Molecular Analysis of a Plasmid-encoded Phenol Hydroxylase from *Pseudomonas* CF600

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Pseudomonas strain CF600 is able to utilize phenol and 3,4-dimethylphenol as sole carbon and energy source. We demonstrate that growth on these substrates is by virtue of plasmid-encoded phenol hydroxylase and a *meta*-cleavage pathway. Screening of a genomic bank, with DNA from the previously cloned catechol 2,3-dioxygenase gene of the TOL plasmid pWW0, was used in the identification of a clone which could complement a phenol-hydroxylase-deficient transposon insertion mutant. Deletion mapping and polypeptide production analysis identified a 1·2 kb region of DNA encoding a 39·5 kDa polypeptide which mediated this complementation. Enzyme activities and growth properties of *Pseudomonas* strains harbouring this fragment on a broad-host-range expression vector indicate that phenol hydroxylase is a multicomponent enzyme containing the 39·5 kDa polypeptide as one component.

INTRODUCTION

Pseudomonads and other soil micro-organisms are able to metabolize the vast majority of natural and synthetic organic compounds (Gibson, 1984). In recent years there has been considerable interest in their ability to degrade and detoxify the increasing amounts of aromatic compounds which enter the environment as a result of man's industrial and agricultural activities. Many catabolic pathways for the degradation of aromatic compounds have been elucidated in detail in terms of their biochemistry. Although these bacteria employ a range of enzymes for the initial attack of the different substrates, the catabolic pathways tend to converge on just a few key intermediates such as catechol or substituted catechols (Ornston & Yeh, 1982; Dagley, 1986). These key intermediates can be further metabolized by two distinct sets of enzymes: those of the *ortho*-cleavage pathway (β-ketoadipate pathway) and those of the *meta*-cleavage pathway (α-ketoacid pathway) (for review see Dagley, 1986).

With rare exceptions, hydroxylation of the benzene ring is a rate-limiting first step in aerobic pathways for the catabolism of aromatic compounds (Chakrabarty, 1982; Dagley, 1986). The specificity of enzymes that catalyse hydroxylation is one of the factors which determine the type of compounds metabolized by the cell (Dagley, 1986; Gibson, 1984; Timmis *et al.*, 1985). Therefore, molecular genetic studies of hydroxylase genes will be of considerable importance for subsequent purification of the enzyme and for manipulation of the pathways with respect to the level of substrate utilization and/or substrate specificity.

Table 1. *Bacterial strains and plasmids*

	Relevant properties*	Reference/source†
E. coli strains		
S17.1	$r^- m^+$ Tp^r Sm^r Mob^+	Simon *et al.* (1983)
SK1592	$r^- m^+$	Kushner (1978)
CSR603	Maxicell strain	Sancar *et al.* (1979)
Pseudomonas strains		
CF600	Hg^r phenol/3,4-dmp degrader	Frey *et al.* (1983)
PB2442	$r^- m^+$ Rif^r derivative of KT2440	Franklin *et al.* (1981)
PB2701	$r^- m^+$ Sm^r derivative of KT2440	MBSC
PB2751	Rif^r derivative of PAO1	MBSC
PaW258	TOL *xylE* mutant	Worsey *et al.* (1978)
Plasmids		
pKT240	Ap^r Km^r RSF1010-based vector	Bagdasarian *et al.* (1983)
pKT502	*xylE* gene of TOL, RSF1010-based vector	Franklin *et al.* (1981)
pMMB66	Ap^r RSF1010-based *tac* expression vector	Fürste *et al.* (1986)
pMG5	Km^r Su^r Tm^r Hg^r IncP-2	Jacoby (1974)
Rms159	Cm^r Sm^r Tc^r Hg^r IncP-2	Jacoby (1977)

* r and m refer to host restriction and modification systems respectively. Antibiotic resistance abbreviations are: Ap^r, ampicillin; Cm^r, chloramphenicol; Hg^r, mercuric chloride; Km^r, kanamycin; Rif^r, rifampicin; Sm^r, streptomycin; Su^r, sulphonamide; Tc^r, tetracycline; Tm^r, tobramycin; Tp^r, trimethoprim.
† MBSC, M. Bagdasarian strain collection.

We are at present studying a strain of *Pseudomonas* (CF600) which is able to efficiently metabolize phenol and its methylated derivatives, *m*-, *p*-, and *o*-cresol and 3,4-dimethylphenol (3,4-dmp), as sole carbon and energy sources. In this communication, we demonstrate that the pathway for phenol degradation by this strain is encoded on a large IncP-2 plasmid designated pVI150. Furthermore, we report biochemical characterization of the pathway and evidence that the phenol hydroxylase is a multicomponent enzyme.

METHODS

Bacterial strains and plasmids. Previously described bacterial strains and plasmids used are listed in Table 1.

Media and culture conditions. *E. coli* and *Pseudomonas* strain PB2751 were cultured at 37 °C; the other *Pseudomonas* strains were cultured at 30 °C. LB was used as rich medium (Kahn *et al.*, 1979), minimal medium was M9-salts (Kahn *et al.*, 1979) supplemented with carbon sources in the following concentrations: phenol (2·5 mM), 3,4-dmp (2·5 mM), benzoate (5 mM) and acetate (5 mM). Selection for resistance markers was at the following concentrations for *E. coli* and *Pseudomonas* strains respectively (µg ml⁻¹): ampicillin (100, –); carbenicillin (–, 1000); kanamycin (100, 100); streptomycin (100, 2000); trimethoprim (100, –); rifampicin (100, 100); mercuric chloride (–, 25).

Enzyme assays. Phenol hydroxylase assays were performed on resting cells. Oxygen consumption was measured in the presence and absence of 2·5 mM-phenol using a Clark oxygen electrode as described by Sala-Trepat *et al.* (1972). Enzyme activity is expressed as the increase above basal levels of oxygen consumption in nmol h⁻¹ at a culture density of $OD_{650} = 0·5$ upon addition of phenol. Other enzyme activities were determined spectrophotometrically on cell-free extracts as described by Sala-Trepat *et al.* (1972), except that the NADase used was purchased from Sigma and solutions of 2-hydroxymuconic semialdehyde were prepared enzymically from catechol by using cell-free extracts of *E. coli* SK1592 harbouring plasmid pMMB26, which overproduces catechol 2,3-dioxygenase of the TOL plasmid pWW0. The unit of these enzyme activities is expressed as the amount of enzyme required to convert 1 µmol of substrate to product min⁻¹.

Conjugal transfer. Transfer of plasmids between strains was performed by applying cells, in the ratio 1:10 donor to recipient, to Millipore filters (HAWP01300) placed on the surface of LB agar plates, and incubating them at 30 °C. Cells were resuspended in 1 ml lambda diluent (10 mM-MgSO₄, 10 mM-Tris/HCl pH 7·4, 8·5 mM-NaCl, 0·01% w/v gelatin) and plated on selective media. RSF1010-based plasmids were transferred into *Pseudomonas* strains from *E. coli* S17.1.

Transposon mutagenesis. *P. putida* strain PB2701 DMP⁺ was mutagenized with Tn5 by transfer of a Tn5-carrying suicide donor plasmid, pSUP2021, from a mobilizing *E. coli* helper strain, S17.1. Kanamycin-resistant transconjugants, resulting from transposition of Tn5, were selected at 30 °C on LB plates containing streptomycin and kanamycin, and subsequently screened for their ability to grow on phenol.

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

DNA manipulations and transformation. Plasmid DNA was isolated by either the method of Hansen & Olsen (1978) or the rapid boiling method of Holmes & Quigley (1981). For transformation of *E. coli* strains the method of Kushner (1978) was followed. Restriction enzymes and DNA-modifying enzymes were purchased from Boehringer Mannheim or New England BioLabs, and used as recommended by the supplier. DNA fragments were isolated from agarose gels by electrophoresis onto DEAE membranes (NA45, Schleicher and Schuell) and subsequently eluted by incubation of the membranes in 50 mM-Tris/HCl pH 7·5, 1 M-NaCl (1–2 h at 70 °C). The eluate was extracted with buffered phenol and with diethyl ether, and the DNA collected by 2-propanol precipitation.

Plasmid constructs. Plasmids pCF100–103 and pVI199 were constructed as described in the text. Plasmids pVI200 and 201 were constructed by insertion, in different orientations, of T4 DNA polymerase treated 2·75 kb *Cla*I–*Xho*I fragment from pCF100 in the *Sma*I site of pMMB66. pVI216 and 217 were obtained by insertion of the 2·47 kb *Eco*RI fragment of pVI201, in different orientations, in the *Eco*RI site of pMMB66. pVI218 was obtained by cloning the 2·1 kb *Eco*RI–*Pst*I fragment of pVI201 between these sites of pMMB66. pVI219 and 220 were obtained by insertion, in different orientations, of the 1·53 kb *Sma*I fragment of pVI200 in the *Sma*I site of pMMB66. pVI221 was constructed by transfer of the 1·22 kb *Eco*RI–*Nru*I fragment from pVI220 between the *Eco*RI and *Sma*I sites of pMMB66. pVI222 was constructed by cloning of the 1·51 kb *Pvu*II–*Hind*III fragment of pVI220 between the *Hind*III sites of pMMB66.

To construct plasmids suitable for use in polypeptide analysis, a vector designated pMMB66Δ, which lacks expression of the *lac*I^Q gene, was made. This was accomplished by deletion of a 0·3 kb *Bss*HII fragment from pMMB66. Plasmids pVI218Δ and 220Δ–222Δ were generated by cloning of the insert DNA from the respective plasmids on *Eco*RI–*Hind*III fragments between these sites in pMMB66Δ. pVI216Δ–218Δ were obtained as for pVI216–218, but using pMMB66Δ. pVI201Δ was obtained by cloning the 0·65 kb *Pst*I fragment from pVI201, in the same orientation as it lies in pVI201, into the *Pst*I site of pVI218Δ. pVI200Δ was constructed by transferring the 2·47 kb *Eco*RI–*Hind*III fragment of pVI200 between these sites of pMMB66Δ, followed by the cloning, in the correct orientation, of the 0·28 kb *Eco*RI fragment of pVI200.

Colony hybridization procedure. Radiolabelled probe DNA fragments were prepared using [α-^{32}P]dCTP (Amersham) and hexanucleotides (Pharmacia). Colonies were transferred to nitrocellulose filters (Schleicher and Schuell type 402 016) and subsequently treated and hybridized as described by Maniatis *et al.* (1982). Hybridization was performed at low-stringency temperature (55 °C) with radiolabelled probe at > 10^6 c.p.m. ml^{-1}. Washes were done at 60 °C in salt concentrations from 2 × SSC/0·1% sodium dodecyl sulphate (SDS) to 0·5 × SSC/0·1% SDS (1 × SSC is 0·15 M-NaCl, 0·015 M-trisodium citrate, pH 7·0). Dry filters were subject to autoradiography with Dupont Cronex X-ray film.

RESULTS AND DISCUSSION

Growth and enzyme activities of Pseudomonas CF600

Pseudomonas CF600 was isolated by selection for growth on phenol and subsequently tested for its ability to grow on a variety of methylated phenols including cresols, 3,4-, 3,5-, 2,3-, 2,4-, 2,5- and 2,6-dmp. It was found that CF600 was able to grow on M9-salts containing *m*-, *p*- and *o*-cresol, and 3,4-dmp as sole carbon and energy sources.

Earlier studies with soil bacteria have demonstrated that phenol is metabolized to catechol via phenol hydroxylase (PH) and subsequently to tricarboxylic acid intermediates through a *meta*-cleavage pathway (Fig. 1; reviewed by Dagley, 1986). To determine if CF600 degrades phenol in an analogous manner and also possesses both branches of the *meta*-cleavage pathway, enzyme activities of PH, catechol 2,3-dioxygenase (C23O), hydroxymuconic semialdehyde dehydrogenase (HMSD) and hydroxymuconic semialdehyde hydrolase (HMSH) were measured. The data presented in Table 2 indicate that this is indeed the case. Comparison of enzyme activities in cells grown in minimal M9 medium containing acetate as sole carbon source with those in cells grown in the presence of phenol clearly demonstrated that all four enzymes were induced above basal levels in the presence of phenol.

The activity of catechol 1,2-dioxygenase (C12O), the first enzyme of the *ortho*-cleavage pathway, in CF600 was also measured (Table 2). There was only a 6-fold increase in C12O activity in the presence of phenol, while the C23O activity showed a 190-fold induction. The
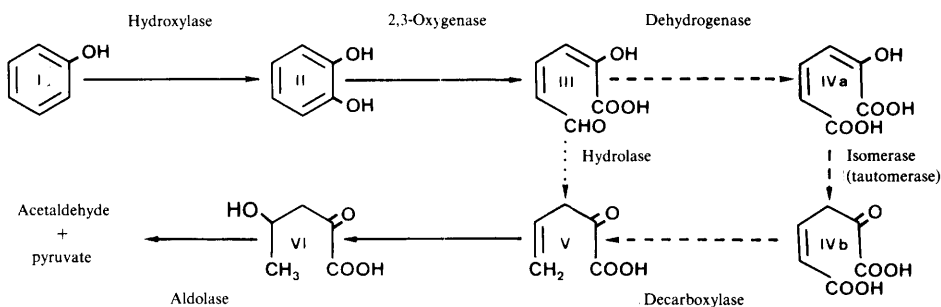
Fig. 1. Diagram of the phenol catabolic pathway of soil bacteria by a *meta*-cleavage pathway, illustrating the hydrolytic (. . . . . .) and oxalocrotonate (- - - - - -) branches (modified from Sala-Trepat *et al.*, 1972). Compounds: I, phenol; II, catechol; III, 2-hydroxymuconic semialdehyde; IVa, 4-oxalocrotonic acid (enol form); IVb, 4-oxalocrotonic acid (keto form); V, 2-oxopent-4-enoic acid; VI, 4-hydroxy-2-oxovalerate.

Table 2. *Enzyme activities of Pseudomonas strains*

Cells were grown overnight in M9-salts containing acetate, diluted and grown into late exponential phase in the same medium in the presence or absence of phenol or benzoate. The experiment was performed three times; one representative set of results is shown.

Strain	Medium*	Enzyme activity†				
		PH	C23O	HMSD	HMSH	C12O
CF600	A + P	3000	747	204	21	6
	A	<100	4	1	5	1
	A + B	<100	99	ND	ND	11
PB2701 DMP⁺	A + P	2900	895	296	18	13
	A	<100	5	1	5	3
	A + B	<100	88	ND	ND	9
PB2442 (pVI150::Tn*5*/1)	A + P	<100	<1	<1	<1	3
	A	<100	<1	<1	<1	3
	A + B	<100	ND	ND	ND	242
PB2701 (pVI150::Tn*5*/1)	A + P	<100	<1	<1	<1	2
	A	<100	<1	<1	<1	3
	A + B	<100	ND	ND	ND	ND
PB2701	A + P	<100	<1	ND	ND	ND
	A	<100	<1	ND	ND	2
	A + B	<100	<1	ND	ND	278

* A, acetate; B, benzoate; P, phenol.

† PH activity is expressed as increase above basal levels of nmol O_2 consumed h^{-1} at 30 °C and an initial culture density of $OD_{650} = 0.5$. Other enzyme activities are expressed as mU (mg protein)$^{-1}$. ND, Not determined.

difference in induction levels of C12O and C23O, along with induction of other *meta*-pathway enzymes and PH (Table 2), demonstrates that the major route for phenol degradation is via PH and *meta*-cleavage. The slight increase in C12O activity in the phenol-induced cells is probably due to a limited amount of 'overspill' of catechol into the *ortho*-pathway.

Ability to catabolize phenol and 3,4-dmp is plasmid encoded

Existing genetic evidence suggests that a significant proportion of the pathways specifying catabolism of aromatic compounds by *Pseudomonas* are encoded on plasmids (Frantz & Chakrabarty, 1986). That this might be the case for phenol degradation encoded by CF600 was

first indicated by the observation that the ability to degrade phenol could be transferred to both *P. putida* and *P. aeruginosa* strains, at a frequency of approximately 1 in 10^7–10^8 donor cells. The resulting transconjugants also obtained the ability to grow on 3,4-dmp. A *P. putida* strain obtained by this means (PB2701 DMP$^+$) was shown to transfer the ability to utilize phenol and 3,4-dmp at a similar frequency, to have a similar catabolic enzyme induction profile (Table 2), and to be able to grow as efficiently on phenol as sole carbon and energy source, as the original strain (generation time 90–100 min in minimal medium containing M9-salts and 2·5 mM-phenol).

Physical isolation of plasmid DNA from CF600 revealed the presence of two plasmids, one of approximately 45 kb (pVI45) and one of considerably higher molecular mass, designated pVI150. The large plasmid appears to be of low or single copy number and its DNA proved difficult to isolate consistently, even when using the method of Hansen & Olsen (1978), which allows isolation of large plasmids in intact circular form. To determine which of the plasmids was responsible for conferring the ability to grow on phenol and 3,4-dmp, a range of plasmids from different incompatibility groups were transferred to a restriction-minus *P. putida* carrying both of the plasmids from CF600 (PB2701 DMP$^+$). Only two plasmids, Rms159 and pMG5, both of the IncP-2 group, were found to have an effect on phenol utilization of this strain. In each case over 90% of the transconjugants tested had been cured of the ability to grow on phenol and 3,4-dmp. Analysis of plasmid DNA from such strains showed that all retained pVI45, suggesting that the large plasmid, pVI150, belongs to the IncP-2 group and encodes the phenol/3,4-dmp pathway.

PB2701 DMP$^+$ was mutagenized with Tn*5*, which specifies resistance to kanamycin, using the suicide donor system of Simon *et al.* (1983). Seven independent transposon mutants were isolated which had lost the ability to grow on both phenol and 3,4-dmp. These derivatives had Tn*5* located on the large pVI150 plasmid and were designated pVI150::Tn*5*/1–7. After several rounds of transfer of these plasmids between *Pseudomonas* strains PB2701 and PB2442, with selection for resistance to kanamycin, two strains were identified which had lost pVI45 while still harbouring derivatives pVI150::Tn*5*/2 or pVI150::Tn*5*/7. Since kanamycin-sensitive revertants of both these strains were able to grow on phenol, we conclude that the IncP-2 plasmid pVI150 encodes all the information required to allow growth on phenol.

Identification of a pmo gene

Given the difficulty of isolating the pVI150 DNA, a genomic bank of CF600, constructed in the broad-host-range cosmid pMMB33 (Frey *et al.*, 1983), was used. The genomic bank was screened by using as a probe the 2·25 kb *Xho*I fragment of pKT502, which carries the *xylE* gene (C23O) of TOL plasmid pWW0. This strategy was adopted with the following three considerations in mind. (i) Studies of other catabolic plasmids, notably TOL, NAH and SAL (Chakrabarty, 1972; Williams & Murray, 1974; Yen & Gunsalus, 1985), have demonstrated that the genes specifying utilization of the respective aromatic substrates are often clustered (reviewed by Frantz & Chakrabarty, 1986). (ii) Considerable homology exists between the *meta*-pathway genes encoded by different plasmids (reviewed by Frantz & Chakrabarty, 1986). (iii) Plasmid pVI150 encodes a C23O gene and product that is homologous to that of TOL on both the DNA and functional level; the *xylE* gene of TOL only hybridizes to DNA of strains harbouring pVI150, not to strains cured of this plasmid (data not shown), and pVI150 can complement a *P. putida* strain (PaW258) harbouring a TOL plasmid *xylE* mutant so that it can once again grow on *m*-toluate.

From 600 clones tested, one was detected which gave positive hybridization to the pWW0-derived *xylE* gene. The plasmid content of this strain revealed a mixed population of plasmids. However, a stable deleted derivative (pCF100) was isolated and purified which, when transferred into *P. putida* strains harbouring the pVI150::Tn*5* derivatives 1–7, restored the ability of three of the strains (Tn*5*/1, 4 and 5) to grow on phenol. The restriction map of the 22·7 kb deleted cosmid clone pCF100 is depicted in Fig. 2. We propose to call the gene responsible for this complementation *pmo* (for *p*henol *mo*nooxygenase) and assign cistron designations when the organization of the gene is elucidated.
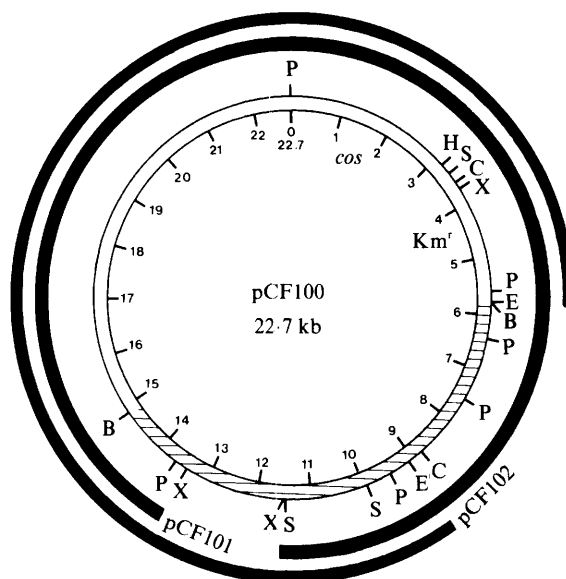
Fig. 2. Physical map of pCF100. Shaded regions indicate the extent of CF600 DNA in pMMB33. ▬▬ Illustrates the extent of DNA in two deletion derivatives of pCF100 (pCF101 and pCF102). Restriction sites: *Bam*HI, B; *Cla*I, C; *Eco*RI, E; *Hin*dIII, H; *Pst*I, P; *Sma*I, S; *Xho*I, X.

Mapping of the pmo cistron

To define the DNA region encoding the ability to complement the pVI150::Tn5 mutants, the CF600-derived insert region of pCF100 was subjected to deletion and subcloning analysis. Two deletion derivatives of pCF100, prepared by deletion of the 1·8 kb *Xho*I fragment (pCF101) and the 3·3 kb *Eco*RI fragment (pCF102), are depicted in Fig. 2. Both these derivatives were still able to mediate the complementation, suggesting that the gene involved is located between the regions deleted in these plasmids. This was confirmed by subcloning the 2·75 kb *Cla*I–*Xho*I fragment of pCF100 between these sites in the broad-host-range plasmid pKT240 (Fig. 3) since this plasmid, designated pVI199, could mediate the complementation.

Plasmids pVI200, 201 and 216–222 are a series of plasmids containing different-sized subclones of the 2·75 kb *Cla*I–*Xho*I fragment, in the two possible orientations, in the polycloning cartridge of pMMB66. Plasmid pMMB66 is a broad-host-range expression vector which transcribes DNA cloned into the polycloning site from the *tac* promoter, which is regulated by the plasmid-located *lacI*^{Q} gene. The extent and orientation of the DNA in the different derivatives is shown in Fig. 3, along with the ability of the plasmids to mediate complementation of *P. putida* PB2701(pVI150::Tn5/1) to grow on phenol. The results show that a 1·2 kb *Pvu*II–*Nru*I fragment contains sufficient information to complement the pVI150::Tn5/1 mutation. However, positive complementation is detected only if the DNA is orientated such that transcription from the *tac* promoter proceeds in the direction from the *Pvu*II site towards the *Nru*I site (left to right in Fig. 3). These results locate the *pmo* gene, determine its transcriptional orientation, and indicate that the normal promoter for this gene is located upstream of the *Xho*I site.

Polypeptide analysis of the pmo gene

To determine the polypeptide responsible for the complementation of the pVI150::Tn5/1 mutation, we analysed plasmid-encoded polypeptides produced by maxicell strain CSR603 harbouring different plasmids. Since the *lacI*^{Q} gene product obscured the results of electrophoretic analysis of proteins of interest, we constructed analogous plasmids to those of the
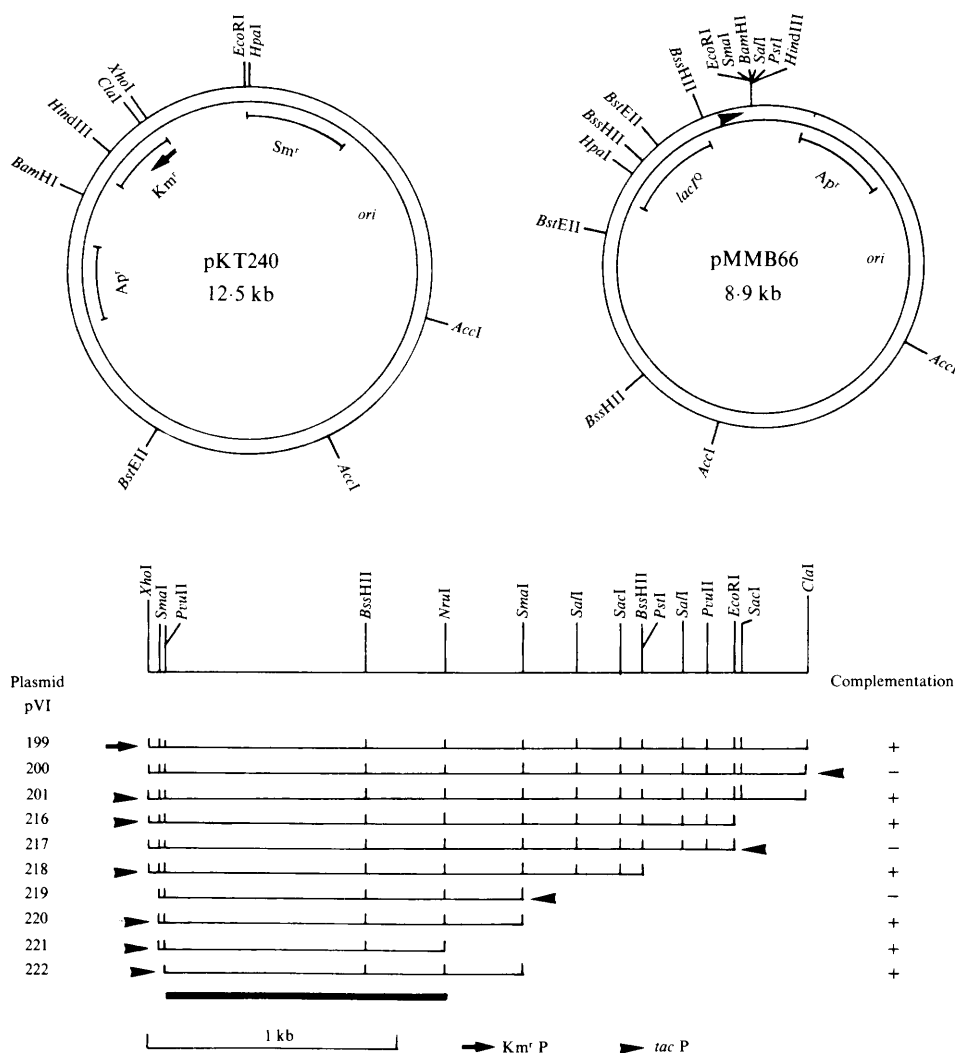
Fig. 3. Extent and orientation of fragments, derived from the 2·75 kb *Cla*I–*Xho*I fragment of pCF100, inserted in the vectors pKT240 and pMMB66. The effect of the plasmids in complementation experiments is also indicated.

pVI200 series, designated pVI200Δ, 201Δ and 216Δ–222Δ, carrying a deletion of the *lacI*^{Q} gene. The plasmid-encoded polypeptides of CSR603 harbouring these plasmids are shown in Fig. 4. Strains harbouring plasmids that encode and express the DNA required for complementation all expressed a 39·5 kDa polypeptide (Fig. 4, lanes 2, 4, 6, 7, 8 and 9), while those harbouring plasmids carrying this DNA, but in an orientation so that the complementing activity was not expressed, did not (Fig. 4, lanes 3 and 5). It seems therefore reasonable to assume that the 39·5 kDa polypeptide is the product of the *pmo* gene and is responsible for the complementation of the mutation in pVI150::Tn5/1.

Interestingly, deletion derivatives PVI220Δ–222Δ all produce the 39·5 kDa polypeptide in elevated amounts and have in common a 50–70 bp deletion in the promoter-proximal region of the inserted DNA. Two possible mechanisms involving deletion of a small region of DNA that might account for elevated production of the polypeptide are: (i) the deletion may have removed
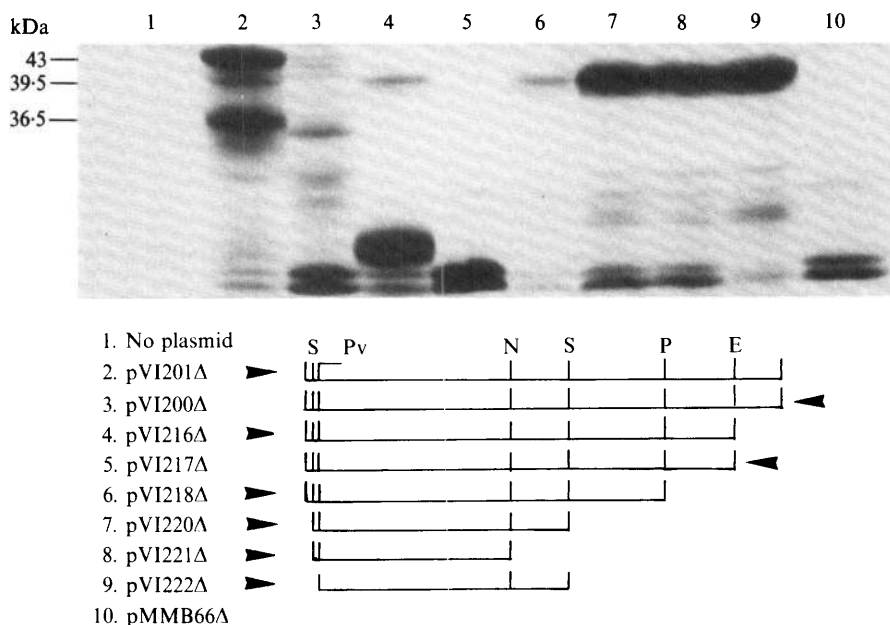
Fig. 4. Polypeptides expressed by maxicells harbouring plasmids of the pVI200Δ series. Polypeptides were separated on 12·5% polyacrylamide gels and visualized by autoradiography. Restriction sites: PvuII, Pv; NruI, N; and as in Fig. 2.

Table 3. *Enzyme activities of PB2701 (pVI150::Tn5/1) harbouring complementing plasmids*

Enzyme activities are expressed as in Table 2. The results shown are from a single experiment, performed twice with similar results.

Plasmid	Enzyme activity		
	PH	C23O	C12O
pCI201	1600	<1	41
pVI216	2100	<1	23
pVI220	1900	<1	11
pVI221	2400	<1	28

a partial transcriptional stop site and/or (ii) a promoter firing in the opposite direction to that of the *tac* promoter. Increase in polypeptide production upon deletion of a promoter firing in the opposite direction to the *tac* promoter has recently been demonstrated during expression of the *xylS* gene of TOL (Spooner *et al.*, 1987).

Enzyme activities of the 39·5 kDa polypeptide

Enzyme activities of *P. putida* PB2701 harbouring pVI150::Tn5/1 (Table 2) show that the Tn5 insertion results in loss of activity of PH and the three *meta*-pathway enzymes tested. When this strain harbours various plasmids which complement the mutation, permitting growth on phenol, PH activity is restored (50–80% of wild-type activity) but not the activity of the test *meta*-pathway enzyme, C23O (Table 3). Catechol appears to be further degraded in these strains by the chromosomally encoded *ortho*-pathway (C12O activity, Table 3). Although the cloned gene restored the ability of *P. putida* strains harbouring pVI150::Tn5/1 to grow on phenol, it did not restore the ability of the strains to grow on 3,4-dmp. This result is consistent with the enzyme activity data, since utilization of methylcatechols usually requires the activities of *meta*-cleavage pathway enzymes; either the first enzyme for the respective *ortho*-cleavage pathway has low

affinity for methylcatechol or catabolism by an *ortho*-cleavage pathway results in dead-end intermediates (reviewed by Dagley, 1986). Even though the complemented strains did not grow on 3,4-dmp, the PH activity produced by complementation appeared to be still capable of acting on 3,4-dmp, since replacement of phenol by 3,4-dmp in the assay for PH on phenol-induced resting cells generated results similar to those obtained with phenol (data not shown). Hence, these results show that complementation of the Tn*5* mutation by the *pmo* gene results in a strain with more limited substrate range than the wild-type, and whose growth on phenol involves a switch from a *meta-* to an *ortho*-cleavage pathway. This is a similar situation to that of a *Pseudomonas* strain harbouring the catabolic plasmid pWW60-1. This strain is capable of growing on naphthalene, but not methylnaphthalene, by expression of plasmid-encoded enzymes for the conversion of naphthalene to salicylate, and a chromosomally encoded *ortho*-cleavage pathway. Derivatives of this strain capable of growth on methylnaphthalene could be selected, which possess this property by virtue of expression of a previously silent plasmid-encoded *meta*-cleavage pathway (Cane & Williams, 1986).

If the cloned *pmo* gene encodes all the information for the PH enzyme, then transfer of plasmids that express this gene to the plasmid-free *P. putida* strain PB2701 should generate strains capable of growth on phenol. However, PB2701 harbouring pVI218 or pVI220, on which the *pmo* DNA is expressed from the *tac* promoter, was not able to grow on phenol. Moreover, these strains produced no detectable PH activity, even when grown in the presence of phenol and/or an inducer of the *tac* promoter, IPTG (data not shown). These results are interpreted to mean that the 39·5 kDa polypeptide of the *pmo* gene represents only one component of the active enzyme. Presumably other component(s) required for activity are provided in strains that harbour pVI150::Tn*5*/1, but not in strains lacking this plasmid. Hence, the PH of *Pseudomonas* CF600 appears to be composed of two or more different polypeptides.

Expression of catabolic operons in *Pseudomonas* is often subject to positive regulation (reviewed by Frantz & Chakrabarty, 1986). In such systems mutation of a positive regulator results in loss of several inducible enzyme activities. Likewise, transposon insertion into an operon can have polar effects and cause similar pleiotropic loss of enzyme activities. However, loss of even basal levels of enzyme activities in strains carrying pVI150::Tn*5*/1, together with restoration of only PH activity in complemented strains, argues against the complementing activity being a positive regulator. Enzyme assays and cloning of the Tn*5*-harbouring DNA fragments from all the mutants are presently being performed to determine if the transposon is located in a structural or regulatory gene in each case. The pleiotropic effect of the Tn*5*/1 insertion on the four enzymes tested is consistent with the corresponding genes being encoded in an operon. Investigation of this possibility using the other Tn*5* insertion mutant described in this paper, along with cloning and expression analysis of other phenol catabolic pathway enzymes, is underway.

The data presented in this paper demonstrate that the phenol catabolic pathway of *Pseudomonas* CF600 is similar to that of other soil bacteria and is encoded on a newly described catabolic plasmid, pVI150. This plasmid, like two other catabolic plasmids, CAM (Rheinwald *et al.*, 1973) and OCT (Fennewald *et al.*, 1978), belongs to the P-2 incompatibility group and is very large. Molecular cloning, polypeptide analysis and enzyme activity measurements demonstrate that the PH encoded by this plasmid is composed of more than one component. The cloning and expression of one of the components provides a probe with which to isolate the other component(s) of PH and genes for other enzymes involved in phenol catabolism. Sequencing of the cloned *pmo* gene should shed light on the function of the 39·5 kDa polypeptide in PH activity.

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