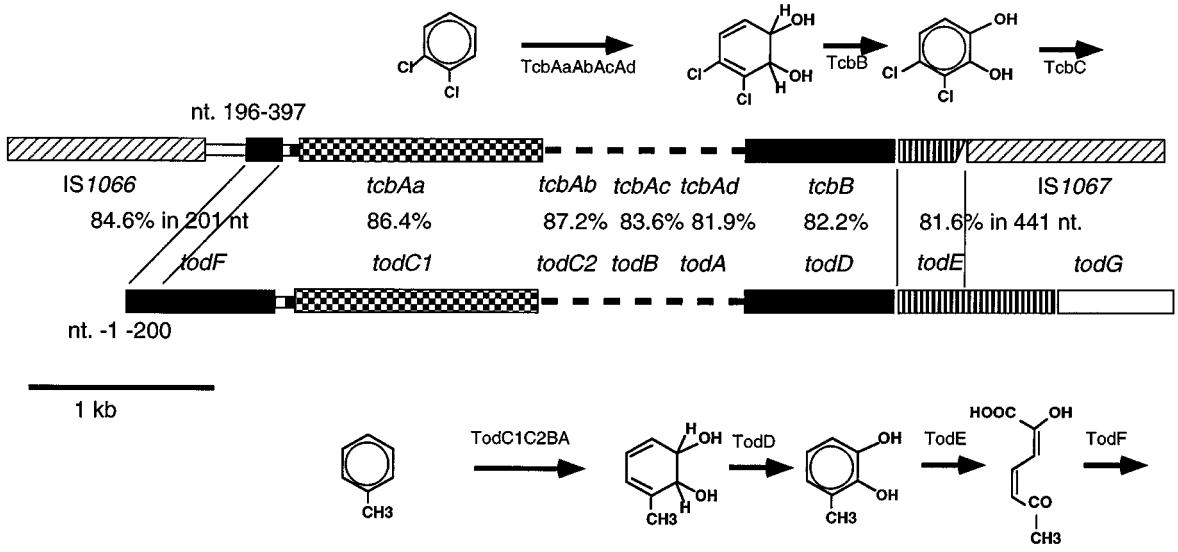

FIG. 7. **Comparison of the *tcbAB* genes and part of the *tod* gene clusters.** Shown is a physical map of the most important features in this comparison. *Blocks* indicate size and location of ORFs on the DNA encoding the different proteins. Similar *shadings* or *hatchings* mean homologous regions in both gene clusters. Directly upstream of *tcbAa* there are 35 bp (indicated in *black*) with homology to the upstream region of *todC1*. Some 30 bp further upstream is a region of 201 bp with homology to *todF*, although from the start of this gene. Percentages of identity in nucleotide sequence between the different gene regions are given in the figure. Downstream of *tcbB* is the part of the gene with homology to *todE*. The sizes and locations of the ORFs for *tcbAb*, *tcbAc*, and *tcbAd*, respectively, *todC2*, *todB*, and *todA* are not shown in detail here.

to improper signals on the DNA or changed stability and structure of the mRNA. 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/ferredoxin/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, ferredoxin, 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 by the activity of two insertion elements (19). The relics of this process can still be seen on the DNA (Fig. 7). Upstream of the gene *tcbAa* there are parts of a gene similar to *todF* (46), and downstream of *tcbB* there is a large part of a gene for a *meta*-cleavage enzyme similar to *todE* (23). It may have been a necessity for the organism to inactivate such a *meta*-cleavage enzyme since these are known in some microorganisms to interfere with the metabolically productive *ortho*-cleavage of chlorinated catechols, which arise as intermediates in chlorobenzene degradation (47). We believe that the strong genetic and biochemical similarity between the *tcbAB* and the *tod* system is good evidence to assume that the *tcbAB* genes originated in a microorganism which could use toluene or benzene as the sole carbon and energy source.

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