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Genetic and Molecular Organization of the Alkylbenzene Catabolism Operon in the Psychrotrophic Strain *Pseudomonas putida* 01G3

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The 11-kb sequence encompassing the alkylbenzene upper pathway in *Pseudomonas putida* 01G3, a psychrotrophic strain able to degrade alkylbenzenes at low temperatures, was characterized. Together with a potential regulator (EbdR), six putative enzymes (EbdAaAbAcAdBC) were identified, and they exhibited highly significant similarities with enzymes implicated in the equivalent pathway in *P. putida* RE204. *ebd* genes appeared to be preferentially induced by ethylbenzene. Multiple-alignment data and growth rate measurements led us to classify 01G3 and closely related strains in two groups with distinct substrate specificities. Close to identified genes, remnants of IS*5*-like elements provided insight into the evolution of catabolic sequences through rearrangements from a less complex ancestral cluster.

To some extent, the *xyl*, *tod*, and *bph* operons in the aromatic compound-degrading strains *Pseudomonas putida* PaW1 (27) and F1 (28) and *Pseudomonas pseudoalcaligenes* KF707 (22), respectively, can be considered the prototypes of *meta*-aromatic compound degradative pathways. Both *tod* and *bph* operons initiate the degradation steps with an aromatic oxidation catalyzed by a multicomponent dioxygenase. As is the case for strains F1 and KF707, most of the degradative pseudomonads that have been studied are mesophilic. Since much of our planet is often exposed to temperatures below 5°C, it is likely that psychrotrophic and psychrophilic strains play an important role in bioremediation of polluted cold environments. We previously isolated *P. putida* 01G3 for its ability to metabolize toluene at 4°C (5). In this study, using an insertional mutagenesis approach, we performed molecular characterization of the alkylbenzene catabolic pathway responsible for this low-temperature degradation. Comparisons with previously identified pathways led us to distinguish two subfamilies of pathways. Possible implications for regulation of an adjacent cloned gene are discussed.

Isolation and characterization of mini-Tn*5lacZ1* mutants. Using a *P. putida* 01G3 spontaneous Sm^r mutant strain (01G3S) as the recipient and the transposon delivery vector pUTmini-Tn*5lacZ1* (Km^r) (7) harbored by *Escherichia coli* S17-1 (24), we performed a random mutagenesis experiment (9) to generate clones affected in the alkylbenzene catabolic pathway. Approximately 25,000 tranposon insertion mutants were grown on selective MMO medium (18) and screened for both reductive dioxygenase and catechol 2,3-dioxygenase activities by using indigo (Ind) (14) and catechol (Cat) tests (26). Fifteen clones (Ind$^-$ and/or Cat$^-$) were unable to grow on MMO medium supplemented with toluene (0.1%, vol/vol) and were kept and used for further analysis.

A 0.9-kb *Hin*dIII fragment of the kanamycin resistance gene was used as a probe to subclone two genomic fragments, A1 (a 7.8-kb *Sac*I fragment from G3A1, a Ind$^-$ Cat$^+$ mutant) and A32 (a 6.7-kb *Bam*HI fragment from G3A32, a Ind$^-$ Cat$^-$ mutant), both containing transposon insertion breakpoints, into the pBluescript SKII+ phagemid, and the fragments were sequenced. A1 contained a putative open reading frame (ORF) truncated by a transposon insertion whose deduced translation product exhibited the highest level of similarity (98% identity, 104 of 106 amino acids) with the C-terminal portion of the α-subunit of isopropylbenzene dioxygenase (IpbAa) encoded by plasmid pRE4 in *P. putida* RE204 (8, 9). A32 also contained a truncated ORF whose deduced protein sequence exhibited 88% identity (120 of 136 amino acids) with the C-terminal part of IpbR, the *ipb* positive regulator encoded by the same plasmid.

Cloning alkylbenzene catabolism genes. An extensive search for plasmid DNA in 01G3 by various extraction methods was negative, suggesting that the genes encoding the alkylbenzene catabolic pathway in 01G3 are located on the chromosome. This practically excludes the possibility that *P. putida* 01G3 and RE204 represent close variants of the same strain. Nevertheless, on the basis of the high level of similarity between 01G3 partial sequences and RE204 partial sequences, the following primers were designed to generate overlapping 01G3 DNA amplicons: forward primers P1 (5′-GGGTGAGAAAC TGGTCTTCG-3′; positions 7571 to 7590), P2 (5′-CCTTTTT GTGCTCAGATGGGGGTCG-3′; positions 8745 to 8770), and P5 (5′-GCATGGAGATCTTTCCTCTC-3′; positions 12758 to 12777); and reverse primers P3 (5′-CTCCATCGCCTTG TTTCGGG-3′; positions 9320 to 9339), P4 (5′-GGTGCTG CTTTTATCTTGCCCGTGC-3′; positions 10481 to 10505), P6 (5′-TGACCCCACATATCGATCCG-3′; positions 13586 to 13605), and P7 (5′-CTGGTGACGCCGATTTTCTTGC-3′; po-

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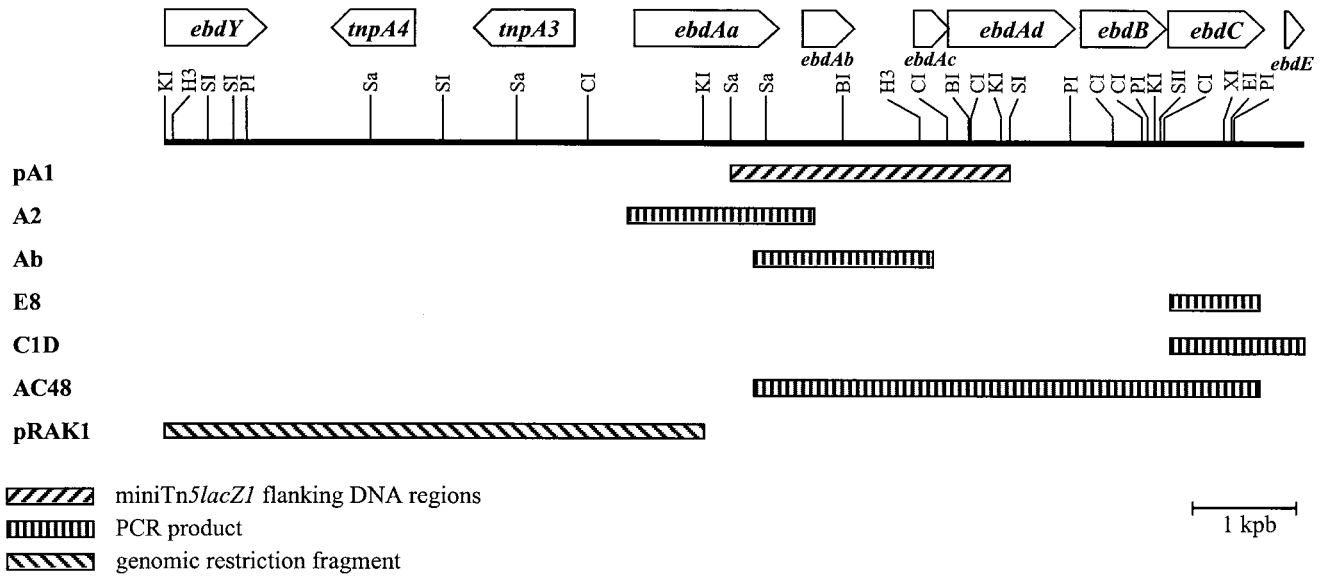

FIG. 1. Cloning and sequencing strategy for the genes encoding catabolism of alkylbenzene in *P. putida* 01G3 (11,023-bp fragment). The directions of transcription are indicated by arrowheads. The pBluescriptII SK-derived plasmids (pA1 and pRAK1) and amplicons (A2, Ab, E8, C1D, and AC48) used for sequencing are shown. Abbreviations: CI, *Cla*I; BI, *Bam*HI; EI, *Eco*RI; H3, *Hin*dIII; KI, *Kpn*I; PI, *Pst*I; SI, *Sac*I; SII, *Sac*II; Sa, *Sal*I; XI, *Xho*I; kpb, kilobase pair.

sitions 14047 to 14068) (numbering based on the sequence deposited under accession no. AF006691). Four amplicons (A2 [1.77 kb] obtained with primers P1 and P3, Ab [1.70 kb] obtained with primers P2 and P4, C1D [1.31 kb] obtained with primers P5 and P7, and AC48 [4.86 kb] obtained with primers P2 and P6) were cloned into the pGEM-T vector (Promega) and sequenced which allowed us to reconstitute a unique data set covering 6,381 bp (Fig. 1).

Protein sequence analysis of the pathway. When the sequence was analyzed, six putative proteins could be directly related to alkylbenzene catabolism. Four of them (EbdAa, EbdAb, EbdAc, and EbdAd) exhibited the highest levels of identity with the *ipb* dioxygenase components of RE204 (98, 88, 87, and 94% with IpbAa, IpbAb, IpbAc, and IpbAd, respectively [Table 1]). The other proteins (EbdB and EbdC) were found to be very similar to RE204 IpbB (*cis*-dihydrodiol dehydrogenase) and IpbC (3-isopropylcatechol-2,3-dioxygenase) (96 and 95% identity, respectively). The sequence identified was presumed to encode all the enzymes necessary for alkylbenzene upper pathway degradation in 01G3. Another ORF, designated *ebdE*, which may encode the 54 N-terminal amino acids of a 2-hydroxypenta-2,4-dienoate hydratase, was also detected further downstream (Fig. 1). The previously reported (19) internal gene *orf3*, whose functionality remains to be demonstrated, was not found in *P. putida* 01G3. The general organization of the *ebd* ORFs along the chromosome was found to be identical to the organization observed in the *ipb* operon in RE204 (9), as well as the organizations observed in

TABLE 1. Catabolic genes of *P. putida* 01G3 involved in biotransformation of alkylbenzene to corresponding catechol derivatives

P. putida 01G3 *ebd* upper pathway				Similar sequences	
Gene	Location (positions)	G+C content (%)	Mol wt (amino acid residues)[a]	Sequence	% Identity[b]
ebdAa	4596–5969	52.1	52,283 (459)	RE204 IpbAa	98
				JR1 IpbA1	90
ebdAb	6202–6696	49.1	19,467 (166)	RE204 IpbAb	85
				JR1 IpbA2	76
ebdAc	7266–7593	48.8	11,688 (109)	RE204 IpbAc	87
				JR1 IpbA3	74
ebdAd	7592–8821	59.1	43,390 (411)	RE204 IpbAd	93
				JR1 IpbA4	79
ebdB	8868–9695	54.8	29,093 (276)	RE204 IpbB	95
				JR1 IpbB	85
ebdC	9703–10638	51	34,693 (312)	RE204 IpbC	94
				JR1 IpbC	81

[a] Predicted molecular weight (number of amino acid residues) of the corresponding protein based on the gene sequence data.
[b] Levels of identity between *P. putida* 01G3 *ebd* gene products and related catabolic enzymes. The sources of enzyme sequences were *P. putida* RE204 (accession no. AF006691) and *Pseudomonas* sp. strain JR1 (U53507).

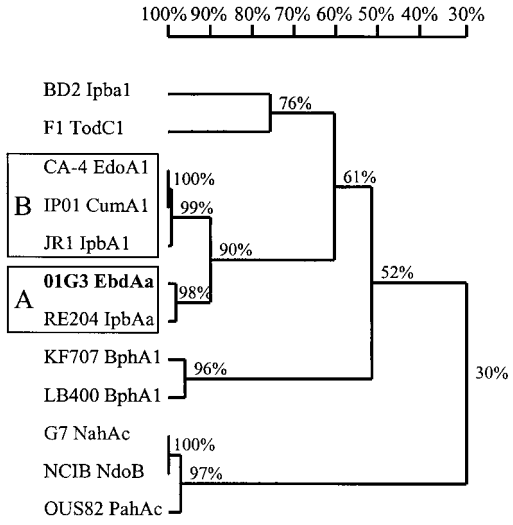

FIG. 2. Relationships of 01G3 α-subunit (EbdAa) with α-subunits of related multicomponent dioxygenases. The values at the nodes are pairwise alignment identity values. The following proteins and bacterial strains (GenBank accession numbers) are included: BD2 IpbA1, isopropylbenzene dioxygenase from *R. erythropolis* BD2 (U24277); F1 TodC1, toluene dioxygenase from *P. putida* F1 (J04996); CA-4 EdoA1, ethylbenzene dioxygenase from *P. fluorescens* CA-4 (AF049851); IP01 CumA1, cumene dioxygenase from *P. fluorescens* IP01 (D37828); JR1 IpbAa, isopropylbenzene dioxygenase from *Pseudomonas* sp. strain JR1 (U53507); 01G3 EbdAa, alkylbenzene dioxygenase from *P. putida* 01G3; RE204 IpbAa, isopropylbenzene dioxygenase from *P. putida* RE204 (AF006691); KF707 BphA1, biphenyl dioxygenase from *P. pseudoalcaligenes* KF707 (AF049345); LB400 BphA1, biphenyl dioxygenase from *Burkholderia* sp. strain LB400 (M86348); G7 NahAc, naphthalene dioxygenase from *P. putida* G7 (M83949); NCIB NdoB, naphthalene dioxygenase from *P. putida* NCIB 9816 (M23914); OUS82 PahAc, polycyclic aromatic hydrocarbon dioxygenase from *P. putida* OUS82 (D16629).

other aromatic compound catabolism operons (1, 10, 19, 20, 22, 28).

Sequence and growth rate comparison. Using the DNAMAN analysis package (Lynnon Biosoft), we aligned α-subunit sequences from aromatic ring-hydroxylating dioxygenases (16) with the EbdAa sequence (Fig. 2). EbdAa appeared to be more closely related (98% identity) to the RE204 dioxygenase α-subunit IpbAa than to any other sequence (group A). The α-subunits of *Pseudomonas* sp. strain JR1 (19) and *Pseudomonas fluorescens* IP01 (1) isopropylbenzene dioxygenases (IpbA1 and CumA1, respectively), as well as of *P. fluorescens* CA-4 ethylbenzene dioxygenase (EdoA1) (6), formed a distinct group (group B) exhibiting 99% identity. The level of identity between groups A and B was 90%. Similar results were obtained with all other proteins (data not shown). Surprisingly, when β-subunits were compared, the EbdAb sequence lacked 20 amino acids (positions 210 to 229 in IpbAa). This deletion, however, did not seem to affect the mineralizing activity of 01G3 (Table 2). When data from all six proteins were taken into account, the group A and B peptide sequences exhibited 93 and 97.6% identity, respectively, on average, and the level of identity between groups A and B was only 80%. At the DNA level (6,045 bp; positions 4596 to 10640 in 01G3) 01G3 and RE204 (group A) exhibited only 77% identity. The level of identity between IP01 and JR1 (group B) was 98%. For the same region, groups A and B exhibited only 63% identity. This means that 01G3 and RE204 could not represent variants of the same strain and shows that group B is more homogeneously conserved than group A.

TABLE 2. Growth rates of alkylbenzene-degrading *Pseudomonas* strains on different substrates

Strain	Growth rate (h^{-1}) on[a]:		
	Toluene	Ethylbenzene	Isopropylbenzene
P. putida 01G3	0.38 ± 0.03	0.44 ± 0.01	0.18 ± 0.03
P. putida RE204	0.43 ± 0.02	0.49 ± 0.02	0.19 ± 0.01
P. fluorescens IP01	0.50 ± 0.07	0.28 ± 0.03	0.32 ± 0.01
Pseudomonas sp. strain JR1	0.36 ± 0.04	0.30 ± 0.05	0.26 ± 0.02

[a] Kinetics were determined at 30°C in MMO medium (40 ml) supplemented with an aromatic compound (0.1%, vol/vol) in the gas phase. The results are means based on at least three distinct experiments.

The capacities of groups A and B to metabolize various potential substrates were compared by measuring the growth rates at 30°C (Table 2). Strains were grown on MMO medium, and toluene, ethylbenzene, or isopropylbenzene was supplied in the gas phase at a concentration of 0.1% (vol/vol) as the sole source of carbon and energy. All strains were able to metabolize the substrates tested. The highest growth rates were observed with ethylbenzene for group A strains and with toluene for group B strains. These results confirmed the dichotomy observed with the multiple-sequence alignments. The finding that two groups of closely related proteins apparently discriminated between closely related substrates is intriguing and needs to be confirmed biochemically.

Characterization of *ebd* promoter. Using a 900-bp DNA fragment from *ebdAa* as a probe, we cloned a *Kpn*I 5.5-kb fragment overlapping the 611-bp *ebdAa* ORF (pRAK1) (Fig. 1). Three other ORFs, *tnpA3*, *tnpA4*, and *ebdY* (*ebdY* was interrupted at a *Kpn*I cloning site), were detected in this fragment. Compared to sequences from protein databases, the TnpA3 sequence (326 amino acids) showed 95% identity with transposases described for *P. putida* H (accession no. AF052751), *Pseudomonas stutzeri* AN10 (2), and *P. putida* RE204. The TnpA4 sequence (271 amino acids) exhibited only 63% identity with a putative transposase of *Pseudomonas syringae* (accession no. M14366). The *ebdY* partial translation product exhibited 54% identity (181 of 325 amino acids) with NahY, a protein implicated in naphthalene chemotaxis in *P. putida* G7 (13) and with no equivalent described for RE204.

By analogy with putative −35 and −10 boxes of *XylOmOp* (11) and *IpbOp* (8), putative −35 and −10 boxes were identified upstream of *ebdAa* (positions 471 and 448 upstream of ATG, respectively [Fig. 3A]). In addition, an almost perfect (14 of 15 bp) direct repeat motif separated by 6 bp was found to overlap the −35 box. An identical motif (36 of 36 bp) has been described previously at the same position in the *ipb* operator-promoter region (*ipbOP*) in RE204 (8), and a closely related motif (22 of 36 bp) has been found upstream of *xyl* genes (*xylOmOp*) in *P. putida* PaW1 (15). No putative XylS activation-related sequence (11) could be detected in *ebdOp* or in *ipbOp*. This supports the apparent conservation noticed previously in the two clusters. No other promoter sequences could

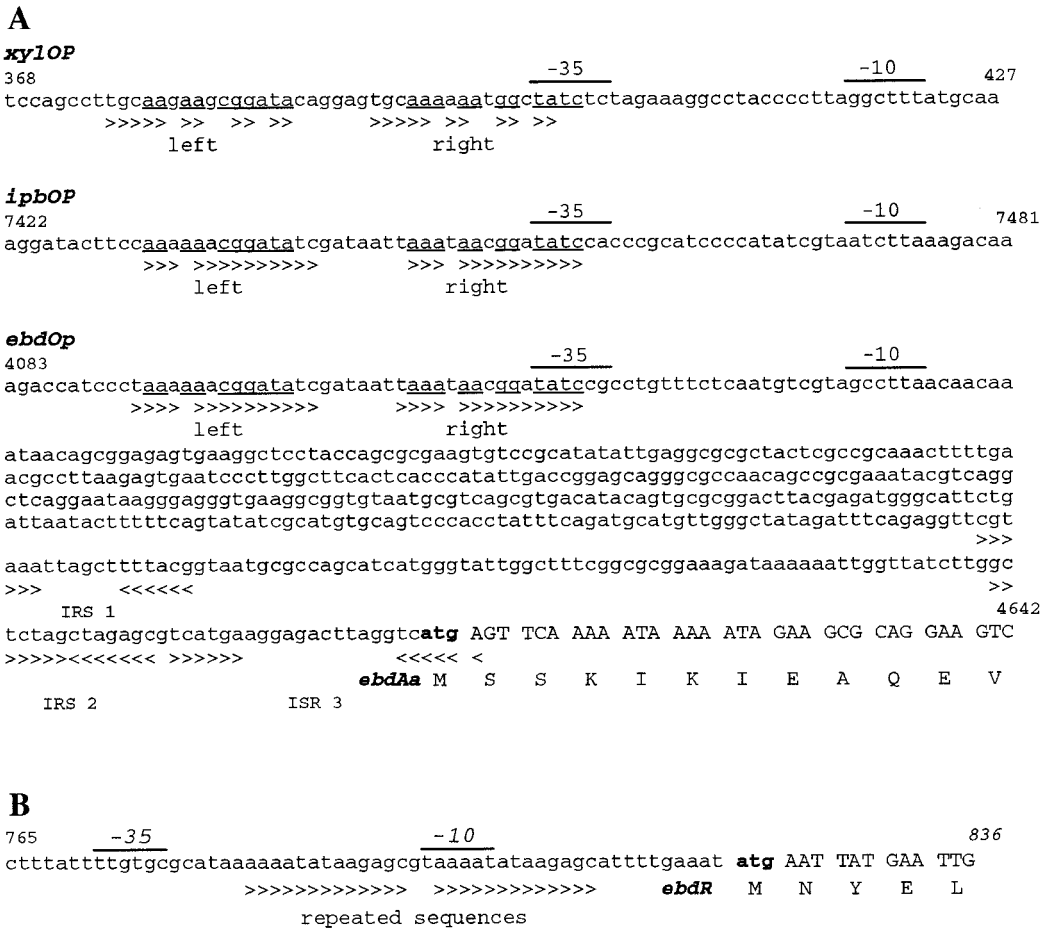

FIG. 3. Regulatory elements of the alkylbenzene catabolism operon. (A) Comparison of the *ebd* upstream region with outstanding elements harbored by the TOL (pWWO) and pRE4 plasmids. (B) Promoter region of the potential regulator gene *ebdR*. Putative −35 and −10 boxes are indicated. Regions where potential *ebd*, *ipb*, and *xyl* operators are identical are underlined. The greater-than symbols indicate conserved bases in tandem repeats, and the greater-than and less-than symbols together indicate conserved bases in inverted repeat sequences (IRS). The translation starting points for *ebdAa* and *ebdR* are indicated by boldface type.

be detected upstream of the other ORFs, indicating that the *ebd* genes may be expressed as a single operon. This finding does not exclude the possibility that there are cryptic alternative internal initiations.

Cloning and sequence analysis of an upper pathway putative regulator, *ebdR*. Since the A32 translation product is a good candidate for a regulator of the *ebd* pathway, a 700-bp *Sac*II subfragment of pA32 was used as a probe to clone a single approximately 3-kb *Bam*HI fragment (pR2B) containing the complete *ebdR* ORF (Fig. 4). EbdR exhibited 90% identity (299 of 332 amino acids) with the IpbR regulatory protein from RE204 and 67% identity (222 of 332 amino acids) with its equivalent (IpbR) recently identified in JR1 (accession no. AF155505). Multiple-sequence alignments (data not shown) confirmed the division into two groups described above. At the DNA level, identity was restricted to the sole *ebdR* coding sequence. EbdR was also found to be similar (50% identity on average) to many regulatory proteins of the XylS-AraC family, all of which have been implicated in regulation of aromatic compound catabolic pathways (12) and all of which have the helix-turn-helix motifs involved in DNA binding (4).

Analysis of *ebdR* upstream sequence. Analysis of the 822-bp upstream region of the *ebdR* gene did not reveal any significant similarity with the corresponding region in *ipbR* or *xylS*, apart from the putative −35 and −10 boxes (Fig. 3B). A 13-bp

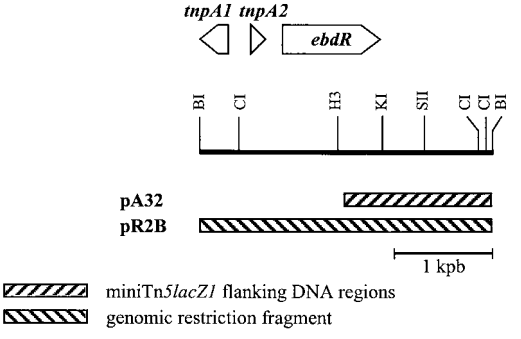

FIG. 4. Cloning and sequencing strategy for the genes encoding catabolism of alkylbenzene in *P. putida* 01G3 (3,013-bp fragment). The directions of translation are indicated by arrowheads. pA32 and pR2B are pBluescriptII SK$^+$ derivatives used for sequencing. Abbreviations: CI, *Cla*I; BI, *Bam*HI; H3, *Hin*dIII; KI, *Kpn*I; SII, *Sac*II; kpb, kilobase pair.

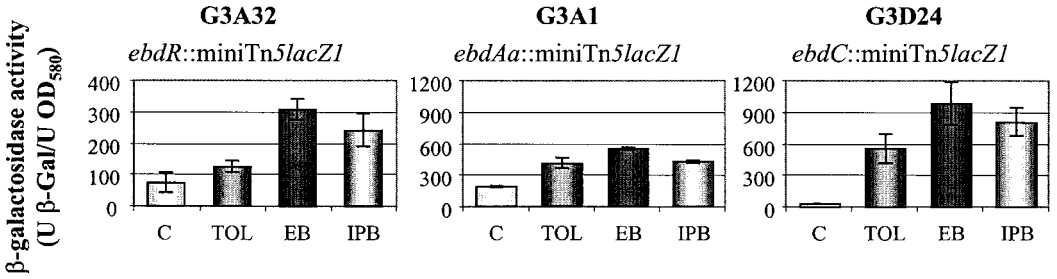

FIG. 5. Activity of the *lacZ* reporter gene in *ebd* insertional mutants. Cells were grown at 17°C in selective Luria-Bertani medium supplemented with toluene (TOL), ethylbenzene (EB), or isopropylbenzene (IPB) until the end of exponential growth. C, unsupplemented control. The results are means based on triplicate determinations. OD_{580}, optical density at 580 nm.

perfect direct repeat separated by only 2 bp was found to partially overlap the putative −10 element. No equivalent feature was found either in *ipbR* or in *xylS* upstream sequences. However, we cannot rule out the possibility that this site may represent a new type of regulator binding site.

Two incomplete transposase-like genes (*tnpA1* and *tnpA2*) apparently transcribed in opposite directions were detected. The TnpA1 protein exhibited 94% identity (90 of 96 amino acids) with TnpA3 encoded upstream of *ebdAa*. A similar value was obtained for the 96 N-terminal residues of the three transposases encoded upstream of the naphthalene-degrading operon in *P. stutzeri* AN10 (2, 3). TnpA2, interrupted by an EbdR sequence, was only 48 amino acids long and thus was considered nonfunctional. No significant similarities with a putative transposase described for *P. putida* RE204 could be found.

Isolation of an *ebdC* mutant. The promoterless *lacZ* gene of miniTn*5lacZ1* was introduced into the ORF of *ebdC* together with the kanamycin marker. Briefly, a 830-bp fragment containing part of *ebdC* was isolated from a PCR product (E8 amplicon) obtained with primers P5 and P6 by using *Bgl*II and *Cla*I restriction enzymes (corresponding to the underlined bases in the primer sequences in the "Cloning alkylbenzene catabolism genes" section) and subcloned. The plasmid was linearized with *Eco*RI at an *ebdC* internal *Eco*RI site and ligated with a 5.2-kb *Eco*RI fragment from pUTmini-Tn*5lacZ1*. The construct was excised from the vector as a *Xba*I-*Pst*I fragment (5.8 kbp) and subcloned in the pME3087 (Tc^r) mobilizable vector (21). The resulting pME8-17 plasmid was then transfected into the S17-1 donor strain (24) prior to conjugation. Integration of the plasmid into the 01G3S recipient yielded Km^r Tc^r Sm^r transconjugants. All $LacZ^+$ merodiploids lacked the characteristic yellow color after catechol spraying, thus showing inactivation of *ebdC* after insertional recombination and eliminating the possibility of a duplication. All clones still exhibited reductive dioxygenase activity, as determined with the indole test (14). Subcloning and sequencing of the G3D24 mutant DNA insert clearly showed that the intact *lacZ* gene was effectively inserted 618 bp downstream of the *ebdC* initiation site (data not shown).

Influence of aromatic substrate on *ebd* expression level. We showed previously that 17°C is a hinge temperature for 01G3 growth (5). G3A32, G3A1, and G3D24 mutants (inactivated in *ebdR*, *ebdAa*, and *ebdC*) were grown at 17°C in selective Luria-Bertani medium supplemented with toluene, ethylbenzene, and isopropylbenzene as potential inducers. β-Galactosidase activity was directly assayed in cell lysates (17). All mutants displayed a marked increase in β-galactosidase activity for all compounds tested compared to a nonsupplemented control (Fig. 5). This result supports the hypothesis that positive regulation occurs. For each mutant, although *ebd* cluster expression appeared to be enhanced by various related compounds, ethylbenzene was the preferential inducer.

Catabolic sequence evolution. It has been suggested previously that random recruitment and assembly of preexisting sequences may lead to acquisition or variegation of catabolic pathways (2, 23, 25). Recurrent remnants of IS*5*-like elements were considered to be supporting evidence that transposition events occur in the actual *nah* operon structure (2, 3, 8). The presence close to the *ebd* degradative cluster of four putative transposases exhibiting an average level of identity of 49% with the IS*5* functional enzyme supports this hypothesis. One of these transposases, TnpA2, appeared to be truncated by a fragment encoding TnpA1 and EbdR. This may be interpreted as a relic of either the imbrication of two successive transpositions or a failed transposition process. Whatever the mechanism, our data support the idea that like the *nah* pathway genes of *P. stutzeri* AN10, the alkylbenzene degradation pathway genes of *P. putida* 01G3 may have evolved from a less complex ancestral cluster. Bosch et al. have recently suggested that the *nah* pathway could represent an evolutionary step towards specificity initiated with a large-spectrum catabolic pathway (2). Given this, the *ipb* pathway carried by plasmid pRE4 in RE204 could be a good candidate for an earlier plasmid pathway that led to emergence of the genomic *ebd* cluster in 01G3 after chromosomal integration.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the EMBL database under accession numbers AJ293587 and AJ293588.

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