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Characterization of Five Genes in the Upper-Pathway Operon of TOL Plasmid pWW0 from *Pseudomonas putida* and Identification of the Gene Products

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The upper operon of the TOL plasmid pWW0 of *Pseudomonas putida* encodes a set of enzymes which transform toluene and xylenes to benzoate and toluates. The genetic organization of the operon was characterized by cloning of the upper operon genes into an expression vector and identification of their products in *Escherichia coli* maxicells. This analysis showed that the upper operon contains at least five genes in the order of *xylC-xylM-xylA-xylB-xylN*. Between the promoter of the operon and *xylC*, there is a 1.7-kilobase-long space of DNA in which no gene function was identified. In contrast, most of the DNA between *xylC* and *xylN* consists of coding sequences. The *xylC* gene encodes the 57-kilodalton benzaldehyde dehydrogenase. The *xylM* and *xylA* genes encode 35- and 40-kilodalton polypeptides, respectively, which were shown by genetic complementation tests to be subunits of xylene oxygenase. The structural gene for benzyl alcohol dehydrogenase, *xylB*, encodes a 40-kilodalton polypeptide. The last gene of this operon is *xylN*, which synthesizes a 52-kilodalton polypeptide of unknown function.

The TOL plasmid pWW0 of *Pseudomonas putida* encodes a set of enzymes for the metabolism of toluene and its substituted derivatives. Degradation of these compounds is initiated by progressive oxidation of the methyl side chain of the aromatic ring (the upper pathway) to produce carboxylic acid (Fig. 1), which is then transformed to substrates of the Krebs cycle through the *meta* cleavage of the aromatic ring (the *meta* cleavage pathway). Genetic studies have shown that genes for these catabolic enzymes are organized into two operons: one encoding enzymes for the upper pathway (the upper operon) and the other encoding enzymes for the *meta* cleavage pathway (the *meta* operon; 4, 7, 8, 19).

The upper pathway is composed of three enzymes: xylene monooxygenase (XO), benzyl alcohol dehydrogenase (BADH), and benzaldehyde dehydrogenase (BZDH) (29). We have recently demonstrated that XO has a broad substrate specificity; it oxidizes not only toluene to benzyl alcohol but also benzyl alcohol to benzaldehyde (8). Therefore, benzyl alcohol can be oxidized both by XO and by BADH. To better understand the physiological roles of the upper-pathway enzymes, it will be necessary to (i) isolate mutants defective in one of the three enzymes and examine their phenotypes and (ii) purify the catabolic enzymes and determine their kinetic parameters and substrate specificities.

The structural genes for these enzymes have been localized to a region of about 8 kilobases (kb) on TOL pWW0 DNA in the order of *xylC* (the structural gene for BZDH), *xylA* (the structural gene for XO), and *xylB* (the structural gene for BADH) (8, 15, 16). However, these three genes make up only about 50% of the available DNA in this region, suggesting that other pathway genes may be present. In this report we present results on (i) identification of the products of five genes in the upper operon and (ii) genetic evidence that XO is a multicomponent enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *Escherichia coli* K-12 derivatives and are shown in Table 1. A Rec$^-$ derivative of K12ΔH1Δtrp was constructed by mating this strain with NK6659 as described previously (10). The plasmids used are shown in Table 1. The pWW0-161::Tn*5-68* plasmid is a derivative of TOL plasmid pWW0 carrying two transposons, Tn*401* and Tn*5*, in two different regions of DNA nonessential for catabolic functions (8). pLV85 is an expression vector derived from pBR322 containing the lambda leftward promoter, p_L (8, 21). Clones of TOL plasmid DNA in pLV85 were transformed into OT99(λ), and LE392 was used as a transformation host for other plasmids.

DNA manipulation. TOL plasmid DNA was isolated by the method of Hansen and Olsen (5), whereas DNAs of hybrid plasmids were prepared either by the method of Holmes and Quigley (11) for the miniscale preparations or by that of Clewell and Helinski (3) for the large-scale preparations. Methods for digestion of DNA with restriction endonucleases or BAL 31 and filling in the single-stranded DNA using DNA polymerase I large fragment (Klenow) have been described previously (18).

Tn*1000* insertion mutagenesis. Mutagenesis of pLV85-based hybrid plasmids by Tn*1000* was carried out by the method described by Sancar and Rupp (26) with the following modifications. Each hybrid plasmid to be mutagenized was transformed into OT99(λ), and a clone containing the monomeric form of the plasmid was isolated. Then the F′ *lac*::Tn*10* plasmid was conjugally transferred into this clone, and about 100 independent transconjugants containing both the hybrid and F′ *lac*::Tn*10* plasmids were isolated. These

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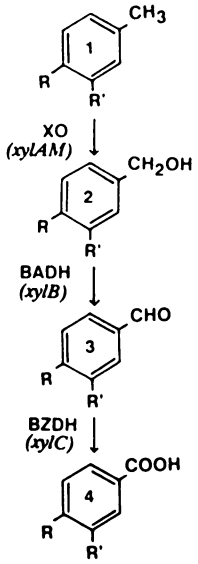

FIG. 1. TOL plasmid-encoded metabolic pathway for degradation of toluene and xylenes to benzoate and toluates showing pathway intermediates, enzymes, and their structural genes. Enzyme abbreviations: XO, xylene monooxygenase (multicomponent); BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; *xylA* to *xylM* designate the structural genes for the catabolic enzymes. When R and R′ are H, compound 1 is toluene, compound 2 is benzyl alcohol, and compound 3 is benzaldehyde. When R is H and R′ is CH_3, compound 1 is *m*-xylene, compound 2 is *m*-methylbenzyl alcohol, and compound 3 is *m*-methylbenzaldehyde. When R is CH_3 and R′ is H, compound 1 is *p*-xylene, compound 2 is *p*-methylbenzyl alcohol, and compound 3 is *p*-methylbenzaldehyde.

were used as donors and mated with K12ΔH1Δtrp as a recipient at 30°C. It was important to use Rec$^-$ donors containing a monomeric hybrid plasmid, because when a multimeric hybrid plasmid is mobilized through the Tn*1000*-mediated cointegrate formation, only one of the multiple copies of the hybrid plasmid is mutagenized by Tn*1000*. Transconjugants from these matings resistant to streptomycin (100 μg/ml) (counterselection against donor) and ampicillin (selection for pLV85-based hybrid plasmids) were isolated at 30°C on antibiotic medium 3 (AM3; Difco Laboratories) plates containing high concentrations of ampicillin (100 to 200 μg/ml) to avoid the growth of satellite colonies. The transconjugants were subsequently purified on AM3 plates containing ampicillin at a concentration of 20 μg/ml. One transconjugant from each mating was kept and the site of Tn*1000* insertion on their plasmid was determined by restriction endonuclease cleavage analysis as described previously (7).

Identification of gene products using maxicells. In the MCL22 strain containing two compatible plasmids, pCI857 and a pLV85 derivative, genes cloned into pLV85 downstream of the p_L promoter are inducible at 42°C. Therefore, cells of these strains were treated by the maxicell method of Sancar and Rupp (27) with the following modifications. Cells were grown overnight at 30°C in L broth (18) containing 100 mM KCl (MCL22 is defective in potassium transport), ampicillin (20 μg/ml) (selection for a pLV85 derivative), and kanamycin (25 μg/ml) (selection for pCI857). Twenty microliters of the overnight culture was transferred into 10 ml of the same medium, and the culture was grown to about 3×10^8 cells per ml. Cells were washed and suspended in 10 ml of 100 mM Tris hydrochloride buffer (pH 7.3) containing 10 mM $MgSO_4$ and irradiated for 20 s by a germicidal lamp (15 W) at a distance of 30 cm. Irradiated cells were suspended in 10 ml of L broth containing 100 mM KCl and incubated at 30°C for 1 h, and then D-cycloserine was added to 100 μg/ml. The incubation at 30°C was continued overnight, and the next morning, the same amount of D-cycloserine was added. The incubation at 30°C was continued for 2 h, and the cells were pelleted, washed, and resuspended to 4×10^9 cells per ml in methionine assay medium (Difco) containing M9 base salt and 0.2% glucose. The cells were incubated at 30°C for 5 min and then further incubated for 10 min either at 30 or 42°C. [^{35}S]methionine (1.2 Ci/μmol; Amersham Corp.) at a final concentration of 1 μM was added to bacterial suspensions thus treated, and the suspensions were incubated at the same temperature for 5 min. Unlabeled methionine at a final concentration of 10^{-4} M was then added, and after a 20-s chase, trichloroacetic acid to a final concentration of 2% was added to stop the reaction. The cells were then pelleted, washed, and suspended in 40 μl of sample buffer (8% sodium dodecyl sulfate, 1% β-mercaptoethanol, 5% glycerol, 0.004% bromophenol blue, 0.1 M Tris hydrochloride [pH 7.3]).

Gel electrophoresis, autoradiography, and limited proteolysis using *Staphylococcus aureus* V8 protease of proteins isolated from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as previously outlined (6).

Enzyme assays. A 1-liter sample of K12ΔH1Δtrp cells containing a pLV85 derivative was grown at 30°C in L broth containing ampicillin (100 μg/ml). When the cell density reached about 2×10^8 cells per ml, the cultures were transferred to 42°C and a 250-ml culture was withdrawn every hour for 4 h. Cell culture samples were pelleted, washed, and suspended in 30 ml of 0.1 M potassium phosphate buffer (pH 7.3). Preparation of cell extracts and assays for BADH and BZDH were carried out as described previ-

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Genotype or phenotype	Reference(s)
Bacterial strains		
LE392	*hsdR514 metB1 lacY1 supE44 supF58 galK2 galT22 trpR55*	9
K12ΔH1Δtrp	*lacZ*(Am), Δ(*bio-uvrB*) Δ*trp*(*E-A*)*2* λ[*N*am7 *N*am53 *c*I857 Δ*H1*]	24
OT99(λ)	*thr-1 leu-6 thi-1 supE44 lacY1 tonA21 recA1* λ$^+$	9
MCL22	*trkA405 trkD1 thi rha rpsL31* Δ(*kdp-phr*)*214* Δ(*gal-uvrB*) Δ(*srl-recA*)*306*	17
NK6659	Hfr KL16 *srl-3000*:Tn*10* *recA56 ilv-318 thi-1 thr-300 relA1 spc-300*	10
K12ΔH1Δtrp *recA*	*lacZ*(Am) Δ(*bio-uvrB*) Δ*trp*(*E-A*)*2* λ[*N*am7 *N*am53 *c*I857 Δ*H1*] *recA56 srl-3000*::Tn*10*	This study
Plasmids		
pWW0-161::Tn*5-68*	TOL$^+$ Ap^r Km^r	4, 8
pLV85	Ap^r lambda p_L	8, 21
pACYC177	Ap^r Km^r	2
pCI857	Ap^r *c*I857	25
pHP45Ω	Ap^r, streptomycin and spectinomycin resistant	23
pUC7	Ap^r	28
pKT231	Km^r, streptomycin resistant	1

ously (29). The assay for XO was carried out with intact cells. Cells were suspended in 0.1 M potassium phosphate buffer to a concentration of about 10^{11} cells per ml and incubated at 30°C for 5 min, and then 10 μl of toluene-saturated phosphate buffer was added to 0.5 ml of the bacterial suspension. The cell suspension was further incubated for 60 min at 30°C, then chilled, and centrifuged for 2 min in an Eppendorf centrifuge. The supernatant was transferred to another Eppendorf tube and centrifuged for 15 min. Toluene, benzyl alcohol, benzaldehyde, and benzoate in the sample were separated by reversed-phase chromatography, using a Nucleosil C18 column (300-Å pore size, 5-μm particle size, 25 cm by 4 mm [inner diameter]) (Macherey-Nagel) with a mobile phase of 39.9% H_2O–60% acetonitrile–0.1% H_3PO_4 at a flow rate of 0.7 ml/min. Detection was done by UV A_{254}.

RESULTS

Identification of the *xylC* product, BZDH, as a 57-kDa polypeptide. The plasmid pGSH2872 carrying the structural genes for BZDH and XO was constructed as shown in Fig. 2. From this plasmid, a series of deletion derivatives were constructed (Fig. 3) and transformed into the maxicell strain MCL22 containing pCI857; the polypeptides synthesized from the hybrid plasmids were examined. At 30°C a polypeptide of 26 kilodaltons (kDa) in size, which is most likely neomycin phosphotransferase, was identified as the sole product from these plasmids. At 42°C maxicells containing pGSH2872 synthesized only this polypeptide (Fig. 4, lane 1); however, other plasmids also synthesized a 57-kDa polypeptide (p57) or a 35-kDa polypeptide (p35) or both (Fig. 4, lanes 2 through 6). The reason why pGSH2872 did not synthesize p57 and p35 may be that a transcription stop signal exists between the *Hin*dIII site at the 0-kb coordinate and the *Xho*I site at the 5.5-kb coordinate of Fig. 3. Both pGSH2918 (Fig. 4, lanes 2 and 6) and pGSH2919 (Fig. 4, lane 4) synthesized p57 and p35, whereas pGSH2916 (Fig. 4, lane 3) and pGSH2958 (Fig. 4, lane 5) synthesized only p35. From this observation, the start point of the structural gene for p57 was localized between the *Cla*I site at the 6-kb coordinate and the *Eco*RI site at the 7-kb coordinate and the structural gene for p35 was localized downstream of the p57 gene. A 13-kDa polypeptide was synthesized not only in MCL22 containing pGSH2918 (Fig. 4, lanes 2 and 6) but also in MCL22 containing other hybrid plasmids (e.g., see Fig. 4, lane 3; the 13-kDa bands were also visible in lanes 4 and 5 in the original autoradiograms). As described below, this polypeptide may be a truncated product of a gene downstream to the p35 gene.

The activity of BZDH in K12ΔH1Δtrp containing either pGSH2872, pGSH2918, pGSH2919, pGSH2916, or pGSH3151 was examined. The BZDH activity was induced at 42°C in cells harboring pGSH2918 or pGSH2919, which synthesize p57, but was not induced in cells containing pGSH2872, pGSH2916, or pGSH3151, which do not synthesize p57 (Table 2). Therefore, p57 was identified as BZDH. This observation is in agreement with the result of Lebens and Williams (16) that *xylC*, the structural gene for BZDH, is located between two *Sal*I sites at the 5.8- and 7.9-kb coordinates. In induced cells of *P. putida*, BZDH is expressed at a level of 150 mU per mg of cellular protein. In this experiment, the activity of BZDH expressed in *E. coli* from the strong lambda p_L promoter at 42°C was only 10% of the induced level in *P. putida*. The reason for this is unknown, but one possibility is that the folding or the activity of BZDH is temperature sensitive.

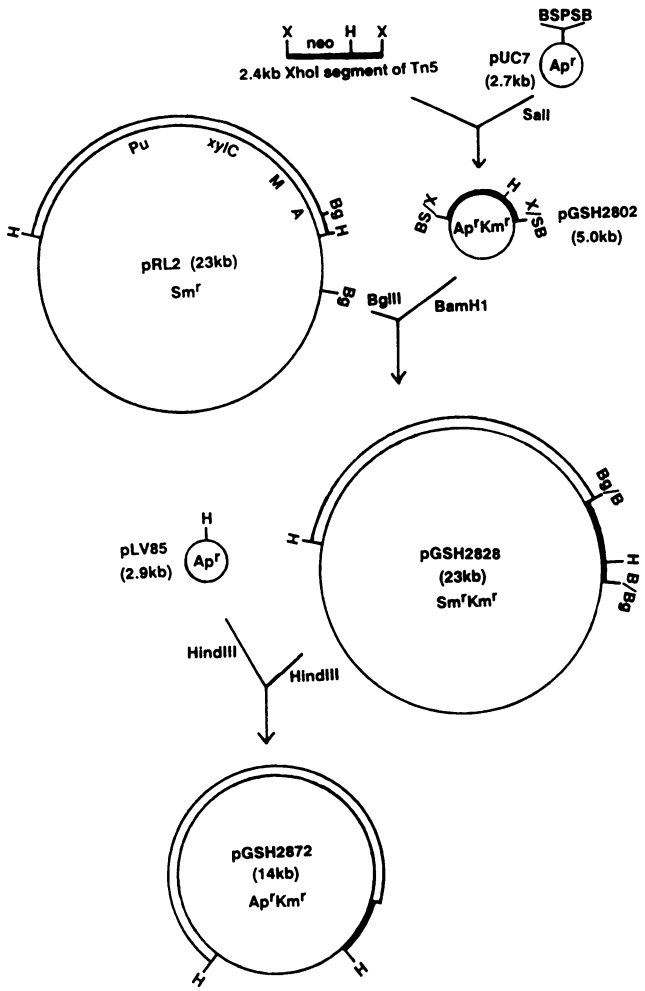

FIG. 2. Construction of pGSH2872. pRL2 is a pKT231 derivative containing a 10-kb *Hin*dIII segment of TOL pWW0. This segment contains, as shown in this study, three catabolic genes, *xylC*, *xylM* (M), and *xylA* (A). pGSH2828 was constructed by replacing a 2-kb-long *Bgl*II-*Bgl*II segment of pRL2 with a 2.4-kb-long *Bam*HI-*Bam*HI segment of pGSH2802 which encodes the *neo* (Km^r) gene. The 11-kb-long *Hin*dIII segment of pGSH2828 was subsequently cloned into pLV85 to construct pGSH2872. Restriction site abbreviations: X, *Xho*I; H, *Hin*dIII; S, *Sal*I; B, *Bam*HI; P, *Pst*I; Bg, *Bgl*II.

The promoter-proximal region of the upper operon may be silent. Inouye et al. (13) have demonstrated that the promoter of the upper pathway operon, Pu, is located about 200 base pairs (bp) downstream of the *Sma*I site at the 4.2-kb coordinate (Fig. 3). Therefore, between the p57 gene and the Pu promoter there is enough space to code for other polypeptide(s). To identify gene products encoded in the DNA region upstream of the p57 gene, pGSH2917 and pGSH2971 were constructed (Fig. 3). No polypeptide synthesis was detected in maxicells containing pGSH2971 (Fig. 4, lane 7). This observation suggested that a transcriptional stop signal exists in the 200-bp-long DNA sequence between the *Sma*I site and the Pu promoter. Therefore, derivatives of pGSH2971 which carry deletions of about 200 bp from the *Sma*I site were constructed (Fig. 5). DNA sequencing of the area around the Pu promoter has shown the presence of a putative Shine-Dalgarno sequence lying at the *Bst*EII site 230 bp downstream from the *Sma*I site (13). pGSH3151 (Fig.

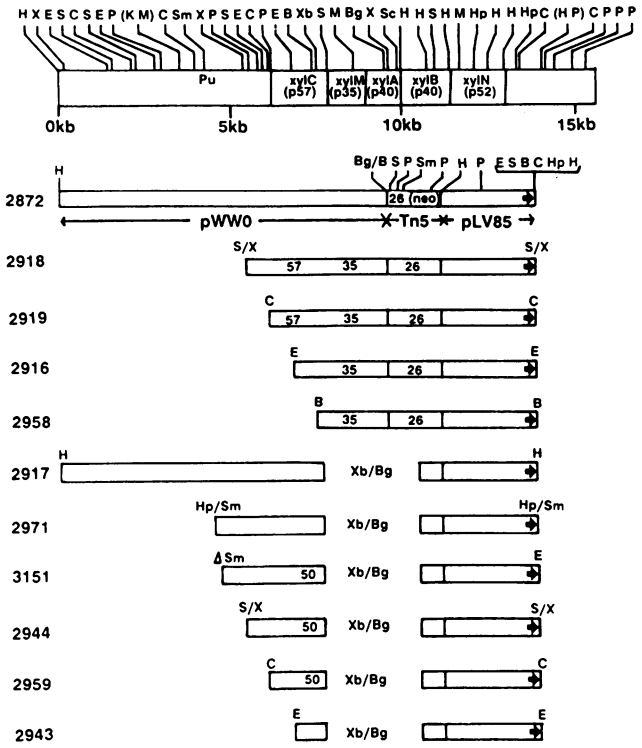

FIG. 3. pLV85-based hybrid plasmids containing *xylC* or *xylM*. The uppermost part of the figure shows the restriction sites of the TOL plasmid pWW0 and locations of the upper-pathway genes. The functions of *xylC*, *xylM*, *xylA*, and *xylB* are shown in Fig. 1, whereas the function of *xylN* is not known. Gene products are indicated in parentheses. Pu is the upper operon promoter. The structures of hybrid plasmids (indicated by only the numbers in the plasmid designations) used in this study are shown below. The method for the construction of pGSH2872 is shown in Fig. 2. pGSH2918, pGSH2919, pGSH2916, pGSH2958, and pGSH2917 were constructed from pGSH2872 by deletion of its *Sal*I-*Xho*I, *Cla*I-*Cla*I, *Eco*RI-*Eco*RI, *Bam*HI-*Bam*HI, and *Xba*I-*Bgl*II segments, respectively. Similarly, pGSH2971, pGSH2944, pGSH2959, and pGSH2943 were isolated from pGSH2917 by deleting its *Hpa*I-*Sma*I, *Sal*I-*Xho*I, *Cla*I-*Cla*I, and *Eco*RI-*Eco*RI segments, respectively. The pGSH3151 plasmid was constructed from pGSH2917, as shown in Fig. 5. Solid arrows indicate the lambda p_L promoter and the direction of its transcription. Restriction site abbreviations: H, *Hin*dIII; X, *Xho*I; E, *Eco*RI; S, *Sal*I; C, *Cla*I; P, *Pst*I; K, *Kpn*I; M, *Mlu*I; Sm, *Sma*I; B, *Bam*HI; Xb, *Xba*I; Bg, *Bgl*II; Sc, *Sac*I; Hp, *Hpa*I.

3 and 5) carries the *Bst*EII site. From cells containing this plasmid, synthesis of a polypeptide of 50 kDa in size (p50) was observed (Fig. 4, lane 11). This polypeptide was also synthesized from pGSH2944 (Fig. 4, lane 9) and pGSH2959 (Fig. 4, lane 8) but not from pGSH2943 (Fig. 4, lane 10). These results indicated that the p50 gene starts between a *Cla*I site at the 6-kb coordinate and an *Eco*RI site at the 7-kb coordinate (Fig. 3), which is the region occupied by *xylC*. Therefore, p50 is the truncated version of p57.

The upper operon comprises five genes: *xylC-xylM-xylA-xylB-xylN*. To explore the region downstream of the p35 gene, the 11.4-kb *Bam*HI segment of pWW0::Tn*5-68* containing a part of the upper-pathway genes and the Km^r determinant (*neo*) was cloned into pLV85 by selecting Ap^r Km^r transformants. Figure 6 shows the pGSH2819 plasmid isolated from one of these transformants and its deletion derivatives. Both pGSH2817 (Fig. 7, lane 3) and pGSH2836

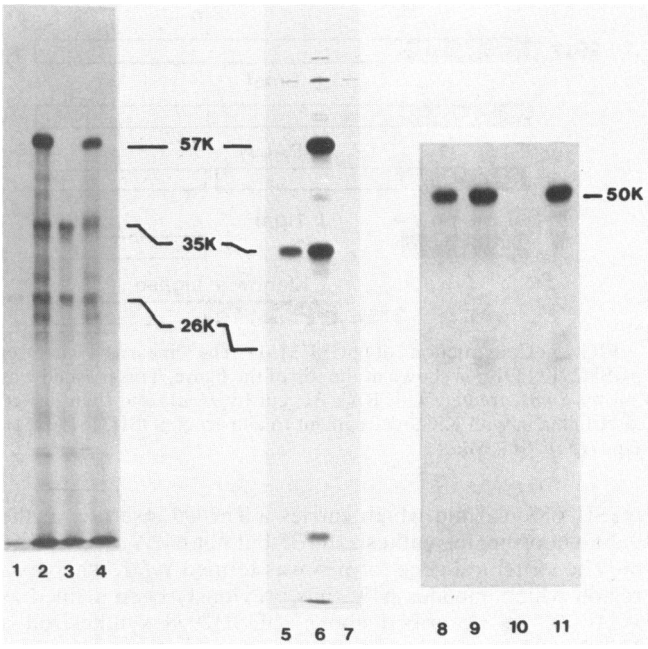

FIG. 4. Autoradiograms of sodium dodecyl sulfate gels containing [^{35}S]methionine-labeled proteins synthesized in maxicells containing a pLV85-based hybrid plasmid. Lane 1, pGSH2872; lane 2, pGSH2918; lane 3, pGSH2916; lane 4, pGSH2919; lane 5, pGSH2958; lane 6, pGSH2918; lane 7, pGSH2971; lane 8, pGSH2959; lane 9, pGSH2944; lane 10, pGSH2943; lane 11, pGSH3151. Numbers indicate molecular weights in thousands.

(Fig. 7, lanes 1 and 4) were shown to encode two polypeptides of 35 and 40 kDa in size (p35 and p40A, respectively), whereas pGSH3141 only programmed the synthesis of p40A (Fig. 7, lane 2). Therefore, the p35 gene starts downstream of the *Sal*I site at the 7.8-kb coordinate and encompasses the *Mlu*I site at the 8.5-kb coordinate, whereas the p40A gene is positioned downstream of the p35 gene and ends upstream of the *Hin*dIII site at the 10-kb coordinate. This result was confirmed by analysis of Tn*1000* insertion derivatives of pGSH2816 (Fig. 8). Two of these derivatives, pGSH2887 and pGSH2885, which have Tn*1000* insertions just downstream of the *xylC* gene, did not encode for the synthesis of any polypeptides (Fig. 7, lanes 9 and 10). pGSH2892, which carries a Tn*1000* insertion at the 8.5-kb coordinate, synthesized neither p35 nor p40A but a 14-kDa polypeptide which may be a truncated version of p35 (Fig. 7, lane 5). The

TABLE 2. BZDH activity expressed in K12ΔH1Δtrp containing a hybrid plasmid[a]

Plasmid	BZDH activity (mU/mg of protein)	
	30°C	42°C
pGSH2872	0	0
pGSH2918	0	27
pGSH2919	NT[b]	14
pGSH2916	NT	0
pGSH3151	NT	0

[a] Cells of the K12ΔH1Δtrp strain containing a hybrid plasmid were grown at 30°C to the cell density of 2×10^8 cells per ml and then shifted to either 42 or 30°C and grown for an additional 2 h. Cell extract was prepared from the individual culture and the BZDH activity in the extract was measured as described in Materials and Methods.

[b] NT, Not tested.

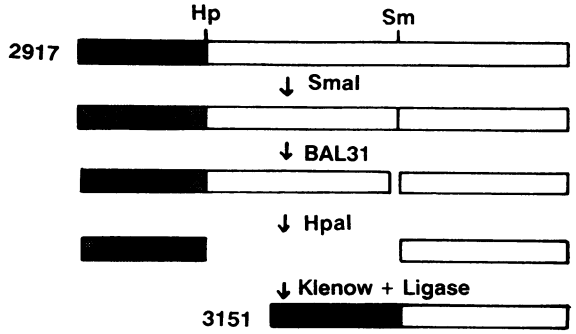

FIG. 5. Construction of pGSH3151. The linearized map of pGSH2917 (2917) is shown at the top of the figure. This plasmid was cut by *Sma*I, treated with BAL 31, cut by *Hpa*I, and then ligated after filling in with Klenow fragment to construct pGSH3151 (3151). Hp, *Hpa*I; Sm, *Sma*I.

pGSH2883 plasmid which carries a Tn*1000* insertion at the 9.2-kb coordinate synthesized p35 but not p40A (Fig. 7, lane 6). The structural gene for p35 was termed *xylM*, whereas a region which encodes p40A has previously been defined as *xylA* (8). As described above, pGSH2958 synthesized a 13-kDa polypeptide. This polypeptide was not synthesized from pGSH2817 (Fig. 3, lane 3) despite the fact that the DNA region cloned in pGSH2958 is completely included in pGSH2917. From this observation, we concluded that the 13-kDa polypeptide synthesized from pGSH2918, pGSH2919, pGSH2916, and pGSH2958 (Fig. 4) is a truncated version of the *xylA* gene product.

Both pGSH2816 and pGSH2819 (Fig. 6) programmed at least three different polypeptides sized 35, 40, and 52 kDa (Fig. 7, lane 7, and Fig. 9, lane 1). In addition, a 47-kDa polypeptide (p47) was detected in some experiments (Fig. 9, lanes 1, 3, and 4). This polypeptide is thought to be a degradative product of the 52-kDa polypeptide (p52), because partial digestion of p47 and p52 with *S. aureus* V8 protease yielded similarly sized fragments (data not shown). pGSH2873 specified polypeptides of 26, 35, and 40 kDa in size (Fig. 7, lane 8). Comparing the polypeptides synthesized from pGSH2816 or pGSH2819 and pGSH2873, we assumed that the 26-kDa polypeptide (p26) is a truncated product of p52 and that the *Hpa*I site at the 12-kb coordinate is in the middle of the structural gene for p52. pGSH2833 (Fig. 6) directed synthesis of three polypeptides of 52, 47, and 40 kDa (Fig. 9, lane 3). Therefore, the upper-pathway operon encodes two 40-kDa polypeptides. The polypeptide specified by pGSH2833 was called p40B to distinguish it from p40A specified by pGSH2817 (Fig. 6). In fact, the mobility of p40A (Fig. 9, lane 2) was slightly different from that of p40B (Fig. 9, lane 3). Analysis of pGSH2848 (Fig. 6) showed the presence of p40B and p26 (Fig. 9, lane 5). This result determined the location of the p40B upstream of the p52 gene. As expected, pGSH2847 (Fig. 6) synthesized only p52 and its degradative product p47 (Fig. 9, lane 4).

Inouye et al. (14) have mapped the structural gene for BADH between the 10- and 13.2-kb coordinates. This region encodes two proteins, p40B and p52. To identify the BADH protein, its activity was measured in *E. coli* K12ΔH1Δtrp cells containing pGSH2816 or its Tn*1000* insertion derivatives. The BADH activity was expressed from pGSH2816

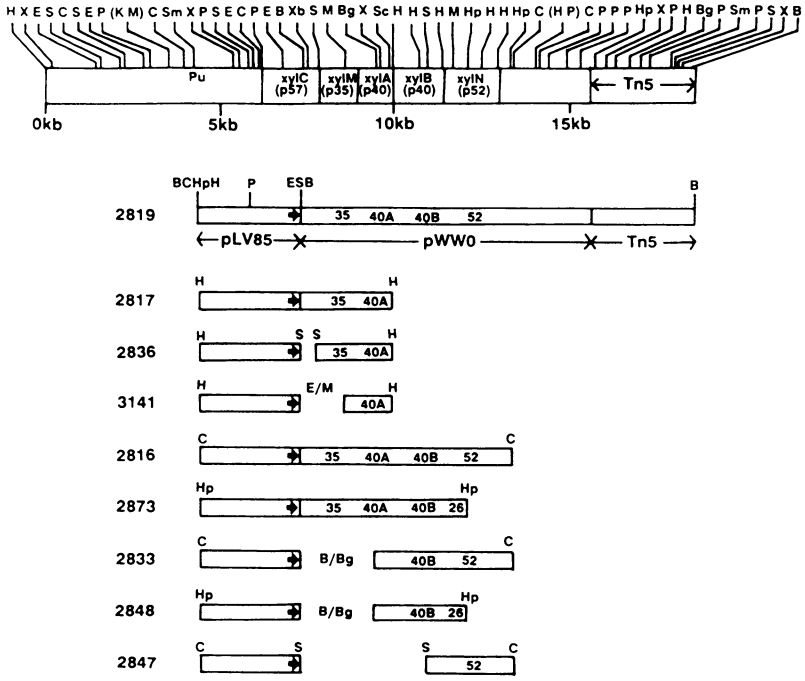

FIG. 6. Hybrid plasmids based on pLV85 containing upper-pathway genes *xylM*, *xylA*, *xylB*, or *xylN*. The uppermost part of the figure shows the upper-pathway genes in pWW0-161::Tn*5-68*. The map is identical to that of Fig. 3, except that a Tn*5* insertion in pWW0-161::Tn*5-68* which is located downstream of the upper operon is also indicated. pGSH2819 was constructed by cloning of a 11.4-kb *Bam*HI segment of pWW0-161::Tn*5-68* into pLV85. pGSH2817 was constructed from pGSH2819 by deletion of seven small *Hin*dIII segments of pLV85. pGSH2836 and pGSH3141 were constructed from pGSH2817 by deletion of its 0.7-kb *Sal*I and 1.3-kb *Eco*RI-*Mlu*I segments, respectively. pGSH2816 and pGSH2873 were constructed from pGSH2819 by deletion of its 5.2-kb *Cla*I segment and 6.5-kb *Hpa*I segment, respectively. pGSH2833 and pGSH2848 were constructed from pGSH2816 and pGSH2873 by deletion of their 2.1-kb *Bam*HI-*Bgl*II segment. pGSH2847 was constructed from pGSH2833 by deletion of its 3.6-kb *Sal*I-*Sal*I segment. See the legend to Fig. 3 for restriction site abbreviations.

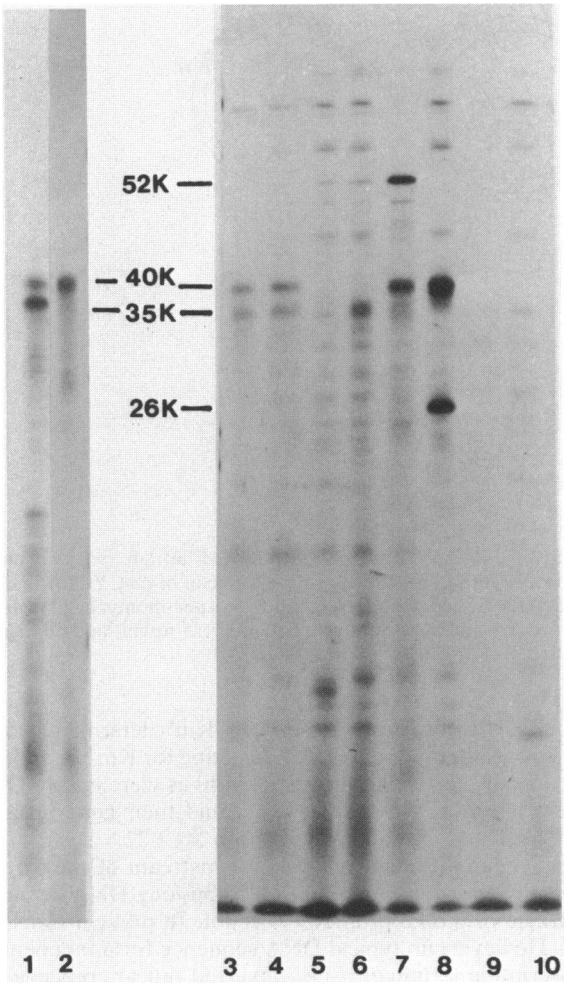

FIG. 7. Autoradiograms of sodium dodecyl sulfate gels containing [^{35}S]methionine-labeled proteins synthesized from pLV85-based hybrid plasmids. Lane 1, pGSH2836; lane 2, pGSH3141; lane 3, pGSH2817; lane 4, pGSH2836; lane 5, pGSH2892; lane 6, pGSH2883; lane 7, pGSH2816; lane 8, pGSH2873; lane 9, pGSH2887; lane 10, pGSH2885. Numbers indicate molecular weights in thousands.

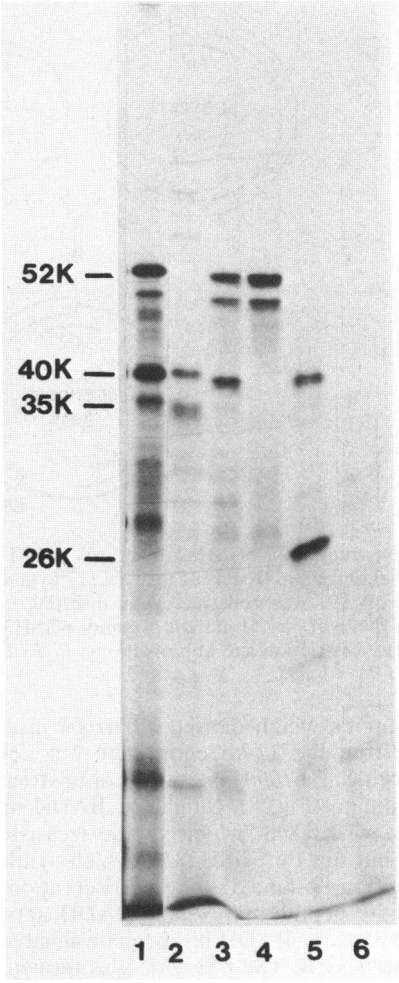

FIG. 9. *xylB* and *xylN* products programmed by hybrid plasmids. Lane 1, pGSH2819; lane 2, pGSH2817; lane 3, pGSH2833; lane 4, pGSH2847; lane 5, pGSH2848; lane 6, pLV85. Numbers indicate molecular weights in thousands.

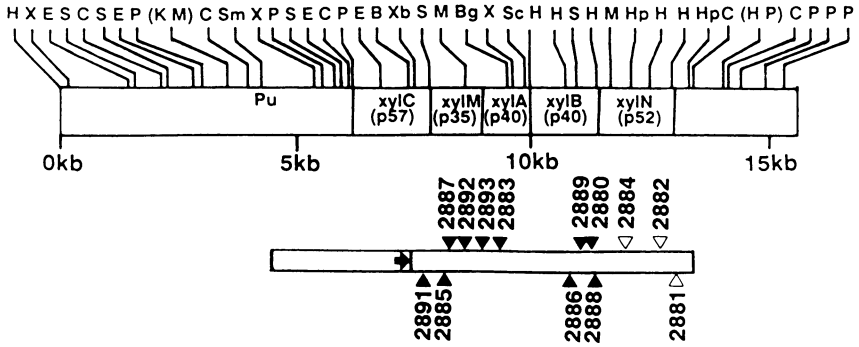

FIG. 8. Sites of Tn*1000* insertion in mutant derivatives of pGSH2816. The upper part of the figure shows the restriction enzyme and functional maps of the upper operon genes, whereas the lower part of the figure shows the structure of pGSH2816 and the sites of Tn*1000* insertion in its mutant derivatives. Triangles on the upper side of the pGSH2816 DNA indicate an orientation of γ to δ, whereas the triangles on the lower side indicate the reverse orientation (δ to γ). Insertions which inactivated BADH activity (▲, ▼) and insertions which did not affect this enzyme activity (△, ▽) are shown. See the legend to Fig. 3 for restriction site abbreviations.

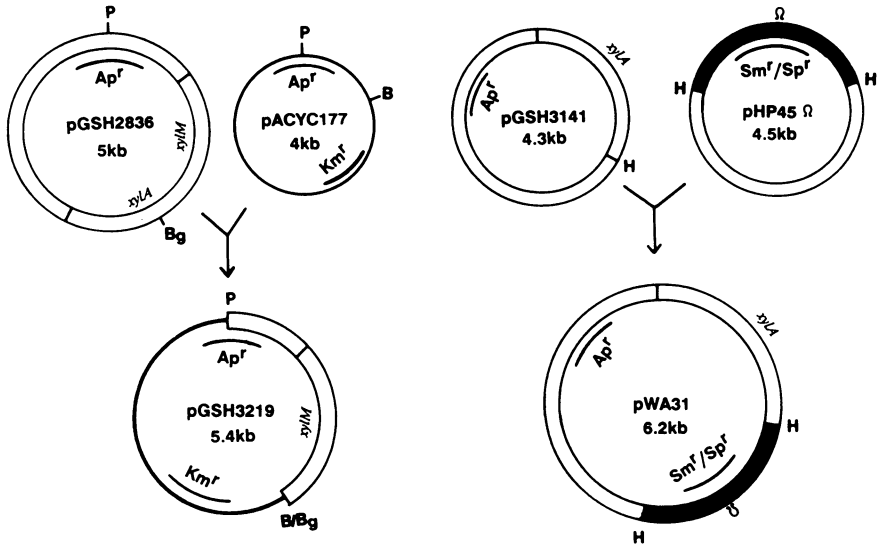

FIG. 10. Construction of pGSH3219 and pWA31. The *Pst*I-*Bgl*II segment of pGSH2836 containing intact *xylM* but not *xylA* was cloned between the *Pst*I and *Bam*HI sites of pACYC177 to generate pGSH3219. GSH3219 thus constructed has the replicon of pACYC177 and was Ap^r and Km^r. pWA31 was constructed by inserting the omega fragment of pHP45Ω, conferring resistance to spectinomycin (23), into a *Hin*dIII site of the *xylA*$^+$ *xylM* mutant plasmid, pGSH3141. pWA31 is therefore Ap^r and spectinomycin resistant (25 μg/ml) and compatible with pGSH3219. Restriction site abbreviations: P, *Pst*I; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II.

and its derivatives which carried a Tn*1000* insertion at or downstream from the 12-kb coordinate but not from the plasmids carrying a Tn*1000* insertion at or upstream from the 11.4-kb coordinate (Fig. 8). Similarly, BADH activity was expressed by pGSH2848, which synthesizes p40B but not p52 (Fig. 6), but not by pGSH2847, which synthesizes p52 but not p40A (Fig. 9, lane 5). These observations indicated that p40B is the peptide expressing BADH activity. Since the structural gene for BADH has been designated *xylB* (14), the p40B gene is *xylB*. The p52 gene was termed as *xylN*.

XO is composed of two different polypeptides coded for by *xylM* and *xylA*. pGSH2836 which encodes both p35 and p40A (Fig. 6) expressed XO activity, but its derivatives, pGSH3219 which synthesizes only p35 and pGSH3141 which synthesizes only p40A (Fig. 10), did not express it. This result suggested that both the *xylM* (p35) and *xylA* (p40A) products are required for the XO activity. To confirm this supposition, genetic complementation of the XO activity between *xylM* and *xylA* mutations was examined. The pGSH3219 and pWA31 plasmids (Fig. 10) were transformed into K12ΔH1Δtrp *recA*. The strain containing pGSH3219 or pWA31 alone did not oxidize toluene whereas the strain containing both pGSH3219 and pWA31 oxidized toluene to benzyl alcohol, benzaldehyde, and benzoate (data not shown). We have demonstrated that XO oxidizes toluene to benzyl alcohol and benzyl alcohol to benzaldehyde (8). In this experiment, we could not determine whether XO oxidizes benzaldehyde to benzoate, since *E. coli* K-12 expresses a chromosomally encoded NADP-dependent dehydrogenase which transforms benzyl alcohol to benzoate (J. Shaw, unpublished results). The physiological role of this enzyme is not yet known.

DISCUSSION

In this study, the upper-pathway genes were cloned into the expression vector pLV85 in two steps. Initially, a Km^r determinant was inserted downstream of the upper-pathway genes to be cloned, and then a segment of DNA containing the upper-pathway genes and the Km^r determinant were cloned together into pLV85 by selecting for Km^r. A series of deletion and Tn*1000* insertion derivatives were isolated from parent plasmids thus constructed, and their gene products were analyzed in the maxicell strain MCL22.

In the gene product analysis downstream of the Pu promoter, we demonstrated that a 200-bp-long DNA sequence upstream of the Pu promoter was able to prevent transcription. However, no typical DNA sequence for ρ-independent transcription terminator, i.e., inverted repeats preceded by AT-rich sequences (22), was found in the DNA sequences of this region determined by Inouye et al. (13).

No polypeptide synthesis from the 1.7-kb-long sequence between the Pu promoter and the start of *xylC* was identified in maxicells. Thus, the region either encodes no polypeptide or a polypeptide(s) present only in a small quantity. Since all the three upper-pathway enzymes were mapped downstream of this silent region, the function(s) of this 1.7-kb-long promoter-proximal region is not clear at present.

It was demonstrated that the catabolic genes are organized in the upper operon in the order of *xylCMABN*, and the products of each gene were identified. The *xylC* gene encodes BZDH, a protein of 57 kDa. Two proteins, namely, the 35-kDa *xylM*-encoded protein and the 40-kDa *xylA*-encoded protein, were shown to constitute XO. The *xylB* gene product is a 40-kDa peptide that corresponds to BADH. The *xylN* product is a 52-kDa protein which is processed to a 47-kDa polypeptide, the physiological role of which is unknown. No polypeptide synthesis from a region downstream of *xylN* was detected; therefore, *xylN* may be the last gene of the upper operon. When the size of each catabolic gene was calculated from the size of each product, most of the DNA sequence between *xylC* and *xylN* was determined to be coding region.

XO catalyzes the hydroxylation of the carbon side chain of toluene and benzyl alcohol (8). Such hydroxylation is often the first step of the decomposition of organic compounds in bacteria and usually catalyzed by monooxygenases (20).

From these observations, we infer that XO is a monooxygenase. However, *p*-cresol hydroxylase from *P. putida* NCIB 9866 (12), which is made from a cytochrome *c* and a flavoprotein, catalyzes incorporation of the hydroxyl group from water into a substrate. Further characterization of XO is therefore required to understand its reaction mechanisms. Overproducers of XO constructed in this study will be useful for such a project.

Although XO is capable of carrying out the first two steps of the upper reactions, the physiological importance of oxidation of (methyl)benzyl alcohol by XO is not clear. Subcloning of the upper genes and their localization on the restriction map done in this study provide the possibility of construction and characterization of nonpolar deletion mutants of the TOL pWW0 plasmid in the silent region between Pu and *xylC*, *xylM*, *xylA*, *xylB*, and *xylN*. We are currently isolating such mutants to aid in the further understanding of the functions of the upper enzymes.

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