Conservation of regulatory and structural genes for a multi-component phenol hydroxylase within phenol-catabolizing bacteria that utilize a *meta*-cleavage pathway

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Pseudomonas sp. strain CF600 can degrade phenol and some of its methylated derivatives via a plasmid (pVI150)-encoded pathway. The metabolic route involves hydroxylation by a multi-component phenol hydroxylase and a subsequent *meta*-cleavage pathway. All 15 structural genes involved are clustered in an operon that is regulated by a divergently transcribed transcriptional activator. The multi-component nature of the phenol hydroxylase is unusual since reactions of this type are usually accomplished by single component flavoproteins. We have isolated and analysed a number of marine bacterial isolates capable of degrading phenol and a range of other aromatic compounds as sole carbon and energy sources. Southern hybridization and enzyme assays were used to compare the catabolic pathways of these strains and of the archetypal phenol-degrader *Pseudomonas* U, with respect to known catabolic genes encoded by *Pseudomonas* CF600. All the strains tested that degraded phenol via a *meta*-cleavage pathway were found to have DNA highly homologous to each of the components of the multi-component phenol hydroxylase. Moreover, DNA of the same strains also strongly hybridized to probes specific for pVI150-encoded *meta*-pathway genes and the specific regulator of its catabolic operon. These results demonstrate conservation of structural and regulatory genes involved in aromatic catabolism within strains isolated from diverse geographical locations (UK, Norway and USA) and a range of habitats that include activated sludge, sea water and fresh-water mud.

Introduction

Soil and water micro-organisms, notably of the genus *Pseudomonas*, are capable of degrading a wide variety of naturally occurring and synthetic aromatic compounds. During aerobic metabolism of these compounds the bacteria express a battery of distinct enzyme systems to convert the substrate to di-hydroxylated destabilized ring structures, such as catechols. Catechol is an intermediate in the degradation of compounds such as benzoate, naphthalene, salicylate and phenol. Substituted catechols are intermediates in the catabolism of methylated and chlorinated derivatives of these compounds (Dagley, 1986; Reineke & Knackmuss, 1988).

These catechol intermediates serve as the substrates for ring fission dioxygenases and are further metabolized to Krebs cycle intermediates by one of two metabolic alternatives: those of the *ortho*- and *meta*-cleavage pathways (Dagley, 1986).

Amongst the earliest studies of phenol and cresol catabolism were those involving *Pseudomonas* U (Dagley & Gibson, 1965), whose enzymes have served in many of the classical biochemical studies of the *meta*-cleavage pathway enzymes. We have recently reported the isolation and characterization of *Pseudomonas* sp. strain CF600 from activated sludge. *Pseudomonas* CF600 can grow efficiently with phenol, cresols or 3,4-dimethylphenol as the sole carbon and energy source (Shingler *et al.*, 1989). The catabolic pathway for these substrates is encoded on an Inc P2 megaplasmid designated pVI150 and involves hydroxylation followed by a *meta*-cleavage pathway. The fifteen genes for the enzymes of this pathway are clustered in a single operon (Bartilson *et al.*, 1990; Nordlund *et al.*, 1990; Nordlund & Shingler, 1990;

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Abbreviations: C12O, catechol 1,2-dioxygenase; C23O, catechol 2,3-dioxygenase.

Fig. 1. Restriction map of the *dmp*-operon and its regulatory gene *dmpR* (Shingler *et al.*, 1993). Arrows indicate the divergent transcription of the two genetic units. Coordinates of restriction endonuclease recognition sites are given in kb; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; P, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sm, *Sma*I.

Shingler *et al.*, 1992; Powlowski *et al.*, 1993; see Fig. 1) that is regulated by a divergently transcribed transcriptional activator (Shingler *et al.*, 1993). Nucleotide sequences have been reported for all sixteen of these genes, making the *meta*-cleavage pathway and associated enzymes of *Pseudomonas* sp. strain CF600 the first for which sequence information is complete.

The first step in aerobic catabolism of phenol is hydroxylation to form catechol. In *Pseudomonas* CF600 this conversion involves six polypeptides, P0 to P5, that are encoded by the *dmpKLMNOP* genes (Nordlund *et al.*, 1990). However, only the polypeptide products of the *dmpLMNOP* genes, namely P1, P2, P3, P4 and P5, are required for *in vitro* activity of this multi-component enzyme (Powlowski & Shingler, 1990).

The multi-component nature of this phenol hydroxylase is intriguing. As a general rule, mono-hydroxylated ring structures such as phenol are oxygenated by single component flavoprotein monooxygenases (Ballou, 1982). For example, genes encoding flavoprotein hydroxylases (monooxygenases) induced by growth on phenol have recently been identified and characterized in a number of micro-organisms, including *Pseudomonas pickettii* (Kukor & Olsen, 1992), *Pseudomonas* EST1001 (Nurk *et al.*, 1991) and the yeast *Trichosporon cutaneum* (Kälin *et al.*, 1992). The 2,4-dichlorophenol hydroxylases of *Acinetobacter* and *Alcaligenes* species have also been shown to be flavoproteins, although these enzymes show no activity with unsubstituted phenol (Beadle & Smith, 1982; Liu & Chapman, 1984). On the other hand, studies of phenol degradation in a *Bacillus* species suggest that the phenol hydroxylase of this organism may not be a simple single component flavoprotein (Gurujeyalakshmi & Oriel, 1989).

Here we report gene-probing experiments performed on 12 phenol-utilizing bacterial isolates that demonstrate that genes encoding the unusual multi-component CF600 phenol hydroxylase are present in other phenol-degrading bacteria. Moreover, these strains also possess DNA highly homologous to the *meta*-pathway genes and the specific regulator of phenol catabolism encoded by plasmid pVI150 in *Pseudomonas* sp. strain CF600.

Methods

Bacterial strains. Marine bacterial strains were isolated from sea-water samples taken downstream of the discharge from a wood pulping mill located on the west coast of Norway. Water samples from a depth of 1 m were taken at approximately 5 km intervals from eight different stations and immediately plated onto marine agar (Difco) or minimal M9-salts media containing benzoate (5 mM). Approximately 150 individual colonies from each station were subsequently tested for their ability to grow on phenol as sole carbon and energy source. The ten isolates chosen for analysis in this study (MI1 to MI10), grew on solid and in liquid media with phenol as sole carbon and energy source. Other bacterial strains used are the phenol-utilizing strains *Pseudomonas* U (Dagley & Gibson, 1965) and *Pseudomonas* sp. strain CF600 (Shingler *et al.*, 1989). *Pseudomonas putida* PB2701, a streptomycin resistant derivative of KT2440 (Franklin *et al.*, 1981), was used as a control strain that is unable to utilize phenol.

Growth conditions. *Pseudomonas* U, *Pseudomonas* sp. strain CF600 and *P. putida* PB2701 were cultured at 30 °C, while marine isolates were grown at ambient temperature. Luria broth (LB) and marine agar (Difco) were used as rich medium for the *Pseudomonas* strains and marine strains, respectively. The ability to utilize different aromatic compounds as sole carbon and energy source was tested on minimal M9-salt plates supplemented with the different carbon sources at the concentrations indicated.

Induction of catabolic enzyme activities of marine isolates for crude extract preparations was performed by growth for 2 d on solid minimal media supplemented with 0.5% Casamino acids and 2.0 mM-phenol, *o*-cresol or aniline.

Crude extract preparations and enzyme assays. Cells used for making crude extracts were prepared as described by Shingler *et al.* (1992). Catechol 2,3-dioxygenase (C23O) and catechol 1,2-dioxygenase (C12O) enzyme assays were performed spectrophotometrically at 25 °C as described by Sala-Trepat *et al.* (1972). Extracts used for C12O activity measurements were pre-treated for 10 min with H₂O₂ at a final concentration of 10 mM to inactivate (Nozaki, 1970), and thus avoid interference by any C23O present in the protein extract. Units of activity are defined as the amounts of enzyme required to convert 1 µmol of substrate to product min⁻¹. Protein concentration in cell extracts was estimated using the BCA assay (Pierce Chemical Co.) with BSA as the standard using the 60 °C protocol described by the manufacturers.

Southern hybridization and DNA probes. DNA extractions and Southern analysis were performed as described by Sambrook *et al.* (1989). Washing stringency was as follows: two rinses of 10 and 20 min duration in 2 × SSC/0.1% SDS at room temperature, two washes of 15 min in 1 × SSC/0.1% SDS at 60 °C, followed by two 10 min washes in 0.25 × SSC/0.1% SDS at 60 °C. Filters were stripped for re-probing by boiling for 3 min in 0.5% SDS, followed by cooling to room temperature in the SDS solution.

Table 1. DNA probes

Strains and genes	Oligonucleotide or DNA fragment*	Reference for bp coordinates
Universal probe A	Oligonucleotide 5'-CGTATTACCGCGGCTGCT-3'	Lane <i>et al.</i> (1985)
<i>Pseudomonas</i> CF600 derived probes		
<i>dmpR</i>	PCR1: 246 to 351 as an <i>NdeI</i> - <i>PstI</i> fragment	Shingler <i>et al.</i> (1992)
<i>dmpK</i>	<i>PvuII</i> 547 to <i>BamHI</i> 1105	
<i>dmpL</i>	<i>BamHI</i> 1105 to <i>PstI</i> 1755	
<i>dmpM</i>	<i>PstI</i> 2040 to <i>XhoI</i> 2330	
<i>dmpN</i>	<i>KpnI</i> 2884 to <i>BglII</i> 3728	
<i>dmpO</i>	<i>XhoI</i> 4139 to <i>EcoRV</i> 4514	
<i>dmpP</i>	<i>EcoRV</i> 4514 to <i>StyI</i> 5218	
<i>dmpQ</i>	<i>NruI</i> 5445 to <i>SmaI</i> 5715	
<i>dmpB</i>	<i>SaII</i> 5902 to <i>PstI</i> 6239	
<i>dmpC</i>	<i>PstI</i> 7713 to <i>HpaI</i> 8044	
<i>dmpD</i>	520 bp <i>Bal31</i> to <i>SaII</i> 8787 fragment	
<i>dmpE</i>	<i>BstEII</i> 9281 to <i>BstEII</i> 9580	
<i>dmpF</i>	<i>NruI</i> 9866 to <i>EcoRI</i> 10046	
<i>dmpG</i>	<i>SaII</i> 10924 to <i>SaII</i> 11461	
<i>dmpH</i>	<i>SaII</i> 12987 to <i>SaII</i> 12320	
<i>dmpI</i>	PCR2: 12674 to 12880 as an <i>NdeI</i> - <i>HpaI</i> fragment	
<i>P. pickettii</i> derived probes		
<i>ibuD</i>	<i>EcoRI</i> 283 to <i>BglII</i> 519	Kukor & Olsen (1992)
<i>ibuD</i>	<i>BglII</i> 519 to <i>Clal</i> 1432	
<i>Pseudomonas</i> sp. EST1001 derived probe		
<i>pheA</i>	<i>BglI</i> 329 to <i>BamHI</i> 1614	Nurk <i>et al.</i> (1991)
<i>P. putida</i> PB2701 derived probe		
<i>rpoN</i>	PCR3: a 282 bp fragment†	This study

* PCR fragments were generated using the following primers: PCR1 primers 5'-CGCCATATGCCGATCA-AGTACAAG-3' and 5'-GCTGAAAACCTGCAGCAACAGC-3'; PCR2 primers 5'-CGCCATATGCCGATTGCTCAGCT-3' and 5'-CAAACCTGTTAACGAACACTCTC-3'; PCR3 primers 5'-ATGCACGAATCGACCATTTC-3' and 5'-TACTTGGCGACGGTGCGACG-3'.

† The primers used were designed on the basis of conserved regions found upon sequence comparisons of *rpoN* genes which encode σ^{54} factors (Inouye *et al.*, 1989; Kullik *et al.*, 1991).

Initially, 10 μ g total *PstI* digested DNA from each strain was used and hybridized to a radiolabelled oligonucleotide, universal probe A (Lane *et al.*, 1985), that spans part of the constant region of the 16S rRNA. To ensure even loading of genomic equivalents from the 13 strains, some of which harbour plasmids, the resulting autoradiograph was scanned using a Helena laboratories Quick Scan apparatus. Sample loading was adjusted to give an equal intensity signal with the universal probe A ($\pm 15\%$). Six essentially identical Southern transfers were made and hybridized with the DNA probes listed in Table 1.

The universal probe A oligonucleotide was 5'-end labelled using [γ - 32 P]dATP (Amersham) and polynucleotide kinase (Boehringer). Double stranded DNA restriction fragments and polymerase chain reaction (PCR)-generated DNA fragments were purified from agarose gels. The resulting DNA was subsequently random-prime labelled using [α - 32 P]dCTP (Amersham) and hexanucleotides (Pharmacia).

Results

Isolation and growth substrate range of phenol-utilizing marine isolates

The ten phenol-utilizing marine isolates, MI1 to MI10, were isolated from eight sea-water samples and were all

found to be Gram-negative rods. The growth substrate range for each of these bacterial isolates was determined and compared with those of three reference strains in Table 2. These bacteria were found to degrade a range of aromatic compounds including methyl-substituted phenols, but none of the chloro-substituted compounds tested. The isolates could be divided into six groups on the basis of their growth substrate range: I, MI1 and MI2; IIa, MI3; IIb, MI5; III, MI8; IVa, MI4, MI6 and MI9; IVb, MI7 and MI10. Groups IIa and IIb differed only in their ability to utilize *p*-cresol, while groups IVa and IVb differed in their ability to utilize aniline. As indicated in Table 2, with the exception of groups IVa and IVb, the members of the different groups were isolated from different samples. The five members of group IVab were isolated from two different water samples on two types of isolation media and therefore represent a minimum of two independent isolates. However, hybridization experiments (see below) using an oligonucleotide complementary to the constant region of

Table 2. Growth substrate range

Strains were grown at room temperature on minimal M9-media containing the growth substrates, at the indicated concentrations, as sole carbon and energy sources. Groups: (I: MI1, MI2), (IIa: MI3), (IIb: MI5), (III: MI8), (IVa: MI4, MI6, MI9), (IVb: MI7, MI10).

Strain...	MI1	MI2	MI3	MI4	MI5	MI6	MI7	MI8	MI9	MI10	PB 2701	CF 600	Pseu. U
Isolate(s) from water sample...	8	6	5	4	4	3	3	1	3	3			
Original isolation medium*...	MA	MA	MA	MA	MA	MA	MA	MA	MB	MB			
Carbon source													
Phenol (2 mM)	+	+	+	+	+	+	+	+	+	+	-	+	+
Catechol (2 mM)	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>m</i> -Toluate (2 mM)	-	-	-	+	-	+	+	-	+	+	-	-	-
Benzoate (5 mM)	+	+	+	+	+	+	+	-	+	+	+	+	+
Aniline (2 mM)	-	-	-	+	-	+	-	-	+	-	-	-	-
3-Chlorobenzoate (1 mM)	-	-	-	-	-	-	-	-	-	-	-	-	-
Monochloro-phenols (1 mM)	-	-	-	-	-	-	-	-	-	-	-	-	-
Dichloro-phenols (1 mM)	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>p</i> -Hydroxybenzoate (2 mM)	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicylate (2 mM)	-	-	-	-	-	-	-	+	-	-	-	-	-
Naphthalene (vapour)	-	-	-	-	-	-	-	-	-	-	-	-	-
3,4-Dimethylphenol (2 mM)	-	-	-	-	-	-	-	-	-	-	-	+	+
<i>p</i> -Cresol (2 mM)	+	+	-	+	+	+	+	-	+	+	-	+	+
<i>m</i> -Cresol (2 mM)	+	+	-	+	-	+	+	-	+	+	-	+	+
<i>o</i> -Cresol (2 mM)	-	-	-	+	-	+	+	-	+	+	-	+	+

* MA, marine agar; MB, minimal benzoate containing medium.

Table 3. Ring-fission enzyme activities of marine isolates

Values shown are means of duplicate determinations. Assays were performed a minimum of two times; one representative set of results is shown. The specific activity values of duplicate assays performed on the same crude extract were within 5%, while assays performed on the same strain on different days were usually within twofold.

Strain	Group	Growth substrate...	Enzyme activity [mU (mg protein) ⁻¹]					
			Phenol		<i>o</i> -Cresol		Aniline	
			C23O	C12O	C23O	C12O	C23O	C12O
MI1	I		230	390				
MI2	I		240	410				
MI3	IIa		< 10	160				
MI4	IVa		800	< 30	450	< 30	< 10	330
MI5	IIb		< 10	260				
MI6	IVa		710	< 30	460	< 30	< 10	280
MI7	IVb		910	< 30	300	< 30		
MI8*	III		< 10	< 30				
MI9	IVa		760	< 30	640	< 30	< 10	320
MI10	IVb		710	< 30	310	< 30		

* Repeated attempts to prepare crude extracts and detect enzyme activities were unsuccessful.

the 16S rRNA and 17 gene-specific DNA probes did not reveal any polymorphism.

Expression of ring-cleavage enzymes

C23O and C12O catalyse the ring-fission step of the *meta*- and *ortho*-cleavage pathways respectively. The enzyme activities in crude extracts from the marine isolates were measured to determine which of these two

pathways is used for growth on different aromatic substrates. Table 3 shows a summary of the results. Group I isolates are unusual in that they simultaneously expressed both C23O and C12O in response to growth on phenol. Group IIa and IIb expressed only C12O when grown on phenol, while groups IVa and IVb expressed only C23O when grown on phenol or *o*-cresol (2-methylphenol). Group IVa grew on aniline while group IVb could not; however, growth on aniline elicited

Fig. 2. Autoradiographs of Southern analysis of total *Pst*I digested DNA of bacterial strains hybridized with radiolabelled probes (*dmpK*, *L*, *M*, *N*, *O* and *P*) specific for the multi-component phenol hydroxylase of *Pseudomonas* sp. strain CF600. Molecular size standards are given in kb on the left, and the sizes of major hybridizing bands are indicated with arrows on the right.

expression of C12O, as opposed to C23O. Therefore, it appeared that while groups IVa and IVb both utilize a *meta*-cleavage pathway to dissimilate phenol and *o*-cresol, only group IVa used an *ortho*-cleavage pathway to dissimilate aniline. Despite repeated attempts, neither C23O nor C12O activity could be detected in crude extracts from MI8. This appears to be an unusual isolate since its DNA did not hybridize with any of the probes directed to either catabolic or non-catabolic genes (see below), although it did hybridize an oligonucleotide complementary to the constant region of the 16S rRNA (data not shown).

Southern analysis using phenol hydroxylase specific probes

Southern analysis was performed to compare the phenol catabolic genes of the marine isolates with those of phenol-utilizing *Pseudomonas* CF600 and *Pseudomonas* U. Hybridization and stringency conditions were the same for all probes used in the analysis. In intact cells,

the multi-component phenol hydroxylase activity of *Pseudomonas* CF600 involves six polypeptides, P0 to P5, encoded by *dmpKLMNOP* (see Fig. 1). Fig. 2 shows the results of Southern analysis using specific probes directed towards the individual genes encoding the multi-component phenol hydroxylase. DNA derived from all the strains that utilize a *meta*-cleavage pathway for dissimilation of phenol, groups IVa, IVb, *Pseudomonas* U and *Pseudomonas* CF600, strongly hybridized the six probes with approximately equal intensity (lanes 4, 6, 7, 9, 10, 12 and 13, Fig. 2). DNA derived from *P. putida* PB2701, which cannot catabolize phenol, from MI8, or from group I isolates, did not hybridize with any of the probes (lanes 1, 2, 8 and 11; Fig. 2). DNA derived from group IIa and IIb isolates showed low level hybridization with some of the probes (lanes 3 and 5, Fig. 2). Hence, the results demonstrate that DNA highly homologous to the genes encoding P0 to P5, *dmpKLMNOP*, is present in all the bacterial strains tested that utilize a *meta*-cleavage pathway to catabolize phenol.

In order to investigate the possible nature of the

Fig. 3. Autoradiographs of Southern analysis of total *Pst*I digested DNA of bacterial strains hybridized with radiolabelled probes specific for genes encoding *meta*-pathway enzymes of *Pseudomonas* sp. strain CF600. Molecular size standards are given in kb on the left, and the sizes of major hybridizing bands are indicated with arrows on the right.

phenol hydroxylase exhibited by groups I, IIab and III, probes for two other phenol hydroxylases were used. These probes, derived from *tbuD* from *Pseudomonas pickettii* and *pheA* from *Pseudomonas* EST1001, are specific to phenol hydroxylase genes that encode single component flavoprotein hydroxylases. Despite the fact that these two genes both encode flavoprotein hydroxylases, they share little overall sequence homology at the nucleotide level (Kukor & Olsen, 1992). None of the three probes tested gave specific hybridization signals with DNA from any of the phenol-degrading isolates (data not shown). Therefore, the nature of the phenol hydroxylase encoded by these strains remains unclear.

Southern analysis using *meta*-pathway specific probes

To investigate further the genetic composition of groups IVa and IVb in terms of their *meta*-cleavage pathway, nine *dmp*-operon probes were used. The *dmp*-operon genes lie in the order *dmpQBCDEFGHI* (see Fig. 1).

DmpQ is a small ferredoxin-like protein whose function is as yet unknown (Shingler *et al.*, 1992). The *dmpBCDEFGHI* genes encode the *meta*-cleavage pathway enzymes (see Shingler *et al.*, 1992; Powlowski *et al.*, 1993). Southern analysis demonstrated that each probe hybridizes a single fragment of each of the strains that utilized a *meta*-cleavage pathway to dissimilate phenol (lanes 4, 6, 7, 9, 10, 12, and 13, Fig. 3), but not DNA of the other strains tested. The sizes and pattern of hybridization using the *dmpC*, *D*, *E*, *F*, *G*, *H* and *I* probes were indistinguishable; therefore, only the results obtained using the *dmpQ*, *B*, *C* and *I* probes are shown.

Group I isolates produced both C23O and C12O activities in response to growth on phenol, but did not hybridize with the *dmpB*–C23O probe or the other *meta*-pathway probes used above. Analysis of C23O-encoding genes from a number of different bacterial genera has identified different classes of this enzyme. Southern hybridization and nucleotide sequence determinations have demonstrated that homology between different C23O-encoding genes varies from very low to approximately 85% for the *dmpB*, *nahH* and *xylE* genes (Bartilson & Shingler, 1989; Benjamin *et al.*, 1991). In an attempt to identify the C23O-gene of group I isolates, a range of C23O-gene probes were used; *mpcI* (Kabisch & Fortnagel, 1990a); *mpcII* (Kabisch & Fortnagel, 1990b); C23DO (Keil, 1990) and C23O II (Keil *et al.*, 1985). Only the *mpcII*-probe hybridized to DNA from group I isolates, albeit poorly. However, this probe also hybridized, with equal intensity, to DNA fragments from all but MI8 (data not shown). Therefore, the nature of the C23O-encoding gene of group I isolates remains obscure.

Southern analysis using gene-expression probes

In view of the homology seen between groups IVa and b, *Pseudomonas* U and *Pseudomonas* CF600 to the multi-component phenol hydroxylase and *meta*-pathway genes, Southern analysis was also performed using components of the *dmp*-operon regulatory system. The *dmp*-operon is positively regulated by the divergently transcribed *dmpR*-gene product (see Fig. 1). DmpR shares homology with the NtrC family of transcriptional activators. However, only XylR, a regulator of the catabolic pathway of pWW0, was found to share homology with the N-terminal domain of DmpR (Shingler *et al.*, 1993). Therefore, we performed Southern analysis using a probe derived from the 5'-end of *dmpR*. The results, shown in Fig. 4, demonstrate that this probe specifically hybridizes to DNA of group IVab, *Pseudomonas* U and *Pseudomonas* CF600, but not to DNA of the other marine isolates or *P. putida* PB2701.

DmpR and other members of the NtrC family regulate transcription from promoters recognized by RNA

Fig. 4. Autoradiographs of Southern analysis of total *Pst*I digested DNA of bacterial strains hybridized with radiolabelled probes specific for genes involved in transcriptional regulation. The *dmpR*-probe was derived from *Pseudomonas* sp. strain CF600 and the *rpoN*-probe from *P. putida* PB2701 (see Methods). Molecular size standards are given in kb on the left, and the sizes of major hybridizing bands are indicated with arrows on the right.

polymerase employing the alternative sigma factor (σ^{54}) encoded by *rpoN* or its homologues (see Inouye *et al.*, 1989). Southern analysis, using an *rpoN*-PCR fragment generated using *P. putida* PB2701 DNA, is shown in Fig. 4. DNA from all strains, except MI8, was found to hybridize with this gene probe.

Discussion

The multi-component phenol hydroxylase of *Pseudomonas* CF600 comprises five polypeptides, P1 to P5. The P5 polypeptide contains FAD and a ferredoxin-type iron-sulphur centre, and can catalyse the transfer of electrons from NADH, which is required for oxygenase activity. P2 is a small essential polypeptide of unknown function, while the P1, P3 and P4 polypeptides form an oxygenase complex (Powlowski & Shingler, 1990, and unpublished data). The multi-component phenol hydroxylase is novel in a number of ways. Firstly, the multi-component nature of this enzyme was unexpected since reactions of this type are usually catalysed by single component flavoproteins (Ballou, 1982). Secondly, the multi-component phenol hydroxylase system, surprisingly, shows organizational similarity and low level

sequence identity with components of oxygenases for unactivated compounds such as methane monooxygenase and toluene-4-monooxygenase (Powlowski & Shingler, 1990; Harayama *et al.*, 1991; Yen *et al.*, 1991). The unusual nature of the multi-component phenol hydroxylase prompted us to ask in this study if similar systems are used in other phenol-utilizing bacteria. In some other *Pseudomonas* species, it is clear that a multi-component phenol hydroxylase is not involved (Kukor & Olsen, 1992; Nurk *et al.*, 1991). However, it is conceivable that multi-component phenol hydroxylases have previously been overlooked due to the difficulties associated with the *in vitro* assay (Powlowski & Shingler, 1990).

Southern hybridization analysis, using DNA probes specific for the components of the phenol hydroxylase of CF600, demonstrated that five out of ten phenol-utilizing marine bacterial isolates (groups IVa and b), and the archetypal phenol-degrader *Pseudomonas* U, all possessed DNA highly homologous to each of the components of *Pseudomonas* CF600 phenol hydroxylase. All of these strains utilize a *meta*-pathway and were also found to possess DNA highly homologous to all nine *meta*-cleavage pathway genes of *Pseudomonas* CF600, and to the specific regulator of phenol catabolism of this strain. Examination of the sizes and order of the hybridizing DNA fragments (Figs 2 to 4) suggests that not only are the structural genes and regulatory genes for phenol catabolism conserved, but they are probably also encoded in the same order in all the strains.

The phenol catabolic pathway of *Pseudomonas* CF600 is located on the megaplasmid pVI150, which has a copy number of one to two (from unpublished data), while that of *Pseudomonas* U is believed to be chromosomally encoded. The genetic locations of the phenol catabolic pathway genes of the five marine isolates of groups IVa and IVb are not known. However, on the basis of the intensity of hybridization signals of genomic equivalents, they are probably located on either the chromosome or a low copy number plasmid.

Clustering and homology of *meta*-cleavage pathway genes of different bacterial genera isolated from diverse geographical locations has been documented for a number of years (Assinder & Williams, 1990). Many aromatic catabolic pathways are encoded on plasmids, and in some cases have been found to be either wholly or partially encoded within large transposons. The genetic events, including conjugation, transposition and recombination, that probably lead to adaptation for growth on aromatic compounds have recently been reviewed by van de Meer *et al.* (1992). Conservation of central pathways (i.e. *meta*- or *ortho*-pathways) has led to the idea that catabolic pathways are built up piecemeal by the acquisition of pre-evolved 'metabolic modules' (Assinder & Williams, 1990). Hence, different degrada-

tive bacteria might be expected to possess homologous central pathways for the dissimilation of catechol(s). However, the genetic location, the enzyme systems for conversion of substrates to catecholic intermediate, and catabolic regulatory systems, are usually different.

Pseudomonas CF600 was originally isolated from an activated sludge sample from the British Carbonisation Research Association, *Pseudomonas* U from the mud of a stream in Urbana, USA, and the marine bacterial isolates from sea water collected off the coast of Norway. These strains represent an example of conservation of both a specific regulatory gene and structural genes whose products are involved in both substrate conversion to a central intermediate and in the subsequent meta-cleavage pathway. Hence, it seems likely that these strains inherited both structural and specific regulatory genes involved in phenol utilization as a single metabolic unit.

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