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Location and organization of the dimethylphenol catabolic genes of *Pseudomonas* CF600

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Summary. The gene organization of the phenol catabolic pathway of *Pseudomonas* CF600 has been investigated. This strain can grow on phenol and some methylated phenols by virtue of an inducible phenol hydroxylase and *meta*-cleavage pathway enzymes. The genes coding for these enzymes are located on pVI150, an IncP-2 degradative mega plasmid of this strain. Twenty-three kilobases of contiguous DNA were isolated from lambda libraries constructed from strains harbouring wild type and Tn*5* insertion mutants of pVI150. A 19.9 kb region of this DNA has been identified which encodes all the catabolic genes of the pathway. Using transposon mutagenesis, polypeptide analysis and expression of subfragments of DNA, the genes encoding the first four enzymatic steps of the pathway have been individually mapped and found to lie adjacent to each other. The order of these genes is the same as that for isofunctional genes of TOL plasmid pWWO and plasmid NAH7.

Key words: *Pseudomonas* – Catabolic pathway – Phenol biodegradation – Gene organization

Introduction

Soil microorganisms exhibit extensive metabolic diversity enabling them to degrade a large variety of aromatic compounds (Gibson 1984). Although bacteria employ a range of enzymes for the initial attack on different aromatic substrates, the catabolic pathways tend to converge on just a few key intermediates such as catechol or substituted catechols (Ornston and Yeh 1982; Dagley 1986). These central intermediates serve as substrates for cleavage of the aromatic ring and can be further metabolized by two distinct sets of enzymes, those of the *ortho*- and *meta*-cleavage pathways (for review see Dagley 1986).

A substantial number of aromatic catabolic pathways are plasmid encoded. Detailed studies of the organization and regulation of the pathway genes have been performed in a few cases, such as toluene plasmid pWWO (Franklin et al. 1981; Spooner et al. 1986, 1987; Inouye et al. 1987, 1988) and naphthalene plasmid NAH7 (Yen and Gunsalus 1982, 1985; Schell and Wender 1986). These and other studies using transposon mutagenesis have revealed that pathway genes are often encoded in co-ordinately regulated operons (reviewed in Frantz and Chakrabarty 1986).

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Pseudomonas CF600 can metabolize phenol, cresols, and 3,4-dimethylphenol (3,4-dmp) as sole carbon sources. The pathway for dissimilation of these compounds involves hydroxylation and a subsequent *meta*-cleavage pathway (Fig. 1). The regulatory and catabolic genes of the pathway are located on an Inc P-2 mega plasmid designated pVI150 (Shingler et al. 1989). We have investigated the gene organization of the phenol/3,4-dmp catabolic pathway of this strain. In this communication we report the cloning of 23 kb of pVI150 DNA, spanning all the catabolic genes, and the clustered location of the genes encoding the first 4 enzymatic steps of the pathway. Evidence is presented that the genes of the pathway may be encoded in a single operon.

Materials and methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown at 37° C, whereas *Pseudomonas* strains were grown at 30° C. Luria broth (LB, Maniatis et al. 1982) was used as complete media; M9-salts (Kahn et al. 1979) supplemented with 2.5 mM carbon source was used as minimal media, and methionine assay media (Difco) was used in maxicell analysis of polypeptides. Ampicillin (Ap, 100 μg/ml) and carbenicillin (Cb, 2 mg/ml) were used for selection of the plasmid-encoded β-lactamase in *E. coli* and *Pseudomonas*, respectively.

Enzyme assays. Enzyme assays were performed spectrophotometrically as described previously (Shingler et al. 1989). The units of activity are expressed as the amount of enzyme required to convert 1 μmole of substrate to product per min.

DNA manipulations. DNA used in the construction of lambda libraries was isolated by the method of Hansen and Olsen (1978). Plasmid DNA was prepared by the rapid boiling method of Holmes and Quigley (1981), and lambda DNA by the method of Loenen and Brammer (1980). Restriction enzymes and DNA-modifying enzymes were purchased from Boehringer Mannheim or New England Bio-Labs, and used as recommended by the manufacturer. Plasmid constructs were generated by standard recombinant DNA techniques as described previously (Shingler et al. 1989).

Introduction of plasmids into bacterial strains. E. coli strains were transformed with plasmid DNA using the procedure

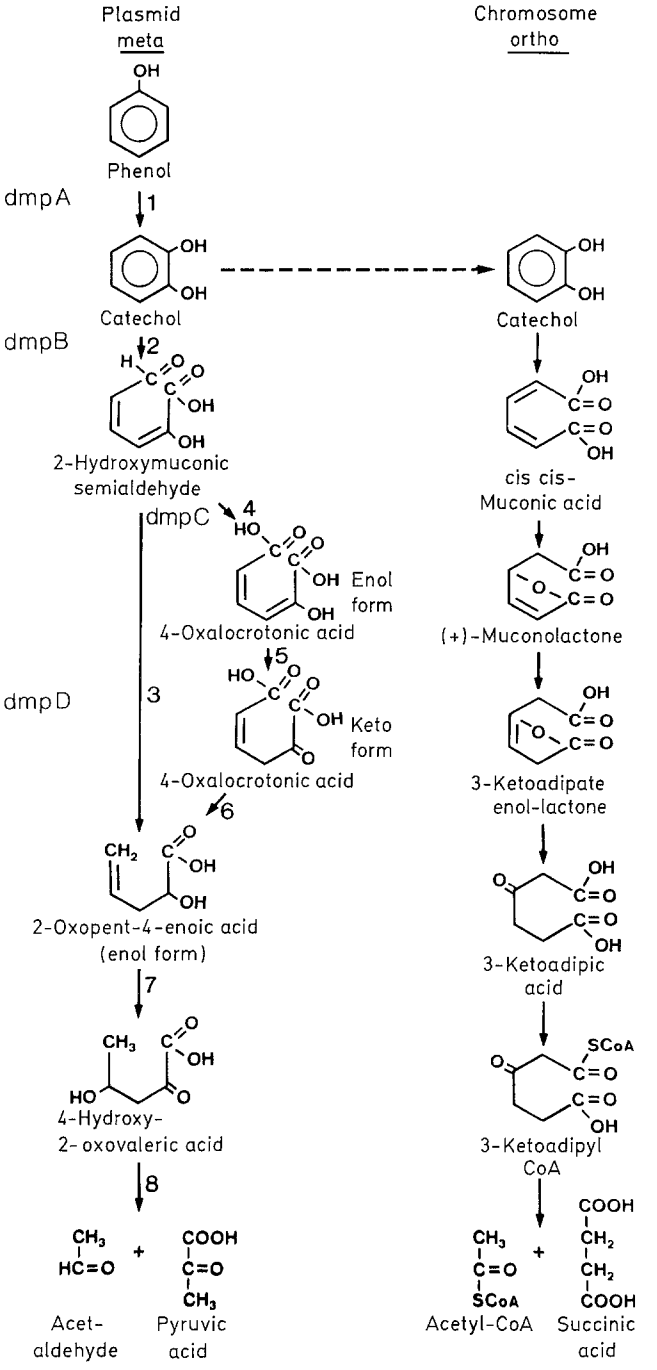


Fig. 1. Plasmid encoded *meta*-cleavage pathway, and chromosome encoded *ortho*-cleavage pathway, for the metabolism of phenol and catechol, respectively. 1, phenol hydroxylase; 2, catechol 2,3-dioxygenase; 3, 2-hydroxymuconic semialdehyde hydrolase; 4, 2-hydroxymuconic semialdehyde dehydrogenase; 5, isomerase; 6, decarboxylase; 7, hydratase; 8, aldolase. The *dotted line* indicates how catechol can enter the chromosomal *ortho*-pathway after its production from phenol by the action of phenol hydroxylase

of Kushner (1978). Plasmids were introduced into *Pseudomonas* strains by conjugation from *E. coli* S17-1, or by electroporation using a Bio-Rad Gene Pulser.

Cloning of Tn5-carrying Eco*RI fragments.* The Tn*5*-carrying *Eco*RI fragments from *Pseudomonas* strains harbouring pVI150::Tn*5*/1–7 were cloned into pUC19 using either of the following strategies: (i) total DNA cut with *Eco*RI was cloned directly into the *Eco*RI site of pUC19, followed by selection for Km^r, or via (ii) cloning into a lambda vector. Lambda libraries were constructed by cloning of *Eco*RI-cut total DNA between the *Eco*RI sites in EMBL4 (Frischauf et al. 1983). Libraries of approximately 1×10^6 were obtained using the in vitro packaging kit (Amersham) and *E. coli* NM539 as recommended by the manufacturer. The desired phages were detected by screening using a Tn*5*-specific probe (the two internal *Xho*I fragments of Tn*5*).

Cloning of the 19 kb Bam*HI fragment.* Total CF600 DNA was digested with *Bam*HI and ligated to EMBL3 (Frischauf et al. 1983) by using *Bam*HI arms purchased from Promega. The library was generated as described above, and screened by hybridization to a pathway-specific probe (the 1.5 kb *Sma*I fragment from pCF100).

Plaque hybridization procedure. Plaques were transferred to nitrocellulose filters (Amersham Hybond-c) and subsequently alkali treated and hybridized to DNA probes (Maniatis et al. 1982). The DNA probes were labelled and detected using the Boehringer Mannheim nonradioactive DNA labelling and detection kit.

Analysis of plasmid-encoded polypeptides. Plasmids were introduced into maxicall strain CSR603 by transformation. Preparation, labelling and analysis of maxicells with L-(^{35}S) methionine (Amersham) were as described by Sancar et al. (1979).

Results

Cloning of the catabolic genes

Due to the large size of *Pseudomonas* CF600 degradative plasmid pVI150 and the inherent difficulty in isolating its DNA, it was not feasible to clone directly from the plasmid. In a previous study one component of the phenol hydroxylase (PH), which catalyses the first enzymatic step of the pathway, was isolated on a cosmid clone. This component is located on a 1.2 kb *Pvu*II-*Nru*I fragment, which is completely encompassed by a 1.5 kb *Sma*I fragment (Shingler et al. 1989). However, this cosmid clone, designated pCF100, contained an internal deletion and was not suitable for further mapping of the degradative genes. Thus, to isolate this region of DNA in its wild-type configuration a lambda library of total CF600 DNA was constructed. Approximately 2×10^4 plaques were screened by hydridization to the pathway-specific 1.5 kb *Sma*I fragment derived from pCF100. The restriction map of 19 kb *Bam*HI fragment present in 1 of the 3 hybridizing phages is shown in Fig. 2. The location of the probe and the *dmp*B gene within the 19 kb *Bam*HI fragment is also shown. *dmp*B encodes the catechol 2,3-dioxygenase (C230) which catalyses the second enzymatic step of the pathway. The nucleotide sequence of this gene has been determined (Bartilson and Shingler 1989), allowing unambiguous location within the fragment.

Seven Tn*5* pathway mutants, isolated on the basis of their inability to grow on phenol and 3,4-dmp, were generated previously. These strains have Tn*5* located in genes of pVI150 that are involved in the catabolism of these substrates (Shingler et al. 1989). The positions of the Tn*5* inser-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Reference or source
Escherichia coli		
S17-1	r^-m^+ Tp^r Sm^r Mob^+	Simon et al. (1983)
DH5	r^-m^+ *rec*A1	Hanahan (1985)
CSR603	Maxicell strain	Sancar et al. (1979)
NM539	r^-m^+ P2 lysogen	Karn et al. (1980)
Pseudomonas		
CF600	Phenol/3,4-dmp degrader	Shingler et al. (1989)
PB2701	r^-m^+ Sm^r derivative of KT2440	MBSC[b]
Plasmids:		
pMMB66HE and EH	Ap^r, RSF1010 based *tac* promoter expression vectors carrying $lacI^Q$	Fürste et al. (1986)
pMMB66HE*Δ* and EH*Δ*	Derivatives of pMMB66HE and EH without the $lacI^Q$ repressor gene	Shingler et al. (1989)
pCF100	Cosmid carrying *pmo*	Shingler et al. (1989)
pVI150	Catabolic plasmid of CF600	Shingler et al. (1989)
pVI150::Tn*5*/*1–7*	Tn*5* insertion derivatives of pVI150	Shingler et al. (1989)
pVI1:18	*dmp*B cloned in pMMB66EH*Δ*	Bartilson and Shingler (1989)
pUC19	Ap^r cloning vector	Yanisch-Perron et al. (1985)

[a] r and m refer to host restriction and modification systems, respectively. Antibiotic resistance abbreviations are: Ap^r, ampicillin; Sm^r, streptomycin; Tp^r, trimethoprim
[b] MBSC, M. Bagdasarian strain collection

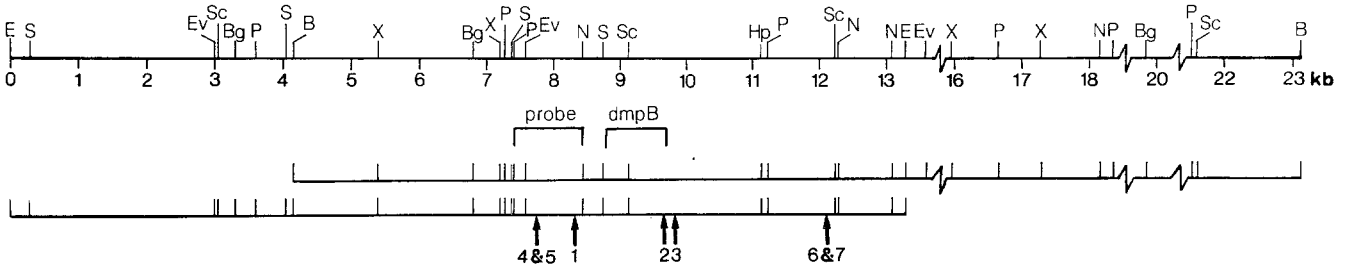


Fig. 2. Restriction map of cloned pVI150 DNA. The position of the 1.5 kb *Sma*I fragment used in the cloning and the previously located *dmp*B gene are indicated. The positions of Tn*5* insertions are indicated by *arrows*. *Kinks* indicate discontinuity in the scale. Restriction endonuclease recognition sites are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; Hp, *Hpa*I; N, *Nru*I; P, *Pvu*II; S, *Sma*I; Sc, *Sac*I; and X, *Xho*I

tions were determined after the Tn*5*-carrying *Eco*RI fragments from these strains were cloned into pUC19. Figure 2 shows that three of the Tn*5* insertions map in the previously defined component of PH, one maps in *dmp*B and three are located downstream of these regions.

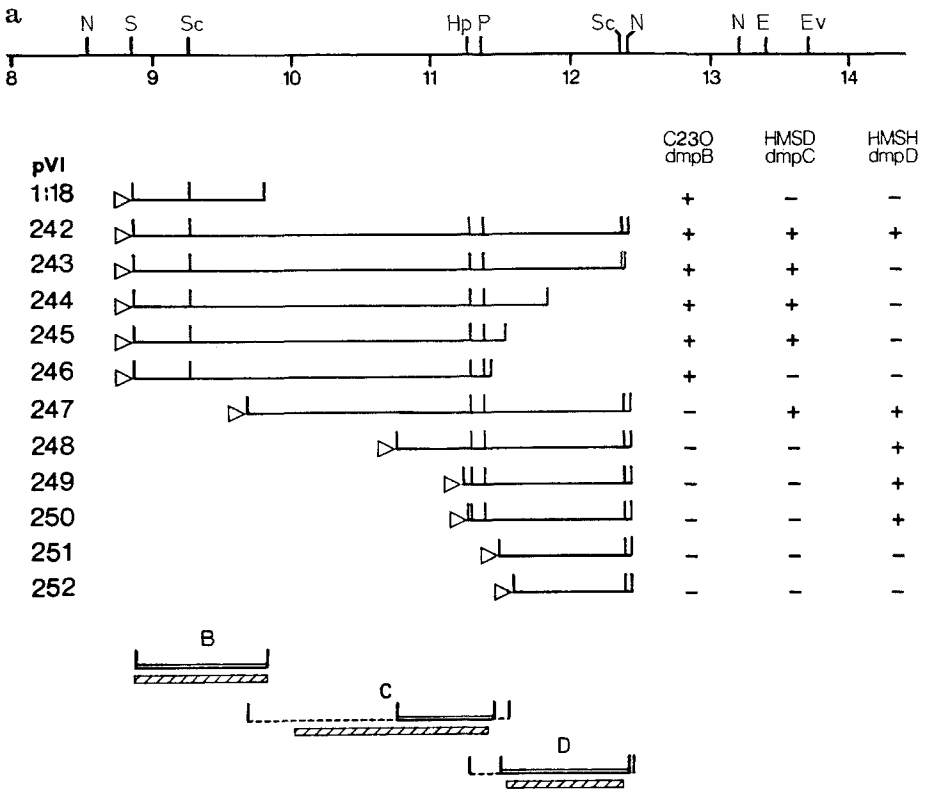
Location and polypeptide products of the dmp*C and* dmp*D genes*

The location of Tn*5* insertions 3, 6 and 7 downstream of *dmp*B indicated that other genes involved in the catabolic pathway may be located in this region. The 3.65 kb *Sma*I-*Nru*I fragment, (Fig. 2, coordinates 8.8–12.45) was subcloned into the *tac* expression vector pMMB66HE*Δ* to generate pVI242. *E. coli* DH5 harbouring pVI242 was found to express C230, 2-hydroxymuconic semialdehyde dehydrogenase (HMSD, *dmp*C) and 2-hydroxymuconic semialdehyde hydrolase (HMSH, *dmp*D) (see Table 2). To locate the *dmp*C and *dmp*D genes more precisely, *Bal*31 deletions of pVI242 were generated that carry various portions of the 3.65 kb *Sma*I-*Nru*I fragment. The extent of the insert DNA in these plasmids, pVI243–pVI252, and in a previously constructed plasmid, pVI1:18, which encodes only

Table 2. Enzyme activities of *E. coli* DH5 harbouring various plasmids

Plasmid	Enzyme activity (mU/mg protein)		
	C230 *dmp*B	HMSD *dmp*C	HMSH *dmp*D
pMMB66HE*Δ*	–	–	–
pVI242	10500	580	200
pVI243	17300	1030	–
pVI244	18500	1100	–
pVI245	19900	930	–
pVI246	19000	–	–
pVI247	–	820	410
pVI248	–	–	160
pVI249	–	–	140
pVI250	–	–	90
pVI251	–	–	–
pVI252	–	–	–

Cells were grown overnight in LB containing Ap, harvested and crude extracts prepared. The assays were performed a minimum of two times; one representative set of results is shown; – indicates values less than or equal to 20



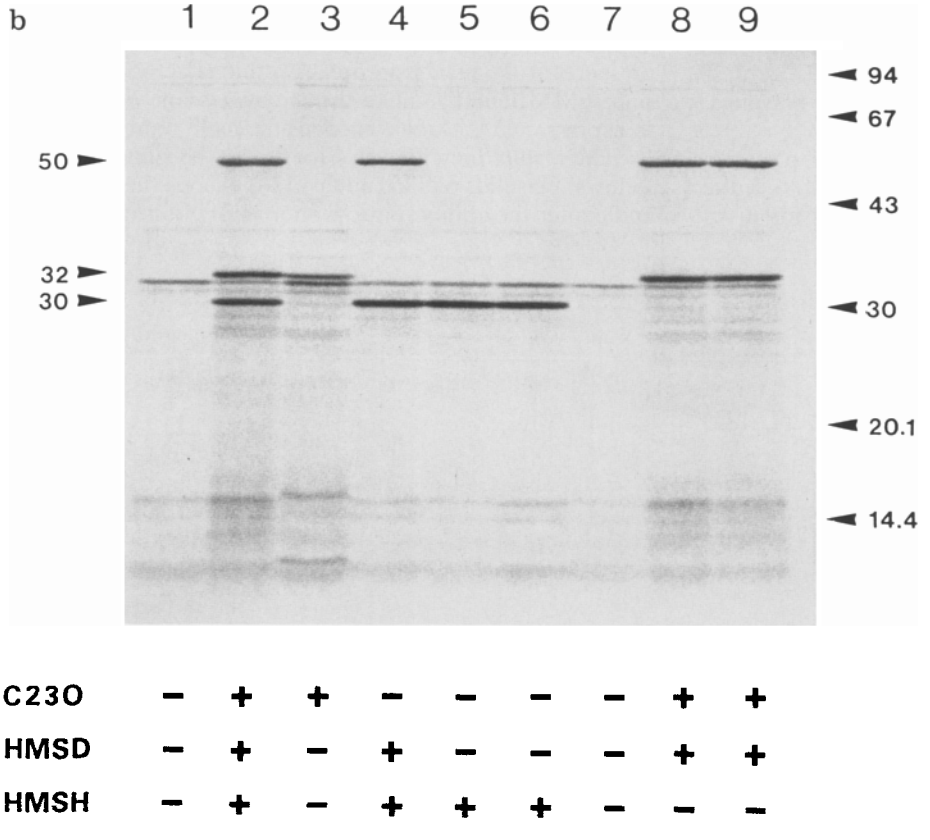


Fig. 3a and b. Mapping and identification of the products of *dmp*C and *dmp*D. **a** Constructs used to determine the location of *dmp*C and *dmp*D. *Arrowheads* indicate the direction of transcription from the *tac* promoter of the expression vector. Coordinates and restriction endonuclease recognition sites are as in Fig. 2. The extent of *dmp*B, C and D genes are shown with regions of ambiguity indicated by *dotted lines*. *Hatched boxes* indicate the sizes of the genes, calculated assuming a molecular weight of 110 daltons per amino acid residue, required to encode polypeptides of the observed sizes (see below). **b** Maxicell analysis of plasmid encoded polypeptides separated on a 10%–20% polyacrylamide gel. Lane 1, vector control – pMMB66HEΔ; lane 2, pVI242; lane 3, pVI1:18; lane 4, pVI247; lane 5, pVI248; lane 6, pVI249; lane 7, pVI252; lane 8, pVI244; lane 9, pVI245. Sizes of mol. wt. markers are given in kDa

C230 activity, is illustrated in Fig. 3a. Correlation of enzyme activities (Table 2) with the DNA carried on the plasmids demonstrates that the genes for these enzymes are clustered in the order *dmp*B, C and D (Fig. 3a).

The *dmp*B gene mediates the production of a M_r 32000 polypeptide (Bartilson and Shingler 1989). To identify the polypeptide products of the *dmp*C and *dmp*D genes we analysed plasmid-encoded polypeptides produced by maxicell strain CSR603 harbouring various plasmids. The results of this analysis (Fig. 3b) demonstrate that the presence or

absence of the enzyme activities correlates with the presence or absence of new polypeptides. C230 activity (*dmp*B) correlates with a M_r 32000 polypeptide as previously described, HMSD activity (*dmp*C) correlates with a M_r 50000 polypeptide and HMSH activity (*dmp*D) correlates with the production of a M_r 30000 polypeptide. The size of the polypeptides (assuming a molecular weight of 110 daltons per amino acid residue) indicates that they could be encoded in tandem within the defined region of DNA (Fig. 3a), with gene sizes of approximately 1.0, 1.4 and 0.8 kb, respectively. The gene order and size correlates well with those of isofunctional enzymes of TOL plasmid pWWO and plasmid NAH7 (Harayarma et al. 1987).

Location of the phenol hydroxylase-encoding dmp*A genes*

The first enzymatic step of the phenol/3,4-dmp catabolic pathway of *Pseudomonas* CF600 is catalysed by a multicomponent phenol hydroxylase. One component of PH, designated *pmo*, has been shown to encode a M_r 39500 polypeptide (Shingler et al. 1989). The coding region for this polypeptide is within a 1.2 kb *Pvu*II-*Nru*I fragment (Fig. 2, coordinates 7.3–8.5). The close proximity of *dmp*B, C and D genes downstream from *pmo* suggested that the remainder of the DNA required to express PH activity might be located upstream from the *Pvu*II site. The DNA from the *Bam*HI site (Fig. 2, coordinate 4.2) to the end of the 1.2 kb *Pvu*II-*Nru*I fragment, was previously shown to be insufficient to encode PH activity (Shingler et al. 1989). Hence, to generate plasmids suitable for testing PH activity, various portions of DNA from coordinates 1.3–8.5 were cloned into pMMB66HE*Δ* and pMMB66EH*Δ* expression vectors. The extent of the DNA in these plasmids, pVI254–pVI258, is shown in Fig. 4a.

PH activity was monitored by the ability of plasmids to mediate growth of *P. putida* PB2701 on phenol via the chromosomally-encoded *ortho*-cleavage pathway for dissimilation of catechol (Fig. 1). The results demonstrate that plasmids pVI254–256 all mediated growth on phenol, whereas plasmids pVI257 and pVI258 did not. Therefore, the DNA between *Bgl*II (coordinate 3.3) and *Bam*HI (coordinate 4.2) defines the start of the PH-encoding region and the DNA between this *Bgl*II and *Nru*I (coordinates 3.3–8.5) is sufficient to encode PH activity. Nearly all the *Pvu*II-*Nru*I fragment would be required to encode the M_r 39000 polypeptide; thus, a DNA region of 4.3–5.2 kb is required to encode PH activity. Analysis of the polypeptides produced from the PH-encoding region shows production of 5 new polypeptides including the M_r 39000 polypeptide (manuscript in preparation).

Expression of the complete phenol/3,4-dmp meta*-cleavage pathway*

P. putida PB2701 harbouring plasmids encoding PH can grow on phenol due to dissimilation of catechol via the chromosomal *ortho*-cleavage pathway. Such strains cannot grow on methylated phenols since utilization of these substrates requires expression of the whole of the *meta*-cleavage pathway, i.e. the *ortho*-cleavage enzyme catechol 1,2-dioxygenase has low affinity for methylcatechols or produces dead-end intermediates. To assess whether the entire phenol/3,4-dmp pathway is encoded within the 23 kb of DNA isolated, plasmids pVI260–pVI265 were constructed using *tac* expression vector pMMB66HE (Fig. 4b). These plasmids were introduced into *P. putida* PB2701 and the resulting strains tested for their ability to grow on phenol, *m*-, *p*- and *o*-cresol, and 3,4-dmp. Tests were performed in the presence of 0.5 mM isopropyl-*β*-D-thiogalactoside (IPTG) since pMMB66HE, unlike the *tac* expression vectors used to express *dmp*A–D, also encodes the *lacI*^{Q} repressor gene.

The results presented in Fig. 4b can be summarized as follows: plasmids pVI260 and pVI261 encode the PH region and confer the ability to grow on phenol but not its methy-

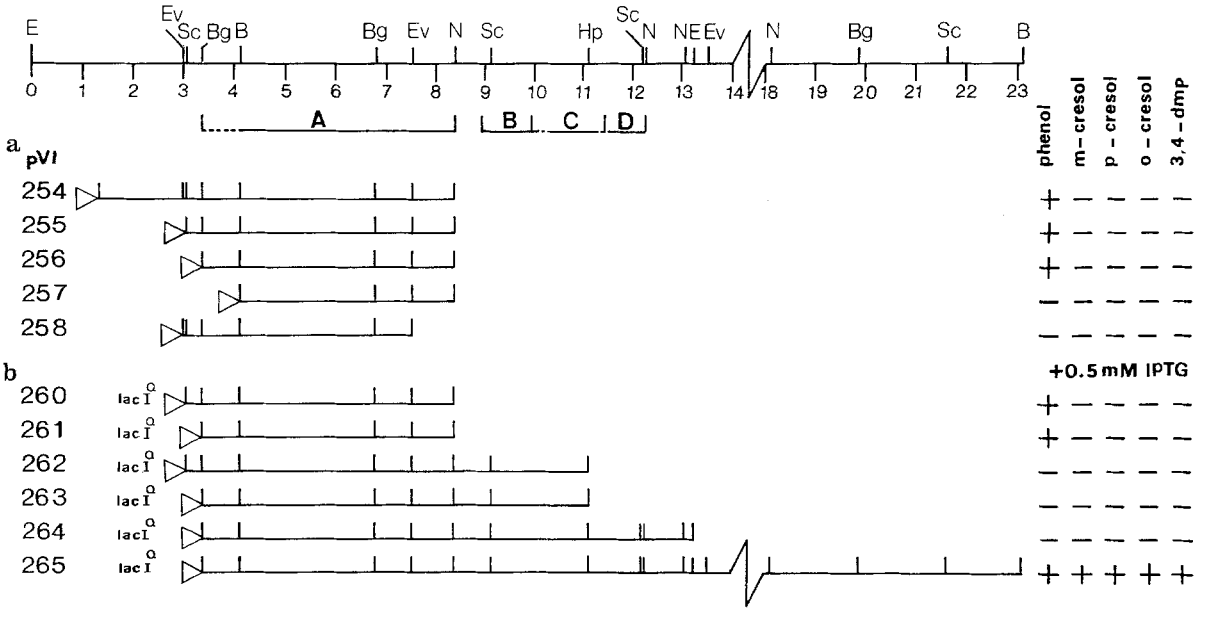


Fig. 4. **a** Mapping of *dmp*A genes and **b** location of the *meta*-pathway. *Arrowheads* indicate the direction of transcription from the *tac* promoter of the expression vector. Coordinates and restriction endonuclease recognition sites are as in Fig. 2. The ability of *Pseudomonas putida* PB2701, harbouring the plasmids, to grow on M9-salts supplemented with different carbon sources is indicated

lated derivatives. Plasmids pVI262–264 encode PH and some of the *meta*-cleavage enzymes. These plasmids do not allow growth of any of the substrates tested. This is presumably the result of the inability of the intermediates of the *meta*-pathway, as opposed to catechol, to be metabolized by the chromosomal *ortho*-cleavage pathway. Plasmid pVI265 contains all the DNA from coordinates 3.3 to 23.2, and confers the ability to grow on all the substrates tested. These data suggest that the enzymes required for the utilization of phenol and its methylated derivatives are the same, and that all the genes encoding these enzyme activities are encoded within a 19.9 kb region. Growth on phenol was found to be dependent on the presence of IPTG in the media, indicating that transcription of the genes is mediated by the *tac* promoter of the vector.

Discussion

In this study we have investigated the gene organization of the phenol/3,4-dmp catabolic pathway of *Pseudomonas* CF600. These genes are located on a plasmid, pVI150, where they are efficiently regulated, with low basal levels of the enzymes expressed in the absence of pathway intermediates (Shingler et al. 1989). A 19.9 kb region (located at 3.3–23.2 kb in Fig. 2) that encodes all the catabolic genes of this pathway has been identified. Expression of the pathway genes of this DNA on plasmid pVI265 is dependent on the *tac* promoter of the vector. This suggests that either the natural promoter or a positive regulator is not present on this DNA fragment. We favour the latter explanation, since possession of a further 0.25 kb of 5′ DNA did not alleviate the dependence of transcription on the *tac* promoter (compare plasmids pVI260 and pVI261). Within the 19.9 kb region, the genes for the first four enzymatic steps have been mapped. The genes lie in a cluster and are transcribed in the same direction. These results, together with the observation that the Tn*5/1* insertion in *dmp*A has polar effects on *dmp*B, C and D (Shingler et al. 1989), suggest that these genes, and possibly the whole of the pathway, are encoded in a single coordinately regulated operon.

It was at first surprising that expression of all the DNA originally isolated (coordinates, 0–23.2 kb) did not mediate growth on any of the substrates tested (data not shown). However, this suggested that a strong transcriptional termination signal was located between the *Eco*RI and *Sac*I sites (coordinates 0–3.05). This interpretation is supported by the finding that deletion of this region in pVI265 results in efficient growth on all the phenolic substrates tested. Prevention of readthrough transcription by such a termination signal would be required for efficient regulation of a catabolic operon. These features suggest that the promoter for the pathway genes is located between the transcription termination signal and the start of *dmp*A. Future experiments, using RNA/DNA hybridization and fusion of the putative promoter region to a reporter gene, should define the promoter region and determine whether all the enzymes of the pathway are translated from a single transcript. Moreover, fusion of the promoter region to a reporter gene provides a system whereby DNA encoding a positive regulator may be identified.

The finding that expression of the 19.9 kb DNA region confers the ability to grow on phenol and methylphenols suggests that the same enzymes are used for the dissimilation of these substrates in *Pseudomonas* CF600. Cloning of the entire phenol/3,4-dmp catabolic pathway, along with location of the HMSD- and HMSD-encoding genes, *dmp*C and *dmp*D, opens up the possibility of determining the role of the branches of the *meta*-pathway (Fig. 1) in dissimilation of methylphenols.

For bacteria there are clear biological benefits of channelling diverse compounds into a few central pathways: these include reduced genetic load, simplification of regulatory circuits and economization of energy. Two possible mechanisms may account for the occurrence of common biochemical routes for the dissimilation of aromatic compounds: (i) divergent evolution, in which a few primordial pathways have been selected and expanded, or (ii) convergent evolution, in which individual pathways have evolved common routes due to biochemical constraints. Common ancestry of *ortho*- and *meta*-cleavage pathways has been suggested by DNA sequence homology and comparison of amino acid sequence of isofunctional enzymes (Harayarma et al. 1987 and references therein). Comparison of the *meta*-pathways of TOL plasmid pWWO and plasmid NAH7 demonstrate DNA sequence homology and conservation of the gene order and size within the operon (Harayarma et al. 1987). We have determined the gene order of the phenol/3,4-dmp *meta*-pathway of pVI150 to be the same as that of pWWO and NAH7. The sizes of the *dmp*B–D genes are also similar to the sizes of isofunctional genes of pWWO and NAH7. Further, there is considerable homology between the C230-encoding genes of these pathways (of approximately 80% at the nucleotide level and 85% at the amino acid level, Bartilson and Shingler 1989). It seems likely, therefore, that the *meta*-cleavage genes of these three plasmid-encoded catabolic pathways have a common origin.

The catabolic pathway of TOL plasmid pWWO is encoded within two alternative large transposons (Tsuda et al. 1989). The location of pathway genes within a transposon provides a mechanism whereby pathways can be shuttled between different plasmids and inherited as metabolic "modules" (Cane and Williams 1986). In the pathways discussed above, the enzymes for conversion to form a catechol differ both in number and the chemical reaction that they catalyse. Therefore, gene recruitment and/or replacement must have occurred to give the present-day gene organization. It will be of interest to learn whether the catabolic pathways of NAH7 and pVI150 are also located on large transposons.

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