Identification of a self-sufficient cytochrome P450 monooxygenase from Cupriavidus pinatubonensis JMP134 involved in 2-hydroxyphenylacetic acid catabolism, via homogentisate pathway

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Summary
The self-sufficient cytochrome P450 RhF and its homologues belonging to the CYP116B subfamily have attracted considerable attention due to the potential for biotechnological applications based in their ability to catalyse an array of challenging oxidative reactions without requiring additional protein partners. In this work, we showed for the first time that a CYP116B self-sufficient cytochrome P450 encoded by the ohpA gene harboured by Cupriavidus pinatubonensis JMP134, a β-proteobacterium model for biodegradative pathways, catalyses the conversion of 2-hydroxyphenylacetic acid (2-HPA) into homogentisate. Mutational analysis and HPLC metabolite detection in strain JMP134 showed that 2-HPA is degraded through the well-known homogentisate pathway requiring a 2-HPA 5-hydroxylase activity provided by OhpA, which was additionally supported by heterologous expression and enzyme assays. The ohpA gene belongs to an operon including also ohpT, coding for a substrate-binding subunit of a putative transporter, whose expression is driven by an inducible promoter responsive to 2-HPA in presence of a predicted OhpR transcriptional regulator. OhpA homologues can be found in several genera belonging to Actinobacteria and α-, β- and γ-proteobacteria lineages indicating a widespread distribution of 2-HPA catabolism via homogentisate route. These results provide first time evidence for the natural function of members of the CYP116B self-sufficient oxygenases and represent a significant input to support novel kinetic and structural studies to develop cytochrome P450-based biocatalytic processes.

Introduction
Cytochrome P450 enzymes are a large superfamily of cysteine thiolate-coordinated b-type haem proteins that bind oxygen generating highly reactive iron-oxo species, which frequently catalyse reactions of monooxygenation, incorporating one oxygen atom to the substrate while the other oxygen atom is reduced to H2O (Denisov et al., 2005; Hrycay and Bandiera, 2015). These enzymes are broadly distributed in all life kingdoms having the ability to catalyse a vast number of chemically challenging reactions, making them powerful biocatalytic tools for biotechnology and synthetic biology (Bernhardt and Urlacher, 2014; Urlacher and Girhard, 2019). Cytochromes P450 act on a wide range of structurally different substrates, such as fatty acids, alkanes, steroids, vitamins, antibiotics, and diverse xenobiotics and drugs, playing key roles in biosynthesis and detoxification of a huge array of compounds (Van Bogaert et al., 2011; Peschke
Electron equivalents required in cytochrome P450 reactions are usually derived from NAD(P)H through an electron transfer chain represented by two main class of redox partners (Munro et al., 2007; McLean et al., 2015). Class I systems are represented by the first discovered microbial cytochrome P450: The Pseudomonas putida camphor hydroxylase P450cam (CYP101A1), a three-component camphor-hydroxylating system able to transfer electrons from NADH through a FAD-binding protein (pseudomonas reductase) into the iron–sulphur (2Fe-2S) cluster-binding ferredoxin (pudaredoxin) to the cytochrome P450 haem iron for catalysis (Mueller et al., 1995). Class II systems, such as membrane-bound mammalian hepatic two-component P450 enzymes, are associated with a di flavin reductase redox partner (named cytochrome P450 reductase; Murataliev et al., 2004; Pandey and Flück, 2013). Remarkably, it has been demonstrated that P450 systems are wider than was initially supposed, including cytochromes P450 bypassing redox partners, P450 fusions with non-electron transfer domains, and P450 fusions with several types of redox partner proteins, apparently facilitating efficient electron transport between protein domains (Munro et al., 2007; McLean et al., 2015). In this context, one of the most interesting systems due to its potential biotechnological applications is the P450-redox partner fusion systems represented by the catalytically self-sufficient P450 BM3 from Bacillus megaterium, that is a single-polypeptide enzyme comprising haem-, FAD- and FMN-binding domains, in which a soluble P450 fatty acid hydroxylase belonging to CYP102A subfamily is fused to a NADPH-dependent reductase (class II), displaying a high catalytic efficiency and a well-coupled reaction (Munro et al., 2002). This enzyme has a rather limited substrate range beyond its putative natural substrate, and it has been extensively engineered to catalyse oxidation of non-physiological substrates such as pharmaceutical metabolites, monosaccharides, alkanes, aromatic compounds, short-chain fatty acids and steroids (Meinhold et al., 2006; Lewis et al., 2009; Kille et al., 2011; Di Nardo and Gilardi, 2012; Whitehouse et al., 2012; Munday et al., 2016; O’Hanlon et al., 2017). Moreover, precise physiological role of the enzyme is still debatable since P450 BM3 expression is not induced in B. megaterium by saturated straight-chain fatty acids (Whitehouse et al., 2012). Another type of catalytically self-sufficient P450 with a distinctive redox partner was identified in Rhodococcus sp. NCIMB 9784 (Roberts et al., 2002), in which a soluble P450 domain is fused at the C-terminus to a FMN and 2Fe-2S ferredoxin containing reductase domain that resembles phthalate dioxygenase reductase (PDIR) from Burkholderia cepacia (Gassner et al., 1995; Chang and Zylstra, 1998). This clearly distinct Rhodococcus cytochrome was termed P450 RhF being classified in the CYP116B subfamily (De Mot and Parret, 2002). Close homologues of P450 RhF have been found in several species non-related to Actinobacteria as the metal-tolerant bacterium Cupriavidus metallidurans CH34 (Warman et al., 2012), the marine bacterium Labrenzia aggregata IAM 12614 (Yin et al., 2013), the alkane-degrading Acinetobacter radioresistens S13 (Minerdi et al., 2015) and the halophile Halomonas sp. NCIMB 172 (Porter et al., 2018), suggesting that CYP116B subfamily of P450s is present in phylogenetically distant genera. The P450 RhF and their homologues show an apparent high level of substrate promiscuity (but with low activity), catalysing a range of O-dealkylations, aromatic hydroxylations, epoxidations and asymmetric sulfoxidations (O’Reilly et al., 2013). The self-sufficient nature of P450 RhF along with its apparent broader substrate range highlights this enzyme as an outstanding starting template for directed evolution and promising alternative to P450 BM3 for protein engineering endeavours (O’Reilly et al., 2013). However, the identification of physiologically relevant substrates of P450 RhF and their homologues is still elusive, hampering further advances in structure-function studies (Roberts et al., 2003; Hunter et al., 2005; Celik et al., 2006).

One bacterial strain in which a close homologue of P450 RhF can be identified is the versatile pollutant degrader Cupriavidus pinatubonensis JMP134 that uses a vast array of aromatic compounds as growth substrate based on the broad diversity of catabolic pathways encoded in its genome (Pérez-Pantoja et al., 2008, 2012; Lykidis et al., 2010). However, a few degradation pathways used by strain JMP134 to metabolize some aromatic compounds such as 2-hydroxyphenylacetate (2-HPA) are still unknown (Pérez-Pantoja et al., 2008). This substrate is a natural product found in plants belonging to genus Astilbe (Kindl and Billek, 1962; Kindl, 1969) and has been proposed as intermediate in phenylacetate catabolism through the well-known homogentisate (2,5-dihydroxyphenylacetate) pathway in Aspergillus nidulans (Mingot et al., 1999). As well, in an earlier report, it has been suggested that 2-HPA could be metabolized via homogentisate in P. fluorescens ST (Baggi et al., 1983), for which it would be necessary an undiscovered 2-hydroxyphenylacetate-5-hydroxylase enzyme. Conversely, the homogentisate-ring cleavage pathway is a well-reported metabolic route, widespread in all life domains because it is used for tyrosine catabolism, starting with the maleylacetoacetate-producing enzyme homogentisate dioxygenase (HmgA) (Fig. 1A;
This product is subsequently isomerized into fumarylacetoacetate by a GSH-dependent enzyme (HmgC) and hydrolysed by a specific hydrolase (HmgB) to generate fumarate and acetoacetate channelled into tricarboxylic acid cycle (Fig. 1A).

Here, we provide genetic evidence that the physiologically self-sufficient cytochrome P450 encoded in strain JMP134 is 2-HPA. This substrate is transformed into homogentisate by a previously unnoticed 2-HPA 5-hydroxylase activity of this P450 RhF homologue, allowing its utilization as a sole carbon source in a way that is transcriptionally controlled by a 2-HPA-inducible promoter; and thus, revealing a landmark for catalytic and structural studies over this biotechnologically relevant enzyme family.

Results and discussion

2-HPA is degraded through homogentisate pathway in C. pinatubonensis JMP134

The metabolically versatile C. pinatubonensis JMP134 bacterium can grow on the o-, m- and p- isomers of HPA as well as to well-known substrates of the homogentisate-ring cleavage pathway such as tyrosine and phenylalanine (Pérez-Pantoja et al., 2008). In this strain, genes encoding homogentisate-degrading enzymes are found distributed along its genome with hmgA encoded in a secondary chromosome, whereas hmgC and a second fumarylacetoacetate hydrolase-encoding gene (hmgB2) located in the main chromosome (Fig. 1B). To get additional evidence for involvement of the homogentisate route in catabolism of 2-HPA, a previously unnoticed 2-HPA 5-hydroxylase activity of this P450 RhF homologue, allowing its utilization as a sole carbon source in a way that is transcriptionally controlled by a 2-HPA-inducible promoter; and thus, revealing a landmark for catalytic and structural studies over this biotechnologically relevant enzyme family.

Fig. 1. Putative 2-hydroxyphenylacetate (2-HPA) degradation pathway and gene clusters involved in 2-HPA catabolism in Cupriavidus pinatubonensis JMP134.

A. C. pinatubonensis JMP134 route channelling degradation of 2-HPA, 3-HPA and 4-HPA through homogentisate producing acetoacetate and fumarate (adapted from Pérez-Pantoja et al., 2008).

B. Genes putatively involved in 2-HPA/homogentisate catabolism in strain JMP134.

C. Protein domain structures of self-sufficient cytochrome P450 enzyme encoded by ohpA gene. The two fused domains are shown in bold. Specific functions are indicated in thin-lines boxes. The protein residue Cystein (haem-iron proximal ligand) is underlined. C1: Main chromosome; C2: Secondary chromosome.
all three HPA isomers, we conducted preliminary experiments on differential gene expression using a sub-genomic DNA microarray of strain JMP134 and found a sharp induction of \textit{hmgA} transcript levels as reported by median of ratios of spot signal intensities (values in brackets) in the presence of 1 mM 2-HPA (23.1 ± 2.9), 3-HPA (7.8 ± 1.5) and 4-HPA (6.5 ± 1.2). These results prompted us to get additional and substantive evidence performing growth tests in 2-, 3-, 4-HPA, tyrosine or phenylalanine as sole carbon and energy sources, with an \textit{hmgA} mutant obtained by insertional inactivation. As expected, none of these substrates allowed growth of the \textit{hmgA} derivative and liquid cultures turned yellow/ brown colouring (see Fig. S1A, for 2-HPA as example) suggesting accumulation and spontaneous polymerization of homogentisate, which produces melanin-like compounds, such as pyomelanin, that confer this characteristic colour to the medium (Rodríguez-Rojas et al., 2009; Han et al., 2015). Homogentisate accumulation in the \textit{hmgA} mutant was confirmed by HPLC / UV detection in resting cells exposed to 2-HPA (Fig. S1B). In contrast, the growth on phenylacetate (PA), a substrate not channelled through the homogentisate pathway (Pérez-Pantoja et al., 2008, 2015) was not affected in the \textit{hmgA} derivative (Fig. S1A). To confirm that the absence of a \textit{hmgA} gene was responsible of the observed phenotype, the mutant strain was transformed with a plasmid expressing the \textit{hmgA} gene controlled by the L-arabinose-inducible P\textsubscript{BAD} promoter. This heterologous promoter was chosen due L-arabinose is non-toxic and is not a carbon source for \textit{C. pinatubonensis} JMP134 allowing reliable growth tests in this strain (Donoso et al., 2011). This construct allowed restoration of growth abilities in all carbon sources channelled through homogentisate in \textit{C. pinatubonensis} JMP134 (see Fig. S1A, including results for 2-HPA as example). These results confirmed that 2-HPA, and 3-HPA and 4-HPA, are channelled into the homogentisate pathway in strain JMP134, as previously proposed by Pérez-Pantoja et al. (2008).

A cytochrome P450-coding gene was differentially expressed by strain JMP134 grown on 2-HPA

Differential expression analyses performed with a sub-genomic DNA microarray of \textit{C. pinatubonensis} JMP134 also revealed a sharp increase in transcripts level (19.5 ± 3.7 as reported by median of ratios of spot signal intensities) of the gene labelled with locus tag Reut_B5278 in cultures exposed to 1 mM 2-HPA, suggesting a role in biodegradation of this aromatic compound. This gene encodes a product with function not previously established but related to cytochrome P450, inferred by conserved domain analysis (Fig. 1C), pinpointing it to be a plausible candidate to encode a 2-HPA 5-hydroxylase activity responsible for homogentisate generation (accession IDs AAZ64624 or WP_011301389; termed \textit{ohpA} by \textit{ortho-hydroxyphenylacetate}). To test if \textit{ohpA} gene is involved in 2-HPA catabolism, a quantitative real-time PCR analysis of RNA extracted from mid-log-phase cells of \textit{C. pinatubonensis} JMP134 growing on 2-, 3-, 4-HPA, PA or fructose (a non-related carbon source as basal level) was carried out. Transcript levels of the \textit{ohpA} gene in 2-HPA-grown cells were at least two orders of magnitude higher than those of cells growing on any of the other compounds (Fig. 2), indicating a quite specific induction profile, and strongly suggesting its participation in 2-HPA catabolism but not in degradation of 3- and 4-HPA. In addition, quantitative analyses also showed that transcript levels of \textit{hmgA} gene were clearly increased in 2-HPA-grown cells, like 3- and 4-HPA-grown cells but not in PA-grown cells (Fig. 2), providing further evidence that catabolism of these three isomers involves the specific activation of the homogentisate-ring cleavage pathway, but most probably recruiting different hydroxylases for channelling each one into this route (Fig. 1A).
Mutational analyses, heterologous expression and enzyme assays confirm the key role of ohpA gene in biodegradation of 2-HPA

As ohpA gene was strongly overexpressed during growth in 2-HPA, we further explored the role of OhpA as a cytochrome P450 system performing a putative 2-HPA 5-hydroxylase activity. A derivative of C. pinatubonensis JMP134 harbouring a defective ohpA gene was generated through insertional inactivation. As expected, this derivative strain was unable to grow on 2-HPA as a sole carbon and energy source (Fig. 3A) but was still able to proliferate in presence of 3- and 4-HPA. Moreover, 2-HPA was not transformed at all in resting cells assays of the mutant derivative, contrasting the consumption profile displayed by the wild-type strain, conversely, 3- and 4-HPA are consumed as the similar rate by the wild type and the mutant derivative strain (Fig. 4A). For further confirmation, this ohpA mutant was complemented with a plasmid construct containing ohpA gene driven by the L-arabinose-inducible P_{BAD} promoter. This derivative recovered the ability to grow on 2-HPA only in presence of L-arabinose (Fig. 3A), indicating that ohpA gene is essential for 2-HPA degradation in strain JMP134. The higher growth rate on 2-HPA displayed by the complemented derivative compared with the parent JMP134 strain would be indicative that the wild-type expression level of ohpA is a limiting step in 2-HPA catabolism. Then, to gain full certainty of the ohpA function the plasmid construct containing such gene was introduced into P. putida KT2440, a well-known aromatic-degrader bacterium that harbours homogentisate pathway but is unable to grow on 2-HPA (Jiménez et al., 2002), and it is also unable to use L-arabinose as carbon source (Wang et al., 2019). The presence of ohpA was sufficient to allow growth of strain KT2440 on 2-HPA exclusively in presence of L-arabinose (Fig. 3B), and a resting cells assay showed complete 2-HPA consumption (and not for 3-HPA or 4-HPA) in this condition (Fig. 4B).

Finally, to indirectly evaluate OhpA enzyme specificity, we assayed the substrate-dependent NADH oxidation in cell extracts of P. putida KT2440 harbouring pBS1-ohpA when grown in presence of 5 mM L-arabinose. The NADH oxidation activities assayed were 951.6 ± 40.7 U g⁻¹, 43.9 ± 12.5 U g⁻¹ and 31.4 ± 11.5 U g⁻¹ when 2-HPA, 3-HPA or 4-HPA were, respectively, added to the reaction mixture, suggesting that 2-HPA is a strongly preferred substrate by OhpA. On the contrary, no measurable 2-HPA-dependent NADH oxidation activity was detected in a cell-free extract of P. putida KT2440 lacking pBS1-ohpA. Altogether, the results of genetic and enzyme assays unequivocally confirmed that OhpA enzyme is key in 2-HPA catabolism.

Expression of ohpA gene is controlled by an inducible promoter responsive to 2-HPA

Visual inspection of the gene context of ohpA gene revealed an adjacent gene, located 46 bp upstream of ohpA, identified by conserved domain analysis as a putative tripartite tricarboxylate transporter substrate-binding protein (Fig. 1B; ohpT gene) that belongs to the Bordetella uptake gene protein family (Antoine et al., 2003), suggesting a putative 2-HPA transporter function. In addition, 130 bp upstream of the ohpT gene, in opposite orientation, a putative transcriptional regulator was found (Fig. 1B; ohpR gene), which may be involved in regulation of 2-HPA catabolism. OhpR belongs to the IclR-type family of regulators that can act as repressors, activators or proteins with dual role and can be involved in multidrug resistance, quorum-quenching, sporation and degradation of aromatic compounds, among other functions (Molina-Henares et al., 2006). The ohpR and ohpT genes were highly and specifically induced by the presence of 2-HPA (Fig. 2), strongly indicating that both genes play a role in 2-HPA catabolism. Concerning control of ohpA gene expression, two upstream sequences might be putatively involved: the ohpA upstream sequence or, more probably, the ohpT upstream region considering that ohpT and ohpA genes would be part of the same transcriptional unit as suggested by two different bioinformatic algorithms for operon prediction based on intergenic distances (http://microbiome.wlu.ca/public/TUpredictions/Predictions/GCF_000203875.tus; Moreno-Hagelsieb and Collado-Vides, 2002) and comparative genomics (http://www.microbesonline.org/operon/gnc264198.html; Price et al., 2005). The operon structure composed by ohpT and ohpA genes was confirmed by quantitative real-time PCR analysis of the extended intergenic region, including boundaries of both genes, that showed a specific induction pattern in presence of 2-HPA (Fig. S2). On the contrary, the extended ohpR-ohpT region was also assayed as a negative control showing no signal (Fig. S2). Consequently, to evaluate the presence of a promoter triggering expression of the ohpT-ohpA operon, a transcriptional fusion was constructed including the ohpR gene and the ohpT upstream sequence to drive the expression of gfp gene (P_{ohpT-GFP}; Fig. 1B). This construct was introduced in C. pinatubonensis JMP134 cells, and the relative levels of GFP fluorescence were recorded growing in different substrates. Results showed that 2-HPA significantly increased P_{ohpT-GFP} activity (Fig. 5) and, on the contrary, PA or aromatics that are degraded through the homogentisate pathway (phenylalanine, tyrosine, or 3-

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HPA or 4-HPA isomers) did not provoke any induction (Fig. 5); strongly suggesting that ohpR-ohpT intergenic sequence contains a promoter that is driving the expression of an ohpT-ohpA operon. This promoter would be specifically responsive to 2-HPA, and not to analogous compounds or shared intermediates of homogentisate pathway.

The OhpA enzyme is a self-sufficient cytochrome P450 that belongs to the CYP116B subfamily

A comparison of the amino acid sequence of OhpA (783 aa) with those of the UniProtKB/Swiss-Prot database showed a 54% sequence identity between residues 19 to 443 with a non-partner fused P450 enzyme CYP116 (also known as ThcB) from Rhodococcus sp. Nl86/21, which is involved in degradation of herbicides S-ethyl dipropylthiocarbamate (EPTC) and atrazine (Nagy et al., 1995a,b). This OhpA region contains cytochrome P450 cysteine haem-iron ligand signature between residues 381 and 390 FGYSHOCMG (PROSITE consensus pattern [F/W]-[S/G/N/H]-[X-[G/D]-[F]-[R/K/H/P/T]-[P]-C-[L/I/V/M/F/A/P]-[G/A/D]; Fig. 1C), indicative that N-terminal region contains haem-binding domain, and that residue C388 is expected to be the haem-iron proximal (fifth) ligand (Werck-Reichhart and Feyereisen, 2000; Denisov et al., 2005). Furthermore, residues 473-771 of the C-terminal portion of OhpA (Fig. 1C) shows 42% amino acid sequence identity to the phenoxybenzoate (POB) dioxygenase beta subunit (PDOR-like) of P. oleovorans, which is the reductase subunit of POB dioxygenase that provides electrons to enable this enzyme to oxygenate 4-carboxydiphenyl ether (Dehmel et al., 1995). This OhpA region comprises at least three key conserved functional parts (Fig. 1C), an FMN-binding domain, a NAD(P)-binding domain and a [2Fe-2S] iron-sulphur cluster-binding domain, deduced by conserved domain analysis. Consequently, ohpA gene apparently encodes a self-sufficient cytochrome P450 like the well-known P450 RhF from Rhodococcus sp. NCIMB 9784, not requiring the involvement of additional proteins to transfer electrons from the reduced pyridine nucleotide to the active site and sharing a 66% amino acid identity on the overall sequence. A previous analysis of prokaryotic genome sequences using as bait P450 RhF from Rhodococcus sp. NCIMB 9784, permitted to find this novel kind of P450 redox system in C. metallidurans CH34, a close relative of C. pinatubonensis JMP134.
allowing assignation of these *Cupriavidus* proteins as new members of the CYP116 family (CYP116B), founded by ThcB (non-partner fused, CYP116A) from *Rhodococcus* sp. NI86/21 (De Mot and Parret, 2002), but whose self-sufficient members lacks a natural substrate properly established. Based on similarity between haem domains of ThcB and *C. metallidurans* CH34 CYP116B1 protein, Warman *et al.* (2012) have tested this protein for activity over thiocarbamate herbicides, showing that EPTC and S-propyl dipropyl diothiocarbamate were hydroxylated on propyl chains, proving thus that CYP116B1 has similar thiocarbamate-oxydizing catalytic properties compared to the rhodococcal CYP116A1 protein, the P450 involved in oxidative degradation of EPTC (Nagy *et al.*, 1995a). Notably, we found that *C. metallidurans* CH34 is also able to use 2-HPA as a sole carbon and energy source (see below), and the amino acid sequence of strain CH34 CYP116B1 has 80% amino acid identity with strain JMP134 OhpA, strongly suggesting that natural substrate of this self-sufficient cytochrome P450 CYP116B1-like enzyme is 2-HPA. A search for OhpA homologues in available bacterial genomes showed that this protein is present in diverse bacterial species mostly belonging to α- and β-proteobacterial classes, and Actinobacteria phylum but also in a few members of the γ-proteobacterial class (Fig. 6). It should be noted from examination of gene clusters of OhpA homologues that some Actinobacteria (*Arthrobacter nitrophenolicus*, *Janibacter indicus*, *Corynebacterium glyciniphilum*, *Saccharomonospora* Fig. 4. Resting cell assays of *Cupriavidus pinatubonensis* JMP134, *Pseudomonas putida* KT2440 and their derivatives in presence of hydrox-phenylacetate isomers. 
A. Resting cells of *C. pinatubonensis* JMP134, and the ohpA mutant (JMP134dohpA) were grown on fructose 20 mM plus 2-HPA 2.5 mM as inducer, washed and subsequently exposed to 1 mM 2-HPA, 3-HPA or 4-HPA. 
B. Resting cells of *P. putida* KT2440, and the derivative expressing ohpA gene driven by the heterologous P_{BAD} promoter (KT2440 pBS1-ohpA) were grown on succinate 30 mM plus arabinose 2.5 mM as inducer, washed and subsequently exposed to 1 mM 2-HPA, 3-HPA or 4-HPA. 
Two biological replicates were performed for growth measurements. Error bars indicate the standard deviation.

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of some strains used in this work, such as those belonging to Cupriavidus species, accomplish such role in addition to 2-HPA biodegradation, since neither JMP134 nor CH34 strains are able to use alkanes as growth substrates (data not shown).

Crystal structures of OhpA homologues from A. radioresistens S13 and Tepidiphilus thermophiles JCM 19170 have been recently solved, providing first bases for the structure-guided design of new biocatalysts with these enzymes (Tavanti et al., 2018; Ciaramella et al., 2019; Zhang et al., 2020). According to the X-ray structure of the homologue harboured by strain JCM 19170, it was revealed that the catalytic pocket of the enzyme is surrounded by 18 residues organized in a 4-tiered system above the haem cofactor that are well conserved among CYP116B members (Tavanti et al., 2018). A sequence alignment analysis using all homologues of OhpA from Fig. 6, showed a well-conserved catalytic pocket (Table S1) and haem-binding domain (Table S2), suggesting a similar substrate profile among analysed strains. Nevertheless, in the case of OhpA homologue from T. thermophiles, a few non-conservative substitutions in comparison with the consensus could reflect a different substrate preference (Tables S1 and S2; Tavanti et al., 2018). Consequently, enzyme assays of the self-sufficient cytochrome P450 in T. thermophiles and A. radioresistens strains using 2-HPA as substrate will provide additional clarification on the physiological substrate of such members of CYP116B subfamily.

Conclusion

The results of this study provide evidence for the thus far unknown function of a self-sufficient cytochrome P450, belonging to the CYP116B family. The 2-HPA 5-hydroxylase activity proposed here is in line with the recent report of proficient 5-hydroxylation on the phenylacetic acid moiety of the synthetic anti-inflammatory drug diclofenac (2-[2,6-dichloroanilino]phenyl)acetic acid) by the founder member of the CYP116B family, the self-sufficient cytochrome P450 RhF from Rhodococcus sp. NCIMB 9784 (Klenk et al., 2017). The identification of 2-HPA as natural substrate of the CYP116B family will allow kinetic and protein engineering studies providing guidelines for improvement and implementation of biocatalytic P450 processes such as those required for production of oxygenfunctionalized high-value compounds in pharmaceutical and related industries (Urlacher and Girhard, 2019).

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacteria and plasmids used in this study are listed in Table 1. C. pinatubonensis JMP134 and P. putida
KT2440, and its derivatives were grown at 30°C in mineral salts medium, supplemented with 5 mM 2-HPA, 3-HPA, 4-HPA, PA, tyrosine, phenylalanine, or fructose, plus the appropriate antibiotics, kanamycin (Km; 50 μg ml⁻¹) or gentamicin (Gm; 30 μg ml⁻¹). Other strains tested for growth on 2-HPA were grown at 30°C in mineral salts medium, supplemented with 2.5 mM 2-HPA.

**Fig. 6.** Evolutionary relationships among several OhpA homologues. Maximum likelihood topology provided by IQ-TREE (Nguyen et al., 2015) based on sequence alignments calculated using MAFFT (Katoh et al., 2019) is shown with SH-like approximate likelihood ratio support values (n = 1000) given at each node (values > 50% are shown). Sequences indicated in bold belong to strains tested by its ability to grow in 2-HPA as a sole carbon and energy source. Blue, α-proteobacteria; orange, β-proteobacteria; green, γ-proteobacteria; yellow, Actinobacteria; red, Hydrogenophilalia class.
with a Synergy HTX multimode plate reader (BioTek, Winooski, VT, USA). At least three biological replicates were performed for each growth measurement.

**Chromosomal disruption of gene sequences in C. pinatubonensis JMP134**

Internal fragments of *ohpA* and *hmgA* were amplified by PCR using primer pairs listed in Table 2. The PCR products were cloned using the pCR2.1-TOPO system (Invitrogen, Carlsbad, CA, USA) to generate plasmids listed in Table 1. For gene inactivation, suicidal pCR2.1-dohpA or pCR2.1-dhmgA plasmids (Table 1) were electroporated in *C. pinatubonensis* JMP134 cells to get a one-recombination-event disruption of the target gene resulting in insertional mutants (Table 1), which were selected on LB agar containing 50 \(\mu\)g ml\(^{-1}\) Km. Correct insertions in all mutants were confirmed by PCR using primer pairs located in genomic DNA and suicidal plasmid DNA (Table 2), and subsequent sequencing.

**Detection of transcripts by quantitative real-time PCR**

Cells of *C. pinatubonensis* JMP134 were grown on 5 mM 2-HPA, 3-HPA, 4-HPA, PA or fructose as sole carbon and energy sources. Then, total RNA was obtained from 4 ml of mid-log-phase cells, using RNAprotect bacterial reagent and the RNeasy minikit (Qiagen, Chatsworth, CA, USA). The RNA was quantified using an Eon microplate spectrophotometer (BioTek, Winooski, VT, USA) and treated with the Turbo DNase kit (Ambion, Austin, TX, USA) to remove DNA contamination. The reverse transcription PCR was performed using the ImProm-II reverse transcription system (Promega Corporation, Madison, WI, USA) with 1 \(\mu\)g of RNA in 20-\(\mu\)l reaction mixtures. Real-time PCR was performed using the Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and AriaMx Real-time PCR System (Agilent Technologies). The PCR mixture (15 \(\mu\)l) contained 3.0 \(\mu\)l of template cDNA (diluted 1:10) and 0.2 \(\mu\)M (each) primer. Amplification was performed under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s, and finishing with a melting cycle from 55 to 95°C. Relative gene expression values were calculated using the comparative cycle threshold method (also known as the 2\(^{-\Delta\Delta CT}\) method; Schmittgen and Livak, 2008). 16S rRNA gene sequence (Reut_AR0020) was used as a reference gene (internal control) in these assays. Gene expression levels were normalized to the average value of the gene expression levels determined in the fructose treatment. Genes analysed by quantitative real-time PCR were *ohpA* (Reut_B5278), *ohpR* (Reut_B5276), *ohpT* (Reut_B5277) and *hmgA* (Reut_B3923). Experiments were done in three biological replicates.

**Construction of plasmid derivatives expressing ohpA and hmgA genes**

To obtain pBS1-ohpA and pBS1-hmgA plasmids (Table 1), which contain the *ohpA* and *hmgA* genes under the control of a L-arabinose-inducible promoter, the Gibson et al. (2009) assembly method was used. In brief, PCR products comprising *ohpA*, or *hmgA*, and
Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid          | Relevant phenotype and/or genotype | Reference or source |
|----------------------------|-----------------------------------|---------------------|
| **C. pinatubonensis strains** |                                   |                     |
| JMP134                     | 2-HPA<sup>+</sup>, 3-HPA<sup>+</sup>, 4-HPA<sup>+</sup>, PA<sup>+</sup>, tyrosine<sup>−</sup>, phenylalanine<sup>−</sup>, fructose<sup>−</sup>, arabinose<sup>−</sup> | DSMZ<sup>a</sup>     |
| JMP134<sup>d</sup>          | 2-HPA<sup>−</sup>, fructose<sup>−</sup> | This study          |
| JMP134<sup>dhm</sup>        | 2-HPA<sup>−</sup>, fructose<sup>−</sup> | This study          |
| **Other strains**           |                                   |                     |
| **P. putida**               |                                   |                     |
| KT2440                     | 2-HPA<sup>−</sup>, arabinose<sup>−</sup> | Bagdasarian et al. (1981) |
| R. ruber                   |                                   |                     |
| Chol-4                     | ohpA homologue, locus tag          | DSMZ                |
| DSM                        | RR2_RS22805                        |                     |
| R. ruber R1                | ohpA homologue, locus tag          | Farkas et al. (2020) |
| DSM                        | E2561_01140                        |                     |
| R. jostii RHA1             | No ohpA homologue                 | McLeod et al. (2006) |
| Rhodococcus sp. H-CA8f     | No ohpA homologue                 | Undabarrena et al. (2018) |
| C. metallidurans           |                                   |                     |
| CH34                       | ohpA homologue, locus tag          | DSMZ                |
| B. multivorans             | ohpA homologue, locus tag          | Nishiyama et al. (2010) |
| ATCC 17616                 | Bmul_5958                          |                     |
| P. xenovorans              | No ohpA homologue                 | Chain et al. (2006) |
| LB400                      | No ohpA homologue                 | Weilharter et al. (2011) |
| P. phytofirmans            |                                   |                     |
| PsHN                      |                                   |                     |
| A. radioresistens          | ohpA homologue, locus tag          | DSMZ                |
| DSM 6976                   | ACRAD_RS10630                      |                     |
| A. johnsonii               | No ohpA homologue                 | DSMZ                |
| DSM 6963                   |                                   |                     |
| A. junci DSM               | No ohpA homologue                 | DSMZ                |
| 6964                       |                                   |                     |
| S. wittichii               | ohpA homologue, locus tag          | DSMZ                |
| RW1                        | RS15645                            |                     |
| **Plasmids**               |                                   |                     |
| pCR2.1-TOPO                | Suicide vector in C. pinatubonensis JMP134, Km<sup>R</sup> | Invitrogen, Carlsbad, USA |
| pCR2.1-dohpA               | pCR2.1 derivative containing internal fragment of ohpA gene, Km<sup>R</sup> | This study          |
| pCR2.1-dhmA                | pCR2.1 derivative containing internal fragment of hmgA gene, Km<sup>R</sup> | This study          |
| pBS1                       | Broad host range vector, araC<sup>−</sup>, P<sub>BAD</sub>, Gm<sup>R</sup> | Bronstein et al. (2005) |
| pBS1-ohpA                  | pBS1 derivative expressing ohpA gene, Gm<sup>R</sup> | This study          |
| pBS1-hmgA                  | pBS1 derivative expressing hmgA gene, Gm<sup>R</sup> | This study          |
| pSEVA237M                  | Broad host range msIGFP reporter vector, Km<sup>R</sup> | Svensningsen et al. (2015) |

Table 1. (Continued) Relevant phenotype and/or genotype of ohpT promoter, Km<sup>R</sup>

| Strain or plasmid          | Relevant phenotype and/or genotype | Reference or source |
|----------------------------|-----------------------------------|---------------------|
| P<sub>ohpT</sub>-GFP       | pSEVA237M derivative, msIGFP gene under control of ohpT promoter | This study          |

pBS1 plasmid, were generated using primer pairs ohpA1-ohpA2, hmgA1-hmgA2 and pBS1a-pBS1b, respectively, Table 2. These primers contain a 20-bp terminal sequence homologous to the terminus of the fragment to be linked, and the sequences were combined and ligated to generate a new DNA molecule in a one-step isothermal reaction (Gibson et al., 2009). Plasmid pBS1 derivatives were electroporated in strains JMP134<sup>dohpA</sup>, JMP134<sup>dhm</sup><sub>mgA</sub> or P. putida KT2440 and selected in LB medium plus Gm (30 µg ml<sup>−1</sup>), or Km (50 µg ml<sup>−1</sup>), as appropriate. For expression of ohpA or hmgA genes driven by the heterologous PBAD promoter, these derivatives were exposed to 5 mM L-arabinose. All plasmid constructs obtained by the Gibson et al. (2009) assembly method, were confirmed by DNA sequencing.

NADH oxidation assay in cells extracts

The substrate-dependent oxidation of NADH was used to test specificity of OhpA in cell extracts of P. putida KT2440. The preparation of cell extracts in this bacterium has been previously described (Pérez-Pantoja et al., 2000, 2009). Briefly, P. putida KT2440 and a derivative harbouring the pBS1-ohpA construct were grown to late exponential phase in mineral medium with succinate (30 mM) as sole carbon source in the presence of 5 mM L-arabinose plus antibiotic Gm when required. Then, cells were harvested, centrifuged at 8500 rpm for 10 min, washed twice with 1 vol. Tris-acetate (50 mM, pH 7.5) and suspended in 1 ml Tris-acetate (50 mM, pH 7.5) to be subjected to lysis by sonication with three pulses of 10 s at potency level 15 using an ultrasonic cell disruptor (Microson XL2000; Misonic Inc., Farmingdale, NY, USA). The lysates were subjected to two successive centrifugations at 16 000 rpm for 45 min at 4°C. Finally, supernatants were collected and used for enzyme assays. The substrate-dependent oxidation of NADH was measured spectrophotometrically by the decrease of cofactor NADH at 340 nm (ε<sub>340</sub> = 6300 M<sup>−1</sup> cm<sup>−1</sup>) in a diode-array Hewlett Packard HP 8452-A UV/Vis.
spectrophotometer. The reaction was performed in 1.0 ml quartz cuvettes with a 1 cm light path and the standard assay mixture contained (ml⁻¹) 35 mM Tris-acetate buffer (pH 7.5), 100 µM substrate (2-HPA, 3-HPA or 4-HPA), 1 µM FAD, 0.2 mM NADH and a suitable quantity of cell extract. After non-specific (in the absence of substrate) NADH oxidation at 340 nm was recorded for 2 min, each substrate was added and recording of NADH oxidation was continued for 2 min. Enzyme activity was calculated from the difference between the non-specific and substrate-dependent oxidation rates. One unit of enzyme activity was defined as the amount of crude extract that catalyses the oxidation of 1 µmol of NADH/min. The protein concentration in the cell extracts was estimated as described by Bradford (1976). Bovine serum albumin was used as protein standard.

Construction of msfGFP reporter fusions

Putative promoter region was fused to the monomeric superfolder msfGFP gene of the pBBR1-based broad-host-range vector pSEVA237M (Svenningsen et al., 2015) of the SEVA collection (Silva-Rocha et al., 2013). PCR product comprising 1062 bp of the upstream region contiguous to translational start of the ohpT gene, also including ohpR, was obtained using primer pairs listed in Table 2. The amplified DNA fragments were cloned into the KpnI-XbaI restriction site of pSEVA237M, forming P-ohpT-msfGFP plasmid; transferred into strain JMP134 and selected in LB medium supplemented with 50 µg ml⁻¹ Km. To evaluate promoter induction profiles, bacterial cells carrying promoter construction were grown overnight on LB medium, and then inoculated in cultures containing 5 mM 2-HPA, 3-HPA, 4-HPA, PA, tyrosine, phenylalanine or fructose as the sole carbon and energy source, plus antibiotic Km. The cultures were incubated in a 96-well microplate (Thermo Fisher Scientific, Rochester, NY, USA) at 30°C and the OD600 and the green fluorescence (excitation filter: 485/20, emission filter: 528/20) were measured in a Synergy HTX Multi-Mode Reader (BioTek, Winooski, VT, USA).

Analytical methods

The presence of 2-HPA, 3-HPA and 4-HPA was determined by high-performance liquid chromatography using cell-free supernatants from resting cells grown on 20 mM fructose (JMP134 derivatives) or succinate 30 mM (KT2440 derivatives) plus 2.5 mM 2-HPA or arabinose (as inducers of ohpA expression in JMP134 or KT2440 strains, respectively), washed twice with 1 volume of phosphate buffer (14 g l⁻¹ Na₂HPO₄ 12H₂O, 2 g l⁻¹ KH₂PO₄), and subsequently incubated with 1 mM 2-HPA, 3-HPA and 4-HPA. Samples (2 ml) were
obtained at different times, filtered (0.22 μm) and injected into a JASCO liquid chromatograph LC-4000 (JASCO, Oklahoma City, OK, USA) equipped with a Kromasil 100-3.5-C18 4.6 mm diameter column. A methanol-H₂O (40:60) mixture containing 0.1% (vol/vol) phosphoric acid was used as the solvent, at a flow rate of 0.8 ml min⁻¹. The column effluent was monitored at 210 nm for all compounds. Retention times for 2-HPA, 3-HPA and 4-HPA were 12.1, 11.7 and 11.3 min, respectively.

Bioinformatic tools

The ohpA gene sequences were retrieved from non-redundant protein sequences database of GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi; NCBI Resource Coordinators, 2018). Only proteins displaying at least 45% amino acid identity with the entire ohpA gene of C. pinatubonensis JMP134 were considered for analysis. Protein similarity and conserved domain searches were performed with the BLASTP program and the Conserved Domains Database from the NCBI website using default parameters (Johnson et al., 2008; Lu et al., 2020). Evolutionary relationships were inferred by IQ-TREE web server tools (http://iqtree.cibiv.univie.ac.at/) designed for estimate maximum-likelihood phylogenies (Nguyen et al., 2015) employing ModelFinder as model-selection method (Kalyaanamoorthy et al., 2017) and UFBoot2 for ultrastart bootstrap approximation (Hoang et al., 2018) with the -m TEST, -bb 1000 and -a1it 1000 options. Sequence alignments for phylogenetic reconstruction were calculated with MAFFT online service (https://mafft.t.cbc.jp/alignment/server/) using Auto (FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i; depends on data size) strategy (Katoh et al., 2019). Visualization and edition of phylogenetic trees were performed by the Interactive Tree Of Life (iTOl) online tool (https://itol.embl.de/; Letunic and Bork, 2019).

Chemicals

2-HPA, 3-HPA, 4-HPA, L-phenylalanine and homogentisic acid were purchased from Sigma-Aldrich (Steinheim, Germany). PA, L-tyrosine, d(-)-fructose and L(-)-arabinose were purchased from Merck (Darmstadt, Germany).

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Conflict of interest

Authors declare no conflict of interest.

REFERENCES

Antoine, R., Jacob-Dubuisson, F., Drobecq, H., Willery, E., Lesjean, S., and Locht, C. (2003) Overrepresentation of a gene family encoding extracytoplasmic solute receptors in Bordetella. J Bacteriol 185: 1470–1474.

Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F.C.H., Bagdasarian, M.M., Frey, J., and Timmis, K.N. (1981) Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. Gene 16: 237–247.

Baggi, G., Boga, M.M., Catelani, D., Galli, E., and Treccani, V. (1983) Styrene catabolism by a strain of Pseudomonas fluorescens. Syst Appl Microbiol 4: 141–147.

Bernhardt, R., and Uhlacher, V.B. (2014) Cytochromes P450 as promising catalysts for biotechnological applications: chances and limitations. Appl Microbiol Biotechnol 98: 6185–6203.

Bhattacharya, S.S., and Yadav, J.S. (2018) Microbial P450 enzymes in bioremediation and drug discovery: Emerging potentials and challenges. Curr Protein Pept Sci 19: 75–86.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Bronstein, P.A., Marrichi, M., Cartinhour, S., Schneider, D.J., and DeLisa, M.P. (2005) Identification of a twin-arginine translocation system in Pseudomonas syringae pv. tomato DC3000 and its contribution to pathogenicity and fitness. J Bacteriol 187: 8450–8461.

Celik, A., Roberts, G.A., White, J.H., Chapman, S.K., Turner, N.J., and Flitsch, S.L. (2006) Probing the substrate specificity of the catalytically self-sufficient cytochrome P450 RhF from a Rhodococcus sp. Chem Commun 43: 4492–4494.

Chain, P.S., Denef, V.J., Konstantinidis, K.T., Vergez, L.M., Aguillo, L., Reyes, V.I., et al. (2006) Burkholderia xenovorans LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. Proc Natl Acad Sci USA 103: 15280–15287.

Chang, H.K., and Zylstra, G.J. (1998) Novel organization of the genes for phthalate degradation from Burkholderia cepacia DB01. J Bacteriol 180: 6529–6537.

Ciaramella, A., Catucci, G., Gilaridi, G., and Di Nardo, G. (2019) Crystal structure of bacterial CYP116B5 heme domain: new insights on class VII P450s structural flexibility and peroxygenase activity. Int J Biol Macromol 140: 577–587.

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De Mot, R., and Parret, A.H. (2002) A novel class of self-sufficient cytochrome P450 monoxygenases in prokaryotes. Trends Microbiol 10: 502–508.

Dehmel, U., Engesser, K.H., Timmis, K.N., and Dwyer, D.F. (1995) Cloning, nucleotide sequence, and expression of the gene encoding a novel dioxygenase involved in metabolism of carboxydiplhenyl ethers in Pseudomonas pseudoalcaligenes POB310. Arch Microbiol 163: 35–41.

Denisov, I.G., Makris, T.M., Sigar, S.G., and Schlichting, I. (2005) Structure and chemistry of cytochrome P450. Chem Rev 105: 2253–2277.

Di Nardo, G., and Gilardi, G. (2012) Optimization of the bacterial cytochrome P450 BM3 system for the production of human drug metabolites. Int J Mol Sci 13: 15901–15924.

Donoso, R.A., Pérez-Pantoja, D., and González, B. (2011) Strict and direct transcriptional repression of the pobA gene by benzoate avoids 4-hydroxybenzoate degradation in the pollutant degrader bacterium Cupriavidus necator. Environ Microbiol 13: 1590–1600.

Farkas, C., Donoso, R.A., Melis-Arcos, F., Gárate-Castro, C., and Pérez-Pantoja, D. (2020) Complete genome sequence of Rhodococcus ruber R1, a novel strain showing a broad catabolic potential toward lignin-derived aromatics. Microbiol Resour Announc 9: e00905-19.

Felpeito-Santero, C., Galán, B., Luengo, J.M., Fernández-Canón, J.M., Del Cerro, C., et al. (2019) Identification and expression of the 11↑-steroid hydroxylase from Cochliobolus lunatus in Corynebacterium glutamicum. Microb Biotechnol 12: 856–868.

Gassner, G.T., Ludwig, M.L., Gatti, D.L., Correll, C.C., and Ballou, D.P. (1995) Structure and mechanism of the iron-sulfur flavoprotein phthalate dioxygenase reductase. FASEB J 9: 1411–1418.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A. 3rd, and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6: 343–345.

Han, H., Iakovenko, L., and Wilson, A.C. (2015) Loss of homogentisate 1,2-dioxygenase activity in Bacillus anthracis results in accumulation of protective pigment. PLoS One 10: e0128967.

Hildebrandt, T.M., Nunes Nesi, A., Araújo, W.L., and Braun, H.P. (2015) Amino acid catabolism in plants. Mol Plant 8: 1563–1579.

Hoang, D.T., Chemomor, O., von Haeseler, A., Minh, B.Q., and Ving, L.S. (2018) UFBoot2: improving the ultrafast bootstrap approximation. Mol Biol Evol 35: 518–522.

Hrycay, E.G., and Bandiera, S.M. (2015) Monoxygenases, peroxidase and peroxidase properties and reaction mechanisms of cytochrome P450 enzymes. Adv Exp Med Biol 851: 1–61.

Hunter, D.J.B., Roberts, G.A., Ost, T.W.B., White, J.H., Müller, S., Turner, N.J., et al. (2005) Analysis of the domain properties of the novel cytochrome P450 RhF. FEBS Lett 579: 2215–2220.

Ichinose, H., and Kitaoka, T. (2018) Insight into metabolic diversity of the brown-rot basidiomycete Postia placenta responsible for sesquiterpene biosynthesis: semi comprehensive screening of cytochrome P450 monoxygenase involved in protoilludene metabolism. Microb Biotechnol 11: 952–965.

Jiménez, J.I., Minnabres, B., García, J.L., and Díaz, E. (2002) Genomic analysis of the aromatic catabolic pathways from Pseudomonas putida KT2440. Environ Microbiol 4: 824–841.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., and Madden, T.L. (2008) NCBI BLAST: a better web interface. Nucleic Acids Res 36: W5–W9.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., and Jeromin, L.S. (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods 14: 587–589.

Kato, K., Rozewicki, J., and Yamada, K.D. (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform 20: 1160–1166.

Kille, S., Zilly, F.E., Acevedo, J.P., and Reetz, M.T. (2011) Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution. Nat Chem 3: 738–743.

Kindl, H. (1969) Biosynthesis and metabolism of hydroxyphenylacetic acids in higher plants. Eur J Biochem 7: 340–347.

Kindl, H., and Billek, G. (1962) 2-hydroxyphenylacetic acid, a new constituent of higher plants. Nature 194: 579.

Klenk, J.M., Dubiel, P., Sharma, M., Grogan, G., and Hauer, B. (2019) Characterization and structure-guided engineering of the novel versatile terpene monoxygenase CYP109Q5 from Chondromyces apiculatus DSM436. Microb Biotechnol 12: 377–391.

Klenk, J.M., Nebel, B.A., Porter, J.L., Kulig, J.K., Hussain, S.A., Richter, S.M., et al. (2017) The self-sufficient P450 RhF expressed in a whole cell system selectively catalyses the 5-hydroxylation of diclofenac. Biotechnol J 12: 1–8.

Letunic, I., and Bork, P. (2019) Interactive Tree Of Life (iTOl) v4: recent updates and new developments. Nucleic Acids Res 47(W1): W256–W259.

Lewis, J.C., Bastian, S., Bennett, C.S., Fu, Y., Mitsuda, Y., Chen, M.., et al. (2009) Chemoenzymatic elaboration of monosaccharides using engineered cytochrome P450BM3 demethylases. Proc Natl Acad Sci USA 106: 16550–16555.

Lu, S., Wang, J., Chitsaz, F., Derbyshy, M.K., Geer, R.C., Gonzales, N., et al. (2020) CDD/SPARCLE: the conserved domain database in 2020. Nucleic Acids Res 48: D265–D268.

Lykidis, A., Pérez-Pantoja, D., Ledger, T., Mavromatis, K., Anderson, I.J., Ivanova, N.N., et al. (2010) The complete multipartite genome sequence of Cupriavidus necator JMP134, a versatile pollutant degrader. PLoS One 5: e9729.

McLean, K.J., Luciakova, D., Belcher, J., Tee, K.L., and Munro, A.W. (2015) Biological diversity of cytochrome P450 reduct partner systems. Adv Exp Med Biol 851: 299–317.

McLeod, M.P., Warren, R.L., Hsiao, W.W., Araki, N., Myhre, M., Fernandes, C., et al. (2006) The complete genome of Rhodococcus sp. RH1A provides insights into a catabolic powerhouse. Proc Natl Acad Sci USA 103: 15882–15887.

Meinhold, P., Peters, M.W., Hartwick, A., Hernandez, A.R., and Arnold, F.H. (2006) Engineering cytochrome P450

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2-Hydroxyphenylacetate as cytochrome P450 substrate

BM3 for terminal alkane hydroxylation. Adv Synth Catal 348: 763–774.
Minerdi, D., Sadeghi, S.J., Di Nardo, G., Rua, F., Castrignanò, S., et al. (2015) CYP116B5: a new class VII catalytically self-sufficient cytochrome P450 from Acinetobacter radioresistens that enables growth on alkanes. Mol Microbiol 95: 539–554.

Mingot, J.M., Penalva, M.A., and Fernández-Canón, J.M. (1999) Disruption of phacA, an Aspergillus nidulans gene encoding a novel cytochrome P450 monooxygenase catalyzing phenylacetate 2-hydroxylation, results in penicillin overproduction. J Biol Chem 274: 14454–14455.

Molina-Henares, A.J., Krell, T., Guazzaroni, M.E., Segura, A., and Ramos, J.L. (2006) Members of the IclR family of bacterial transcriptional regulators function as activators and/or repressors. FEBS Microbiol Rev 30: 157–186.

Moreno-Hagelsieb, G., and Collado-Vides, J. (2002) A powerful non-homology method for the prediction of operons in prokaryotes. Bioinformatics 18(Suppl. 1): S329–S336.

Mueller, E.J., Loida, P.J., and Sligar, S.G. (1995) Twenty-five years of P450cam research. In Cytochrome P450: Structure, Mechanism and Biochemistry, 2nd edn. de Montellano, P.R.O. (ed). New York, NY, USA: Plenum Press, pp. 83–124.

Munday, S.D., Shoji, O., Watanabe, Y., Wong, L.L., and Bell, S.G. (2016) Improved oxidation of aromatic and aliphatic hydrocarbons using rate enhancing variants of cytochrome P450BM3 in combination with decay molecules. Chem Commun 52: 1036–1039.

Murataliev, M.B., Feyereisen, R., and Walker, F.A. (2004) Improved expression and functional properties of a modulated, chimeric P450BM3 subunit. J Biol Chem 279: 2466–2473.

Nagy, I., Compernolle, F., Ghys, K., Vanderleyden, J., and de Mot, R. (1995a) A single cytochrome P-450 system is involved in degradation of the herbicides EPTC (S-ethyl dipropylcarbamate) and atrazine by Rhodococcus sp. strain NI86/21. Appl Environ Microbiol 61: 2056–2060.

Nagy, I., Schools, G., Compernolle, F., Proost, P., Vanderleyden, J., and de Mot, R. (1995b) Degradation of the thiocarbamate herbicide EPTC (S-ethyl dipropylcarbamothioate) and biosynthesis of Rhodococcus sp. strain NI86/21 involve an inducible cytochrome P-450 system and aldehyde dehydrogenase. J Bacteriol 177: 676–687.

NCBI Resource Coordinators (2018) Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 46: D8–D13.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., and Minh, B.Q. (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32: 268–274.

Nishiyama, E., Ohtsubo, Y., Nagata, Y., and Tsuda, M. (2010) Identification of Burkholderia multivorans ATCC 17616 genes induced in soil environment by in vivo expression technology. Environ Microbiol 12: 2539–2558.

O’Hanlon, J.A., Ren, X., Morris, M., Wong, L.L., and Robert-son, J. (2017) Hydroxylation of anilides by engineered cytochrome P450(BM3). Org Biomol Chem 15: 8780–8787.

Pandey, A.V., and Flück, C.E. (2013) NADPH P450 oxidoreductase: structure, function, and pathology of diseases. Pharmacol Ther 138: 229–254.

Pérez-Pantoja, D., De la Iglesia, R., Pieper, D.H., and González, B. (2008) Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium Cupriavidus necator JMP134. FEMS Microbiol Rev 32: 736–794.

Pérez-Pantoja, D., Donoso, R.A., Sánchez, M.A., and González, B. (2009) Genuine genetic redundancy in malleylactate-reductase-encoding genes involved in degradation of halooaromatic compounds by Cupriavidus necator JMP134. Microbiology 155: 3641–3651.

Pérez-Pantoja, D., Donoso, R., Agulló, L., Córdova, M., Seeger, M., Pieper, D.H., and González, B. (2012) Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ Microbiol 14: 1091–1117.

Pérez-Pantoja, D., Guzmán, L., Manzano, Mieher, D.H., and González, B. (2000) Role of tfdC(I)D(I)E(I)F(I) and tfdD(II)C(II)E(II)F(II) genes modules in catabolism of 3-chlorobenzoate byRalstoniaeutrophaJMP134(pJP4). Appl Environ Microbiol 66: 1602–1608.

Porter, J.L., Sabatini, S., Manning, J., Tavanti, M., Galman, J.L., Turner, N.J., and Flitsch, S.L. (2018) Cloning, expression and characterisation of P450-Hal1 from Cupriavidus pinatubonensis strain JMP134. Appl Environ Microbiol 81: 3914–3924.

Price, M.N., Huang, K.H., Alm, E.J., and Arkin, A.P. (2005) A novel method for accurate operon predictions in all sequenced prokaryotes. Nucleic Acids Res 33: 880–892.

Roberts, G.A., Çelik, A., Hunter, D.J.B., Ost, T.W.B., White, J.H., Chapman, S.K., et al. (2003) A self-sufficient cytochrome p450 with a primary structural organization that includes a flavin domain and a [2Fe-2S] redox center. J Biol Chem 278: 48914–48920.

Roberts, G.A., Grogan, G., Greter, A., Flitsch, S.L., and Turner, N.J. (2002) Identification of a new class of cytochrome P450 from a Rhodococcus sp. J Bacteriol 184: 3898–3908.
Rodriguez-Rojas, A., Mena, A., Martin, S., Borrell, N., Oliver, A., and Blazquez, J. (2009) Inactivation of the hmgA gene of Pseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology 155: 1050–1057.

Schmittgen, T.D., and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3: 1101–1108.

Silva-Rocha, R., Martinez-Garcia, E., Calles, B., Chavarria, M., Arce-Rodriguez, A., de las Heras, A., et al. (2013) The Standard European Vector Architecture (SEVA): a coherent platform for the analysis and deployment of complex prokaryotic phenotypes. Nucleic Acids Res 41: D666–D675.

Svenningsen, N.B., Perez-Pantoja, D., Nikel, P.J., Nicolaisen, M.H., de Lorenzo, V., and Nybroe, O. (2015) Pseudomonas putida mt-2 tolerates reactive oxygen species generated during matric stress by inducing a major oxidative defense response. BMC Microbiol 15: 202.

Tavanti, M., Porter, J.L., Levy, C.W., Garcia-Silva, R., Svenningsen, N.B., Perez-Pantoja, D., Martínez-Garcia, E., Jaen-Luchoro, D., Castro-Flitsch, S.L., and Turner, N.J. (2018) The crystal structure of CYP450-TT heme-domain provides the first structural insights into the versatile class VII P450s. FEBS J 285: 3383–3393.

Undabarrena, A., Salva-Serra, F., Jaen-Luchoro, D., Castro-Flitsch, S.L., and Turner, N.J. (2018) Complete genome sequence of the marine Rhodococcus sp. H-C49t isolated from Comau fjord in Northern Patagonia, Chile. Mar Genomes 40: 13–17.

Urlacher, V.B., and Girhard, M. (2019) Cytochrome P450 monooxygenases in biotechnology and synthetic biology. Trends Biotechnol 37: 882–897.

Van Bogaert, I.N., Groeneboer, S., Saerens, K., and Soetaert, W. (2011) The role of cytochrome P450 monooxygenases in microbial fatty acid metabolism. FEBS J 278: 206–221.

Zhang, L., Xie, Z., Liu, Z., Zhou, S., Ma, L., Liu, W., et al. (2020) Structural insight into the electron transfer pathway of a self-sufficient P450 monooxygenase. Nat Commun 11: 2676.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Brown color, presumably pyomelanin derived from homogentisate, in liquid cultures of Cupriavidus pinatubonensis JMP134d(hmgA) derivative when exposed to 2-HPA. (A) Growth of C. pinatubonensis wild type (JMP134), hmgA mutant (JMP134d(hmgA) and hmgA mutant expressing hmgA gene driven by a heterologous P_{BAD} promoter (JMP134d(hmgA) pBS1-hmgA) on 2-HPA as sole carbon and energy sources, in the presence (+) or absence (−) of L-arabinose after 8 days. Phenylacetate was used as a control because it is degraded employing a different catabolic pathway, non-related to homogentisate production (Perez-Pantoja et al., 2008). It should be mentioned that in living cells homogentisate polymerizes spontaneously to form pyomelanin, a brown color polymer, at short incubation times (Schmaler-Ripcke et al. 2009). (B) HPLC-UV chromatogram of supernatant from resting cells of strain JMP134d(hmgA) grown on fructose, washed, and subsequently exposed to 1 mM 2-HPA. Sample was obtained at 24 h, filtered (0.22 μm) and injected into a Jasco liquid chromatograph LC-4000 (JASCO, Oklahoma City, OK, USA) equipped with a Kromasil 100-3.5-C18 4.6 mm diameter column. A methanol-H_{2}O (60:40) mixture containing 0.1% (vol/vol) phosphoric acid was used as the solvent, at a flow rate of 1 ml min^{-1}. The column effluent was monitored at 210 nm. Retention time for 2-HPA was 5.6 min. A signal putatively related to homogentisate was found at retention time of 2.3 min. This signal has a UV-Vis spectrum and retention time identical to that of the homogentisate analytical standard showed in (C).

Fig. S2. Transcript levels of putative operon formed by ohpT and ohpA genes from Cupriavidus pinatubonensis JMP134 cells exposed to 2-HPA. Real-time PCR analysis was performed for intergenic zones ohpR-ohpT and ohpT-ohpA, as indicated in Figure, in cells grown on 2-HPA, 4-HP4 or fructose (control) as a sole carbon and energy sources. Transcript levels were normalized to the average value of transcript levels in fructose treatment. Additionally, 16S rRNA levels were used as a reference gene (internal control). All experiments were performed in three biological replicates. Error bars represent SEM. Different letters indicate statistically significant differences between treatments for each gene (one-way analysis of variance, P < 0.05; Tukey’s test, P < 0.05).

Fig. S3. Gene clusters in which homologues to the cytochrome P450-encoding gene (ohpA) are found from different bacterial lineages. Phylogenetic tree of OhpA homologues was constructed using IQ-TREE (Nguyen et al., 2015) based on sequence alignments calculated employing MAFFT (Katoh et al., 2019), and it is displayed with SH-like approximate likelihood ratio support values (n = 1000) given.
at each node (values > 50% are shown). The putative functions of the different genes included in the clusters are indicated on the right. The sizes of genes are to scale. The numbers included in the OhpA-encoding genes (black arrow) are indicating amino acid identity (%) with OhpA from *C. pinatubonensis* JMP134.

**Table S1.** Sequence alignment of the catalytic pocket in OhpA homologues using as reference CUB06540 from *Tepidiphilus thermophilus* JCM 19170.

**Table S2.** Sequence alignment of heme binding domain in OhpA homologues using as reference the protein from *Cupriavidus pinatubonensis* JMP134.