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Localization and organization of phenol degradation genes of *Pseudomonas putida* strain H

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Abstract The genetic organization of the DNA region encoding the phenol degradation pathway of *Pseudomonas putida* H has been investigated. This strain can utilize phenol or some of its methylated derivatives as its sole source of carbon and energy. The first step in this process is the conversion of phenol into catechol. Catechol is then further metabolized via the *meta*-cleavage pathway into TCA cycle intermediates. Genes encoding these enzymes are clustered on the plasmid pPGH1. A region of contiguous DNA spanning about 16 kb contains all of the genetic information necessary for inducible phenol degradation. The analysis of mutants generated by insertion of transposons and cassettes indicates that all of the catabolic genes are contained in a single operon. This codes for a multicomponent phenol hydroxylase and *meta*-cleavage pathway enzymes. Catabolic genes are subject to positive control by the gene product(s) of a second locus.

Key words *Pseudomonas putida* · Phenol degradation · Insertion mutagenesis · Gene organization · DNA sequence

Introduction

Representatives of the genus *Pseudomonas* and related genera exhibit extensive metabolic diversity enabling them to degrade a large variety of aromatic substrates. Whereas bacteria express a range of enzymes for the initial attack on different aromatic compounds, most pathways converge on a few central intermediates, such as catechol or substituted catechols. The key enzymes of these common routes are the catechol dioxygenases, which cleave the aromatic ring either in *meta* or in *ortho* position and thus initiate further degradation by a distinct set of enzymes.

Many such degradative pathways are encoded on large plasmids. The organization of the corresponding genes has been analyzed in some cases, such as the TOL plasmid pWW0 (Nakazawa et al. 1980; Franklin et al. 1981; Harayama et al. 1984) and the NAH plasmid NAH7 (Yen and Gunsalus 1982). Using transposon mutagenesis, these studies have revealed that structural genes are encoded in two operons and are coordinately regulated by one or two plasmid-encoded, positive controlling proteins. Plasmid pVI150, described more recently, encodes the degradation of dimethylphenol. On this plasmid, catabolic genes form one large operon (Bartilson et al. 1990).

Plasmid pPGH1, indigenous to *Pseudomonas putida* strain H, codes for the inducible degradation of phenol and some of its methylated derivatives via the *meta*-cleavage pathway (the Phl^+ phenotype; Herrmann et al. 1987). Using the promiscuous resistance plasmid R68.45 (Haas and Holloway 1976) as a mobilizing plasmid, hybrid plasmids conferring the Phl^+ phenotype were selected via conjugation. These plasmids consist of the complete R68.45 and a segment of pPGH1 (Herrmann et al. 1988). The smallest of the hybrid plasmids, pPGH11, about 100 kb in size, was chosen for localization and analysis of *phl* genes. This report describes the physical and genetic mapping of the pPGH1 DNA carried by pPGH11.

Material and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions

Nutrient broth (NB) and nutrient agar (NA) were supplemented with 0.5% (w/v) yeast extract (Difco). *P. putida* strains were

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Table 1 Bacterial strains and plasmids

Strain, plasmid	Relevant characteristics[a]	Reference or source
Escherichia coli strains		
HB101	*hsdR recA*13*ara*14 *proA*2 *lacY*1 *galK*2 *rspL*20 *xyl*-5 *mtl*-1 *supE*44	Boyer and Roulland-Dussoix 1969
DH5a	*hsdR recA thi*-1 *relA*1 *gyrA*96	Hanahan 1985
S17-1	*thi pro hsdR* chr::RP4-2 (Tc::Mu Km::Tn*7*)	Simon et al. 1983
IE960	J53::Tn*1826*	Collection of Robert-Koch-Institute, Wernigerode; Tietze et al. 1987
Pseudomonas putida strains		
KT2440	Strain mt-2, *hsdR*	Bagdasarian and Timmis (1981)
PG5	KT2440 Rif^r Nal^r	Herrmann et al. 1988
PG125	KT2440 pPGH11	This study
PG5	pPGH11::Tn*5*-B61	This study
PG306	Strain H, pPGH1 (Phl^+)	Herrmann et al. 1987
PG320	PG306 Rif^rNal^r	This study
Plasmids		
pMMB67EH	Ap^r, RSF1010 replicon	Fürste et al. 1986
pPL392	Ap^r, TOL *meta*-cleavage genes	Harayama et al. 1984
pSUP202	Ap^r Tc^r Cm^r Mob^+	Simon et al. 1983
pSUP2021	pSUP202::Tn*5* (Km^rSm^r)	Simon et al. 1983
pSUP202::Tn*5*-B61	Ap^r Tc^r Cm^r Gm^r Mob^+	Simon et al. 1989
pKOK5	Ap^r Km^r *lacZ* (promoterless)	Kokotek and Lotz 1989
pTZ18/19R	Ap^r	Mead al. 1986
pPGH1	Phl^+ Hg^r	Herrmann et al. 1987
pPGH11	R68.45-pPGH1 hybrid plasmid Phl^+ Ap^r Km^r Tc^r	Herrmann et al. 1988
pPGH52	pPGH11::Tn*5*-B61 (Gm^r)	This study
pPGH93	*Sal*I fragment D of pPGH11 cloned in pSUP202	This study
pPGH97	*Pst*I fragment N of pPGH11 cloned in pSUP202	This study
pPGH99	*Sal*I fragment G of pPGH11 cloned in pSUP202	This study
pPGH100	*Kpn*I-*Bam*HI fragment of pPGH11 cloned in pSUP202	This study
pPGH450	*Pst*I/*Bgl*II fragment of pPGH11 cloned in pMMB67EH	This study

[a] *Rif* rifampicin, *Nal* nalidixic acid, *Ap* ampicillin, *Tc* tetracycline, *Cm* chloramphenicol, *Km* kanamycin, *Sm* streptomycin, *Gm* gentamycin, *Hg* $HgCl_2$

grown in NB containing 0.45% (w/v) potassium nitrate (NNB). Minimal medium (agar) was either medium E (Vogel and Bonner 1956) containing 1% (w/v) glucose as carbon source or PAS (phosphate ammonium salt) medium (Chakrabarty et al. 1973) containing 3.7 mM phenol as carbon source. Selective media contained antibiotics at the following concentrations (in μg/ml). For *Escherichia coli*: ampicillin (Ap, 50–100), kanamycin (Km, 100), streptomycin (Sm, 100), streptothricin (St, 100), chloramphenicol (Cm, 25), gentamicin (Gm, 2), rifampicin (Rif, 250). For *P. putida*: carbenicillin (Cb, 500–2000), piperacillin (Pip, 100), Km (500), Sm (500), St (250), Cm (50), Gm (40), Rif (250), nalidixic acid (Nal, 1000), $HgCl_2$ (30).

Biochemical characterization of phenol catabolism

Respirometric analyses of whole-cell suspensions were performed as described by Janke (1987). Preparation of cell-free extracts and determination of the specific activity of catechol 2,3-dioxygenase (C23O) were carried out essentially by the method of Kaminski et al. (1983). Phenol hydroxylase activity was determined by measuring phenol consumption in vivo in a defined reaction mixture. Cells were collected by centrifugation and resuspended in 0.5 ml of 0.1 M TRIS-HCl (pH 8.0) containing 200 μM phenol, 125 μM NADPH, and 100 μM $FeSO_4$. Optical density (OD_{600}) was adjusted to 0.3. The reaction was allowed to proceed for 8 min at 30°C and stopped by adding 0.4 ml of 0.1% 4-aminoantipyrin, followed by 0.2 ml of 1% potassium ferricyanide. The absorption (A_{500}) of the supernatant was measured after centrifugation and compared with that of the phenol standard. β-galactosidase activity was determined as decribed by Miller (1972).

Conjugative transfer and mutagenesis procedures

The spot mating technique used for plasmid transfer has been described elsewhere (Herrmann et al. 1986). Tn*5* and Tn*5*-B61 were introduced into PG125 by using the mobilizable suicide vectors pSUP2021 and pSUP202::Tn*5*-B61, respectively. Transposon-marked derivatives of pPGH11 were constructed by conjugation of pPGH11 into PG5 and selection for the transposon-encoded antibiotic resistance. Sm was used to select for Tn*5*. pPGH11 derivatives mutated by the insertion of Tn*1826* were isolated by triple crosses: logarithmically growing cells of the first donor, PG125 (carrying pPGH11) and stationary phase cells of the second donor, IE960 (carrying Tn*1826*) and the recipient, PG5 were mixed on a NA plate and incubated at 30°C for at least 5 h, harvested and spread on selective media (NA containing Rif and St). To introduce cassettes, filter mating procedures were used. Cells from 1 ml of the donor culture grown to the logarithmic growth phase and from 0.2 ml of recipient grown overnight were harvested by centrifugation, mixed in 5 ml of 10 mM $MgSO_4$ and collected on a nitrocellulose filter. Filters were incubated for 8–12 h at 30°C on NA plates. Cells were then suspended in 10 mM $MgSO_4$ and appropiate dilutions were plated on selective media (NA containing Rif and Km or Nal and Km). Most of the single colonies tested were Pip^r (100 μg/ml) and either Cm^r or Tc^r. This observation suggested that the entire plasmid had integrated into pPGH1. The clones were stored on selective medium at 4°C for 1–2 weeks. After this period they were restreaked for single colony reisolation on NA containing Km and tested for further resistance properties. Most clones segregated colonies which had lost vector-encoded antibiotic resistances.

DNA isolation, analysis and manipulation

Cloning vectors and recombinant plasmids were isolated either by the method of Birnboim and Doly (1979) or using Qiagen columns according to the manufacturer's recommendations. The procedure for isolation of pPGH11 DNA has been described previously (Herrmann et al. 1987). Plasmid pPGH1 DNA was prepared by the method of Nies et al. (1987). Restriction digests were performed as recommended by the suppliers. Phosphatase treatment, ligation, transformation, and gel electrophoresis were as outlined by Sambrook et al. (1989). DNA fragments were isolated from agarose gels using the Qiaex extraction kit (Qiagen). Southern hybridizations (Southern 1975) were performed according to the original protocol. Probes were labeled with digoxigenin using the DIG DNA labeling kit (Boehringer Mannheim) according to the manufacturer's recommendations. Detection was performed with the DIG detection kit (Boehringer Mannheim) as recommended by the manufacturer.

Nucleotide sequence determinations

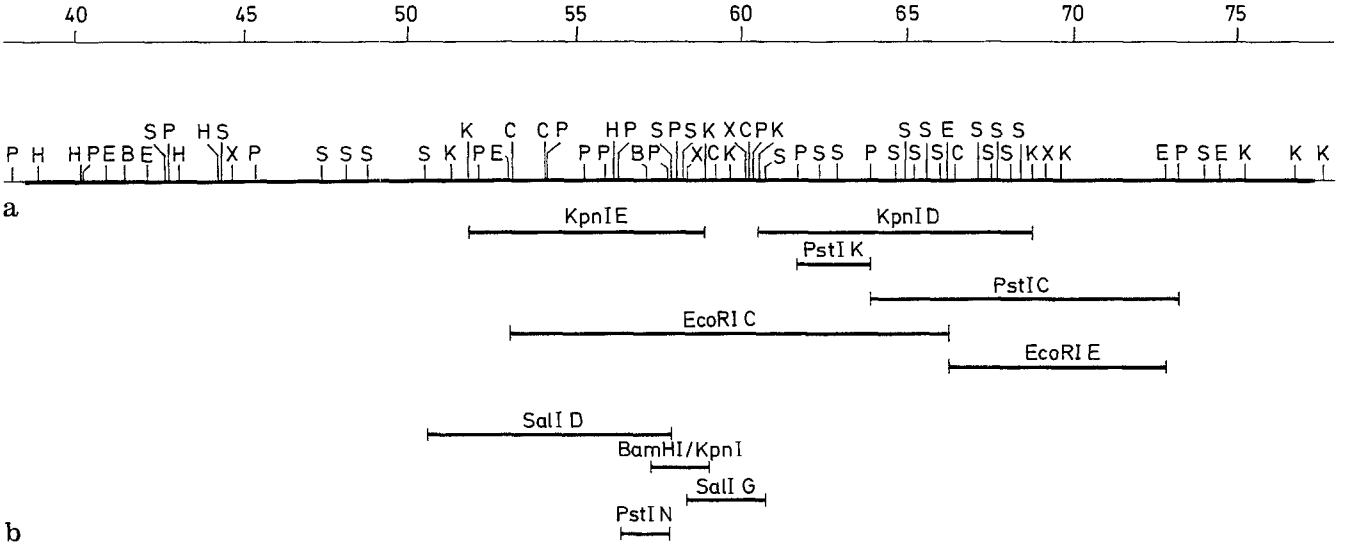
Nucleotide sequences were determined directly from recombinant plasmids by the dideoxy method (Sanger et al. 1977) using the USB sequencing kit. Sequence data have been submitted to the EMBL GenBank data library under accession number X80765.

Results and discussion

Physical mapping of pPGH11

A restriction map of the pPGH1 DNA contained in plasmid pPGH11 is given in Fig. 1a. The map was obtained by a combination of approaches including single, double, and partial restriction digests of the complete hybrid plasmid, and subcloning of the entire insert as smaller restriction fragments, followed by restriction analysis and hybridization to blots of pPGH11 DNA digested with various restriction enzymes.

Fig. 1a,b Physical map of the pPGH1 DNA inserted in pPGH11. **a** Restriction map of pPGH1 DNA with respect to the coordinates in the hybrid plasmid (**b**). Restriction fragments in which transposons were located in the mutants (*top*) and which were used to clone cassettes for gene replacement (*bottom*) are indicated. Restriction sites shown are: *B BamHI*, *C ClaI*, *E EcoRI*, *H HindIII*, *K KpnI*, *P PstI*, *S SalI*, *X XhoI*



Identification of *phl* genes by transposon mutagenesis

Transposon mutagenesis was performed to identify regions of pPGH11 encoding phenol degradation enzymes and to analyze their organization. Transposon-marked derivatives of pPGH11 were selected and tested for their ability to grow on phenol as sole carbon source. Phl$^-$ clones were further analyzed for whole-cell respiratory activity on both phenol and catechol as substrates and catechol 2,3-dioxygenase (C23O) and phenol hydroxylase activity.

Most of the mutants had a reduced oxygen consumption rate on both phenol and catechol and expressed a C23O activity similar to that of the wild type. This phenotype is characteristic for mutants which are blocked in one of the enzymatic steps of the *meta*-cleavage pathway beyond ring cleavage. Restriction analysis of these mutant plamids revealed that all the insertions were clustered in the *KpnI* fragment D between coordinates 60.5 kb and 68.8 kb of pPGH11 (Fig. 1).

Another type of Phl$^-$ mutant (PG220) was generated by the integration of the promoter-out transposon Tn*5*-B61. It showed constitutive expression of C23O gene(s) and constitutive oxygen consumption with catechol as substrate, both at slightly higher levels than in the induced wild type. Phenol hydroxylase activity was not detectable. Following transfer of the mutated plasmid into *E. coli* HB101, C23O activity was inducible by IPTG. This observation is consistent with the conclusion that in this mutant all of the *meta*-cleavage pathway genes are under the control of the transposon-borne *tac* promoter. Furthermore, the phenotype of PG220 suggests that the transposon is integrated into a gene whose

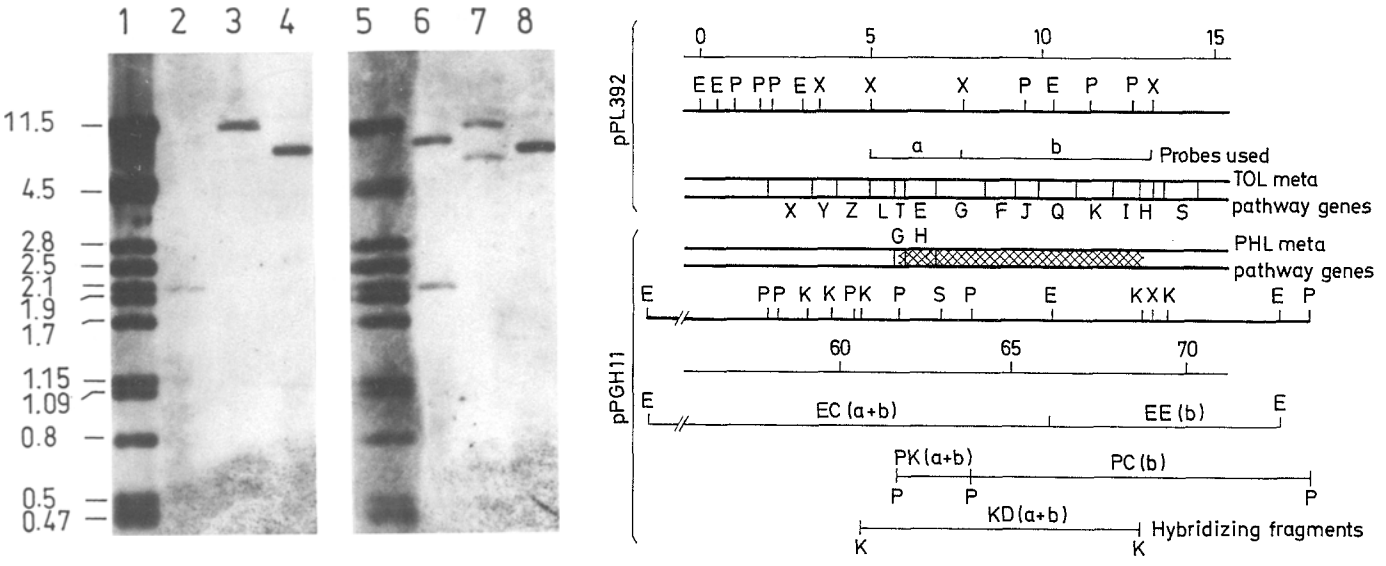


Fig. 2 Identification of *meta*-cleavage pathway genes of pPGH11 by DNA:DNA hybridization. Left. Autoradiograms of Southern hybridization of totally digested pPGH11 DNA probed with fragments of TOL plasmid pWW0 carrying the *meta*-cleavage genes. pPGH11 was digested with *Pst*I (lanes 2, 6), *Eco*RI (lanes 3, 7) and *Kpn*I (lanes 4, 8) and proved with the 2.2 kb *Xho*I fragment (probe a) of pPL392 (lanes 2–4) or to the 5.5 kb *Xho*I fragment (probe b) of pPL392 (lanes 6–8). In lanes 1 and 5 lambda DNA digested with *Pst*I was loaded as a size marker and hybridized to labeled lambda DNA. Right. Physical/genetic map of pPL392 and part of pPGH11. *Xho*I fragments used as labeled probes and pPGH11 fragments which hybridize to the probe(s) are shown. The *phl* gene region homologous to pWW0 DNA is hatched. Abbreviations for restriction enzymes are the same as in Fig. 1

product is necessary for phenol hydroxylase activity. The integration site of the transposon was mapped at one end of the cluster of *meta*-cleavage pathway genes, at coordinate 60.7 kb (Figs. 1, 4).

A third group of mutants did not consume oxygen on either phenol or catechol as substrate, even under induced conditions, and their C23O activity was very low. In these mutants, a transposon was inserted into *Kpn*I fragment E (coordinates 51.8–58.9 kb). The mutant phenotype could be complemented *in trans*, e.g. by the cloned fragments *Kpn*I E or *Sal*I D (50.5–57.9 kb). Based on these observations we suppose that, in these mutants, insertion of the transposon has inactivated the gene(s) coding for protein(s) necessary for the positive regulation of the expression of structural genes for phenol degradation.

Analysis of the *meta*-cleavage pathway genes

DNA:DNA hybridization studies and DNA sequence comparisons of genes for the enzymes of the common *meta*-cleavage route of the catabolic pathways of naphthalene, salicylate, toluene/xylene and dimethylphenol have shown that they all share significant homology (Lehrbach et al. 1983; Cane and Williams 1986; Harayama et al. 1987; Bartilson and Shingler 1989). To analyze the *meta*-cleavage pathway genes of pPGH1 in this context we hybridized total digests of pPGH11 DNA using as labeled probes each of two adjacent *Xho*I fragments of pWW0, which carry most of the TOL *meta*-cleavage pathway genes (Fig. 2, right). The autoradiograms (Fig. 2, left) confirm the expected homology between the genes of the *meta*-cleavage routes of phenol and toluene/xylene degradative pathways. Additionally, hybridization analysis of the various restriction fragments allowed a more precise localization of *meta*-cleavage genes in pPGH11. TOL DNA only shares homology with pPGH11 DNA distal to the *Pst*I site at coordinate 61.7 kb and proximal to the *Kpn*I site at coordinate 68.8 kb (Fig. 2, right). Hybridization between the 2.2 kb *Xho*I fragment of pWW0, containing genes *xylT* and *xylE* (encoding ferredoxin and C23O, respectively) and the 2.1 kb *Pst*I fragment of pPGH11 is rather weak (Fig. 2, left). This might indicate that these genes show less homology to the TOL genes than do the remaining *meta*-cleavage pathway genes. Other C23O genes, in addition to *xylE*, have been found in the TOL plasmid pWW15. They are located outside the degradative operon and show little homology with *xylE*. They have been designated class II enzymes, and express higher specific activity toward methylcatechol than toward catechol (ratio about 1:8; Keil et al. 1985). The specific activity of the C23O encoded by pPGH1 toward methylcatechol is about 6 times higher than toward catechol (Herrmann et al. 1987). Thus, the pPGH1-encoded enzyme might resemble class II enzymes. For further investigations several fragments of *Pst*I fragment K were subcloned and C23O activity of the clones was determined. The smallest fragment expressing C23O activity was located between the *Pst*I site at coordinate 61.7 kb and the *Sal*I site at coordinate 62.9 kb (Fig. 1). Subclones were used to determine the nucleotide sequence. Analysis of the DNA sequence revealed an open reading frame of 921 bp, which was called *phlH*. It corresponds to nucleotides 5252–6175 of the sequence in the EMBL GenBank data library under accession number X80765. The nucleotide sequence of

Fig. 3 Comparison of the amino acid sequences of catechol 2,3-dioxygenases encoded by *phlH*, *dmpB* and *xylE*. The complete sequence of PhlH is shown. Identical amino acids in DmpB and XylE are symbolized by *asterisks*

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PhlH    MKKGVMRPGH VQLRVLNLES ALTHYRDLLG LIETDRDEQG RIYLKAWSEV    50
DmpB    ********** ********** **A******* ***M****** *V*****T**    50
XylE    *N******** ******DMSK **E**VE*** ***M***D** *V*****T**    50

PhlH    DKFSVVLREA AEPGMDFMAF KVLDDATLSS LTDDLIRYGC LVELMPAGEL   100
DmpB    ********** DQ******G* **I*EDC*NR **Q**LN*** *I*TI*****   100
XylE    ****L***** D*******G* **V*EDA*RQ *ER**MA*** A**QL*****   100

PhlH    KDCGRRVRFQ APSGHFFELY ADKEYTGKWG LAEVNPEAWP RNLKGMRAVR   150
DmpB    *G******** T********* ********** *E*I****** **********   150
XylE    NS******** *****H**** ********** *ND******* *D****A***   150

PhlH    FDHCLMYGDE LQATYELFTE VLGFYLAEQV IDDDGTRVAQ FLSLSTKAHD   200
DmpB    *****L**** *****A**** ********** ********** **********   200
XylE    ***A****** *P***D***K ********** L*EN****** **********   200

PhlH    VAFIHCPEKG KFHHVSFFLE TWEDVLRAAD LISMTDTSID IGPTRHGLTH   250
DmpB    ********** ********** ********** ********** **********   250
XylE    *****H**** RL*****H** ****L***** ********** **********   250

PhlH    GKTIYFFDPS GNRNEVFCGG DYNYQDHKPV TWLAKDLGKA IFYHDRVLNE   300
DmpB    ********** ********** ********** ********** **********   300
XylE    ********** ********** ****P***** **TTDQ**** ******I***   300

PhlH    RFMTVLT*
DmpB    **L*****
XylE    ********
```

the coding region is GC rich (61.8%), as has been shown for other genes of *P. putida*. Sequence comparison of *phlH* with *xylE*, the gene encoding the prototype class I enzyme, and *dmpB*, the C23O gene of the dimethylphenol degradation plasmid pVI150, revealed homology of 78% and 90%, respectively. The deduced amino acid sequence homology of C23O encoded by pPGH1 to the corresponding enzymes ranges between 83% (XylE) and 90% (DmpB) (Fig. 3). In spite of their different substrate specificities, PhlH and DmpB are quite homologous to class I enzymes.

Upstream of *phlH* and overlapping the *Pst*I site at 61.7 kb another open reading frame (*phlG*) encoding a ferredoxin was identified (positions 4919–5224 of the sequence X80765). The overall homology of the deduced amino acid sequence of PhlG with XylT, NahT and DmpQ is 62%, 58% and 72%, respectively. Homology is almost 100% with other ferredoxins in the segment including the amino acid motif Cys-X-X-X-Cys-X-X-Cys, characteristic of chloroplast-type ferredoxins (Harayama et al. 1991).

Analysis of the phenol hydroxylase genes

To locate and analyze the gene(s) encoding phenol hydroxylase, the DNA region upstream of the *meta*-cleavage pathway genes was mutated by insertion of the Km^r, *lacZ* cassette from pKOK5 (Kokotek and Lotz 1989). For this purpose, *Sal*I fragment G (coordinates 58.2–60.7 kb) and the *Bam*HI-*Kpn*I fragment (coordinates 57.1–58.9 kb; Fig. 1b) were cloned into pSUP202 to generate plasmids pPGH99 and pPGH100, respectively. The recombinant plasmids were linearized by partial digestion with either *Sau*3A (pPGH99) or *Pst*I (pPGH100) and ligated to the cassette excised from pKOK5. The plasmid populations generated by this procedure carried the cassette inserted into one or other of the *Sau*3A or *Pst*I sites. The integration sites of the cassette were mapped. The cassette was also inserted into the unique *Bam*HI site at position 57.1 kb of fragment *Pst*I N (Fig. 1b). Plasmids carrying appropriately oriented insertions at desired positions (C902, C905, C920, Fig. 4) were transferred via conjugation from *E. coli* S17-1 into *P. putida* strain PG320. This strain harbors plasmid pPGH1. Plasmid pSUP202 cannot replicate in *P. putida* and therefore does not render this organism Km^r unless it becomes integrated into a host replicon by homologous recombination. Gene replacement was verified by the loss of pSUP202-derived antibiotic resistances and confirmed by Southern blot experiments (data not shown). The mutants obtained by this method were phenotypically characterized for

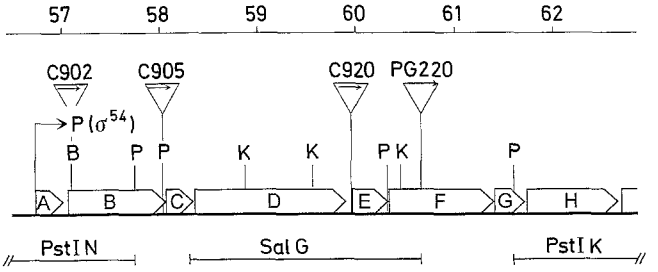

Fig. 4 Physical/genetic map of the promoter-proximal part of the catabolic operon of pPGH1. *Open arrows* show sizes and directions of open reading frames encoding the subunits of phenol hydroxylase (A–F), ferredoxin (G) and catechol 2,3-dioxygenase (H), respectively. Integration sites of cassettes (C902, C905, C920) and transposon Tn*5*-B61 are indicated by *triangles*. Directions of transcription of the promoterless *lacZ* of the cassettes and the Tn*5*-B61-borne *tac* promoter are shown by *small arrows* within the triangles. Abbreviations for restriction enzymes are the same as in Fig. 1. The coordinates of pPGH11 are shown in the upper part

Table 2 Characteristics of phenol hydroxylase mutants

Strain	Phenol (2.5 mM)	Activity[a]		
		Phenol hydroxylase (μmol/min per ml)	Catechol 2,3-dioxygenase (μmol/min per ml)	β-Galactosidase (Miller units)
PG320 (WT)	+	49.2	43.9	9
	−	<2	3.0	nd
C902	+	<2	<2	2231
C905	+	<2	<2	6753
C920	+	<2	<2	1864
	−	<2	<2	44

[a] Enzyme activities were determined at least three times. A representative set of results is shown

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-169                                                    -118          -24          -12          +1
 GCCATAAGCATTTGCTCAAGCGGCCTTGGGCAATTGATCAAATGCTTAAAAA - N94 - TTGGCACAGCCGTTGCTTGATGTCCTG  Pdmp
 :::::: :::::::::::::::::::::::::::::::::::::::::::::        :::::::::: :::::::: :::::::
 GCCATGAGCATTTGCTCAAGCGGCCTTGGGCAATTGATCAAATGCTTAAAAA - N94 - TTGGCACAGCTGTTGCACTTTGTCCTG  Pphl
 ::::::: ::  :   :: : ::   :: :: :::::: :  :::: ::: :          :::::  :: :::::    : : :
 GCCATGATCAAATCGACAGGTGGTTATGCGCGATTGATGATTTGCTCAAATA - N95 - ATGGCATGGCGGTTGCTAGCTATACGA  P1xyl
-170                                                    -119          -24          -12          +1
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Fig. 5 Comparison of the promoter regions of the catabolic operons of pPGH1 (P*phl*), pVI150 (P*dmp*) and TOL plasmid pWW0 upper pathway operon (P1*xyl*). The consensus sequence of the −24, −12 region is written in *boldface*

their ability to grow on phenol and their phenol hydroxylase and C23O activities. All three mutants did not grow on phenol and phenol hydroxylase and C23O activity was not detectable (Table 2). Therefore, we assume that phenol hydroxylase is encoded by the promoter-proximal region of an operon that also contains the *meta*-cleavage pathway genes. Insertion of a cassette into one of these genes also inactivates *meta*-cleavage pathway genes due to its polar effect. This conclusion is confirmed by the direction of transcription (from phenol hydroxylase genes to *meta*-cleavage pathway genes) deduced from the phenol-induced *lacZ* activity of the mutants (Table 2) and by the phenotype of the Tn*5*-B61 mutant. Mutants affected in phenol hydroxylase activity map in a region at least 3.5 kb in size. This large size is inconsistent with a single-component enzyme like that found in *Pseudomonas pickettii* (Kukor and Olsen 1990). It points rather to a multicomponent enzyme such as that described for *Pseudomonas* CF600 (Nordlund et al. 1990).

A recombinant plasmid (pPGH450) was generated which contains the complete DNA region between the *Pst*I site at 61.7 kb and the *Bgl*II site at 56.2 kb under the control of the vector-borne *tac* promoter. This plasmid enables *P. putida* PG5 (expressing the chromosomally encoded *ortho*-cleavage pathway) to grow on phenol. This result demonstrates that all of the structural genes encoding phenol hydroxylase are located promoter-proximal to the *meta*-cleavage pathway genes. Recombinant plasmids carrying smaller fragments of that region did not confer a Phl^+ phenotype.

Several fragments of the DNA region between the *Pst*I site at coordinate 61.7 kb and the *Hind*III site at coordinate 56.1 kb were subcloned in pTZ18R/pTZ19R and used for the determination of the complete DNA sequence. The sequence was evaluated for open reading frames by the codon preference plot (Gribskov et al. 1984) on the basis of the codon usage of *P. putida*. Six open reading frames, designated *phlA–phlF* in order of transcription, were identified. The coding region of *phlA* consists of 276 bp, *phlB* of 1026 bp, *phlC* of 273 bp, *phlD* of 1548 bp, *phlE* of 357 bp, *phlF* of 1059 bp (Fig. 4). The sequence encoding all of the subunits of phenol hydroxylase corresponds to nucleotides 244–4910 of sequence X80765.

Nucleotide sequences in this region share significant homology with the genes encoding the monooxygenase of the dimethylphenol degradation pathway of plasmid pVI150 (Nordlund et al. 1990). The homology of the genes is: *phlA: dmpK* 96%, *phlB: dmpL* 99%, *phlC: dmpM* 94%, *phlD: dmpN* 98%, *phlE: dmpO* 98%, *phlF: dmpP* 86%. DNA sequences of two other phenol hydroxylases have been submitted to gene banks (accession numbers D28864, X79063). Both of these phenol hydroxylases consist of six proteins and show high homology to the enzymes encoded by pPGH1 and pVI150. Thus, similar multicomponent enzymes seem quite common.

The nucleotide sequence was also analyzed for potential regulatory signals. Regions with strong homology to RpoN-dependent promoters and a palindromic sequence as potential activator binding site were identified upstream of *phlA*. Figure 5 shows an alignment of this sequence with that of the catabolic operon of pVI150 and the upper pathway operon of TOL. In the sequence determined so far, no other DNA motif could be found which might serve as a (regulated) promoter. Sequence data are in agreement with the results of genetic experiments and also indicate that in pPGH1 of *P. putida* catabolic genes responsible for the degradation of phenol and methylated phenol form a single, large operon.

All the data presented demonstrate that at least the catabolic genes of pPGH1 of *P. putida* strain H are highly homologous to the catabolic genes of plasmid pVI150 of

Pseudomonas sp. C600, in that they cluster in only one large operon and in their nucleotide sequences (insofar as they are known for pPGH1). Homology may also extend to regulatory mechanisms. Both operons carry a RpoN-dependent promoter and require activation for expression of the corresponding genes.

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