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Structural characterization of the gene and corresponding cDNA for the cytochrome P450rm from *Rhodotorula minuta* which catalyzes formation of isobutene and 4-hydroxylation of benzoate

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Abstract Cytochrome P450rm was previously isolated from the basidiomycete yeast *Rhodotorula minuta* as a bifunctional enzyme with isobutene-forming and benzoate 4-hydroxylase activities. We cloned the gene and corresponding cDNA for P450rm in order to characterize the enzyme in the context of fungal phylogeny and physiology. From the cDNA sequence, P450rm was deduced to have 527 amino acids with a calculated molecular weight of 59 136. P450rm shared 48% amino acid sequence identity with CYP53A1 from *Aspergillus niger*, indicating that the gene belongs to a novel subfamily of CYP53, CYP53B. However, the organization of the P450rm gene, which has eight exons and seven introns, differed completely to that of *CYP53A1*. Northern analysis demonstrated that the level of P450rm mRNA expression increased when L-phenylalanine was used as sole carbon source. These results suggest that P450rm has been well conserved during the evolution of fungi as a benzoate 4-hydroxylase in the dissimilation pathway starting from L-phenylalanine

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The nucleotide sequence data reported in this paper appears in the DDBJ, EMBL, and GenBank Nucleotide Sequence Database with the accession number D63703

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Introduction

We previously found that the basidiomycete yeast *Rhodotorula minuta* produces isobutene from L-leucine present in the medium (Fujii et al. 1987), and reported that isobutene is formed from isovalerate, which is a metabolite of L-leucine, by an isobutene-forming enzyme found in microsomes (Fujii et al. 1989a). It was also demonstrated that synthesis of the isobutene-forming enzyme is induced in microsomes when L-phenylalanine is provided in the medium (Fujii et al. 1987, 1989a; Fukuda et al. 1993, 1994). The protein behaved like a cytochrome P450, responding to several known inhibitors of this class of enzyme (Fujii et al. 1989a). We then isolated a cytochrome P450, tentatively named P450rm, and P450 reductase from microsomes of *R. minuta* (Fukuda et al. 1993). Reconstitution of the purified proteins demonstrated that P450rm could convert isovalerate to isobutene in the presence of NADPH (Fukuda et al. 1994). This series of experiments confirmed that P450rm itself is the isobutene-forming enzyme.

There are several reports that *Rhodotorula* yeasts and certain fungi metabolize L-phenylalanine and other aromatic compounds in the β-ketoadipate pathway *via* benzoate (Cook and Cain 1974; Durham et al. 1984). Thus, these microorganisms have a monooxygenase with benzoate 4-hydroxylase activity. Benzoate 4-hydroxylase was first purified from *Aspergillus niger*, shown to be capable of degrading benzoate, and was considered a soluble and tetrahydropteridine-dependent enzyme (Reddy and Vaidyanathan 1975). Afterward, a gene that complemented a deficiency of benzoate 4-hydroxylase (*bphA*) activity was isolated by self-cloning from another strain of *A. niger*. The protein encoded by the cloned gene had the properties of a cytochrome P450 enzyme and was named CYP53A1 (Gorcom et al. 1990).

No cytochrome P450 enzymes with benzoate 4-hydroxylation activity have been isolated to date, but we presumed that P450rm is involved in the β-ketoadipate pathway in *R. minuta*. As expected, the reconstituted system could stoichiometrically catalyze 4-hydroxylation of benzoate (Fukuda et al. 1996), indicating that P450rm has benzoate 4-hydroxylase in addition to isobutene-forming activity. The P450rm system is the first example of P450 enzyme performing two reactions in lower eucaryotic cells. The enzymological characteristics of CYP53A1 have yet to be elucidated because the CYP53A1 protein has not been isolated. Accordingly, it is not clear whether or not P450rm is similar to CYP53A1. If CYP53A1 and P450rm are related, one may speculate that the benzoate 4-hydroxylase activity has been conserved in genera of fungi as distant as *Aspergillus* and *Rhodotorula*, and that the enzymes have important functions in the life of the fungi. Here, the P450rm gene and the primary structure of the enzyme are characterized. Furthermore, Northern blot analyses were performed, using the cloned cDNA as a probe, to elucidate the physiological roles of P450rm.

Materials and methods

Chemicals

Vent DNA polymerase was purchased from New England Biolabs (Beverly, Mass., USA) and used for PCR. [γ-^{32}P] ATP and [α-^{32}P]-dCTP were from Amersham International plc (Amersham, Bucks., UK). Restriction enzymes and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Microorganisms, plasmid vector, and growth conditions

The strain used for the investigation was *Rhodotorula minuta* IFO 1102. *E. coli* DH5α was used as a host for the plasmid. Plasmid pUC19 was used to clone the gene and the cDNA. *R. minuta* was grown in a complex medium, modified nutrient broth medium (Fujii et al. 1985), for extraction of chromosomal DNA or in a chemically defined medium, CD medium (Fujii et al. 1989b), for isolation of RNA.

Purification of P450rm

P450rm was purified from microsomes of *R. minuta* as previously described (Fukuda et al. 1994).

Cleavage of P450rm with BrCN and fractionation of peptide fragments

The purified P450rm preparation, from which detergents and glycerol had been removed, was lyophilized. Dried P450rm protein was digested with BrCN according to the method of Gross (1967). The resultant peptides were separated by HPLC on a reversed-phase column (AP-802; YMC, Kyoto, Japan).

Amino acid sequence analyses

The peptides purified by HPLC were subjected to sequence analyses on a Model 477A sequency (Applied Biosystems, Foster City, Calif., USA).

Preparation of chromosomal DNA

The cells harvested from the culture broth were disrupted in liquid nitrogen using a mortar and pestle, and then dissolved in 1 M Tris-HCl pH 8.5, containing 0.2 M NaCl, 0.1 M sodium EDTA and 5% SDS. The suspension obtained was treated with proteinase K and was subjected to repeated phenol-chloroform extractions. The DNA in the resultant solution was precipitated with ethanol and dissolved in TE buffer.

Procedures for cloning P450rm gene and for cloning P450rm cDNA

Two kinds of degenerate oligonucleotides, 5′-GGNATHGTN-CARGARGC-3′ and 5′-GCNGCRAANGCNCC-3′ (where H = A + C + T, R = A + G, and N = A + G + C + T) were synthesized, based on the N-terminal sequences of P450rm (Fukuda et al. 1993). We amplified sequences by PCR using the oligonucleotides as primers and chromosomal DNA as a template, inserted these into pUC19 and then transformed *E. coli*. The desired DNA fragment was recovered from the clones and used as a probe for the isolation of clones carrying the full-length P450rm gene.

Total RNA was extracted with the RNeasy kit (Qiagen, Chatsworth, Calif., USA) from cells disrupted in liquid nitrogen. Two DNA primers were synthesized based on the 5′- and 3′-flanking regions of the coding sequence present in the cloned genomic sequence. The P450rm cDNA was reverse-transcribed and then amplified by PCR using the two primers. Amplified full-length P450rm cDNA was cloned into pUC19 and sequenced.

DNA Sequencing and identification of putative substrate recognition sites

DNA fragments of appropriate length were subcloned into pUC19 and sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1977). Putative substrate recognition sites were identified as described previously (Gotoh 1992).

Southern blot analysis

The chromosomal DNA was digested with various restriction enzymes. The resulting fragments were electrophoresed in an agarose gel, transferred to a Hybond-N, nylon membrane (Amersham), and then hybridized with a ^{32}P-labeled probe which was synthesized with a Rediprime random primer labeling kit (Amersham) and P450rm cDNA as a template.

Northern blot analysis

The *R. minuta* cells were cultivated in CD medium (Fujii et al. 1989b) or in modified CD medium, in which the carbon source is L-phenylalanine (6 mM) instead of glucose. Total RNA was isolated from the cultivated cells in the media by using RNeasy (Qiagen). Samples were electrophoresed in 1% agarose gel containing 2.2 M formaldehyde, blotted to a Hybond-N, and hybridized with radiolabeled P450rm cDNA in the same way as for Southern analyses.

Other methods

All the cloning procedures followed the guidelines for research involving recombinant DNA molecules issued by the Ministry of Education, Science, Sport and Culture of Japan.

Results

Isolation of the P450rm gene and a P450rm cDNA

We cloned the 4.2-kb fragment thought to carry the full-length P450rm gene. Analysis of the fragment revealed the sequence encoding the N-terminal amino acids. Nevertheless, no open reading frame (ORF) long enough to encode the full-length P450rm could be found in the sequence. Hence, we assumed that the P450rm gene has a segmented structure, and attempted to isolate a P450rm cDNA. The cloned cDNA had an ORF which coded for a protein of 527 residues with a calculated molecular weight of 59 136. As expected, the deduced sequence encoded the N-terminal amino acids and the four peptides generated by BrCN cleavage of P450rm. Furthermore, we observed that the deduced sequence encodes motifs characteristic of all the P450 proteins, such as the distal helix region and the heme-binding domain. Therefore, we concluded that the cDNA indeed encodes P450rm.

Structure of the P450rm gene

Comparison of the sequences of the cDNA and the cloned fragment of genomic DNA allowed us to localize the sites of introns and exons, and to confirm the boundaries between the coding and the 5′ and 3′-flanking regions in the gene. The P450rm gene has seven introns and eight exons within a span of ca. 2-kb. The numbers of exons and introns are among the largest found in fungal cytochrome P450 genes reported to date. All the intron-exon boundaries conform to the canonical GT-AG rule. The length of introns ranged from 71 bp to 142 bp. The sizes of the exons ranged widely from the 20 bp of the third exon to the 597 bp of the eighth exon. We did not find any consensus promoter sequence within 278 bp upstream of the coding region.

To confirm that the cloned P450rm gene is not a pseudogene, Southern blot analysis of genomic DNA was performed by using the P450rm cDNA as a probe. Figure 1 shows the restriction map of the cloned P450rm gene and the result of the Southern blot analysis. Only one hybridizing band was found in the lanes containing DNA digested with *Pst*I and *Sac*I, for which there are no sites in the coding region of the cloned P450rm gene. This suggests that P450rm cDNA can hybridize with only one region of the genome. The enzymes *Eco*RI and *Bam*HI, for which one site is available, generated two bands. The hybridization patterns mentioned above are consistent with those expected from the restriction map of the cloned gene (Fig. 1A). These results demonstrate that the cloned gene is derived from the single-copy P450rm gene.

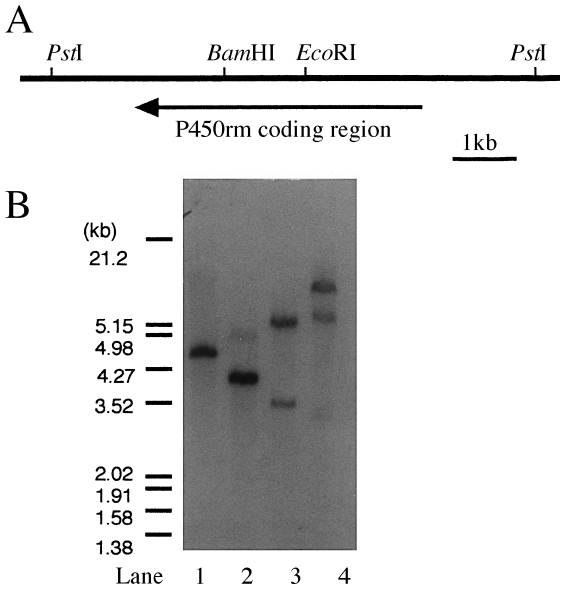


Fig. 1A, B Partial restriction map of a cloned DNA fragment (**A**), and Southern analysis of a genomic DNA using P450rm cDNA (**B**). **A** The DNA fragment was cloned in pUC19. Part of the P450rm coding region was contained in a *Pst*I fragment 4.2-kb. *Pst*I, *Eco*RI, and *Bam*HI sites are indicated. **B** Southern blot analysis. *R. minuta* genomic DNA was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Pst*I (lane 3), and *Sac*I (lane 4). The result fragments were fractionated by electrophoresis in 1% agarose. Then DNAs were blotted on to a nylon membrane and subjected to Southern analysis with P450rm cDNA as a probe. The positions of size markers are indicated on the left

Comparison of the P450rm cDNA and P450rm gene with other forms of P450s

The primary structure of P450rm was compared with those of other molecular species of cytochrome P450s by computer analysis. The results are summarized in Table 1. P450rm shared 48% amino acid sequence identity with CYP53A1 from *A. niger* (Gorcom et al. 1990), the closest relative of P450rm. This suggests that P450rm belongs to a novel subfamily of the CYP53 family. Accordingly, P450rm was named CYP53B1 (Nelson et al. 1996). P450rm and two pisatin hydroxylase cytochrome P450s, CYP57A1 and A2, from *Nectria haematococca* (Maloney and VanEtten 1994; Reimmann and VanEtten 1994) show 25.7% and 25.2% sequence identity, respectively. Two other fungal P450s, trichodiene oxygenase CYP58A from *Fusarium sporotrichioides* (Hohn et al. 1995), and a gene product from *Uromyces fabae* (GenBank accession no. U81793) are slightly more remotely related to P450rm than the CYP57s. However, these six fungal cytochrome P450s are not closely related to any other family of cytochrome P450 and thus seem to constitute an independent group within the P450 gene superfamily.

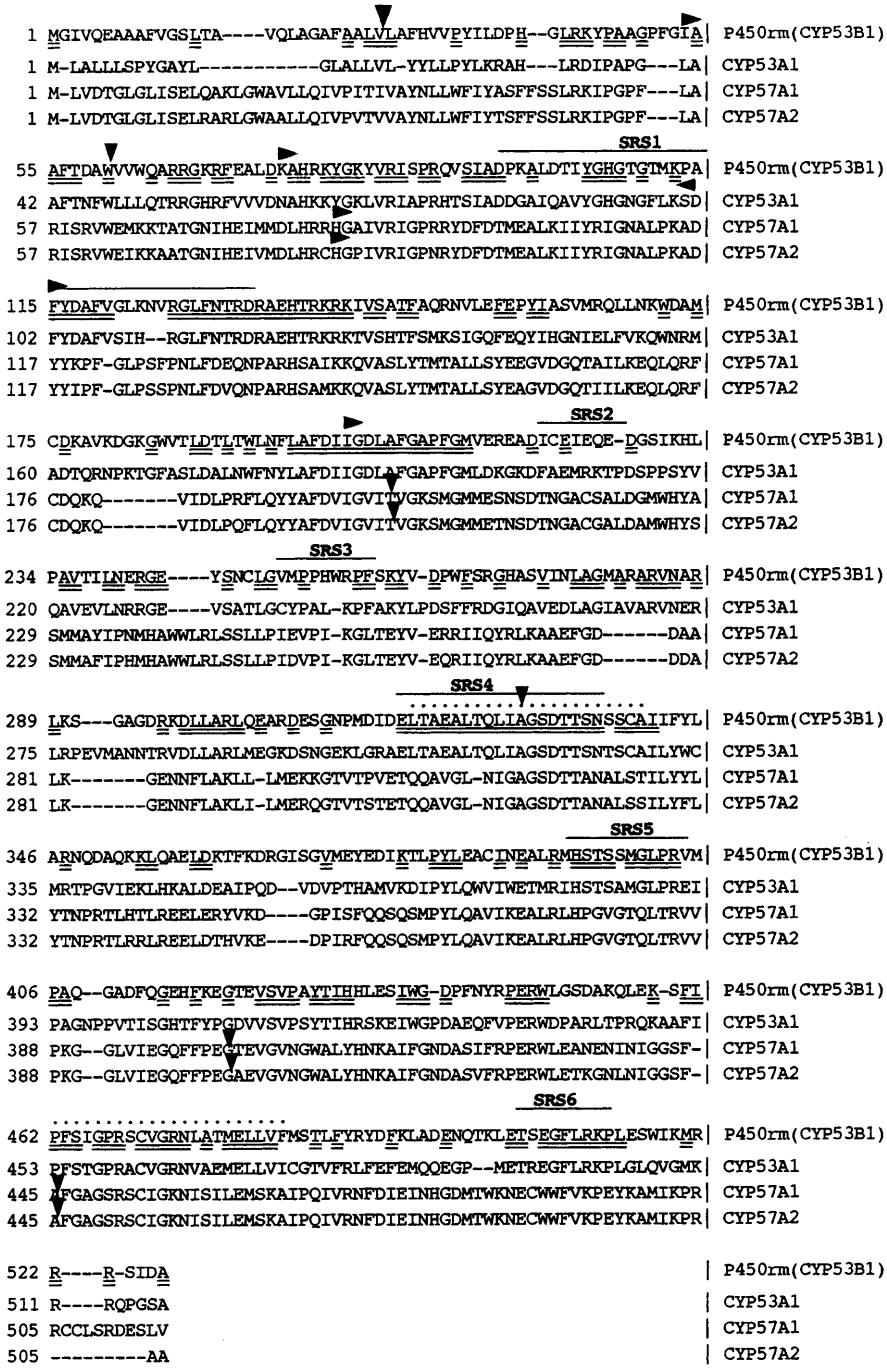
Figure 2 shows alignment of the amino acid sequences among P450rm, CYP53A1, CYP57A1 and CYP57A2. P450rm and CYP53A1 appear to share identical amino acids largely in the heme-binding domain

Table 1 Degrees of amino acid sequence identity between P450rm, CYP53A1, CYP57A1, and CYP57A2, CYP58 and a P450 from *Uromyces fabae*

	Percent identity[a]					
	P450rm	CYP53A1	CYP57A1	CYP57A2	P450* of *U. fabae*	CYP58
P450rm	–	48.08	25.73	25.20	25.51	20.89
CYP53A1		–	24.36	24.40	24.30	19.80
CYP57A1			–	89.74	24.20	21.10
CYP57A2				–	24.40	21.31
P450 of *U. fabae*					–	21.01

[a]Identity values were calculated based on the alignment shown in Fig. 2. Each gap was counted as a single mismatch. P450* *of U. fabae* appears in the EMBL/genbank database under the accession number U81793

Fig. 2 Alignment of the amino acid sequences of P450rm, CYP53A1, CYP57A1, and CYP57A2. The amino acid sequences of P450rm, CYP53A1, CYP57A1, and CYP57A2 were deduced from the nucleotide sequences of their respective cDNAs. The alignment was performed by means of a program utilizing the randomized iterative refinement strategy that optimizes the weighted sum-of-pairs scores (Gotoh 1995). *Single lines* show substrate recognition sites (SRSs). *Double underlines* represent sequence identities between P450rm and CYP53A1. The orientation of the *triangles* indicates the position of the intron within or between codons: (▼) the intron lies between flanking codons, (►) lies between the first and second nucleotides of the following codon, and (◄) lies between the second and third nucleotides of the preceding codon. Dotted sequences indicate the putative distal helix region and the heme-binding domain



and putative substrate recognition sites (SRSs) 1, 4, 5 and 6. SRSs 1, 4, 5 and 6 in both P450rm and CYP53A1 might be important for interaction with benzoate as a common substrate. Locations of introns in the genes encoding each protein are indicated above the amino acid sequences in Fig. 2. The introns in the P450rm gene are located at entirely different sites from those in CYP57A1, A2, and CYP53A1.

Northern blot analysis of expression of P450rm mRNA

It is likely that the expression of the P450rm mRNA having the activity of benzoate 4-hydroxylase increases when L-phenylalanine is utilized as a carbon source by *R. minuta*. We performed Northern blot analysis on total RNA extracted from the cells to confirm the inducibility of P450rm by L-phenylalanine. Total RNA was prepared from cells grown in CD medium containing glucose as a sole carbon source or in a modified CD medium supplemented with L-phenylalanine instead of glucose. The total RNAs prepared were subjected to agarose gel electrophoresis and then blotted to a nylon membrane. The mRNA encoding P450rm was detected with the P450rm cDNA as a probe. As shown in Fig. 3, the presence of L-phenylalanine in the medium increased the expression of the P450rm gene transcript, a 2.2 kb species, by about 20-fold as compared to the case in which glucose was used as a carbon source. This suggests that L-phenylalanine induces the transcription of P450rm gene when it is used as a carbon source.

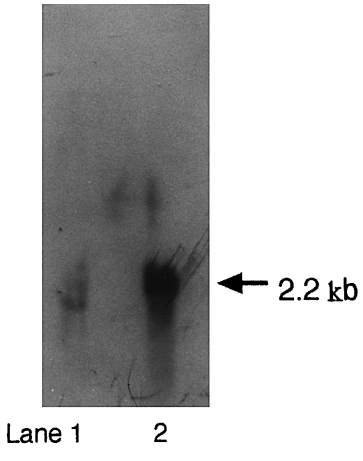


Fig. 3 Northern blot analysis of RNA from *Rhodotorula minuta*. Total RNA was extracted from cells grown on glucose or L-phenylalanine (1 g/l) as sole carbon source for 15 h. Thirty micrograms of total RNA from each sample was electrophoresed with RNA size markers (RNA Ladder, Gibco-BRL Gaithersburg, Md., USA) in formaldehyde-containing 1% agarose gel, and blotted onto a nylon membrane. P450rm cDNA was used as a probe. Lane 1, RNA from cells grown on glucose; lane 2, RNA from cells grown on L-phenylalanine

Discussion

Comparison of the primary structure of P450rm with those of other known cytochrome P450s indicated 48% amino acid identity with CYP53A1, a benzoate 4-hydroxylase from *A. niger*. These results indicate that orthologous cytochrome P450 enzymes with benzoate 4-hydroxylase activity are distributed in distant genera of higher fungi, such as *Rhodotorula* and *Aspergillus*. The heme-binding domain and the putative substrate recognition sites, SRS 1, 4, 5 and 6, as well conserved between CYP53A1 and CYP53B1. These regions might be important for the benzoate 4-hydroxylase activity. However, it is not clear whether the regions are also important for formation of isobutene or whether isobutene-forming activity is an intrinsic property of the CYP53 family.

We did not find any promoter consensus sequences, such as TATA or CAAT boxes, in a 278-nucleotide stretch of upstream sequence. The potential CAAT-box sequences CTTT and CATC have been reported in *pyr3*, *ums2* and a GAPDH-encoding gene of a basidiomycete fungus *Ustilago maydis* (Spanos et al. 1992). It is also reported that a repetitive CTTC motif functions as a promoter in *U. maydis* (Bölker et al. 1995). These motifs are also observed in non-coding regions of P450rm and might function as a promoter in *Rhodotorula minuta* instead of TATA or CAAT boxes.

No highly conserved sequences at the branch points of introns such as the TACTAAC in *Saccharomyces cerevisiae* introns (Teem et al. 1984), were present in any introns of the P450rm gene. However, the vertebrate consensus sequence YYRAY (Keller and Noon 1984), where Y represents C or T, and R, A or G, was observed 19 to 44 bp upstream from the 3′ splice boundary, although the pyrimidine-rich stretch near the 3′ end was absent. Intron positions in the coding region of P450rm gene were entirely different from those in CYP53A1 (Fig. 1), although it is known that the exon-intron organization is conserved among genes within a P450 family (Gotoh 1993). This finding raises two possibilities; that the intron-exon organization changes at a comparatively rapid rate in fungi, or that the primary structures of CYP53B1 and CYP53A1 have been well conserved so that intron-exon organization has changed extensively. As yet, we have no evidence to confirm either. However, it is, at least, estimated that divergence between basidiomycetes and ascomycetes occurred at an early stage of higher fungal evolution (Wilmotte et al. 1993). Aoyama et al. reported that CYP51s known as house-keeping enzymes, lanosterol 14-demethylases, are well conserved in fungal and mammalian cells (Aoyama et al. 1996). According to their results, CYP51s of the ascomycete yeasts *S. cerevisiae* and *Candida tropicalis* share 47% amino acid identity with those of the *basidiomycete* fungus *Ustilago maydis*. This suggests that CYP53s are another well conserved species of

cytochrome P450 and have an important physiological role in fungal cells.

We reported previously that isobutene-forming activity and expression of a cytochrome P450 were induced in microsomes when the cells were cultivated in medium containing L-phenylalanine (Fujii et al. 1992). Figure 3 shows that expression of P450rm gene was enhanced when L-phenylalanine was used as a carbon source, suggesting that P450rm is involved in the catabolism of L-phenylalanine via the β-ketoadipate pathway. Benzoate is basically toxic to fungi, and is widely used as a food preservative. An accumulation of benzoate during the catabolism of L-phenylalanine could damage *R. minuta*. As a key enzyme in the β-ketoadipate pathway, P450rm might function, in part, to detoxify benzoate.

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