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Isopropylbenzene catabolic pathway in *Pseudomonas putida* RE204: nucleotide sequence analysis of the *ipb* operon and neighboring DNA from pRE4 *

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Abstract

Pseudomonas putida RE204 employs a set of plasmid-specified enzymes in the catabolism of isopropylbenzene (cumene) and related alkylbenzenes. A 21,768 bp segment of the plasmid pRE4, whose sequence is discussed here, includes the *ipb* (isopropylbenzene catabolic) operon as well as associated genetic elements. The *ipb* operon, *ipbAaAbAcAdBCEGFHD*, encodes enzymes catalyzing the conversion of isopropylbenzene to isobutyrate, pyruvate, and acetyl-coenzyme A as well as an outer membrane protein (IpbH) of uncertain function. These gene products are 75 to 91% identical to those encoded by other isopropylbenzene catabolic operons and are somewhat less similar to analogous proteins of related pathways for the catabolism of mono-substituted benzenes. Upstream of *ipbAa*, *ipbR* encodes a positive regulatory protein which has about 56% identity to XylS regulatory proteins of TOL (xylene/toluate) catabolic plasmids. This similarity and that of the DNA sequence in the proposed *ipb* operator-promoter region (*ipbOP*) to the same region of the *xyl meta* operon (*xylOmPm*) suggest that, although the IpbR and XylS regulatory proteins recognize very different inducers, their interactions with DNA to activate gene expression are similar. Upstream of *ipbR* is an 1196 bp insertion sequence, IS*1543*, related to IS*52* and IS*1406*. Separating *ipbR* from *ipbAa* are 3 additional tightly clustered IS elements. These are IS*1544*, related to IS*1543*, IS*52*, and other members of the IS*5* family; IS*1545*, related to IS*1240*; and IS*1546*, related to IS*1491*. Encompassing the *ipb* catabolic genes and the other genetic elements and separated from each other by 18,492 bp, are two identical, directly repeated 1007 bp DNA segments. Homologous recombination between these segments appears to be responsible for the occasional deletion of the intervening DNA from pRE4.

Introduction

Aromatic hydrocarbons and their alkyl-substituted homologues are found in fossil fuels such as petroleum and coal and their derivatives (Radke 1987). Mono-substituted aromatic hydrocarbons such as isopropylbenzene (cumene), toluene, and biphenyl are degraded by bacteria through essentially isofunctional pathways (Figure 1) to pyruvate, acetyl-coenzyme A, and an aromatic acid (isobutyrate, acetate, and benzoate, respectively) (Gibson & Subramanian 1984; Ribbons & Eaton 1982). The best studied of these pathways is the toluene catabolic pathway of *Pseudomonas putida* F1. The DNA sequence of the *tod* operon encoding the complete toluene catabolic pathway has been determined (Lau et al. 1994; Menn et al. 1991; Wang et al. 1995; Zylstra & Gibson 1989) and many of the enzymes (Gibson & Subramanian 1984; Jiang et al.

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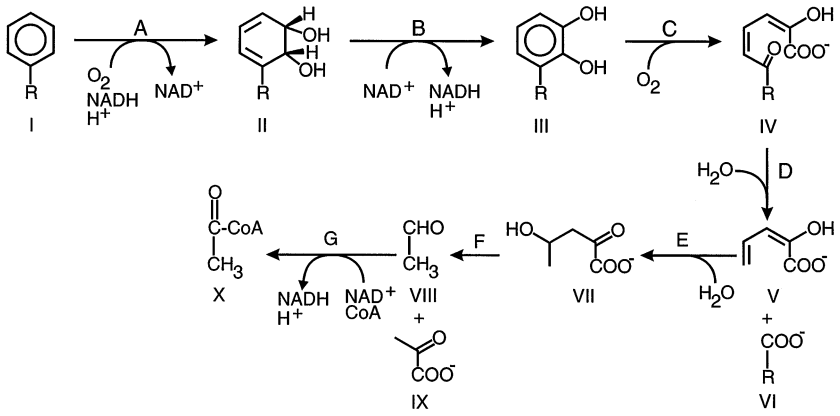

Figure 1. Pathway for the catabolism of mono-substituted benzenes. Compounds are, for R= isopropyl, methyl, and phenyl, respectively: I, isopropylbenzene (cumene), toluene, and biphenyl; II, *cis*-2,3-dihydroxy-2,3-dihydroisopropylbenzene, *cis*-2,3-dihydroxy-2,3-dihydrotoluene, and *cis*-2,3-dihydroxy-2,3-dihydrobiphenyl; III, 3-isopropylcatechol, 3-methylcatechol, and 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate, 2-hydroxy-6-oxo-hepta-2,4-dienoate, and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; V, 2-hydroxypenta-2,4-dienoate; VI, isobutyrate, acetate, benzoate; VII, 4-hydroxy-2-oxovalerate; VIII, acetaldehyde; IX, pyruvate; and X, acetyl-coenzyme A. Enzymes are: A, dioxygenase; B, dihydrodiol dehydrogenase; C, ring-cleavage dioxygenase; D, hydrolase; E, hydratase; F, aldolase; g, dehydrogenase (acylating).

1996; Yeh et al. 1977) have been purified and studied in some detail. Genes (*bph*) encoding the degradation of biphenyl and the biotransformation of chlorinated biphenyls in several bacterial strains have also been sequenced (Erickson & Mondello 1993; Furukawa 1994; Hofer et al. 1993, 1994). More recently the DNA sequences of the complete *cum* (cumene catabolic) operon of *Pseudomonas fluorescens* IP01 (Aoki et al. 1996; Habe et al. 1996a, 1996b) and the genes encoding the first three enzymes of the nearly identical isopropylbenzene catabolic operon of *Pseudomonas* sp. JR1 (Pflugmacher et al. 1996) have been determined. All of these pathways have a common origin; similarities in the DNA sequences of genes and amino acid sequences of pathway enzymes are readily evident (Pflugmacher et al. 1996; Williams & Sayers 1994).

Pseudomonas putida RE204 was isolated in 1982 from the Rhone river in Geneva, Switzerland by enrichment with isopropylbenzene (cumene) as sole carbon and energy source (Eaton & Timmis 1984, 1986a). The isopropylbenzene catabolic pathway, encoded by the plasmid pRE4 in strain RE204, was studied previously by using transposon Tn*5* to isolate mutants unable to grow with isopropylbenzene (Eaton & Timmis 1986a). These mutants were characterized by growing them in the presence of isopropylbenzene and identifying the pathway intermediates which accumulated as well as by assaying pathway enzymes in extracts of uninduced and isopropylbenzene-induced cells. In this way mutants having Tn*5* inserted into the gene *ipbR* encoding a positive regulatory protein as well as into genes encoding enzymes that catalyze the first five steps of the pathway (Figure 1, A-E) were identified. Tn*5*-carrying DNA fragments were subsequently cloned from all of the mutants and used to construct a functional map of the isopropylbenzene catabolism region of pRE4 (Eaton & Timmis 1984, 1986a). This region, which occupies over 15 kbp of pRE4 and includes the genes *ipbR* and *ipbABCED*, is part of an approximately 20 kbp DNA fragment which occasionally undergoes spontaneous deletion from pRE4 (Eaton & Timmis 1984, 1986b). Southern hybridization experiments demonstrated possible involvement of homologous DNA segments at the ends of the easily-deleted segment (Eaton & Timmis 1986b).

Strain RE204 and its mutant and recombinant derivatives have potential uses in chemical syntheses as well as in bioremediation. The enzymes of the isopropylbenzene catabolic pathway can act on such diverse non-growth substrates as the simple sulfur heterocycle, benzothiophene (Eaton & Nitterauer 1994), the cyclopentadiene derivative, dimethylfulvene (Eaton & Selifonov 1996), and the chlorinated solvent, trichloroethylene (Dabrock et al. 1992, 1994; Eaton unpubl.).

Regulation of the *ipb* operon of pRE4 has been studied by constructing a plasmid, pOS25, which contains the regulatory elements of the *ipb* operon (*ipbR*

Table 1. List of bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or references(s)
Pseudomonas putida RE204	grows with isopropylbenzene, other alkylbenzenes	Eaton & Timmis 1986a
Escherichia coli JM109	*recA endA1 gyrA96 thi hsdR17 sup E44 relA1 Δ(lac-proAB)* (F′ *traD36 pro AB lacI^q ZΔM15)*	Yanisch-Perron et al. 1985
Escherichia coli LE392	F-, *hsdR514 supE44 supF58 lacY1 (lacI24) galK2 gal T22, metB1 trpR55,* λ^-	Maniatis et al. 1982
pMMB277	Cm^r, Mob$^+$, derived from RSF1010, lacI^q, multiple cloning site in *lacZα* downstream from P*tac*	Morales et al. 1991
pBluescript II SK	Ap^r, multiple cloning in *lacZα*	Alting-Mees & Short 1989
pRE4	105 kbp plasmid, encodes isopropylbenzene catabolism (Ipb$^+$, Ind$^+$[a]	Eaton & Timmis 1984, 1986a
pRE47	3.35 kbp *Cla*I fragment (map coordinates[b] 7.45 to 10.8) of pRE4 inserted into pBR322, Ind$^+$	Eaton & Timmis 1986a
pRE48	3.35 kbp *Cla*I fragment (map coordinates 7.45 to 10.8) of pRE4 inserted into pACYC184, Ind$^+$	Eaton & Timmis 1986a
pRE49	2.73 and 13.17 kbp *Eco*RI fragments (map coordinates 1.58 to 4.31 and 4.33 to 17.5) of pRE4 inserted into pBR322, Ind$^+$	Eaton & Timmis 1986a
pRE50	2.73 and 13.17 kbp *Eco*RI fragments (map coordinates 1.58 to 4.31 and 4.33 to 17.5) of pRE4 inserted into pACYC184, Ind$^+$	Eaton & Timmis 1986a
pRE51	13.17 kbp *Eco*RI fragment (map coordinates 4.33 to 17.5) in pACYC184, made by recutting and ligating pRE50, Ind$^-$	Eaton & Timmis 1986a
pRE52	2.7 kbp *Eco*RI fragment (map coordinates 1.58 to 4.31) in pACYC184, made by recutting and ligating pRE50, Ind$^-$	Eaton & Timmis 1986a
pRE58	11.0 kbp *Bgl*II fragment of pRE4 (map coordinates 12.67 to 23.7) inserted into pLV59, Ind$^-$	Eaton & Timmis 1986a
pRE60	9.98 kbp *Bgl*II fragment (map coordinates 2.69 to 12.67) of pRE4 inserted into pLV59, Ind$^+$	Eaton & Timmis 1986a
pRE61	9.31 kbp *Bam*HI fragment (map coordinates 1.47 to 10.78) of pRE4 inserted into pBR322, Ind$^+$	Eaton & Timmis 1986a
pRE650	23.3 kbp and 7 kbp *Xma*I fragments from pRE4 inserted into pMMB277, Ind$^+$	This study
pRE658	pRE650 recut with *Xma*I and ligated, contains 23.3 kbp *Xma*I fragment (map coordinates −0.7 to 22.65) in pMMB277, Ind$^+$	This study
pRE659	pRE650 recut with *Xma*I and ligated, contains 23.3 kbp *Xma*I fragment (map coordinates −0.7 to 22.65) in pMMB277 in orientation opposite to that in pRE658, Ind$^+$	This study
pOS25	5.71 kbp *Bgl*II-*Apa*I fragment (map coordinates 2.69 to 8.4) from pRE61 inserted as an *Eco*RI-*Xba*I fragment upstream of the *luxCDABE* genes of pRB28	Selifonova & Eaton 1996
pRG13	3.3 kbp *Hind*III fragment (map coordinates 0.54 to 3.84) from pRE658 inserted into pBluescriptII SK	This study

[a] Ind$^+$: encodes the conversion of indole to indigo.
[b] In kilobase pairs (kbp) (Figure 2).

and the operator/promoter region, *ipbOP*) inserted upstream of the genes (*luxCDABE*) from *Vibrio fischeri* encoding luciferase (Selifonova & Eaton 1996). When *E. coli* carrying this plasmid is exposed to an inducer of the *ipb* operon, it produces light which can be readily measured. While isopropylbenzene and other alkylbenzene growth substrates appear to be the normal physiological inducers of the pathway, a variety of other hydrophobic compounds including naphthalene and trichloroethylene also act as inducers. Bacteria

carrying pOS25 or similar plasmids have potential use as indicators of these environmental pollutants and of their bioavailability. The *ipb* regulatory elements

may also have value in the construction of expression vectors that respond to these hydrophobic inducers.

Because of the demonstrated and potential value of the isopropylbenzene catabolism operon, its components, and its gene products and in order to better understand the basis for its genetic instability, the sequence of the isopropylbenzene catabolism region of pRE4 was determined. The features of that DNA sequence are discussed here.

(This work was presented in a preliminary form at *Pseudomonas* '97 in Madrid, Spain, 4–8 September 1997.)

Materials and methods

Plasmids and bacterial strains used in this study are listed in Table 1. *Escherichia coli* JM109 was routinely used as the host in cloning experiments. Cultivation of bacteria, DNA isolation and cloning, and analyses of clones were carried out as previously described (Eaton & Timmis 1986a; Selifonova & Eaton 1996). Recombinant bacteria were identified as follows: Bacteria carrying isopropylbenzene dioxygenase genes converted indole to indigo and appeared as blue colonies on selective media containing 1 mm indole. *E. coli* JM109 carrying different DNA fragments, inserted into the multiple cloning site within the *lacZá* gene of the plasmid pBluescriptII SK, formed white colonies on selective media containing IPTG (0.25 mm) and X-gal (0.02% wt/vol).

DNA sequence determination

Plasmids that were sources of DNA fragments for subcloning and sequencing are listed in Table 1. Both strands of a DNA segment of 21,768 bp were sequenced by the dideoxy chain-termination method with double-stranded DNA as template (Sanger et al. 1977). Sequencing reactions were initiated with vector-complementary primers and by using newly-synthesized primers whose sequences were chosen based on previous sequence data. DNA primers were synthesized by the ICBR DNA Synthesis Core Laboratory, University of Florida, Gainesville. All sequencing reactions and analyses were carried out by the ICBR DNA Sequencing Core Laboratory, University of Florida, Gainesville, using an Applied Biosystems, Inc., Model 373a sequencer as previously described (Eaton 1994). Sequence data were aligned and edited by using DNASTAR (DNASTAR, Inc., Madison, Wis.). Searches for specific nucleotide or amino acid sequences in the GenBank database were carried out by using the BLAST program (Altschul et al. 1990; Benson et al. 1993). Sequences were retrieved from GenBank and compared to sequences obtained here by using DNASTAR COMPARE, ALIGN, and AALIGN programs (Needleman & Wunsch 1970; Wilbur & Lipman 1983).

The DNA sequence obtained in this study is available from GenBank (accession number AF006691).

Results and discussion

Cloning isopropylbenzene catabolic genes

The genes (*ipb*) encoding catabolism of isopropylbenzene in *P. putida* RE204 are located on plasmid pRE4. These genes are encompassed by DNA segments having a high degree of sequence homology to each other; recombination between these homologous DNA segments was thought to be responsible for the observed deletion of the *ipb* operon from pRE4 at a high frequency (Eaton & Timmis 1984, 1986a, 1986b). From previous mapping of the region of pRE4 encoding isopropylbenzene catabolism, it was also thought that the *ipb* operon and the homologous DNA segments would be located on an *Xma*I fragment of approximately 25 kbp. Because bacteria containing isopropylbenzene dioxygenase can convert indole to the water-insoluble blue dye, indigo, cloning the *Xma*I fragment carrying the genes encoding this enzyme and the rest of the isopropylbenzene catabolic pathway was straightforward. The plasmid pRE4 was digested with *Xma*I and ligated to the similarly digested plasmid vector, pMMB277. The ligation mixture was used to transform *E. coli* JM109 which was subsequently spread on LB agar plates supplemented with chloramphenicol and indole. A blue colony that appeared on one of these plates was formed by strain JM109 carrying pRE650 which contains 23.3 kbp and 7 kbp *Xma*I fragments inserted into pMMB277. To remove the 7 kbp fragment, pRE650 was recut with *Xma*I and ligated. The resulting plasmids, pRE658 and pRE659, contain only the 23.3 kbp fragment inserted in both orientations in pMMB277.

Spontaneous deletions

When pRE658 or pRE659 was introduced into the $RecA^+$ strain *E. coli* LE392, most of the inserted fragment was deleted leaving only a 3.85 kbp *Xma*I fragment inserted in pMMB277 (data not shown). This

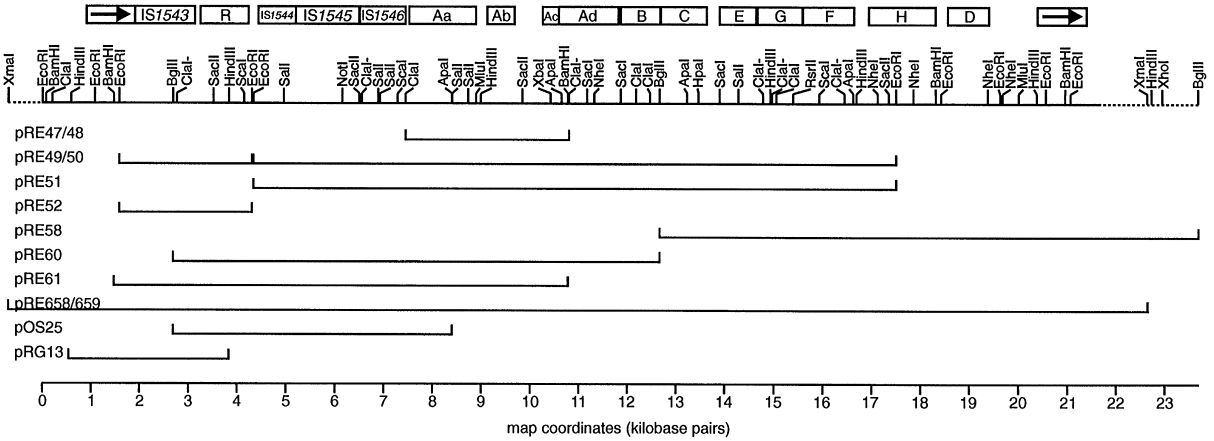

Figure 2. DNA from the plasmid pRE4 of *P. putida* RE204 encoding the catabolism of isopropylbenzene by *P. putida* RE204: restriction map of a 24.4 kbp DNA segment. The sequence from map coordinate 0 to 21.768 was determined in this study. The locations of the genes encoding pathway enzymes (see legend to Figure 1) and other genetic elements are indicated at the top. The boxed arrows indicate the 1007 bp direct repeats. Below the map are shown cloned DNA fragments referred to in this paper. Names of plasmids containing these fragments are indicated at the left. *Cla*I$^-$ indicates sites where *Cla*I cuts in DNA prepared from *dam*$^-$ but not *dam*$^+$ hosts.

deletion also occurred in the RecA$^-$ strain JM109 but only at a low frequency. The size of the deletion (19.45 kbp) is similar to the approximately 20 kbp deletions of pRE4 previously observed (Eaton & Timmis 1984, 1986a, 1986b).

Nucleotide sequence

The sequence of the isopropylbenzene catabolism region of pRE4 was obtained using previously constructed plasmids (Table 1, Figure 2), pRE658, and various plasmid derivatives (most of these are not shown). One of these derivatives, pRG13, containing a 3.3 kbp *Hin*dIII fragment (map coordinates 0.54 to 3.84) was constructed specifically for use as the source of DNA for sequencing the upstream direct repeat, in order to avoid any ambiguity once it was determined that the upstream and downstream direct repeats might be identical. The 21,768 bp DNA segment contains the complete 11 gene isopropylbenzene catabolism (*ipb*) operon, a gene, *ipbR*, encoding a regulatory protein, several genetic elements related to IS elements, and, at each end, two large, identical, directly repeated elements (Figure 2, Table 2, Table 3).

Isopropylbenzene catabolic pathway

The proteins encoded by the *ipb* operon are related to a large number of proteins performing analogous functions in other pathways for the catabolism of toluene, biphenyl, and other aromatic compounds. They are most closely related to proteins of two isopropylbenzene catabolic pathways (Table 2). Activities of most pathway enzymes have been demonstrated previously with *P. putida* RE204 and its Tn*5*-mutant derivatives (Eaton & Nitterauer 1994; Eaton & Selifonov 1996; Eaton & Timmis 1986a); the similarity of these enzymes to well-studied enzymes of other aromatic catabolic pathways serves to confirm their functions.

Ipb and Cum proteins are not only closely related to each other (75% to 91% identity, Table 2) but the genes encoding them are also arranged identically in their respective operons (Habe et al. 1996b). The relative locations of these genes (Figure 2) are entirely consistent with the original functional map of the isopropylbenzene catabolic operon of pRE4 established following Tn*5* mutagenesis of strain RE204 (Eaton & Timmis 1986a).

Isopropylbenzene dioxygenase

The first step in the isopropylbenzene catabolic pathway (Figure 1, A) is catalyzed by a three-component reductive dioxygenase. In related dioxygenases, electrons are transferred from NADH through a flavoprotein reductase to a ferredoxin and then to a two-subunit terminal dioxygenase. The reduced terminal dioxygenase then interacts with aromatic substrate and molecular oxygen to introduce two hydroxyl groups into the aromatic ring (Jiang et al. 1996).

A low level of isopropylbenzene dioxygenase activity was previously demonstrated in *E. coli* carrying

Table 2. Products of *ipb* genes; comparison to proteins having similar functions in other aromatic catabolic pathways

Gene	Location[a]	Gene product	Deduced mol wt (amino) acid residues)	Correponding proteins, % identity				
				P. fluorescens IP01[b]	*Pseudomonas sp.* LB400	*P. putida* F1	Other strains	Other proteins
ipbAa	7525 to 8902	Isopropylbenzene dioxygenase, large subunit	52,322 (459)	CumA1, 91	BphA(A1), 73	TodC1, 63	JR1	IpbA1, 90
ipbAb	9130 to 9690	Isopropylbenzene dioxygenase, small subunit	21,754 (186)	CumA2, 86	BphE(A2), 59	TodC2, 50	JR1	IpbA2, 86
ipbAc	10264 to 10593	Isopropylbenzene dioxygenase, ferredoxin	11,539 (109)	CumA3, 81	BphF(A3), 76	TodB, 56	JR1	IpbA3, 80
ipbAd	10590 to 11825	Isopropylbenzene dioxygenase, ferredoxin reductase	43,349 (411)	CumA4, 79	BphG(A4), 73	TodA, 53	JR1	IpbA4, 79
ipbB	11866 to 12696	2,3-dihydroxy-2,3-dihydroisopropylbenzene dehydrogenase	29,071 (276)	CumB, 90	BphB, 81	TodD, 60	JR1	IpbB, 88
ipbC	12707 to 13639	3-isopropylcatechol dioxygenase	34,300 (310)	CumC, 75	BphC, 59	TodE. 48	JR1	IpbC, 78
ipbE	13875 to 14657	2-hydroxypenta-2,4-dienoate hydratase	27,695 (260)	CumE, 90	BphH, 79	TodG, 43	CF600	DmpE, 66
ipbG	14675 to 15595	Acetaldehyde dehydrogenase (acylating)	32,837 (306)	CumG, 91	BphJ, 80	TodI, 54	NCIB 9816	NahO, 74
ipbF	15616 to 16650	4-Hydroxy-2-oxovalerate aldolase	37,161 (344)	CumF, 82	BphI, 82	TodH, 54	NCIB 9816	NahM, 76
ipbH	16954 to 18432	Outer membrane protein	52,411 (492)	CumH, 79	—	TodX,38	mt-2 F1	XyIN, 48 CymD, 45
ipbD	18573 to 19421	2-Hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase	31,202 (282)	CumD, 83	BphD, 35	TodF, 62	CF600 mt-2	DmpD, 63 Xy1F, 57
ipbR	3248 to 4246	Regulatory protein	37,387 (322)	—	—	—	mt-53 HS1 mt-53 TMB mt-2	XylS3, 56 XylS1, 56 XylS1, 55 TmbS, 55 XylS, 55

[a] Location (map coordinates, Figure 2) in bp derived from the DNA sequence (GenBank AF006691).
[b] Bacterial strains and GenBank numbers (references): *P. fluorescens* IP01, D37838, D63377, D83955 (Aoki et al. 1996; Habe et al. 1996a, 1996b); *P. putida* F1, J04996, U09250, M64080, U24215 (Eaton 1997; Lau et al. 1994; Menn et al. 1991; Wang et al. 1995; Zylstra & Gibson 1989); *Pseudomonas* sp. LB400, X66122, X66123, M86348, X76500 (Erickson & Mondello 1993; Hofer et al. 1993, 1994); *Pseudomonas* sp. JR1, U53507 (Pflugmacher et al. 1996); *P. putida* CF600, X60835, X52805 (Nordlund & Shingler 1990; Shingler et al. 1992); *P. putida* NCIB9816, U13232 (Platt et al. 1995); *P. putida* mt-2, D63341, M64747 (Horn et al. 1991; Inouye et al. 1986; Spooner et al. 1986); *P. putida* HS1, L02358 (Assinder et al. 1993); *P. putida* mt-53, L02357 (Assinder et al. 1993); *P. putida* TMB, U41301.

the 3.35 kbp *Cla*I fragment (Figure 2, map coordinates 7.45 to 10.8) in pRE47 and pRE48 (Eaton & Timmis 1986a). However, this fragment is too small to encode all four components of the dioxygenase. Minicell expression experiments with pRE47 and pRE48 showed plasmid-encoded products of approximate mol wt 51,000 and 25,000 corresponding to the expected sizes of the two terminal dioxygenase subunits. It was suggested at that time that the reductase and possibly the ferredoxin might be provided by the *E. coli* host (it was not possible to detect the 11,539 mol wt ferredoxin protein in the minicell experiment). As evident from the DNA sequence, *ipbAa*, *ipbAb*, and *ipbAc* are located on the 3.35 kbp *Cla*I fragment, while most (81%) of the reductase gene, *ipbAd*, is not. Other oxygenases were subsequently shown to be similarly complemented; thus several incomplete naphthalene dioxygenases and toluene-4-monooxygenase (encoded by recombinant plasmids lacking the respective reductase genes) exhibit oxygenase activity in *E. coli* (Denome et al. 1993; Ensley et al. 1988; Kurkela et al. 1991; Yen & Karl 1992).

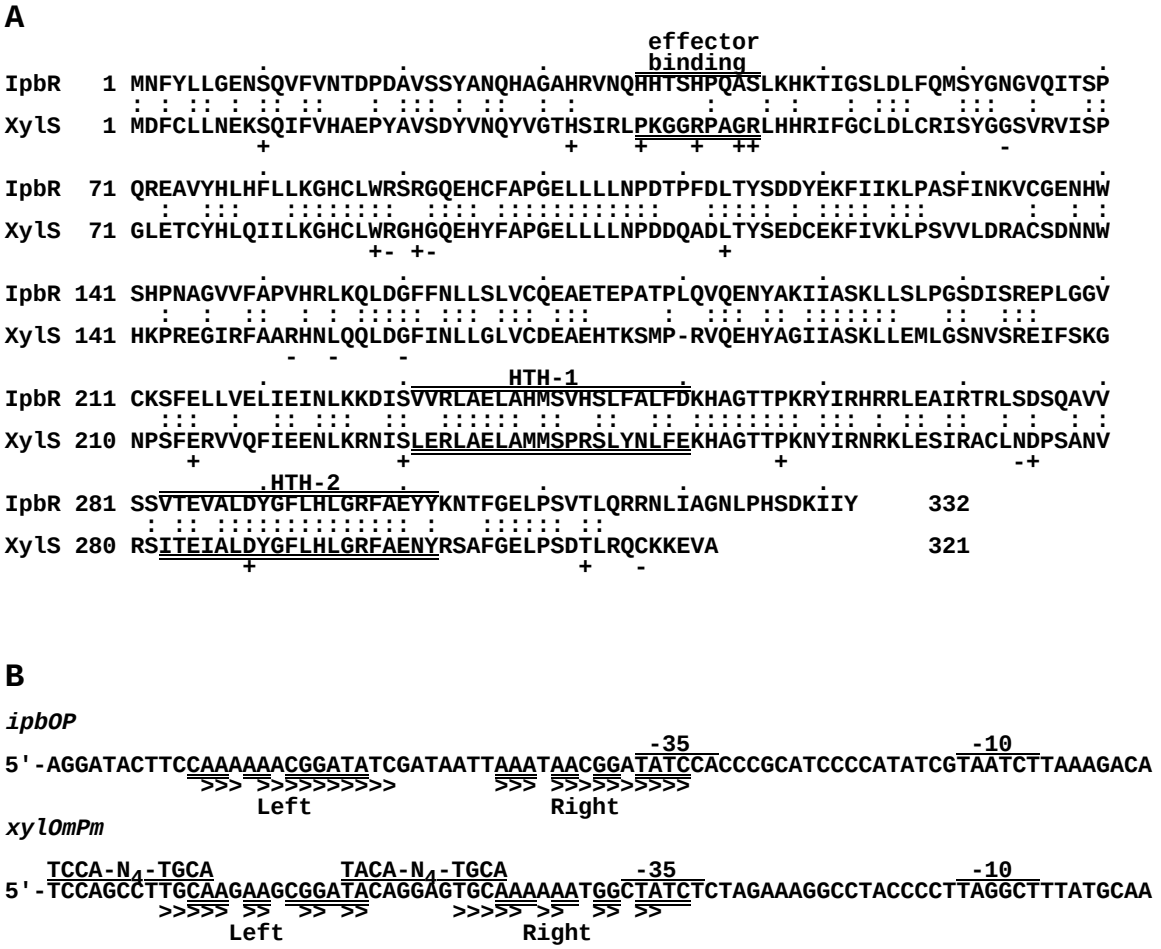

Figure 3. Comparison of regulatory elements of the isopropylbenzene catabolism operon with those of the TOL plasmid (pWW0)-xylene/toluate catabolism *meta*-operon (see text for references). (A) Comparison of IpbR with XylS. Structural features (a possible inducer [effector] binding site and helix-turn-helix DNA binding sites [HTH-1 and HTH-2]) identified in XylS are shown. A + sign below an amino acid residue indicates that an alteration of that residue has been shown to cause a significant change in the properties of XylS; a – sign indicates an amino acid that, when changed, did not result in altered XylS function. (B) Comparison of *ipbOP* with *xylOmPm*. The > symbols indicate bases conserved in left and right tandem repeats. Double underlines indicate homology between putative *ipb* and *xyl* operators. The *ipb* sequence extends from bp 7422 to bp 7500; the first gene of the *ipb* operon, *ipbAa*, begins at bp 7525.

The enzyme responsible for this ferredoxin reductase activity in *E. coli* has not been identified, however there are at least two possibilities: (1) The *E. coli* genome sequencing project (Blattner et al. 1997) revealed an operon containing genes encoding the components of a dioxygenase (large and small terminal dioxygenase subunits, ferredoxin, ferredoxin reductase) and a dihydrodiol dehydrogenase. These gene products may be involved in the conversion of phenylpropionate to 2,3-dihydroxyphenylpropionate; the activity of the reductase toward ferredoxins of other dioxygenases has not been studied. (2) NAD(P)H:flavin oxidoreductase produces free reduced flavins which, in turn, reduce ferric iron in ferrisiderophores and in such proteins as ribonucleotide reductase (Fontecave et al. 1994); these reduced flavins may also be responsible for a low level of ferredoxin reduction.

Other ipb operon-encoded proteins

The sequence confirms the identities of previously located genes *ipbB*, *ipbC*, *ipbD*, and *ipbE*, which encode 2,3-dihydroxy-2,3-dihydroisopropylbenzene dehydrogenase (Figure 1, enzyme B), 3-isopropylcatechol dioxygenase (enzyme C), 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase (enzyme D), and 2-hydroxypenta-2,4-dienoate hydratase (enzyme E) re-

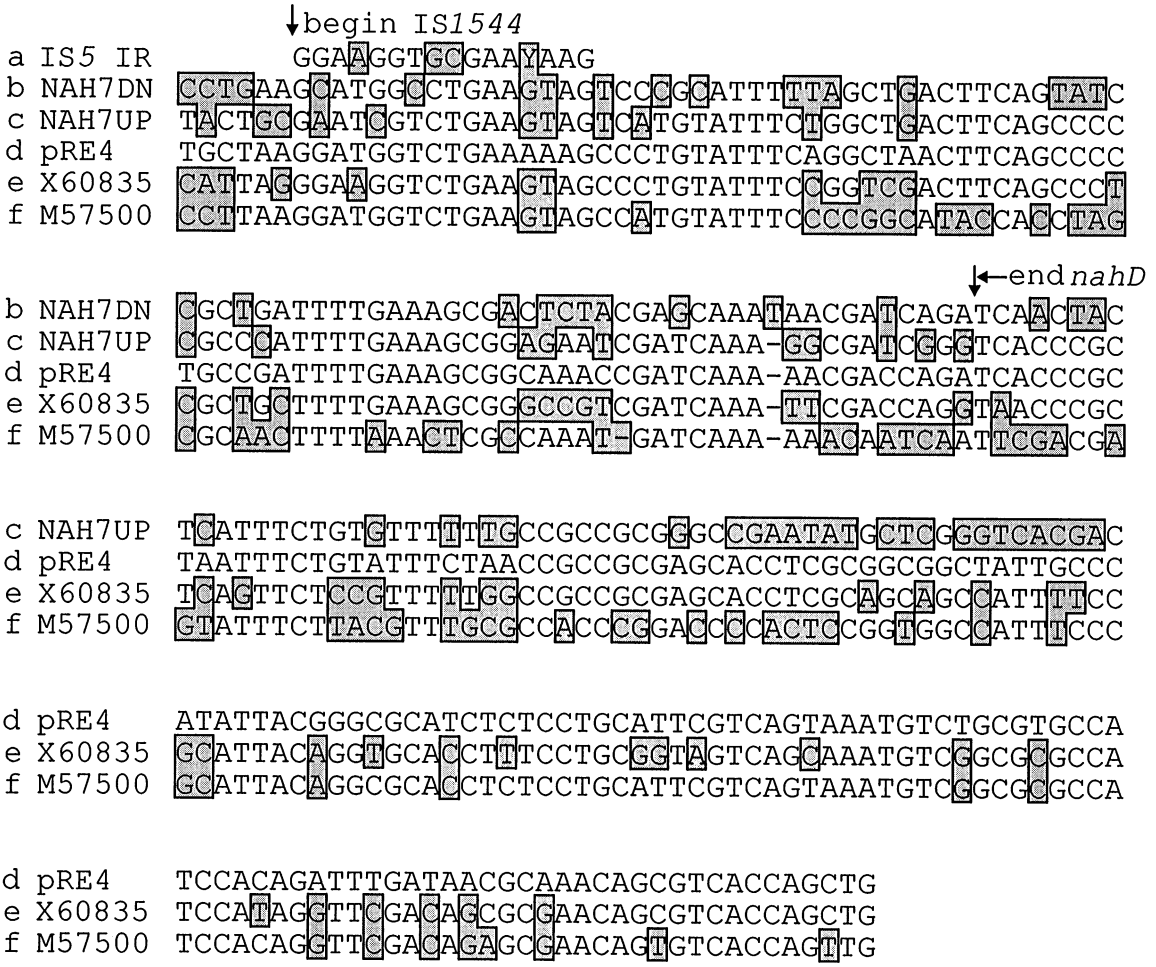

Figure 4. Comparison of pRE4 DNA upstream of and including part of IS*1544* with related DNA sequences (described in text and in Table 3). Sources of DNA sequences are: *a*, bp 1 to 16 and 1195 to 1180 of GenBank J01735 (the inverted repeat sequence located at the ends of IS*5*) (Kroger & Hobom 1982); *b*, bp 2172 to 2073 of GenBank U09057; *c*, bp 751 to 603 of GenBank M83949 (Eaton 1994); *d*, bp 4435 to 4670 of GenBank AF006691; *e*, bp 4242 to 4477 of GenBank X60835; and *f*, bp 1449 to 1215 of GenBank M57500. Shaded boxes indicate nucleotides that are not identical to the corresponding nucleotide in pRE4.

spectively, and also reveals genes (*ipbGFH*) encoding three additional proteins. Although mutants, having Tn*5* insertions in two of these genes, *ipbG* and *ipbF*, encoding acetaldehyde dehydrogenase (acylating) and 4-hydroxy-2-oxovalerate aldolase, were previously isolated and their insertions mapped (Eaton & Timmis 1986a), the resulting metabolic defects had not been determined. The third gene product, IpbH, has a high degree of similarity to putative outer membrane proteins encoded by genes occurring in operons encoding other alkyl-substituted aromatic hydrocarbon catabolic pathways (Table 2), but not biphenyl catabolic pathways. The function of IpbH in isopropylbenzene metabolism is unknown, however, a possible physiological role for the related outer membrane protein TodX in facilitating toluene movement across the membrane at low concentrations has been demonstrated (Wang et al. 1995).

Regulation of the isopropylbenzene catabolic pathway

IpbR was previously shown to be a positive regulatory protein, required for expression of the *ipb* operon and inducible by isopropylbenzene and a variety of additional hydrophobic chemicals (Eaton & Timmis 1996a; Selifonova & Eaton 1996). It has a high degree of similarity (54 to 56%) to the XylS regulator from several TOL plasmids (IpbR is compared to XylS from pWW0 in Figure 3, A); consequently, some un-

Table 3. Genetic elements associated with the *ipb* operon of pRE4

pRE4 element	Location[a]	Homologous element[b]	Range of homology[c]	% Homology
Upstream	912 to 1918	Downstream direct	912 to 1918	100
Direct repeat		Repeat		
IS*1543*	1923 to 3119	IS*52*	2095 to 3043	65
		gb M57500	2092 to 2740	64
		IS*1544*	2102 to 2676	65
		IS*1406*	2094 to 2873	66
IS*1544*	4441 to 5218	NAH7 down repeat[d]	4441 to 4528	67
		NAH7 up repeat[d]	4441 to 4562	80
		gb M57500	4441 to 5218	77
		gb X60835	4441 to 4670	78
		IS*52*	4602 to 5207	62
		IS*1543*	4624 to 5207	65
		IS*1406*	4573 to 5169	68
IS*1545*	5219 to 6501	IS*1240*	5219 to 6501	69
IS*1546*	6502 to 7406	IS*1491*	6502 to 7406	80
		IS*1162*	6748 to 7301	61
Downstream	20410 to 21416	Upstream direct	20410 to 21416	100
Direct repeat		Repeat		
(IS*1406*)[e]	(at approx. 19522)	IS*52*		91

[a] Location of the genetic element (map coordinates in bp, Figure 2), derived from the DNA sequence (GenBank AF006691).
[b] Genetic element, GenBank (gb) accession number (reference): IS*52*, M14366 (Yamada et al. 1986); IS*1406*, D83955 (Habe et al. 1996a); IS*1240*, L48985; IS*1162*, X79443 (Beltrametti et al. 1997; Solinas et al. 1995); NAH7 repeated segments, U09057, M83949, (Eaton 1994); IS*1491*, U84154.
[c] Location of detectable DNA sequence homology in the pRE4 map between the pRE4 genetic element and the homologous genetic element.
[d] DNA segments located upstream and downstream of the naphthalene catabolic pathway upper operon on the plasmid NAH7.
[e] IS*1406*, 1206 bp, is not present in pRE4 but is included here to draw attention to its location downstream from the closely related *cum* operon of *P. fluorescens* IP01 (Habe et al. 1996a).

derstanding of the regulation of the isopropylbenzene catabolic operon by IpbR may be gained from the many previous studies of regulation by XylS (Gallegos et al. 1993, 1996; Kessler et al. 1993, 1994; Michan et al. 1992a, 1992b; Ramos et al. 1986, 1990a, 1990b; Zhou et al. 1990). XylS activates transcription of the *xyl meta* operon, which encodes the catabolism of *m*- and *p*-toluate, by binding to the operator/promoter, *xylOmPm*. Kessler et al. (1993, 1994), demonstrated that tandemly repeated DNA sequences (11/15 identical bp) separated by six bp and overlapping the -35 region of the *Pm* promoter (Figure 3, B) are recognized and bound by XylS. Equivalent bases of these repeats are separated by 21 bp (two DNA helix turns); mutational analyses of *Om* and XylS indicate that XylS may bind to *Om* as a tandem dimer (Kessler et al. 1994). Similarly (Figure 3, B), the putative *ipbO* consists of two tandemly repeated DNA sequences of 14 bp (13/14 identical) which are separated from each other by seven bp and which overlap the -35 region of *ipbP*; as with *xylOm*, equivalent bases are separated by 21 bp. The left and right tandem repeats of *ipbO* also have some similarity to the corresponding repeats of *xylOm* (indicated by the double underline, Figure 3, B). More recently, Gallegos et al. (1996) have proposed that the sequence, T(C/A)CAN$_4$TGCA (Figure 3, B), is the minimum requirement for XylS activation of *OmPm*; however, their results also indicate that deletion of the left tandem repeat of *xylOm* results in a substantial decrease in promoter activity of *xylPm*. A sequence similar to that proposed by Gallegos et al. (1996) for *xylOmPm* is not readily apparent in the *ipbOP* region.

The XylS protein is part of the XylS/AraC family of more than 20 regulatory proteins (Gallegos et al. 1993; Ramos et al. 1990). Both XylS and IpbR have about 16% identity to the 292 amino acid *E. coli* AraC protein (Ramos et al. 1990). Two regions of these pro-

teins where similarity is highest are thought to resemble the DNA-binding helix-turn-helix region (Brennan & Matthews 1989) of the bacteriophage λ Cro protein and many other regulatory proteins. They are referred to as HTH-1 and HTH-2 (Figure 3, A). The salient features of the twenty amino acid helix-turn-helix motif as represented by that of λ Cro (QTKTAKDLGVYQSAINKAIH) are that residue 9 is glycine which permits the turn between the α-helices, residues 3 to 8 and 15 to 20 should not be proline since they are within α-helices, residues 4 and 15 should not be charged since they are buried in Cro, and residue 5 should not be a β-branched amino acid as it is wedged between two helices. Neither HTH-1 nor HTH-2 completely satisfies these criteria. HTH-2 of both IpbR and XylS has a charged amino acid (arginine) as residue 15, while HTH-1 of most members of the XylS/AraC family lacks the important (although not essential) glycine at residue 9 (Brennan & Matthews 1989; Harrison & Aggarwal 1990).

While IpbR responds to a diverse group of hydrophobic compounds, including isopropylbenzene, naphthalene, and trichloroethylene (Selifonova & Eaton 1996), XylS responds to many different substituted benzoates. Various mutant XylS proteins having altered responses to inducers or affinity for operator DNA have now been isolated (Kessler et al. 1994; Michan et al. 1992a, 1992b; Ramos et al. 1986, 1990a; Zhou et al. 1990). From studies with these mutants, it appears that inducer (effector) binding occurs in the amino terminus of XylS while the carboxy terminus binds to operator DNA (Michan et al. 1992a, 1992b). Although a specific effector binding region (amino acid residues 37 to 45) and DNA-binding region (HTH-1) have been proposed for XylS, the changes in many of these XylS mutant proteins are not within these designated regions. Thus, XylS proteins having altered inducer specificity have been obtained by changing amino acid residues 37, 41, 44, 45, 88, 152, 155, 229, 256, 274, and 288; other XylS proteins having an increased basal level of activity were obtained by altering amino acid residues 37, 41, 44, 88, 91, 229, 256, and 274 (Kessler et al. 1994; Michan et al. 1992a, 1992b; Ramos et al. 1990a; Zhou et al. 1990). Mutant XylS proteins that did not respond to inducers were obtained by altering single (residues 41, 91, 113, 312) and multiple (residues 10/11, 32/33, 213/214/215) amino acid residues (Kessler et al. 1994; Michan et al. 1992a, 1992b).

The designation of HTH-1 as an important element in DNA binding in the XylS/AraC family is most strongly supported by studies carried out by Brunelle and Schleif (1989) using 'missing contact probing'. They demonstrated that specific residues within HTH-1 of AraC contact specific bases in *araI*, the operator which controls the *araBAD* operon. However, other mutant AraCs with altered operator affinities resulting from changes in amino acid residues within HTH-2 as well as outside of either helix-turn-helix have also been obtained (Brunelle & Schleif 1989; Cass & Wilcox 1986; Francklyn & Lee 1988). While the designated regions of the XylS/AraC family of regulatory proteins clearly have primary roles in effector recognition and DNA binding, other regions also seem to be directly involved.

Genetic elements associated with isopropylbenzene catabolic genes

Several genetic elements, not directly involved in isopropylbenzene catabolism are associated with *ipb* genes in pRE4 (Figure 2, Table 3).

Encompassing the *ipb* catabolic genes, extending from bp 912 to 1918 and bp 20410 to 21416, are two identical directly repeated DNA segments of 1,007 bp. They have no homology to any other known DNA elements. Homologous recombination between these elements in pRE4 would result in a deletion of 19,498 bp as was experimentally observed previously (Eaton & Timmis 1984, 1986b). In plasmids pRE658 and pRE659, this would be expected to leave a 3.85 kbp *Xma*I fragment inserted in the vector, as was observed here. Since the segments are identical, their duplication is probably a recent event; the mechanism for this duplication is not evident.

There are four additional DNA elements upstream of the *ipb* operon which are related to insertion sequence (IS) elements.

Next to the upstream direct repeat is an insertion sequence (IS*1543*) having a high degree of homology to IS*52* (65%) (Yamada et al. 1986) and other members of the IS*5* family. IS*1543* is 1196 bp in length, about the same size as IS*52*, and has 13 bp inverted repeats (5′-GCTGATTTATTCG-3′) at its ends. IS*1543* does not appear to encode a complete transposase; however, a protein deduced from the DNA sequence, encoded by two ORFs extending from bp 2870 to 2095 with one frame shift (not shown) is 70% similar to the proposed transposase of IS*52*. In their study of the closely related *cum* operon, Habe et al. (1996a) identified an insertion sequence, IS*1406*, which has 91% homology to IS*52* and 66% homology to IS*1543* over

779 bp. IS*1406* is located 100 base pairs downstream from the last gene of the *cum* operon, *cumD* (analogous to *ipbD*), and in the orientation opposite to that of IS*1543*. There is no evidence for IS*1406* downstream from the *ipb* operon; the DNA sequences of *ipb* and *cum* operons begin to differ significantly at bp 19,390, 31 bp before the end of *ipbD*. Habe et al. (1996a) sequenced the DNA up to about 2 kbp upstream of the *cum* operon but did not identify sequences having homology to IS*1406* or related IS elements. These two IS*52*-like elements may be remnants, along with the associated *ipb* and *cum* operons, of a composite (class I) isopropylbenzene (cumene) catabolic transposon (Wyndham et al. 1994) in which one of the IS elements has been deleted (IS*1543* from *P. fluorescens* IP01 and IS*1406* from pRE4).

The regulatory gene *ipbR*, which follows IS*1543*, is separated from the *ipb* operon by three additional IS elements. The first of these, IS*1544*, is a member of the IS*5* family, having extensive homology to IS*52*, IS*1543*, and IS*1406*. It is most closely related to DNA segments found in unpublished sequences located downstream from the *dmp* (dimethyl phenol catabolism) operon of *P. putida* CF600 (Shingler et al. 1992) and upstream of phenol catabolism genes, *pheBA*, located on plasmid pEST1226 of *Pseudomonas* sp. EST1001 (Kivisaar et al. 1991) (GenBank numbers X60835 and M57500, respectively) (Figure 4, Table 3). The deduced amino acid sequence of a partial protein encoded by bp 5222 to 4860 of IS*1544* has 94% identity to a putative transposase of the insertion sequence present in GenBank M57500. The left end of IS*1544* also has homology to short DNA segments previously found at either end of the upper operon of the naphthalene catabolic plasmid NAH7 and related naphthalene catabolic operons (Eaton 1994). In NAH7 there is 76% homology between these short segments which serve to define the ends of the DNA encoding the naphthalene upper catabolic pathway. From the comparisons in Figure 4, it would seem that the NAH7 repeated DNA segments are remnants (86 bp and 122 bp) of an insertion sequence element of the IS*5* family. IS*1544* is itself only a fragment (777 bp) of an IS element. The right end of IS*1544* may have been deleted by the insertion of the adjacent IS*1545*, a large insertion sequence fragment (1284 bp) which has 69% homology to an unpublished insertion sequence, IS*1240*, from *P. syringae*. The intact left end of IS*1545* is nearly identical (26/27 bp) to the proposed right end of IS*1240* while the right end of IS*1545* has been deleted.

IS*1546*, located between IS*1545* and the *ipb* operon, has 80% homology to IS*1491* of *P. alcaligenes*. However, IS*1546* has a 668 bp deletion (relative to IS*1491*) at sequence position bp 6750 and lacks inverted repeat sequences corresponding to those at the ends of IS*1491*. It has somewhat less homology to other IS elements including IS*1162*, previously identified in the chromosome of *P. fluorescens* ST next to styrene catabolic genes and also as two copies on plasmid pEG in that strain (Beltrametti et al. 1997; Solinas et al. 1995).

Unrelated IS elements have been known for many years to occur together, although this is not very common (Hänni et al. 1982; Iida et al. 1983). For example, in an early report, adjacent IS*2* and IS*5* elements were observed in mutant derivatives of bacteriophage Mu (Chow and Broker 1978) while more recently three different IS elements were found clustered near dichloromethane catabolic genes in *Methylobacterium* sp. DM4 (Schmid-Appert 1997). All of the clustered IS elements located upstream of the *ipb* operon appear to have become defective. Except for IS*1543*, they lack inverted repeat sequences corresponding to those at the ends of homologous IS elements and they carry only partial transposase genes. Deletions at the ends of IS elements and transposons have been well-documented (Ahmed 1984; Jilk et al. 1993; Roberts et al. 1991), and presumably result from insertion of one transposon into another or from failed transposition events.

Conclusion

Analysis of the DNA sequence of the isopropylbenzene catabolism region of pRE4 strengthens and extends previous biochemical and genetic studies with *P. putida* RE204 and its transposon-generated mutant derivatives. In addition, several previously undetected IS elements, associated with *ipbR* and the upstream end of the *ipb* operon, were identified. While these elements may have been involved in the assembly of pRE4, the nature of their involvement is not yet clear. It is somewhat surprising that identical 1007 bp DNA segments, bracketing these IS elements and genes encoding isopropylbenzene catabolism, and previously implicated in the deletion of these genes from pRE4, do not appear to be related to IS elements. The most interesting result of this study may be the demonstration that the regulatory system, IpbR/*ipbOP* is related to the well-studied XylS/*xylOmPm* system. Future

comparative studies of these two regulatory systems including construction of hybrid regulatory proteins should provide useful information about their function and evolution.

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