JOURNAL OF BACTERIOLOGY, Mar. 1993, p. 1467–1474
0021-9193/93/051467-08$02.00/0

Vol. 175, No. 5

Two Independently Regulated Cytochromes P-450 in a *Rhodococcus rhodochrous* Strain That Degrades 2-Ethoxyphenol and 4-Methoxybenzoate

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Received 20 October 1992/Accepted 28 December 1992

A red-pigmented coryneform bacterium, identified as *Rhodococcus rhodochrous* strain 116, that grew on 2-ethoxyphenol and 4-methoxybenzoate as sole carbon and energy sources was isolated. Phylogenetic analysis based on the 16S rDNA sequences indicates that the strain clusters more closely to other rhodococci than to other gram-positive organisms with a high G+C content. Each of the abovementioned growth substrates was shown to induce a distinct cytochrome P-450: cytochrome P-450$_{RR1}$ was induced by 2-ethoxyphenol, and cytochrome P-450$_{RR2}$ was induced by 4-methoxybenzoate. A type I difference spectrum typical of substrate binding was induced in cytochrome P-450$_{RR1}$ by both 2-ethoxyphenol (K_s = 4.2 ± 0.3 μM) and 2-methoxyphenol (K_s = 2.0 ± 0.1 μM), but not 4-methoxybenzoate or 4-ethoxybenzoate. Similarly, a type I difference spectrum was induced in cytochrome P-450$_{RR2}$ by both 4-methoxybenzoate (K_s = 2.1 ± 0.1 μM) and 4-ethoxybenzoate (K_s = 1.6 ± 0.1 μM), but not 2-methoxyphenol or 2-ethoxyphenol. A purified polyclonal antiserum prepared against cytochrome P-450$_{RR1}$ did not cross-react with cytochrome P-450$_{RR2}$, indicating that the proteins are immunologically distinct. The cytochromes appear to catalyze the O-dealkylation of their respective substrates. The respective products of the O-dealkylation are further metabolized via *ortho* cleavage enzymes, whose expression is also regulated by the respective aromatic ethers.

The design of novel efficient microbial catabolic pathways to degrade otherwise recalcitrant environmental compounds involves not only the recruitment of enzymes with desirable catalytic activities from different organisms but also the modification of such enzymes to improve their catalytic abilities. Cytochromes P-450 are good candidates for such recruitment, as this class of heme monooxygenases catalyze a remarkably broad range of chemical reactions on an equally broad range of substrates (29). In bacterial systems, P-450s generally occur as part of a soluble three-component system, consisting of a flavin-containing reductase and an iron-sulfur electron transfer protein in addition to the cytochrome (37). Bacterial P-450s often occur in catabolic pathways, frequently catalyzing an initial hydroxylation reaction of the growth substrate, as in the case of P-450$_{cam}$ (21) and P-450$_{lin}$ (42). P-450's have also been reported to catalyze the O-dealkylation of *para*-substituted benzoates (3), veratrole (41), and *ortho*-substituted alkoxyphenols (5). The characteristic absorbance peak at approximately 450 nm of the CO-difference spectrum of the reduced P-450 provides a ready means to screen for the presence of these enzymes in whole cells and crude extracts (31). For these reasons, interest in recruiting P-450's into both biotransformation and biodegradation processes has increased rapidly (6, 29, 37).

A structural element that contributes to both the recalcitrance and the toxicity of such pollutants as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is the ether bond. To investigate the mechanism of ether bond cleavage in aromatic compounds, microorganisms were screened for enzymes capable of cleaving ether bonds in simpler aromatic ethers. In screening soil isolates for their ability to grow on ether bond-containing aromatic compounds such as 2-ethoxyphenol and 4-ethoxybenzoate, we isolated a strain of *Rhodococcus rhodochrous* which expresses two different P-450 isozymes that O-dealkylate these substrates. The 16S rDNA sequence of the organism was determined and used to establish the organisms's phylogenetic relationship to other bacteria. We report here the differential induction of the two cytochromes as well as the different substrate-binding specificities of the two enzymes. The further degradation of these compounds was also investigated.

MATERIALS AND METHODS

Strain isolation and growth. Cultures were enriched for organisms able to degrade ethoxyaromatics by batch cultivation at 30°C with rotary shaking. M9 minimal medium (36) containing 10% activated sewage sludge from a Geneva municipal sewage treatment plant was amended with either 2-ethoxyphenol or 4-ethoxybenzoate on the following schedule: 0.5 mM for 2 months, 1 mM for 1 month, and 2 mM for 1 month. After this time, portions of the culture fluid were plated on M9 minimal medium agar plates containing the enrichment compound at a concentration of 5 mM. The

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TABLE 1. Growth of *R. rhodochrous* 116 and induction of P-450 (if tested)[a]

Carbon source (isomers tested)	Growth[b]	P-450 induced
Glucose	++	No
Succinate	++	No
Methanol	−	
Ethanol	+	
Glycerol	+	No
Acetate	+	
Formate	−	
Hexane	+	
Formaldehyde	−	
Acetaldehyde	−	
Ethoxybenzene	−	
Dimethoxybenzene (1,2-, 1,3-)	−	
Phenol	+	
2-Chlorophenol	+	No
Chlorophenol (3-, 4-)	−	
Dichlorophenol (2,4-, 3,4-)	−	
Trichlorophenol (2,3,5,-, 2,4,5-)	−	
2,3,4,5-Tetrachlorophenol	−	
Methylphenol (2-, 3-, 4-)	+	
Hydroxyphenol (2-,3-,4-)	−	
2-Methoxyphenol	++	Yes
2-Ethoxyphenol	++	Yes
3-Methoxyphenol	−	
4-Methoxyphenol	+	
4-Ethoxyphenol	+	
4-Propoxyphenol	+	
4-Butoxyphenol	+	
Anisole	+	Yes
2-Methylanisole	[+]	
Methylanisole (3-, 4-)	−	
o-Aniside	−	
Aniside (*m*-, *p*-)	+	
Benzoate	+	
Chlorobenzoate (2-, 3-, 4-)	−	
Hydroxybenzoate (2-, 4-)	+	
3-Hydroxybenzoate	−	
Methoxybenzoate (2-, 3-)	−	
4-Methoxybenzoate	++	Yes
4-Ethoxybenzoate	++	Yes
4-Butoxybenzoate	+	
4-Propoxybenzaldehyde	−	

[a] Cells were grown on minimal medium supplemented with the listed carbon sources at 1 mM.

[b] Symbols: ++, rapid growth; +, growth; [+], slow growth; −, no growth.

resulting colonies were streaked to purity. Glycerol-containing stocks of the cultures were stored at −70°C (13).

Partial biochemical characterization of the strain was performed with the API-20E system (API Systems, Montalieu-Vercieu, France) with incubation at 30°C. Positive reactions were scored at 24 and 48 h. A sample of the strain was sent to the German Collection of Microorganisms and Cell Cultures (DSM), Braunschweig, Germany, for identification and storage.

The organism was routinely cultivated at 30°C in M9g (M9 minimal medium supplemented with 2.5 ml of a trace element solution [1] per liter) and one of the following carbon sources: 5 mM 2-ethoxyphenol, 5 or 10 mM 4-ethoxybenzoate, or 5 mM succinate. Cells were harvested in the late log growth phase by centrifugation, washed, and resuspended in buffer A (20 mM Tris-HCl [pH 7.4]). The substrate range of the organism was determined by cultivation of the organism in minimal medium supplemented with a 1 mM concentration of various hydrocarbons (Table 1). Bacterial growth was determined by monitoring the optical density of the culture at 500 nm, with that of the sterile medium used as a reference.

Isolation of genomic DNA. Approximately 0.1 g of cells were washed and resuspended with 560 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]). The cell suspension was treated overnight with 2.5 mg of lysozyme (Boehringer) and 0.1 mg of proteinase K (Sigma). Complete cell lysis was then accomplished by the addition of 30 μl of 10% sodium dodecyl sulfate (SDS). DNA extraction and precipitation were performed by the CTAB miniprep protocol for bacterial genomic DNA preparations (44).

PCR amplification of DNA. The 16S rDNA was targeted for amplification by polymerase chain reaction (PCR) by standard protocols (34) with a Hans Landgraf model 5.92 thermocycler. The forward primer (16F27; 5′-AGAGTTTG ATCMTGGCTCAG-3′) annealed at positions 8 to 27 (*Escherichia coli* numbering), and the reverse primer (16R1488; 5′-CGGTTACCTTGTTAGGACTTCACC-3′) annealed at the complement of positions 1511 to 1488. The PCR mixture consisted of 10 mM Tris-Cl (pH 8.3), 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.1 mg of gelatin, 2.0 μl of formamide, 200 μM each of the four deoxynucleoside triphosphates, 0.6 μM each of the two primers, 1.0 μg of genomic DNA, and 2.5 U of *Taq* DNA polymerase (Promega) in a 100-μl reaction volume overlaid with sterile mineral oil. The PCR used 30 cycles with the following profile: 1 min of denaturation at 94°C, 1 min of primer annealing at 50°C, and 2 min of primer extension at 72°C. Reaction products were elongated with a final incubation of 10 min at 72°C. Amplified DNA was extracted with chloroform-isoamyl alcohol and purified with a Centricon-100 microconcentrator (Amicon). The sample was resuspended in 85 μl of water, of which 5 μl was used to check the quality of the PCR-amplified DNA by electrophoresis on a 1% agarose gel.

Sequencing of PCR-amplified DNA. The PCR-amplified 16S rDNA was sequenced directly on an Applied Biosystems, Inc., model 373A automated DNA sequencer by the standard protocols of the manufacturer for *Taq* DNA polymerase-initiated, cycle-sequencing reactions with fluorescently labeled dideoxynucleotide terminators. The primers used for 16S rRNA gene sequence determinations have been described previously (24).

16S rDNA sequence analysis. Sequence data were aligned with known 16S rDNA sequences maintained on an in-house data base by using conserved regions and secondary-structure characteristics as references (15, 45). Once the sequences were optimally aligned, homology and evolutionary distance values were calculated for sequence-pair comparisons using only unambiguous, homologous nucleotide positions. A sequence "mask" (24) that allows comparison of up to 1,353 positions was used. Similarity values and evolutionary distances were calculated with a correction factor for reverse mutations (20). Phylogenetic trees were generated by using a pairwise, weighted, least-squares distance method (28).

Oxygen uptake measurements. Cells were diluted to ca. 10^8/ml with buffer A. Five hundred microliters of this dilution was placed in a barrel-type continuously stirred oxygen electrode equipped with a cover (Bachofer, Reutlingen, Germany). After equilibration, the appropriate test substrate, dissolved in 10 μl of either Millipore MilliQ purified water or dimethyl sulfoxide, was added to a final concentration of 1 mM. Dimethyl sulfoxide and water alone were injected for baseline determinations. Oxygen consumption

was calculated from the slope of the recorder tracing of dissolved O_2 level versus time during the first minute after injection. Oxygen consumption was measured by using cells grown on 2-ethoxyphenol, 4-ethoxybenzoate, and succinate. Each of these compounds was used as a test substrate in these measurements.

Cellular extracts. All manipulations were performed at 4°C unless otherwise noted. Approximately 2.5 g (wet weight) of freshly harvested cells were washed and resuspended in 5 ml of buffer B (20 mM Tris-HCl [pH 7.4], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The cells were disrupted in a horizontal shaker-type mixer mill (model MM2; K. Retsch GmbH, Haan, Germany), equipped with a 12.5-ml stainless steel grinding chamber (38). The chamber was filled with the cell suspension and 10 g of glass beads (0.25 to 0.50 mm in diameter). The filled chamber was operated for 15 min at a mixing frequency of 1,800 min^{-1}. The resulting homogenate was centrifuged at 30,000 × *g* for 20 min. The supernatant fluid, referred to as the cell extract, was carefully decanted and used immediately. The protein concentration of the extract was determined by the Folin reaction (16) after the sample was heated to 90°C in 0.25 M sodium hydroxide for 10 min.

Cytochrome P-450 assay. Cell extracts were diluted 10-fold in buffer A, reduced with several grains of sodium dithionite, and divided equally between two optically matched cuvettes. The contents of the sample cuvette were exposed to carbon monoxide (CO) for 20 s by gently bubbling the gas through the cuvette. Immediately afterwards, the difference spectrum against the non-CO-exposed (reference) cuvette was determined on a Uvicon 810 spectrophotometer with a recorder (Kontron Instruments, Zürich, Switzerland). The presence of P-450 was ascertained from the characteristic absorption peak at about 450 nm. *Moraxella* sp. strain GU2, an organism known to contain P-450 (5), served as a control.

Substrate binding spectra. The binding of different aromatic ether compounds to cytochrome P-450 was quantified by the known type I response (19). Cell extracts were diluted in buffer B and divided equally between two optically matched cuvettes. Increasing amounts of the substrate, dissolved in buffer B, were mixed into the sample cuvette in a stepwise manner. A corresponding amount of buffer was added to the reference cuvette. After each addition, the difference spectrum from 340 to 500 nm was recorded on a Uvicon 810 spectrophotometer equipped with a chart recorder (Kontron Instruments). Substrate binding was quantitated by the difference in absorbance at 386 and 417 nm. Substrates investigated for binding were 2-ethoxyphenol, 4-ethoxybenzoate, 2-methoxyphenol, and 4-methoxybenzoate. Substrate binding was determined in extracts from cells grown on 5 mM 2-ethoxyphenol and 5 mM 4-ethoxybenzoate.

Purification of P-450 and preparation of antibodies. $P\text{-}450_{RR1}$ was purified to homogeneity from *R. rhodochrous* grown on 5 mM 2-ethoxyphenol as described elsewhere (10). The concentration of the cytochrome was determined spectrophotometrically, using a difference in extinction coefficients $De^{450-490}$ of 92.8 cm^{-1} mM^{-1} for the reduced CO-bound species (14). New Zealand White rabbits were injected with 1 mg of purified $P\text{-}450_{RR1}$ three times at 3-week intervals with Freund's complete adjuvant in the first injection and incomplete adjuvant in the subsequent injections. The rabbits were then killed, and serum samples were collected. Preimmune sera and immune sera were tested for immunoreactivity with purified $P\text{-}450_{RR1}$ as the antigen in an enzyme-linked immunosorbent assay. Polyclonal antibodies specific against $P\text{-}450_{RR1}$ were purified from the antiserum by affinity column chromatography on a gel consisting of $P\text{-}450_{RR1}$ coupled to cyanogen bromide-activated Sepharose 4B (4). Antibodies were stored at −20°C in phosphate-buffered saline–0.02% sodium azide.

Gel electrophoresis and Western immunoblots. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a Bio-Rad (Richmond, Calif.) Miniprotein II apparatus by standard procedures (22). Proteins were either visualized with Coomassie R250 brilliant blue or electroblotted on a nitrocellulose filter (Schleicher & Schüll). Antigens were detected by indirect immunolabeling with the diluted (1:1,000 in phosphate-buffered saline) purified $P\text{-}450_{RR1}$ antiserum and a dilution (1:1,000) of peroxidase-linked goat anti-rabbit immunoglobulin G (heavy and light chains) (Dianova GmbH, Hamburg, Germany) by standard procedures (36).

Enzymatic activities. Dioxygenase activities were measured at 30°C in 1-ml quartz cuvettes in a Beckman DU-70 spectrophotometer interfaced to an IBM Personal System/2 model 55SX microcomputer. Extracts of cells grown on 2-ethoxyphenol or 4-ethoxybenzoate were stored on ice until diluted as appropriate in buffer A and used immediately. Reactions were initiated with the addition of substrate. Catechol-1,2-dioxygenase activity was measured by monitoring the formation of muconate at 260 nm (17) after blocking muconate cycloisomerase with 1.2 mM EDTA, using an extinction coefficient of 16.8 mM^{-1} cm^{-1} for *cis,cis*-muconate (8). Catechol-2,3-dioxygenase was determined by monitoring the formation of 2-hydroxymuconic acid semialdehyde at 375 nm, using an extinction coefficient of 34.0 mM^{-1} cm^{-1} (35). The activity of protocatechuate-3,4-dioxygenase was measured by monitoring the disappearance of substrate at 290 nm, using a difference in extinction coefficients between substrate and product (3-carboxymuconate) at 2.3 mM^{-1} cm^{-1} (39). The activity of protocatechuate-4,5-dioxygenase was determined by monitoring the formation of 2-hydroxy-4-carboxymuconic acid semialdehyde at 410 nm, using an extinction coefficient of 9.7 mM^{-1} cm^{-1} (43).

Nucleotide sequence accession number. The *R. rhodochrous* 165 rRNA sequence been assigned EMBL accession number X70295.

RESULTS

A red-pigmented bacterium was isolated from activated sludge by growth on ethoxylated aromatics and was shown to grow on either 2-ethoxyphenol or 4-ethoxybenzoate as the sole carbon and energy source. The strain was determined to be Gram stain reaction positive, exhibiting an elementary rod-coccus life cycle. The red pigmentation was affected by neither the presence of light, the growth temperature (22 to 37°C), nor the richness of the growth substrate (Luria broth versus supplemented M9 medium). The strain was positive for β-galactosidase, lysine decarboxylase, citrate utilization, urease, and gelatinase. It was negative for hydrogen sulfide production, tryptophan deaminase, indole production, and acetoin production. Acid is weakly produced from glucose, mannose, sorbitol, saccharose, and amylose but not from inositol, rhamnose, or arabinose. In cells of this morphological type, the presence of *N*-glycolylmuramic acid in the cell wall polymers is a key characteristic of members of the *Rhodococcus* genus (22). This strain contained glycolic muramic acid, as demonstrated by a positive reaction in the glycolate test. The organism was subsequently identified as *Rhodococcus rhodochrous* strain 116 by the DSM.

The use of PCR primers 16F27 and 16R1488 allowed amplification of the entire gene encoding the 16S rRNA of

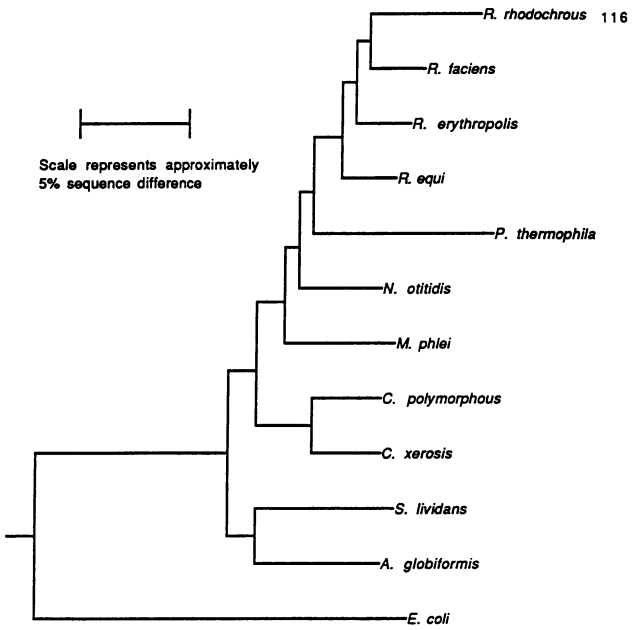

FIG. 1. Estimated phylogenetic position of *R. rhodochrous* 116 within a group of representative species of the gram-positive, high-G+C group. Species included are *Rhodococcus faciens*, *R. erythropolis*, *R. equi*, *Pseudonocardia thermophila*, *Nocardia otitidis*, *Mycobacterium phlei*, *Corynebacterium polymorphous*, *C. xerosis*, *Streptomyces lividans*, and *Arthrobacter globiformis*.

the organism, except for 27 and 54 nucleotides at the 5′ and 3′ termini of the gene, respectively. More than 1,300 nucleotide positions of the 16S rDNA of *R. rhodochrous* 116 were used in a masked sequence comparison with sequences from more than 900 organisms (7). This comparison determined that *R. rhodochrous* 116 clusters with the gram-positive bacteria with high G+C DNA content. A rooted phylogenetic tree based on a least-squares distance analysis of the estimated relatedness of *R. rhodochrous* 116 to other gram-positive, high-G+C species is depicted in Fig. 1. It can be seen that within this group, *R. rhodochrous* 116 clusters most closely with the other species of rhodococci, possessing sequence similarities of approximately 95% with each of the other species of this genus.

It was established that a variety of *ortho*-substituted alkoxyphenols and *para*-substituted alkoxybenzoates could support growth, whereas phenols and benzoates substituted in other positions could not (Table 1). The organism could also be cultivated on succinate, glucose, and Luria broth. Growth was also observed on 4-hydroxybenzoate but not on either catechol or veratrole. Cell yields suitable for experimental work were achieved in full medium and in M9 medium supplemented with trace elements and either glucose, succinate, 2-methoxyphenol, 2-ethoxyphenol, 4-methoxybenzoate, or 4-ethoxybenzoate. Typical doubling times in liquid cultures containing 5 mM substrate grown at 30°C and shaken at 250 rpm were about 24 h. Substrate depletion occurred after approximately 36 h.

Metabolic activity, as measured by oxygen uptake by resting cells (Table 2), was observed only after injection of the respective substrate on which the cells had been cultivated. The rate of oxygen uptake by resting cells in the presence of succinate was only one-quarter to one-third of the rate observed in the presence of 2-ethoxyphenol or 4-ethoxybenzoate.

TABLE 2. Oxygen uptake by resting cells of *R. rhodochrous* 116[a]

Carbon source	Oxygen consumption (nmol/ml/min)		
	Succ	2EP	4EB
Succ	13.8	0	0
2EP	0	64.8	0
4EB	0	0	46.4

[a] *R. rhodocrous* was grown in M9g minimal medium supplemented with 5 mM succinate (succ), 2-ethoxyphenol (2EP), or 4-ethoxybenzoate (4EB) as the carbon source. Each substrate was then injected during measurements of metabolic activity.

The electronic absorption spectra of reduced extracts of cells grown on either 2-ethoxyphenol, 2-methoxyphenol, 4-methoxybenzoate, or 4-ethoxybenzoate, recorded as the difference in absorption of matched samples in the presence and absence of CO, showed an absorption maximum at about 450 nm, indicating the presence of P-450 in the cell extracts (Fig. 2). This maximum was not observed when the extracts contained 0.5 mM metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone). Inspection of the difference spectra revealed that the observed maxima in extracts grown on either 2-ethoxyphenol or 2-methoxyphenol occurred at 447 nm, while that of extracts from cells grown on either 4-ethoxybenzoate or 4-methoxybenzoate occurred at 450 nm. Of the growth substrates tested, the presence of P-450 was detected only in extracts of cells grown on 2-methoxyphenol, 2-ethoxyphenol, 4-methoxybenzoate, or 4-ethoxybenzoate. P-450 was not detected in extracts of cells grown on succinate, glucose, or Luria broth. For the remainder of this study, 2-ethoxyphenol and 4-ethoxybenzoate, chosen to represent the two structural classes of aromatic ethers that supported rapid growth of the organism and that induced the synthesis of P-450, were used as the growth substrates.

The spectra of extracts of cells grown on either 2-ethoxyphenol or 4-ethoxybenzoate, recorded as the difference in absorption of matched samples in the presence and absence of substrate, were characteristic of the type I response of P-450. A family of difference spectra obtained by titrating the

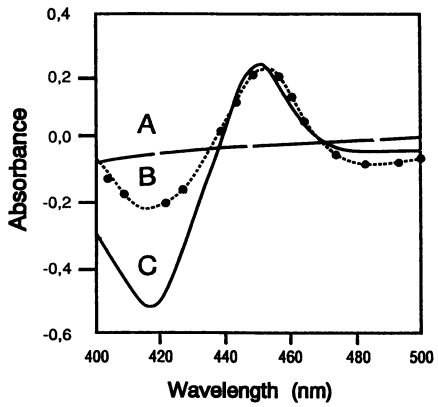

FIG. 2. CO-difference spectra of reduced cell extracts of *R. rhodochrous* 116 grown on M9g minimal medium amended with (A) 5 mM succinate, (B) 10 mM 4-ethoxybenzoate, or (C) 5 mM 2-ethoxyphenol. Protein concentrations were 10 mg/ml (A and B) or 2.5 mg/ml (C).

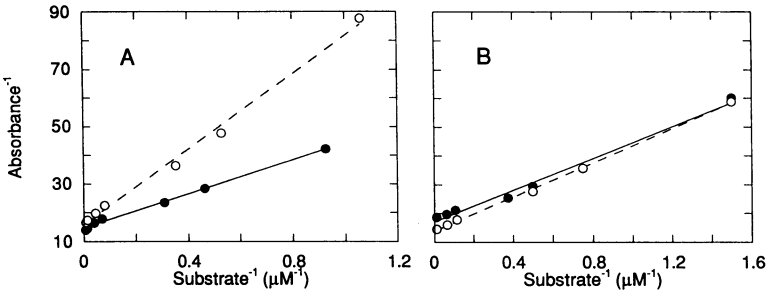

FIG. 3. Titration of cellular extracts of *R. rhodochrous* 116. (A) Cells grown on 5 mM 2-ethoxyphenol. The extract (protein concentration, 0.7 mg/ml) was titrated with 2-methoxyphenol (●) or 2-ethoxyphenol (○). (B) Cells grown on 5 mM 4-ethoxybenzoate. The extract (protein concentration, 2.0 mg/ml) was titrated with 4-methoxybenzoate (●) or 4-ethoxybenzoate (○).

cytochrome solution with the substrate provided a measure of substrate binding. Double-reciprocal plots of changes in absorption versus substrate concentration determined by using extracts obtained from cells grown on 2-ethoxyphenol and 4-ethoxybenzoate are presented in Fig. 3. The binding constants calculated from these data (Table 3) clearly show that the P-450 induced by 2-ethoxyphenol binds 2-methoxyphenol more strongly than it does 2-ethoxyphenol. Binding of 4-ethoxybenzoate and 4-methoxybenzoate to the cytochrome was not detected in this extract. Conversely, the P-450 induced by 4-ethoxybenzoate binds 4-ethoxybenzoate slightly more strongly than 4-methoxybenzoate, while no binding of 2-ethoxyphenol and 2-methoxyphenol was found.

When a preparation of purified anti-P-450$_{RR1}$ polyclonal antibodies was used to stain a Western blot from a PAGE separation of a homogenate of cells grown on 2-ethoxyphenol, a strong band was visible in the vicinity of 44.5 kDa (Fig. 4), which corresponded to the band in a parallel lane of the blot loaded with purified P-450$_{RR1}$. As the purified cytochrome runs as a single band on a silver-stained polyacrylamide gel (10), the additional bands seen in the lane loaded with an extract of cells grown on 2-ethoxyphenol probably arise from insufficient purification of the polyclonal antiserum and/or degradation of P-450$_{RR1}$ in the cellular extract. No band was visible in parallel lanes that contained homogenates of cells grown on either 4-ethoxybenzoate or Luria broth medium. The presence of P-450 in extracts of cells grown on 4-ethoxybenzoate was confirmed by CO difference spectra. Coomassie blue staining of the lanes of the same PAGE separation indicated the presence of similar quantities of total cellular protein in each of the three cell extracts.

In experiments performed with the purified cytochrome, it has been shown that P-450$_{RR1}$ catalyzes the O-dealkylation of 2-ethoxyphenol and 2-methoxyphenol to form catechol (10). It was conjectured that P-450$_{RR2}$ catalyzes the O-dealkylation of 4-ethoxybenzoate and 4-methoxybenzoate, producing *p*-hydroxybenzoate. This could be hydroxylated to form protocatechuate. The metabolic fates of catechol and protocatechuate were investigated in extracts of cells grown on 2-ethoxyphenol and 4-ethoxybenzoate. Catechol-1,2-dioxygenase activity was detected in both 2-ethoxyphenol- and 4-ethoxybenzoate-grown cells, but was approximately twice as high in the former (Table 4). Protocatechuate-3,4-dioxygenase activity was also found in extracts of cells grown on 2-ethoxyphenol or 4-ethoxybenzoate but, in this case, was significantly higher in cells grown on 4-ethoxybenzoate. In both cases, catechol-2,3-dioxygenase and protocatechuate-4,5-dioxygenase activities were either negligible or below the detection limit.

TABLE 3. Substrate binding constants of P-450$_{RR1}$ and P-450$_{RR2}$ as determined by difference spectroscopy at 25°C[a]

Cells and substrate	K_s (μM)[b]
Cells grown on 2-ethoxyphenol	
2-Ethoxyphenol	4.2 (0.3)
2-Methoxyphenol	2.0 (0.1)
4-Ethoxybenzoate	ND
4-Methoxybenzoate	ND
Cells grown on 4-ethoxybenzoate	
2-Ethoxyphenol	ND
2-Methoxyphenol	ND
4-Ethoxybenzoate	1.6 (0.1)
4-Methoxybenzoate	2.1 (0.1)

[a] The cell extracts were diluted with 20 mM Tris-HCl–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride (pH 7.4).

[b] Standard errors are indicated in parentheses. ND, no change in absorption was detected in the extract after addition of the substrate.

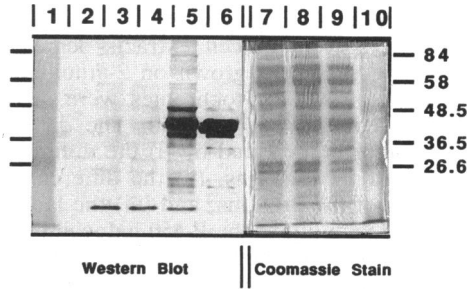

FIG. 4. SDS-PAGE of total cellular protein of *R. rhodochrous* 116 grown on Luria broth medium (lanes 3 and 7), M9g plus 5 mM 4-ethoxybenzoate (lanes 4 and 8), and M9g plus 5 mM 2-ethoxyphenol (lanes 5 and 9). The quantity of P-450, as determined by CO-difference spectra of these preparations, was 0 μg in lanes 3 and 7, 0.25 μg in lanes 4 and 8, and 0.04 μg in lanes 5 and 9. Lanes 1 and 10 contained prestained marker proteins purchased from Sigma (6 × 0.4 μg) (sizes in kilodaltons). Lane 2 contained 250 μg of total cellular protein from *E. coli*. Lane 6 contained 0.2 μg of purified P-450$_{RR1}$. Lanes 7 through 10 were stained with Coomassie blue. Lanes 1 through 6 were electroblotted onto a nitrocellulose filter and stained with goat anti-rabbit immunoglobulin G diluted 1:1,000 after reaction with a 1:1,000 dilution of purified rabbit anti-P-450$_{RR1}$ polyclonal antiserum.

TABLE 4. Ring cleavage activities measured in cell extracts of *R. rhodochrous* 116

Carbon source	Protein (μg/ml)	Enzyme assayed[a]	Initial substrate concn (μM)	Turnover (μM/min)
2-Ethoxyphenol	3,500	C23D	1,000	0.3
	700	C12D	500	13.9
	3,500	P45D	200	0.0
	3,500	P34D	400	2.2
4-Ethoxybenzoate	800	C23D	1,500	0.0
	800	C12D	500	7.1
	1,600	P45D	800	0.0
	1,600	P34D	400	26.1

[a] C23D, catechol-2,3-dioxygenase; C12D, catechol-1,2-dioxygenase; P45D, protocatechuate-4,5-dioxygenase; P34D, protocatechuate-3,4-dioxygenase. Enzymatic activities were determined as described in the text.

DISCUSSION

An actinomycete identified as *R. rhodochrous* 116 that grew on both 2-ethoxyphenol and 4-ethoxybenzoate as a sole source of carbon and energy was isolated. *Rhodococcus* is a poorly genetically characterized genus of increasing importance to the processes of biodegradation and biotransformation (11). Our partial 16S rDNA sequence characterization of *R. rhodochrous* is the first such analysis of this species and as such precludes confirmation of the identity of the organism based on the 16S rDNA sequence. However, the sequence similarities between *R. rhodochrous* 116 and the other *Rhodococcus* species that have been analyzed to date (*R. equi*, *R. erythropolis*, and *R. faciens*) demonstrate that *R. rhodochrous* 116 is more closely related to other rhodococci than to any other members of the gram-positive, high-G+C group (Fig. 1). The sequence similarity values observed for 16S rDNAs of *R. rhodochrous* 116 and the other *Rhodococcus* species (approximately 95%) is within the range of sequence similarity values observed between the 16S rDNAs of species of the same genus. Despite the small number of *Rhodococcus* species that have been analyzed by 16S rDNA sequences, it appears that this genus comprises closely related species.

CO-difference spectra of cell extracts demonstrate the presence of P-450s in cells grown on 2-ethoxyphenol and 4-ethoxybenzoate. These cytochromes were shown to be distinct and independently regulated. The distinctness of these cytochromes is indicated by (i) the different spectral characteristics of the proteins, (ii) the different substrate-binding abilities of the proteins, and (iii) the lack of immunological cross-reactivity of the P-450 induced by 4-ethoxybenzoate with polyclonal antibodies prepared against the P-450 induced by 2-ethoxyphenol. The cytochromes are induced in a growth substrate-dependent manner, and the compounds that induce the expression of each P-450 also bind specifically to the cytochromes. The following nomenclature is proposed for the enzymes: P-450$_{RR1}$ is induced by and binds 2-ethoxyphenol, and P-450$_{RR2}$ is induced by and binds 4-ethoxybenzoate.

While multiple, independently regulated P-450s are ubiquitous in eukaryotic organisms, there are but two reported examples of this in prokaryotes, in *Streptomyces griseolus* and *Bacillus megaterium*. In the former organism, a constitutive P-450 exists in addition to two cytochromes, P-450$_{SU1}$ and P-450$_{SU2}$, that are induced by and catalyze various oxidations of sulfonylurea herbicides (27, 33). In *B. megaterium*, at least three P-450s are induced by phenobarbital. P-450$_{BM-1}$ has been cloned (18) but as yet has no ascribed catalytic function. P-450$_{meg}$ has been demonstrated to catalyze the 15β-hydroxylation of 3-oxo-steroids (2). P-450$_{BM-3}$ is a unique multidomain cytochrome that catalyzes the hydroxylation of fatty acids (26).

Through high-pressure liquid chromatography measurements of appropriately induced whole cells, we were able to demonstrate the consumption of substrate but not the appearance of the expected intermediates, catechol and 4-hydroxybenzoate (results not shown). This is probably due to the rapid catabolism of these intermediates following their generation by the respective O-dealkylation reactions. P-450-catalyzed O-dealkylation reactions have been reported for 2-ethoxyphenol (5) and 4-methoxybenzoate (3) as well as for veratrole (41). Although we were not able to demonstrate directly that P-450$_{RR1}$ and P-450$_{RR2}$ catalyze the O-dealkylation of 2-ethoxyphenol and 4-ethoxybenzoate, respectively, several lines of evidence point to this conclusion. First, the cytochromes are specifically induced in a growth substrate-dependent manner. Second, these compounds induce a type I change in the electronic absorption difference spectrum of the cytochromes. This change is characteristic of substrate binding and reflects a transition in the spin state of the heme iron. In P-450$_{LM2}$, the degree of spin state transition induced by a compound has been correlated with the intrinsic ability of the substrate to convert the cytochrome to its biologically active conformation (32). The observations that 2-ethoxyphenol and 4-ethoxybenzoate bind strongly to P-450$_{RR1}$ and P-450$_{RR2}$, respectively, and that these compounds induce substantial transitions in the heme iron spin state of the respective cytochromes imply that 2-ethoxyphenol and 4-ethoxybenzoate are excellent substrates for P-450$_{RR1}$ and P-450$_{RR2}$, respectively. Third, we have identified enzymatic activities in the cell extracts that catalyze the ring cleavage of the expected products of the postulated O-dealkylation reactions. Finally, in an in vitro system consisting of purified P-450$_{RR1}$ and chemical reductants, we have demonstrated that P-450$_{RR1}$ catalyzes the O-dealkylation of 2-ethoxyphenol to produce catechol (10).

The aromatic ring cleavage activities present in the cell extracts indicate that the aromatic ring of the growth substrate is further catabolized via *ortho*-cleavage mechanisms. Furthermore, the levels of expression of catechol-1,2-dioxygenase and protocatechuate-3,4-dioxygenase activities in cells grown on 2-ethoxyphenol and 4-ethoxybenzoate (Table 4) suggest that in *R. rhodochrous* 116, these compounds not only specifically induce the expression of the respective P-450s but also induce the expression of appropriate intradiol dioxygenases. Catechol-1,2-dioxygenases show almost no protocatechuate cleavage activity (25), and protocatechuate-3,4-dioxygenases demonstrate very little ability to cleave catechol (40). Thus, it is probable that irrespective of whether cells are grown on 2-ethoxyphenol or 4-ethoxybenzoate, they contain both a catechol-1,2-dioxygenase and a protocatechuate-3,4-dioxygenase. As succinate is a catabolite expected to occur at a stage subsequent to *ortho*-ring cleavage, it is perhaps surprising that this compound is not metabolized by cells grown on 2-ethoxyphenol or 4-ethoxybenzoate (Table 2). This may be due to the fact that this gram-positive organism requires a transport system for succinate uptake, and without succinate in the growth medium, no such transport system is induced.

We propose that 2-ethoxyphenol and 2-methoxyphenol are catabolized via P-450$_{RR1}$-catalyzed O-dealkylation and

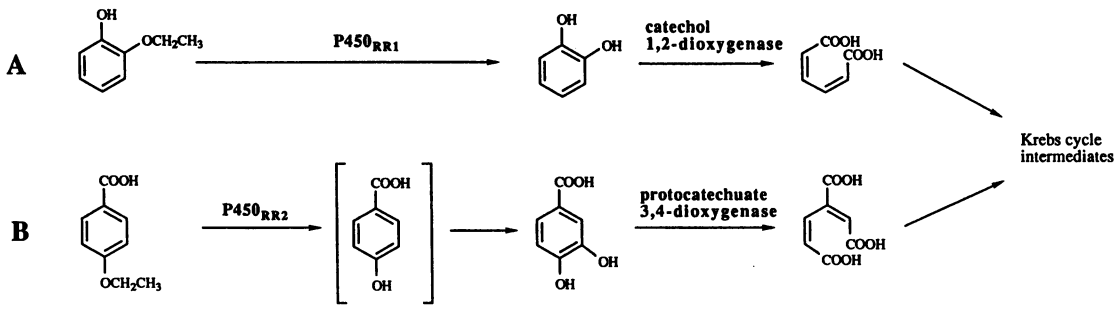

FIG. 5. Proposed metabolic pathways induced by (A) 2-alkoxyphenols and (B) 4-alkoxybenzoates in *R. rhodochrous* 116.

that the resulting catechol is further catabolized via *ortho*-cleavage of the aromatic ring. 4-Methoxybenzoate and 4-ethoxybenzoate are catabolized via P-450_{RR2}-catalyzed O-dealkylation, followed by hydroxylation to protocatechuate and *ortho*-cleavage of this intermediate by protocatechuate-3,4-dioxygenase (Fig. 5). While each of the P-450s is specifically induced in a growth substrate-dependent manner, the regulation of the intradiol dioxygenases appears to be more complex.

As part of a study to characterize and modify the enzymatic activities of these ether bond-cleaving cytochromes, we have purified both proteins to apparent homogeneity (10, 12). The polyclonal antibodies described in this study are being used to screen an expression library in order to clone the structural gene of P-450_{RR1}. Further characterization of the enzymes, together with cloning of the genes, should facilitate the manipulation of the substrate specificities of these cytochromes, as is being done for P-450_{cam} (29).

ACKNOWLEDGMENTS

Moraxella sp. strain GU2 was a generous gift from Jean Pelmont. The assistance of Jürgen Wehland and Silke Fischer in preparing the antibodies and performing the Western blots is gratefully acknowledged. We thank Joan Timmis for skilled technical assistance.

This work was supported in part by the German Ministry for Research and Technology (BMFT grant 0319378A3) and the Fonds der Chemischen Industrie.

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