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Molecular Cloning of TOL Genes *xylB* and *xylE* in *Escherichia coli*

SACHIYE INOUYE, ATSUSHI NAKAZAWA, AND TERUKO NAKAZAWA*

Department of Biochemistry, Yamaguchi University School of Medicine, Ube, 755 Japan

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The *xylB* and *xylE* genes in the TOL plasmid of *Pseudomonas putida* mt-2, which code for benzyl alcohol dehydrogenase and catechol 2,3-oxygenase, respectively, were cloned onto plasmid pBR322 in *Escherichia coli* for detailed mapping. The *xylB* gene was mapped in a 2.9-kilobase region within the *Bam*HI BC fragment of pTN2, an in vivo RP4-TOL recombinant, whereas the *xylE* gene was mapped in a 1.8-kilobase region within the *Bam*HI BD fragment. The directions of transcription of these genes were deduced from the expression of the cloned genes which had been ligated in orientations opposite pBR322 at its *Bam*HI site within the tetracycline resistance gene. The *xylB* and *xylE* genes are inducible by a specific inducer of the TOL pathway genes in the RP4-TOL recombinant, whereas they are not inducible in the pBR322-TOL hybrids. The regulatory regions involved in expression of the *xylB* and *xylE* genes do not appear to be located in the vicinity of the structural genes. Catechol 2,3-oxygenase formed in *E. coli* carrying an *xylE*-containing plasmid is similar, or identical, to that formed in *P. putida* carrying the TOL plasmid.

The TOL plasmid of *Pseudomonas putida* mt-2 codes for a set of enzymes responsible for the degradation of toluene, which proceeds through benzyl alcohol, benzaldehyde, benzoate, catechol, and 2-hydroxymuconic semialdehyde (19). These enzymes are inducible by a specific inducer, and a model for the regulation of their synthesis has been proposed (18): the genes of the early enzymes of the pathway are in two regulatory blocks, *xylABC* and *xylDEFG*, which are under the control of two regulatory genes, *xylR* and *xylS*. To understand the molecular basis of the regulation of *xyl* genes, a recombinant plasmid of TOL and RP4, designated pTN2, was isolated in vivo in *Pseudomonas aeruginosa*, which is transmissible to, and replication proficient in, *Escherichia coli* (10). The induction of the TOL pathway enzymes in cells of *P. putida* carrying pTN2 is similar to that of the wild-type TOL plasmid. Cells of *E. coli* carrying pTN2 also showed induction of benzyl alcohol dehydrogenase and catechol 2,3-oxygenase (EC 1.13.11.2), which are encoded by the *xylB* and *xylE* genes, respectively, although the enzyme levels are lower than in *P. putida*. We have recently reported a restriction endonuclease cleavage map of pTN2 (111 kilobase pairs [kb] in length) and its derivatives (11). Physical and functional mapping of deletion and insertion mutations allowed us to estimate regions containing some *xyl* genes on the pTN2 map. On the other hand, Downing and Broda (4) reported a cleavage map for restriction endonucleases *Xho*I and *Hin*dIII of the TOL plasmid (117 kb). The maps of the corresponding regions of pTN2 and TOL showed good agreement except for the reversed location of two *Xho*I fragments.

In the present study, we carried out molecular cloning of the *xyl* genes in *E. coli* to map further the TOL plasmid. The *Bam*HI fragments of pTN2, which were considered to contain *xylB* and *xylE*, were isolated and inserted into the *Bam*HI site of pBR322. The directions of transcription of these genes were determined by analyzing the expression of the cloned genes with plasmids in which the genes were ligated in orientations opposite the vector. The cloned genes were not inducible and were expressed mainly from the vector promoter. Thus, the detailed physical and functional maps of the *xylB* and *xylE* genes were constructed.

MATERIALS AND METHODS

Bacterial strain and plasmids. The bacterial strain used was *E. coli* 20SO (*thi lac mal mtl ara xyl rpsL*) (1), a derivative of *E. coli* K-12. The plasmids used were pBR322 (2), pACYC184 (3), and an RP4-TOL recombinant, pTN2 (10).

Media and culture conditions. The medium used throughout the experiment was L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Agar was added to 1.5% in the L broth medium to prepare L agar plates. Antibiotic concentrations used for selection of trans-

formants were 100 μg of ampicillin, 10 μg of tetracycline, and 10 μg of chloramphenicol per ml. All incubations were carried out at 37°C.

Enzyme assays. Cells were grown overnight in L broth containing ampicillin (20 μg/ml) at 37°C, and crude extracts were prepared in 0.05 M potassium phosphate (pH 7.5) containing 10% acetone (acetone phosphate buffer) (12). Benzyl alcohol dehydrogenase, catechol 2,3-oxygenase, and β-lactamase were assayed with crude extracts as described previously (11). Proteins were determined by the biuret method, using bovine serum albumin as a standard.

Purification of plasmid DNA. Isolation of covalently closed circular plasmid DNA has been described previously (11). Crude plasmid DNA was prepared on a reduced scale by the same procedure, except for omitting cesium chloride-ethidium bromide equilibrium centrifugation.

Digestion of DNA by restriction endonucleases and electrophoresis of digested DNA fragments. Restriction endonucleases *Bam*HI, *Xho*I, *Eco*RI, and *Hin*dIII, were purchased from Bethesda Research Laboratories, Bethesda, Md., or Boehringer Mannheim Corp., New York, N.Y. *Sal*I was purchased from Takara Shuzo Co. Ltd., Kyoto, Japan. DNA was digested with *Sal*I in the buffer containing 10 mM Tris-hydrochloride (pH 7.5), 7 mM $MgCl_2$, 0.1 M NaCl, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, and 0.1% bovine serum albumin. Digestion with other restriction endonucleases, purification of digested DNA fragments by electrophoretic elution, and analysis of restriction fragments by agarose or polyacrylamide gel electrophoresis were done as previously described (11).

Ligation and transformation. The T4 ligase was purified from *E. coli* ED1150 lysogenized with λT4*lig* (NM989), a phage containing the T4 ligase gene (9) by a modification of the method described for the purification of restriction endonucleases (5). The purified preparation was nuclease-free and could be used for molecular cloning. Ligation was carried out by using the T4 ligase at 4°C for 20 h in a ligation buffer containing 90 mM Tris-hydrochloride (pH 7.9), 60 mM $MgCl_2$, 100 mM dithiothreitol, and 0.5 mM ATP (7). The ligation mixture was used directly to transform $CaCl_2$-treated cells of *E. coli* (8). Transformants were selected on L agar containing ampicillin or chloramphenicol and replicated onto L agar containing tetracycline to isolate ampicillin-resistant (Ap^r) and tetracycline-sensitive (Tc^s) clones or chloramphenicol-resistant (Cm^r) and Tc^s clones. A solution of 0.1 M catechol was sprayed over the colonies for detection of catechol 2,3-oxygenase.

Purification of catechol 2,3-oxygenase. The purification of catechol 2,3-oxygenase from *E. coli* 20SO carrying a hybrid plasmid, pTS92, or *P. putida* mt-2 was carried out by the method of Nozaki (13). Cells were grown overnight in L broth at 37°C for *E. coli* and at 27°C for *P. putida* with aeration, and the crude extract was prepared in the acetone phosphate buffer by sonic oscillation followed by centrifugation to remove cell debris. The supernatant solution was fractionated with acetone. The precipitates between 50 to 66% acetone were collected and dissolved in the acetone phosphate buffer. The solution was then applied onto a DEAE-cellulose column equilibrated with the acetone phosphate buffer. The enzyme was eluted with a linear gradient from 0 to 5% saturation of ammonium sulfate in the acetone phosphate buffer. The active fractions were collected by precipitation with 66% acetone followed by dialysis against the acetone phosphate buffer. Purity of the enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Immunological analysis. A rabbit was injected subcutaneously two times with 500 μg of the purified catechol 2,3-oxygenase of *E. coli* emulsified in Freund complete adjuvant (Iatron Laboratories Co., Tokyo, Japan) over a 2-week period. One week after the second injection, antiserum was prepared. Ouchterlony double-diffusion analysis was performed as described previously (15).

Containment conditions. The experiments were carried out under P1-EK1 containment conditions as specified by the Guideline for Recombinant DNA Research published by the Ministry of Education, Science and Culture of Japan.

RESULTS

Molecular cloning of *xyl* genes. We previously described the restriction endonuclease cleavage map of pTN2 (11). The locations of *xylB*, the structural gene of benzyl alcohol dehydrogenase, and *xylE*, the gene of catechol 2,3-oxygenase, were suggested to be *Bam*HI fragments BC and BD of pTN2 DNA, respectively. To confirm the physical map and to make a detailed functional map of *xyl* genes, molecular cloning of *xylB* and *xylE* was carried out. Figure 1 summarizes the physical and functional map of pTN2 and the constructed plasmids containing TOL fragments based on previous and present studies. A mixture of BC and BD fragments purified by electrophoretic elution from the *Bam*HI-digested pTN2 DNA was ligated to the unique *Bam*HI site of pBR322. Since the *Bam*HI site in the vector is within the gene for tetracycline resistance (2), transformants with *Bam*HI inserts are recognized as Ap^r Tc^s clones. Seven such transformants were obtained. Crude plasmid DNAs were digested with *Bam*HI, and the sizes of pTN2 inserts were determined by agarose gel electrophoresis. Two transformants had a hybrid plasmid (pTS7) containing an insert of 15.4 kb which was equal in size to the BC fragment. Five transformants had a hybrid plasmid (pTS1) containing a 13.5-kb insert which was equal in size to the BD fragment. All of these five transformants produced catechol 2,3-oxygenase, as judged by the yellow color after a solution of catechol was sprayed on the colonies. Cleavage of the hybrid plasmids with *Eco*RI established the orientation of the fragments cloned into the *Bam*HI site of pBR322.

To isolate hybrid plasmids in which the orientations of the cloned *Bam*HI fragments were different from those of pTS7 and pTS1, plasmid

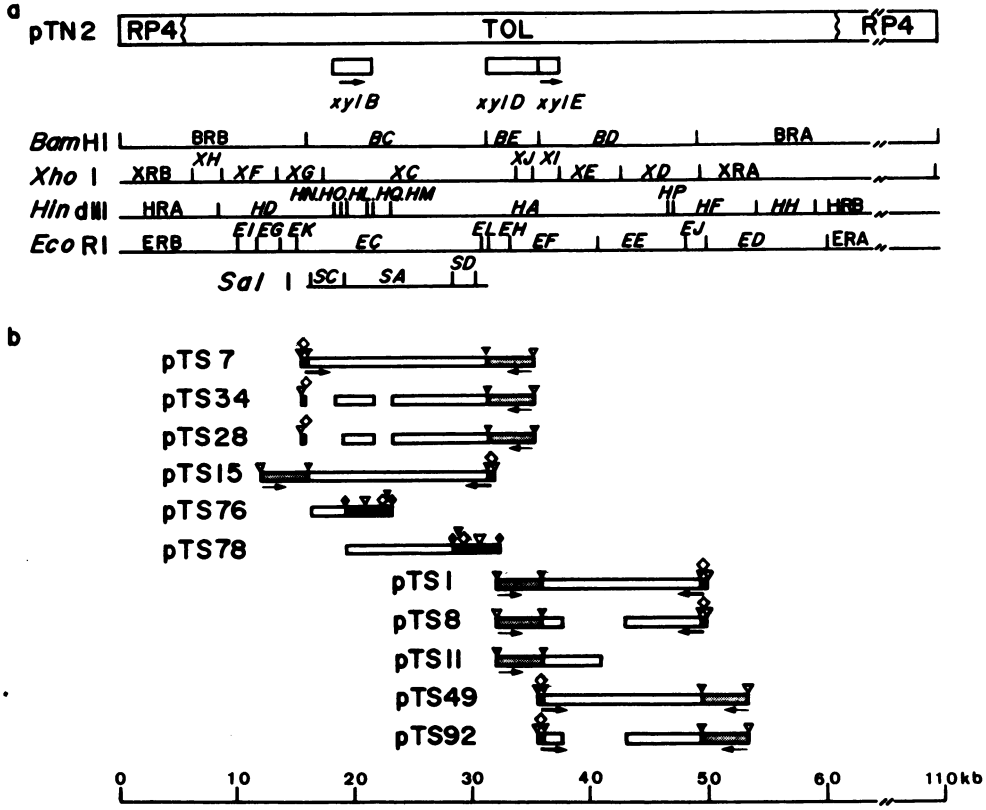

FIG. 1. *Map of xyl genes. (a) Physical map of the TOL region of pTN2, including the positions of the genes of benzyl alcohol dehydrogenase (xylB), toluate oxygenase (xylD), and catechol 2,3-oxygenase (xylE). The arrows indicate the direction of transcription for each gene. For each of the restriction endonucleases indicated on the left, the relative locations of the cleavage sites are marked by a vertical line (11). (b) Physical maps of pBR322 and pACYC184 derivatives containing inserted TOL fragments. Open, stippled, and closed boxes indicate the TOL, pBR322, and pACYC184 regions, respectively. The thick and thin arrows indicate the direction of transcription of tetracycline resistance and β-lactamase genes, respectively. Symbols for restriction sites in pBR322 and in pACYC184 are as follows: ▽, EcoRI; ▼, BamHI; ◇, HindIII; ◆, SalI. The scale is shown in kilobases, starting from the BamHI site of RP4.*

DNAs were cleaved with *Bam*HI, precipitated with ethanol, and ligated again with the T4 ligase. Ap^r Tc^s transformants were selected, and a pTS7-derived plasmid (pTS15) and a pTS1-derived plasmid (pTS49) were obtained. The orientations of the inserted BC fragment in pTS15 and the BD fragment in pTS49 were opposite to those of the corresponding fragments in the parent plasmids as judged by cleavage with *Eco*RI.

Physical map of the BC fragment and deletion derivatives of pTS7. The *Bam*HI BC fragment contains five small *Hin*dIII fragments which form a cluster in pTN2. Their order, however, had not previously been established (4, 11). To map this region, the cleavage map for *Sal*I of the BC fragment was constructed. By digestion of pTS7 DNA with *Sal*I, five fragments were generated: 9.0 kb (SA), 4.6 kb, 3.2 kb (SC), 1.8 kb (SD), and 1.25 kb. By double digestion with *Sal*I and *Bam*HI, the 4.6- and 1.25-kb fragments were cleaved, indicating that these are the junction fragments of pBR322 and the TOL insert. By double digestions with *Sal*I and either *Xho*I, *Eco*RI, or *Hin*dIII, the order of the *Sal*I fragments in BC was deduced to be SC, SA, SD from left to right on the pTN2 map (Fig. 1a). Double digestion with *Sal*I and *Hin*dIII also showed that the HO fragment had a *Sal*I site which is the junction between SC and SA.

To locate other *Hin*dIII fragments of pTS7, the plasmid was digested with *Sal*I and ligated to pACYC184 at its unique *Sal*I site that is within the tetracycline gene (3). Among Cm^r Tc^s transformants, those carrying a hybrid plasmid containing the SA fragment (pTS78) or SC fragment (pTS76) were isolated. By digestion with

*Hin*dIII, pTS76 generated HN, whereas pTS78 generated HL, HM, and HQ. To determine the arrangement of the latter fragments, pTS7 was partially digested with *Hin*dIII, ligated, and transformed. Plasmids harbored by Ap^r Tc^s transformants were analyzed by cleavage with *Hin*dIII. Thus, nine derivatives were obtained in which one, two, or three *Hin*dIII fragments were absent. pTS34 and pTS28 are such derivatives (Fig. 1b). Assuming that neighboring fragments remained together upon cleavage and ligation, HO is closer to HL than to HQ and is far from HM. Based on these results, the order of the small *Hin*dIII fragments in BC of pTN2 is deduced to be HN, HO, HL, HQ, HM from left to right (Fig. 1a).

Deletion derivatives of pTS1. Digestion of pTS1 DNA with *Xho*I generated a 5.3-kb fragment equal in size to that of XE, whereas digestion with *Eco*RI generated a 7.8-kb fragment equal in size to that of EE. To map the *xylE* gene in the BD fragment, deletion derivatives of pTS1 were prepared by digestion with *Xho*I or *Eco*RI followed by ligation and transformation. Thus, pTS8 was made after the XE fragment was removed, and pTS11 was constructed after the EE fragment and a part of EJ fragment were removed. By an analogous procedure, pTS92 was constructed from pTS49 by removal of the XE fragment.

Locations of *xylB* and *xylE* on the pTN2 map. To locate the *xylB* and *xylE* genes, benzyl alcohol dehydrogenase and catechol 2,3-oxygenase were assayed in crude extracts of *E. coli* carrying pBR322-TOL hybrids (Table 1). The presence of the BC fragment in the hybrid plasmid (pTS7 and pTS15) resulted in production of benzyl alcohol dehydrogenase. Hybrid pTS34, but not pTS28, expressed the enzyme. pTS34 had the *Hin*dIII fragments HN, HO, HL, and HQ, whereas pTS28 had HO, HL, and HQ. Therefore, the HN fragment is essential for the enzyme synthesis, and the region extending from HN to HQ contains sufficient information to direct the synthesis of the functional enzyme. The cells carrying pTS76 that contains HN and a part of HO or those carrying pTS78 that contains HL, HQ, HM, and a part of HO could not synthesize the enzyme. Furthermore, plasmids without HO or HL which were prepared by cleaving pTS7 partially with *Hin*dIII and ligating again, could not express the functional enzyme. Thus, the HO and HL fragments are required for the synthesis of benzyl alcohol dehydrogenase. The requirement for the HQ fragment is not clear at present. It is concluded that the structural gene of benzyl alcohol dehydrogenase *xylB* is located in the 2.9-kb region extending from the HN fragment to the HQ fragment of pTN2 DNA (Fig. 1a).

TABLE 1. *TOL enzyme levels in E. coli carrying recombinant plasmids*[a]

Plasmid	Sp act (mU/mg)[b]		
	Benzyl alcohol dehydrogenase	Catechol 2,3-oxygenase	β-Lactamase
pTN2	13	3	1,950
pTN2	24[c]	290[c]	2,160[c]
pTS7	970	0	4,800
pTS34	110	0	2,540
pTS28	0	0	ND[d]
pTS15	245	0	4,500
pTS1	0	42	2,580
pTS8	0	36	ND
pTS11	0	26	ND
pTS49	0	6,240	2,610
pTS92	0	6,320	3,260

[a] Cells were grown in L broth at 37°C.
[b] One milliunit (mU) corresponds to the formation of 1 nmol of the product per min at 27°C.
[c] Cells of *E. coli* carrying pTN2 were grown in the presence of 2.5 mM *m*-methylbenzyl alcohol.
[d] ND, Not determined.

Strains bearing plasmids containing the BD fragment (pTS1 and pTS49) produced catechol 2,3-oxygenase. Since deletion of XE or EE did not cause a loss of the enzyme, the structural gene of catechol 2,3-oxygenase (*xylE*) is located in the 1.8-kb region extending from left end of the BD fragment to the right end of the XI fragment (Fig. 1a).

Quantitative expression of the *xyl* genes of pTN2 and of pBR322-TOL plasmids. Quantitative expression of *xyl* genes in *E. coli* carrying pTN2 was determined under induced or noninduced conditions (Table 1). The *xylE* expression was activated markedly by an inducer, *m*-methylbenzyl alcohol, as in *P. putida*, but its induced and noninduced levels were only 4 and 1% of those in *P. putida*, respectively (11). On the other hand, the *xylB* expression was activated only twofold by the inducer, and the noninduced expression was more than five times higher than in *P. putida*.

The results described in the previous section demonstrated that the 1.8-kb region of the BD fragment contains sufficient information to direct the synthesis of catechol 2,3-oxygenase. A striking difference at the quantative level of the *xylE* expression, however, was observed when the inserted genes in the hybrid plasmids were in opposite orientations. The level of catechol 2,3-oxygenase produced in cells carrying pTS49 was more than 100-fold of that in cells carrying

pTS1, although the levels of β-lactamase in these strains were similar (Table 1). These results suggest that the expression of the cloned *xylE* gene of pTS49 is a result of the read-through transcription initiated at a promoter of pBR322. Since the cloned gene is inserted into the *Bam*HI site within the tetracycline gene of the vector, the above-mentioned findings indicate that transcription of the *xylE* gene is initiated at the tetracycline promoter (16, 17). If this is the case, the direction of transcription of the *xylE* gene should be from left to right on the pTN2 map. The same conclusion was reached in the previous studies on a polarity mutation caused by an insertion of a DNA segment into the BD fragment of pTN2 DNA (11).

The cells carrying pTS7 produced four times more benzyl alcohol dehydrogenase than those carrying pTS15, although the levels of β-lactamase were essentially the same (Table 1). These plasmids contain the BC fragment in opposite orientations in the *Bam*HI site of pBR322. pTS34, a derivative of pTS7, lost the promoter region of the tetracycline gene that is between the *Eco*RI and *Bam*HI sites of pBR322 (16, 17). The cells carrying pTS34 produced less benzyl alcohol dehydrogenase than those carrying pTS7. The difference in the expression of *xylB* of these plasmids could also be interpreted by the read-through transcription from the tetracycline promoter, although the effect was not so remarkable as in the case of *xylE*. The above interpretation is compatible with the previous results, suggesting that the direction of transcription of *xylB* proceeds from left to right on the pTN2 map (11).

Catechol 2,3-oxygenase synthesized in *E. coli*. The level of catechol 2,3-oxygenase in the crude extract of *E. coli* carrying pTS92 was almost equivalent to that in the crude extract of *P. putida* carrying pTN2 grown in the presence of an inducer (11). To determine whether the product of the cloned *xylE* gene in *E. coli* had the same properties as that of *xylE* in *P. putida*, catechol 2,3-oxygenase was purified from *E. coli* carrying pTS92. The specific activity of the purified enzyme was 227 U/mg of protein, which was higher than that of the *P. putida* enzyme purified from the mt-2 strain (110 U/mg of protein) (13).

The purified preparation of catechol 2,3-oxygenase from *E. coli* was essentially homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Coelectrophoresis of the *E. coli* enzyme with the *P. putida* enzyme gave a single band. Therefore, the *E. coli* enzyme appears to be identical to the *P. putida* enzyme which consists of four identical subunits with a molecular weight of 35,000 (14). An antiserum against the *E. coli* enzyme showed only one fused precipitation line to both the *E. coli* and the *P. putida* enzymes on Ouchterlony immunodiffusion analysis (data not shown). The above-mentioned results indicate that pTS92 contains the sequence representing essentially the complete coding region of *xylE*, and neither polypeptide fusion nor shortening occurs as a result of the cloning.

DISCUSSION

Catabolic versatility of *Pseudomonas* spp. has been attracting the attention of many researchers, but its genetic background has rarely been clarified. Some catabolic pathways are controlled by plasmids that encode for a set of enzymes functioning in sequential steps of degradation. The TOL plasmid is one such degradative plasmids found in strains of *P. putida*. In a previous report, we described a restriction endonuclease cleavage map of pTN2, an in vivo recombinant consisting of the entire RP4 genome and a part of the TOL plasmid (11). We have described here molecular cloning of two *xyl* genes onto pBR322 in *E. coli*. The endonuclease cleavage map of the cloned fragments confirmed our previous physical map. The cloned *xylB* and *xylE* genes were expressed in this host, and benzyl alcohol dehydrogenase and catechol 2,3-oxygenase were produced. By in vitro recombination, we located *xylB* in the 2.9-kb region of the BC fragment on the pTN2 map ranging from the HN to HQ fragment (Fig. 1a). Similarly, *xylE* was located in the 1.8-kb region which is shared by fragments BD and XI. Catechol 2,3-oxygenase of *P. putida* mt-2 has a molecular weight of 140,000 in a native form and consists of four identical subunits of a 35,000-dalton polypeptide (14). A DNA region equivalent to the polypeptide is about 0.9 kb in length, which corresponds to about one-half of the mapped *xylE* region. The *xylE* gene product formed in *E. coli* is similar, or identical, to that formed in *P. putida* as judged by the catalytic activity, molecular weight of the subunit, and antigenicity of the gene product.

Plasmid pTN2 conferred inducible synthesis of catechol 2,3-oxygenase in *E. coli* cells, but both induced and noninduced levels of the enzyme were very low as compared with those of *P. putida*. These results can be interpreted as inefficient transcription of the foreign gene in *E. coli*, although inefficient translation is not excluded. Cells of *E. coli* carrying pTS1 produced 10 times more catechol 2,3-oxygenase than those carrying pTN2 in the absence of the inducer. The enzyme encoded by pTS1, however, was not inducible. The operator-promoter region of the

xylDEFG operon appears to be absent in pTS1 because xylD, the proximal gene of the operon, is located in the BE fragment (Fig. 1a) (11). The *xylE* gene in pTS1, therefore, should be transcribed from a promoter which is not the natural promoter of the *xylDEFG* operon and is located in the BD fragment or in the vector. A hundred-fold-higher level of catechol 2,3-oxygenase was observed in cells carrying pTS49, the plasmid containing *xylE* in the opposite orientation from pTS1, than that in cells carrying pTS1. Transcription of the tetracycline gene proceeds in the direction from the *Eco*RI site to the *Bam*HI site of pBR322 (17), whereas transcription of the *xylE* gene proceeds from left to right on the pTN2 map (11). Restriction analysis revealed that the tetracycline promoter can be used for the *xylE* transcription of pTS49. The efficient expression of *xylE* in pTS49, therefore, is due to the efficient transcription from the tetracycline promoter by *E. coli* RNA polymerase.

The expression of *xylE* of pTN2 in *E. coli* was almost entirely dependent on the inducer, whereas that of *xylB* was essentially independent. According to the model of Worsey et al. (18), both *xylABC* and *xylDEFG* operons are activated by the *xylR* product in the presence of *m*-methylbenzyl alcohol in *P. putida*. If the expression of *xylB* and *xylE* in *E. coli* were controlled in a manner similar to that in *P. putida*, the induction of benzyl alcohol dehydrogenase and catechol 2,3-oxygenase might have occurred to a similar extent. Therefore, the transcription of *xylB* in *E. coli* may depend on a promoter which is not the natural promoter of the *xylABC* operon. Such a transcription could account for, in part, the high level of the *xylB* gene product in the noninduced cells of *E. coli*.

Cells of *E. coli* carrying pTS15 produced 20 times more benzyl alcohol dehydrogenase than those carrying pTN2, which might be due to the gene-dose effect of the multicopy vector pBR322 (16) against the few-copy vector RP4 (6). The effect of the orientation of *xylB* on its expression was also observed between pTS7 and pTS15 but was not so remarkable as in the case of *xylE*. The inserted fragment of these plasmids might contain an internal promoter of *xylB* which is used for the transcription in *E. coli* and cause the effect of the orientation of the gene less prominent.

Our results demonstrated that molecular cloning is useful for analyzing the gene expression of TOL in *E. coli*. To understand the molecular basis of the control of the gene expression, it is necessary to clone separately the structural genes with intact operator-promoter regions and the regulatory gene. The location of gene *xylE* and its direction of transcription presented in this paper, as well as the location of *xylD* previously described (11), provide information to locate the operator-promoter region of the *xylDEFG* operon. Molecular cloning of the regulatory gene *xylS* is now in progress in our laboratory.

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