JOURNAL OF BACTERIOLOGY, Apr. 1991, p. 2724–2728 Vol. 173, No. 8
0021-9193/91/082724-05$02.00/0
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Nucleotide Sequence of *xylE* from the TOL pDK1 Plasmid and Structural Comparison with Isofunctional Catechol-2,3-Dioxygenase Genes from TOL pWW0 and NAH7

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Received 14 September 1990/Accepted 10 January 1991

Detailed restriction and nucleotide sequence analysis of the *Pseudomonas putida* TOL plasmid pDK1 *xylE* gene revealed significant homology with isofunctional *xylE* (81.5%) and *nahH* (78.0%) genes from the TOL pWW0 and NAH7 plasmids. The highest degrees of nucleotide and apparent amino acid conservation (82.2 and 86.4%, respectively) among all three genes were found to exist within a region comprising 264 nucleotides encoding the C terminus. A comparison of localized regions revealed significantly greater homology between $xylE_{pWW0}$ and $xylE_{pDK1}$ within the C-terminal region, whereas $xylE_{pWW0}$ and *nahH* showed greater similarity at the N terminus. The possibility that $xylE_{pWW0}$ may represent a genetic hybrid of $xylE_{pDK1}$ and *nahH* is discussed.

TOL plasmids, which encode for the degradation of toluene and related aromatic hydrocarbons, have been the subject of much recent intensive study. The genes in TOL plasmids are arranged in two operons, one encoding enzymes for initial hydrocarbon oxidation (the upper operon) and the other specifying enzymes for aromatic ring cleavage and the *meta* fission pathway (the lower operon) (for reviews, see references 1, 6, and 22). Despite extensive mapping studies aimed at resolving the genetic fine structure of TOL-encoded operons, little information on the nucleotide sequence of relevant genes has yet to emerge. The one exception to this is *xylE* from TOL plasmid pWW0 ($xylE_{pWW0}$), which encodes the aromatic-ring-cleavage enzyme, catechol 2,3-dioxygenase (C23O) (metapyrocatechase) (EC 1.13.11.2) (13). The *xylE* gene product is a 307-amino-acid polypeptide that shares 74% sequence homology with its isofunctional counterpart *nahH* from plasmid NAH7, conferring naphthalene degradation (4, 5). The two genes are 80% homologous in nucleotide sequence, and therefore the possibility that they evolved from a common ancestor has been proposed (5). A comparison of isofunctional *xylE* genes from separate TOL plasmids has, however, not been undertaken despite the fact that some TOL plasmids may encode for more than a single C23O (2, 10, 14). The present work describes the nucleotide sequence of *xylE* from the TOL plasmid pDK1, which is geographically remote in origin from that of TOL pWW0. In addition, its structure is compared with that of $xylE_{pWW0}$ and *nahH*. A preliminary account of this work appeared previously (20).

The TOL plasmid pDK1 was originally isolated from *Pseudomonas putida* HS1 in Minnesota by Kunz and Chapman (11). The catabolic genes were subsequently subcloned by Shaw and Williams (18) into plasmid RP1 and shown to exist in two separate operons similar to the situation in TOL pWW0 (22). We acquired the RP1-TOL pDK1 recombinant, designated pDKR1, from the above investigators and subjected it to further subcloning procedures to isolate the *xylL-E* region of the lower operon. pBR325 and pUC19 cloning vectors and pDKR1 DNA from *P. putida* PaW630 (Trp^- Str^r) were prepared by established methods (8, 19). DNA from an *Eco*RI digest of pDKR1 was subcloned into pBR325, generating plasmid pBK188 containing a 5.1-kb insert spanning the *xylL-E* region. This plasmid was then digested with *Xho*I, and DNA was further subcloned into the *Sal*I cloning site of pUC19 to generate pBK189 carrying the *xylE* gene on a 2.2-kb fragment. Cells carrying the latter plasmid expressed C23O activity as evidenced by the formation of yellow ring-fission product (2-hydroxymuconic semialdehyde) when suspended in catechol-containing (1 mM) phosphate buffer (pH 7.0). The 2.2-kb *Xho*I fragment from pBK189 was isolated and digested with *Taq*I, *Bst*EII, *Rsa*I, *Sal*I, or multiple digests thereof, and individual fragments were purified before being subjected to Maxam and Gilbert (12) or M13 (16, 17, 23) sequencing techniques. The $xylE_{pDK1}$ gene was identified by comparing preliminary sequence data with published sequences for $xylE_{pWW0}$ and *nahH* (4, 5, 13). Figure 1 shows a detailed restriction map of the $xylE_{pDK1}$ region and the sequencing strategy used.

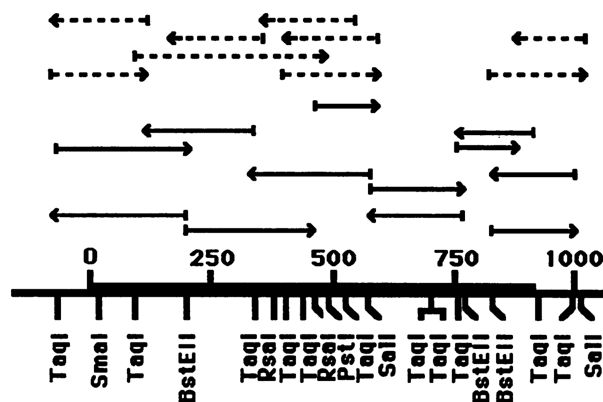

FIG. 1. Restriction map and DNA sequencing strategy used for the TOL plasmid pDK1 *xylE* gene. Solid and broken arrows represent DNA sequenced by the methods of Maxam and Gilbert (12) and Sanger et al. (16), respectively. The location of $xylE_{pDK1}$ is shown superimposed on the bottom line (thick line), and relative distances in base pairs are indicated.

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                                                                   -10
-78 CGCGCTGATCTGTACATCGAATGCCTTCGCCAAGTGCGAACGAATCCCTGACAACAAGAAACATTAAGAGGTGTCGTG

  1 ATG AAA AAA GGA GTT ATG CGC CCG GGC CAC GTC CAG CTT CGC GTG TTG AAC CTG GAG GCT
    met lys lys gly val met arg pro gly his val gln leu arg val leu asn leu glu ala

 61 GCG CTA ACG CAT TAC CGC GAC CTG CTC GGC CTG ATC GAG ATG GAC CGC GAC GAG CAG GGC
    ala leu thr his tyr arg asp leu leu gly leu ile glu met asp arg asp glu gln gly

121 CGC GTC TAT CTG AAG GCC TGG AGC GAG GTG GAC AAA TTT TCC GTG GTG CTG CGT GAG GCC
    arg val tyr leu lys ala trp ser glu val asp lys phe ser val val leu arg glu ala

181 GAC CAG CCG GGC ATG GAT TTC ATG GGC TTC AAG GTG ACC GAC GAT GCC TGC CTG ACC CGC
    asp gln pro gly met asp phe met gly phe lys val thr asp asp ala cys leu thr arg

241 CTG GCC GGC GAG CTG CTG GAG TTC GGC TGC CAG GTG GAG GAG ATT CCT GCC GGC GAA CTC
    leu ala gly glu leu leu glu phe gly cys gln val glu glu ile pro ala gly glu leu

301 AAG GAC TGC GGG CGT CGG GTG CGC TTC CTG GCA CCA TCC GGG CAT TTC TTC GAG CTC TAT
    lys asp cys gly arg arg val arg phe leu ala pro ser gly his phe phe glu leu tyr

361 GCC GAG AAG GAG TAC ACC GGC AAG TGG GGC ATC GAG GAG GTC AAC CCT GAA GCC TGG CCA
    ala glu lys glu tyr thr gly lys trp gly ile glu glu val asn pro glu ala trp pro

421 CGT GAC CTG AAG GGT ATG CGC GCG GTG CGC TTC GAC CAC TGT CTG ATG TAC GGC GAC GAA
    arg asp leu lys gly met arg ala val arg phe asp his cys leu met tyr gly asp glu

481 CTG CAG GCG ACC TAC GAA CTG TTC ACT GAG GTG CTC GGT TTC TAC CTG GCC GAG CAG GTG
    leu gln ala thr tyr glu leu phe thr glu val leu gly phe tyr leu ala glu gln val

541 ATC GAA GAC AAC GGC ACG CGC ATC TCC CAG TTC CTC AGC CTG TCG ACC AAG GCG CAC GAC
    ile glu asp asn gly thr arg ile ser gln phe leu ser leu ser thr lys ala his asp

601 GTG GCC TTT ATC CAG CAC GCG GAG AAG GGC AAG TTC CAT CAC GTC TCA TTC TTC CTG GAA
    val ala phe ile gln his ala glu lys gly lys phe his his val ser phe phe leu glu

661 ACC TGG GAA GAC GTG CTT CGC GCC GCC GAC CTG ATC TCC ATG ACC GAC ACC TCG ATC GAT
    thr trp glu asp val leu arg ala ala asp leu ile ser met thr asp thr ser ile asp

721 ATC GGC CCG ACC CGT CAC GGT CTG ACC CAC GGC AAG ACC ATC TAC TTC TTC GAC CCG TCC
    ile gly pro thr arg his gly leu thr his gly lys thr ile tyr phe phe asp pro ser

781 GGT AAC CGC AAC GAA GTG TTC TGC GGT GGA GAT TAC AAC TAC CCG GAC CAC AAA CCG GTG
    gly asn arg asn glu val phe cys gly gly asp tyr asn tyr pro asp his lys pro val

841 ACC TGG ACC GCC GAC CAA CTG GGC AAG GCG ATC TTT TAC CAC GAC CGC ATT CTC AAC GAA
    thr trp thr ala asp gln leu gly lys ala ile phe tyr his asp arg ile leu asn glu

901 CGA TTC ATG ACC GTG CTG ACC TGA AGGCCCGGTTCGACTTATTGCAGAGATTGCGCAGATGAAAGAAATCA
    arg phe met thr val leu thr OPA

972 AGCATTTCATTAACGGCGAATACGTCGAATCGGCCAGCGGCAAGCTGTTCGATAACGTCAACCCGGCCAACGGCCAGGT
```

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *xylE* gene from the TOL plasmid pDK1. The sequence shown includes the 921-bp open reading frame and flanking regions. The putative ribosome binding site at −10 is shown in boldface type. The *Taq*I sites immediately flanking the gene (see Fig. 1) are underlined.

A comparison of restriction sites for *Sma*I, *Bst*EII, *Sal*I, and *Pst*I with those published for other C23O genes (2, 10) indicates that $xylE_{pDK1}$ encodes a class Ib type C23O. It should be noted, however, that two *Bst*EII sites were detected 50 bp apart at the 3′ end of the gene (Fig. 1) instead of the single site reported for other class Ib C23Os. The nucleotide and predicted amino acid sequences of $xylE_{pDK1}$ are illustrated in Fig. 2. As expected, the gene was found to have an identical size and a similar restriction pattern, G+C content (55%), and nucleotide sequence as $xylE_{pWW0}$ and *nahH* had. A comparison of the sequence with those of $xylE_{pWW0}$ and *nahH* (Fig. 3 and Table 1) shows that 72% of the nucleotides and 78% of the amino acids have been conserved for all three genes. A total of 258 variable nucleotides, affecting 199 codons, were found in the three genes. Most of the changes are silent (131 of 199) or result in neutral amino acid substitutions (eight are glutamate-to-aspartate switches and eight involve valine-isoleucine-leucine exchanges), a phenomenon not unlike that observed for other isofunctional bacterial genes (3, 9). The DNA homology for any pair of genes ranges from 78 to 82%. Comparisons made within arbitrarily subdivided localized regions (I to VI)

```
           -30        -20        -10        1          11         21         31         41         51
pWW0       TGACAACATG AACTATGAAG AGGTGACGTC ATGAACAAAG GTGTAATGCG ACCGGGCCAT GTGCAGCTGC GTGTACTGGA CATGAGCAAG
NAH7       gac     ac   ag -   t       tt                         C  C     C      A                     g
pDK1               a    ac  t         t   g      a      A  T      C        C   C     T   C  GT  a   t  gaggct

61         71         81         91         101        111        121        131        141        151
GCCCTGGAAC ACTACGTCGA GTTGCTGGGC CTGATCGAGA TGGACCGTGA CGACCAGGGC CGTGTCTATC TGAAGGCTTG GACCGAAGTG GATAAGTTTT
   T                        T                    T            A                  C       T  G  T   C  A  C
  G  Aacg   T   cg    cC                            C       g       C                      g           C  A

161        171        181        191        201        211        221        231        241        251
CCCTGGTGCT ACGCGAGGCT GACGAGCCGG GCATGGATTT TATGGGTTTC AAGGTTGTGG ATGAGGATGC TCTCCGGCAA CTGGAGCGGG ATCTGATGGC
  g        G     A  C   T     A   T                          G  C   C  A   ag    Aaat gc   Ca   at       c  aa
  g        G  T     C     c                  C     C          Gacc   C  t cctg c  Gacc gc T           g   c   a

261        271        281        291        301        311        321        331        341        351
ATATGGCTGT GCCGTTGAGC AGCTACCCGC AGGTGAACTG AACAGTTGTG GCCGGCGCGT GCGCTTCCAG GCCCCCTCCG GGCATCACTT CGAGTTGTAT
c t        ctga a  Aa  cg cg        A     C   ag g      T                            T
g tc     C cag  G   g   a t  t   C  C     C   gcac  C   G  T  G          t    A  A         tt          C C

361        371        381        391        401        411        421        431        441        451
GCAGACAAGG AATATACTGG AAAGTGGGGT TTGAATGACG TCAATCCCGA GGCATGGCCG CGCGATCTGA AAGGTATGGC GGCTGTGCGT TTCGACCACG
  T            C         A       g   g                    T                                 G          T  T  Tt
  C  g      G  C  C   C        C a cg g         C  T   A  T     A   T  C      G      cg c  G     C          t

461        471        481        491        501        511        521        531        541        551
CCCTCATGTA TGGCGACGAA TTGCCGGCGA CCTATGACCT GTTCACCAAG GTGCTCGGTT TCTATCTGGC CGAACAGGTG CTGGACGAAA ATGGCACGCG
g   Gc a      T       C A aa  C   T     gT     T   g           C   T  C        G  A   g c  T ccg  c  T ta
gt  G      C          C   a         C  a         Tg                    C  A       G      a c  a  c   C

561        571        581        591        601        611        621        631        641        651
CGTCGCCCAG TTTCTCAGTC TGTCGACCAA GGCCCACGAC GTGGCCTTCA TTCACCATCC GGAAAAAGGC CGCCTCCATC ATGTGTCCTT CCACCTCGAA
 c g            A  CT                     T      T  T   C  T   g     G  G    aagt         cc   A   tt      t
 a  t         C     C                G              T   C  g Cg     G  G    aagt        C  c  A   tt  T G

661        671        681        691        701        711        721        731        741        751
ACCTGGGAAG ACTTGCTTCG CGCCGCCGAC CTGATCTCCA TGACCGACAC ATCTATCGAT ATCGGCCCAA CCCGCCACGG CCTCACTCAC GGCAAGACCA
        G   Tg    G      T             AG             C  G               G     a            G
   c          g                                       C  G               G       T       T  G  C

761        771        781        791        801        811        821        831        841        851
TCTACTTCTT CGACCCGTCC GGTAACCGCA ACGAAGTGTT CTGCGGGGGA GATTACAACT ACCCGGACCA CAAACCGGTG ACCTGGACCA CCGACCAGCT
 T  T                   C      t g   G            C  G a           T     T    T  G         T   ttgg   a gg tg
                                                  T                                                g        A

861        871        881        891        901        911        921        931        941        951
GGGCAAGGCG ATCTTTTACC ACGACCGCAT TCTCAACGAA CGATTCATGA CCGTGCTGAC CTGATGGTCC GGTACGACTT ATTGCAGAGA TTGTGCAGAT
                C  T        Gg   g                         Ta        a a  c   a ct                    ac
                                                                       a  c   g  t                     c
```

FIG. 3. Comparison of the nucleotide sequences of C23O genes encoded by TOL pWW0, NAH7, and TOL pDK1 plasmids. Positions in $xylE_{pDK1}$ and *nahH* which are different from those in $xylE_{pWW0}$ are indicated below the pWW0 sequence. Lowercase letters refer to nucleotide changes that alter the amino acid sequence. Nucleotide changes giving rise to degenerate codons with no accompanying amino acid changes are indicated in uppercase letters.

revealed that the 3′ 471 nucleotides encoding the carboxy half of the protein were somewhat more conserved than the 5′ 450 nucleotides comprising the amino terminus (76 and 68%, respectively). Furthermore, the highest degree of nucleotide conservation (82.2%) was found within the 264 nucleotides that comprise the C terminus (nucleotides 658 to 921 [region VI]). Importantly, this region also showed the greatest homology when nucleotides from any pair of genes were compared (average, 87.9%). These results find analogy with recent work suggesting that it is the carboxy terminus of C23O which plays a key role in substrate binding and catalysis (21).

Further intimations were revealed when comparisons of additional regions were made. For example, a comparison of both DNA and amino acid sequence homologies within the amino-terminal portion of the gene gave the unexpected result that $xylE_{pWW0}$ and *nahH* were most similar (84.7% similarity for nucleotides and 86.7% similarity for amino acids). This level of homology existed despite the fact that regions I and III of the amino terminus are separated by a segment (II) of 99 nucleotides that exhibit the least conservation for the entire gene (48.5% at the nucleotide level). Analysis of variable positions within region II further revealed no consistent pattern of homology, with the most common observation being that all three genes were different. We interpret these observations as implying that evolutionary constraints on both nucleotide and amino acid sequences within this region are reduced and therefore the usefulness of the homology comparisons is decreased. In contrast to the N terminus, within the carboxy half of the gene, $xylE_{pDK1}$ and $xylE_{pWW0}$ were found to be most similar (88.5% for nucleotides, 89.2% for amino acids); it is noteworthy that the final 264 nucleotides comprising the 3′ end of the gene (VI) showed a striking 95.5% homology. Contrary to this, in region V, *nahH* and $xylE_{pDK1}$ exhibited the highest homology; however, the significance of this comparison is

TABLE 1. Comparison of nucleotide and predicted amino acid sequences of C23O genes

Region	Nucleotides		Amino acids	
	No.	% Homology	No.	% Homology
Complete gene (regions I to VI)	1 to 921		1 to 307	
Nonidentical positions	258/921	72.0	68/307	77.9
$xylE_{pWW0}/xylE_{pDK1}$ match	88/258	81.5	15/68	82.7
$xylE_{pDK1}/nahH$ match	55/258	78.0	16/68	83.1
$xylE_{pWW0}/nahH$ match	90/258	81.8	21/68	84.7
All 3 genes different	25/258		16/68	
N-terminal portion (regions I to III)	1 to 450		1 to 150	
Nonidentical positions	143/450	68.2	38/150	74.7
$xylE_{pWW0}/xylE_{pDK1}$ match	27/143	74.2	2/38	76.0
$xylE_{pDK1}/nahH$ match	24/143	73.6	6/38	78.7
$xylE_{pWW0}/nahH$ match	74/143	84.7	18/38	86.7
All 3 genes different	18/143		12/38	
C-terminal portion (regions IV to VI)	451 to 921		151 to 307	
Nonidentical positions	115/471	75.6	30/157	80.9
$xylE_{pWW0}/xylE_{pDK1}$ match	61/115	88.5	13/30	89.2
$xylE_{pDK1}/nahH$ match	31/115	82.2	10/30	87.3
$xylE_{pWW0}/nahH$ match	16/115	79.0	3/30	82.8
All 3 genes different	7/115		4/30	
Region I	1 to 213		1 to 71	
Nonidentical positions	56/213	73.7	12/71	83.1
$xylE_{pWW0}/xylE_{pDK1}$ match	15/56	80.8	0/12	83.1
$xylE_{pDK1}/nahH$ match	8/56	77.5	1/12	84.5
$xylE_{pWW0}/nahH$ match	33/56	89.2	10/12	97.2
All 3 genes different	0/56		1/12	
Region II	214 to 312		72 to 104	
Nonidentical positions	51/99	48.5	19/33	42.4
$xylE_{pWW0}/xylE_{pDK1}$ match	9/51	57.6	2/19	48.5
$xylE_{pDK1}/nahH$ match	13/51	61.6	4/19	54.5
$xylE_{pWW0}/nahH$ match	13/51	61.6	4/19	54.5
All 3 genes different	16/51		9/19	
Region III	313 to 450		105 to 150	
Nonidentical positions	36/138	73.9	7/46	84.8
$xylE_{pWW0}/xylE_{pDK1}$ match	3/36	76.1	0/7	84.8
$xylE_{pDK1}/nahH$ match	3/36	76.1	1/7	87.0
$xylE_{pWW0}/nahH$ match	28/36	94.2	4/7	93.5
All 3 genes different	2/36		2/7	
Region IV	451 to 606		151 to 202	
Nonidentical positions	49/156	68.6	12/52	76.9
$xylE_{pWW0}/xylE_{pDK1}$ match	25/49	84.6	3/12	82.7
$xylE_{pDK1}/nahH$ match	12/49	76.3	4/12	84.6
$xylE_{pWW0}/nahH$ match	8/49	73.7	2/12	80.8
All 3 genes different	4/49		3/12	
Region V	607 to 657		203 to 219	
Nonidentical positions	19/51	62.7	6/17	64.7
$xylE_{pWW0}/xylE_{pDK1}$ match	1/19	64.7	1/6	70.6
$xylE_{pDK1}/nahH$ match	13/19	88.2	4/6	88.2
$xylE_{pWW0}/nahH$ match	4/19	70.6	1/6	70.6
All 3 genes different	1/19		0/6	
Region VI	658 to 921		220 to 307	
Nonidentical positions	47/264	82.2	12/88	86.4
$xylE_{pWW0}/xylE_{pDK1}$match	35/47	95.5	9/12	96.6
$xylE_{pDK1}/nahH$ match	6/47	84.5	2/12	88.6
$xylE_{pWW0}/nahH$ match	4/47	83.7	0/12	86.4
All 3 genes different	2/47		1/12	

less clear given that this region comprises only 5% of the gene.

Although the overall structure of *xylE*$_{pDK1}$ appears to be very similar to that of *xylE*$_{pWW0}$ and *nahH*, it was surprising to find that the relatedness between the two *xylE* genes was no greater than that between either *xylE* gene and *nahH*. A priori, one may have hypothesized that the two *xylE* genes would be more similar given that they occur in isofunctional operons. The finding that, at the N terminus, the resemblance is greater for *xylE*$_{pWW0}$ and *nahH* while, at the C terminus, *xylE*$_{pWW0}$ and *xylE*$_{pDK1}$ are more related could be interpreted to mean that *xylE*$_{pWW0}$ represents a genetic hybrid of the other two C23Os, i.e., having borrowed the N-terminal half from *nahH* and the C-terminal half from *xylE*$_{pDK1}$. This hypothesis is certainly consistent with the well-recognized propensity of bacterial plasmid genes to undergo genetic recombination. This further finds analogy with the notion that proteins may evolve piecemeal via the interchange of DNA encoding separate domains (7, 15). Alternatively, it is possible that *xylE*$_{pWW0}$ and *xylE*$_{pDK1}$ diverged after the separation of *xylE* and *nahH* ancestors, but, because of selective pressure exerted on the hosts of *xylE*$_{pWW0}$ and *nahH*, the nucleotide sequences encoding the N-terminal half of these two genes were better conserved than those of *xylE*$_{pDK1}$. In any event, the present work suggests that the evolution of C23Os may have occurred independently of the plasmid-encoded pathway (TOL, NAH) in which they are found. At the same time, evolutionary constraints appear to have been greater on the structural conservation of the C terminus than on other regions of the gene, a conclusion consistent with recent work indicating that it is this part of C23O which is most important catalytically (21).

This research was supported by grant 003594-030 from the Texas State Higher Education Coordinating Board.

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