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

Miguel A. Providenti,1,2 Jörg Mampel,2 Scott MacSween,1 Alasdair M. Cook2 and R. Campbell Wyndham1

Author for correspondence: R. Campbell Wyndham. Tel: +1 613 520 2600 ext. 3651. Fax: +1 613 520 3539. e-mail: cwyndham!ccs.carleton.ca.

1 Institute of Biology, College of Natural Sciences, Carleton University, Ottawa, Ontario, Canada, K1S 5B6
2 Faculty of Biology, The University, D-78457, Konstanz, Germany

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 funneled through this compound (m- and p-hydroxybenzoate, p-sulfofenzoate, phthalate, isophthalate, terephthalate, vanillate, isovanillate and veratrates). Growth on benzolate 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; HCM5, 2-hydroxy-4-carboxyemunocate semialdehyde; HCMSD, HCMS dehydrogenase; Km, kanamycin; MMA, minimal medium A; OCA, 4-oxalocitratamale 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.
enzymes are listed.

\[
\text{Nsi} \quad \text{CTCT AGAGTCGACC TGCAGGCATG CAAGCTTGCG GCCGC-3}
\]

\[\alpha\]

The MCS of pUC18Not (de Lorenzo & Timmis, 1994) was modified by ligating an oligonucleotide into the pUC18Not but with a modified MCS*.

\[\text{Swa} \text{I}, \text{Sma} \text{I}, \text{Bam} \text{HI, } \text{Sac} \text{I}, \text{Hin} \text{II, } \text{Xba} \text{I, } \text{Sal} \text{I, } \text{Hind} \text{III, } \text{Bgl} \text{II, } \text{Bsd} \text{I, } \text{Sph} \text{I}]

\[\text{Bgl} \text{II site so that the } p\text{md} \text{A fragment into the BamHI/NotI site of pCR-Script SK+}
\]

\[\text{Ap}^*, \text{Cm}^*, \text{source of Cm}^* \text{ gene and pRR1 backbone}
\]

\[\text{Ap}^*, \text{mobilizable R6K-based vector; constructed by digesting pUTCM with SaI and religating the fragment containing oriV, oriT and Ap}^* \text{ gene}
\]

\[\text{Ap}^*, \text{Cm}^* \text{; intermediate in the construction of the } p\text{md} \text{A crossover cassette; } Cm^* \text{ gene from pUTCM cloned as a } Bgl\text{II–NotI fragment into the BamHI/NotI site of pCR-Script SK+}
\]

\[\text{Ap}^*, \text{portion of } p\text{md} \text{ locus spanning nt 1~7600 cloned into pUC18Not,1}
\]

\[\text{Ap}^*, \text{portion of } p\text{md} \text{ locus spanning nt 1500~8800 cloned into pUC18Not,1}
\]

\[\text{Ap}^*, \text{portion of } p\text{md} \text{ locus spanning nt 2800~10848 cloned into pUC18Not,1}
\]

\[\text{Ap}^*, \text{806 bp } \text{NotI–SaI} \text{ fragment from pLIB20G12 subcloned into pUC128}
\]

\[\text{Ap}^*, \text{Cm}^* \text{; gene cloned as a } Bsa\text{HI fragment into pSM99,7}
\]

\[\text{Ap}^*, \text{Cm}^* \text{; knock-out cassette for } p\text{md} \text{A cloned into pRR1 as a NotI fragment}
\]

\[\text{C. testosteroni}
\]

\[\text{BR6020 Derivative of wild-type BR60 cured of plasmid pBR60; originally identified as an } \text{Alcaligenes} \text{ sp. but reclassified based on biochemical features and sequencing of the 16S rRNA gene}
\]

\[\text{BR6020::pmdA Cm}^* \text{; BR6020 with a disrupted } p\text{md} \text{A}
\]

\[\text{C. testosteroni}
\]

\[\text{BR6020 Derivative of wild-type BR60 cured of plasmid pBR60; originally identified as an } \text{Alcaligenes} \text{ sp. but reclassified based on biochemical features and sequencing of the 16S rRNA gene}
\]

\[\text{BR6020::pmdA Cm}^* \text{; BR6020 with a disrupted } p\text{md} \text{A}
\]

*The MCS of pUC18Not (de Lorenzo & Timmis, 1994) was modified by ligating an oligonucleotide into the SacI/BamHI site so that the new MCS (5’-GGGCGCGCGG GAATTCGAGC TCCAGCGCGG TGCGCGCGGA TGCATATTTA AATCCCGCGG GGGATC-CTCT AGAGTCGACC TGCAGGCATG CAAGCTTGCG GCCGC-3’) contains restriction sites for NotI, EcoRI, SacI, SaI, EsgI, NstI, ScaI, SmI, BamHI, XbaI, SalI, SbfI/PstI, SbfI, HindIII and NotI when read 5’ to 3’. Note that not all sites for 6 bp-recognizing enzymes are listed.

genes have been studied in the z-proteobacterium <i>Sphingomonas paucimobilis</i> 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 <i>C. testosteroni</i> strains, metabolic pathways channel distinct aromatic compounds, some of which are pollutants, via Pca. Examples include <i>m-</i> and <i>p-</i> hydroxybenzoate (Michalover et al., 1973; Wheelis et al., 1967), chlorobenzoates (Nakatsu & Wyndham, 1993; Nakatsu et al., 1995b, 1997), <i>m-</i>nitrobenzoate (Nadeau & Spain, 1995), pthalates (Nakazawa & Hayashi, 1977, 1978; Schlaffi et al., 1994; Wang et al., 1995), methoxylated benzoates (Kersten et al., 1982, 1985; Ribbons, 1971), <i>p-<i>toluolate (Locher et al., 1991),<i> p-</i>toluensulfonate (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 <i>C. testosteroni</i>. We therefore undertook a study to clone and characterize PMD genes from <i>C. testosteroni</i> BR6020 and discovered the whole <i>meta</i> pathway in one locus (Fig. 1b).

**METHODS**

**Chemicals, bacteria, plasmids and growth conditions.** All 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, <i>Escherichia coli</i> 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

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (nkat)</th>
<th>Specific activity [mkat (kg protein)−1]</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1653</td>
<td>11·1</td>
<td>6·9</td>
<td>100</td>
<td>1·0</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>1610</td>
<td>10·9</td>
<td>7·8</td>
<td>98</td>
<td>1·1</td>
</tr>
<tr>
<td>Ultracentrifugation</td>
<td>544</td>
<td>5·1</td>
<td>9·3</td>
<td>45</td>
<td>1·4</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>25</td>
<td>0·75</td>
<td>30</td>
<td>6·7</td>
<td>4·3</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>0·7</td>
<td>0·22</td>
<td>316</td>
<td>2</td>
<td>52</td>
</tr>
</tbody>
</table>

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 108 kb locus harbouring *pmd* pathway genes cloned from the chromosome of *C. testosteroni* BR6020. No sites for BamHI, HindIII, SacI, Swal or XbaI 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 *SalI/NotI* 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 107 kb *EcoRI* 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 Pallikon 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: 30 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...
The column Amicon) was run with a Pharmacia apparatus. The anion exchange column of activity. FPLC was performed at room temperature with Tris-buffered eluents (pH 7–8) as described elsewhere (Junker et al., 1994). Reversed-phase HPLC of 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 dyeideoxy method and an ABI Prism Automated Sequencer (Biotechnology Research Institute, University of Ottawa, Ottawa, Canada). The locus was designated pmdA (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 pSMpmdACm5. 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 CCI182pir 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, 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).

**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 conjugal transfer (oriT) and the inverted repeat recognized by the transposase of Tn5 (O end). The latter is a remnant of the plasmid from which these vectors are derived and serves no purpose.

200000 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 O₂-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 Superose 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 (Schlafly 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).
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- or p-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>
<thead>
<tr>
<th>pmd gene product</th>
<th>Derived molecular mass (kDa)</th>
<th>Homologue (percentage identity)</th>
<th>GenBank accession no.</th>
<th>Function of homologue, if known</th>
<th>Reference</th>
<th>Inferred function of pmd gene product based on sequence homology and/or other features (see text for more details)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PmdA 168</td>
<td>LigA (56)</td>
<td>M34835</td>
<td>α-subunit of PMD</td>
<td>Noda et al. (1990)</td>
<td>α-subunit of PMD</td>
<td></td>
</tr>
<tr>
<td>PmdB 317</td>
<td>LigB (61)</td>
<td>M34835</td>
<td>β-subunit of PMD</td>
<td>Noda et al. (1990)</td>
<td>β-subunit of PMD</td>
<td></td>
</tr>
<tr>
<td>PmdC 352</td>
<td>LigC (76)</td>
<td>AB035122, CbaC (23)</td>
<td>U18133</td>
<td>1-Carboxy-3-chloro-3,4-dihydroxy-cyclohexa-1,5-diene dehydrogenase</td>
<td>Masai et al. (2000)</td>
<td>HCMSD</td>
</tr>
<tr>
<td>PmdD 344</td>
<td>LigD (55)</td>
<td>AB015964</td>
<td>2-Pyrone-4,6-dicarboxylic acid hydrolase</td>
<td>Masai et al. (1999)</td>
<td>2-Pyrone-4,6-dicarboxylic acid hydrolase</td>
<td></td>
</tr>
<tr>
<td>PmdE 382</td>
<td>LigE (63)</td>
<td>AB035121</td>
<td>4-Oxalomesaconate hydratase</td>
<td>Hara et al. (2000)</td>
<td>4-Oxalomesaconate hydratase</td>
<td></td>
</tr>
<tr>
<td>PmdF 240</td>
<td>FldZ (54)</td>
<td>AJ277295</td>
<td>Putative acyl transferase</td>
<td>Unpublished</td>
<td>OCA</td>
<td></td>
</tr>
<tr>
<td>PmdH 22</td>
<td>Hps-1 (22)</td>
<td>AE001045</td>
<td>d-Arabinose-3-hexulose-6-phosphate formaldehyde lyase-like protein</td>
<td>Klenk et al. (1997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PmdI 489</td>
<td>PcaK (42)</td>
<td>Q51955</td>
<td>Transporter for p-hydroxybenzoate and Pca</td>
<td>Harwood et al. (1994); Nichols &amp; Harwood (1997)</td>
<td>Aromatic transporter</td>
<td></td>
</tr>
<tr>
<td>PmdJ 29</td>
<td>BenK (29)</td>
<td>AAC46425</td>
<td>Benzoate transporter</td>
<td>Collier et al. (1997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PmdK 25</td>
<td>TfdK (25)</td>
<td>U16782</td>
<td>2,4-Dichlorophenoxyacetate transporter</td>
<td>Leveau et al. (1998)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3. For legend see facing page.
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 represent 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. testosterone* 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. testosterone*, 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,

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

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

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 kDa and 34 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-oxalocitrimalate aldolase (OCA) based on a similar derived amino acid composition (Table 4) and molecular mass (24 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. ochraceae* 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.
(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 ~4.3 kb upstream of and divergently transcribed from ligJABC. With respect to arrangement, while ligI 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., 1995a; Peel & Wyndham, 1999; Tralau et al., 1997; Nakatsu & Wyndham, 1993; Wyndham et al., 2001). The 4-β-ketoacid pathway genes for metabolism of catechol, generated from benzoate by a 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.

ACKNOWLEDGEMENTS

We are grateful to R. Rashid for supplying pRR1 and to K. Denger and P. J. Vierula for technical assistance and helpful discussion. M. A. P. was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) grant (to R. C. W.) and by a post-doctoral fellowship from the Alexander von Humboldt Foundation. J. M. was supported by a grant from the Deutsche Forschungsgemeinschaft (to A. M. C.). S. M. was the recipient of a NSERC summer scholarship.

REFERENCES


Received 29 January 2001; revised 18 April 2001; accepted 26 April 2001.