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Cytochrome P450rm from *Rhodotorula minuta* Catalyzes 4-Hydroxylation of Benzoate

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***Rhodotorula minuta*, a red yeast, produces a cytochrome P450, tentatively named P450rm, catalyzing the formation of isobutene from isovalerate. We found that P450rm interacted with benzoate and the dissociation constant of P450rm for benzoate was 36 μM. A reconstituted system that consisted of purified P450rm and cytochrome P450 reductase catalyzed the 4-hydroxylation of benzoate in addition to the formation of isobutene; the turnover rate was ~40 nmol/min/nmol P450rm. The P450rm-monooxygenase system was specific for benzoate and did not catalyze hydroxylation of other aromatic carboxylates. Since only a benzoate 4-hydroxylase that requires tetrahydropteridine has been isolated to date, P450rm appears to be the first isolated cytochrome P450 that acts as a benzoate 4-hydroxylase. The P450rm-monooxygenase system in microsomes of *R. minuta* might function in the degradation of L-phenylalanine on the pathway to β-ketoadipate.**

Key words: benzoate 4-hydroxylase, cytochrome P450, formation of isobutene, P450rm, *Rhodotorula minuta*.

We have been studying a cytochrome P450, tentatively named P450rm, of *Rhodotorula minuta* (*1*), which produces a C4-hydrocarbon, isobutene, at the stationary phase of growth (*2*). We reported previously the purification of P450rm (*3*) and we showed that isobutene is formed from isovalerate by a P450rm-monooxygenase system (*4*). During our studies, we noted that P450rm is induced by L-phenylalanine in the medium used for cultivation of the microorganism (*5*). It has been shown that L-phenylalanine is dissimilated to cinnamate and then to benzoate, which is further metabolized *via* the β-ketoadipate pathway in *Rhodotorula*, a yeast (*6, 7*). Some other yeasts and filamentous fungi can also dissimilate aromatic compounds, such as L-phenylalanine, mandalate and benzoate, *via* protocatechuate, to β-ketoadipate (*8-13*). However, no cytochrome P450 has been considered to be involved in the degradation of L-phenylalanine or of other relevant aromatic compounds. In spite of a possible conflict with the generally accepted characteristics of enzymes on the β-ketoadipate pathway, we postulated that P450rm might function in the degradation of L-phenylalanine, in addition to forming isobutene from isovalerate. As reported herein, we have demonstrated that P450rm possesses benzoate 4-hydroxylase activity, and might function in the β-ketoadipate pathway.

MATERIALS AND METHODS

Materials—*R. minuta* var. *texensis* IFO 1102 was used throughout this study. DLPC was purchased from Sigma Chemical, St. Louis, MO, USA. NADPH was from Oriental Yeast, Tokyo. The other reagents were from Wako Pure Chemical Industries, Osaka.

Analysis of Benzoate 4-Hydroxylation by the Reconstituted System—P450rm and cytochrome P450 reductase were purified according to the methods reported in our reports (*3, 4*). One milliliter of the standard reaction mixture (the reconstituted system) contained 0.177 nmol of purified P450rm, 0.8 unit of purified cytochrome P450 reductase, 69 nmol of DLPC, 50 μg of sodium cholate, 6 mg of bovine serum albumin, 10 μmol of potassium phosphate buffer, pH 7.5, 150 nmol of sodium benzoate, and 0.4 μmol of NADPH. After preincubation of the mixture without NADPH and benzoate at 25°C for 1 min, NADPH and benzoate were added. The mixture was incubated at 25°C for 10 min with shaking (120 rpm, 7 cm amplitude), then the reaction was stopped by the addition of 60 μl of 30% trichloroacetic acid and 40 μl of 2 N HCl. After centrifugation (1,500 × *g*, 10 min) of the mixture, the supernatant was used directly for analysis. Consumption of benzoate and the formation of a product were evaluated by HPLC on a system (Tosoh, Tokyo) equipped with an octacylsilica gel column (Cosmosil 5C8-300w, 4.6 mm i.d. × 150 mm; Nacalai Tesque, Kyoto) and an ultraviolet detector. Benzoate and its product were eluted with 10% acetonitrile containing 0.1% phosphate at 0.4 ml/min of flow rate.

Analyses for Oxidation of NADPH and Consumption of Oxygen in the Reaction Mixture—Oxidation of NADPH in the reaction mixture was determined by measuring the decrease of absorbance at 340 nm with a spectrophotometer (model UV-160A, Shimadzu, Kyoto). Consumption of oxygen was measured with a Clark type oxygen electrode (YSI model 5300 Biological Oxygen Monitor, Yellow Springs Instrument, Yellow Springs, O., USA).

Analysis of Benzoate 4-Hydroxylation by Microsomes and Soluble Fraction—Microsomes and a soluble fraction

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Abbreviations: DLPC, dilauroylphosphatidylcholine; DTT, 1,4-dithiothreitol; EDTA, disodium ethylenediaminetetraacetate.

were prepared from an appropriate volume of cell-free extract as described previously (*1*). Microsomes were resuspended, to give the original volume, in 100 mM potassium phosphate buffer, pH 8.0, that contained 0.5 mM DTT, 3 mM EDTA, 0.4 μg/ml pepstatin A, and 20% glycerol. The reaction mixture (final volume, 1 ml) consisted of 100 μl of the microsomal suspension or the soluble fraction, 40 mM potassium phosphate buffer, pH 7.5, 1 mM potassium cyanide, 150 μM benzoate, and 0.4 mM NADPH. The reaction mixture was incubated at 25°C for 10 min with gentle shaking, and the reaction was stopped by the addition of 60 μl of 30% trichloroacetic acid and 40 μl of 2 N HCl. Determination of the product was carried out by the same method as described for the reconstituted system.

Inhibition of Microsomal Benzoate 4-Hydroxylase by P450rm-Specific Antiserum—Antiserum was raised against P450rm in a rabbit by Wako Pure Chemical Industries. Microsomes were preincubated at 25°C for 30 min with the antiserum or with serum from an unimmunized rabbit. Then the reactions were carried out as described above.

RESULTS

Benzoate-Induced Spectral Change in P450rm—If P450rm acts as an enzyme in the β-ketoadipate pathway, it seems to be a benzoate 4-hydroxylase or a 4-hydroxybenzoate 3-hydroxylase. When oxidized cytochromes P450 interact with various compounds including substrates, they exhibit spectral changes known as "substrate-induced difference spectra" (*14*). In order to detect the interaction of P450rm with benzoate and 4-hydroxybenzoate, these compounds were separately added to the preparation of purified P450rm. Although no spectral change was observed after the addition of 4-hydroxybenzoate, Type I spectral change, which is caused by a change in the spin state of cytochrome P450 from low to high spin (*14*), was observed upon the addition of benzoate (Fig. 1). The dissociation constant of P450rm for benzoate was estimated to be 36 μM from the inset of Fig. 1.

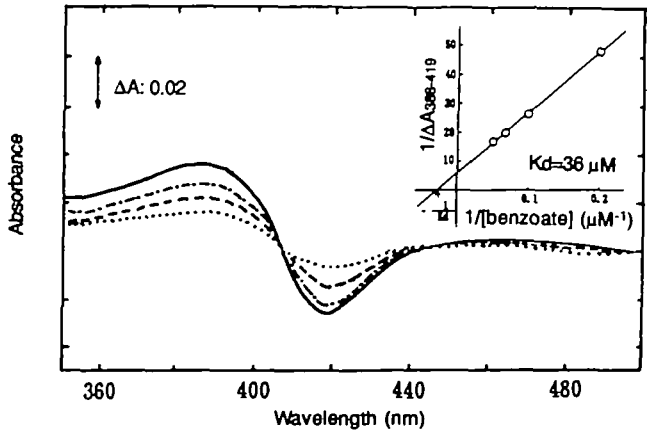

Fig. 1. **Benzoate-induced difference spectra of P450rm.** Purified P450rm was dissolved in 150 mM potassium phosphate buffer, pH 8.0, that contained 0.2% sodium cholate, 0.2% octylglucoside, 0.1 mM DTT, and 20% glycerol. P450rm was present at a concentration of 1.99 μM. Sodium benzoate was added in aliquots to give a concentration of ····· 5 μM, ---- 10 μM, -·-·- 15 μM, or — 20 μM. Spectra of the solutions that contained sodium benzoate were recorded against that of the solution without sodium benzoate. The inset shows the reciprocal of the difference in absorbance between 388 and 419 nm plotted against the reciprocal of the concentration of benzoate. The ΔA_{max} value that should be observed at infinite concentration of benzoate was estimated to be 0.167 from the intercept of the *y* axis with the line on the inset.

Hydroxylation of Benzoate by the Reconstituted System—We examined whether sodium benzoate is hydroxylated by the reconstituted system. The standard reaction mixture described in "MATERIALS AND METHODS" was incubated for 10 min, and after termination of the reaction and removal of a precipitate, the resultant supernatant was directly loaded onto an octacylsilica gel column and analyzed by HPLC. Compounds in eluates from the column were detected by measuring the absorbance at 255 nm. Three peaks were detected. The first and largest peak, and the last and smallest peak were derived from NADPH and benzoate in the reaction mixture, respectively. The middle peak with a retention time of 6.8 min appeared to be a reaction product. To identify the product, the following experiment was carried out. Authentic 4-hydroxybenzoate, which was expected to be the product, was subjected to HPLC under the same conditions as described above. A peak was observed with a retention time of 6.8 min, which was consistent with that of the product. Authentic 4-hydroxybenzoate and the reaction product after elution from the column were mixed and analyzed by HPLC. They yielded a single peak. Furthermore, the UV spectrum (above 200 nm) of the product showed a peak at 255 nm (Fig. 2). The spectrum was completely coincident with that of authentic 4-hydroxybenzoate dissolved in 10% acetonitrile plus 0.1% phosphate, and it was different from those of benzoate (λ_{max}: 231.5 nm), 2-hydroxybenzoate (λ_{max}: 204, 237.5, and 305.5 nm), and 3-hydroxybenzoate (λ_{max}: 208, 237, and 293 nm). These results indicate that the product was 4-hydroxybenzoate.

4-Hydroxybenzoate was formed at a constant rate for more than 10 min under the standard reaction conditions as described in "MATERIALS AND METHODS." Incubation without P450rm, the reductase, or benzoate yielded no metabolites. Varying the concentration of P450rm in the standard reaction mixture in the range of less than 0.2 nmol/ml, we observed that the amount of 4-hydroxybenzo-

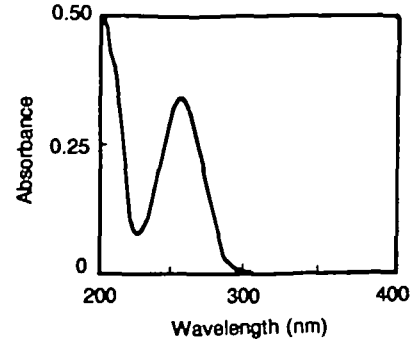

Fig. 2. **Spectrum of the reaction product eluted from HPLC.** The standard reaction mixture was incubated at 25°C for 10 min. After the reaction, the compounds in the mixture were isolated by HPLC as described in "MATERIALS AND METHODS" and the UV spectrum of the product eluted at 6.8 min from the column was measured with a spectrophotometer.

ate formed was virtually proportional to the concentration of P450rm. Under these conditions, it was estimated that 1 nmol of P450rm catalyzed the conversion of about 40 nmol of benzoate to the metabolite per min.

We examined the stoichiometry of this reaction. The reaction conditions were the same as described in "MATERIALS AND METHODS," except that P450rm was used at the concentration of 0.08 nmol/ml reaction mixture. After 10 min, the consumption of benzoate and oxygen, the oxidation of NADPH and the formation of 4-hydroxybenzoate were measured. The results are summarized in Table I. Table I shows that 1 nmol of 4-hydroxybenzoate was formed for 1 nmol consumption of benzoate and oxygen, and 1 nmol oxidation of NADPH.

Interaction of Several Compounds with P450rm and the Effects on Them of P450rm—Several relevant compounds were tested as possible substrates for P450rm. An appropriate amount of each compound was added to the preparation of purified P450rm. Table II shows that L-phenylalanine, 4-hydroxybenzoate, and 3-hydroxybenzoate did not induce any spectral change, while benzoate, cinnamate, and 2-hydroxybenzoate (salicylate) each induced a spectral change of P450rm. The K_d values of P450rm for cinnamate and salicylate were estimated to be 330 and 25 μM, respectively. Since the former value was tenfold higher than that for benzoate, the affinity of P450rm for cinnamate appeared to be low. Three millimolar cinnamate and 0.3 mM salicylate were added separately as substrates in place of benzoate to the standard reaction mixture and the compounds formed were analyzed by HPLC. While no detectable product was generated from salicylate, cinnamate caused the appearance of a small peak having 11.5 min retention time, which is consistent with that of authentic 4-coumarate. However, the turnover rate of formation of 4-coumarate was calculated to be less than 0.3 nmol/min/nmol P450rm. These results indicate that the P450rm-monooxygenase system works exclusively as a benzoate 4-hydroxylase.

Metabolism of Benzoate by Microsomes and Soluble Fraction—To investigate whether only P450rm functioned as a benzoate 4-hydroxylase in cells of *R. minuta*, we prepared microsomes and a soluble fraction as described in our previous paper (*1*). The microsomes were resuspended in the same volume of potassium phosphate buffer as that of

TABLE I. **Stoichiometry of benzoate 4-hydroxylase activity of the reconstituted system.** The experiments were carried out two times under standard reaction conditions as described in "MATERIALS AND METHODS," except that 0.08 nmol/ml of P450rm was contained in the reaction mixture. The reaction mixture (1 ml) was incubated at 25°C for 10 min. All values were expressed in nmol per ml of reaction mixture after 10 min reaction.

Experiment	Benzoate utilized (nmol/ml)	NADPH oxidized (nmol/ml)	Oxygen consumption (nmol/ml)	4-Hydroxybenzoate formed (nmol/ml)
1	41.3	40.9	—[a]	37.2
2	42.9	41.0	42.7	42.3

[a]—, not determined.

TABLE II. **Interaction of various compounds with P450rm and the effects on them of the P450rm-monooxygenase system.** Spectral changes induced by addition of each compound at various concentrations into a preparation of purified P450rm were recorded with a spectrophotometer. The apparent dissociation constant of each compound, K_d, was determined from a reciprocal plot of the absorbance difference between 388 and 419 nm and the concentration of each compound. Each product in the reconstituted system was analyzed by HPLC.

Compound	Spectral change (Type I)	K_d (μM)	Product
L-Phenylalanine	No	—[a]	—[a]
Cinnamate	Yes	330	4-Coumarate (<0.3)[b]
Benzoate	Yes	36	4-Hydroxybenzoate (~40)[b]
4-Hydroxybenzoate	No	—[a]	—[a]
3-Hydroxybenzoate	No	—[a]	—[a]
2-Hydroxybenzoate (Salicylate)	Yes	25	Not detectable

[a]—, not determined; [b]turnover rate (nmol/min/nmol P450rm).

TABLE III. **Effects of additives on the benzoate 4-hydroxylase activity in microsomes and the soluble fraction.** 4-Hydroxybenzoate formed for 10 min at 25°C in the reaction mixture that contained microsomes or soluble fraction was analyzed by HPLC as described in "MATERIALS AND METHODS." The reaction was carried out in an atmosphere of carbon monoxide and oxygen (90 : 10) and also in the presence of tetrahydropteridine and ferrous sulfate according to the reaction system for *Aspergillus niger* (*15*).

Additives	Activity of benzoate 4-hydroxylase (relative value[c] %)	
	Microsomes[a]	Soluble fraction[b]
None	0	0
Benzoate (150 nmol/ml)	100	6.1
Benzoate + CO/O_2	12	0
Benzoate + dimethyl tetrahydropteridine (0.02 mM) + ferrous sulfate (0.01 mM)	91	6.1

[a]Microsomes: microsomal suspension, 9.6 mg protein/ml, 1.4 nmol P450/ml; [b]Soluble fraction: 9.9 mg protein/ml, P450 was not detectable. [c]Relative value: activity of microsomes in the presence of benzoate was 87.8 nmol/min/ml microsomal suspension, which was taken as 100%.

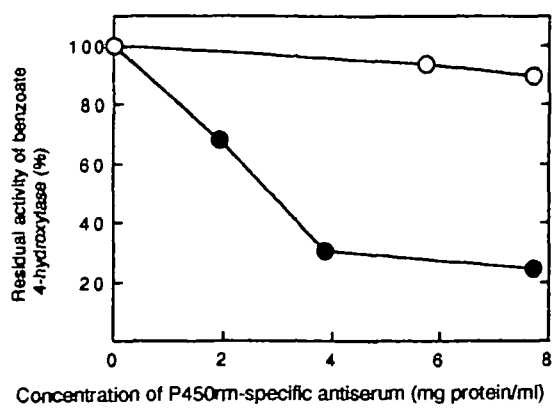

Fig. 3. **Inhibition of the microsomal activity of benzoate 4-hydroxylase in microsomes by P450rm-specific antiserum.** Microsomes were preincubated at 25°C for 30 min with the indicated concentrations of P450rm-specific antiserum (●) or of serum isolated from an unimmunized rabbit (○). Each mixture was then incubated with benzoate and NADPH for 10 min. Formation of 4-hydroxybenzoate was monitored as described in "MATERIALS AND METHODS." Microsomes were suspended at a concentration of about 1 mg protein/ml, such that cytochrome P450 was present at a concentration of 0.17 nmol/ml.

the soluble fraction obtained. The activities of benzoate 4-hydroxylase in microsomes and the soluble fraction were measured. As shown in Table III, no product was formed by either fraction without the addition of benzoate; 4-hydroxybenzoate was formed at 87.8 nmol/min/ml microsomal solution by microsomes in the presence of benzoate, while the soluble fraction had only 6.1% of the microsomal activity. Carbon monoxide strongly inhibited the benzoate 4-hydroxylase activities of both fractions. The activity in the soluble fraction may have been due to the presence of a small amount of microsomes. It was reported that benzoate 4-hydroxylase from *Aspergillus niger* (*15*) required dimethyl tetrahydropteridine and ferrous sulfate. Therefore, we added these reagents to the reaction mixture. Tetrahydropteridine had, however, no effect on the benzoate 4-hydroxylase activity of *R. minuta*. Only cytochrome(s) P450 in the microsomes appears to catalyze the 4-hydroxylation of benzoate. Inhibition of the activity of microsomes by P450rm-specific antiserum was examined. Figure 3 shows that most of the activity of microsomes was inhibited by P450rm-specific antiserum. These results indicated that the 4-hydroxylation of benzoate was entirely catalyzed by a P450rm-monooxygenase system in the microsomes of *R. minuta*.

DISCUSSION

We carried out experiments with a reconstituted system which consisted of P450rm and cytochrome P450 reductase purified from cells of *R. minuta* and demonstrated that the system catalyzed the 4-hydroxylation of benzoate. Data on the stoichiometry (Table I) suggest that the overall reaction proceeds according to the following equation:

$$\text{Benzoate} + O_2 + \text{NADPH} + H^+ \longrightarrow 4\text{-hydroxybenzoate} + \text{NADP}^+ + H_2O$$

Benzoate 4-hydroxylase was previously isolated from *A. niger* (*15*). However, this enzyme from *A. niger* is a soluble protein requiring tetrahydropteridine as a coenzyme. These characteristics are completely different from those of the P450rm-monooxygenase system. The P450rm is the first isolated cytochrome P450 catalyzing the 4-hydroxylation of benzoate.

Among aromatic compounds tested, cinnamate and salicylate interacted with P450rm (Table II). Although we detected formation of 4-coumarate from cinnamate in a reaction catalyzed by the reconstituted system, the activity might not be physiological because of the high K_d of P450rm for cinnamate and the low turnover rate for the formation of 4-coumarate. Salicylate had high affinity for P450rm, but no compound generated from it by the P450rm-monooxygenase system could be detected. Upon addition of both salicylate and benzoate at the same concentration, 150 μM, to the standard reaction mixture, the rate of formation of 4-hydroxybenzoate was reduced to about 40% of that formed on addition of benzoate alone (data not shown). Salicylate might competitively inhibit benzoate 4-hydroxylase. Salicylate is considered to be metabolized *via* a pathway from catechol to β-ketoadipate in cells of *Rhodotorula* (*7*). The interaction between salicylate and P450rm might have a physiological meaning, but this remains to be examined.

In experiments with microsomes and the soluble fraction (Table III), more than 90% of the activity of benzoate 4-hydroxylase was found in microsomes; carbon monoxide strongly inhibited the activities in the microsomes and the soluble fraction; and tetrahydropteridine did not enhance the activity in microsomes or the soluble fraction (Table II). A benzoate 4-hydroxylase that requires tetrahydropteridine as its coenzyme appears to be absent and the activity is exclusively due to a cytochrome P450 enzyme in the cells of *R. minuta*. The turnover rate (~60 nmol/min/nmol of cytochrome P450 in microsomes) could be calculated from the data in Table III. The value was consistent with that (~40 nmol/min/nmol P450rm) in the reconstituted system. The activity of microsomes was reduced by ~80% by P450rm-specific antiserum. These results demonstrate that the activity in microsomes can be entirely explained by that of the P450rm-monooxygenase system.

R. minuta is hardly able to utilize benzoate or related aromatic compounds as a sole source of carbon (data not shown). P450rm might have a physiological role in the dissimulation of L-phenylalanine, as shown in Fig. 4. P450rm is induced by L-phenylalanine in the growth medium at the stationary phase of growth of *R. minuta* (*1*). This observation is well explained by our finding that P450rm has the activity of benzoate 4-hydroxylase. Benzoate 4-hydroxylase might be repressed while the cells are assimilating glucose as their source of carbon and energy, but it might be induced to exploit L-phenylalanine in the medium and function as one of the enzymes on the β-ketoadipate pathway after the disappearance of glucose.

The first report on benzoate 4-hydroxylase indicated that the enzyme could be purified from *A. niger* as described above (*15*). An attempt was made by Gorcom *et al.* to clone the gene (*bphA*) for the pterin-linked benzoate 4-hydroxylase of *A. niger* (*16*). The protein coded by *bphA* gene has not yet been isolated. However, the gene complemented a

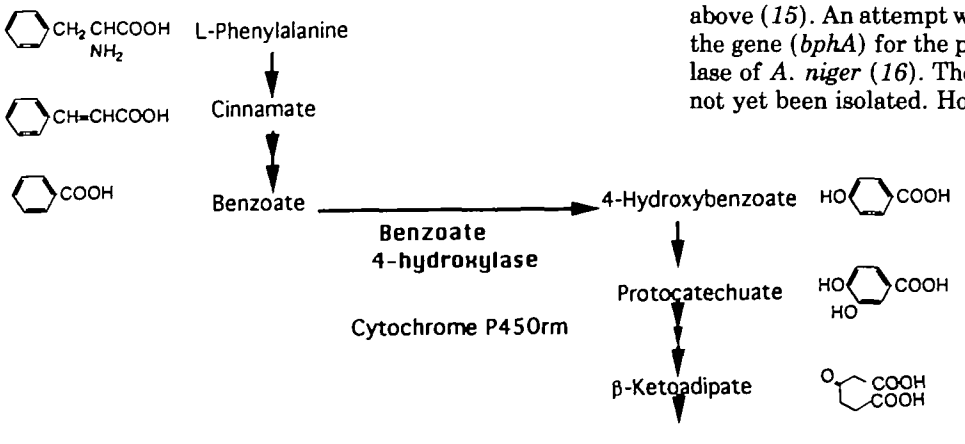

Fig. 4. The proposed role of P450rm in the dissimilation of L-phenylalanine.

deficiency in benzoate 4-hydroxylase in mutants of the microorganism. The amino acid sequence deduced from the isolated *bphA* gene indicated that the protein, bph A, encoded by the gene belongs to the superfamily of cytochrome P450 enzymes, although this result conflicts with the finding that the enzyme requires tetrahydropteridine as a coenzyme. Since *Rhodotorula* is taxonomically apart from genus *Aspergillus*, it is considered that cytochromes P450 that catalyze 4-hydroxylation of benzoate, such as P450rm, might be widespread in microorganisms.

REFERENCES

1. Fujii, T., Ogawa, T., and Fukuda, H. (1989) The relationship between isobutene-forming activity and a cytochrome P450 in *Rhodotorula minuta*. *J. Ferment. Bioeng.* **68**, 174-177
2. Fukuda, H., Ogawa, T., and Fujii, T. (1985) Production of isobutene by *Rhodotorula* yeasts. *Agric. Biol. Chem.* **49**, 1541-1543
3. Fukuda, H., Fujii, T., Daimon, H., Iwata, M., Ogawa, T., Tanase, S., and Morino, Y. (1993) Purification and characterization of cytochrome b_5 from *Rhodotorula minuta*. *Biosci. Biotech. Biochem.* **57**, 1599-1601
4. Fukuda, H., Fujii, T., Sukita, E., Tazaki, M., Nagahama, S., and Ogawa, T. (1994) Reconstitution of the isobutene-forming reaction catalyzed by cytochrome P450 and P450 reductase from *Rhodotorula minuta*. *Biochem. Biophys. Res. Commun.* **201**, 516-522
5. Fujii, T., Ogawa, T., and Fukuda, H. (1992) Induction of cytochrome P450rm in *Rhodotorula minuta* by L-phenylalanine in *Proceedings of 7th International Conference on Biochemistry and Biophysics of Cytochrome P450* (Archakov, A.I. and Bachmanova, G.I., eds.) pp. 516-518, INCO-TNC, Joint Stock Company, Moscow, Russia
6. Cook, K.A. and Cain, R.B. (1974) Regulation of aromatic metabolism in the fungi: Metabolic control of the 3-oxoadipate pathway in the yeast. *J. Gen. Microbiol.* **85**, 37-50
7. Durham, D.R., Mcnamee, C.G., and Stewart, D.B. (1984) Dissimilation of aromatic compounds in *Rhodotorula graminis*: Biochemical characterization of pleiotropically negative mutants. *J. Bacteriol.* **160**, 771-777
8. Harris, G. and Ricketts, R.W. (1962) Metabolism of phenolic compounds by yeasts. *Nature* **195**, 473-474
9. Moore, K., Subba Rao, P.V., and Towers, G.H.N. (1968) Degradation of phenylalanine and tyrosine by *Sporobolomyces roseus*. *Biochem. J.* **106**, 507-514
10. Cain, R.B., Bilton, R.F., and Darrah, J.A. (1968) The metabolism of aromatic acids by micro-organisms. *Biochem. J.* **106**, 797-828
11. Jamaluddin, M., Subba Rao, P.V., and Vaidyanathan, C.S. (1970) Involvement of the protocatechuate pathway in the metabolism of mandelic acid by *Aspergillus niger*. *J. Bacteriol.* **101**, 786-793
12. Gall, A. and Naujahr, H.Y. (1979) Metabolism of phenol and resorcinol in *Trichosporon cutaneum*. *J. Bacteriol.* **137**, 13-21
13. Anderson, J.J. and Dagley, S. (1980) Catabolism of aromatic acids in *Trichosporon cutaneum*. *J. Bacteriol.* **141**, 534-543
14. Imai, Y. and Sato, R. (1967) Studies on the substrate interaction with P-450 in drug hydroxylation by liver microsomes. *J. Biochem.* **62**, 293-249
15. Reddy, C.C. and Vaidyanathan, C.S. (1975) Purification, properties and induction of a specific benzoate-4-hydroxylase from *Aspergillus niger* (UBC 814). *Biochim. Biophys. Acta* **384**, 46-57
16. Gorcom, R.F.M., Boschloo, J.G., Kuijvenhoven, A., Lange, J., Vark, A.J., Bos, C.J., Balken, J.A.M., Pouwels, P.H., and Hondel, C.A.M.J.J. (1990) Isolation and molecular characterization of the benzoate-para-hydroxylase gene (*bphA*) of *Aspergillus niger*: A member of a new gene family of the cytochrome P450 superfamily. *Mol. Gen. Genet.* **223**, 192-197