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Genetic and Biochemical Analyses of the *tec* Operon Suggest a Route for Evolution of Chlorobenzene Degradation Genes

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The TecA broad-spectrum chlorobenzene dioxygenase of *Burkholderia* sp. strain PS12 catalyzes the first step in the mineralization of 1,2,4,5-tetrachlorobenzene. The catabolic genes were localized on a small plasmid that belongs to the IncPβ incompatibility group. PCR analysis of the genetic environment of the *tec* genes indicated high similarity to the transposon-organized catabolic *tcb* chlorobenzene degradation genes of *Pseudomonas* sp. strain P51. Sequence analysis of the regions flanking the *tecA* genes revealed an upstream open reading frame (ORF) with high similarity to the *todF* 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase gene of *Pseudomonas putida* F1 and a discontinuous downstream ORF showing high similarity to the *todE* catechol 2,3-dioxygenase gene of strain F1. Both homologues in strain P51 exist only as deletion remnants. We suggest that different genetic events thus led to inactivation of the perturbing *meta*-cleavage enzymes in strains P51 and PS12 during the evolution of efficient chlorobenzene degradation pathways. Biochemical characterization of TodF-like protein TlpF and a genetically refunctionalized TodE-like protein, TlpE, produced in *Escherichia coli* provided data consistent with the proposed relationships.

Considerable quantities of xenobiotics are released each year into the environment. Bacteria that are able to use many of these compounds as sole sources of carbon and energy have been isolated from natural habitats (33). The aerobic metabolism of aromatic compounds is frequently initiated by dioxygenases, followed by a dehydrogenation reaction catalyzed by a *cis*-dihydrodiol dehydrogenase to give catechols or substituted catechols (17), which serve as substrates for oxygenolytic cleavage of the aromatic ring (21). Chlorocatechols are usually channelled into the Krebs cycle by modified *ortho*-cleavage pathways, whereas methylcatechols are most commonly metabolized by *meta*-cleavage routes (27, 32, 34).

Experimental combination of genes encoding the first two enzymes of a toluene degradation pathway with those coding for chlorocatechol degradation produced a functional metabolic sequence for the mineralization of chlorobenzenes via a modified *ortho* pathway (31). It has been proposed that the 1,2,4-trichlorobenzene degrader *Pseudomonas* sp. strain P51 evolved by recruitment of the *tod* pathway genes of the toluene-degrading bacterium *Pseudomonas putida* F1, followed by mutational drift of the *todCBA* toluene dioxygenase and *todD* dehydrogenase genes to yield the *tcb* genes encoding the first two enzymes in the transformation of chlorobenzenes (40–44). Sequences flanking the *tcb* genes were suggested to be evolutionary remnants of the *todE* extradiol dioxygenase and *todF* hydrolase genes, which had become inactivated by major DNA deletions such that misrouting of catechol into the unproductive *meta* pathway would no longer be possible (44).

Although a number of chlorobenzene-degrading bacteria have been isolated over the past few years, very few, such as *Burkholderia* sp. strain PS12, are able to degrade tetrachlorobenzene (5). The evolution of such bacteria able to degrade higher chlorinated xenobiotics is of interest not only from the environmental and biotechnological points of view but also from the genetic point of view. We investigated the localization, organization, and sequences of genes that flank the *tecAB* chlorobenzene degradation genes of strain PS12, which led us to propose an evolutionary route from the *tod* toluene degradation gene to the *tec* and *tcb* chlorobenzene degradation genes.

Southern analysis of *Bam*HI-, *Bgl*II-, and double-digested PS12 total DNA revealed a single band, indicating that the *tecA* gene is present at only one locus (Fig. 1A). To analyze whether the *tec* genes reside on a plasmid, PS12 total DNA prepared as described previously (38) was separated in a 0.9% agarose gel by pulsed-field gel electrophoresis (PFGE) (36). The presence of three plasmids, designated pPS12-1, pPS12-2, and pPS12-3, all of which were present in two forms and all of which are smaller than the 50-kbp reference plasmid of *Comamonas testosteroni* T-2 used as a size marker (Fig. 1B), was observed. The *tecA* genes were localized by Southern blotting (Hybond N; Amersham) of the pulsed-field gel and hybridization under stringent conditions with the *tecA1* gene probe derived by PCR amplification from plasmid pSTE7 with primers prSTB1 and prSTB2 (forward, atgaatcacaccgacacctcccct; reverse, tcagcgtgtggcgttcagcgcggc). Positive signals were obtained with the two forms of plasmid pPS12-1 (Fig. 1C).

Plasmids of the IncP incompatibility group often carry catabolic genes. In order to determine if strain PS12 contains an IncP plasmid and to classify it, PCR was carried out on PS12 total DNA and reference plasmids RP4 (IncPα) (10) and R751 (IncPβ) (26) by using as templates the previously described conserved primers prSTB59 and prSTB60 (forward, cgaaatt crtrtgggagaagta; reverse, cgyttgcaatgcaccaggtc); prSTB61 and prSTB62 (forward, atgaagaaacggctnaccga; reverse, ttcctgtt tyytcttggcgtc), and prSTB63 and prSTB64 (forward, cagcctcgc agagcaggat; reverse, cagccgggcaggataggtgaagt) (18), which are based on replicon-specific DNA regions (*trfA2*, *korA*, and *oriT*). Pairwise sequence alignments (data not shown) of all three PS12-derived PCR products showed higher similarities to the corresponding sequences of IncPβ plasmid R751 (90, 90, and 91% nucleotide sequence identity, respectively) than to those of IncPα plasmid RP4 (85, 77, and 70% nucleotide sequence identity, respectively).

The PFGE Southern blot membrane, after removal of *tecA1*,

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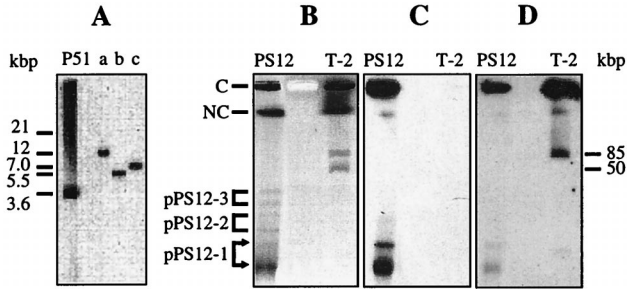


FIG. 1. Southern analysis of PS12 DNA. Total DNA of 1,2,4,5-tetrachlorobenzene-grown *Burkholderia* sp. strain PS12 (lane PS12) was digested with *Bam*HI (lane a), *Bam*HI-*Bgl*II (lane b), and *Bgl*II (lane c) and electrophoretically separated on an agarose gel. The *tcbAaAbAcAd* dioxygenase genes (lane P51) from *Pseudomonas* sp. strain P51 were amplified from plasmid pTBCB60 (42) with primers prSTB1 and prSTB4 (forward, **atg**aatcacaccgacacctcccct [the *tecA1* start codon is in boldface]; reverse, **tca**tgctgagtctccttgttgtgc [the *tcbAd* stop codon is in boldface]) and were used as a positive control. The gel was analyzed by Southern hybridization with the *tecA1* gene probe of PS12 (A). Total DNAs from strain PS12 and toluenesulfonate-grown *C. testosteroni* T-2 (lane T-2) were subjected to PFGE, stained with ethidium bromide (B), and analyzed by Southern hybridization with the *tecA1* gene probe of PS12 (C) and the IncPβ-specific *trfA2* gene probe derived from plasmid R751 (D). The bracket arrow indicates the positions of the two PS12-1 plasmid species which gave signals with both probes. The positions of other plasmids are indicated by horizontal bars. The reference plasmids of strain T-2 (pTSA [85 kb, IncPβ] and pT2T [50 kb, unknown incompatibility group]) served as DNA molecular size markers and as controls for probe specificity in Southern experiments. In addition, the positions of the slots containing chromosomal (C) and nicked chromosomal (NC) DNAs are indicated. Excess blank lanes between the PS12 and T-2 samples were digitally removed with Photoshop software (Adobe).

was reprobed under stringent conditions with the IncPβ-specific *trfA2* probe. The probe hybridized strongly with the positive control (85-kbp plasmid pTSA) and with both forms of plasmid pPS12-1 (Fig. 1D), indicating that the plasmid on which the *tec* genes reside belongs to the IncPβ subgroup.

A PCR strategy was used to analyze the genetic organization flanking the previously described 5.5-kb gene cluster of *Burkholderia* sp. strain PS12 containing the *tecA* genes, an open reading frame (ORF) encoding a putative protein with similarity to the TodF hydrolase and another ORF for a truncated TecB dehydrogenase (5). Oligonucleotide primers were designed on the basis of known sequences from strain PS12 (5) and *Pseudomonas* sp. strain P51 (40, 41, 43, 44). The sizes of fragments obtained by PCR with genomic DNA from PS12 as the template (Fig. 2B) suggest an organization in the vicinity of the *tec* genes similar to that of transposon Tn*5280* (43) (Fig. 2C). This transposon (Fig. 2A) harbors the genes encoding TcbA chlorobenzene dioxygenase and TcbB dehydrogenase of strain P51 (42, 44) in the vicinity of the chlorocatechol degradation genes (40) and a LysR-type regulator gene (41). Our experiments indicated that an entire *tecB* gene is located downstream of the *tecA* chlorobenzene dioxygenase genes. The distance between *tecB* and the end of a putative insertion element is approximately 0.4 kb more than was found in strain P51. This suggested the presence of a full-length version of a catechol 2,3-dioxygenase gene, in contrast to the structure in strain P51, which has only a gene remnant. Also, a complete *tlpF* hydro-

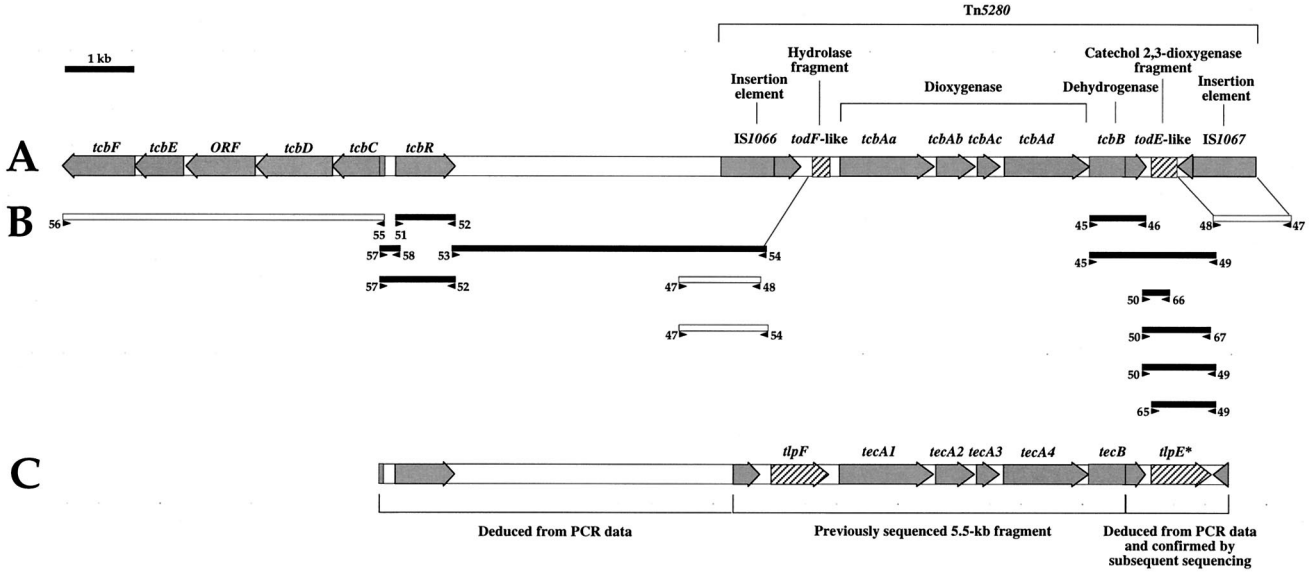


FIG. 2. PCR analysis of the genetic environment of the *tecA* genes of *Burkholderia* sp. strain PS12. The sequence context of a previously characterized 5.5-kb genomic DNA fragment from strain PS12 (5), which is similar to the corresponding sequence of *Pseudomonas* sp. strain P51 (A), was analyzed by PCR (B). The PS12 sequence comprises the *tecA1A2A3A4* genes encoding the tetrachlorobenzene dioxygenase, the truncated *tecB* gene encoding the *cis*-chlorobenzene dihydrodiol dehydrogenase, the truncated IS*1066* homologue, the *tlpF* 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase gene, and the inactivated *tlpE** catechol 2,3-dioxygenase pseudogene (C). The primers were designed on the basis of known sequences of *Pseudomonas* sp. strain P51 and *Burkholderia* sp. strain PS12. PCR products are drawn as black boxes, whereas white boxes indicate failure to obtain a product of the expected size. The numbers and arrows below the boxes refer to prSTB primer numbers and priming direction, respectively, as follows: prSTB45, atgaaactcaaaggtgaagtg, (containing the *tecB* start codon); prSTB46, **tca**gcgaaatgcttgtcgag (containing the *tecB* stop codon [boldface]); prSTB47, ctagtgtttaattcgtcatg (containing the IS*1066* inverted repeat [underlined]); prSTB48, ctagtg**ttta**attccgtaaattg (containing the IS*1066* inverted repeat [underlined] and stop codon [boldface]); prSTB49, caatttacggaat**taa**acaatag (containing the IS*1067* inverted repeat [underlined] and stop codon [boldface]); prSTB50, gctcgacaagcatttcgct**tga** (containing the *tecB* stop codon [boldface]); prSTB51, **atg**gaattccggcagctcaag (containing the *tcbR* start codon [boldface]); prSTB52, **tca**gtccttcgcggatcgccgc (containing the *tcbR* stop codon [boldface]); prSTB53, gcggcgatccgcgaaggac**tga** (containing the *tcbR* stop codon [boldface]); prSTB54, gatcgccgcaatgtggttgcat (containing nucleotides −60 to −82 of the *tlpF* gene); prSTB55, **atg**aacgaacgagtgaagcagg (containing the *tcbC* start codon [boldface]); prSTB56, g**tca**gggttgcggtggctcc (containing the *tcbF* stop codon [boldface]); prSTB57, cctgcttcactcgttcgtt**cat** (containing the *tcbC* start codon [boldface]); prSTB58, cttgagagctg ccggaattc**cat** (containing the *tcbR* start codon [boldface]); prSTB65, c**atg**agcattcaaagattgggctac (containing the *todE* start codon [boldface]); prSTB66, gtgacctaagccct ggtctccag (containing the middle region of *todE*); prSTB67, g**tca**ggcgggcgcctggaac (containing the *todE* stop codon [boldface]). The deduced genetic environment of the 5.5-kb fragment of strain PS12 is indicated 5′ and 3′ with respect to the previously obtained sequence. Gene names are shown above the respective ORFs, which are shown as grey arrows. Hatched boxes indicate sequences exhibiting high similarity to the *meta*-cleavage pathway *todE* and *todF* genes of *P. putida* F1.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Sources, reference(s), or structure
Strains		
E. coli DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+)	Clontech
DH5α	*recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ*80*δ*lacZ*Δ*M15* F^- λ^-	
E. coli J53	Met Pro; host for IncP reference plasmids	9
Burkholderia sp. strain PS12	Growth on 1,2,4,5-tetrachlorobenzene	5, 37
C. testosteroni T-2	pTSA (85 kb, IncPβ), pT2T (50 kb)	39
Host vectors		
pCR2.1	Ap^r Km^r	Invitrogen
pBluescript II KS(+)	Ap^r	Stratagene
Plasmids used for cloning and genetic refunctionalization of *tlpE** catechol 2,3-dioxygenase pseudogene		
pCR12	*tecB* dehydrogenase gene and *tlpE** pseudogene	PCR-amplified 1.8-kb fragment from PS12 total DNA with primers prSTB45 and prSTB49 in pCR2.1
pCR18	*tlpE* catechol 2,3-dioxygenase gene	0.9-kb fragment obtained from third round of splicing by overlap extension-pCR in pCR2.1
pSTE3	*tlpF* hydrolase gene, *tecA1A2A3A4* chlorobenzene dioxygenase gene, 3′-truncated *tecB* dehydrogenase gene	5
pSTE56	Expression of *tlpE* catechol 2,3-dioxygenase gene	1.0-kb *Kpn*I-*Xba*I fragment of pCR18 inserted into *Kpn*I-*Xba*I site of pBluescript II KS(+)
Reference plasmids used for incompatibility group determination		
RP4	60 kb; IncPα; Ap^r Km^r Tc^r	10
R751	53 kb; IncPβ; Tp^r	26

[a] Antibiotic resistances (solutions in milligrams per liter): Ap^r, ampicillin (100); Km^r, kanamycin (50); Tc^r, tetracycline (20); Tp^r, trimethoprim (20). Met, methionine dependent; Pro, proline dependent.

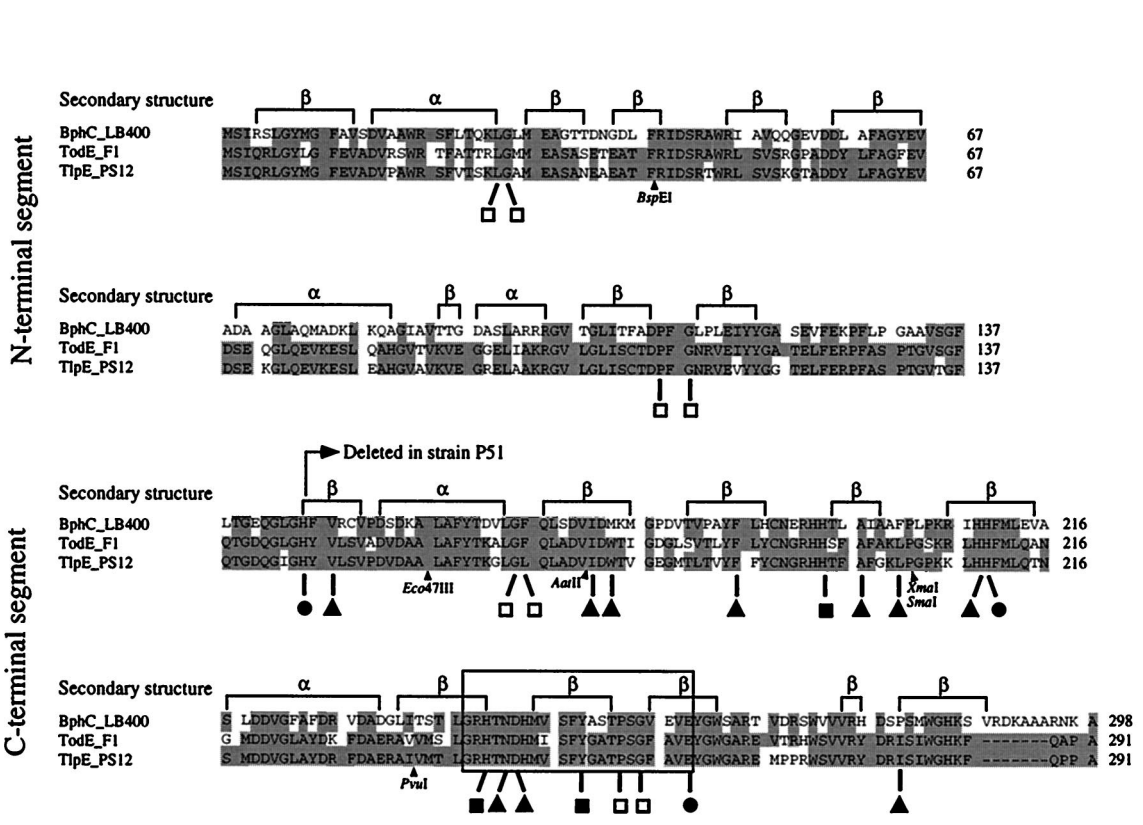
lase homologue was found upstream of the *tecA* genes, in contrast to the remnant found in strain P51.

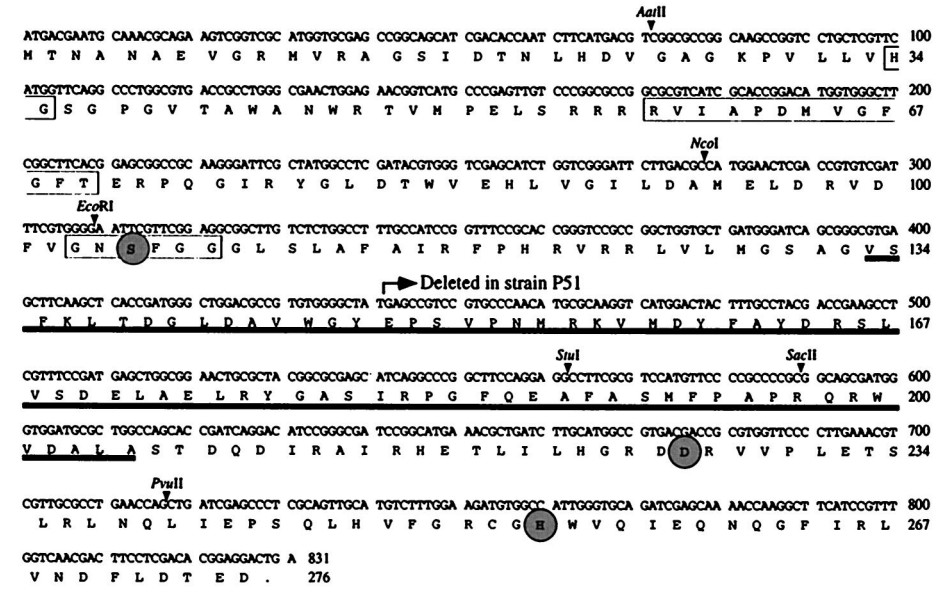
Biochemical analysis of the products of the *tcb* flanking regions in strain P51 was not possible due to the deleted sequences. However, the presence of complete flanking sequences in strain PS12 (Fig. 2C) permitted such studies to assess evolutionary relationships among strains PS12, P51, and F1.

The TecB dehydrogenase located downstream of the *tecA* gene and encoded on plasmid pCR12 (Table 1) has been shown to be active against several *cis*-chlorobenzene dihydrodiols (unpublished data). Downstream of the *tecB* gene is a 0.9-kb sequence which exhibits high similarity (82%) to the *todE* catechol 2,3-dioxygenase gene of *P. putida* F1 (45). This *tlpE** (TodE-like protein E) pseudogene is, however, inactive, and the putative functional gene has apparently been inactivated by mutations introducing one frameshift and two premature translational stops. In order to determine whether corrections of these three defects would result in a functional dioxygenase, we repaired them with equivalent sequences from the *todE* gene by three successive rounds of splicing by overlap extension-PCR (25) using pCR12 as the template and appropriate overlapping oligonucleotide primers prSTB116 and prSTB118 (forward, gaaggagagacaac**atg**agcattcaaagg [the *tlpE** start codon is in boldface]; reverse, ctgaccgatagccg**cca**ggtgcgcg [the codon for Trp that replaces the *tlpE** stop codon is in boldface]), prSTB117 and prSTB120 (forward, ccggatcgactcgc gcacc**tgg**cggctatc [with the *tlpE** stop codon replaced with a codon for Trp [boldface]); reverse, gccgaacgggtc**cgt**acaggaaat aag [with the *tlpE** frameshift cgct replaced with cg-t [boldface]); prSTB119 and prSTB122 (forward, gggcttatttcctgt**acg**g acccgttc [with the *tlpE** frameshift agcg replaced with a-cg [boldface]; reverse, cccagcccttggt**ata**gaaggcgag with the *tlpE** stop codon replaced with a codon for Tyr [boldface]); and prSTB121 and prSTB123 (forward, cagcgctcgccttc**tat**accaagg ggc [with the *tlpE** stop codon replaced with a codon for Tyr [boldface]; reverse, **tca**tgcgggcggctggaacttgtgc [with the *tlpE** stop codon [boldface]), containing the corresponding *todE* wild-type sequences of strain F1. Gel-purified PCR products served as megaprimers in the subsequent amplification reactions. The resulting refunctionalized catechol 2,3-dioxygenase gene, designated *tlpE* (TodE-like protein E), was subsequently cloned into pCR2.1 to produce plasmid pCR18 and subcloned into pBluescript II KS(+) to produce plasmid pSTE56.

The polypeptide sequences of the refunctionalized TlpE catechol 2,3-dioxygenase and the TodE enzyme exhibited 83% identity and clustered phylogenetically with *meta*-cleavage enzymes preferring polycyclic aromatic substrates (14). Comparison of the polypeptide sequence with the sequence (24) of the corresponding structure-solved BphC enzyme (14, 19) from *Burkholderia* sp. strain LB400 (6) revealed that all residues that are iron ligands or play a structural and direct catalytic role (Fig. 3A) are conserved.

Cell extracts of *Escherichia coli* DH5α(pSTE56) cultures


A
N-terminal segment
C-terminal segment
Secondary structure
BphC_LB400
TodE_FI
TlpE_PS12
BspEI
Deleted in strain P51
Eco47III
AatII
XmaI
SmaI
PvuI


B
AatII
NcoI
EcoRI
Deleted in strain P51
StuI
SacII
PvuII

grown overnight in Luria-Bertani medium (36) containing 0.1-mg/ml ampicillin and 1.0 mM isopropyl-β-D-thiogalactopyranoside were prepared as previously described, and the protein concentration was determined (7). Enzyme activities were assayed spectrophotometrically by monitoring the product formation from catechol (2-hydroxymuconic semialdehyde [2HMSA]; $\varepsilon_{375} = 36\ mM^{-1}\ cm^{-1}$), 3-methylcatechol (2-hydroxy-6-oxohepta-2,4-dienoate [HOHDA]; $\varepsilon_{388} = 16.8\ mM^{-1}\ cm^{-1}$), or 2,3-dihydroxybiphenyl (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate [HOPDA]; $\varepsilon_{434} = 13.2\ mM^{-1}\ cm^{-1}$) (15, 35), respectively, in phosphate buffer (50 mM, pH 7.5).

Refunctionalized TlpE* catechol 2,3-dioxygenase was able to oxidize catechol, 3-methylcatechol, and 2,3-dihydroxybiphenyl to products with the expected absorption maxima at 376, 388, and 436 nm, respectively. The highest V_{max} was obtained with 3-methylcatechol (1,200 ± 60 μM min^{-1} g^{-1}), followed by catechol (715 ± 16 μM min^{-1} g^{-1}) and 2,3-dihydroxybiphenyl (365 ± 38 μM min^{-1} g^{-1}), which is the same order found with the TodE of strain F1 (23). However, TlpE dioxygenase shows the highest substrate preference, expressed by the relative specificity constant V_{max}/K_m (16), for 2,3-dihydroxybiphenyl (given as 100), followed by 3-methylcatechol (65) and catechol (0.6), which reflects the low K_m values for 3-methylcatechol (1 ± 0.2 μM) and especially 2,3-dihydroxybiphenyl (0.2 ± 0.1 μM) compared to catechol (57 ± 4 μM). As high concentrations of 3-methylcatechol inhibited the TlpE enzyme, a substrate inhibition model (1, 22) was used to calculate the kinetic parameters of 3-methylcatechol oxidation. The inhibition constant K_{ss} was determined to be 130 ± 25 μM. Transformation of 3-chlorocatechol by the TlpE dioxygenase was not observed, although 67 nM 3-chlorocatechol reduced the initial oxidation rate of 50 μM catechol by 50% and 1 μM 3-chlorocatechol completely abolished catechol transformation (4, 28). This inhibition indicates that its original role was in the metabolism of methylbenzenes rather than chlorobenzenes.

An ORF identified upstream of the *tecA* gene had high similarity, with 87 and 89% identity on the nucleotide and polypeptide levels, respectively (5), to the *todF* gene from *P. putida* F1 (30). This gene, designated *tlpF*, is very similar to the corresponding *meta*-cleavage pathway gene fragment found in *Pseudomonas* sp. strain P51 (99.5% similarity in 201 nucleotides) (44). Pairwise sequence comparison with other bacterial hydrolases involved in the degradation of aromatic compounds and a human serine hydrolase indicates that the TlpF hydrolase belongs to the group of serine hydrolases involved in the *meta*-cleavage pathway for mononuclear aromatics (Fig. 3B).

Functional expression of the recombinant TlpF HOHDA hydrolase in *E. coli* DH5α was shown by the conversion of 2HMSA, HOHDA, and HOPDA in reaction mixtures containing an extract of *E. coli* DH5α(pSTE3) cells prepared as described above for *E. coli* DH5α(pSTE56) cells. The *meta*-cleavage products which were used as TlpF substrates were produced from catechol, 3-methylcatechol, and 2,3-dihydroxybiphenyl by transformation of 1 mM solutions in phosphate buffer with *E. coli* DH5α(pSTE56) cells grown and washed as described above and resuspended to an A_{600} of 10.

The highest V_{max} was obtained with HOHDA (3,350 ± 250 μM min^{-1} g^{-1}), followed by 2HMSA (800 ± 40 μM min^{-1} g^{-1}), which is the same order found with the 2-hydroxymuconic semialdehyde hydrolase from TOL plasmid pWW0 of *P. putida* mt-2 (13). The K_m values were 4.4 ± 1.1 and 70 ± 5 μM, respectively. Hydrolysis of HOPDA (<5 μM min^{-1} g^{-1}) was too low for kinetic analysis. In contrast to the substrate preference observed with TlpE dioxygenase, Tlp hydrolase prefers the monocyclic aromatic-derived substrates. Therefore, both sequence analysis and biochemical properties show that the TodF and TlpF proteins cluster with hydrolases involved in the degradation of monocyclic aromatics, whereas the TodE and TlpE enzymes cluster with dioxygenases involved in the degradation of bicyclic aromatics. If polycyclic aromatics that transform enzymes have evolved from those that oxidize monocyclic aromatics, as suggested by Harayama and Rekik (20), the TodF and TlpF hydrolases may be evolutionarily older than the TodE and TlpE dioxygenases. The *tod* operon itself may thus be a mosaic of genes derived from different pathways for mono- and bicyclic substrates.

In summary, both primary structure analysis and biochemical data strongly suggest that the *tec* and *tcb* chlorobenzene degradation genes are closely related and that they diverged rather recently. They seem to have their common origin in the *tod* genes (or a common precursor) of the toluene degradation pathway and have adapted for efficient chlorobenzene transformation. The flanking evolutionary gene relics are most likely descendants of the corresponding *todE* and *todF* genes. Characterization of these evolutionary relics revealed different mutational events—deletions in the case of P51 and point mutations leading to a frameshift and the introduction of stop codons in the case of PS12—having caused inactivation of the *meta*-cleavage genes disadvantageous for chlorobenzene degradation during subsequent divergence of the two strains.

Nucleotide sequence accession numbers. The new nucleotide sequences presented here have been deposited in the GenBank database under accession no. AF073901 (*trfA2*), AF073902 (*korA*), and AF073903 (*oriT*) for the IncPβ-specific sequences of plasmid pPS12-1.

The sequence of the refunctionalized *tlpE* dioxygenase gene is available under GenBank accession no. AF073900. The previously deposited 5.5-kb sequence containing the *tlpF* HOHDA hydrolase, the *tecA* chlorobenzene dioxygenase, and the partial *tecB cis*-chlorobenzene dihydrodiol genes (U78099) has been updated and now includes the entire *tecB* gene and the *tlpE** catechol 2,3-dioxygenase pseudogene.

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FIG. 3. Sequence alignment and structural features of catechol 2,3-dioxygenases and nucleotide and deduced amino acid sequences of the TlpF hydrolase. (A) Alignment of extradiol dioxygenases of *Burkholderia* sp. strain LB400 (BphC_LB400) and *P. putida* F1 (TodE_F1) and the refunctionalized TlpE protein of *Burkholderia* sp. strain PS12 (TlpE_PS12). Based on the solved structure of the BphC enzyme, two domains (N and C terminal) with similar secondary structures can be distinguished (19), as indicated above the alignment. Identical residues are shaded. The amino acid ligands of the catalytic Fe(II) are marked (●), and those playing a direct catalytic role in the LB400 enzyme are indicated (■). Additional residues that form the substrate binding site in the LB400 enzyme are marked (▲), and conserved residues that play a structural role are indicated (□). The boxed fingerprint region contains the consensus sequence (**G** or N or T or I or V)-$\mathbf{X_1}$-**H**-$\mathbf{X}_{5\ or\ 7}$-(L or I or V or M or **F**)-**Y**-$\mathbf{X_2}$-(D or E or N or **T** or A)-**P**-$\mathbf{X_1}$-(**G** or P)-$\mathbf{X_3}_{\ or\ 4}$-**E** (14), where X_n indicates *n* residues of any type, parentheses enclose residues found at one position, and boldface letters indicate the residues found in the TlpE protein. The positions of relevant restriction sites of the corresponding *tlpE* gene are indicated. (B) The dipeptide His-Gly of the putative oxanion hole, the RVIAPDXXGXGXS motif, and the so-called hydrolase or lipase box with the nucleophile motif Gly103-Xaa-Ser105-Xaa-Xaa-Gly108 (3, 11, 12) of the TlpF hydrolase are boxed. The catalytic residues Ser105, Asp226, and His254, representing the catalytic triad of hydrolases (2, 12, 29) and lipases (8, 11), are circled and in boldface. The region proposed to be involved in determination of substrate specificity (12) is underlined. Relevant restriction sites are indicated.

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