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Selection of *Pseudomonas* sp. Strain HBP1 Prp for Metabolism of 2-Propylphenol and Elucidation of the Degradative Pathway

HANS-PETER E. KOHLER,[1]* MARC J. E. C. van der MAAREL,[1]† and DORIS KOHLER-STAUB[2]

Swiss Federal Institutes of Technology Zürich and Swiss Federal Institute for Water Resources and Water Pollution Control, CH-8600 Dübendorf,[1] and Department for Microbiology, Swiss Federal Institutes of Technology, ETH-Zentrum, CH-8092 Zürich,[2] Switzerland

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A mutant of *Pseudomonas* sp. strain HBP1, originally isolated on 2-hydroxybiphenyl, was selected for the ability to grow on 2-propylphenol as the sole carbon and energy source. In the mutant strain, which was designated as *Pseudomonas* sp. strain HBP1 Prp, the cellular induction mechanism involved in the synthesis of the NADH-dependent monooxygenase is changed. 2-Propylphenol, which is known to be a substrate of the monooxygenase, does not induce formation of the monooxygenase in the wild type but does have an induction effect in the mutant strain. Furthermore, in contrast to the wild type, mutant strain HBP1 Prp constitutively produces a small amount of monooxygenase and metapyrocatechase. The enzymes from strain HBP1 Prp catalyzing the first three steps in the degradation of 2-propylphenol—the NADH-dependent monooxygenase, the metapyrocatechase, and the *meta* fission product hydrolase—were partially purified, and their activities were measured. The product of the monooxygenase activity was identified by mass spectrometry as 3-propylcatechol. The metapyrocatechase used this compound as a substrate and produced a yellow *meta* fission product that was identified by mass spectrometry as 2-hydroxy-6-oxo-nona-2,4-dienoate. Butyrate could be detected as a product of the *meta* fission product hydrolase in crude cell extract of 2-propylphenol-grown cells, as well as an intermediate in culture broths during growth on 2-propylphenol. All three enzymes expressed highest activities for the metabolites of the degradation of 2-hydroxybiphenyl.

Methyl-substituted phenols are known to be metabolized in bacterial cells either by an initial attack on the side chain or by an initial hydroxylation of the aromatic structure, whereby a catechol derivative is produced (1, 5). 4-Ethylphenol is catabolized by bacteria via both routes (3). *ortho*- and *meta*-substituted methylphenols seem to be preferentially metabolized by a *meta* cleavage pathway starting with hydroxylation of methylphenol to form 3-methylcatechol. Subsequent cleavage of the carbon bond between the hydroxyl-substituted C-2 and the methyl-substituted C-3 carbon of 3-methylcatechol yields 2-oxopenta-4-enoate and acetate (1, 13). The same general scheme is observed for the metabolism of 2- and 3-hydroxybiphenyl (2- and 3-phenylphenol) by *Pseudomonas* sp. strain HBP1 and *Pseudomonas* sp. strain FH12 (7, 9). The hydroxylating monooxygenases exhibited strict regioselectivity for the position of the hydroxyl group in both cases, such that the strain isolated on 2-hydroxybiphenyl was not able to metabolize 3-hydroxybiphenyl and vice versa. On the other hand, the 2-hydroxybiphenyl monooxygenase was quite relaxed about the structure of the hydrophobic side chain and was able to turn over various 2-alkylphenols. It is known that dioxygenases needed for the cleavage of 3-alkylcatechol intermediates generally exhibit a broad substrate spectrum (11, 14). Although inducible monooxygenase was formed only in cells growing with 2-hydroxy- and 2,2′-dihydroxybiphenyl (9), strain HBP1 was assumed to have the potential to grow on 2-alkylphenols, and it was of interest to confirm the metabolism of such compounds and to elucidate the pathway involved in their degradation. The present article reports the selection of *Pseudomonas* sp. strain HBP1 Prp, which has the ability to grow on 2-propylphenol as the sole carbon and energy source, and the metabolic route employed in its utilization.

MATERIALS AND METHODS

Media and growth conditions. *Pseudomonas* sp. strain HBP1 has been described in a previous article (9). The mineral salts medium used for growth of strain HBP1 and HBP1 Prp consisted of 20 mM phosphate buffer (KH_2PO_4-$Na_2HPO_4 \cdot 2H_2O$, pH 7.2), 0.125 g of $(NH_4)_2SO_4$, 0.025 g of $MgSO_4 \cdot 7H_2O$, and 0.03125 g of $Ca(NO_3)_2 \cdot 4H_2O$ per liter of deionized water, supplemented with 1 ml of a trace element stock solution containing the following (in grams per liter): $FeSO_4 \cdot 7H_2O$, 1.00; $MnSO_4 \cdot H_2O$, 1.00; $Na_2MO_4 \cdot 2H_2O$, 0.25; H_3BO_3, 0.10; $CuSO_4 \cdot 5H_2O$, 0.25; $ZnSO_4 \cdot 7H_2)$, 0.25; NH_4VO_3, 0.10; $Co(NO_3)_2 \cdot 6H_2O$, 0.5; $NiSO_4 \cdot 6H_2O$, 0.010. The stock solution also contained 5.00 ml of H_2SO_4.

2-Hydroxybiphenyl and 2-propylphenol were added directly from the reagent bottles (500 mg liter^{-1}, if not mentioned otherwise) after sterilization to minimize volatile losses. This method was found to be satisfactory since no growth in uninoculated flasks could be observed.

Agar plates were prepared by adding 2-hydroxybiphenyl and 2-propylphenol directly to the hot minimal salts medium containing 15 g of agar liter^{-1} just after sterilization. In order to obtain a homogeneous substrate distribution in the case of 2-hydroxybiphenyl plates, the compound was added as a concentrated methanol solution and the fine particles that precipitated were mixed well before the plates were poured.

* Corresponding author.
† Present address: Department of Microbiology, University of Groningen, Kerklaan 30, 9750 AA Haren, The Netherlands.

2-Propylphenol plates were incubated in large closed glass cylinders.

Chemicals. 2-Propylphenol, 3-methylcatechol, and 4-methylcatechol were obtained from Aldrich-Chemie, Steinheim/Albuch, Germany. 2,3-Dihydroxybiphenyl was obtained from Wako Chemicals GmbH, Neuss, Germany. Sodium benzoate was purchased from Merck, Darmstadt, Germany. All other chemicals used were purchased from Fluka Chemie AG, Buchs, Switzerland.

Preparation of washed cell suspensions and cell extract. Cells were grown with the mineral salts medium supplemented with the appropriate substrate in indented Erlenmeyer flasks (500- or 1,000-ml capacity) on a rotary shaker (140 to 150 rpm) at 30°C. Alternatively, cells were grown in 20-liter carboys equipped with a magnetic stirring bar, with forced aeration. In this case, 1 drop of sterile polypropylene glycol was added after sterilization to prevent foaming. Cells were harvested in the late exponential growth phase by centrifugation (15 min at 6,000 × *g*) at 4°C, washed with an excess amount of phosphate buffer (20 mM, pH 7.2), and resuspended in the same buffer (0.2 g [wet weight] ml^{-1}). For preparation of crude cell extract, washed cells were broken by means of a French press (two passages, 20,000 lb in^{-2}) and centrifugation (40 min at 40,000 × *g*) at 4°C.

Oxygen uptake with washed cell suspension. Oxygen uptake was measured polarographically with an oxygen electrode (Rank Brothers, Cambridge, England). This electrode was mounted to a reaction vessel (2 ml) and held at a constant temperature (30°C). The assay mixture contained 1.79 ml of phosphate buffer (20 mM, pH 7.2) and 0.2 ml of washed cell suspension (optical density at 546 nm in the reaction vessel, approximately 1.9). Reactions were started by adding 10 μl of a substrate as a concentrated methanol or buffer solution (0.2 mM in the assay). Methanol in the concentration range used did not have an effect on the assay. Specific oxygen uptake rates were corrected for endogenous rates. Absolute rates are expressed as nanomoles of O_2 per milligram of protein per minute (an optical density of 1 absorbance unit corresponds to 174 mg of protein $liter^{-1}$).

Separation of enzyme activities. The clarified crude extract of strain HBP1 Prp contained all the enzymes for metabolizing 2-hydroxybiphenyl to benzoate and 2-propylphenol to butyrate. In order to separate the different activities, partial purification by protamine sulfate precipitation and ion-exchange chromatography was necessary. First, 0.33 ml of a protamine sulfate solution (12 mg ml^{-1} in 20 mM phosphate buffer, pH 7.2) per ml was added to crude cell extract and stirred for 30 min at 4°C. The precipitated biopolymers were removed by centrifugation (30 min at 40,000 × *g*) at 4°C. After the protamine sulfate-treated extract was desalted on a Sephadex G-25M column (Pharmacia, Uppsala, Sweden), it was fractioned by anion-exchange chromatography with a fast protein liquid chromatography system consisting of a 2152 LC controller, two 2150 high-performance liquid chromatography (HPLC) pumps, and a 2212 Helirac sample collector (all from LKB-Pharmacia). The sample was applied on a Mono-Q anion-exchange column (HR5/5; LKB-Pharmacia) and eluted with a linear gradient of NaCl, 0 to 1 M in 40 ml of triethanolamine buffer (10 mM, pH 7.5; 1 ml min^{-1}). The protein profile was measured at 280 nm with a 655A variable-wavelength UV monitor (Hitachi). After fractions were collected, enzyme activities were determined as described below.

Enzyme assays. One unit of enzyme activity was defined as the amount of enzyme converting 1 μmol of substrate per min.

NADH-dependent monooxygenase activity was measured spectrophotometrically by monitoring NADH disappearance at 340 nm. The reaction mixture contained 20 mM phosphate buffer (pH 7.2), 0.2 mM NADH, 50 μl of the appropriate protein fraction, and 0.1 mM a substrate. The reaction was usually started by the addition of a substrate (5 μl of a methanol solution).

Metapyrocatechase activity in phosphate buffer (20 mM, pH 7.5) was measured spectrophotometrically by monitoring the increase of absorption corresponding to the accumulation of the ring *meta* cleavage products. The following extinction coefficients for various *meta* cleavage products were used: 2-hydroxymuconic semialdehyde (*meta* cleavage product of catechol) (λ_{max}, 375 nm), 36,000 M^{-1} cm^{-1} (15); 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta* cleavage product of 2,3-dihydroxybiphenyl) (λ_{max}, 434 nm), 22,000 M^{-1} cm^{-1} (15); 2-hydroxy-6-oxo-nona-2,4-dienoic acid (*meta* cleavage product of 3-propylcatechol) (λ_{max}, 383 nm), 20,000 M^{-1} cm^{-1} (4). Metapyrocatechase activity was also measured polarographically with the same device as described above for O_2 uptake measurements with washed cell suspensions.

meta cleavage product hydrolase was assayed spectrophotometrically by monitoring the decrease in the absorption of the different *meta* cleavage products.

Analytical procedures. (i) HPLC. The disappearance of 2-propylphenol (ε_{270}, 1,593 M^{-1} cm^{-1}) and formation of metabolites (catechols) were monitored by HPLC. Samples containing cells and protein were pretreated either by centrifugation (15 min at 20,000 × *g*) or by the addition of trichloroacetic acid (0.2 ml of a 3 M solution per ml of sample) and subsequent centrifugation. The samples were analyzed by injecting 50 μl of the supernatant onto a Waters-Millipore (Milford, Mass.) 625LC HPLC consisting of a Rheodyne 9125 injector, a gradient controller, a low-pressure mixing fluid handling unit, a computer-controlled Waters 991 photodiode array detector, and an LKB-Pharmacia Redifrac fraction collector. Reverse-phase separation was achieved on a Spherisorb OD SII column (25 cm by 4.6 mm) of 5-μm particle size from Bischoff GmbH (Leonberg, Germany) by applying a linear gradient of 60% 10 mM H_3PO_4 to 70% 90% methanol–10% 10 mM H_3PO_4 with a flow rate of 1 ml min^{-1}. The column was equilibrated at initial conditions before each injection.

(ii) GC analysis. Butyric acid was analyzed on an HP 5890 series II (Hewlett-Packard Co., Palo Alto, Calif.) gas chromatograph (GC) equipped with a flame ionization detector and an OV-351 capillary column (length, 30 m; inner diameter, 0.32 mm; film thickness, 0.25 μm; J&W, Carlo Erba Instruments). The temperature program was run from 80 to 140°C with initial and final holding times of 2.0 and 4.74 min, respectively, and a rate of 15°C min^{-1}. The injector and detector temperatures were 200 and 250°C, respectively. At the end of each run, the oven was heated to 195°C and the temperature was held for 2 min. Helium was the carrier gas, with a linear flow velocity of 50 cm s^{-1} at 100°C. Splitless injection (1 μl) was done with an automatic sampler (HP 7673A; Hewlett-Packard). The aqueous samples were pretreated by centrifugation (15 min at 20,000 × *g*) and were acidified (5% [vol/vol] formic acid [final concentration]) just prior to analysis.

(iii) GC-MS. Mass spectra of the catechols were obtained on a Carlo Erba QMD 1000 GC-mass spectrometer (MS) (Carlo Erba Instruments) equipped with an SE-54 glass capillary column. Electron ionization was used. The injection was administered on column at 58°C. The temperature

TABLE 1. Induction of monooxygenase and metapyrocatechase activities with 2-hydroxybiphenyl and 2-propylphenol in *Pseudomonas* sp. strain HBP1 and *Pseudomonas* sp. strain HBP1 Prp

Strain	Growth substrate[a]	Inducing compound[b]	Monooxygenase activity (U/mg) with:		Metapyrocatechase activity (U/mg) with 2,3-dihydroxybiphenyl
			2-Hydroxybiphenyl	2-Propylphenol	
HBP1	2-Hydroxybiphenyl		0.166	0.075	6.26
	Succinate		0	0	0
	Succinate	2-Hydroxybiphenyl	0.043	0.016	1.64
	Succinate	2-Propylphenol	0	0	>0.01
HBP1 Prp	2-Hydroxybiphenyl		0.207	0.132	12.09
	2-Propylphenol		0.427	0.195	5.91
	Succinate		0.015	0.007	0.20

[a] Starting substrate concentrations were 10 mM for succinate and 500 ppm for 2-hydroxybiphenyl and 2-propylphenol. Growth occurred overnight at 30°C in 1-liter Erlenmeyer flasks with 200 ml of liquid medium.
[b] After 12 h of growth on succinate, 200 ppm each of 2-hydroxybiphenyl and 2-propylphenol was added to the liquid cultures. Additionally, 1.25 mM succinate was supplemented. The cultures were then incubated for 12 h.

program was run from 58 to 120°C with a rate of 21°C min^{-1} and from 120 to 200°C with a rate of 8°C min^{-1}. Mass spectra of the trimethylsilyl derivatives of the *meta* cleavage compounds were obtained on a Finnigan MAT (San Jose, Calif.) ITD 800 (ion trap detection) MS coupled to a Carlo Erba HRGC 5160 mega series GC equipped with a 10-m PS090 (80% dimethyl, 20% diphenyl) glass capillary column. Electron and chemical ionizations were used. The injection (1 μl) was administered on column at 80°C. The temperature program was run from 80 to 100°C with a rate of 20°C min^{-1} and from 100 to 220°C with a rate of 5°C min^{-1}. Trimethylsilyl derivatives were produced by silylating the hydroxy groups with *N,O-bis*(trimethylsilyl)trifluoroacetamide.

(iv) UV-VIS spectroscopy. UV-VIS spectra were taken with a Uvikon 860 spectrophotometer (Kontron AG, Zurich, Switzerland).

(v) Protein determination. Protein contents in cell extracts and whole cells were determined by the methods of Bradford (2) and Lowry et al. (12), respectively, with bovine serum albumin as a standard.

Production of 3-propylcatechol. A mixture containing 500 μl of the protein fraction containing the monooxygenase activity, 100 μl of NADH (0.2 mM), 8.75 ml of phosphate buffer (20 mM, pH 7.2), and 50 μl of 2-propylphenol (10 mM) was incubated at 30°C. After 30 min, another 200 μl of the monooxygenase activity and another 100 μl of NADH were added and the reaction mixture was reincubated for an additional 30 min. Then, the pH was adjusted to 1 with H_3PO_4 (1 M) and the reaction mixture was extracted with 20 ml of ethyl acetate. The organic fraction was evaporated to dryness and taken up in 3 ml of methanol. This methanol solution was subsequently used for GC-MS analysis.

Production of 2-hydroxy-6-oxo-nona-2,4-dienoate. One milliliter of 3-propylcatechol (0.1 mM) was incubated with 10 μl of the protein fraction containing the *meta* cleavage activity. After the intensity of the yellow color was at a maximum, the mixture was acidified (pH 1) with H_3PO_4 (1 M). The *meta* cleavage compound was extracted with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4, subsequently silylated with *N,O-bis*(trimethylsilyl)trifluoroacetamide, and used for GC-MS analysis.

RESULTS

Selection for growth on 2-propylphenol. Initial growth experiments with 500 mg of 2-propylphenol $liter^{-1}$ as the substrate failed. Only when the 2-propylphenol concentration was reduced to 100 mg $liter^{-1}$ and after a selection period of about 2 weeks could growth be noticed. After a sample (2%) from such a culture was transferred to fresh medium containing 2-propylphenol (500 mg $liter^{-1}$), growth occurred after a short lag period and an optical density at 546 nm of 0.5 was reached within 38 h. After these two transfers, a mutant strain, designated as *Pseudomonas* sp. strain HBP1 Prp, had apparently been selected. Strain HBP1 Prp was still able to grow on 2-hydroxybiphenyl, and, when a culture grown on 2-hydroxybiphenyl was brought back to 2-propylphenol, growth commenced immediately. Table 1 shows the results of an experiment in which the efficiencies of 2-hydroxybiphenyl and 2-propylphenol as inducers for monooxygenase and metapyrocatechase in strains HBP1 and HBP1 Prp were tested. In mutant strain HBP1 Prp, the cellular induction mechanism involved in the induction of the monooxygenase and metapyrocatechase appears to be changed. First, while 2-propylphenol does not induce the formation of the monooxygenase in the wild type it does have such an effect in the mutant strain, in which the highest specific monooxygenase activities were reached during growth on 2-propylphenol. Second, during growth on a carbon source such as succinate, which is not an enzyme substrate and does not share any degradation steps with 2-hydroxybiphenyl, there is no monooxygenase or metapyrocatechase activity detectable in the wild type whereas mutant strain HBP1 Prp constitutively produces a small amount of monooxygenase and metapyrocatechase (Table 1).

Detection of 2-propylphenol-degrading mutants on agar plates. In order to determine a spontaneous rate of mutation of strain HBP1 for growth on propylphenol, succinate- and 2-hydroxybiphenyl-grown liquid cultures containing 6.9×10^9 and 2.0×10^9 2-hydroxybiphenyl-utilizing cells per ml, respectively, were spread onto 2-propylphenol plates. The largest numbers of cells spread were 6.9×10^{10} and 2.0×10^{10}, respectively. No mutant colonies appeared within 3 days of incubation on any of the plates. After 4 days, colonies started to appear (Fig. 1). Interestingly, further incubation of the plates resulted in the continuing appearance of new colonies, as shown in Fig. 1. Streaking out single colonies onto fresh 2-propylphenol plates led to detectable growth within 1 day. After 2 days, the colony diameter amounted to 1 mm, and a maximum diameter of 1.5 mm was reached after 3 days. These colony growth characteristics of the mutants appearing on 2-propylphenol plates were identical to those of wild-type strain HBP1 as well as to those of strain HBP1 Prp growing on 2-hydroxybiphenyl plates.

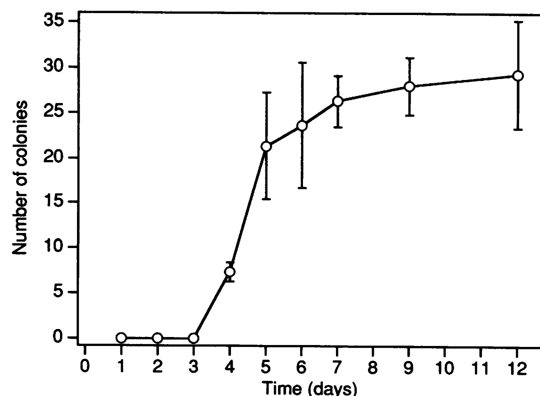

FIG. 1. Accumulation of mutant colonies (able to grow on 2-propylphenol) appearing on minimal 2-propylphenol plates incubated at 30°C as a function of time. About 2.0×10^7 cells, grown in 2-hydroxybiphenyl minimal liquid medium for 48 h, were spread onto each of three plates. Error bars represent 95% confidence intervals (*t* distribution, $n = 3$). The first three points have no error bars because the standard deviation was zero.

Growth characteristics. The growth of *Pseudomonas* sp. strain HBP1 Prp on 2-propylphenol resulted in the disappearance of the substrate, the formation of biomass, and the formation of butyric acid (Fig. 2) and a yellow compound as intermediates. The growth rate of strain HBP1 Prp growing on 2-propylphenol in batch culture was $0.17\ h^{-1}$ ($t_d = 4.1$ h), with a yield of 4.5 mg of protein per mmol of substrate C. Residual concentrations of 2-propylphenol and butyric acid amounted to 0.2 and 0.12 mM, respectively. At the beginning of the stationary growth phase, a significant increase in the concentration of butyric acid could be seen. During growth, the formation of a yellow metabolite was observed. The UV-VIS spectrum above 300 nm revealed an absorption maximum at 320 nm and a shoulder at 380 nm. The A_{320} and A_{380} reached a maximum at 26.5 h and decreased thereafter.

Oxygen uptake with washed cell suspensions. Cells of strain HBP1 Prp grown on 2-hydroxybiphenyl were induced moderately for oxygen uptake with benzoate and only slightly for oxygen uptake with butyrate, whereas cells grown on 2-propylphenol were highly induced for oxygen uptake with butyrate (Table 2). This suggests that butyrate is formed as an intermediate when cells grow on 2-propylphenol. Butyrate- and 2-propylphenol-grown cells of strain HBP1 Prp were highly induced for oxygen uptake with butyrate, but succinate-grown cells were not induced at all. This indicates that enzymes associated with the metabolism of butyrate are inducible. In contrast to the wild type (9), succinate- and benzoate-grown cells of strain HBP1 Prp were induced for oxygen uptake with 2-hydroxybiphenyl and 2-propylphenol.

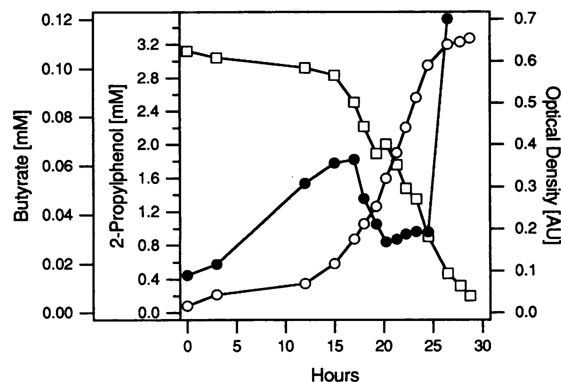

FIG. 2. Growth of *Pseudomonas* sp. strain HBP1 Prp on 2-propylphenol. Symbols: ○, optical density at 546 nm; □, 2-propylphenol; ●, butyrate. AU, absorbance units.

TABLE 2. Oxygen uptake rates with various compounds by resting cell suspensions of *Pseudomonas* sp. strain HBP1 Prp grown on different substrates

Substrate	% Oxygen uptake[a] of cells grown on:				
	2-Hydroxybiphenyl	2-Propylphenol	Butyrate	Benzoate	Succinate
2-Propylphenol	100 (123)	100 (25)	100 (229)	100 (140)	100 (8)
2-Hydroxybiphenyl	147	207	108	198	120
Butyrate	11	136	134	8	0
Benzoate	21	34	4	66	0

[a] Numbers in parentheses are absolute rates in nanomoles of O_2 per minute per milligram of protein.

Conversion of 2-propylphenol to 3-propylcatechol. NADH-dependent monooxygenase activity toward 2-propylphenol was exhibited by crude extracts. The product of the activity was immediately consumed in crude cell extracts and converted to a yellow *meta* fission compound, which also could be observed only as an intermediate. In order to characterize the product of the monooxygenase activity, it was necessary to partially purify the monooxygenase by protamine sulfate precipitation and anion-exchange chromatography. The activity could be well separated from the metapyrocatechase activity, but the hydrolase and the monooxygenase activities overlapped somewhat (Fig. 3). The fractions containing monooxygenase were pooled and used in the following experiments. First, assays were performed by monitoring NADH consumption spectrophotometrically and by monitoring 2-propylphenol conversion by HPLC. The concentration of 2-propylphenol (retention time, 12.7 min) decreased, and the increase of a more hydrophilic metabolite (retention time, 8.6 min) was observed. The metabolite was enriched as described in Materials and Methods and subsequently characterized by GC-MS. The MS spectrum of the metabolite (Fig. 4) agrees well with the spectrum of 3-propylcatechol (8), with ion peaks at *m/e* (major fragment ions) as follows: 153, $(M + 1)^+$; 152, $(M)^+$; 124, loss of CO; 123 (base peak), loss of CHO; 110, loss of C_3H_6; 105, loss of CHO and H_2O; 95, loss of CHO and CO; 94, loss of CHO and CHO; 89, $C_5H_9O_2^+$; 81, loss of CHO and C_3H_6; 79, $C_6H_7^+$; 78, $C_6H_6^+$; 77, $C_6H_5^+$. The UV-VIS spectrum (solvent, 50% methanol, 50% 0.01 M H_3PO_4) shows a maximum at 273 nm and a minimum at 243 nm, with a A_{273}/A_{243} ratio of 4.3. This is in accordance with a catechol structure.

The specific activity of the partially purified monooxygenase was 1.11 μmol of NADH min^{-1} mg of $protein^{-1}$ when measured spectrophotometrically and 0.92 μmol of O_2 min^{-1} mg of $protein^{-1}$ when measured polarographically. This corresponds to a 1:1 stoichiometry of NADH to O_2. The addition of catalase (6,500 U) at the end of a polarographical assay did not give an increase in O_2, indicating that no H_2O_2 was formed during the reaction.

Metapyrocatechase activity. When cell extracts from 2-propylphenol-grown cells were tested for metapyrocatechase activity with 2,3-dihydroxybiphenyl, rapid formation of a yellow intermediate with an absorption maximum at 430 nm

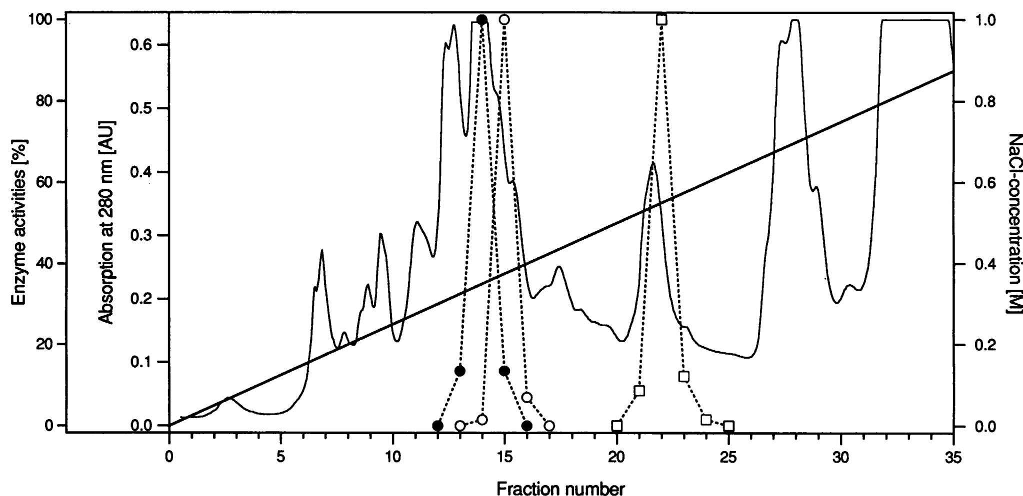

FIG. 3. Mono-Q ion-exchange chromatography of a protamine sulfate-precipitated and desalted cell extract of *Pseudomonas* sp. strain HBP1 Prp grown on 2-propylphenol. The protein content of the eluent was detected photometrically at 280 nm. The rates of enzyme activities in the eluted fractions (1 ml) were measured and are depicted as follows: ●, rate of monooxygenase (100% = 0.75 U ml^{-1}); ○, rate of *meta* fission product hydrolase (100% = 4.7 U ml^{-1}); □, rate of metapyrocatechase (100% = 111.9 U ml^{-1}).

could be observed. The yellow compound was identified as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid on the grounds of the mass spectrum of its trimethylsilyl derivative; *m/e* (major fragment ions) were as follows: 347, loss of CH_3; 319, loss of CH_3 and CO; 273, loss of C_3H_9OSi; 257, loss of C_7H_5O; 245 (base peak), loss of $C_4H_9O_2Si$; 147, $C_5H_{15}OSi_2^+$; 105, $C_7H_5O^+$; 73, $C_3H_9Si^+$.

Partial purification of the enzymes (Fig. 3) revealed the presence of a metapyrocatechase eluting from the Mono-Q column as a single peak at 0.5 M NaCl. Table 3 shows activities of the metapyrocatechase for a series of catechol derivatives. The activity for 3-propylcatechol amounts to about 12% of the activity for 2,3-dihydroxybiphenyl. When partially purified metapyrocatechase was incubated with 3-propylcatechol, a yellow *meta* fission product was formed. The electron impact mass spectrum of its trimethylsilyl derivative (Fig. 5) can be interpreted as follows: *m/e* (major fragment ion) 328, M^+ (0.3%); 313, loss of CH_3; 285, loss of C_3H_7 or loss of CH_3 and CO; 257, loss of C_3H_7 and CO; 239, loss of C_3H_9OSi; 211 (base peak), loss of $C_4H_9O_2Si$; 147, $C_5H_{15}OSi_2^+$; 95, $C_5H_3O_2^+$; 73, $C_3H_9Si^+$. The compound is suggested to be 2-hydroxy-6-oxo-nona-2,4-dienoic acid, as the MS spectrum showed the expected pattern and all the major fragment ions could be rationalized. The relative abundance (0.3%) of the molecular ion $(M)^+$ in the electron impact mass spectrum was rather low. That is why chemical ionization was employed to reconfirm the molecular weight of the trimethylsilyl derivative of the *meta* cleavage compound. The spectrum showed the characteristic peak of $(M + H)^+$ at *m/e* 329, confirming the molecular weight to be 328.

The enzyme could not be enhanced by the addition of Fe^{2+} ($FeSO_4$, 0.04 mM). The specific activity of the partially purified metapyrocatechase was 61.0 μmol of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid min^{-1} mg of $protein^{-1}$ when measured spectrophotometrically and 65.7 μmol of O_2 min^{-1} when measured polarographically. This corresponds to a 1:1 stoichiometry of formation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid to disappearance of O_2.

During enzyme assays, a rapid irreversible inactivation of the metapyrocatechase could be observed. Within about 30 s activity ceased completely, although there was no limitation of oxygen or 2,3-dihydroxybiphenyl. This inactivation could

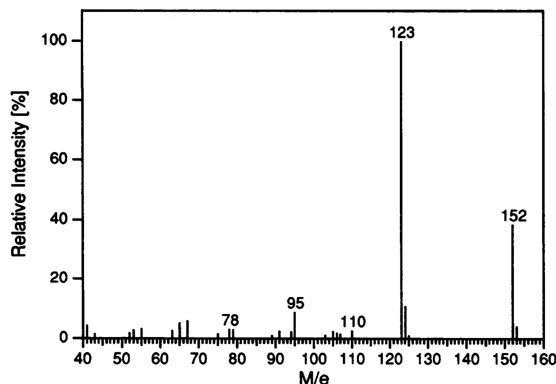

FIG. 4. Electron ionization mass spectrum of 3-propylcatechol, the product of the NADH-dependent monooxygenase activity, with 2-propylphenol as the substrate. See the text for a discussion of the fragmentation pattern.

TABLE 3. Enzyme activities of partially purified metapyrocatechase and partially purified *meta* cleavage product hydrolase in 2-propylphenol-grown *Pseudomonas* sp. strain HBP1 Prp

Substrate[a]	Activity of metapyrocatechase		Activity of hydrolase	
	U/mg of protein	Relative (%)	U/mg of protein	Relative (%)
2,3-Dihydroxybiphenyl	66.7	100.0	4.9	100.0
3-Propylcatechol	8.0	12.0	0.06	1.2
Catechol	2.5	3.7	0	0
3-Methylcatechol	3.9	5.9	ND[b]	ND
4-Methylcatechol	0.2	0.3	0	0

[a] For the hydrolase, the substrates are the *meta* cleavage products of the compounds.
[b] ND, not determined.

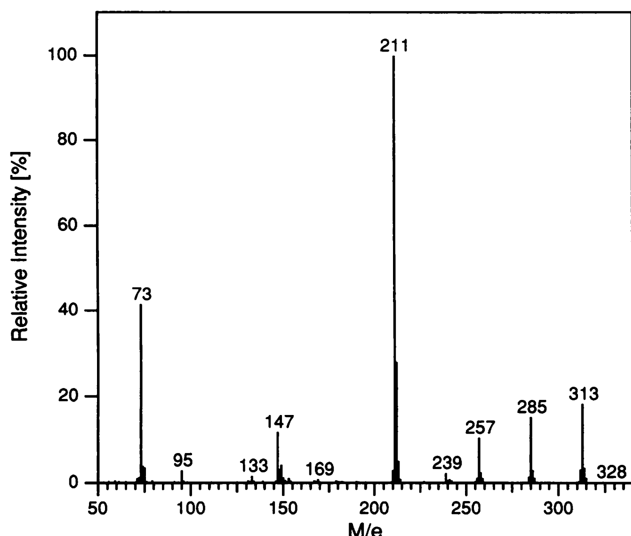

FIG. 5. Electron ionization mass spectrum of the trimethylsilyl derivative of 2-hydroxy-6-oxo-nona-2,4-dienoic acid, the *meta* cleavage product of 3-propylcatechol. See the text for a discussion of the fragmentation pattern.

partially be overcome by the addition of *meta* cleavage product hydrolase, indicating that irreversible product inhibition occurred. Even so, in such assays time-dependent inactivation was evident, in spite of the fact that the *meta* fission product did not accumulate.

***meta* fission product hydrolase activity.** Incubation of 2-propylphenol with cell extract led to the formation of butyrate (yield of about 8%). The enzyme responsible for the last reaction in this sequence could be located in the ion-exchange chromatography run (Fig. 3) as a single peak that was eluted just after the monooxygenase. Table 3 shows activities of the partially purified, cofactor-independent *meta* fission product hydrolase toward a series of *meta* fission compounds that were prepared from the corresponding catechols by the action of the partially purified metapyrocatechase. The highest activity could be observed for the *meta* cleavage product of 2,3-dihydroxybiphenyl. The *meta* cleavage products of catechol and 4-methylcatechol did not act as substrates for the hydrolase at all.

DISCUSSION

Pseudomonas sp. strain HBP1 Prp appears to be a regulatory mutant of strain HBP1. Wild-type strain HBP1 was not able to grow on 2-propylphenol because this compound did not induce monooxygenase and metapyrocatechase, the first two enzymes in the catabolic pathway. In mutant strain HBP1 Prp, the cellular induction mechanism involved in the synthesis of monooxygenase and metapyrocatechase has changed (Table 1) in such a way that growth on 2-propylphenol yields the highest specific monooxygenase activities and that succinate-grown cells constitutively produce a small amount of monooxygenase and metapyrocatechase. The enzyme synthesis in the mutant is still regulated, as the specific activities in extracts of succinate-grown cells are only 5 to 10% of the activities in extracts of cells grown on the enzyme substrates 2-hydroxybiphenyl and 2-propylphenol, respectively. This observation suggests that the mutation did not simply affect a repressor in a negatively controlled gene. No spontaneous mutations leading to the described phenotype of fast growth on 2-propylphenol could be detected among as many as 10^{10} wild-type cells. Only after selection had been applied did mutants appear after a lag period of 4 days, and they continued to appear for several more days (Fig. 1). Similar observations, especially with *Escherichia coli* Lac^- cells spread on lactose agar on which Lac^+ revertants continued to appear, have been interpreted as consequences of directed mutation (6). A deeper understanding of the mutational changes that occurred will be needed to decide whether this explanation holds in our case. This will be the goal of future genetic studies.

2-Propylphenol is metabolized via the same route as described for 2-hydroxybiphenyl (9), involving initial hydroxylation of the aromatic ring by an NADH-dependent monooxygenase (Fig. 6). The product of the monooxygenase activity was identified as 3-propylcatechol (Fig. 4) by using mass and UV-VIS spectra. Our assignment of the ion at *m/e* 123 to $(M\text{-}CHO)^+$ contrasts with the assignment that has been published (8) but agrees well with our experience with structurally different 3-alkyl- and 3-phenylcatechols. The catechol was further metabolized to the yellow *meta* fission product 2-hydroxy-6-oxo-nona-2,4-dienoic acid (Fig. 5) by a metapyrocatechase with high activity for 2,3-dihydroxybiphenyl and low activity for catechol and 3-methylcatechol (Table 3). With respect to activity toward these substituted catechols, the metapyrocatechase resembles the type I enzyme that has been found in dibenzofuran-grown cells of *Brevibacterium* sp. strain DPO 1361 (15), although in our case the addition of Fe^{2+} did not stimulate activity of the partially purified enzyme. During enzymatic assays, the enzyme was rapidly inactivated. This was also noted by Strubel et al. (15) for the metapyrocatechase (type II) acting on 2′,2,3-trihydroxybiphenyl. Further studies of the enzyme will be needed to establish the exact nature of this inactivation.

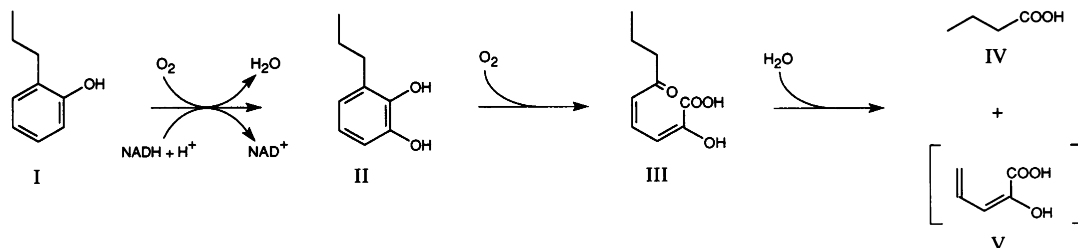

FIG. 6. Pathway proposed for the metabolism of 2-propylphenol by *Pseudomonas* sp. strain HBP1 Prp. Compounds: I, 2-propylphenol; II, 3-propylcatechol; III, 2-hydroxy-6-oxo-nona-2,4-dienoic acid; IV, butyric acid; V, 2-hydroxypenta-2,4-dienoic acid.

The partially purified *meta* fission product hydrolase, which was independent of any cofactors, exhibited higher activity for the *meta* cleavage product of 2,3-dihydroxybiphenyl than for the *meta* fission product of 3-propylcatechol (Table 3). Calculations have shown that the measured activity of the hydrolase for the *meta* fission product of 3-propylcatechol gives rise to a flux of this metabolite (2.4 μM min^{-1}) that is in the same order of magnitude (3.3 μM min^{-1}) as needed for growth of strain HBP1 Prp at the observed growth rate. The other *meta* cleavage products tested did not act as substrates. Therefore, the enzyme is distinct from the 2-hydroxymuconic semialdehyde hydrolase encoded by TOL plasmid pWWO from *Pseudomonas putida* mt-2, for which the *meta* cleavage product of 2,3-dihydroxybiphenyl did not act as a substrate (4) although the *meta* cleavage product of 3-propylcatechol did serve as a substrate. The enzyme rather resembles the hydrolase described by Smith and Ratledge (14) from alkylbenzene-grown *Pseudomonas* sp. NCIB 10643. This hydrolase also preferred the *meta* cleavage product from 2,3-dihydroxybiphenyl as a substrate and was able to turn over the *meta* cleavage products of various 3-alkylcatechols. As an explanation, the authors put forth the suggestion that the organism may have evolved as a biphenyl degrader that can also degrade a range of alkylbenzenes by a pathway analogous to that proposed for biphenyl.

The metabolism of butyrate, which is the product of the hydrolase activity, is not yet clear. In contrast to bacterial catabolism of β-chlorinated four-carbon aliphatic acids (10), an ATP-dependent butyryl-coenzyme A-synthetase seems not to be involved in its metabolism, as such an activity could not be measured in cell extracts of 2-propylphenol-grown cells of strain HBP1 Prp (data not shown). The accumulation of butyrate at the beginning of the stationary growth phase is somewhat perplexing. One possible explanation might be that the butyrate-activating enzymes are inactivated while the first three enzymes in the degradation pathway are still active.

All the enzymes involved in 2-propylphenol degradation to butyrate in strain HBP1 Prp have a higher affinity for the metabolites of the degradation of 2-hydroxybiphenyl as substrates. Because the activities for 2-hydroxybiphenyl degradation copurify with the activities for 2-propylphenol degradation upon ion-exchange chromatography, there is strong evidence that strain HBP1 Prp uses the same set of enzymes to degrade 2-hydroxybiphenyl to benzoic acid as it uses to degrade 2-propylphenol to butyric acid. Therefore, this catabolic pathway is another example of the generally assumed broad specificity (11) of *meta* pathways with regard to their enzymic function. The regulatory system involved in the synthesis of monooxygenase and metapyrocatechase in the wild type appears to be more discriminating than the catabolic enzymes themselves. This adds an additional layer of complexity to the efforts of finding versatile strains with biodegradative capacity, as theoretically functioning pathways might not be expressed because of regulatory inadequacies.

Further investigations will be needed to show whether the organism is able to grow on other 2-alkylphenols and to get a better insight into the exact nature of the regulation of the pathway.

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