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Genetics and biochemistry of phenol degradation by *Pseudomonas* sp. CF600

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Abstract

Pseudomonas sp. strain CF600 is an efficient degrader of phenol and methylsubstituted phenols. These compounds are degraded by the set of enzymes encoded by the plasmid located *dmp*operon. The sequences of all the fifteen structural genes required to encode the nine enzymes of the catabolic pathway have been determined and the corresponding proteins have been purified. In this review the interplay between the genetic analysis and biochemical characterisation of the catabolic pathway is emphasised. The first step in the pathway, the conversion of phenol to catechol, is catalysed by a novel multicomponent phenol hydroxylase. Here we summarise similarities of this enzyme with other multicomponent oxygenases, particularly methane monooxygenase (EC 1.14.13.25). The other enzymes encoded by the operon are those of the well-known *meta*-cleavage pathway for catechol, and include the recently discovered *meta*-pathway enzyme aldehyde dehydrogenase (acylating) (EC 1.2.1.10). The known properties of these *meta*-pathway enzymes, and isofunctional enzymes from other aromatic degraders, are summarised. Analysis of the sequences of the pathway proteins, many of which are unique to the *meta*-pathway, suggests new approaches to the study of these generally little-characterised enzymes. Furthermore, biochemical studies of some of these enzymes suggest that physical associations between *meta*-pathway enzymes play an important role. In addition to the pathway enzymes, the specific regulator of phenol catabolism, DmpR, and its relationship to the XylR regulator of toluene and xylene catabolism is discussed.

Introduction

Utilisation of phenol as a source of microbial nourishment has perhaps been overshadowed by awareness of its generally unhealthy effects on humans. Although the use of phenol as an antiseptic for treating wounds and wound dressings was of unquestionable value in helping to establish the importance of sterile techniques in medicine, and probably helped save the lives of many patients, the toxicity of the compound was evident from, amongst other things, its contemporaneous use as a popular orally-administered suicidal agent (Lister 1867; McGrew 1985). While the use of phenol as an antiseptic dwindled by the turn of the century, its application as a general disinfectant continued, as did development of new uses for phenol and its derivatives. Today approximately 1700 million kilograms of phenol are synthesised annually in the United States alone (Anonymous 1993), and countless quantities of phenol and its derivatives are produced as industrial by-products.

Considering the large scale on which this highly toxic compound and related chemicals are produced, its degradability is of obvious interest. Persistence of phenol itself in the environment is not a particular problem, with both physico-chemical and biological agents contributing to its degradation (Howard et al. 1991). However, as with any metabolic process, biodegradability can be affected by many variables, including, for example, the presence of alternative carbon sources or substituents on the phenol ring. While such limitations may be overcome by genetic manipulation of the catabolic pathway involved, dissimilatory mechanisms and their regulation must first be characterized

at the molecular level in individual microbial species. Since phenol is a relatively simple, unadorned, aromatic compound for which much information on biodegradation is already available, phenol-degrading bacteria provide good model systems for these kinds of studies.

Microbial phenol degradation is unlikely to be a recently-acquired trait. While the use of phenol on a large scale by humans is relatively recent, microbes have long been exposed to biologically generated phenols and methyl-substituted phenols produced, for example, by enterobacteria from tyrosine (Kumagai et al. 1970; Spoelstra 1977) and by the break-down of plant materials. The isolation of phenol-degrading bacteria was achieved as early as 1932 (Happold & Key 1932), and numerous others have been unearthed since then. While many of the most-studied phenol degraders are pseudomonads, other bacterial genera, such as *Bacillus*, as well as yeast, are also represented (Gurujeyalakshmi & Oriel 1989; Neujahr & Gaal 1973).

Work during the nineteen sixties and seventies with phenol-degrading *Pseudomona putida* U was responsible for much of the progress in understanding the biochemistry of the *meta*-cleavage pathway (reviewed in Bayly & Barbour 1984). In this pathway, ring cleavage adjacent to two aromatic hydroxyls (Fig. 1) is the distinguishing feature, with the subsequent series of reactions dictated by the nature of the ring-fission product. This contrasts with the *ortho*-cleavage pathway, used by some phenol degraders, in which catechol (1,2-dihydroxybenzene) produced from phenol is cleaved *between* the catechol hydroxyls, with an entirely different set of enzymes required for metabolism of the distinctive ring-cleavage product (reviewed by Ornston & Yeh 1982). One consequence of the enzymologies of the two pathways is that while the *ortho*-cleavage pathways of pseudomonads cannot tolerate methyl-substituents, all three mono-methyl substituted phenols can be degraded via the *meta*-cleavage pathway. *Ortho*-cleavage degraders can, however, circumvent this problem by first oxidizing the methyl group to a carboxyl, and then using a separate set of enzymes for the resulting hydroxybenzoic acid (see Dagley 1986).

The main emphasis in this review is work done on the *dmp* system which encodes the phenol degradation pathway of *Pseudomonas* sp. strain CF600. In addition to phenol, this organism metabolizes all monomethylphenols, as well as 3,4-dimethylphenol, via the *dmp*-encoded hydroxylation and *meta*-cleavage pathway enzymes. This review highlights the interplay between the analysis of the genes of this and other pathways, and the biochemical studies of the enzymes involved.

Genetic location and organisation of the dmp system

Like many other pathways for the catabolism of aromatic compounds, the phenol/dimethylphenol (*dmp*) pathway of *Pseudomonas* sp. strain CF600 is plasmid encoded. The plasmid, designated pVI150, belongs to the P-2 incompatibility group (Shingler et al. 1989). Like two other IncP-2 catabolic plasmids, OCT and CAM (Fennewald et al. 1978; Rheinwald et al. 1973), pVI150 is very large, and exceeds 200 kb in size. Due to the large size of pVI150 and attendant difficulties in its purification, initial isolation of the pathway-encoding DNA necessitated utilization of methods more often associated with isolation of chromosomally-encoded genes (Shingler et al. 1989; Bartilson et al. 1990).

The results of genetic dissection of the 15 kb DNA region encoding the *dmp* system, and the locations of the sixteen genes involved, are summarised in Fig. 1. This figure also illustrates the biochemical route, via hydroxylation and a *meta*-cleavage pathway, for the catabolism of the pathway substrates. The fifteen genes encoding the enzymes of the pathway (Table 1) are clustered in the *dmp* operon. Our attention initially focused on the DNA encoding the first enzyme of the pathway, phenol hydroxylase, that catalyses the conversion of phenol to catechol. DNA encoding this enzyme activity was distinguished from that encoding the remaining pathway enzymes by expressing different parts of the *dmp* operon in a strain of *Pseudomonas* that possesses an *ortho*-cleavage pathway capable of further catabolism of catechol, but not methylcatechols. Subsequent testing of the growth range of the resulting strains functionally defined the DNA for the phenol hydroxylase (growth on phenol only) and the entire pathway (growth on phenol and methylphenols).

A combination of nucleotide sequencing and polypeptide analysis identified six genes, *dmpKLMNOP*, and their corresponding products, P0, P1, P2, P3, P4 and P5, within the phenol hydroxylase-coding region. Deletions within each gene in turn, and expression of each individual open reading frame, coupled with polypeptide analysis, confirmed the assignment of the polypeptide products to individual genes (Nordlund et al. 1990a). As discussed below, the find-

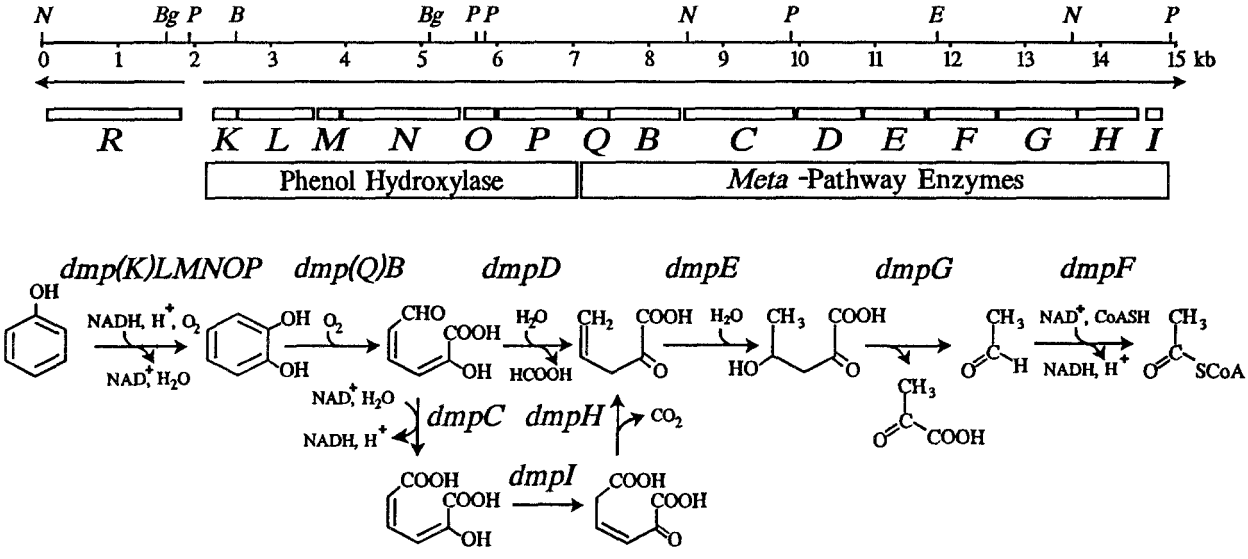

Fig. 1. Schematic representation of the phenol/dimethylphenol pathway encoded by a 15 kb region of the pVI150 catabolic plasmid of *Pseudomonas* sp. strain CF600. The arrows indicate the divergent transcription of the *dmpR* regulatory gene and the *dmpKLMNOPQBCDEFGHI*-operon. Enzyme functions are as shown in Table 1. Both the hydrolytic (*dmpD*-encoded) and the 4-oxalocrotonate (*DmpCIH*-encoded) branches of the *meta*-cleavage pathway are shown. Restriction enzyme recognition site are: *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *N*, *Not*I; *P*, *Pvu*II.

Table 1. Summary of the genes and gene products of the *dmp* operon.

Gene	Amino acid residues	Molecular mass (kDa) predicted/estimated	Product	Function	Reference
dmpK	92	10.6/12.5	P0	Unknown	Nordlund et al. 1990
dmpL	331	38.2/34.0	P1	Phenol hydroxylase component	Nordlund et al. 1990
dmpM	90	10.5/10.0	P2	Phenol hydroxylase component	Nordlund et al. 1990
dmpN	517	60.5/58.0	P3	Phenol hydroxylase component	Nordlund et al. 1990
dmpO	119	13.2/13.0	P4	Phenol hydroxylase component	Nordlund et al. 1990
dmpP	353	38.5/39.0	P5	Phenol hydroxylase component	Nordlund et al. 1990
dmpQ	112	12.2/12.0	DmpQ	Ferredoxin-like protein	Shingler et al. 1992
dmpB	307	35.2/32.0	C23O	Catechol 2,3-dioxygenase, EC 1.13.11.2	Bartilson & Shingler 1989
dmpC	486	51.7/50.0	HMSD	2-Hydroxymuconic semialdehyde, dehydrogenase, EC 1.2.1.–	Nordlund & Shingler 1990
dmpD	283	31.0/30.0	HMSH	2-Hydroxymuconic semialdehyde hydrolase	Nordlund & Shingler 1990
dmpE	261	27.9/28.0	OEH	2-Oxopent-4-dienoate hydratase, EC 4.2.1.80	Shingler et al. 1992
dmpF	312	32.7/35.0	ADA	Aldehyde dehydrogenase (acylating), EC 1.2.1.10	Shingler et al. 1992
dmpG	345	37.5/39.0	HOA	4-Hydroxy-2-oxovalerate aldolase, EC 4.1.3.–	Shingler et al. 1992
dmpH	264	28.4/28.5	4OD	4-Oxalocrotonate decarboxylase, EC 4.1.1.–	Shingler et al. 1992
dmpI	63	7.1/6.7	4OI	4-Oxalocrotonate isomerase, EC 5.3.2.–	Shingler et al. 1992

ing that the phenol hydroxylase encoded by pVI150 is multicomponent was initially surprising and prompted further studies of this unusual enzyme.

The remaining nine genes of the operon, *dmpQBCDEFGHI*, encode the *meta*-cleavage pathway enzymes for conversion of catechol to pyruvate and acetyl-CoA (Table 1, Fig. 2). Assignment of a function to each of these genes was achieved using a combination of expression of open reading frames, both alone and in combination, nucleotide sequencing, and

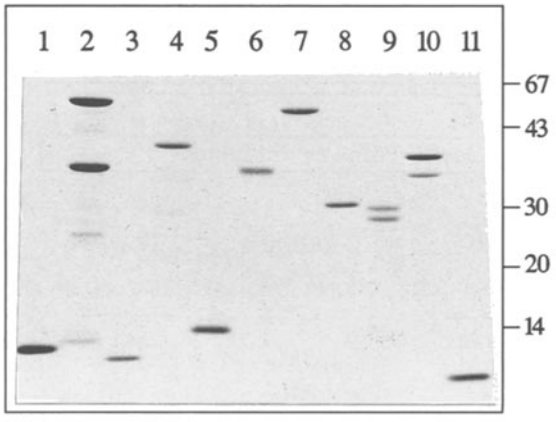

Fig. 2. Proteins encoded by the *dmp*operon. 2 to 5 μg of protein were analysed on a gradient (10 to 20%) SDS polyacrylamide gel. Lane 1, DmpK (P0); 2, DmpLNO (P1-P3-P4); 3, DmpM (P2); 4, DmpP (P5); 5, DmpQ; 6, DmpB (C230); 7, DmpC (HMSD); 8, DmpD (HMSH); 9, DmpEH (OEH/4OD); 10, DmpFG (ADA/HOA); 11, DmpI (4OI): Molecular mass standards are given in kDa on the right-hand side. All polypeptides were purified from phenol-grown *Pseudomonas* sp. strain CF600 except DmpK (lane 1), DmpQ (lane5) and DmpC (lane7), which were purified from separate *E. coli* strains expressing the corresponding genes. Enzyme abbreviations and estimated molecular masses are given in Table 1.

biochemical techniques (Bartilson & Shingler 1989; Nordlund & Shingler 1990; Shingler et al. 1992).

Since many pathways for the catabolism of aromatic compounds include *meta*-cleavage pathway enzymes, the *dmp* genes and proteins have counterparts in other systems. The most extensively studied of these are those from pseudomonads harbouring the pWW0-encoded toluene/xylene *xyl* pathway, the NAH7- and pWW60-1-encoded naphthalene/salicylate *nal* (*sal*) pathways, and the phenol degradation pathway of *P. putida* U. All these systems have both branches of the *meta*-cleavage pathway as depicted in Fig. 1 (see Assinder & Williams 1990; Williams & Sayer 1994 for recent reviews). Using gene-specific probing, this has also been shown to be true for the *meta*-pathway genes of the *Pseudomonas* sp. strain IC biphenyl degradation pathway (Carrington et al. 1994). Recently, nucleotide sequences have been completed for two chromosomally encoded *meta*-cleavage pathways involved in the catabolism of (chloro-)biphenyl and toluene respectively: the *Pseudomonas* sp. strain KKS102 *bph* system (Kikuchi et al. 1993), and the *P. putida* F1 *tod* system (Lau et al. 1993). In these two systems, like that of *Pseudomonas* sp. strain CF600, the genes for conversion of growth substrates to catechols appear to be clustered in a single operon together with the *meta*-pathway genes. However, the *bph* and *tod* operons only code for an abridged version of the *meta*-cleavage pathway and do not include genes for the 4-oxalocrotonate branch (see Table 2), which is not necessary for the metabolites of these two pathways. Comparisons between all of these pathways and the *dmp*-encoded *meta*-cleavage pathway will be included. However, before discussion of the *meta*-cleavage pathway, the *dmp*-encoded phenol hydroxylase that feeds it will be considered.

Initial hydroxylation of phenol

Although some of the first well-studied bacterial aromatic degraders included phenol-degrading pseudomonads that hydroxylate the ring as a prelude to ring-cleavage, little biochemical information about phenol hydroxylases from these bacteria was published. Work that required measurements of phenol hydroxylase activity relied on oxygen-uptake experiments using whole cells. In fact, the first reported purification of a hydroxylase specific to a microbial phenol degradation pathway was for phenol hydroxylase from the yeast *Trichosporon cutaneum* (Neujahr & Gaal 1973). This enzyme is a flavoprotein hydroxylase with properties similar to those of many bacterial flavoprotein hydroxylases used for catabolism of such compounds as *p*-hydroxybenzoate and melilotate (reviewed by Ballou 1982) (Fig. 3).

All of these flavoprotein hydroxylases monooxygenate the aromatic ring at a position *ortho* (or in some cases *para*) to a pre-existing hydroxyl group. The role of the flavin in these enzymes is the activation of molecular oxygen, which is normally unreactive with organic compounds. Activation initially involves reaction of 2-electron reduced flavin with O_2 to form a flavin 4a-hydroperoxide, which is an electrophilic, relatively weak, oxygenating agent (Entsch et al. 1976). Oxygenation by this enzyme-bound species is aided by delocalization of electrons into the ring from the hydroxyl group of the substrate. Conversion of phenolics to dihydroxylated ring-fission substrates by proteins containing flavin as the sole prosthetic group is thus a well-documented occurrence in microbial aromatic metabolism.

This contrasts with oxygenases for compounds, such as benzene, toluene, or phthalate, that are less susceptible to oxygenation by virtue of the lack of an electron-donating substituent on the ring. For these compounds, a more potent enzyme-generated oxy-

Table 2. Compilation of sequence homologies between the *meta*-cleavage pathway proteins of the *dmp*[a]-, *xyl*[b]-, *tod*[c]- and *bph*[d]-systems.

Ferredoxin-like proteins	DmpQ	XylT (64)	–	–	
2,3-dioxygenases, EC 1.13.11.–	DmpB	XylE (84)	TodE (22)	BphC (22)	TodE/BphC (50)
Dehydrogenases (HMSD), EC 1.2.1.–	DmpC	XylG (84)	–	–	
Hydrolases (HMSH)	DmpD	XylF (75)	TodF (64)	BphD (32)	
Hydratases (OEH), EC 4.2.1.80	DmpE	XylJ (89)	TodG (41)	BphE (43)	
CoA-dependent dehydrogenases (ADA), EC 1.2.1.10	DmpF	XylQ (90)	TodI (75)	BphG (78)	
Aldolases (HOA), EC 4.1.3.–	DmpG	XylK (87)	TodH (78)	BphF (81)	
Decarboxylases (4OD), EC 4.1.1.–	DmpH	XylI (89)	–	–	
Isomerases (4OI), EC 5.3.2.–	DmpI	XylH (78)	–	–	

The percent identity of the deduced amino acid sequences when compared with those of the *dmp* system are shown in parenthesis.

[a] The phenol/dimethylphenol pVI150-encoded pathway of *Pseudomonas* sp. CF600: references as in Table 1.

[b] The toluene/xylene pWW0-encoded pathway of *Pseudomonas putida* mt-2: Nakai et al. (1983), Harayama et al. (1991), Harayama & Rekik (1993), Horn et al. (1991), Chen et al. (1992).

[c] The chromosomally-encoded toluene pathway of *Pseudomonas putida* F1: Zylstra & Gibson (1989), Menn et al. (1991), Lau et al. (1993) and Lau (pers. comm.).

[d] The chromosomally-encoded (chloro-)biphenyl pathway of *Pseudomonas* sp. KKS102: Kimbara et al. (1989), Kikuchi et al. (1993).

Homologies not determined in the above references were analysed using the GCG Wisconsin sequence analysis software package.[2]

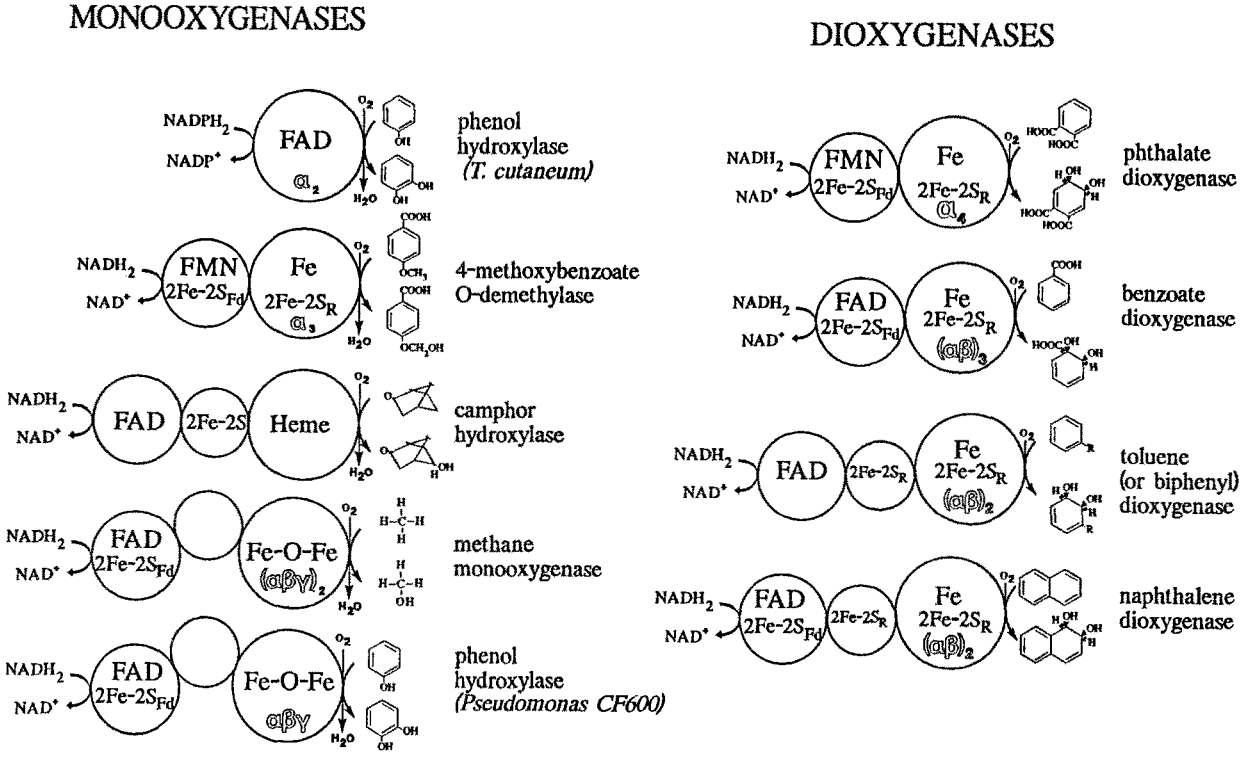

Fig. 3. Composition of representative hydroxylating mono- and di-oxygenases involved in microbial metabolism. All are *Pseudomonas* enzymes except phenol hydroxylase (*T. cutaneum*), and methane monooxygenase (*M. capsulatus* (Bath), *M. trichosporium*). Subscripts denote ferredoxin (Fd) or Reiske (R)- type 2Fe-2S centres. Original references may be found in: Mason & Cammack (1992), Haryama et al. (1992), and Neujahr & Gaal (1973).

genating agent is required. This is commonly thought to be generated by complexation of O_2 with iron in some form at the active site of the enzyme (Fig. 3), although in most cases shown here the nature of the activated oxygen species is poorly defined. One or more electron-transferring proteins are associated with the oxygenating component of each enzyme in order to shunt electrons from NAD(P)H for complete reduction of O_2 (Fig. 3). While these enzyme systems dioxygenate, some examples of multicomponent monooxygenases used for unactivated non-aromatic compounds are also shown in Fig. 3.

In the light of the above, it was quite surprising to find that *Pseudomonas* sp. strain CF600 elaborates a multicomponent phenol hydroxylase that has little in common with single-component flavoprotein hydroxylases. As will be discussed below, while flavoprotein phenol hydroxylases are now known to be used by some other phenol-degrading species of *Pseudomonas*, the multicomponent variety is not restricted to *Pseudomonas* sp. strain CF600.

Phenol hydroxylase: A new aromatic oxygenase

As described earlier, the *dmpKLMNOP* gene products, P0 to P5, were all found to be essential for growth of the catechol-degrading test strain on phenol. Furthermore, a series of strains harbouring plasmids lacking expression of each of the genes in turn confirmed that all six of these genes are required (Nordlund et al. 1990a). However, these results did not rule out the possibility that one or more of these gene products is involved in something other than hydroxylase activity, for example phenol transport.

At the time the nucleotide sequences of these genes were determined, database searches revealed similarity only for the amino terminal of P5 (38.5 kDa) with other proteins. These are plant-type ferredoxins, which are small (11 kDa) electron-transfer proteins containing an iron-sulfur centre that is liganded by four cysteine residues with characteristically well-conserved spacing. Purification of P5 revealed the presence of flavin adenine dinucleotide (FAD) in addition to the expected [2Fe-2S] centre, which was confirmed by visible absorbance spectroscopy to be of the ferredoxin type (Powlowski & Shingler 1990). The purified protein catalyzes the NAD(P)H-dependent reduction of cytochrome *c*, a property that is shared with other proteins containing these prosthetic groups (reviewed in Mason & Cammack 1992). The role of the flavin in these reductases is to accept electrons from the obligate 2-electron donor, NAD(P)H, and presumably to then pass them one at a time to the oxygenase active site on another polypeptide via the [2Fe-2S] centre. In some multicomponent oxygenases the flavin and iron-sulfur centre prosthetic groups may be present on separate polypeptides (Fig. 3).

Since this work was published, sequences for a number of these electron-transferring proteins have appeared, and up-to-date comparisons including P5 can be found in another review in this volume (Williams & Sayers 1994). Possible evolutionary relationships between ferredoxin-$NADP^+$ reductases and P5, as well as other oxygenase reductases, are discussed by Andrews et al. 1992.

Further characterization of phenol hydroxylase, and the roles of the five other polypeptides, P0-P4, required the development of an *in vitro* assay and purification of the individual components. *In vitro* activity of the enzyme was found to be dependent on the addition of NADH, or, less effectively, NADPH, to an assay mixture, in addition to Fe^{+2} (with Fe^{+3} stimulating a lower level of activity).[1] The polypeptides required for hydroxylase activity were identified by assaying crude extracts from strains in which genes for each polypeptide in turn had been deleted. These experiments revealed that of the six polypeptides demonstrated to be required for growth on phenol by a catechol-degrading test strain, only P0 was dispensable for hydroxylase activity (Powlowski & Shingler 1990). The function of P0, whose primary structure does not resemble that of any protein currently in the data bases,[2] is not yet known but roles in phenol transport or hydroxylase regulation are two possibilities.

Purification of P1-P4 (J Powlowski & V Shingler, unpubl.) resolves these polypeptides into two components, one consisting of P2 alone, and the other a complex of P1-P3-P4 (see Fig. 2). The purified proteins are active in the *in vitro* assay when supplemented with the reductase (P5) (Powlowski & Shingler 1990, unpubl.). Visible absorbance spectroscopy showed that none of the purified P1-P4 components appear to contain any of the prosthetic groups commonly associated with microbial aromatic mono- and di-oxygenases (see Fig. 3). However, Fe^{+2} is still required in the assay, raising the possibility that one of the components had lost an intact iron centre: since S^{-2} was not added to the assay, reconstitution of an iron-sulfur centre is not a likely explanation. The presence of a Reiske-type iron-sulfur centre, common in numerous oxygenase components (Fig. 3), is also unlikely, based on sequence

comparisons with other Reiske-centre containing proteins (Powlowski & Shingler 1990).

On the basis of these studies a general similarity between the phenol hydroxylase from this organism and methane monooxygenase was noted (Powlowski & Shingler 1990). Thus, both enzymes are made up of five polypeptides of similar sizes: one is a reductase with FAD and a ferredoxin-type [2Fe-2S] centre; three co-purify, and in the case of methane monooxygenase have been demonstrated to comprise the oxygenase itself; and one is a low molecular-weight polypeptide that can be resolved from the others and which possesses no chromophoric prosthetic group (P2 in phenol hydroxylase, component B in methane monooxygenase) (see Fig. 3). The function of the latter component in methane monooxygenase is somewhat controversial, but appears to be involved in regulation of the oxygenase activity (Green & Dalton 1985; Froland et al. 1992). The sequence similarities between components of these two oxygenases include 28% identity between the reductases of the two systems (Stainthorpe et al. 1989, 1990; Nordlund et al. 1990a). The next highest sequence similarity is found between the P2 and MmoB components of the two systems, which share 27% identity if two large gaps are included to optimise alignment. However, little overall sequence identity is evident between the other polypeptides of phenol hydroxylase and methane monooxygenase, even after inclusion of multiple gaps.

Among the properties not yet known about phenol hydroxylase is the identity of the oxygen-activating prosthetic group. One possible candidate, despite the lack of overall sequence identity, is the binuclear iron centre like that of methane monooxygenase (Fox et al. 1988; Ericson et al. 1988; Rosenzweig et al. 1993). While variants of this centre are found in a number of different enzymes, that found in the α-subunit of the A-component of methane monooxygenase (MmoX) is similar to the binuclear iron centre of ribonucleotide reductase, to which methane monooxygenase appears to be very distantly related (Nordlund et al. 1992). The crystal structure of ribonucleotide reductase revealed that the binuclear iron centre is liganded by two histidine, one aspartate, and three glutamate residues (Nordlund et al. 1990b), while the crystal structure of methane monooxygenase showed ligation by two histidine and four glutamate residues (Rosenzweig et al. 1993). The spacing of these ligands is conserved in methane monooxygenase and ribonucleotide reductase, as well as in the P3 polypeptide of phenol hydroxylase (Fig. 4). Despite the lack of overall sequence identity between ribonucleotide reductase and methane monooxygenase, the active site structures of the two enzymes are quite similar (Nordlund et al. 1992; Rosenzweig et al. 1993). Therefore the lack of strong sequence identity around the putative binuclear iron centre ligands of P3 is not particularly worrisome. It is interesting to note that Thr-213 of methane monooxygenase, thought to play an important role in some dioxygen-activating centers is conserved as Thr-204 in P3 (Fig. 4; Rosenzweig et al. 1993, and references therein).

Several polypeptides of phenol hydroxylase also share low-level sequence homology with polypeptides of toluene-4-monooxygenase from *Pseudomonas mendocina* KR1. The sequence similarities between these two proteins have been noted previously and include homologies with the P1, P2, P3 and P5 polypeptides of phenol hydroxylase (Yen et al. 1991; Yen & Karl 1992). It is interesting to note that putative binuclear iron centre ligands are also found in TmoA of toluene monooxygenase, which shares 35% overall sequence identity with P3 (Fig. 4). Although the presence of a binuclear iron centre in toluene and phenol hydroxylases must be confirmed by biochemical studies of the purified proteins, it appears that oxygenases containing this centre are not limited to methane monooxygenase.

Phenol hydroxylases from other bacterial strains

The oxygenation of electron-rich phenol is a considerably easier task than the oxygenation of toluene or methane. Considering that a single FAD-binding polypeptide is so commonly used by microbes for hydroxylation of phenolics, it is at the moment puzzling that *Pseudomonas* sp. strain CF600 elaborates such a complex phenol hydroxylase. Indeed, two other species of *Pseudomonas*, *P. pickettii* PKO1 and *Pseudomonas* EST1001, have clearly been shown to utilize phenol hydroxylases that are single-component flavoproteins related to other aromatic flavoprotein hydroxylases (Kukor & Olsen 1992; Nurk et al. 1991). Further study of the multicomponent hydroxylase might hint at what advantages are conferred to compensate for the energetic expense of synthesizing six polypeptides rather than one.

These considerations prompted us to ask whether the multicomponent phenol hydroxylase is an orphan or whether other phenol degrading bacteria also use related multicomponent phenol hydroxylases. Reports in the literature indicated that not all bacterial phenol

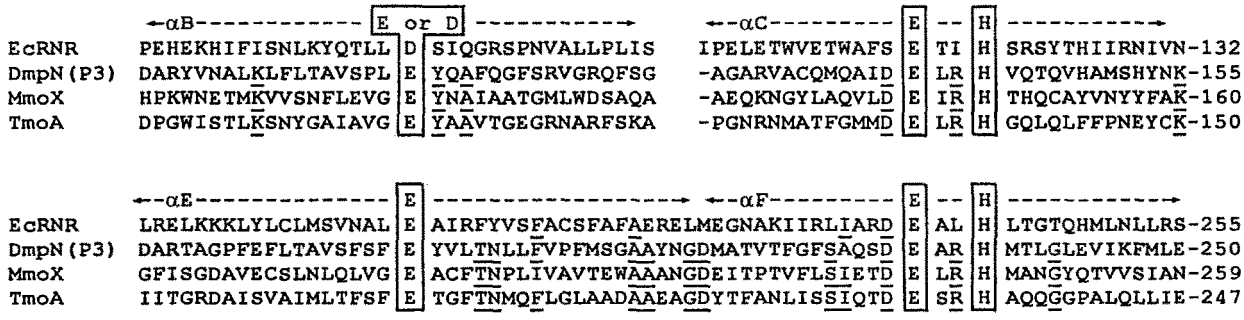

Fig. 4. Conservation of ligands for the binuclear iron centre of different enzymes. The consensus sequence and extent of helices of ribonucleotide reductase are taken from Nordlund et al. (1990b). *E. coli* ribonucleotide reductase (EcRNR, Carlson et al. 1984), DmpN (P3, Nordlund et al. 1990a), MmoX (Stainthorpe et al. 1990; Rosenzweig et al. 1993), TmoA (Yen et al. 1991). Ligating residues are boxed, other residues present in at least three out of the four sequences shown are underlined.

hydroxylases are simple flavoproteins (Gurujeyalakshmi & Oriel 1989), and that some multicomponent oxygenases for compounds like toluene are capable of turning over phenols (Spain & Gibson 1988). Considering the surprising complexity of the phenol hydroxylase revealed by our studies, it seemed unlikely that similar enzymes would readily have been isolated, especially if a single-component enzyme was expected. In order to address this question, DNA from a collection of phenol-degrading bacteria was probed for the presence of each of the phenol hydroxylase genes.

Gene-probing experiments were performed on eleven phenol-utilizing bacteria in addition to *Pseudomonas* sp. strain CF600. One of these strains was *P. putida* U, the archetypal phenol-degrader whose phenol hydroxylase has not been characterised. The other ten were marine strains collected off the Norwegian coast. Southern hybridization analysis demonstrated that five of the marine isolates as well as *P. putida* U all possess DNA highly homologous to each of the components of the *dmp*-encoded phenol hydroxylase. Furthermore, all of the strains that tested positive were also found to posses DNA highly homologous to all nine *meta*-cleavage pathway genes of the *dmp*-operon, and to the specific regulator of phenol catabolism of this strain (Nordlund et al. 1993).

The nucleotide sequence of the chromosomally-encoded phenol hydroxylase region from another phenol-degrading pseudomonad, *P. putida* P35X, has also recently been determined (Ng et al. 1993). This strain, like *Pseudomonas* sp. strain CF600, utilizes a multicomponent phenol hydroxylase and a *meta*-cleavage pathway. The deduced amino acid sequences from the six open reading frames of the phenol hydroxylase region share between 89% and 99.7% identity with those of P0 to P5 (LC Ng, pers. comm.).

It is interesting to note that all of these organisms catabolize phenol using a *meta*-cleavage pathway, while marine isolates that degrade phenol via an *ortho*-cleavage pathway lack DNA homologous to the multicomponent phenol hydroxylase. However, this arrangement does not always hold, as a single-component flavoprotein phenol hydroxylase has been shown to be associated with the *meta*-cleavage pathway of *P. pickettii* PKO1 (Kukor & Olsen 1991). Nevertheless, it is now clear that the multicomponent phenol hydroxylase is not unique to *Pseudomonas* sp. strain CF600. In all likelihood related enzymes will turn out to make up a rather significant fraction of the hydroxylases used in aromatic metabolism.

The meta-cleavage pathway for catechol

The biochemical route of the *meta*-cleavage pathway following phenol hydroxylation is illustrated in Fig. 1, and the function of the *dmp*-encoded enzymes involved are summarised in Table 1. With the exception of isofunctional genes homologous with *dmpB* and *dmpQ*, no other *meta*-cleavage enzyme gene sequences had been published prior to work with the *dmp* system. Furthermore, only the amino acid sequence of DmpC was similar enough to other sequences in the databases to deduce a function with any confidence. Hence assignment of the functions of individual genes relied almost exclusively on correlating enzymatic activity with expression of individual genes. Although the majority of the enzyme activities of the *meta*-cleavage pathway had been discovered by the mid-seventies, this

type of analysis of the *dmp* operon led to the discovery of a new *meta*-cleavage pathway enzyme, aldehyde dehydrogenase (acylating) (ADA, see Fig. 1, Table 1).

In the following sections we will review the individual enzymes of the pathway, and where appropriate, or where evidence is lacking for the *dmp*-encoded enzymes, isofunctional enzymes of other *meta*-cleavage operons. In particular, reference will be made to the four *meta*-cleavage pathway systems shown in Table 2, for which the sequences of all *meta*-cleavage pathway genes are known.

Catechol-2,3-dioxygenase: Cleavage of the aromatic ring

This enzyme catalyzes the critical ring-opening step of the *meta*-cleavage pathway (Fig. 1), and contains non-heme Fe^{+2} at the active site (reviewed by Yamamoto & Ishimura 1991). The *dmpB*-encoded enzyme shares 83–87% sequence identity with catechol 2,3-dioxygenases from other *meta*-cleavage pathways of different *Pseudomonas* species (Bartilson & Shingler 1989). Much lower levels of sequence similarity are shared with other *meta*-cleavage dioxygenases (Table 2) that form a different evolutionary subgroup. Evolutionary relationships, and up-to-date comparisons of different ring cleavage 2,3-dioxygenases are reviewed in this volume by Williams & Sayers (1994). Despite the fact that catechol-2,3-dioxygenase was the first *meta*-cleavage pathway enzyme to be isolated and crystallized, no structure or detailed mechanism has yet been elucidated for any catechol-2,3-dioxygenase. However, some progress has been made in manipulating the substrate specificities of the *xylE*-encoded catechol 2,3-dioxygenase using genetically-selected mutants and chimeric proteins (Wasserfallen et al. 1991; Williams et al. 1990).

DmpQ: A protein with homology to plant-type ferredoxin

The *dmpQ* gene, located between the genes encoding phenol hydroxylase and catechol-2,3-dioxygenase, encodes a polypeptide about the size of, and with up to 41% sequence identity with, plant-type ferredoxins (Shingler et al. 1992). Homologous proteins, encoded in analogous locations of the *xyl* and *nah* operons, share 64% and 52% identity with DmpQ (Harayama & Rekik 1993; Harayama et al. 1991; You et al. 1991). Studies with strains in which *dmpQ* has been deleted from the operon indicate that expression of this protein is required to allow strains to grow at the expense of 4-methylphenol or 3,4-dimethylphenol, but not phenol, 2-methylphenol or 3-methylphenol (V Shingler & J Powlowski unpubl.).

This growth pattern is reminiscent of that recently reported for mutants of the *xyl* homologue (*xylT*), namely lack of growth on 4-methyl substituted substrates. The *xyl* homologue has been shown, using *in vivo* studies, to be involved in reactivation of catechol-2,3-dioxygenase (Polissi & Harayama 1993). This enzyme is particularly sensitive to inactivation during turnover with 4-substituted catechols, probably by inadvertent oxidation of the active-site ferrous iron. Reactivation is postulated to involve transfer of electrons from an as-yet unidentified donor through XylT to the oxidized iron. Considering the high degree of similarity between the *dmp* and *xyl* operons, and the qualitatively similar growth patterns of *dmpQ* and *xylT* mutants, it is likely that DmpQ performs the same function as XylT. It is interesting to note, that while the sequence identity between the catechol 2,3-dioxygenases in the two strains is 84%, XylT and DmpQ share only 64% identity. It is possible that these ferredoxins have evolved to accommodate interaction with different electron donors in the two strains. Hence the greater divergence of XylT and DmpQ might reflect these accommodations rather than a response to structural differences between the corresponding catechol-2,3-dioxygenases with which they presumably interact.

The presence of the ferredoxin-like protein in the *xyl*-, *dmp*- and *nah*-encoded pathways supports the idea that inclusion of these redox proteins represents a bacterial strategy to expand the substrate specificity of the *meta*-cleavage pathway to include compounds chanelled through the otherwise suicidal 4-methylcatechol (Polissi & Harayama 1993). In this respect the observation that neither the *tod*- nor the *bph*-abridged *meta*-pathway operon appears to encode a similar protein is illuminating (Lau et al. 1993; Kikuchi et al. 1993). In both of these enzyme systems, no compounds that would be metabolised via *para*-substituted intermediates have been shown to be substrates of the respective pathways.

2-Hydroxymuconic semialdehyde dehydrogenase (HMSD) and hydrolase (HMSH): gatekeepers at the pathway branch point

These enzymes both use ring cleavage products as substrates, which are 2-hydroxymuconic semialdehyde from catechol, 5-methyl-2-hydroxymuconic semialdehyde from 4-methylcatechol, and 2-hydroxy-6-oxo-2,4-heptadienoate from 3-methylcatechol. Since the ring-cleavage product of 3-methylcatechol is a ketone, rather than an aldehyde, it cannot be further oxidized by the dehydrogenase, and must therefore be metabolised via the hydrolytic route (Fig. 1). This was originally shown using hydrolase-defective strains of *P. putida* U that failed to grow at the expense of phenols that are channelled via the *meta*-cleavage pathway through 3-methylcatechol (Bayly & Wigmore 1973). Despite the potential for use of either branch for the ring-cleavage products of catechol or 4-methylcatechol, these compounds were shown to be preferred substrates for the dehydrogenase, rather than the hydrolase of the *meta*-cleavage pathway of *P. putida* U (Sala-Trepat et al. 1972). Conclusions from these in vitro results were reinforced by the observation that mutants of *P. putida* U defective only in the 4-oxalocrotonate branch failed to grow at the expense of phenols channelled through catechol or 4-methylcatechol (Bayly & Wigmore 1973).

These conclusions are also apparently valid for the *dmp*-encoded *meta*-cleavage pathway. Thus, a deletion within the HMS-dehydrogenase gene (*dmpC*), or either of the genes for the other two enzymes of the 4-oxalocrotonate branch (*dmpI* and *dmpH*), resulted in strains that grew on 2-methyl, 3-methyl and 3,4-dimethylphenols, but not on phenol or 4-methylphenol. On the other hand, strains deleted within *dmpD*, the gene encoding the HMS-hydrolase, grew on phenol and 4-methylphenol, but not at the expense of the other substrates (V Shingler & J Powlowski unpubl.).

Despite the structural similarity of the substrates of both enzymes, the lack of sequence homology between HMS-hydrolase and HMS-dehydrogenase indicates different evolutionary origins. The amino acid sequence of HMS-dehydrogenase shows approximately 40% identity with various eukaryotic aldehyde dehydrogenases (Nordlund & Shingler, 1990), and 84% identity with *xyl*-encoded HMS-dehydrogenase (Horn et al. 1991). Speculations about the possible evolutionary significance of this similarity have been presented for the *xylG*-encoded enzyme (Horn et al. 1991), and can equally well be applied to the *dmpC*-encoded counterpart. Sequence comparison searches for HMS-hydrolase, on the other hand, only readily identify isofunctional hydrolases including the *xyl* and *tod*-encoded enzymes, as well as hydrolases for phenyl-substituted 2-hydroxymuconic semialdehyde, which is an intermediate in microbial biphenyl metabolism (Table 2). In the latter case, the sequence identity is considerably lower than that for the other HMS-hydrolases, possibly reflecting differences in substituent bulkiness on the substrate.

While information about structure-function relationships for any HMS-hydrolase or HMS-dehydrogenase is non-existent, the sequence comparisons suggest avenues for further experimentation. Consider first the striking similarity between HMS-dehydrogenase and eukaryotic aldehyde dehydrogenases. While chemical modification studies have implicated a number of residues in enzyme activity, including Cys-302 and Glu-268 of horse aldehyde dehydrogenase (conserved as Cys-288 and Glu-254 in *dmp*-encoded HMS-dehydrogenase), assignment of definite roles for these residues has been difficult (reviewed in Hempel & Jörnvall 1989). By analogy with glyceraldehyde-3-phosphate dehydrogenase, the mechanism of aldehyde dehydrogenase has often been assumed to involve formation of a thiohemiacetal enzyme-substrate intermediate that transfers a hydride equivalent to NAD^+, and is then hydrolyzed from the enzyme. However, compelling evidence has been presented that an active-site serine residue of sheep-liver aldehyde dehydrogenase is involved in formation of an acyl-enzyme intermediate (Loomes et al. 1990). It is interesting that the serine residue implicated in these studies is conserved in aldehyde dehydrogenase isozymes (Johansson et al. 1988), while it is not conserved in the HMS-dehydrogenase sequence. HMS-dehydrogenase thus provides a new variation with which to test roles proposed for the various conserved aldehyde dehydrogenase residues, and to probe the involvement of an active-site serine residue in catalysis.

The participation of an active site serine residue in HMS-hydrolase catalysis has also been suggested on the basis of low sequence identity (20% overall) of the isofunctional *xyl*-encoded enzyme with atropine esterase, a serine hydrolase (Horn et al. 1991). This observation can be extended to note that Ser-107 of the *dmp*-encoded HMS-hydrolase lies within an active-site lipase consensus sequence GXSXG (reviewed by Derewenda & Sharp 1993). HMS-hydrolase shares 24% overall identity with a lipase from *Moraxella* (Feller et al. 1991). The serine residue within the lipase

consensus sequence is coupled in lipases to Asp (or Glu) and His, much like the archetypal catalytic triad of serine proteases (see Derewenda & Sharp 1993). On the basis of these comparisons it is conceivable that the mechanism of HMS-hydrolase, which cleaves a carbon-carbon bond, is similar to the mechanisms of esterases, in which a carbon-oxygen bond is cleaved. It is interesting to note that the lipase consensus sequence with a Cys replacing Ser is a motif conserved in the *ortho*-cleavage pathway enzyme dienelactone hydrolase (Pathak & Ollis 1990).

4-Oxalocrotonate isomerase (4OI): a small but essential participant in the HMS-dehydrogenase initiated branch

4-oxalocrotonate isomerase (4OI) catalyzes the isomerization of 4-oxalocrotonate, the product of HMS-dehydrogenase, to 2-keto-3-hexenedioate (Fig. 1). Studies using mutants of *P. putida* U lacking 4OI activity showed that this enzyme is necessary to support growth on phenols channelled through catechol or 4-methylcatechol, which are metabolized via this branch (Wigmore et al. 1974). As indicated earlier, this is also the case for the *dmp*-encoded pathway, since a deletion within the 4OI-encoding gene (*dmpI*) of the operon prevented the resulting strain from growing at the expense of phenol and 4-methylphenol. These results demonstrate the indispensability of 4OI, which is significant since the non-enzymatic isomerization reaction is rather fast and could have been sufficient to support growth in the absence of a specific enzyme (Sala-Trepat & Evans 1971).

The sequence of 4OI shares 78% identity with the isofunctional *xylH*-encoded enzyme, but no similarity is found with other protein sequences. 4-Oxalocrotonate isomerase encoded by the *xyl* operon has been isolated and partially characterized (Chen et al. 1992). Mechanistic studies suggest that the isomerization of 2-oxo-4-*trans*-hexenedioate proceeds via the enol form of the substrate shown in Fig. 1, and in this respect at least, 4OI resembles 3-oxo-Δ^5-steroid isomerase, as well as other isomerases (Bayly & Barbour 1984; Whitman et al. 1991). It will be interesting to compare the properties of these two isofunctional 4OIs, as 22% of the amino acid sequence is not identical, in order to see what conclusions can be drawn about structure-function relationships. X-ray structures of both the *xyl*-encoded enzyme (Davenport & Whitman 1993) and the *dmp*-encoded enzyme (D. Roper unpubl.) are currently being sought.

4-Oxalocrotonate decarboxylase (4OD) and 2-oxopent-4-dienoate hydratase (OEH): A complex of related polypeptides

4OD catalyzes the final step in the 4-oxalocrotonate branch, while OEH is involved in the subsequent reaction, which occurs after the 4-oxalocrotonate and hydrolytic branches merge (Fig. 1). The first attempted purification of OEH, from phenol-grown *P. putida* U, was reported by Collinsworth et al. (1972). The enzyme proved difficult to purify, and could not be resolved from some persistent contaminants. One of the contaminants was undoubtedly 4OD, as years later it was shown that *xyl* operon-encoded 4OD and OEH were tightly enough associated with each other to co-purify. As the unstable product of 4OD is the substrate for OEH, it has been proposed that the physical association between the two enzymes has arisen to channel the unstable intermediate (Harayama et al. 1989). However, as these workers pointed out, HMS-hydrolase of the hydrolytic branch generates the same intermediate, and this enzyme is not tightly associated with the hydratase (OEH) in the *xyl*-encoded system. A loose association that still allows metabolite channelling has not been ruled out.

Sequencing of the genes for OEH and 4OD from the *dmp*operon suggested a possible evolutionary explanation for the association of these two polypeptides (Shingler et al. 1992). As is the case for the *xyl*-encoded homologues, the *dmp*-encoded enzymes are also tightly associated with each other, and co-purify (J. Powlowski unpubl., but see Fig. 2). When optimally aligned, their deduced amino acid sequences show 37% identity, indicating common ancestry. It is therefore possible that these enzymes evolved from a protein that was originally multimeric, and this may be why they are so tightly associated with each other. The importance of channelling, which could result from such an arrangement, remains to be demonstrated. If metabolite channelling is indeed important it may have played a role in maintaining selective pressure on the multimeric form of the enzymes.

It seems rather remarkable that an enzyme that catalyzes carbon-carbon bond cleavage is related to one that involves hydration of a double bond. However, similar electron shifts can reasonably be postulated for these two reactions (Fig. 5A, Dagley 1975), so evolution of the two enzymes from a common ancestor would perhaps not be too surprising. In both cases, the keto group of the substrate could act as an electron sink, especially when complexed with a metal

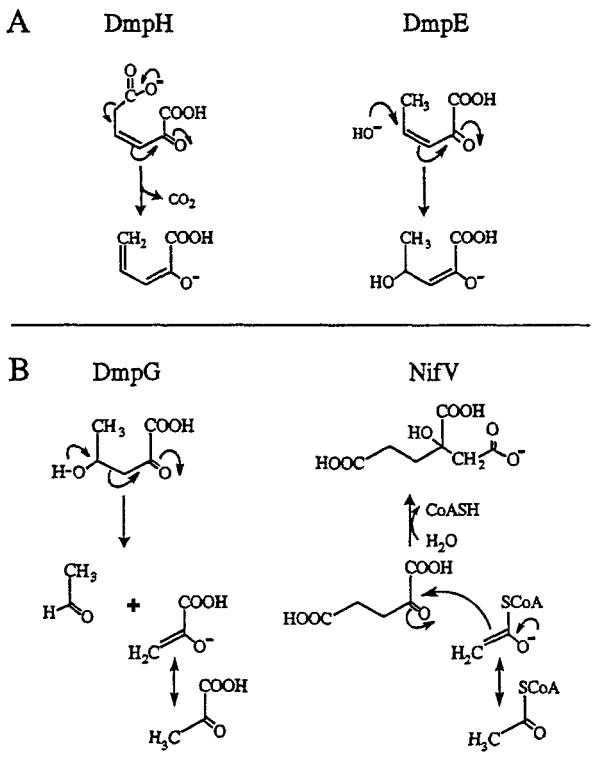

Fig. 5. Comparisons of reactions catalyzed by DmpE (OEH) and DmpH (4OD) (A), and DmpG (HOA) and NifV (B). While the mechanisms of these enzymes are as yet not known, these reasonable possibilities are illustrated for comparative purposes (see text).

ion, a requirement for which has been demonstrated for both the decarboxylase (4OD, Sala-Trepat & Evans 1971; Harayama et al. 1989) and the hydratase (OEH, Collinsworth et al. 1972; Harayama et al. 1989). However, it is not currently known whether the isomer of the hydratase substrate shown in Fig. 5A is catalytically competent. 2-Keto-4-pentenoate, which is the form shown in Fig. 1, is in rapid equilibrium with the enol form, 2-hydroxy-2,4-pentadienoate (Marcotte & Walsh 1978), which is competent for turnover by OEH (Harayama et al. 1989). A solution of these isomers decays to *trans*-2-keto-3-pentenoate (Marcotte & Walsh 1978) which is probably the form not metabolised by the enzyme (Harayama et al. 1989). The involvement of *cis*-2-keto-3-pentenoate (the isomer shown in Fig. 5A), as a substrate or transient intermediate in the OEH catalyzed reaction, has not been specifically addressed.

It is worth emphasizing that from comparisons of the sequences of all *meta*-pathway proteins, *dmpE* (hydratase, OEH) and *dmpH* (decarboxylase, 4OD) represent the only example of possible evolution by gene duplication. None of the other protein sequences show significant similarity with each other. Hence, gene duplication and divergence were not foremost in evolution of this pathway (Shingler et al. 1992). In this respect the comparatively low homology between the hydratase (OEH)-encoding genes of the *dmp*-encoded *meta*-pathway and those of the abridged *tod*- and *bph*-encoded *meta*-pathways is notable (Table 2). Metabolism of the substrates of the *tod* and *bph*-systems does not require the 4-oxalocrotonate branch of the pathway, and in neither case do the respective operons include genes for this branch (Lau et al. 1993; Kikuchi et al. 1993, pers. comm.). Therefore, in these abridged *meta*-pathways the hydratase counterparts, which use exactly the same substrate as the *dmp*-encoded enzyme, would have evolved under different conditions i.e., in the absence of a physically-associated decarboxylase partner.

4-Hydroxy-2-ketovalerate aldolase (HOA) and aldehyde dehydrogenase (acylating) (ADA): A second example of physical association and a new meta-cleavage pathway enzyme

Close physical association has also been conclusively demonstrated for the last two enzymes of the *dmp*-encoded pathway, 4-hydroxy-2-ketovalerate aldolase (HOA) and aldehyde dehydrogenase (acylating) (ADA), which generate the end-products pyruvate and acetyl-CoA, respectively. Like the two preceeding enzymes, HOA and ADA purify to homogeneity (Powlowski et al. 1993, Fig. 2), but unlike them do not share common ancestry. Close association in this case may be required to channel the aldehyde produced by the aldolase either to reduce toxicity or to ensure that it is metabolized by ADA, and not some less efficient route (see below). A consequence of this association appears to be that the activity of HOA is regulated by NAD^+, and to a lesser extent NADH, which are used not by the aldolase itself, but by its partner, ADA (Powlowski et al. 1993). The mechanism by which this occurs has not yet been elucidated, but conceivably involves allosteric interactions. This phenomenon, and the possibility of channelling, may be significant for modulation of pathway activity at the enzyme level.

Aldolases in general can be divided into two classes: those that require a metal ion, and those that rely on an active-site lysine, for interaction with the keto-group of the substrate to provide an efficient electron sink (Walsh 1979). It has long been known that the

meta-cleavage aldolase, HOA, requires a metal ion for activity. While that from *P. putida* U is apparently stimulated by Mg^{+2} (Dagley & Gibson 1965), the *dmp*-encoded HOA is stimulated by Mn^{+2}, with no increase in activity observed using Mg^{+2} (Powlowski et al. 1993). However, the enzyme is still active in the absence of added metal ions, and no attempt has yet been made to correlate metal content of the enzyme with activity.

The *dmp*-encoded HOA shares low sequence homology with enzymes that catalyze reactions that are mechanistically the reverse of the aldol cleavage reaction catalyzed by HOA (Shingler et al. 1992, and references therein). One example is NifV, which is thought to encode homocitrate synthase that catalyzes Claisen condensation of acetyl-CoA and α-ketoglutarate (Fig. 5B).

For years the *meta*-cleavage pathway has been depicted as ending at pyruvate and acetaldehyde (or propionaldehyde in the case of 4-methyl-substituted growth substrates). Work with the *dmp* operon has uncovered the existence of a new *meta*-cleavage operon encoded enzyme that converts the aldehyde to the acyl-CoA derivative using NAD^+ and coenzyme A (ADA; Shingler et al. 1992; Powlowski et al. 1993). Metabolism in other organisms involves oxidation of acetaldehyde to acetate, followed by ATP-dependent conversion to the CoA ester (reviewed in Nunn 1987). The ATP-independent reaction catalyzed by ADA thus represents an energetically efficient mechanism for catabolism of the short-chain aldehyde formed by the pathway. However, *Pseudomonas* CF600 grown at the expense of phenol also has low levels of enzymes involved in an ATP-dependent pathway, so some metabolism of acetaldehyde or propionaldehyde by these enzymes is possible. It is difficult to test directly whether or not the *dmp*-enzyme is absolutely necessary, as the close coupling of ADA activity with HOA activity means that mutations or deletions within the ADA-gene also affect HOA activity second-hand (Shingler et al. 1992).

Apart from recently-discovered isofunctional enzymes (Table 2), the sequence of aldehyde dehydrogenase (acylating) (ADA) is not similar to any other proteins so far entered into the databases. However, the amino-terminal of the protein may encompass the NAD^+-binding site, as it contains a characteristic ADP-binding fingerprint sequence (Wierenga et al. 1986, Shingler et al. 1992).

Sequence information is available for at least one other bacterial CoA-dependent dehydrogenase, namely methylmalonate semialdehyde dehydrogenase (Steele et al. 1992), and its rat counterpart (Kedishvili et al. 1992). It is interesting that these sequences show higher similarity to the CoA-independent eukaryotic aldehyde dehydrogenase, than to the *dmp*-encoded CoA-dependent dehydrogenase, ADA. This suggests at least two separate evolutionary origins for the CoA-dependent dehydrogenases, possibly involving one ancestral NAD^+-binding protein and another that bound CoA. It is perhaps even more intriguing that the CoA-independent HMS-dehydrogenase from the *dmp*-encoded pathway is also related to eukaryotic aldehyde dehydrogenases but not to the CoA-dependent dehydrogenase (ADA). When the two *dmp*-encoded dehydrogenases are directly compared they show $<$ 20% identity even with insertion of sixteen gaps to optimise the alignment. Thus, while these two dehydrogenases of the *meta*-cleavage pathway might have been predicted to have evolved from a common ancestor, sequence comparisons indicate they have not. It will be interesting to compare the sequences of other biochemically-characterized bacterial CoA-dependent dehydrogenases, e.g., *Clostridium* acetaldehyde dehydrogenase (acylating) (Burton & Stadman 1953), when they become available.

Transcriptional regulation of the dmp operon

Transcription of the *dmp* operon is tightly regulated by the divergently transcribed *dmpR* gene product (see Fig. 1). As with other regulators of aromatic catabolism, the activity of the regulator is itself modulated by the presence of aromatic compounds to allow expression of the specialized catabolic enzymes only when the substrates of the pathway are present (Shingler et al. 1993). In this capacity the aromatic compound serves as an effector molecule. For a given compound to support growth as the sole carbon and energy source, it must therefore serve as both an effector for the regulator and as a substrate for the enzymes of the pathway. Hence, the effector recognition specificity of the regulator, in addition to specificities of the catabolic enzymes, is intimately involved in determining the range of compounds that can be degraded by a metabolic pathway. This has been most elegantly exploited in manipulation of the pWW0-encoded XylS regulator of benzoate metabolism. Studies of this regulator have shown that new substrates for the pWW0-encoded pathway could be selected by mutational expansion of the effector specificity range of XylS (Abril et al. 1989; Ramos et al. 1986, 1990).

DmpR, like the pWW0-encoded XylR regulator of toluene and xylene catabolism, belongs to the extensive NtrC-family of transcriptional activators (Shingler et al. 1993). Members of this family respond to diverse environmental signals to regulate genes involved in a variety of physiological processes. These transcriptional activators act by binding to enhancer-like elements, and regulating transcription from a distinct set of promoters recognised by σ^{54}-holoenzyme RNA polymerase (reviewed in North et al. 1993). As with eukaryotic enhancer-binding proteins, members of the bacterial NtrC family are composed of distinct functional domains. The central and carboxy-terminal domains of these activators are conserved among all members of the family and are believed to be involved in interaction with the σ^{54} RNA polymerase and in DNA binding, respectively. In many members of the family the amino-terminal A domains are the sites of transfer of the signals that are received via sensory proteins. However, the A domain of DmpR shares 64% homology with the equivalent domain of XylR, but does not share homology with other members of the NtrC family or other proteins in the data bases (Shingler et al. 1993). The unique A-domain homology shared by DmpR and XylR, in conjunction with the signal receptor function of the A-domains in other members of the family, suggested that this region is involved in the activation of DmpR and XylR by their respective aromatic effectors. Studies of the response of hybrid DmpR/XylR regulators, in which all or parts of the A-domain of DmpR were exchanged with that of XylR, have now clearly shown that the distinct effector recognition specificities of the two regulators spans and completely resides within the amino terminal 234 residues (Shingler & Moore 1994).

Both DmpR and XylR have broad effector specificity, but respond differentially depending on the position and nature of the substituent(s) on the aromatic ring of the effector (Abril et al. 1989; Shingler & Moore 1994). In the case of DmpR the response to phenolic compounds substituted in the *para*-position is relatively poor, suggesting that the inefficient growth observed on these compounds might be due to low expression of the catabolic enzymes. This is apparently the case, since a *Pseudomonas* strain carrying the *dmp* system on an RSF1010-based plasmid of 16–20 copies/cell has a generation time of 66 minutes in minimal media containing 4-methylphenol as the carbon source. This compares favourably with the 132 minute generation time of this strain harbouring the 1–2 copy number pVI150 wild-type plasmid (H. Pavel & V. Shingler unpubl.). However, the improved 4-methylphenol degrader was unable to grow on phenol, a phenomenon associated with hyperproduction of the phenol hydroxylase in response to the efficient effector property of phenol. Although not specifically tested, growth inhibition in this case may be due to interference with aromatic amino acids, a possibility suggested by the observation that expression of phenol hydroxylase in *E. coli* results in accumulation of a brown mixture of pigments produced from aromatic amino acids in the media (Powlowski & Shingler 1990). The detrimental effects of wholesale over-expression in this case suggests that more subtle modifications, such as effector specificity mutants, are likely to be more successful in construction of strains with all-round improved degradative properties.

Most aromatic catabolic regulators so far analysed are members of one of three families of bacterial transcriptional regulators: the LysR family e.g., NahR, CatM, CatR, TcbR, TfdS and ClcR (see Coco et al. 1993, and references therein), the AraC family e.g., XylS (Inouye et al. 1986; Ramos et al. 1990), or the NtrC family e.g., XylR and DmpR (Inouye et al. 1988; Shingler et al. 1993). For a long time XylR was the sole example of an NtrC-like regulator. However, as described earlier, gene-probing experiments using a *dmpR* A-domain gene probe identified highly homologous DNA associated with the phenol catabolic genes of *P. putida* U and five marine isolates (Nordlund et al. 1993). Similarly, a regulatory region with high homology to *xylR* has been located upstream of the phenol catabolic genes of *P. putida* P35X (Ng et al. 1993). Therefore, the NtrC-like transcriptional activators promise to feature more prominently in the future with respect to regulation of aromatic catabolism.

Concluding remarks

Current knowledge of *meta*-cleavage pathway enzymology, and to some extent chemistry, lags behind genetic characterization of the pathway. However, analysis of the genes of the complete pathway, and comparisons of the amino acid sequences of related proteins, have identified potentially important residues for many of these enzymes. Moreover, cloning and over-expression of these genes can help provide large quantities of the proteins for enzymological and structural studies. In the absence of structural data, the information gained from genetic analysis provides a rational basis to begin testing the importance of residues iden-

tified as, for example, potentially ligating prosthetic groups, interacting with co-factors, or being involved in catalysis. One caveat is that since a number of the enzymes of the *meta*-cleavage pathway appear to be unrelated to other proteins, and others are associated with each other, it is particularly important to initially characterize the enzymes from the parent strain before proceeding to large-scale over-expression systems.

A fuller understanding of *meta*-cleavage pathway enzymology also depends on better characterization of the lower pathway chemistry. At the very least, better methods for preparation of some of the enzyme substrates are required (e.g., Powlowski et al. 1993). In addition, since many of these substrates can exist as keto-enol tautomers and/or *cis-trans* isomers, they must be very carefully characterized. In this respect the recent work of Whitman and colleagues with 4-oxalocrotonate isomerase is exemplary (Whitman et al. 1991; Chen et al. 1992).

One of the particularly interesting properties of some of the *meta*-cleavage pathway enzymes, shown in this and other work, is the formation of tight multienzyme complexes. In the *dmp* system tight associations between the related polypeptides DmpH and DmpE (4OD and OEH), and the unrelated DmpF and DmpG polypeptides (ADA and HOA) have been identified (see Fig. 2). In both cases the enzyme pairs catalyse sequential steps of the pathway and the member of each pair that makes the substrate for its partner is apparently dependent on the presence of that partner for activity (Shingler et al. 1992; Harayama et al. 1989). In the case of the DmpF/DmpG pair, cross-regulation of enzyme activity is possible (Powlowski et al. 1993). These properties suggest that metabolic channelling may be important for pathway efficiency, thus providing additional evolutionary impetus for clustering of the genes involved into an operon. Co-purification of enzyme activities through multiple steps of purification only identifies tight associations and it may well be that other enzymes of the pathway are also associated, albeit loosely. The existence of these associations, and the so-far unproven idea of metabolite channelling, certainly merit further attention.

The application of microbial metabolic activities to detoxification and clean-up has stimulated much interest in construction of strains with improved degradative efficiency or expanded catabolic capacity. While changes in substrate specificities of enzymes or effector recognition by regulators have been successful, little has been reported on genetic manipulation of strains for improved efficiency. One possible strategy is simple over-expression of the enzymes involved. The observation of complexing between *meta*-cleavage pathway enzymes has obvious practical implications for this type of work. In addition, the cross-regulation evident between the DmpF/DmpG pair highlights the fact that very little is really known about (potential) regulation at the enzyme level in this pathway. This will undoubtedly be remedied as progress on pathway enzymology is made. Finally, the toxic effects of over-expression of some of the enzymes (e.g., phenol hydroxylase) suggest further hindrances to increasing strain efficiency by brute force over-expression.

It is when manipulations for practical applications are considered that the importance of a comprehensive knowledge of pathway genetics, enzymology, and chemistry is realized. The current boom in knowledge of the genetics of the *meta*-cleavage pathways, and their associated oxygenases, contributes enormously to bringing this goal closer for at least one type of model system.

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Notes

1. In the original reference (Powlowski & Shingler 1990), the metal ion and NADH concentrations in the *in vitro* assay was given in mM. This was a typographical error, and the concentrations should have read 100μM and 280μM respectively.

2. Computer searches of the EMBL/GenBank (release 35) were made using the TFastA program of the GCG Wisconsin sequence analysis software package. One to one protein sequence alignments were performed using the BestFit program.

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