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Comamonas testosteroni BR6020 possesses a single genetic locus for extradiol cleavage of protocatechuate

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A key intermediate for biodegradation of various distinct aromatic growth substrates in *Comamonas testosteroni* is protocatechuate (Pca), which is metabolized by the 4,5-extradiol (*meta*) ring fission pathway. A locus harbouring genes from *C. testosteroni* BR6020 was cloned, dubbed *pmd*, which encodes the enzymes that degrade Pca. The identity of *pmdAB*, encoding respectively the α- and β-subunit of the Pca ring-cleavage enzyme, was confirmed by N-terminal sequencing and molecular mass determination of both subunits from the separated enzyme. Disruption of *pmdA* resulted in a strain unable to grow on Pca and a variety of aromatic substrates funnelled through this compound (*m*- and *p*-hydroxybenzoate, *p*-sulfobenzoate, phthalate, isophthalate, terephthalate, vanillate, isovanillate and veratrate). Growth on benzoate and *o*-aminobenzoate (anthranilate) was not affected in this strain, indicating that these substrates are metabolized via a different lower pathway. Tentative functions for the products of other *pmd* genes were assigned based on sequence identity and/or similarity to proteins from other proteobacteria involved in uptake or metabolism of aromatic compounds. This study provides evidence for a single lower pathway in *C. testosteroni* for metabolism of Pca, which is generated by different upper pathways acting on a variety of aromatic substrates.

Keywords: aromatic, biodegradation, *meta* ring fission, *lig* genes

INTRODUCTION

Bacteria possess three widely known ring-cleavage mechanisms for the diol metabolites generated during aerobic degradation of aromatic compounds: *ortho* (intradiol), *meta* (extradiol) and gentisate (Harwood & Parales, 1996; Lipscomb & Orville, 1992). Whereas the genetic determinants of the *ortho* pathways for catechol, chlorocatechols and protocatechuate (Pca) (Harwood & Parales, 1996; Reineke, 1998; van der Meer *et al.*, 1992) and the *meta* cleavage(s) of catechols (Harayama *et al.*, 1992; Spence *et al.*, 1996) have been studied intensively in various proteobacteria, the genetics of the Pca *meta* cleavage pathway have received less attention. With the realization, though, that the Pca extradiol ring fission pathway is crucial in the metabolism of aromatic pollutants and lignin-derived compounds by some bacteria, this pathway has been subject to renewed interest.

Comamonas (formerly *Pseudomonas*) *testosteroni*, a β-proteobacterium, was used in pioneering studies on the Pca *meta* pathway (Dagley *et al.*, 1968; Dennis *et al.*, 1973; Wheelis *et al.*, 1967) and is the organism from which the ring cleavage enzyme, Pca 4,5-dioxygenase (PMD), was first purified and characterized (Arciero *et al.*, 1990) and in which the metabolic pathway via the pyrone to oxaloacetate and pyruvate (Fig. 1a) was elucidated (Kersten *et al.*, 1982). Four other Pca *meta* pathway enzymes were also purified and characterized from the non-fluorescent bacterium *Pseudomonas ochraceae* (Maruyama, 1979, 1983a, b, 1985, 1990a, b; Maruyama *et al.*, 1978) and five of the corresponding

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; HCMS, 2-hydroxy-4-carboxymuconate semialdehyde; HCMSD, HCMS dehydrogenase; Km, kanamycin; MMA, minimal medium A; OCA, 4-oxalocitramalate aldolase; Pca, protocatechuate; PDCH, 2-pyrone-4,6-dicarboxylic acid hydrolase; PMD, Pca 4,5-dioxygenase.

The GenBank accession number for the sequence reported in this paper is AF305325.

Table 1. Bacteria and plasmids used in this study

Bacteria and plasmids	Relevant characteristics	Reference
E. coli		
MM294 (pRK2013)	Km^r, RK2 *tra*; used in triparental matings to mobilize conjugatable vectors	Figurski & Helinski (1979)
DH5α (pUC128)	Ap^r, α-*lacZ*/MCS; general purpose cloning vector	Keen *et al.* (1988)
XL-1Blue (pCR-Script SK+)	Ap^r, α-*lacZ*/MCS; general purpose cloning vector	Stratagene
JM109 (pUC18Not.1)	Ap^r, α-*lacZ*/MCS; general purpose cloning vector; based on pUC18Not but with a modified MCS*	This study
CC118::λ*pir* (pUTCm)	Ap^r, Cm^r; source of Cm^r gene and pRR1 backbone	de Lorenzo *et al.* (1990)
CC118::λ*pir* (pRR1)	Ap^r; mobilizable R6K-based vector; constructed by digesting pUTCm with *Sal*I and religating the fragment containing *oriV*, *oriT* and Ap^r gene	This study
JM109 (pMP141.1)	Ap^r, Cm^r; intermediate in the construction of the *pmdA* cross-over cassette; Cm^r gene from pUTCm cloned as a *Bgl*II–*Not*I fragment into the *Bam*HI/*Not*I site of pCR-Script SK+	This study
Clones from plasmid library and vector for disrupting *pmdA*		
JM109(pLIB8H4)	Ap^r; portion of *pmd* locus spanning nt 1–~ 6700 cloned into pUC18Not.1	This study
JM109(pLIB20G12)	Ap^r; portion of *pmd* locus spanning nt ~ 1500–~ 8800 cloned into pUC18Not.1	This study
JM109(pLIB20F2)	Ap^r; portion of *pmd* locus spanning nt ~ 2800–10848 cloned into pUC18Not.1	This study
JM109(pSM99.7)	Ap^r; 806 bp *Not*I–*Sal*I fragment from pLIB20G12 subcloned into pUC128	This study
JM109(pSM99.7CmA)	Ap^r, Cm^r; Cm^r gene cloned as a *Bss*HII fragment into pSM99.7	This study
CC118::λ*pir*(pSMpmdACm5)	Ap^r, Cm^r; knock-out cassette for *pmdA* cloned into pRR1 as a *Not*I fragment	This study
C. testosteroni		
BR6020	Derivative of wild-type BR60 cured of plasmid pBRC60; originally identified as an *Alcaligenes* sp. but reclassified based on biochemical features and sequencing of the 16S rRNA gene	Wyndham *et al.* (1988); M. A. Providenti, M. A. & R. C. Wyndham, unpublished results
BR6020::*pmdA*	Cm^r; BR6020 with a disrupted *pmdA*	This study

* The MCS of pUC18Not (de Lorenzo & Timmis, 1994) was modified by ligating an oligonucleotide into the *Sac*I/*Bam*HI site so that the new MCS (5′-GCGGCCGCGC GAATTCGAGC TCCACCGCGG TGGCGGCCGA TGCATATTTA AATCCCCCGG GGGATC-CTCT AGAGTCGACC TGCAGGCATG CAAGCTTGCG GCCGC-3′) contains restriction sites for *Not*I, *Eco*RI, *Sac*I, *Sac*II, *Eag*I, *Nsi*I, *Swa*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Sbf*I/*Pst*I, *Sph*I, *Hin*dIII and *Not*I when read 5′ to 3′. Note that not all sites for 6 bp-recognizing enzymes are listed.

genes have been studied in the α-proteobacterium *Sphingomonas paucimobilis* SYK-6, an organism used to investigate degradation of model lignin compounds (Hara *et al.*, 2000; Masai *et al.*, 1999, 2000; Noda *et al.*, 1990). This bacterium also served as the source of PMD in crystal structure studies (Sugimoto *et al.*, 1999).

In various *C. testosteroni* strains, metabolic pathways channel distinct aromatic compounds, some of which are pollutants, via Pca. Examples include *m*- and *p*-hydroxybenzoate (Michalover *et al.*, 1973; Wheelis *et al.*, 1967), chlorobenzoates (Nakatsu & Wyndham, 1993; Nakatsu *et al.*, 1995b, 1997), *m*-nitrobenzoate (Nadeau & Spain, 1995), phthalates (Nakazawa & Hayashi, 1977, 1978; Schläfli *et al.*, 1994; Wang *et al.*, 1995), methoxylated benzoates (Kersten *et al.*, 1982, 1985; Ribbons, 1971), *p*-toluate (Locher *et al.*, 1991), *p*-toluenesulfonate (Locher *et al.*, 1989), and naphthalene, phenanthrene and anthracene (Goyal & Zylstra, 1996). PMD is thus central to the complete biodegradation of many aromatic substrates by this bacterium, yet no appropriate genetic data are available for the enzyme in *C. testosteroni*. We therefore undertook a study to clone and characterize PMD genes from *C. testosteroni* BR6020 and discovered the whole *meta* pathway in one locus (Fig. 1b).

METHODS

Chemicals, bacteria, plasmids and growth conditions. All antibiotics and chemicals were obtained from Sigma-Aldrich. Bacteria and plasmids used in this study are listed in Table 1. Unless otherwise stated, *Escherichia coli* strains were grown at 37 °C in Luria–Bertani medium (LB: 1 %, w/v, tryptone; 0·5 %, w/v, yeast extract; 0·5 %, w/v, NaCl) containing ampicillin (Ap; 250 mg l^{-1}), kanamycin (Km; 40 mg l^{-1}) or chloram-

Table 2. Separation of PMD from *C. testosteroni* BR6020

Step	Total protein (mg)	Total activity (nkat)	Specific activity [mkat (kg protein)$^{-1}$]	Yield (%)	Purification (-fold)
Crude extract	1653	11·1	6·9	100	1·0
Heat treatment	1610	10·9	7·8	98	1·1
Ultracentrifugation	544	5·1	9·3	45	1·4
DEAE-Sepharose	25	0·75	30	6·7	4·3
Superdex 200	0·7	0·22	316	2	52

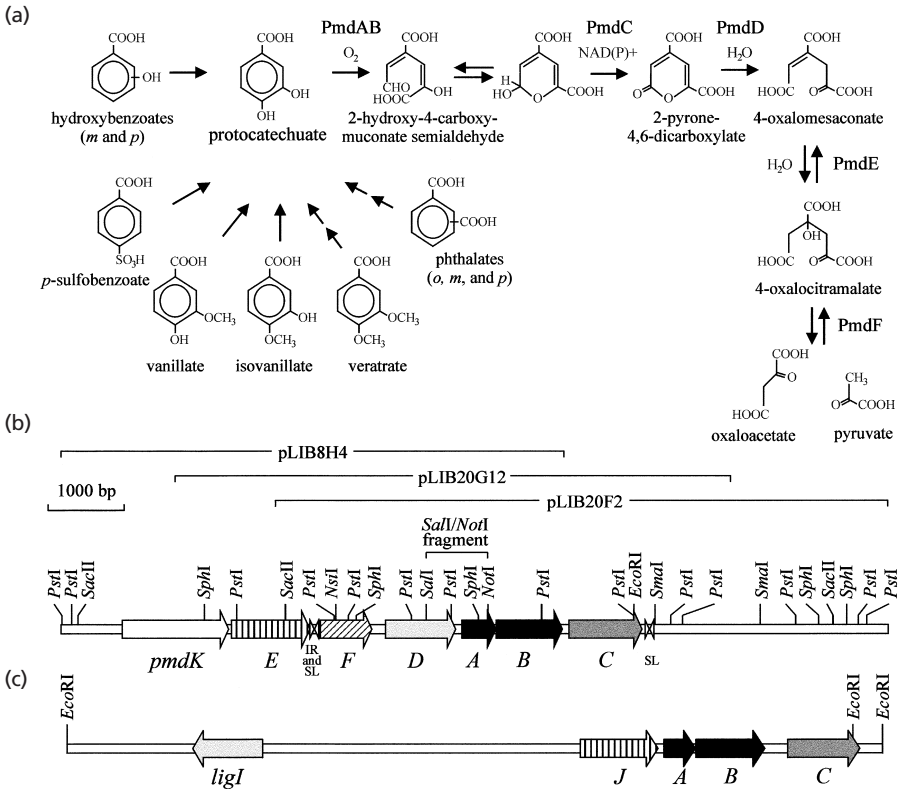

Fig. 1. (a) Catabolic pathways in *C. testosteroni* funnel various aromatic compounds towards Pca. The *meta* ring fission pathway then cleaves Pca at the 4,5 position and converts the product, which cyclizes spontaneously, into Krebs cycle intermediates. Based on Kersten *et al*. (1982). (b) Physical and restriction map of a 10·8 kb locus harbouring *pmd* pathway genes cloned from the chromosome of *C. testosteroni* BR6020. No sites for *Bam*HI, *Hin*dIII, *Sac*I, *Swa*I or *Xba*I were detected. The region present in each of the three *pmd*$^+$ library clones is indicated above the map. In this study, the α- and β-subunit of PMD are shown to be encoded by *pmdA* and *pmdB*, respectively. The putative functions of the other gene products are shown in (a) or discussed in the text. Also shown are the positions of an inverted repeat structure (IR) and two potential stem–loops (SL). The indicated *Sal*I/*Not*I fragment was subcloned and manipulated as described in Fig. 2. (c) Physical map of *lig* genes encoding the Pca *meta* pathway enzymes of *S. paucimobilis* SYK-6, present in a 10·7 kb *Eco*RI fragment from the chromosome. Based on Hara *et al*. (2000). Homologous *lig* and *pmd* genes are shaded identically.

phenicol (Cm; 50 mg l^{-1}) as required. *C. testosteroni* strains were routinely grown at 32 °C in minimal medium A (MMA) (Wyndham, 1986) amended with succinate (10 mM), aromatic compounds (4 mM) and Cm (100 mg l^{-1}) as required. When necessary, growth media were solidified by the addition of agar to a final concentration of 1·6 % (w/v).

Purification of PMD. *C. testosteroni* BR6020 was grown to mid-exponential phase on 3 mM Pca-salts medium in a 12·5 l fermenter with a 9 l working volume (Biostat V; B. Braun). Cells were harvested with a Pellikon cassette filtration system (Millipore), washed in potassium phosphate buffer (50 mM, pH 7·5) and stored at −20 °C. Crude extract was prepared as follows: 25 g cells (wet wt) were resuspended in 25 ml Tris/HCl buffer (20 mM, pH 7·5) containing DNase I (0·02 mg ml^{-1}) and disrupted by three passages through a French pressure cell at 135 MPa. The suspension was then incubated at 45 °C for 2 min, centrifuged at 36000 ***g*** (30 min, 4 °C) to remove cell debris, followed by ultracentrifugation at

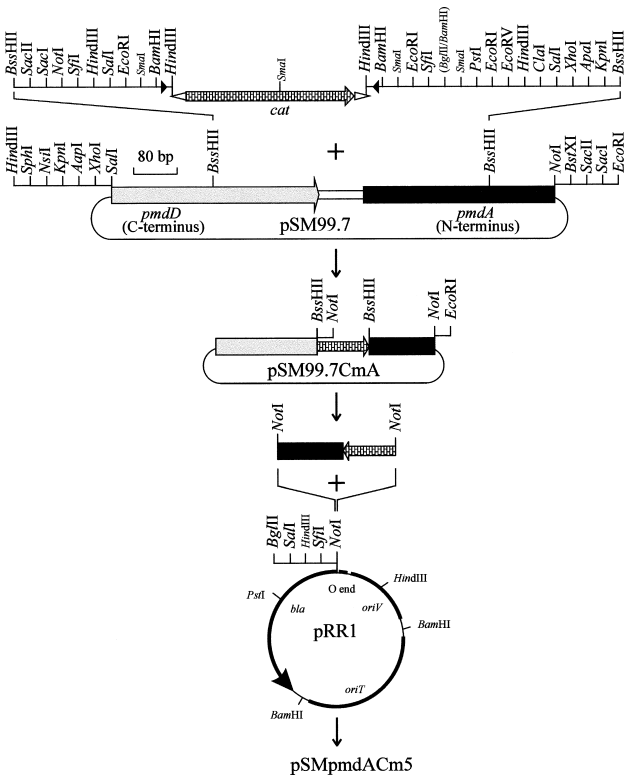

Fig. 2. Schematic diagram of the cloning steps for the construction of pSMpmdACm5, a vector for disruption of *pmdA* by site-specific recombination. Beyond the first step, only selected restriction sites are indicated. The Cm resistance gene (*cat*; not to scale) was obtained from pMP141.1 and is flanked by transcriptional (dark triangles) and translational terminators (white triangles). Indicated on suicide delivery vector pRR1 is the multiple cloning site and, in smaller type, sites unsuitable for cloning because they are not unique. Also indicated are the relative positions of the β-lactamase gene (*bla*), the R6K-based origin of vegetative replication (*oriV*), the RP4-based origin of conjugative transfer (*oriT*) and the inverted repeat recognized by the transposase of Tn*5* (O end). The latter is a remnant of the plasmid from which these vectors are derived and serves no purpose.

200 000 ***g*** (1 h, 4 °C). The supernatant could be stored frozen at −70 °C for at least 4 weeks without significant loss of activity. FPLC was performed at room temperature with a Pharmacia apparatus. The anion exchange column (300 × 26 mm, DEAE-Sepharose CL6B; Pharmacia) was run with Tris-buffered eluents (pH 7·5) as described elsewhere (Junker *et al*., 1994). Fractions containing significant activity, as judged by Pca-induced O_2-uptake (Locher *et al*., 1989), were combined and concentrated by membrane filtration (30 kDa exclusion limit, Diaflo; Amicon) in a stirring cell (model 8050; Amicon). Gel filtration chromatography was done with a Superformance cartridge (600 × 10 mm; Merck) filled with Superdex 200 prep grade material (Pharmacia). The column was operated at a flow rate of 0·8 ml min^{-1} with a Tris/HCl buffer (50 mM, pH 7·5) containing 150 mM NaCl. Active fractions were combined and concentrated as described above.

N-terminal amino acid sequences of blotted proteins (gel filtration step) were determined after Edman degradation (Schläfli *et al*., 1994). Reversed-phase HPLC of the ring cleavage product, 2-hydroxy-4-carboxymuconate semialdehyde (HCMS), was done with the method established by Locher *et al*. (1989) with an apparatus described by Laue *et al*. (1996). HCMS was generated in an oxygen-dependent conversion of Pca catalysed by partially purified PMD (DEAE step) from strain BR6020 (see Table 2).

Construction of chromosomal DNA library and screening for PMD genes. All enzymes used for cloning purposes were purchased from New England Biolabs. Purified chromosomal DNA (Ausubel, 1992) from strain BR6020 was partially digested with *Sau*3AI and a pool of DNA in the size range of ∼16·5–∼6·8 kb was ligated into the *Bam*HI site of pUC18Not.1 (Table 1). The ligation mix was electroporated into *E. coli* JM109 and transformants were recovered on LB agar with Ap and X-Gal. Approximately 2300 white colonies (selected at random) were transferred to fresh LB agar with Ap and grown overnight at 32 °C. An alkaline aqueous solution of Pca (500 mM) was then sprayed over the cells and the ring cleavage product, HCMS (Fig. 1a), was visible as a yellow colour. The inserts from three positive clones were restriction-enzyme-mapped and yielded a contiguous region spanning 10·8 kb (Fig. 1b). Both DNA strands of this region were sequenced by primer walking using the chain-terminating dideoxy method and an ABI Prism Automated Sequencer (Biotechnology Research Institute, University of Ottawa, Ottawa, Canada). The locus was designated *pmd* (for Pca *meta* dioxygenase) and the sequence was analysed for similarities to entries in the GenBank non-redundant database using the BLAST network service (Altschul *et al*., 1997) of the National Centre for Biotechnology Information (NCBI) at Bethesda, Maryland, USA (http://www.ncbi.nlm.nih.gov). ORFs identified by BLAST analysis were then scanned for conserved domains and signature sequences of protein families using the CD-Search network service of NCBI and the Prosite ProfileScan network service of the Swiss Institute of Bioinformatics (http://www.expasy.ch).

Generation of BR6020 with a disrupted *pmdA*. A summary of steps for the construction of a recombinational disruption cassette for *pmdA* is provided in Table 1 and a schematic diagram is provided in Fig. 2. The cassette contained a site-specific cross-over region and a Cm resistance marker and was cloned into the suicide-delivery vector, pRR1, resulting in pSMpmdACm*5*. The latter can be transferred by conjugation but possesses an R6K *oriV* and can thus only be maintained as an independent plasmid in hosts encoding *λpir* (de Lorenzo *et al*., 1990). Recombination at the cross-over region results in duplication of this section, complete integration of the plasmid and insertional inactivation of the gene. The knock-out vector was mobilized from *E. coli* CC118*λpir* into *C. testosteroni* BR6020 via tri-parental filter mating (Nakatsu & Wyndham, 1993) and transconjugants were recovered on succinate-MMA agar with Cm. Some spontaneous Cm resistance was observed in controls, but a true *pmdA* mutant, designated BR6020::*pmdA*, was recognized by its inability to grow after being patched to MMA containing *p*-hydroxybenzoate and Cm. Proper integration of the knock-out vector was confirmed by Southern blotting. To test whether disruption of *pmdA* affected complete metabolism of various aromatic growth compounds (see Results), BR6020::*pmdA* was cultured initially on succinate-MMA agar with Cm, patched to MMA agar containing Cm and an aromatic growth substrate and scored for growth after 2 to 7 d incubation.

Assay for Pca production. The ability of BR6020 and BR6020::*pmdA* to generate Pca when grown on succinate in the presence of various aromatic substrates was determined using the method of Parke for detection of vicinal diols (Parke, 1992), with minor modifications. In brief, bacteria were

cultured for 48 h at 32 °C on MMA agar (~ 20 ml medium per plate) containing succinate, an aromatic substrate, Cm for BR6020::*pmdA* and spread onto plates prior to addition of bacteria, 70 µl of a 50 mM aqueous $FeCl_3$ solution (filter-sterilized) and 100 µl of a 0·1 M *p*-toluidine solution in dimethylformamide. Production of Pca resulted in a dark reddish-brown halo around colonies.

RESULTS

Aromatic substrate range of *C. testosteroni* BR6020

BR6020 is able to grow on the aromatic substrates benzoate, *o*-aminobenzoate, *m*- and *p*-hydroxybenzoate, *p*-sulfobenzoate, all three phthalate isomers, vanillate, isovanillate, veratrate and the diol intermediates Pca and gentisate. It cannot grow on *m*- or *p*-aminobenzoate, *o*-hydroxybenzoate (salicylate), *m*-sulfobenzoate, *m*-nitrobenzoate, any of the three toluate and anisate isomers, nor the diol intermediate catechol.

Separation and analysis of PMD from *C. testosteroni* BR6020

A low level of PMD activity [0·2 mkat (kg protein)$^{-1}$] was observed in extracts of succinate-grown cells, while high activity was observed in Pca-grown cells [6·9 mkat (kg protein)$^{-1}$]. The inducible enzyme is unstable and initial purification attempts used the protective buffers described by Arciero *et al.* (1990), but they had little effect. The protocol presented here is a modified version of an established procedure used for purification of PMD from *C. testosteroni* T-2 (Mampel, 2000). It allowed us to separate sufficiently pure, active enzyme such that we could determine the relative molecular

Table 3. Comparison of *pmd* products to entries in the GenBank database

pmd gene product	Derived molecular mass (kDa)	Homologue (percentage identity)	GenBank accession no.	Function of homologue, if known	Reference	Inferred function of *pmd* gene product based on sequence homology and/or other features (see text for more details)
PmdA	16·8	LigA (56)	M34835	α-subunit of PMD	Noda *et al.* (1990)	α-subunit of PMD
PmdB	31·7	LigB (61)	M34835	β-subunit of PMD	Noda *et al.* (1990)	β-subunit of PMD
PmdC	35·2	LigC (76)	AB035122	HCMSD	Masai *et al.* (2000)	HCMSD
		CbaC (23)	U18133	1-Carboxy-3-chloro-3,4-dihydroxy-cyclohexa-1,5-diene dehydrogenase	Nakatsu *et al.* (1997)	
		OphB (22)	AF095748	2-Phthalate dihydrodiol dehydrogenase	Chang & Zylstra (1998)	
PmdD	34·4	LigI (55)	AB015964	2-Pyrone-4,6-dicarboxylic acid hydrolase	Masai *et al.* (1999)	2-Pyrone-4,6-dicarboxylic acid hydrolase
PmdE	38·2	LigJ (63)	AB035121	4-Oxalomesaconate hydratase	Hara *et al.* (2000)	4-Oxalomesaconate hydratase
		LigY (35)	AB018415	2,2′,3-Trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl hydrolase	Peng *et al.* (1999)	
PmdF	24·0	FldZ (54)	AJ277295	Putative acyl transferase	Unpublished	OCA
		MenG (30)	AL021411	*S*-Adenosylmethionine:2-demethylmenaquinone methyltransferase-like protein	Redenbach *et al.* (1996)	
		Hps-1 (22)	AE001045	D-Arabino-3-hexulose-6-phosphate formaldehyde lyase-like protein	Klenk *et al.* (1997)	
PmdK	48·9	PcaK (42)	Q51955	Transporter for *p*-hydroxybenzoate and Pca	Harwood *et al.* (1994); Nichols & Harwood (1997)	Aromatic transporter
		BenK (29)	AAC46425	Benzoate transporter	Collier *et al.* (1997)	
		TfdK (25)	U16782	2,4-Dichlorophenoxyacetate transporter	Leveau *et al.* (1998)	

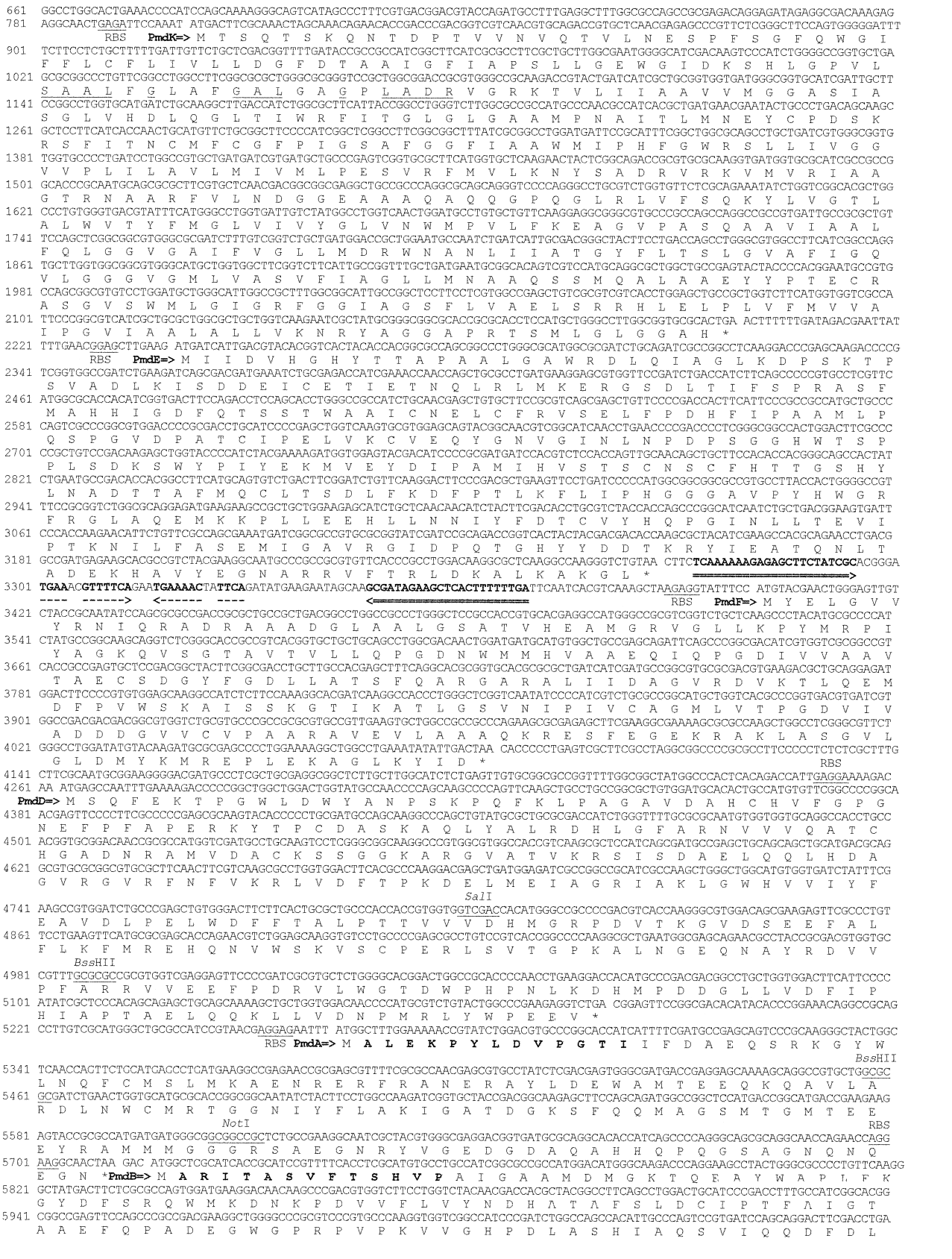

Fig. 3. For legend see facing page.

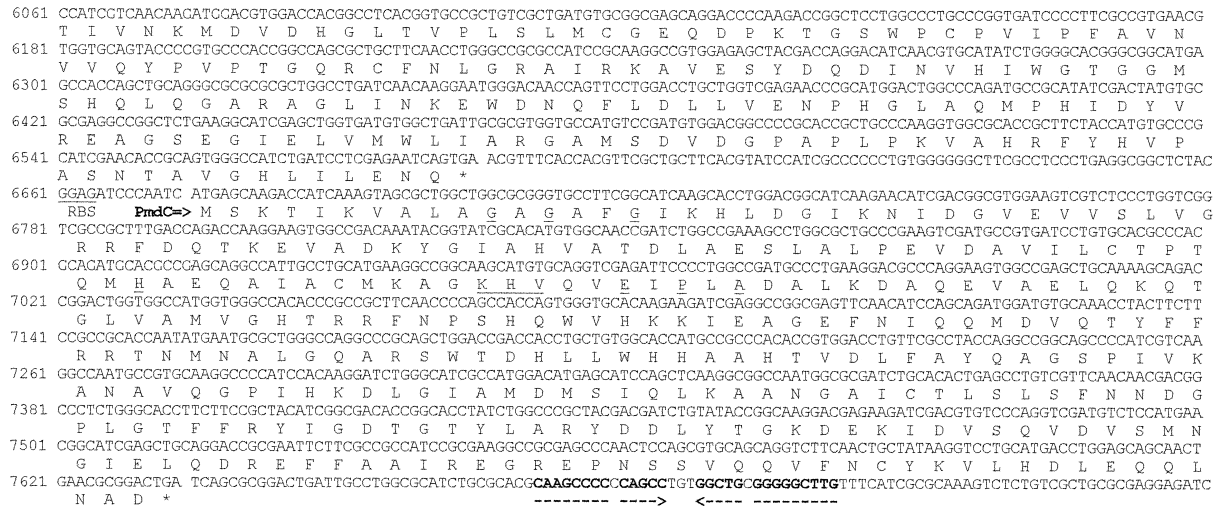

Fig. 3. DNA sequence of the upper strand of *pmdKEFDABC* spanning nucleotides 661–7740 of the 10·8 kb region cloned from *C. testosteroni* BR6020. Nucleotide 1 was arbitrarily defined as the first base of the insert in clone pLIB8H4 (see Table 1). Indicated are the translation products, potential ribosome-binding sites (RBS), the location of select restriction sites used for subcloning purposes and an inverted repeat structure as well as two potential stem–loops (nucleotides in bold with arrows beneath). Amino acids in PmdA and PmdB confirmed by N-terminal sequencing of the two subunits from the separated protein are shown in bold, while amino acids underlined in PmdK and PmdC show similarity to conserved motifs for the family of proteins to which these putative proteins belong. See text for additional detail.

masses (18 and 31 kDa, respectively) and N-terminal amino acid sequences of the α- and β-subunits (ALEKPYLDVPGTI and ARITASVFTSHVP, respectively). The reaction product from separated enzyme, HCMS, was identified by co-chromatography and identical UV-visible spectra with authentic material generated by whole cells of *C. testosteroni* BR6020. Analyses were at pH 2·2 (λ_{max} 283 nm) and pH 6·7 (λ_{max} 411 nm).

Cloning and sequence analysis of the *pmd* locus in *C. testosteroni* BR6020

Three clones in the plasmid library, pLIB8H4, pLIB20G12 and pLIB20F2 (Table 1 and Fig. 1b), were positive for PMD activity, as judged by conversion of Pca to HCMS on plates. Based on restriction mapping, the three clones represented a contiguous 10·8 kb chromosomal region and the complete sequence was determined. Seven ORFs in an area spanning nt 661–7740 of this locus were identified based on homologies to entries in the GenBank database (Table 3) and these were designated *pmdKEFDABC* (Figs 1b and 3). The conceptual translation of the N-termini of *pmdA* and *pmdB* (Fig. 3) and the derived molecular masses of the products (Table 3) corresponded to data obtained from separated PMD (see above), thus confirming that these genes encode the two subunits of this enzyme. Tentative functions for products of the other ORFs from the *pmd* locus (Fig. 1a) were attributed based on sequence identity to entries in the GenBank database and similarity to purified proteins of the Pca *meta* pathway from *S. paucimobilis* SYK-6 and *P. ochraceae* (see Table 3 and Discussion). A 22 nt inverted repeat and a potential stem–loop structure were detected between *pmdF* and *pmdE*, and another potential stem–loop was found following *pmdC* (Fig. 3). However, their significance remains to be elucidated. The regions flanking the *pmd* locus (Fig. 1b) presumably encode proteins for other aspects of bacterial metabolism and are not discussed here. In all three clones, the *pmd* genes are read in the same direction as the plasmid promoter (P_{lac}). We attempted to IPTG-induce *pmd* expression in liquid cultures of *E. coli* to determine whether Pca was converted to pyruvate by whole cells or cell-free extracts, but negligible metabolism of Pca was measured. The colour change observed on plates by cultures exposed to Pca presumably reflected a low level of initial conversion of the substrate.

Two sets of conserved motifs were identified in *pmd* products. PmdK possesses the N-terminal motif from the aromatic acid:H^+ symporter subclass of the major facilitator superfamily described by Pao *et al.* (1998) that is believed to encompass the hydrophilic loop between hydrophobic transmembrane spanning domains 1 and 2 (Fig. 3). PmdC possesses the two conserved motifs of the glucose-fructose oxidoreductase family of dehydrogenases described by Nakatsu *et al.* (1997). One is found at the N terminus and is thought to mediate NAD(P) binding, while the other is found internally and has no known function (Fig. 3).

Effects of disrupting *pmdA* on aromatic metabolism by *C. testosteroni* BR6020

Strain BR6020::*pmdA*, containing an interrupted gene for the α-subunit of PmdAB (see Methods), was not able to grow with Pca, nor were any residual levels of PMD

activity detected in succinate-grown cells. In addition, the mutant could not grow with the aromatic growth substrates shown in Fig. 1(a), although each of the compounds could still be converted to Pca. The mutation obviously affected the lower pathway for degradation of Pca and not the upper pathways that generate this compound from a range of aromatic precursors. Strain BR6020::*pmdA* was able to grow normally with benzoate and *o*-aminobenzoate, indicating that these are not degraded via Pca. In addition, no vicinal diols were detected when BR6020 or BR6020::*pmdA* were grown on succinate in the presence of these compounds, indicating that they are not converted to catechol, a known metabolite for these substrates in many other bacteria (see Discussion). Strain BR6020::*pmdA* also grew with gentisate.

DISCUSSION

In this study, genes encoding enzymes of the Pca *meta* pathway of *C. testosteroni* BR6020 were cloned, characterized and tentatively assigned to one locus. Direct evidence linked the product of *pmdAB* to the presumed function (Table 3) because the N-terminal amino acid sequences of the separated subunits of PMD from BR6020 (see Results) were identical to the derived sequences of PmdA and PmdB (Fig. 3). Furthermore, the derived relative molecular masses corresponded to the observed data (see Results). Finally, recombinational disruption of *pmdA* resulted in a strain unable to grow on Pca and various aromatic substrates that are funnelled through Pca (Fig. 1a). In this strain, growth on benzoate and *o*-aminobenzoate (anthranilate) was unaffected, indicating that these two compounds are not funnelled through Pca. Wheelis *et al.* (1967) originally suggested that in *C. testosteroni*, benzoate is metabolized via *m*-hydroxybenzoate to Pca, but our data do not support the second part of that hypothesis. Two different types of aerobic pathways have been reported for metabolism of benzoate and anthranilate: those that funnel them through catechol (Harwood & Parales, 1996) and those that funnel them via CoA-esters through gentisate (Altenschmidt *et al.*, 1993; Ziegler *et al.*, 1989). Disruption of *pmdA* did not affect growth on gentisate and no vicinal diol intermediates were detected when BR6020 or BR6020::*pmdA* were cultured on succinate medium containing benzoate or *o*-aminobenzoate, nor is BR6020 able to grow on catechol (see Results), so we are exploring the hypothesis that this organism metabolizes these substrates via gentisate.

Other ORFs physically linked to *pmdAB* were identified (Figs 1b and 3) and potential roles were inferred based on high identity to proteins of known function (summarized in Fig. 1a and Table 3). PmdK shows similarity to members of the aromatic acid:H^+ symporter subclass of the major facilitator superfamily (Pao *et al.*, 1998). Other examples of this subclass are responsible for transport of Pca, *p*-hydroxybenzoate, benzoate and 2,4-dichlorophenoxyacetate (Table 3), and although this remains to be shown, PmdK may mediate uptake of Pca. PmdC, PmdD and PmdE appear to be, respectively, the HCMS dehydrogenase (HCMSD), 2-pyrone-4,6-dicarboxylic acid hydrolase (PDCH) and 4-oxalomesaconate hydratase of BR6020 based on similarity to LigC, LigI and LigJ, respectively, of *S. paucimobilis* SYK-6 (Table 3). In addition to high sequence identity as evidence of proposed functions, the derived amino acid compositions (Table 4) and molecular masses of PmdC and PmdD (35·2 and 34·4 kDa, respectively) are similar to those reported, respectively, for the 35 kDa monomer of the HCMSD and the 33 kDa PDCH from *P. ochraceae* (Maruyama, 1983b; Maruyama *et al.*, 1978). Moreover, with respect to PmdD, we have generated a BR6020 strain with a disrupted *pmdD* using a method similar to the one described here for *pmdA* and the growth characteristics of this strain on various aromatic substrates were identical to those obtained with BR6020::*pmdA* (unpublished data), further evidence showing that the product of *pmdD* is involved in the Pca *meta* pathway of BR6020. PmdF appears to be 4-oxalocitramalate aldolase (OCA) based on a similar derived amino acid composition (Table 4) and molecular mass (24·0 kDa) to the 26 kDa monomer of the homohexameric OCA from *P. ochraceae* (Maruyama, 1990a). This aldolase differs from typical Schiff's base-forming (Class I) aldolases

Table 4. Comparison of experimentally determined amino acid compositions of HCMSD, PDCH and OCA from *P. ochracea* to the derived compositions of PmdC, PmdD and PmdF, respectively

The amino acid compositions of HCMSD, PDCH and OCA are found in Maruyama *et al.* (1978), Maruyama (1983b) and Maruyama (1990a), respectively. The reproducibility of the reported values was not discussed.

Amino acid	HCMSD*	PmdC	PDCH	PmdD	OCA	PmdF
Ala	32	36	26	27	35	33
Cys	3	4	4	5	4	3
Asx	34	36	28	33	22	18
Glx	34	37	26	28	12	19
Phe	13	13	15	18	8	4
Gly	22	23	18	19	25	22
His	12	13	10	11	3	2
Ile	17	19	5	6	12	12
Lys	16	18	15	17	13	12
Leu	24	26	22	25	22	19
Met	7	9	6	7	9	9
Pro	9	8	23	23	10	9
Arg	12	12	15	18	13	12
Ser	12	13	11	11	12	10
Thr	13	16	11	12	13	9
Val	21	25	22	31	24	25
Trp	3	3	11	8	3	2
Tyr	6	8	6	6	7	7

* The amino acid composition of HCMSD was originally reported for the dimer and the numbers presented here were obtained by halving the published values.

(Maruyama, 1990a) and instead shares biochemical features with an *E. coli* methyltransferase (Maruyama, 1990b). PmdF does not possess any of the consensus signature sequences of Class I aldolases but instead shows sequence homology to hypothetical transferases (Table 3).

The arrangement and orientation of *pmd* genes in *C. testosteroni* BR6020 relative to homologous *lig* genes of *S. paucimobilis* SYK-6 show some interesting contrasts and the schematics in Fig. 1(b) and (c) summarize the differences. *pmd* genes are arranged more compactly and read in the same direction, and although this remains to be shown, they could conceivably be transcribed as one polycistronic mRNA. In contrast, relevant *lig* genes are spread out over a larger area, with *ligI* located $\sim 4{\cdot}3$ kb upstream of and divergently transcribed from *ligJABC*. With respect to arrangement, while *ligJ* immediately precedes *ligABC*, its homologue *pmdE* is ~ 2 kb upstream of the homologous cluster *pmdABC*, which is instead preceded by *pmdD*, the *ligI* homologue. Variations in the relative arrangement of homologous genes for *ortho* metabolism of catechol, chlorocatechol and Pca, and *meta* metabolism of catechol have also been reported (Harwood & Parales, 1996; Reineke, 1998; van der Meer *et al.*, 1992).

This study provides evidence for a single lower pathway in *C. testosteroni* for the metabolism of Pca, which is generated by a variety of upper pathways acting on many aromatic substrates. This contrasts with the situation sometimes observed in other bacteria, which can possess alternative lower pathways for metabolism of the same diol. Examples include *Pseudomonas putida*, which metabolizes catechol generated from benzoate by an *ortho* pathway (Harwood & Parales, 1996), but catechols generated from toluates by a *meta* pathway (Assinder & Williams, 1990); or the presence of three dedicated lower *meta* pathways in *Alcaligenes* sp. O-1 for metabolism of the catechols generated by distinct upper pathways (Junker *et al.*, 1994). The upper pathways of BR6020 addressed in this study are all chromosomally encoded (Table 1), but plasmid-encoded upper pathways in some *C. testosteroni* strains for conversion of aromatic compounds to Pca have also been reported, such as *cba* for chlorobenzoates, *tsa* for *p*-toluenesulfonate and *psb* for *p*-sulfobenzoate (Junker *et al.*, 1997; Nakatsu & Wyndham, 1993; Wyndham *et al.*, 1988). These plasmid-encoded upper pathways, which are widespread in the environment and can be acquired by horizontal gene transfer (Nakatsu *et al.*, 1995a; Peel & Wyndham, 1999; Tralau *et al.*, 2001), require a functional Pca *meta* pathway for complete metabolism of the respective aromatic substrates. In the case of *cba*-encoded metabolism of *m*-chlorobenzoate, a disrupted *pmdA* also results in growth defects on this compound (unpublished data) and we are currently investigating whether the same occurs with the latter two pathways. As well, we are studying the distribution and degree of conservation of the *pmd* locus in other *C. testosteroni* strains and various aromatic-degrading environmental isolates.

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REFERENCES

Altenschmidt, U., Oswald, B., Steiner, E., Herrmann, H. & Fuchs, G. (1993). New aerobic benzoate oxidation pathway via benzoyl-coenzyme A and 3-hydroxybenzoyl-coenzyme A in a denitrifying *Pseudomonas* sp. *J Bacteriol* **175**, 4851–4858.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Arciero, D. M., Orville, A. M. & Lipscomb, J. D. (1990). Protocatechuate 4,5-dioxygenase from *Pseudomonas testosteroni*. *Methods Enzymol* **188**, 89–95.

Assinder, S. J. & Williams, P. A. (1990). The TOL plasmids: determinants of the catabolism of toluene and the xylenes. *Adv Microb Physiol* **31**, 1–69.

Ausubel, F. M. (1992). *Short Protocols in Molecular Biology: a Compendium of Methods from Current Protocols in Molecular Biology*. New York: Wiley.

Chang, H. K. & Zylstra, G. J. (1998). Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. *J Bacteriol* **180**, 6529–6537.

Collier, L. S., Nichols, N. N. & Neidle, E. L. (1997). *benK* encodes a hydrophobic permease-like protein involved in benzoate degradation by *Acinetobacter* sp. strain ADP1. *J Bacteriol* **179**, 5943–5946.

Dagley, S., Geary, P. J. & Wood, J. M. (1968). The metabolism of protocatechuate by *Pseudomonas testosteroni*. *Biochem J* **109**, 559–568.

Dennis, D. A., Chapman, P. J. & Dagley, S. (1973). Degradation of protocatechuate in *Pseudomonas testosteroni* by a pathway involving oxidation of the product of *meta*-fission. *J Bacteriol* **113**, 521–523.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76**, 1648–1652.

Goyal, A. K. & Zylstra, G. J. (1996). Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from *Comamonas testosteroni* GZ39. *Appl Environ Microbiol* **62**, 230–236.

Hara, H., Masai, E., Katayama, Y. & Fukuda, M. (2000). The 4-oxalomesaconate hydratase gene, involved in the protocatechuate 4,5-cleavage pathway, is essential to vanillate and syringate degradation in *Sphingomonas paucimobilis* SYK-6. *J Bacteriol* **182**, 6950–6957.

Harayama, S., Kok, M. & Neidle, E. L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Annu Rev Microbiol* **46**, 565–601.

Harwood, C. S. & Parales, R. E. (1996). The β-ketoadipate pathway and the biology of self-identity. *Annu Rev Microbiol* **50**, 553–590.

Harwood, C. S., Nichols, N. N., Kim, M. K., Ditty, J. L. & Parales, R. E. (1994). Identification of the *pcaRKF* gene cluster from *Pseudomonas putida*: involvement in chemotaxis, biodegradation, and transport of 4-hydroxybenzoate. *J Bacteriol* **176**, 6479–6488.

Junker, F., Leisinger, T. & Cook, A. M. (1994). 3-Sulphocatechol 2,3-dioxygenase and other dioxygenases (EC 1.13.11.2 and EC 1.14.12.-) in the degradative pathways of 2-aminobenzenesulphonic, benzenesulphonic and 4-toluenesulphonic acids in *Alcaligenes* sp. strain O-1. *Microbiology* **140**, 1713–1722.

Junker, F., Kiewitz, R. & Cook, A. M. (1997). Characterization of the *p*-toluenesulfonate operon *tsaMBCD* and *tsaR* in *Comamonas testosteroni* T-2. *J Bacteriol* **179**, 919–927.

Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**, 191–197.

Kersten, P. J., Dagley, S., Whittaker, J. W., Arciero, D. M. & Lipscomb, J. D. (1982). 2-Pyrone-4,6-dicarboxylic acid, a catabolite of gallic acids in *Pseudomonas* species. *J Bacteriol* **152**, 1154–1162.

Kersten, P. J., Chapman, P. J. & Dagley, S. (1985). Enzymic release of halogens or methanol from some substituted protocatechuic acids. *J Bacteriol* **162**, 693–697.

Klenk, H. P., Clayton, R. A., Tomb, J. F. & 22 other authors (1997). The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature* **390**, 364–370.

Laue, H., Field, J. A. & Cook, A. M. (1996). Bacterial desulfonation of the ethanesulfonate metabolite of the chloroacetanilide herbicide metazachlor. *Environ Sci Technol* **30**, 1129–1132.

Leveau, J. H. J., Zehnder, A. J. B. & Van Der Meer, J. R. (1998). The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP134 (pJP4). *J Bacteriol* **180**, 2237–2243.

Lipscomb, J. D. & Orville, A. M. (1992). Mechanistic aspects of dihyroxybenzoate dioxygenases. In *Degradation of Environmental Pollutants by Micro-organisms and their Metalloenzymes*, pp. 243–298. Edited by H. Sigel & A. Sigel. New York: Marcel Dekker.

Locher, H. H., Leisinger, T. & Cook, A. M. (1989). Degradation of *p*-toluenesulphonic acid via sidechain oxidation, desulphonation and *meta* ring cleavage in *Pseudomonas* (*Comamonas*) *testosteroni* T-2. *J Gen Microbiol* **135**, 1969–1978.

Locher, H. H., Malli, C., Hooper, S., Vorherr, T., Leisinger, T. & Cook, A. M. (1991). Degradation of *p*-toluic acid (*p*-toluenecarboxylic acid) and *p*-toluenesulphonic acid via oxygenation of the methyl sidechain is initiated by the same set of enzymes in *Comamonas testosteroni* T-2. *J Gen Microbiol* **137**, 2201–2208.

de Lorenzo, V. & Timmis, K. N. (1994). Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. *Methods Enzymol* **235**, 386–405.

de Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K. N. (1990). Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J Bacteriol* **172**, 6568–6572.

Mampel, J. (2000). *Transport- und Regulationsphänomene beim Abbau von 4-Toluolsulfonat in Comamonas testosteroni T-2*. PhD thesis, University of Konstanz, Konstanz, Germany.

Maruyama, K. (1979). Isolation and identification of the reaction product of α-hydroxy-γ-carboxymuconic ε-semialdehyde dehydrogenase. *J Biochem* **86**, 1671–1677.

Maruyama, K. (1983a). Enzymes responsible for degradation of 4-oxalmesaconic acid in *Pseudomonas ochraceae*. *J Biochem* **93**, 567–574.

Maruyama, K. (1983b). Purification and properties of 2-pyrone-4,6-dicarboxylate hydrolase. *J Biochem* **93**, 557–565.

Maruyama, K. (1985). Purification and properties of γ-oxalomesaconate hydratase from *Pseudomonas ochraceae* grown with phthalate. *Biochem Biophys Res Commun* **128**, 271–277.

Maruyama, K. (1990a). Purification and properties of 4-hydroxy-4-methyl-2-oxoglutarate aldolase from *Pseudomonas ochraceae* grown on phthalate. *J Biochem* **108**, 327–333.

Maruyama, K. (1990b). Activation of *Pseudomonas ochraceae* 4-hydroxy-4-methyl-2-oxoglutarate aldolase by inorganic phosphate. *J Biochem* **108**, 334–340.

Maruyama, K., Ariga, N., Tsuda, M. & Deguchi, K. (1978). Purification and properties of α-hydroxy-γ-carboxymuconic ε-semialdehyde dehydrogenase. *J Biochem* **83**, 1125–1134.

Masai, E., Shinohara, S., Hara, H., Nishikawa, S., Katayama, Y. & Fukuda, M. (1999). Genetic and biochemical characterization of a 2-pyrone-4,6-dicarboxylic acid hydrolase involved in the protocatechuate 4,5-cleavage pathway of *Sphingomonas paucimobilis* SYK-6. *J Bacteriol* **181**, 55–62.

Masai, E., Momose, K., Hara, H., Nishikawa, S., Katayama, Y. & Fukuda, M. (2000). Genetic and biochemical characterization of 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase and its role in the protocatechuate 4,5-cleavage pathway in *Sphingomonas paucimobilis* SYK-6. *J Bacteriol* **182**, 6651–6658.

van der Meer, J. R., de Vos, W. M., Harayama, S. & Zehnder, A. J. B. (1992). Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol Rev* **56**, 677–694.

Michalover, J. L., Ribbons, D. W. & Hughes, H. (1973). 3-Hydroxybenzoate 4-hydroxylase from *Pseudomonas testosteroni*. *Biochem Biophys Res Commun* **55**, 888–896.

Nadeau, L. J. & Spain, J. C. (1995). Bacterial degradation of *m*-nitrobenzoic acid. *Appl Environ Microbiol* **61**, 840–843.

Nakatsu, C. H. & Wyndham, R. C. (1993). Cloning and expression of the transposable chlorobenzoate-3,4-dioxygenase genes of *Alcaligenes* sp. strain BR60. *Appl Environ Microbiol* **59**, 3625–3633.

Nakatsu, C. H., Fulthorpe, R. R., Holland, B. A., Peel, M. C. & Wyndham, R. C. (1995a). The phylogenetic distribution of a transposable dioxygenase from the Niagara River watershed. *Mol Ecol* **4**, 593–603.

Nakatsu, C. H., Straus, N. A. & Wyndham, R. C. (1995b). The nucleotide sequence of the Tn*5271* 3-chlorobenzoate 3,4-dioxygenase genes (*cbaAB*) unites the class IA oxygenases in a single lineage. *Microbiology* **141**, 485–495.

Nakatsu, C. H., Providenti, M. & Wyndham, R. C. (1997). The *cis*-diol dehydrogenase *cbaC* gene of Tn*5271* is required for growth on 3-chlorobenzoate but not 3,4-dichlorobenzoate. *Gene* **196**, 209–218.

Nakazawa, T. & Hayashi, E. (1977). Phthalate metabolism in *Pseudomonas testosteroni*: accumulation of 4,5-dihydroxyphthalate by a mutant strain. *J Bacteriol* **131**, 42–48.

Nakazawa, T. & Hayashi, E. (1978). Phthalate and 4-hydroxyphthalate metabolism in *Pseudomonas testosteroni*: purification and properties of 4,5-dihydroxyphthalate decarboxylase. *Appl Environ Microbiol* **36**, 264–269.

Nichols, N. N. & Harwood, C. S. (1997). PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *J Bacteriol* **179**, 5056–5061.

Noda, Y., Nishikawa, S., Shiozuka, K. & 7 other authors (1990). Molecular cloning of the protocatechuate 4,5-dioxygenase genes of *Pseudomonas paucimobilis*. *J Bacteriol* **172**, 2704–2709.

Pao, S. S., Paulsen, I. T. & Saier, M. H., Jr (1998). Major facilitator superfamily. *Microbiol Mol Biol Rev* **62**, 1–34.

Parke, D. (1992). Application of *p*-toluidine in chromogenic detection of catechol and protocatechuate, diphenolic intermediates in catabolism of aromatic compounds. *Appl Environ Microbiol* **58**, 2694–2697.

Peel, M. C. & Wyndham, R. C. (1999). Selection of *clc*, *cba*, and *fcb* chlorobenzoate-catabolic genotypes from ground-water and surface waters adjacent to the Hyde Park, Niagara Falls, chemical landfill. *Appl Environ Microbiol* **65**, 1627–1635.

Peng, X., Masai, E., Katayama, Y. & Fukuda, M. (1999). Characterization of the *meta*-cleavage compound hydrolase gene involved in degradation of the lignin-related biphenyl structure by *Sphingomonas paucimobilis* SYK-6. *Appl Environ Microbiol* **65**, 2789–2793.

Redenbach, M., Kieser, H. M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H. & Hopwood, D. A. (1996). A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol Microbiol* **21**, 77–96.

Reineke, W. (1998). Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. *Annu Rev Microbiol* **52**, 287–331.

Ribbons, D. W. (1971). Requirement of two protein fractions for *O*-demethylase activity in *Pseudomonas testosteroni*. *FEBS Lett* **12**, 161–165.

Schläfli, H. R., Weiss, M. A., Leisinger, T. & Cook, A. M. (1994). Terephthalate 1,2-dioxygenase system from *Comamonas testosteroni* T-2: purification and some properties of the oxygenase component. *J Bacteriol* **176**, 6644–6652.

Spence, E. L., Kawamukai, M., Sanvoisin, J., Braven, H. & Bugg, T. D. (1996). Catechol dioxygenases from *Escherichia coli* (MhpB) and *Alcaligenes eutrophus* (MpcI): sequence analysis and biochemical properties of a third family of extradiol dioxygenases. *J Bacteriol* **178**, 5249–5256.

Sugimoto, K., Senda, T., Aoshima, H., Masai, E., Fukuda, M. & Mitsui, Y. (1999). Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. *Struct Fold Design* **7**, 953–965.

Tralau, T., Cook, A. M. & Ruff, J. (2001). Map of the IncP1β plasmid pTSA encoding the widespread genes (*tsa*) for *p*-toluenesulfonate degradation in *Comamonas testosteroni* T-2. *Appl Environ Microbiol* **67**, 1508–1516.

Wang, Y. Z., Zhou, Y. & Zylstra, G. J. (1995). Molecular analysis of isophthalate and terephthalate degradation by *Comamonas testosteroni* YZW-D. *Environ Health Perspect* **103**, 9–12.

Wheelis, M. L., Palleroni, N. J. & Stanier, R. Y. (1967). The metabolism of aromatic acids by *Pseudomonas testosteroni* and *P. acidovorans*. *Arch Microbiol* **59**, 302–314.

Wyndham, R. C. (1986). Evolved aniline catabolism in *Acinetobacter calcoaceticus* during continuous culture of river water. *Appl Environ Microbiol* **51**, 781–789.

Wyndham, R. C., Singh, R. K. & Straus, N. A. (1988). Catabolic instability, plasmid gene deletion and recombination in *Alcaligenes* sp. BR60. *Arch Microbiol* **150**, 237–243.

Ziegler, K., Buder, R., Winter, J. & Fuchs, G. (1989). Activation of aromatic acids and aerobic 2-aminobenzoate metabolism in a denitrifying *Pseudomonas* strain. *Arch Microbiol* **151**, 171–176.

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