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# Dissimilation of Aromatic Compounds in *Rhodotorula graminis*: Biochemical Characterization of Pleiotropically Negative Mutants

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**Microorganisms oxidize many aromatic compounds through the dihydroxylated intermediates catechol and protocatechuate and through the β-ketoadipate pathway. The catabolic sequences used by the yeast *Rhodotorula graminis* for the dissimilation of aromatic compounds were elucidated after biochemical analysis of pleiotropically negative mutant strains. Growth properties of one mutant strain revealed that benzoate-4-hydroxylase was required for the utilization of phenylalanine, mandelate, and benzoate. Analysis of benzoate-4-hydroxylase- and *p*-hydroxybenzoate hydroxylase-deficient mutants provided genetic evidence that benzoate was hydroxylated in the *para* position forming *p*-hydroxybenzoate. Enzyme assays and growth studies with wild-type and mutant strains of *R. graminis* indicated that separate and highly specific hydroxylases oxidized *p*-hydroxybenzoate and *m*-hydroxybenzoate to protocatechuate. Examination of a protocatechuate 3,4-dioxygenase-deficient mutant demonstrated the role of the protocatechuate branch of the eucaryotic β-ketoadipate pathway for the utilization of phenylalanine, mandelate, benzoate, and *m*-hydroxybenzoate. Salicylate, on the other hand, was shown to be metabolized through catechol. Thus, *R. graminis* differs from other yeasts such as *Trichosporon cutaneum* and *Rhodotorula mucilaginosa* in that it contains both branches of the β-ketodipate pathway.**

Numerous aromatic or substituted aromatic compounds are oxidized to catechol or protocatechuate. These diphenolic intermediates can be further dissimilated through a convergent set of analogous enzymatic reactions to β-ketoadipate, which is then metabolized to succinate and acetyl-coenzyme A. The β-ketoadipate pathway has been studied extensively in bacteria (for reviews, see 5, 18, 21) and fungi (2). The ability of yeasts to utilize aromatic compounds has been documented in several laboratories (1, 2, 4, 8, 9, 11, 14). Biochemical studies indicate that most yeasts metabolize aromatic substrates through reactions of the β-ketoadipate pathway that are identical to those described for filamentous fungi (2, 4). One exception is *Trichosporon cutaneum*, which does not possess a ring cleavage enzyme for protocatechuate. Rather, protocatechuate is oxidized to 1,3,4-trihydroxybenzene, which undergoes ring cleavage to maleylacetate (1, 8). Previous biochemical studies with *T. cutaneum*, *Rhodotorula* spp., and *Sporobolomyces roseus* have demonstrated the role of the β-ketoadipate pathway for the degradation of aromatic compounds such as phenylalanine (13, 15), benzoate (2, 9, 14), salicylate (1, 11, 14), and phenol (8, 9, 16).

This report describes a biochemical genetic analysis of pleiotropically negative mutant strains of *Rhodotorula graminis* which indicates that phenylalanine, DL-mandelate, and benzoate are metabolized through *p*-hydroxybenzoate to protocatechuate and that *m*-hydroxybenzoate is oxidized to protocatechuate by a monooxygenase distinct from the one which hydroxylates *p*-hydroxybenzoate to protocatechuate. In addition, salicylate is shown to be metabolized through the catechol branch of the β-ketoadipate pathway.

## MATERIALS AND METHODS

**Organism and growth conditions.** The yeast strain used in this study was isolated from soil by enrichment culture with benzoate as a sole carbon and energy source and has been identified as *Rhodotorula graminis* by the American Type Culture Collection, Rockville, Md. Key characteristics for identifying this isolate as *R. graminis* included pink pigment formation, the inability to ferment any of the sugars tested, and the ability to assimilate glucose, galactose, and sucrose but not maltose and lactose. *R. graminis* strain KGX39 does not form true mycelia or ballistospores, grows on vitamin-free medium, and can utilize nitrate or nitrite as a source of nitrogen.

*R. graminis* was maintained on Sabouraud-Dextrose agar plates (Difco Laboratories) or basal salts (6) agar medium containing 20 mM glucose. Cells cultured in basal salts liquid medium plus 20 mM glucose were used as a source of inoculum (5% [vol/vol]) for growth in basal salts medium containing 2 mM glucose plus 20 mM aromatic substrate. Cultures were grown at 30°C with aeration (250 rpm), harvested at late exponential phase by centrifugation at 4°C, and washed with a volume of 20 mM potassium phosphate buffer (pH 7.6) equal to the original culture volume. Cell pastes were stored at −80°C until use.

For determination of specific activities of benzoate dissimilatory enzymes in mutants of *R. graminis*, strains cultured in basal salts medium containing 20 mM glucose were used to inoculate 500-ml Erlenmeyer flasks containing 150 ml of basal salts medium supplemented with 10 mM glucose. Growth was monitored turbidometrically with a Klett-Summerson colorimeter (no. 66 filter) until the cultures reached ca. 150 Klett units. At this point, aromatic substrate (10 mM) was added, cultures were incubated an additional 12 h to allow for enzyme induction, and cells were harvested as described above. Residual glucose in the medium was determined by a glucose analyzer (YSI 23A; Yellow Springs Instruments). Under these growth conditions, no significant glucose was detected after the 12-h induction period.

**Mutagenesis.** Mutants of *R. graminis* were isolated after ethylmethane sulfonate mutagenesis and nystatin enrichment as described by Cook (3). *p*-Hydroxybenzoate and DL-mandelate were used with nystatin as growth substrates

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TABLE 1. Levels of aromatic dissimilatory enzymes in crude extracts of *R. graminis*[a]

| Growth substrate | Sp act (nmol/min per mg of protein) of: | | | |
|---|---|---|---|---|
| | Benzoate-4-hydroxylase | *p*-Hydroxybenzoate hydroxylase | *m*-Hydroxybenzoate-4-hydroxylase | Protocatechuate 3,4-dioxygenase |
| DL-mandelate | 14 | 37 | ND[b] | 674 |
| Phenylalanine | 7 | 14 | ND | 197 |
| Benzoate | 11 | 36 | 4 | 589 |
| *p*-Hydroxybenzoate | <0.01 | 37 | 1 | 454 |
| *m*-Hydroxybenzoate | <0.01 | 3 | 35 | 157 |
| Protocatechuate | <0.01 | <0.01 | ND | 385 |
| Glucose | <0.01 | <0.01 | <0.01 | <0.01 |

[a] Crude extract preparation and enzyme assays were done as described in the text.
[b] ND, Not determined.

during enrichments. Spontaneous revertants were isolated by culturing mutants overnight in basal salts medium containing 20 mM glucose, harvesting by centrifugation, and suspending the cells in basal salts medium equivalent to 1/10 of the original volume. Viable counts were determined by plating appropriate dilutions of each mutant strain on Luria agar medium. Mutant strains were spread onto basal salts agar medium supplemented with 5 to 10 mM aromatic substrate. Revertants appearing after 120 h were patched onto additional aromatic minimal agar medium to confirm the revertant phenotype.

**Preparation of extracts and enzyme assays.** Frozen cell pellets were suspended in 2 volumes of 20 mM potassium phosphate buffer (pH 7.6) and disrupted by passage through an Aminco French pressure cell at 18,000 lb/in$^2$. Unbroken cells and cell debris were removed by centrifugation at 15,000 × *g* for 15 min at 4°C; the supernatant thus obtained was designated the crude extract.

Benzoate-4-hydroxylase, *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2), and *m*-hydroxybenzoate 4-hydroxylase were measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm. Reaction mixtures contained, in a final volume of 1 ml: 200 μmol of potassium phosphate buffer (pH 7.6), 0.1 μmol of NADPH, 0.5 μmol of substrate, and a suitable amount of crude extract. Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) (7), salicylate hydroxylase (EC 1.14.13.1) (22), catechol 1,2-dioxygenase (EC 1.13.11.1) (10, 17), and muconate lactonizing enzyme (EC 5.5.1.1) (17) were assayed by previously published procedures. The protein concentration was determined by the procedure of Lowry et al. (12).

**Partial purification of salicylate catabolic enzymes.** Salicylate hydroxylase, catechol 1,2-dioxygenase, and muconate lactonizing enzyme were partially purified from salicylate-grown cells of *R. graminis*. Cells (30 g [wet weight]) were suspended in 5 volumes of Tris buffer (20 mM Tris-hydrochloride, pH 7.6, containing 1 mM dithiothreitol), and crude extracts were prepared as described above. Crude extracts were dialyzed against Tris buffer and applied to a DEAE-cellulose column (2.5 by 10 cm) previously equilibrated with the same buffer. The column was washed with 5 volumes of Tris buffer, after which enzymes were eluted with a 1-liter linear gradient of 0 to 0.3 M NaCl in Tris-hydrochloride (pH 7.6) containing 1 mM dithiothreitol. Fractions of ca. 6 ml each were collected at a flow rate of 35 ml/h. Those fractions containing salicylate hydroxylase activity, catechol oxygenase activity, and muconate lactonizing enzyme activity were each pooled and brought to 90% saturation with ammonium sulfate. Enzymes were stored in ammonium sulfate at 4°C until use.

**High-pressure liquid chromatography.** High-pressure liquid chromatography was used to identify the reaction product of salicylate hydroxylase. Reaction mixtures, containing 0.5 μmol of salicylate, 0.1 μmol of NADPH, an NADPH regeneration system (19), and partially purified salicylate hydroxylase in 20 mM potassium phosphate buffer (pH 7.6), were injected into a Waters Associates μBondapak C18 column (30 by 0.39 cm) after a suitable incubation period. Compounds were eluted at a flow rate of 2 ml/min with a linear gradient of 5 to 95% methanol in 100 mM acetic acid and were detected by absorbance at 276 nm.

## RESULTS

**Utilization of aromatic substrates.** The ability of *R. graminis* to utilize aromatic compounds which are normally metabolized through the β-ketoadipate pathway was tested by performing diauxic growth experiments in basal salts medium containing 2 mM glucose and 10 mM aromatic substrate. The most rapid growth on aromatic substrates was obtained on *p*-hydroxybenzoate, which resulted in a doubling time of 5.1 h as compared to 3.4 h for glucose. Other aromatic substrates which supported growth (and their doubling times) included benzoate (5.8 h), *m*-hydroxybenzoate (6.3 h), DL-mandelate (6.6 h), protocatechuate (7.5 h), salicylate (8.3 h), and phenylalanine (9.0 h). The aromatic substrates gentisate, *p*- and *m*-toluate, 2,3-dihydroxybenzoate, cinnamate, resorcinol, phenol, anthranilate, catechol, cresols, and toluene were not utilized at 2.5, 5.0, and 10 mM concentrations and were not inhibitory.

**Induction of aromatic dissimilatory enzymes.** Crude extracts prepared from cells grown on phenylalanine, mandelate, and benzoate contained elevated levels of benzoate-4-hydroxylase, *p*-hydroxybenzoate hydroxylase, and

TABLE 2. Growth phenotype of mutants of *R. graminis*[a]

| Strain | Growth on 20mM aromatic substrate: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Benzoate | *p*-Hydroxybenzoate | Mandelate | Phenylalanine | Protocatechuate | *m*-Hydroxybenzoate | Salicylate |
| KGX39 wt | + | + | + | + | + | + | + |
| KGX39-1 | − | − | − | − | − | − | ± |
| KGX39-5 | − | − | − | − | − | − | + |
| KGX39-2 | − | − | − | − | + | + | ± |
| KGX39-3 | − | − | − | − | + | + | + |
| KGX39-4 | − | + | − | − | + | + | + |

[a] Growth phenotypes of parent and mutant strains were determined in liquid basal salts medium containing 2 mM glucose plus 20 mM aromatic substrate. Symbols: +, Growth; −, no growth; ±, weak growth; wt, wild type.

TABLE 3. Specific activities of benzoate dissimilatory enzymes in mutant strains of *R. graminis*[a]

| Strain | Inducer | Benzoate-4-hydroxylase | *p*-Hydroxybenzoate hydroxylase | Protocatechuate 3,4-dioxygenase |
|---|---|---|---|---|
| KGX39 wild type | Benzoate | 9 | 31 | 281 |
| | *p*-Hydroxybenzoate | ND[b] | 28 | 175 |
| KGX39-1 | Benzoate | 9 | 8 | 3 |
| | *p*-Hydroxybenzoate | ND | 11 | 4 |
| KGX39-2 | Benzoate | 7 | 0.5 | 364 |
| | *p*-Hydroxybenzoate | ND | 0.3 | 298 |
| KGX39-3 | Benzoate | 7 | 0.3 | 334 |
| | *p*-Hydroxybenzoate | ND | 0.1 | 364 |
| KGX39-4 | Benzoate | 1 | 39 | 98 |
| | *p*-Hydroxybenzoate | ND | 31 | 172 |
| KGX39-5 | Benzoate | 0.3 | 5 | 41 |
| | *p*-Hydroxybenzoate | ND | 6 | 36 |

[a] Crude-extract preparation and enzyme assays were done as described in the text.
[b] ND, Not determined.

protocatechuate 3,4-dioxygenase activities. Basal (uninduced) levels of these activities were observed after growth on glucose (Table 1). *p*-Hydroxybenzoate hydroxylase and protocatechuate 3,4-dioxygenase, but not benzoate-4-hydroxylase, were induced after growth on *p*-hydroxybenzoate. Similarly, protocatechuate induced only protocatechuate 3,4-dioxygenase; benzoate-4-hydroxylase and *p*-hydroxybenzoate hydroxylase were not detected (Table 1).

Growth of *R. graminis* on *m*-hydroxybenzoate elicited the induction of an NADPH-dependent hydroxylase specific for *m*-hydroxybenzoate. Nominal activity toward *p*-hydroxybenzoate was observed with extracts from *m*-hydroxybenzoate-induced cells. This enzyme activity was detected at low levels in crude extracts from benzoate- and *p*-hydroxybenzoate-grown cells (Table 1).

**Growth characteristics of aromatic dissimilatory mutants.** Thirty mutant strains of *R. graminis* were selected, after chemical mutagenesis, that were unable to utilize aromatic substrates. Five independent mutant strains, exhibiting three distinctive growth phenotypes, were chosen for further study (Table 2). Growth of mutant strains was compared to growth of parent strain KGX39 by culturing strains in liquid basal salts medium containing 2 mM glucose and 20 mM aromatic substrate. Mutant strains KGX39-1 and KGX39-5 grew on salicylate but failed to utilize any of the other aromatic substrates tested (Table 2). Strains KGX39-2 and KGX39-3 grew on *m*-hydroxybenzoate, protocatechuate ($Pca^+$), and salicylate, but not on benzoate ($Ben^+$), *p*-hydroxybenzoate ($Pob^+$), mandelate, or phenylalanine. Mutant strain KGX39-4 was unable to utilize benzoate, phenylalanine, or mandelate. Mutant strains KGX39-1 and KGX39-2 grew at reduced rates with salicylate as a sole carbon source.

**Levels of aromatic dissimilatory enzymes in mutant strains.** Specific activities of benzoate-4-hydroxylase, *p*-hydroxybenzoate hydroxylase, and protocatechuate 3,4-dioxygenase were determined in crude extracts of wild-type and mutant strains. The inability of mutant strain KGX39-1 to utilize aromatic substrates, excluding salicylate, was due to defi-

TABLE 4. Specific activities of benzoate dissimilatory enzymes in crude extracts of spontaneous revertants[a]

| Strain | Phenotype[b] | Sp act (nmol/min per mg of protein) of: | | |
|---|---|---|---|---|
| | | Benzoate-4-hydroxylase | *p*-Hydroxybenzoate hydroxylase | Protocatechuate 3,4-dioxygenase |
| KGX39 | Wild type | 12 | 40 | 217 |
| KGX39-4 | $Ben^-$ $Pob^+$ $Pca^+$ | <1 | 71 | 138 |
| KGX39-41 | $Ben^+$ | 8 | 37 | 290 |
| KGX39-2 | $Ben^-$ $Pob^-$ $Pca^+$ | 6 | <1 | 417 |
| KGX39-21 | $Ben^+$ $Pob^+$ $Pca^+$ | 6 | 28 | 252 |
| KGX39-3 | $Ben^-$ $Pob^-$ $Pca^+$ | 6 | <1 | 469 |
| KGX39-33 | $Ben^+$ $Pob^+$ $Pca^+$ | 11 | 26 | 190 |
| KGX39-1 | $Ben^-$ $Pob^-$ $Pca^-$ | 5 | 9 | 16 |
| KGX39-12 | $Ben^+$ $Pob^+$ $Pca^+$ | 12 | 43 | 416 |
| KGX39-13 | $Ben^-$ $Pob^-$ $Pca^+$ | 5 | 8 | 462 |
| KGX39-5 | $Ben^-$ $Pob^-$ $Pca^-$ | <1 | 2 | 13 |
| KGX39-52 | $Ben^+$ $Pob^+$ $Pca^+$ | 8 | 37 | 217 |
| KGX39-55 | $Ben^-$ $Pob^-$ $Pca^+$ | 4 | 4 | 482 |

[a] Cells were grown on 10 mM glucose plus 10 mM benzoate and crude extract preparation, and enzyme assays were done as described in the text. Similar results were obtained when cells were grown with phenylalanine or mandelate instead of benzoate.
[b] All $Ben^-$ strains were unable to utilize phenylalanine or mandelate.

TABLE 5. Specific activities of salicylate dissimilatory enzymes in crude extracts of *R. graminis*[a]

| Enzyme | Sp act (nmol/min per mg of protein) on growth substrate: | | |
|---|---|---|---|
| | Glucose | Salicylate | Benzoate |
| Salicylate hydroxylase | <0.02 | 41 | 2 |
| Catechol-1,2-dioxygenase | 0.3 | 161 | 3 |
| Muconate lactonizing enzyme | 2 | 27 | ND[b] |

[a] Crude-extract preparation and enzyme assays were determined as described in the text.
[b] ND, Not determined.

cient expression of protocatechuate 3,4-dioxygenase (Table 3). In addition, reduced levels of *p*-hydroxybenzoate hyroxylase were detected; benzoate-4-hydroxylase activity was equivalent to that activity observed with the parent strain (Table 3). Mutant strains KGX39-2 and KGX39-3 were deficient in *p*-hydroxybenzoate hydroxylase (Table 3); fully induced levels of benzoate-4-hydroxylase and protocatechuate 3,4-dioxygenase activities were observed. Mutant strain KGX39-4 contained induced levels of *p*-hydroxybenzoate hydroxylase and protocatechuate 3,4-dioxygenase, but the level of benzoate-4-hydroxylase activity was markedly reduced (Table 3). Similarly, extracts of KGX39-5 contained negligible benzoate-4-hydroxylase activity. In addition, *p*-hydroxybenzoate hydroxylase and protocatechuate 3,4-dioxygenase were expressed at severely reduced levels.

**Characterization of spontaneous revertants.** Spontaneous revertants of mutant strains of *R. graminis* were obtained by plating each mutant strain onto either benzoate, *p*-hydroxybenzoate, or protocatechuate basal salts agar medium. Growth phenotypes and specific activities of benzoate catabolic enzymes for each mutant strain and respective spontaneous revertant(s) are summarized in Table 4. Mutant KGX39-4 (Ben$^-$) was reverted to Ben$^+$ phenotype; this resulted in the expression of wild-type levels of benzoate-4-hydroxylase and enabled this strain to utilize phenylalanine and mandelate. Spontaneous revertants of Ben$^-$ Pob$^-$ Pca$^+$ mutant strains KGX39-2 and KGX39-3 regained normal levels of *p*-hydroxybenzoate hydroxylase and thus regained the ability to grow on *p*-hydroxybenzoate, benzoate, and compounds dissimilated through benzoate. Two classes of spontaneous revertants were obtained from Ben$^-$ Pob$^-$ Pca$^-$ mutant strains KGX39-1 and KGX39-5. Many of those selected on protocatechuate agar medium were Ben$^-$ Pob$^-$, whereas those revertants obtained on either benzoate or *p*-hydroxybenzoate could utilize all of the aromatic substrates. Ben$^-$ Pob$^-$ Pca$^+$ revertants of mutant strain KGX39-1, which lacked protocatechuate 3,4-dioxygenase and contained reduced levels of *p*-hydroxybenzoate hydroxylase, expressed wild-type levels of protocatechuate 3,4-dioxygenase; the inability of Ben$^-$ Pob$^-$ Pca$^+$ revertants to utilize benzoate or *p*-hydroxybenzoate is presumably due to the reduced expression of *p*-hydroxybenzoate hydroxylase. Ben$^+$ revertant strains of this mutant expressed wild-type levels of these enzymes (Table 4). Mutant strain KGX39-5 appeared to be a regulatory mutant in that it contained uninduced levels of each of these benzoate dissimilatory

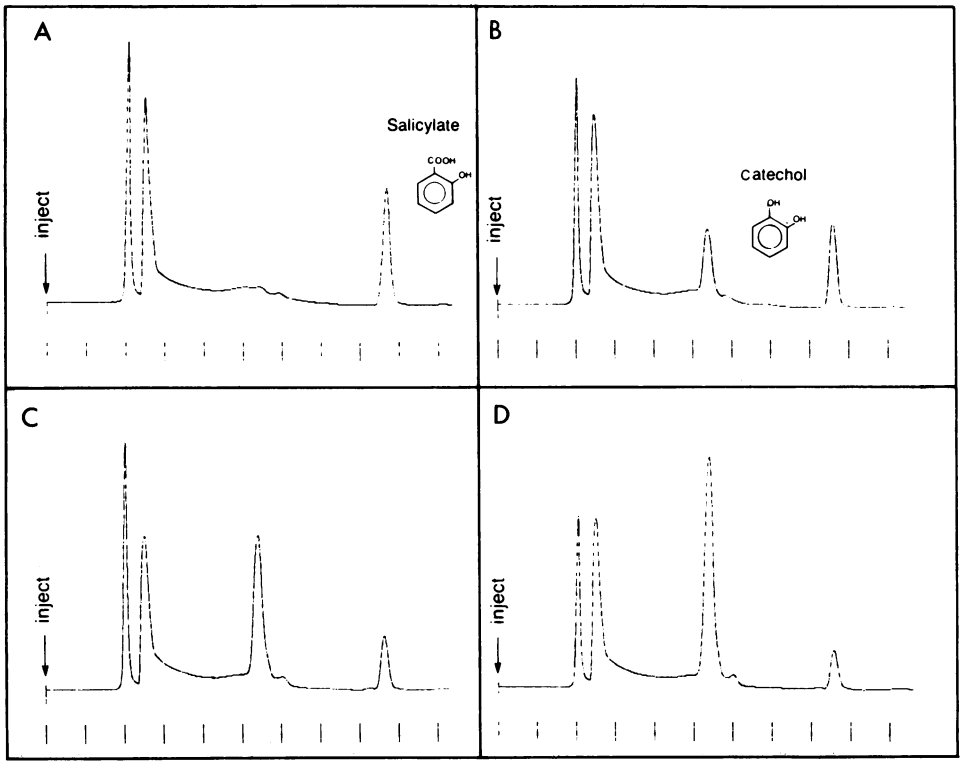


FIG. 1. Enzymatic conversion of salicylate to catechol by partially purified salicylate hydroxylase. Reaction mixture contained 8.3 mU of partially purified salicylate hydroxylase, 0.5 μmol of salicylate, 0.1 μmol of NADPH, and an NADPH-regeneration system (19) in 20 mM phosphate buffer, pH 7.6. (A) 0.4 min after addition of salicylate hydroxylase; (B) 22 min after addition; (C) 83 min after addition; (D) addition of an internal standard of authentic catechol (12 nmol) to the reaction mixture (83 min). High-pressure liquid chromatography separations were as described in the text.

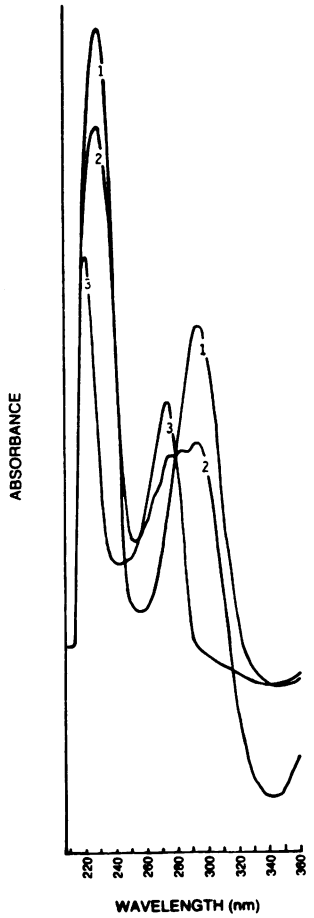


FIG. 2. Absorbance spectra of reaction mixtures demonstrating the oxidation of salicylate to catechol. Reaction mixtures, containing 0.5 μmol of salicylate, 0.1 μmol of NADPH, an NADPH-regeneration system (19), and 8.3 mU of partially purified salicylate hydroxylase in 20 mM phosphate buffer (pH 7.6), were scanned in a Gilford 2400 spectrophotometer at time 0 (curve 1), 10 min (curve 2), and 30 min (curve 3). Absorbance maxima for authentic standards are 231 and 297 nm for salicylate and 214 and 277 nm for catechol.

enzyme activities; $Ben^+$ revertants formed these enzymes. However, $Ben^-$ $Pob^-$ $Pca^+$ revertants expressed protocatechuate 3,4-dioxygenase and benzoate-4-hydroxylase but possessed reduced levels of *p*-hydroxybenzoate hydroxylase. Thus, $Ben^-$ $Pob^-$ $Pca^+$ revertants of mutant strains KGX39-1 and KGX39-5 appeared to be similar, although the original mutant strains possessed different enzymatic deficiencies (Tables 3 and 4).

**Metabolism of salicylate.** Crude extracts prepared from salicylate-grown *R. graminis* contained induced levels of salicylate hydroxylase (NAD[P]H-specific), catechol-1,2-dioxygenase, and muconate lactonizing enzyme (Table 5). Moreover, these extracts oxidized NADPH in the presence of gentisate (data not shown). Thus, it is possible that salicylate was oxidized either to catechol or to gentisate. The latter compound may be oxidatively decarboxylated to 1,3,4-trihydroxybenzene (1), which is a substrate for catechol oxygenase (8). To determine the route of salicylate metabolism in *R. graminis*, the product of salicylate hydroxylase was identified. Salicylate hydroxylase was partially purified from crude extracts of *R. graminis* by DEAE-cellulose chromatography. This procedure was highly reproducible and separated salicylate hydroxylase from subsequent enzymes such that the product of salicylate hydroxylase could be identified. High-pressure liquid chromatography of reaction mixtures containing partially purified salicylate hydroxylase revealed a time-dependent accumulation of a product with a retention time equivalent to catechol (Fig. 1B and C). Furthermore, the addition of an internal standard of authentic catechol to reaction mixtures resulted in a quantitative increase in the peak identified as catechol (Fig. 1D). Spectral scans of reaction mixtures corroborated the identification of catechol as the reaction product (Fig. 2). Salicylate (absorbance maxima, 231 and 297 nm) was oxidized to a compound with absorbance maxima of 214 and 277 nm, which compares favorably to authentic catechol (23).

## DISCUSSION

The results of this study demonstrate that *R. graminis* utilizes the reaction sequences illustrated in Fig. 3 for the dissimilation of aromatic compounds. Others have shown through biochemical analysis that phenylalanine, benzoate, and *p*-hydroxybenzoate are metabolized in yeasts through protocatechuate and the β-ketoadipate pathway (2, 11, 13, 15, 21). Our results concur with these observations; activities of benzoate-4-hydroxylase, *p*-hydroxybenzoate hydroxylase, and protocatechuate 3,4-dioxygenase were induced during growth on phenylalanine, mandelate, and benzoate (Table 1). Biochemical analysis of mutant strains of *R. graminis* provides additional evidence for the physiological significance of the enzymatic sequences depicted in Fig. 3. The inability of strain KGX39-1 to express protocatechuate 3,4-dioxygenase, coupled with the negative phenotypic

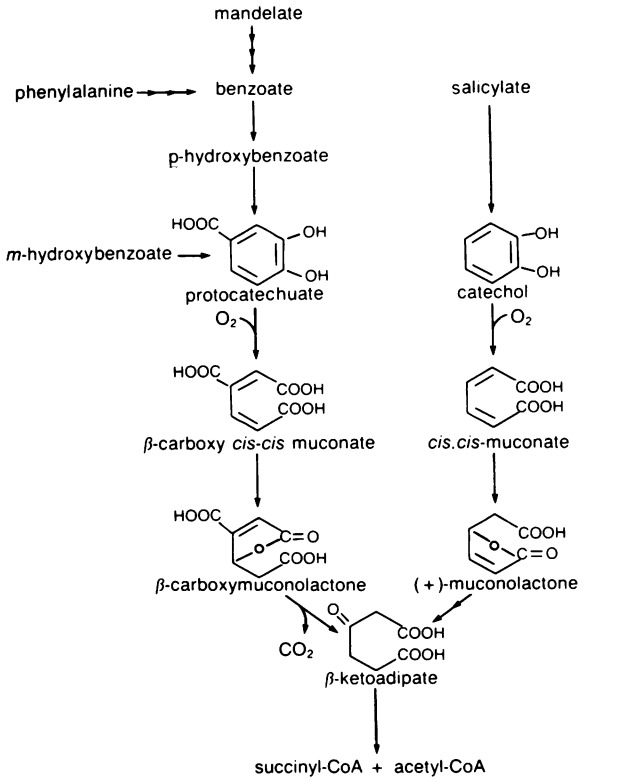


FIG. 3. Metabolic sequences utilized by *R. graminis* for the dissimilation of aromatic compounds.

growth characteristics of this strain (Table 2), clearly elucidates the role of the protocatechuate branch of the β-ketoadipate pathway for the utilization of phenylalanine, mandelate, benzoate, and *m*-hydroxybenzoate. Salicylate, on the other hand, was shown to be metabolized through the catechol branch (Fig. 1 and 3). Growth studies by Mills et al. (14) suggested that *Rhodotorula* spp. may possess both branches of the β-ketoadipate pathway. However, Cook and Cain (4) demonstrated that *R. mucilaginosa* contained only the protocatechuate branch. Through biochemical and genetic means, we have demonstrated the existence of both branches of the β-ketoadipate pathway for the dissimilation of aromatic compounds in *R. graminis*.

Mutant strain KGX39-4 lacks benzoate-4-hydroxylase activity and thus is unable to utilize benzoate, phenylalanine, and mandelate (Tables 2 and 3); spontaneous revertants regain wild-type growth on these compounds. These data favor the conclusion that the catabolic sequences for phenylalanine and mandelate converge at benzoate (Fig. 3). Mutant strains KGX39-2 and KGX39-3 are deficient in *p*-hydroxybenzoate hydroxylase and exhibit a $Ben^-$ $Pob^-$ phenotype. Revertants of these strains isolated on *p*-hydroxybenzoate express normal levels of *p*-hydroxybenzoate hydroxylase (Table 4), concurrent with the ability of these strains to grow on benzoate (or phenylalanine and mandelate). This suggests that benzoate is hydroxylated to *p*-hydroxybenzoate (Fig. 3). Others (1, 13, 15) have shown in vivo that yeasts accumulate *p*-hydroxybenzoate transiently during the metabolism of aromatic compounds, and Reddy and Vaidyanathan (19) have described a benzoate-4-hydroxylase activity in *Aspergillus niger*. We also have identified *p*-hydroxybenzoate as the oxidation product of benzoate by benzoate-4-hydroxylase in *R. graminis* (C. G. McNamee and D. R. Durham, manuscript in preparation).

Several lines of evidence suggest that specific monooxygenases oxidize *p*-hydroxybenzoate and *m*-hydroxybenzoate to protocatechuate in *R. graminis*. Crude extracts of cells grown on either *p*-hydroxybenzoate or *m*-hydroxybenzoate do not oxidize the other substrate to a significant extent (Table 1). Similar results were reported with *Sporobolomyces roseus* (15). Furthermore, mutant strains KGX39-2 and KGX39-3, which do not express *p*-hydroxybenzoate hydroxylase (Table 3), exhibit wild-type growth on *m*-hydroxybenzoate, and $Ben^-$ $Pob^-$ $Pca^+$ revertants of strains KGX39-1 and KGX39-5 grow on *m*-hydroxybenzoate but fail to utilize *p*-hydroxybenzoate (data not shown).

It has been shown in *R. mucilaginosa* that *p*-hydroxybenzoate hydroxylase is induced by its substrate, whereas protocatechuate 3,4-dioxygenase may be induced by either protocatechuate or *p*-hydroxybenzoate (4). Our results demonstrate the sequential induction of the first three enzymes involved in benzoate metabolism (Table 1) and are in agreement with the results of Cook and Cain (4). Mutant strains KGX39-2 and KGX39-3 (*p*-hydroxybenzoate hydroxylase deficient) express protocatechuate 3,4-dioxygenase when cultured in the presence of *p*-hydroxybenzoate (Table 3). Furthermore, analysis of crude extracts from strain KGX39-4 (benzoate-4-hydroxylase deficient) after growth in the presence of benzoate indicates that *p*-hydroxybenzoate hydroxylase and protocatechuate 3,4-dioxygenase are induced (Table 3). These results extend the observations of Cook and Cain (4) by providing evidence that these enzymes may be induced by benzoate as well. It should be noted, however, that the levels of protocatechuate oxygenase were lower in this strain (KGX39-4) when cultured with benzoate (Tables 3 and 4). It is unclear if this is due to a toxic effect of benzoate or to the inability of benzoate to effectively induce the enzyme.

Anderson and Dagley (1) reported that simultaneous adaption was not useful for studying aromatic pathways in *T. cutaneum*. Their results suggest that the modes of induction in yeast differ from those observed in bacteria. For example, they reported that cells adapted to growth on benzoate were also adapted to growth on salicylate, protocatechuate, and gentisate (1). In contrast, enzymes for salicylate utilization are not induced in benzoate-grown cells of *R. graminis* (Table 5), and benzoate dissimilatory enzymes are not induced in salicylate-grown cells (data not shown). *T. cutaneum* also differs from *R. graminis* in that it does not contain a ring cleavage enzyme for protocatechuate (1, 8, 20). Benzoate, gentisate, and resorcinol are metabolized through 1,2,4-trihydroxybenzene to maleylacetate and the β-ketoadipate pathway. We have been unable to demonstrate this pathway in *R. graminis*. Moreover, *R. graminis* is unable to utilize substrates (e.g., gentisate and resorcinol) which are metabolized through this pathway. Thus, it appears that these yeasts have divergent metabolic reactions for the catabolism of aromatic compounds and, perhaps, divergent regulatory controls for these pathways as well.

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## LITERATURE CITED

1. **Anderson, J. J., and S. Dagley.** 1980. Catabolism of aromatic acids in *Trichosporon cutaneum*. J. Bacteriol. **141**:534–543.
2. **Cain, R. B., R. F. Bilton, and J. A. Darrah.** 1968. The metabolism of aromatic acids by microorganisms: metabolic pathways in the fungi. Biochem. J. **108**:797–827.
3. **Cook, K. A.** 1974. Regulation of aromatic metabolism in fungi: selection of mutants of the yeast *Rhodotorula mucilaginosa* with nystatin. J. Gen. Microbiol. **85**:29–36.
4. **Cook, K. A., and R. B. Cain.** 1974. Regulation of aromatic metabolism in the fungi: metabolic control of the 3-oxoadipate pathway in the yeast *Rhodotorula mucilaginosa*. J. Gen. Microbiol. **85**:37–50.
5. **Dagley, S.** 1979. Pathways for the utilization of organic growth substrates. The Bacteria **6**:305–388.
6. **Durham, D. R., and P. V. Phibbs, Jr.** 1982. Fractionation and characterization of the phosphoenolpyruvate: fructose 1-phosphotransferase system from *Pseudomonas aeruginosa*. J. Bacteriol. **149**:534–541.
7. **Durham, D. R., L. A. Stirling, L. N. Ornston, and J. J. Perry.** 1980. Intergeneric evolutionary homology revealed by study of protocatechuate-3,4-dioxygenase from *Azotobacter vinelandii*. Biochemistry **19**:149–155.
8. **Gaal, A., and H. Y. Neujahr.** 1979. Metabolism of phenol and resorcinol in *Trichosporon cutaneum*. J. Bacteriol. **137**:13–21.
9. **Harris, G., and R. W. Ricketts.** 1962. Metabolism of phenolic compounds by yeasts. Nature (London) **195**:473–474.
10. **Hegeman, G. D.** 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. J. Bacteriol. **91**:1140–1154.
11. **Henderson, M. E. K.** 1961. The metabolism of aromatic compounds related to lignin by some hyphomycetes and yeast-like fungi from soil. J. Gen Microbiol. **26**:155–165.
12. **Lowry, O. H., N. J. Rosenbrough, A. C. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–272.
13. **Marusich, W. C., R. A. Jensen, and L. O. Zamir.** 1981. Induction of L-phenylalanine ammonia-lyase during utilization

of phenylalanine as a carbon or nitrogen source in *Rhodotorula glutinis*. J. Bacteriol. **146**:1013–1019.
14. **Mills, S. C., J. J. Child, and J. F. T. Spencer.** 1971. The utilization of aromatic compounds by yeasts. Antonie van Leeuwenhoek J. Microbiol. Serol. **37**:281–287.
15. **Moore, K., P. F. Subba Rao, and G. H. N. Towers.** 1968. Degradation of phenylalanine and tyrosine by *Sporobolomyces roseus*. Biochem. J. **106**:507–514.
16. **Neujahr, H. Y., S. Lindsjo, and J. M. Varga.** 1974. Oxidation of phenols by cells and cell-free enzymes from *Candida tropicalis*. Antonie van Leeuwenhoek J. Microbiol. Serol. **40**:209–216.
17. **Ornston, L. N.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. III. Enzymes of the catechol pathway. J. Biol. Chem. **241**:3795–3799.
18. **Ornston, L. N., and D. Parke.** 1976. The evolution of induction mechanisms in bacteria: insights derived from the study of the β-ketoadipate pathway. Curr. Top. Cell. Regul. **12**:209–262.
19. **Reddy, C. C., and C. S. Viadyanathan.** 1975. Purification, properties and induction of a specific benzoate-4-hydroxylase from *Aspergillus niger* (UBC814). Biochim. Biophys. Acta **384**:46–57.
20. **Sparnins, V. L., D. G. Burbee, and S. Dagley.** 1979. Catabolism of L-tyrosine in *Trichosporon cutaneum*. J. Bacteriol. **138**:425–430.
21. **Stanier, R. Y., and L. N. Ornston.** 1973. The β-ketoadipate pathway. Adv. Microb. Physiol. **9**:89–151.
22. **White-Stevens, R. H., and H. Kamin.** 1972. Studies of a flavoprotein, salicylate hydroxylase. I. Preparation, properties, and the uncoupling of oxygen reduction from hydroxylation. J. Biol. Chem. **247**:2358–2370.
23. **Yamamoto, S., M. Katagin, H. Maeno, and O. Hayaishi.** 1965. Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. I. Purification and general properties. J. Biol. Chem. **240**:3408–3413.