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Gene Organization of the First Catabolic Operon of TOL Plasmid pWW53: Production of Indigo by the *xylA* Gene Product

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The entire operon coding for the enzymes responsible for conversion of toluenes to benzoates has been cloned from TOL plasmid pWW53 and the position of the genes accurately located. The coding region was 7.4 kilobase pairs (kbp) long, and the gene order was operator-promoter region (OP1)–a small open reading frame–*xylC* (1.6 kbp)–*xylA* (2.9 kbp)–*xylB* (1.8 kbp). Within the coding region there was considerable homology with the isofunctional region of the archetypal TOL plasmid pWW0. A central region of 2.9 kbp complemented an *xylA* (for xylene oxygenase) mutant of *Pseudomonas putida* mt-2 and was also capable of conferring the ability to convert indole to indigo on strains of *Escherichia coli* and *P. putida*. This reaction has been reported previously only for dioxygenases involved in aromatic catabolism but not for monooxygenases. It is proposed that the region encodes xylene oxygenase activity capable of direct monohydroxylation of indole to 3-hydroxyindole (oxindole), which then spontaneously dimerizes to form indigo.

In soil isolates of *Pseudomonas* spp. the ability to catabolize toluene and substituted toluenes via benzoate and substituted benzoates (20, 29) appears to be almost always plasmid encoded (27), but the plasmids themselves differ considerably in properties and structure (5, 28).

Horizontal transfer on plasmid vectors through different members of the soil microbial population undoubtedly imposes selection pressures on catabolic genes different from those which are chromosomally located. One way of monitoring and assessing these differences is to compare the structure and organization of catabolic genes on isofunctional plasmids isolated from different geographical locations.

The archetypal TOL plasmid pWW0 (117 kilobase pairs [kbp]) was found in *Pseudomonas putida* mt-2 (23, 27), a strain originally isolated in Japan in the 1950s. The majority of molecular biological studies on the catabolic structural genes of the toluene-xylene pathway have been performed on pWW0 or its derivatives (7, 10, 11, 15, 16, 21). More recently, we isolated *P. putida* MT53 in North Wales and initiated studies on its TOL plasmid, the 105-kbp plasmid pWW53, and its RP4 cointegrate, pWW53-4 (18). The DNA of its second operon (*xylDLEGF. . .*), coding for the enzymes converting benzoic acids to central metabolites, has the same gene order as the corresponding operon on pWW0, and its restriction map shows a number of common sites. This paper reports parallel studies on the first operon of the pathway (*xylCAB*) responsible for the conversion of toluenes to benzoates.

MATERIALS AND METHODS

Bacterial strains and media. The *Escherichia coli* and *P. putida* strains used or constructed in this study are listed in Table 1. The media for their growth and maintenance have been described elsewhere (19, 29). To maintain recombinant plasmids in *E. coli*, streptomycin sulfate, kanamycin, ampicillin, and tetracycline were added to final concentrations of 15, 15, 25, and 7.5 μg/ml, respectively, when appropriate. In *Pseudomonas* hosts, streptomycin and kanamycin were added at 150 and 50 μg/ml, respectively.

Enzyme assays. Cell extracts were prepared, and benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH) were assayed in the extracts by published procedures (29).

Isolation and manipulation of DNA. Plasmid pWW53 DNA was extracted from *P. putida* MT53 by the sucrose gradient method (26). The RP4::pWW53 cointegrate plasmid pWW53-4 was isolated from *E. coli* by CsCl-ethidium bromide gradient centrifugation, as were the vectors and recombinant plasmids. For rapid analysis of recombinant strains, the method of Holmes and Quigley (12) was used. *Hin*dIII fragment HF and *Bam*HI fragment BE were cloned from pWW53 and pWW53-4, respectively, under the conditions for restriction, ligation, and transformation described previously (19). The unstable RP4 derivative plasmid pNJ5000 (9) was used to mobilize recombinant plasmids from *E. coli* into *P. putida*.

DNA-DNA hybridization experiments. Restriction fragments were separated by agarose gel electrophoresis and transferred by Southern blotting (25) to Biodyne filter membranes (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.). Recombinant plasmid pWW53-3052 was labeled by nick translation with [^{32}P]dGTP (24). Hybridization was performed under different conditions of stringency (25 or 50% formamide at 37°C).

Purification and identification of indigo. Some *E. coli* strains carrying recombinant plasmids produced a deep blue coloration after cultivation on Luria broth. Addition of tryptophan or indole (100 μg/ml) increased pigment production. The pigment was identified as indigo by extracting a 50-ml culture with hot $CHCl_3$ (6) and comparing the extracted material with authentic indigo by mass spectrometry; the characteristic molecular ion at M/e = 262 and significant peaks at 234, 205, 131, 104, and 76 were found in both authentic indigo and the extracted sample.

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TABLE 1. Bacterial strains and plasmids

Strains or plasmid	Notes (reference)
P. putida	
MT53(pWW53)	Wild type (18)
PaW130	Plasmid-free derivative of *P. putida* mt-2 (PaW1)
PaW263(pWW0 *xylA*)	8
PaW340	Trp^- Str^r derivative of PaW1
PaW611(pWW53-4)	RP4::pWW53 cointegrate plasmid in PaW340 host (18)
PaW823(pWW0-660)	21
E. coli	
C600	Thi^- Leu^- F^- *supE44*
C600(pNJ5000)	Contains unstable Tet^r derivative of RP4 (9)
C600(pWW53-4)	Transfer of pWW53-4 from PaW611 to C600
Recombinant plasmids	
pKT230	1
pBR322	3
pBR325	2
pWW53-3001	*Hin*dIII fragment HF of pWW53 in pKT230
pWW53-3002	Reverse orientation of pWW53-3001 insert in pKT230
pWW53-3004	*Bam*HI fragment BE of pWW53 in pBR325
pWW53-3005	Reverse orientation of pWW53-3004 insert in pBR325
pWW53-3020	5.8-kbp *Sma*I-*Hin*dIII subfragment of pWW53-3001 generated by deletion of internal 1.6-kbp *Sma*I fragment
pWW53-3030	3.0-kbp *Sma*I-*Bam*HI subfragment of pWW53-3020 generated by deletion of internal 3.6-kpb *Bam*HI fragment
pWW53-3033	*Bam*HI fragment BE cloned into unique *Bam*HI site of pWW53-3030
pWW53-3040	Internal 3.9-kbp *Pst*I subfragment of HF cloned into pBR322
pWW53-3050	Internal 4.3-kbp *Xho*I subfragment of HF cloned into pKT230
pWW53-3052	Internal 2.3-kbp *Xho*I subfragment of BE cloned into pKT230
pWW53-3053	Reverse orientation of pWW53-3052 insert in pKT230
pWW53-3066	Internal 6.1-kbp *Sal*I subfragment of BE cloned in pBR322
pWW53-3067	Reverse orientation of pWW53-3066 insert in pBR322
pWW53-3070	Internal 3.7-kbp *Sst*I subfragment of BE cloned in pKT230
pWW53-3207	3.2-kbp *Hin*dIII fragment of pWW53-3004 cloned into pKT230
pWW53-3208	Reverse orientation of pWW53-3207 insert in pKT230
pWW53-3211	3.7-kbp *Bam*HI fragment of pWW53-3208 cloned into pBR325
pWW53-3212	Reverse orientation of pWW53-3211 insert in pBR325
pWW53-3227	3.7-kbp *Bam*HI fragment of pWW53-3211 cloned into unique *Bam*HI site of pKT230

RESULTS

Molecular cloning of *Hin*dIII fragment HF. Preliminary transposon mutagenesis experiments had shown that the genes of the first operon (*xylCAB*) of the toluene-xylene pathway were located on fragment HF (18). This 7.1-kbp fragment was cloned in both orientations into the *Hin*dIII site of the broad-host-range vector pKT230. The two recombinant plasmids, pWW53-3001 and pWW53-3002 (Fig. 1), were mobilized into the plasmid-free strain *P. putida* PaW130 and strain PaW823 containing pWW0-660, a deletion derivative of TOL plasmid pWW0, lacking the entire *xylCAB* operon but with the regulator genes *xylR* and *xylS* still present (21): neither PaW130 nor PaW823 grows on *m*-xylene. Whereas PaW130 containing either recombinant plasmid was unable to grown on *m*-xylene, both of the corresponding PaW823 derivatives grew on *m*-xylene, although at a slower rate than the wild-type MT53.

Location of *xylC* and OP1. BZDH activity was detected in *E. coli* strains carrying recombinant plasmids with HF inserted in either orientation (pWW53-3001 and pWW53-3002; Table 2), but it was not further induced by *m*-methylbenzyl alcohol in the *E. coli* strains or by *m*-xylene in the PaW130 host, suggesting that there was no regulator gene on the cloned HF fragment. However, *m*-xylene did induce BZDH in PaW823 strains carrying both recombinant plasmids (Table 2), indicating the presence of an operator-promoter (OP) region on HF on which the gene product of the pWW0 *xylR* gene could act in *trans*. The *Sma*I subfragment of pWW53-3001, pWW53-3020 (Fig. 1), showed a similar induction in PaW823, demonstrating that both the OP region (which we call OP1 by analogy with the corresponding region on pWW0 [14]) and the BZDH gene (*xylC*) are located between coordinates 1.4 and 7.1. No induction of BZDH activity by *m*-xylene was observed in PaW823, which carried the cloned *Xho*I fragment on pWW53-3050 (Table 2), pinpointing the OP1 region to between the *Sma*I site (coordinate 1.4) and the *Xho*I site (coordinate 2.0). BZDH activity was also detected in *E. coli* carrying the *Pst*I fragment (pWW53-3040) but not the *Sma*I-*Bam*HI fragment (pWW53-3030) (Table 3) or the 0.8- and 1.6-kbp *Sal*I fragments internal to pWW53-3040. *xylC* must therefore lie between coordinates 2.9 and 6.3 (Fig. 1). Since OP1 appears to be upstream of the *Xho*I site at coordinate 2.0 and the start of the BZDH gene is at least 0.9 kbp downstream of this, it can be assumed that the first reading frame in the operon is not part of *xylC*.

Surprisingly, both the recombinant plasmids containing a functional BZDH gene which did not also have OP1 (pWW53-3040, pWW53-3050) were found to carry the cloned fragment in the orientation in which initiation of transcription could not start from the strong constitutive promoters of

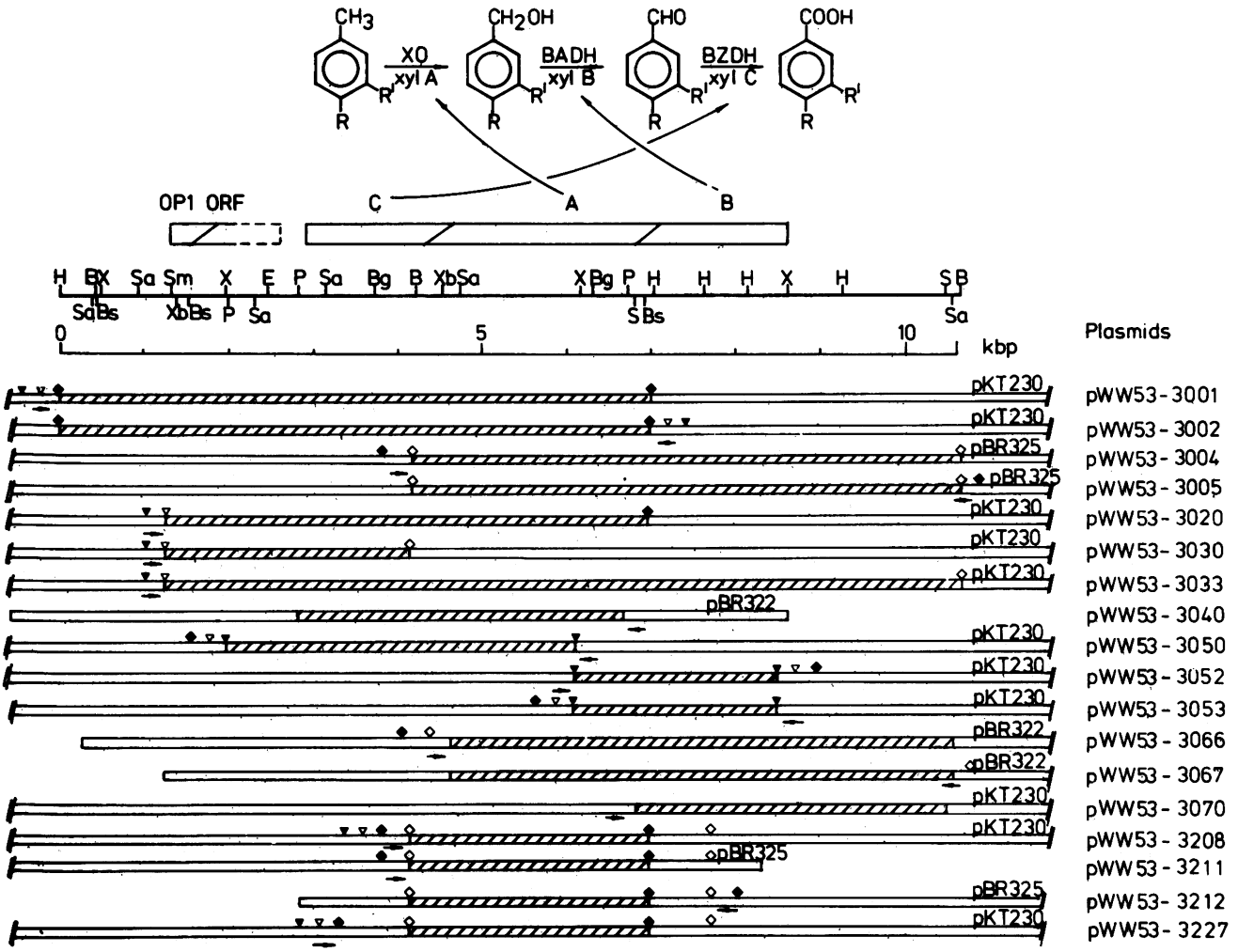

FIG. 1. Location of genes and restriction map of the DNA encoding the first catabolic operon on TOL plasmid pWW53. The enzymatic reactions corresponding to the structural genes are shown above. Fragments of the DNA cloned and subcloned during the study are shown below the map. In these recombinant plasmids the open boxes represent vector DNA and the hatched boxes represent the inserts derived from pWW53. The small arrows below the vector indicate the direction of transcription from the vector promoters. Restriction sites on the vectors: ▼, *Xho*I; ▽, *Sma*I; ◆, *Hin*dIII; ◇, *Bam*HI. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I. OP1 indicates the operator-promoter region for the operon and ORF the unidentified open reading frame (see text).

either pBR322 or pKT230. Several attempts to obtain the cloned DNA in the orientation in which it would be controlled from the vector promoter failed. We take this to mean that high amounts of the *xylC* gene product, which would be expected in that orientation, cannot be tolerated in *E. coli* hosts.

Location of *xylB*. The 6.5-kbp *Bam*HI fragment BE, which overlaps by 2.9 kbp with HF, was cloned in both orientations into pBR325 from pWW53-4 DNA to give pWW53-3004 and pWW53-3005 (Fig. 1). BADH activity was detected in *E. coli* carrying pWW53-3004 and subclones thereof (Table 3). The smallest such plasmids contained the 2.5-kbp *Xho*I (pWW53-3052) and the 3.8-kbp *Sst*I (pWW53-3070) fragments, respectively, showing that the gene is located between coordinates 6.9 and 8.7 on the restriction map (Fig. 1). In contrast to *xylC*, the *xylB* gene could be inserted in either orientation, allowing high specific activities when downstream of the vector promoter.

We compared the homology between the two dehydrogenase genes by DNA-DNA hybridizations. The probe was the 2.5-kbp *Xho*I fragment on pWW53-3052 carrying the functional *xylB* gene. The probe did not hybridize with any of the region between itself and OP1 (for example, the 4.3-kbp *Xho*I insert in pWW53-3050) (Fig. 2). Thus, even though BADH and BZDH have a common cosubstrate and their substrates show structural similarity, their DNA does not seem to have any significant degree of homology.

Expression of the BADH gene was high regardless of the orientation of its insertion; for both the *Sal*I (pWW53-3066 and pWW53-3067) and the *Bam*HI (pWW53-3004 and pWW53-3005) inserts, the specific activity determined by the fragment in reverse orientation to the vector promoter was about half that in the forward orientation (with transcription from the vector promoter) (Table 3). However, the activity was much lower (≈2%) in a clone containing the 2.5-kbp *Xho*I fragment in the reverse orientation (pWW53-3053 compared with pWW53-3052, Table 3). This would appear to indicate a region between coordinates 4.8 (*Sal*I site) and 6.3 (*Xho*I site) which functions as a strong promoter in *E. coli*.

Production of indigo by cloned DNA. Several of the recom-

TABLE 2. Enzyme activities and properties of *E. coli* and *P. putida* strains carrying recombinant plasmids

Plasmid	Host[a]	Activity (mU/mg of protein)						*xylA* complementation	Indigo accumulation[b]
		BADH			BZDH				
		Uninduced	*m*-Methylbenzyl alcohol	*m*-Xylene	Uninduced	*m*-Methylbenzyl alcohol	*m*-Xylene		
pWW53-3001	*E. coli*	1	2	ND[c]	40	50	ND	ND	+
	P. putida	14	ND	15	10	ND	170	+	+
pWW53-3002	*E. coli*	<1	<1	ND	15	20	ND	ND	−
	P. putida	12	ND	11	6	ND	130	+	+
pWW53-3020	*E. coli*	<1	ND	ND	50	70	ND	ND	+
	P.putida	10	ND	10	8	ND	160	+	+
pWW53-3033	*E. coli*	380	300	ND	15	15	ND	ND	±
	P. putida	25	60	550	6	120	200	+	±
pWW53-3050	*E. coli*	4	ND	ND	35	45	ND	ND	−
	P. putida	11	ND	10	15	ND	10	−	−
pWW53-3227	*E. coli*	<1	ND	ND	<1	ND	ND	ND	±
	P. putida	<1	ND	ND	<1	ND	ND	+	±
pWW53	*P. putida*	10	ND	180	3	ND	170	ND	+
pWW0-660	*P. putida*	11	ND	10	4	ND	5	ND	ND

[a] *E. coli* C600 and *P. putida* PaW823.
[b] +, Good; ±, poor; −, none.
[c] ND, Not determined.

binant plasmids made in this study caused the accumulation of indigo when their *E. coli* hosts were grown in Luria broth (Tables 2 and 3). This was first observed with pWW53-3004, carrying the *Bam*HI fragment BE in the orientation downstream from the vector promoter. No indigo was formed with BE in the reverse orientation (pWW53-3005), showing a dependence on a strong promoter. Tryptophan or indole added to the growth medium increased indigo production from pWW53-3004.

Indigo was produced to a lesser extent by cells containing HF (pWW53-3001) and its *Sma*I subclone (pWW53-3020). The reduced indigo formation in these strains could be the result of differences in promoter strength or of the presence of a functional gene product downstream of OP1 interfering with indigo formation. To distinguish between these possibilities, *E. coli* strains carrying pWW53-3004 or pWW53-3211 were complemented in *trans* with the *Sma*I-*Bam*HI fragment (pWW53-3030), which contains OP1 and the open reading frame downstream of it (Fig. 1). No significant reduction in the conversion of indole to indigo was observed in these double transformants, suggesting that the first unidentified gene product of the operon plays no role in this oxidation reaction. We assume therefore that the lower indigo production in *E. coli* carrying pWW53-3001 or pWW53-3020 is due to mixed initiation of transcription from OP1 and the constitutive kanamycin promoter of pKT230.

TABLE 3. Enzyme activities and indigo accumulation in recombinant strains of *E. coli* C600

Plasmid	Activity (mU/mg of protein)		Indigo accumulation[a]
	BADH	BZDH	
pWW53-3004	470	1	+ +
pWW53-3005	240	1	−
pWW53-3030	<1	<1	−
pWW53-3040	3	40	−
pWW53-3052	930	<1	−
pWW53-3053	20	<1	−
pWW53-3066	1,280	<1	−
pWW53-3067	460	<1	−
pWW53-3070	370	<1	−
pWW53-3208	<1	<1	±
pWW53-3211	<1	<1	+ +
pWW53-3212	<1	<1	−
pBR322	3	4	−

[a] + +, High, ±, poor; −, none.

The *Sal*I fragment (pWW53-3066) did not lead to indigo formation, suggesting that the site at coordinate 4.7 is within the gene involved in the oxidation of indole (Fig. 1). Because the only region common to BE and HF was the 2.9-kbp *Bam*HI-*Hin*dIII region from coordinate 4.2 to 7.1, we cloned this region downstream of a strong vector promoter (Table 1). The recombinant plasmid pWW53-3208 contained a 3.2-kbp insert (consisting of 2.9 kbp from BE with 340 bp of pBR325 upstream of it), which led to marginal indigo production. The two plasmids, pWW53-3211 and pWW53-3212, had a 3.7-kbp insert (consisting of the same 2.9 kbp of BE but with 800 bp of pKT230 downstream) in opposite orientations (Fig. 1), the first of which caused strong indigo accumulation (Table 3). The indole-oxidizing activity can therefore be mapped to the 2.9 kbp region common to BE and HF.

When pWW53-3020 and recombinant plasmids containing HF in either orientation were mobilized into *P. putida* PaW823, indigo was formed from added indole only when the operon was induced with *m*-methylbenzyl alcohol. Induced but not uninduced cells of wild-type MT53 were also able to convert indole to indigo.

Location of *xylA*. To locate the gene for xylene oxygenase (*xylA*), subcloned fragments were examined to identify which of them expressed this activity. Since *E. coli* is very sensitive to the hydrocarbons toluene and *m*-xylene, exper-

iments were caried out exclusively in *P. putida* hosts. Recombinant plasmids containing parts of the operon cloned into pKT230 were mobilized into PaW263, a mutant derivative of PaW1 with a nonfunctional xylene oxygenase but with normal activities of subsequent enzymes of the pathway (8). The resulting recombinant strains were then examined for complementation of the mutation in PaW263.

Of the recombinant plasmids mobilized, only HF in either orientation (pWW53-3001 and pWW53-3002), pWW53-3020, and pWW53-3227 (the 2.9-kbp *Bam*HI-*Hin*dIII region) complemented PaW263 for growth on *m*-xylene, confirming that *xylA* is located between coordinates 4.2 and 7.1 (Fig. 1), the region corresponding exactly to that responsible for the indole oxidation.

Mobilization of pWW53-3001, pWW53-3002, and pWW53-3020 carrying both *xylC* and *xylA* into PaW823 led to only slow growth on *m*-xylene due to the lack of the *xylB* gene on these fragments, which cannot be fully compensated for by the presence of the weak isofunctional enzyme which is chromosomally coded.

In vitro construction of the complete operon cloned on a broad-host-range vector. The *Bam*HI fragment BE was inserted into the unique *Bam*HI site of the recombinant plasmid pWW53-3030 (Fig. 1), and the ligated DNA was transformed into *E. coli* containing pNJ5000, selecting for resistance to tetracycline (for pNJ5000) and streptomycin.

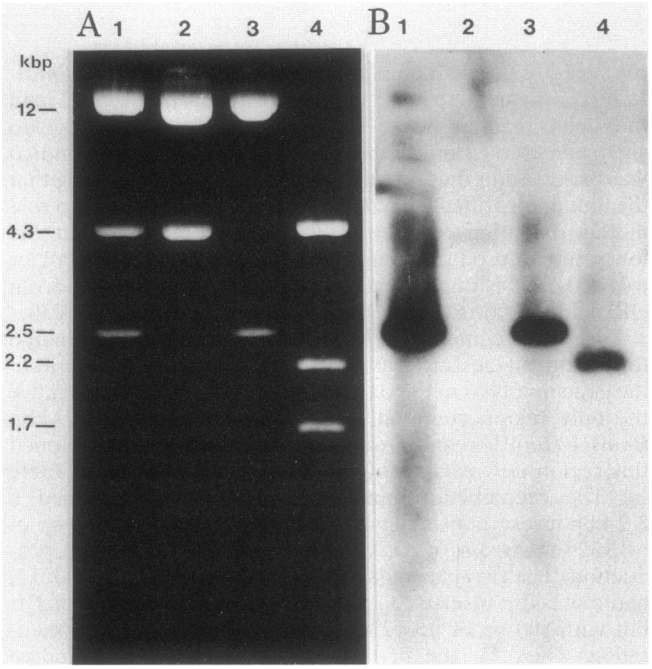

FIG. 2. Hybridization analysis of cloned fragments carrying parts of the *xylCAB* operon of TOL plasmid pWW53. (A) Agarose gel electrophoresis of restriction digests of recombinant plasmids; (B) autoradiogram of the same gel after Southern blotting and hybridization to the 2.5-kbp *Xho*I subfragment of pWW53-3052, which had been extracted from agarose gels prior to radiolabeling with ^{32}P by nick translation. Lanes: 1, pWW53-3033 digested with *Xho*I; 2, pWW53-3050 digested with *Xho*I; 3, pWW53-3052 digested with *Xho*I; 4, pWW53-3040 digested with *Pst*I and *Xba*I. The *xylB*-specific probe hybridized to its corresponding region in lanes 1 and 3 but not to the 4.3-kbp *Xho*I fragment containing *xylC* and part of *xylA* in lanes 1 and 2. In lane 4 the probe hybridized to the 2.2-kbp fragment containing part of *xylA* due to an overlap of 600 bp but not to the 1.7-kbp *Pst*I-*Xba*I fragment containing just the *xylC* gene.

The transformants were mated with PaW823, and the corresponding transconjugants were screened for their ability to grow normally on *m*-xylene. One *E. coli* strain conferring this ability to PaW823 contained a plasmid (pWW53-3033) with an insert stretching from the *Sma*I site at coordinate 1.4 to the right-hand *Bam*HI site at coordinate 10.8 (Fig. 1).

The expression of BADH and BZDH in *E. coli* and *P. putida* was measured as induced and uninduced activity (Table 2). In *E. coli* the activity of BADH was found to be much higher than that of BZDH, presumably due to the promoter sequence recognized by *E. coli* upstream of *xylB*. In *P. putida* PaW823, both *m*-xylene and *m*-methylbenzyl alcohol lead to an induction of 20- to 30-fold. The specific activities found in this constructed strain were in reasonable agreement with the equivalent activities found in wild-type MT53, being about twofold higher.

DISCUSSION

Previous results have shown that although TOL plasmids pWW0 and pWW53 are quite different replicons, they have significant homology within the coding region for the second (*meta* pathway) operon (*xylDLEGF. . .*) of the toluene-xylene pathway (18). This study extends the comparison to the genes of the first operon of the pathway. As shown in Fig. 3, 17 of 22 restriction sites were at the same relative positions on the two plasmids between the start and the end of the operon, but the similarity ended at either end outside the coding region.

The maximum length of coding DNA required for expression of each of the structural genes was 1.6 kbp (*xylC*), 2.9 kbp (*xylA*), and 1.8 kbp (*xylB*), with the downstream end of *xylC* being deduced by analogy with pWW0 (21) because of the conservation of restriction sites.

The high levels of BADH activity in both orientations in recombinant plasmids, assumed to be due to the presence of an *E. coli*-like promoter upstream, agree with similar results of Inouye et al. (15) with the *xylB* gene from pWW0 cloned on a 15.4-kbp *Bam*HI fragment. By contrast, BZDH activities greater than 50 mU/mg of protein could not be exceeded in *E. coli*, whereas in *P. putida* activities of 200 mU/mg of protein were attainable. Apparently, high activities of BZDH cannot be tolerated by some factor in the physiology of *E. coli*. These differences were also reflected in the lack of homology between the two structural genes. It might be expected that these two enzymes, catalyzing dehydrogenation reactions on structurally related substrates as sequential reactions in a pathway, would be ideal candidates for having evolved by tandem duplication from a common ancestral gene (13). This may still be a possibility, but if so, the two copies must have subsequently diverged widely. Only complete DNA sequencing will definitively assess whether there is a common ancestry, but the apparent differences in their effect on *E. coli* hosts and their total lack of detectable homology under conditions of low stringency would seem to make this a remote possibility.

The observation that several cloned fragments caused the accumulation of indigo in *E. coli* and in *P. putida* hosts when the latter were supplemented with indole facilitated the study of *xylA*. The original observation was made with cloned pWW0 DNA (M. R. Lebens, H. Keil, and P. A. Williams, unpublished data) and has also been made independently by Mermod et al. (22): in this study it was extended to pWW53 DNA. We have found that location of the cloned DNA downstream of a strong promoter was essential for high indigo accumulation, but that the *E. coli*

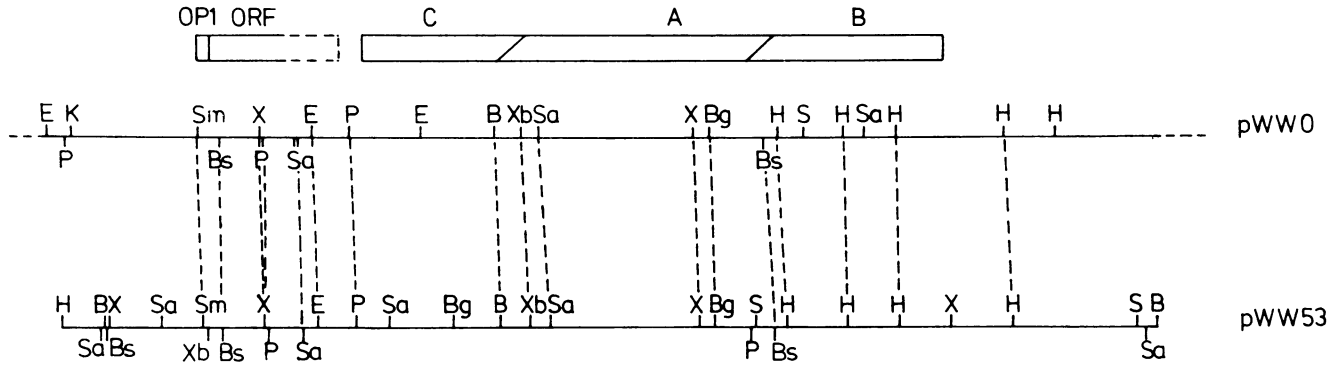

FIG. 3. Comparison of the restriction map of the *xylCAB* operon region from TOL plasmids pWW53 with the corresponding region of pWW0 from reference 21, for which a correction has been made to the *Xba*I and *Sal*I sites. Corresponding restriction sites are connected by broken lines. Location of the *xyl* genes and the symbols used for restriction enzymes are as in Fig. 1.

strains carrying such recombinant plasmids grew more slowly on complete medium, demonstrating the deleterious effect of the reaction on the host cell physiology, presumably because of depletion of endogenous tryptophan.

Complementation of the *xylA* mutant PaW263 by the same 2.9-kbp region of the operon that is responsible for the formation of indigo demonstrates that the indigo reaction is a true indicator for the activity of the *xylA* gene product. Until now the production of indigo from indole, or indirectly from tryptophan in *E. coli* by way of tryptophanase, has been ascribed only to the action of dioxygenases (4, 6, 17): it is proposed that because of their lack of substrate specificity, the dioxygenases convert indole to 2.3-dihydro-2,3-dihydroxyindole, which then undergoes two sequential spontaneous reactions, dehydration to 3-hydroxyindole (oxindole), which subsequently dimerizes to give indigo (6). This mechanism cannot operate for xylene oxygenase, which is a monooxygenase, hydroxylating a methyl group to hydroxymethyl. It seems most likely that the mechanism of this reaction proceeds via the direct monohydroxylation on the reactive 3 position of indole to form 3-hydroxyindole, without the intermediate dihydrodiol proposed for the dioxygenase-catalyzed reactions. This would imply that the enzyme has a very relaxed substrate specificity and is able to oxidize groups as different as the methyl group of toluenes and the aromatic C-3 of indole. What has not yet been ascertained is whether the 2.9-kbp region is sufficient for the physiological conversion of toluenes to benzyl alcohols, since it is possible that the indigo reaction could occur with only part of the xylene oxygenase functional. However, preliminary results indicate that prolonged incubation of *E. coli* strains containing plasmid pWW53-3004 or pWW53-3211 with toluene resulted in significant accumulation of benzoate in the medium (S. Collins, personal communication): the only mechanism by which this could happen is via a functional *xylA* gene product on the recombinant plasmids converting toluene to benzyl alcohol, which is then further oxidized by the nonspecific action of dehydrogenases in the host.

One unexplained feature of the operon is the open reading frame at the start of the operon. We have sequenced this region on pWW0, and it appears to code for a 14-kilodalton polypeptide (M. R. Lebens and P. A. Williams, unpublished data). From the conservation of restriction sites in this region between pWW0 and pWW53, it is reasonable to assume that a homologous gene exists on pWW53. So far we have not been able to ascribe a function to this putative polypeptide, but it is possible that it plays a role in the action

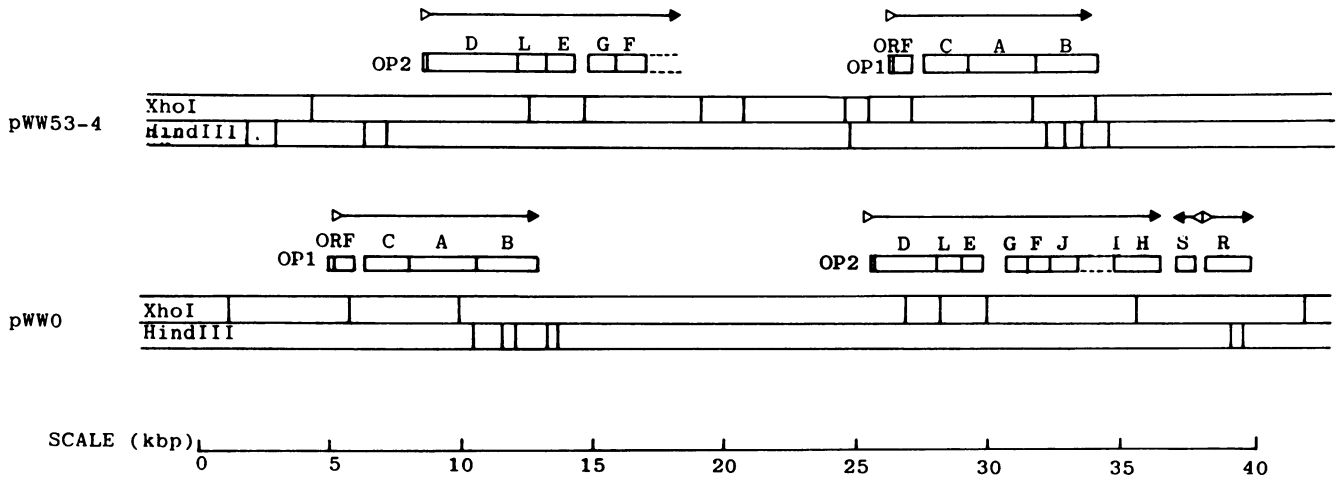

FIG. 4. Physical organization of the *xyl* genes on TOL plasmids pWW53-4 and pWW0. The positions for the genes of the two operons on pWW0 are taken from references 10 and 21, and the position of the genes of the *meta* pathway operon on pWW53 is from reference 18. The arrows indicate the direction of transcription of each operon. For simplicity, only the *Hin*dIII and *Xho*I sites are shown. ORF, Open reading frame.

of xylene oxygenase on toluenes in vivo, although it is apparent from the results of this paper that it is not necessary for the indigo reaction.

The fact that the plasmid containing the complete operon, pWW53-3033, can be induced by the functional *xylR* regulator gene of pWW0-660 in *trans* demonstrates further the strong homology between this operon on pWW53 and pWW0, since the pWW0 *xylR* gene product is able to cross-react with the pWW53 OP1 region. There was no evidence that a regulatory gene(s) is present on pWW53-3033. Data not presented here show that pWW53 has an *xylR* gene and that it is located about 2 kbp upstream of the OP1 region. There is thus the possibility of constructing a recombinant plasmid containing the entire regulated upper operon from pWW53.

Although the two TOL plasmids pWW0 and pWW53 share significant homology within the coding region of the two operons (18; Fig. 3), two main differences clearly emerge. The distance between the two operons is about 12 kbp in pWW0, compared with only about 1 kbp between the end of *xylR* and OP1 in pWW53 (data not shown). Perhaps more significant is the difference in orientations of the two operons relative to their directions of transcription (Fig. 4). It is obvious that the acquisition of the *xyl* genes by the two plasmids is more complex than a simple transposition of a single length of coding DNA from one location to another. This poses some interesting questions on the ways the *xyl* genes have evolved and spread horizontally between different plasmids and host strains.

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