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Elucidation of the 4-Hydroxyacetophenone Catabolic Pathway in *Pseudomonas fluorescens* ACB▽

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The catabolism of 4-hydroxyacetophenone in *Pseudomonas fluorescens* ACB is known to proceed through the intermediate formation of hydroquinone. Here, we provide evidence that hydroquinone is further degraded through 4-hydroxymuconic semialdehyde and maleylacetate to β-ketoadipate. The *P. fluorescens* ACB genes involved in 4-hydroxyacetophenone utilization were cloned and characterized. Sequence analysis of a 15-kb DNA fragment showed the presence of 14 open reading frames containing a gene cluster (*hapCDEFGHIBA*) of which at least four encoded enzymes are involved in 4-hydroxyacetophenone degradation: 4-hydroxyacetophenone monooxygenase (*hapA*), 4-hydroxyphenyl acetate hydrolase (*hapB*), 4-hydroxymuconic semialdehyde dehydrogenase (*hapE*), and maleylacetate reductase (*hapF*). In between *hapF* and *hapB*, three genes encoding a putative intradiol dioxygenase (*hapG*), a protein of the Yci1 family (*hapH*), and a [2Fe-2S] ferredoxin (*hapI*) were found. Downstream of the *hap* genes, five open reading frames are situated encoding three putative regulatory proteins (*orf10*, *orf12*, and *orf13*) and two proteins possibly involved in a membrane efflux pump (*orf11* and *orf14*). Upstream of *hapE*, two genes (*hapC* and *hapD*) were present that showed weak similarity with several iron(II)-dependent extradiol dioxygenases. Based on these findings and additional biochemical evidence, it is proposed that the *hapC* and *hapD* gene products are involved in the ring cleavage of hydroquinone.

Acetophenones are widely found in the environment as degradation products of industrial chemicals. Ring-chlorinated acetophenones originate from the microbial degradation of insecticides (6–8), polychlorobiphenyls (3–5), and chloroxanthones (83). Nonchlorinated acetophenones have been identified as intermediates in the microbial degradation of ethylbenzene (16, 85), 1-phenylethanol (18), 4-ethylphenol (42), and the flame-retardant tetrabromobisphenol A (55, 73).

Several aerobic microorganisms are capable of utilizing acetophenones for their growth (17–19, 32, 34, 35). 4-Hydroxyacetophenone can be converted in two different ways to 4-hydroxybenzoate (28, 35, 48). The latter compound then enters the β-ketoadipate pathway via the formation of the ring cleavage substrate 3,4-dihydroxybenzoate (42). For *Pseudomonas fluorescens* ACB (34), *Pseudomonas putida* JD1 (19), and *Aspergillus fumigatus* ATCC 28282 (41), it was shown that the initial steps of 4-hydroxyacetophenone mineralization comprise a Baeyer-Villiger oxidation to 4-hydroxyphenyl acetate and subsequent ester hydrolysis to hydroquinone (1.4-dihydroxybenzene) (19, 34, 41, 44). However, the subsequent degradation of hydroquinone, a potential human nephrocarcinogen (54), remained elusive.

The aerobic degradation of hydroquinone may proceed in two different ways. Hydroquinone can be hydroxylated to hydroxyhydroquinone (1,2,4-trihydroxybenzene) (24, 87), followed by ring fission by a intradiol hydroxyhydroquinone 1,2-dioxygenase (39, 53, 72). Alternatively, direct ring fission of hydroquinone to 4-hydroxymuconic semialdehyde by a hydroquinone 1,2-dioxygenase may occur (10, 19, 59, 77). Hydroquinone 1,2-dioxygenases have been indicated to be involved in the degradation of 4-ethylphenol (19), *p*-nitrophenol (12, 34, 70), 4-nitrocatechol (12, 13), and the insecticide fenitrothion (33). However, none of these enzymes has been characterized in much detail.

^{19}F nuclear magnetic resonance (NMR) studies have given some clues about the further degradation of hydroquinone in *P. fluorescens* ACB (60, 61). Whole cells converted 4-fluoroacetophenone to 4-fluorophenol. Prolonged incubations showed that 4-fluorophenol is not further degraded, suggesting that 4-hydroxyacetophenone-grown cells of *P. fluorescens* ACB lack phenol hydroxylase activity (60, 61). Without such monooxygenase activity, hydroquinone cannot be converted to hydroxyhydroquinone (24).

In this paper we have addressed the further degradation of hydroquinone in the catabolism of 4-hydroxyacetophenone in *P. fluorescens* ACB. To that end, we studied the conversion of (fluorinated) hydroquinones by (dialyzed) cell extracts and sequenced the *hap* gene cluster involved in 4-hydroxyacetophenone utilization. The biochemical and genetic characterization of the degradation pathway revealed that hydroquinone is metabolized through 4-hydroxymuconic semialdehyde and maleylacetate to β-ketoadipate. Two novel genes were found whose functions are linked to the ring cleavage of hydroquinone.

MATERIALS AND METHODS

Chemicals. NADH and NAD$^+$ were from Boehringer. Phenylmethylsulfonyl fluoride was from Merck. *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) was from Sigma. 2,3-Difluoro-4-hydroxybenzoic acid was synthesized from 2,3-difluorophenol according to Komiyama and Hirai (52) and purified by

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straight-phase high-performance liquid chromatography (HPLC) (Kieselgel 60 μm; Merck) with a 1 to 40% linear gradient of ethanol in petroleum ether (60 to 80°C). The identity of the product was demonstrated by ^{1}H NMR. Other fluorinated 4-hydroxybenzoic acids were synthesized and purified as described before (23, 38). All other chemicals were of analytical grade.

Bacterial strains and culture conditions. *P. fluorescens* ACB (34) was grown on 4-hydroxyacetophenone as reported before (44). Cell extract was prepared as described previously (44).

Enzymatic synthesis and transformation of difluorohydroquinones. 2,3-Difluoro-, 2,5-difluoro-, and 3,5-difluorohydroquinone were prepared from the corresponding difluorinated 4-hydroxybenzoates by incubation with 4-hydroxybenzoate 1-hydroxylase from *Candida parapsilosis* CBS604 (23). The reaction mixtures contained 50 mM air-saturated potassium phosphate (pH 7.0), 10 μM flavin adenine dinucleotide, 1 mM ascorbate, 0.3 mM difluoro-4-hydroxybenzoate, 0.25 mM NADH, and 80 μl of 4-hydroxybenzoate 1-hydroxylase in a total volume of 2.0 ml. The reactions were carried out at 25°C. After 2 h of incubation, the reaction mixtures were frozen in liquid nitrogen. Completion of the reactions was checked with ^{19}F NMR. Next, the difluorohydroquinones (1,800 μl) were incubated for 2 h in an orbital shaker at 30°C in the presence of 20 μl of *P. fluorescens* ACB cell extract. Formation of products was followed with ^{19}F NMR.

Enzyme purification. Partial purification of hydroquinone dioxygenase (HQDO) was performed at 4°C. *P. fluorescens* ACB cells (5 g, wet weight) were suspended in 5 ml of 20 mM BisTris [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)-methane] chloride, pH 7.0, containing 0.1 mM phenylmethylsulfonyl fluoride (buffer A). Immediately after the addition of 1 mM EDTA, 2 mM $MgCl_2$, and 1 mg of DNase, cells were disrupted three times through a precooled French press at 10,000 lb/in^2. After centrifugation (27,000 × *g* for 30 min), the clarified cell extract was adjusted to 25% ammonium sulfate saturation. The precipitate thus formed was removed by centrifugation (27,000 × *g* for 30 min), and the supernatant was loaded onto a phenyl-Sepharose column (1.6 × 11 cm) equilibrated in buffer A containing 25% ammonium sulfate. After a washing step with 3 column volumes of starting buffer, the HQDO activity was eluted with a 100-ml linear gradient of 25 to 0% ammonium sulfate in buffer A. Active fractions were pooled and adjusted to 60% saturation with pulverized ammonium sulfate. The precipitate was collected by centrifugation (27,000 × *g* for 30 min), dissolved in 1 ml of buffer A, and stored at −20°C.

Analytical methods. Absorption spectra were recorded using a Hewlett-Packard 8453 diode array spectrophotometer. HQDO activity was routinely determined by monitoring the formation of 4-hydroxymuconic semialdehyde at 320 nm (ε_{320} = 11.0 mM^{-1} cm^{-1}) (77). The assay mixture (1.0 ml) typically contained 50 μl of cell extract and 10% (wt/vol) glycerol in 20 mM BES, pH 7.0. Reactions were started by the addition of 10 μl of 50 mM hydroquinone in dimethylformamide. For activity tests with iron(II)-treated enzyme, partially purified HQDO preparations were freshly incubated for at least 1 min with 100 μM iron(II) sulfate and desalted over a Biogel P-6DG column (1 × 10 cm), running in 20 mM BisTris, pH 7.0, containing 10% glycerol. One unit of HQDO activity is defined as the amount of enzyme that forms 1 μmol of semialdehyde product per minute. 4-Hydroxymuconic semialdehyde dehydrogenase activity was measured by the addition of 1 mM NAD^+ to the above-produced semialdehyde and following the decrease of absorbance at 320 nm (77).

^{19}F NMR measurements were performed on a Bruker DPX 400 NMR spectrometer, essentially as described elsewhere (88). The sample temperature and sample volume were 7°C and 1.6 ml, respectively. For calibration an insert containing D_2O and a known amount of 4-fluorobenzoate was used, which also served as a deuterium lock for locking the magnetic field. Chemical shifts are reported relative to $CFCl_3$. The resonance of the 4-fluorobenzoate internal standard was set at −114.2 ppm with respect to $CFCl_3$. The detection limit of an overnight ^{19}F NMR measurement (60,000 scans) is 1 μM. The sample volume was 1.6 ml. ^{19}F NMR chemical shift values of the various fluorine-containing compounds were identified on the basis of authentic reference compounds (68) or as described in the present study.

HPLC analysis was performed on a Waters 600 controller system equipped with a reversed-phase Alltima C_{18} column (150 by 4.6 mm) running in 10% (vol/vol) acetonitrile and containing either 1, 0.1, or 0% acetic acid. Products were detected with a Waters 996 photodiode array detector.

LC-mass spectrometry (LC-MS) experiments were carried out on a LCQ Classic mass spectrometer (Thermo-Finnigan, San Jose, CA). Reaction mixtures containing 20 μl of 50 mM hydroquinone and 20 μl of desalted cell extract in 940 μl of sodium bicarbonate, pH 7.8, were incubated for 7 min at 25°C. Separation was achieved on a 150- by 2-mm Alltima C_{18} (Alltech, Breda, The Netherlands) column running in 10% (vol/vol) acetonitrile. MS analysis was performed in the negative atmospheric pressure chemical ionization mode using a vaporizer temperature of 450°C, a discharge voltage of 2 kV, and a capillary temperature of 150°C with nitrogen as a sheath gas (20%) and auxiliary gas (5%). Tandem MS (MS/MS) scans were recorded in the data-dependent mode when an MS base peak signal higher than 10^6 was obtained with a normalized collision energy of 35% and a 2-Da isolation width.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 15% Tris-glycine gels (91). An Amersham Pharmacia Biotech low-molecular-mass calibration kit containing phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) served as a reference. Proteins were stained with Coomassie brilliant blue G250.

N-terminal sequencing. The N-terminal sequence of HQDO was determined by Edman degradation. The contents of a 15% SDS-PAGE gel (140 by 120 by 1.5 mm) loaded with 50 μg of partially purified enzyme were blotted onto a polyvinylidene difluoride Immobilon-P support (Millipore) in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], pH 11.0, containing 10% ethanol. After the gel was stained with 0.1% Coomassie R-250 in 50% methanol, the main band corresponding to a relative molecular mass of 38 kDa was excised. Gas phase sequencing of the polypeptide on the Immobilon support was carried out at the sequencing facility of Leiden University, The Netherlands.

Cloning of the *hap* gene cluster. The *hap* gene cluster from *P. fluorescens* ACB was sequenced from a genomic cosmid library as described previously (44). In brief, by using degenerated primers based on the N-terminal amino acid sequences of 4-hydroxyphenyl acetate esterase and 4-hydroxyacetophenone monooxygenase, a PCR product was obtained that contained the esterase gene (*hapB*). This gene was used as a probe to screen the cosmid library, which yielded two positive clones (44). Sequencing of these cosmid clones resulted in a contiguous segment of 14.8 kbp consisting of 14 complete open reading frames (ORFS).

Sequence comparison. Protein sequence similarity searches were performed using the BLASTP option at www.ncbi.nlm.nih.gov. Multiple sequence alignments were made with the CLUSTAL W program at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw) (81). BioEdit (29) was used to calculate the pairwise identity and similarity scores (PAM250 matrix) from the aligned sequences and for the display of the alignment.

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank sequence databases under accession number AF355751.

RESULTS

Conversion of hydroquinone by desalted cell extract of *P. fluorescens* ACB. To study the conversion of hydroquinone in the degradation pathway of 4-hydroxyacetophenone in *P. fluorescens* ACB, 0.5 mM hydroquinone was incubated with desalted cell extract in 20 mM BES, pH 7.0, at 25°C. Absorption spectral analysis revealed that hydroquinone with a maximum absorption at 290 nm was converted into a product with a maximum absorption at 320 nm (Fig. 1A).

HPLC analysis of the reaction mixture showed a spectrum (Fig. 1B) indicative for the formation of 4-hydroxymuconic semialdehyde (77). HPLC separation of substrate and product was achieved at neutral pH because acidic mobile phase conditions resulted in vanishing of the absorbance at 320 nm, a feature observed with maleyl-substituted ring fission products (11). The identity of the reaction product of the conversion of hydroquinone by *P. fluorescens* ACB was confirmed by LC-MS. The product eluted at 2.65 min, and its mass spectrum gave an *m*/*z* of 141.0 in the negative mode. Collision-induced defragmentation showed an *m*/*z* of 96.9, corresponding to the decarboxylation of 4-hydroxymuconic semialdehyde (77). Addition of NAD^+ to the incubation mixture resulted in a decrease of absorbance at 320 nm, most likely due to conversion of 4-hydroxymuconic semialdehyde to maleylacetate. During this reaction, no increase at 340 nm was observed, suggesting that maleylacetate was rapidly further converted by an NADH-dependent maleylacetate reductase.

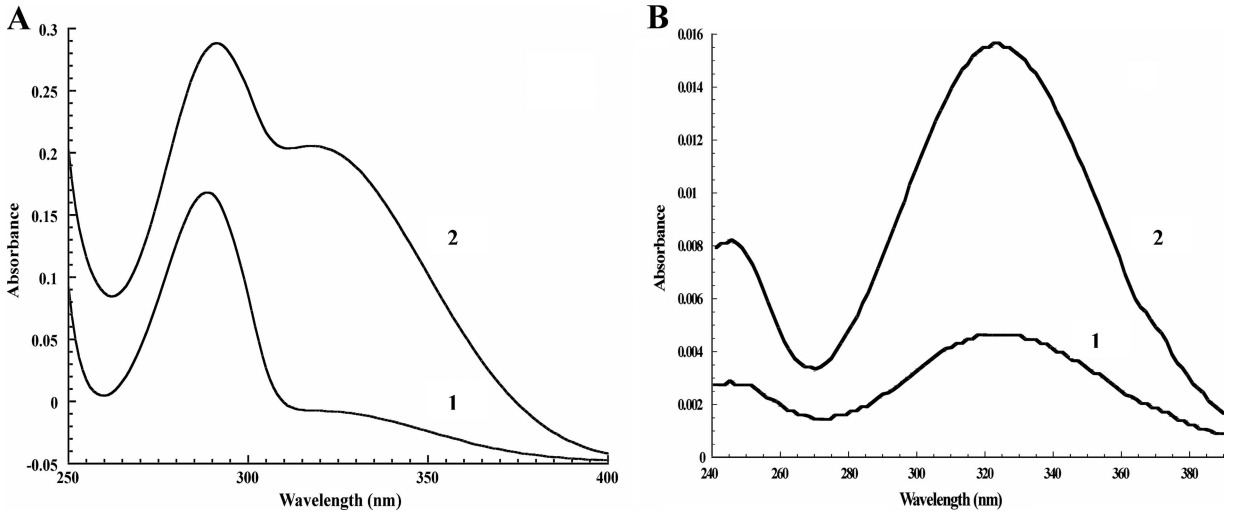

FIG. 1. Product analysis of the conversion of hydroquinone by desalted cell extract of *P. fluorescens* ACB. (A) Absorption spectrum of hydroquinone (1) and after conversion (2). (B) HPLC diode array spectra of the 4-hydroxymuconic semialdehyde product with (1) and without (2) 0.1% acetic acid in the eluent.

Degradation of difluorinated hydroquinones by *P. fluorescens* ACB. To get more information about the degradation of hydroquinones by *P. fluorescens* ACB, several difluorinated hydroquinones were prepared from the corresponding difluorinated 4-hydroxybenzoates by the action of 4-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* CBS604. Table 1 gives an overview of the ^{19}F NMR chemical shift values of the fluorinated 4-hydroxybenzoates and hydroquinones at pH 8.0. It should be mentioned here that difluorinated hydroquinones were selected for analysis because the oxidative ring opening of monofluorinated hydroquinones might result in nonfluorinated products that cannot be detected by ^{19}F NMR.

Figure 2 shows the ^{19}F NMR spectra of the incubation of 2,3-difluorohydroquinone with cell extract of *P. fluorescens* ACB. After 1 h, about 95% of the fluorinated aromatic substrate was converted (Fig. 2B), whereas all substrate was converted after 2 h of incubation (Fig. 2C). Formation of new signals at −129.0, −129.7, −130.3, −142.1, −154.0 and −154.4 ppm was observed to an extent that accounts for the decrease in the amount of 2,3-difluorohydroquinone. The resonance at −123.0 ppm originates from fluoride anions, indicating that some defluorination has occurred. Based on their intensity, the other six signals could be attributed to the formation of three difluorinated products. The first product with chemical shift values at −129.7 and −154.4 ppm was assigned to 2,3-difluoro-4-hydroxymuconic semialdehyde. This assignment is based on the following considerations. The NMR spectra show no formation of monofluorinated products. Furthermore, possible hydroxylation by a hydroquinone monooxygenase can be excluded because the chemical shift values of the newly formed peaks are not consistent with the formation of 5,6-difluoro-1,2,4-trihydroxybenzene (−162.0 ± 1.2 and −174.8 ± 1.7 ppm) (51). 2,3-Difluorohydroquinone can be split by HQDO between C-1 and C-2 or between C-1 and C-6. If ring fission occurred between C-1 and C-2, this would result in the formation of an acyl halide, as described for the conversion of 2-chlorohydroquinone by chlorohydroquinone dioxygenase (10, 67, 94). The acyl fluoride will readily react with water, yielding a monofluorinated maleylacetate. From this we conclude that ring fission occurred between C-1 and C-6 yielding 2,3-difluoro-4-hydroxymuconic semialdehyde. Upon 2,3-difluoro-4-hydroxymuconic semialdehyde formation, the chemical surrounding of the C-2 fluorine atom of 2,3-difluorohydroquinone changes more than that of the C-3 fluorine atom. Thus, the chemical shift of −129.7 ppm is assigned to the 2-fluoro substituent, whereas the chemical shift of −154.4 ppm is assigned to the 3-fluoro substituent of the semialdehyde (Table 1).

TABLE 1. ^{19}F NMR chemical shift values of fluorinated substrates and products

Compound	Chemical shift(s) (ppm)
2,3-Difluoro-4-hydroxybenzoate	−143.9 (F2), −167.4 (F3)
2,3-Difluorohydroquinone	−163.8
2,3-Difluoro-4-hydroxymuconic semialdehyde	−129.7 (F2), −154.4 (F3)
enol-2,3-Difluoromaleylacete	−130.3 (F2), −154.0 (F3)
keto-2,3-Difluoromaleylacete	−129.0 (F2), −142.1 (F3)
2,5-Difluoro-4-hydroxybenzoate	−121.4 (F2), −146.9 (F5)
2,5-Difluorohydroquinone	−144.6
2,5-Difluoro-4-hydroxymuconic semialdehyde[a]	−110.7 (F2), −174.9 (F5)
3,5-Difluoro-4-hydroxybenzoate	−139.2
3,5-Difluorohydroquinone	−136.8

[a] Or *enol*-2,5-difluoromaleylacetate.

The second difluorinated product which is already formed after 1 h but is most abundantly present after 2 h of incubation (Fig. 2C) is assigned to 2,3-difluoromaleylacetate. This assignment is based on the fact that 4-hydroxymuconic semialdehydes are readily converted by 4-hydroxymuconic semialdehyde dehydrogenases to the corresponding maleylacetates (77, 96). Maleylacetates can exist in the keto and enol tautomeric forms (76). Conversion of the 2,3-difluoro-4-hydroxymuconic semialdehyde to *enol*-2,3-difluoromaleylacetate does not change the direct environment of the two fluorine atoms, and only small differences in chemical shift values will occur.

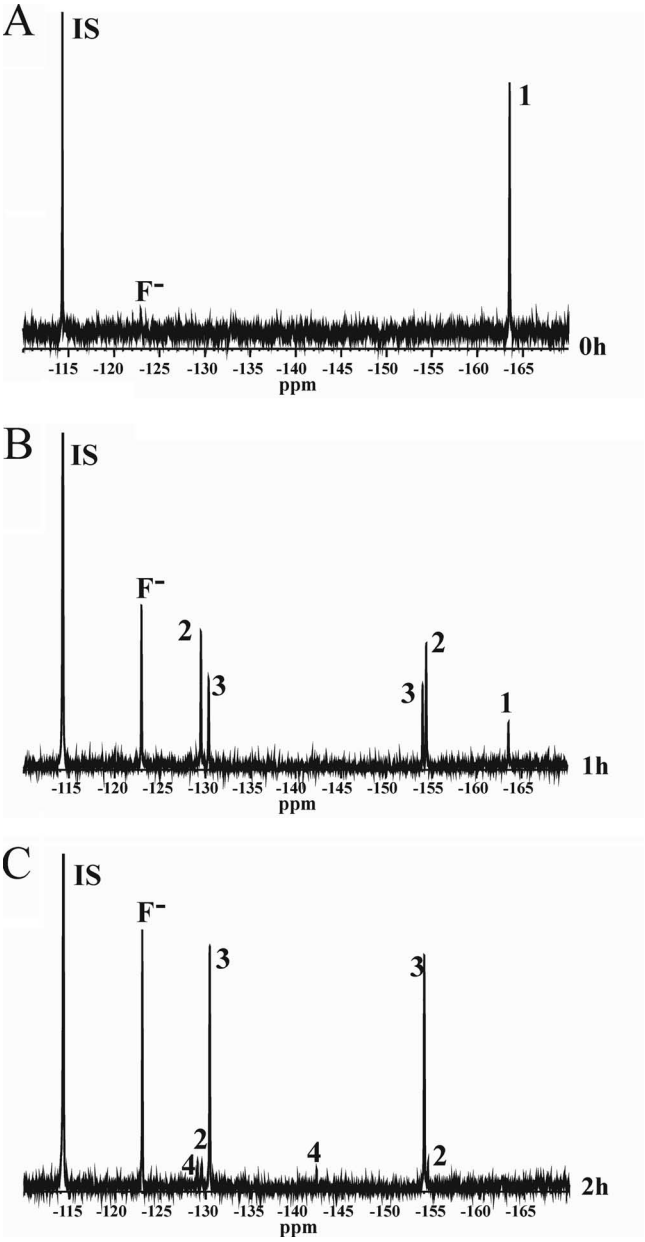

FIG. 2. ^{19}F NMR spectra of the conversion of 2,3-difluorohydroquinone by *P. fluorescens* ACB before the addition of cell extract (A), after 1 h of incubation (B), and after 2 h of incubation (C). Substrate and products are numbered as follows: 1, 2,3-difluorohydroquinone; 2, 2,3-difluoro-4-hydroxymuconic semialdehyde; 3, *enol*-2,3-difluoromaleylacetate; and 4, *keto*-2,3-difluoromaleylacetate. The internal standard 4-fluorobenzoate (IS) and the formed fluoride anion (F^-) are also indicated.

Therefore, the chemical shift values of −130.3 and −154.0 ppm are assigned to *enol*-2,3-difluoromaleylacetate. Figure 2C also shows the formation of a minor compound with resonances at −129.0 and −142.1 ppm. This compound is assigned to *keto*-2,3-difluoromaleylacetate. The keto tautomer of the maleylacetate drastically changes the chemical shift value of the 3-fluoro substituent, whereas the surrounding environment of the 2-fluoro substituent does not significantly change. Thus, the chemical shift value of −129.0 ppm is assigned to the 2-fluoro substituent, whereas the chemical shift value of −142.1 ppm is assigned to the 3-fluoro substituent of *keto*-2,3-difluoromaleylacetate. Halogenated maleylacetates generally are further degraded by reductive dehalogenation to β-ketoadipate (10, 46, 90). Therefore, the strong increase in fluoride anion observed in Fig. 2B and C most likely results from maleylacetate reductase activity present in *P. fluorescens* ACB.

A 2-h incubation of 2,5-difluorohydroquinone with cell extract of *P. fluorescens* ACB resulted in the conversion of about 80% of the aromatic substrate (results not shown). Formation of about 25% fluoride anions (−123.0 ppm) and one main difluorinated aromatic product with resonances at −110.7 and −174.9 ppm was observed, showing ring splitting between C-1 and C-6. This compound is tentatively assigned to 2,5-difluoro-4-hydroxymuconic semialdehyde or *enol*-2,5-difluoromaleylacetate (Table 1).

A 2-h incubation of 3,5-difluorohydroquinone with cell extract of *P. fluorescens* ACB did not show accumulation of fluorinated compounds (results not shown). Under the conditions applied, nearly 100% of the fluorinated aromatic substrate was converted into fluoride anions.

Partial purification of HQDO. Purification of HQDO by ammonium sulfate fractionation and hydrophobic interaction chromatography resulted in an enzyme preparation with a specific activity of about 0.5 U mg^{-1}. The partially purified enzyme was rather unstable and rapidly lost activity. Part of the HQDO activity could be restored by incubation of the enzyme with iron(II) salts. The partially purified enzyme showed two main bands on SDS-PAGE around 38 kDa and 70 kDa. The N-terminal sequence of the 70-kDa protein (SAFNTTLPS LDY) corresponded to that of residues 2 to 13 of 4-hydroxyacetophenone monooxygenase (44). The N-terminal sequence of the 38-kDa protein was found to be AMLEAVETEN. Except for the starting methionine, this sequence corresponds to the N-terminal part of *orf2* of the *hap* gene cluster (see below).

Cloning and sequencing of the *hap* gene cluster. We have sequenced a 15-kb fragment of the *P. fluorescens* ACB genome containing the 4-hydroxyphenyl acetate hydrolase gene (44). Analysis revealed that the DNA sequence contains 14 complete ORFs (Table 2). Except for *orf11*, encoding a putative outer membrane channel protein, all ORFs have the same direction. The upstream region of the sequenced DNA (*orf1* to *orf9*) contains a cluster of genes involved in the degradation of 4-hydroxyacetophenone (*hapCDEFGHIBA*). Downstream of the *hap* genes, three genes encoding putative regulatory proteins (*orf10*, *orf12*, and *orf13*) and two genes coding for proteins possibly involved in a membrane efflux pump (*orf11* and *orf14*) were found. The sequences of the *orf* genes have been deposited in the DDBJ/EMBL/GenBank databases (see Materials and Methods).

The *hapA* gene product, 4-hydroxyacetophenone monooxygenase (EC 1.14.13.84), is the first enzyme in the degradation pathway. HapA (formerly called HapE) (44) converts 4-hydroxyacetophenone to 4-hydroxyphenyl acetate and is active with a wide range of aromatic and aliphatic ketones (43–45). HapA belongs to the family of flavin adenine dinucleotide-containing Baeyer-Villiger monooxygenases (26, 58, 86) and shows the highest sequence identity with steroid monooxygenase from *Rhodococcus rhodochrous* (63) (Table 2).

TABLE 2. ORFs of a 14-kb DNA fragment of *P. fluorescens* ACB

ORF	Frame	Position in sequence (nt)	Gene product		Function	Closest characterized/uncharacterized homolog(s)[b]				
			No. of residues	Mass (Da)		Description	GenBank accession no.	Sequence identity (%)	Reference	Organism[c]
hapC	+3	360–857	165	17,899		Hypothetical protein	ZP_01296719	78		*P. aeruginosa* PA7
hapD	+1	904–1929	341	38,345	Hydroquinone dioxygenase	Hypothetical protein	ZP_01296720	80		*P. aeruginosa* PA7
hapE	+1	2020–3483	487	52,400	4-Hydroxymuconic semialdehyde dehydrogenase	**_p_-Cumic aldehyde dehydrogenase**	**AAB62298**	**47**	22	*P. putida* F1
						Hypothetical protein	ZP_01296721,	85		*P. aeruginosa* PA7
hapF	+3	3492–4559	355	37,612	Maleylacetate reductase	**Maleylacetate reductase**	**Q45072**	**56**	20	*B. cepacia* AC1100
						hypothetical protein	ZP_01296722	76		*P. aeruginosa* PA7
hapG	+1	4603–5478	291	32,159	Hydroxyhydroquinone dioxygenase	**Hydroxyquinol 1,2-dioxygenase**	**BAF44523**	**49**	95	*Rhizobium sp.* MTP-10005
						Hypothetical protein	ZP_01296723	67		*P. aeruginosa* PA7
hapH	+3	5475–5786	103	11,828	Protein YciI family	Putative protein	ZP_01296724	72		*P. aeruginosa* PA7
hapI	+3	5790–6179	129	13,866	Ferredoxin	**Chloroplast-type ferredoxin XylT**	**P23103,**	**17**	31	*P. putida*
						Hypothetical protein	ZP_01296725	58		*P. aeruginosa* PA7
hapB	+1	6169–7086	305	33,262	4-Hydroxyphenyl acetate esterase	**Lipase**	**AAC38151**	**42**	14	*Pseudomonas* sp. strain B11–1
						Esterase/lipase	YP_299651	45		*R. eutropha* JMP134
hapA	+2	7139–9061	640	71,957	4-Hydroxyacetophenone monooxygenase	**Steroid monooxygenase**	**JC7158**	**21**	63	*R. rhodochrous*
						Hypothetical protein	YP_639903	39		*Mycobacterium sp.* strain MCS
orf10	+3	9162–10094	310	35,222	Regulatory protein (LysR family)	**HTH-type transcript. regulator ptxR**	**P72131**	**24**	30	*P. aeruginosa* PA01
						Hypothetical protein	ZP_01296718	67		*P. aeruginosa* PA7
orf11	−3	10166–11626	486	53,006	Outer membrane channel protein	**Outer membrane channel protein ttgF**	**CAB72260**	**55**	64	*P. putida*
						Hypothetical efflux system	ZP_00898203	54		*P. syringae*
orf12	+2	11774–12382	202	22,221	Regulatory protein (TetR family)	**HTH-type transcriptional regulator srpR**	**Q9R9T9**	**57**	92	*P. putida* S12
						Hypothetical transcript. regulator, TetR	ZP_01499416	27		*B. phymatum* STM815
orf13	+3	12450–13241	263	28,189	Regulatory protein (Ic1R family)	**HTH-type transcriptional regulator srpS**	**Q9R9U0**	**57**	92	*P. putida* S12
						Hypothetical regulatory protein	ZP_00898206	55		*P. putida F1*
orf14	+1	13801–14736	311	34,623	Efflux pump (EmrA family)	**Multidrug resistance protein A**	**P27303**	**21**	56	*E. coli*
						Hypothetical secretion protein	YP_574727	48		*C. salexigens*

[a] nt, nucleotides.
[b] Characterized homologs are shown in boldface.
[c] *R. eutropha*, *Ralstonia eutropha*; *P. syringae*, *Pseudomonas syringae*; *B. phymatum*, *Burkholderia phymatum*; *E. coli*, *Escherichia coli*; *C. salexigens*, *Chromohalobacter salexigens*.

HapB (formerly called HapD) (44) is the second enzyme of the 4-hydroxyacetophenone degradation pathway. This esterase (EC 3.1.1.2) efficiently converts 4-hydroxyphenyl acetate to hydroquinone (44). The N-terminal sequence of purified HapB (44) exactly matches the amino acid sequence derived from the *hapB* gene. The HapB amino acid sequence shows significant similarity with several lipases and esterases and contains a consensus motif, GXSXG, that includes the conserved active-site serine (9). HapB shares the highest sequence identity (38%) with a lipase from *Pseudomonas* sp. strain B11-1 (Table 2). This hydrolytic enzyme has been shown to be active with a range of *p*-nitrophenyl esters (14).

The reaction of hydroquinone to 4-hydroxymuconic semialdehyde is catalyzed by an extradiol-type of dioxygenase (19, 77). This conclusion is supported by the fact that the enzymatic activity of partially purified HQDO is stimulated by iron(II) ions. Based on the identification of the N-terminal sequence, the function of HQDO is linked to the *hapD* gene. HapD showed weak sequence identity (6 to 12%) with known non-heme-iron(II)-dependent dioxygenases and no significant sequence similarity with any other characterized protein. A BLASTP search in the current bacterial genome database (848 genomes) revealed that only nine homologous ORFs can be found that are highly similar in sequence (>59% sequence

identity). All of these *hapD* homologs are upstream, flanked by a gene that shows significant sequence homology (>49% sequence identity) with *hapC*. In the accompanying paper (62), biochemical evidence is provided that *hapC* and *hapD* encode the α- and β-subunits of HQDO.

The deduced amino acid sequence of the fourth protein in the degradation pathway, HapE, shows 45% sequence identity with CymC, a *p*-cumic aldehyde dehydrogenase, from *P. putida* (21) (Table 2). Based on the hydroquinone degradation experiments reported above, HapE is assigned to be an NAD^+-dependent dehydrogenase (EC 1.2.1.61) that converts 4-hydroxymuconic semialdehyde to maleylacetate. Interestingly, HapE shows 37 to 43% sequence identity with 2-hydroxymuconic semialdehyde dehydrogenases, which are involved in the *meta*-cleavage pathways of catechol degradation (66, 80). There is no other sequence of a 4-hydroxymuconic semialdehyde dehydrogenase known. However, genome sequencing projects have revealed putative proteins that have up to 85% sequence identity with HapE from *P. fluorescens* ACB (Table 2).

HapF shows the highest sequence identity (56%) with the characterized maleylacetate reductase (EC 1.3.1.32) from *Pseudomonas cepacia* (20). This enzyme converts maleylacetate to β-ketoadipate, a well-known step in many degradation pathways, and it is present in the degradation of both hydroquinone and hydroxyhydroquinone (47).

The *hap* gene cluster contains three genes that are not directly involved in the degradation of 4-hydroxyacetophenone. The *hapG* gene product shows 43% sequence identity with 6-chlorohydroxyhydroquinone 1,2-dioxygenase from *Wautersia eutropha* JMP134, an iron(III)-dependent intradiol ring cleavage enzyme that converts 6-chlorohydroxyhydroquinone to chloromaleylacetate (47, 57). HapG is also closely related to other hydroxyhydroquinone 1,2-dioxygenases (1, 25, 50, 65).From this and the crystal structure of hydroxyhydroquinone dioxygenase from *Nocardioides simplex* 3E (Protein Data Bank code 1TMX) (25), it can be inferred that the strictly conserved residues Tyr160, Tyr194, His218, and His220 of HapG are involved in coordination of the active-site ferric ion.

hapH codes for a conserved protein of the Yci1 family (pfam03795), but its function is unknown. *hapI* encodes a ferredoxin that might be involved in reduction of the iron-metal cofactor of extradiol dioxygenases (27, 36, 37, 69, 84).

Besides the *hap* gene cluster and *orf5-orf7*, there are five additional ORFs in the 15-kb DNA fragment of *P. fluorescens* ACB. Sequence similarity searches suggest that *orf10*, *orf12*, and *orf13* code for putative regulatory proteins while *orf11* encodes a putative outer membrane channel protein and *orf14* codes for a putative efflux pump.

DISCUSSION

This paper describes the biochemical and genetic characterization of the 4-hydroxyacetophenone catabolic pathway in *P. fluorescens* ACB. Earlier studies revealed that catabolism of 4-hydroxyacetophenone is initiated by a Baeyer-Villiger oxidation to 4-hydroxyphenyl acetate and then proceeds through the formation of hydroquinone (34, 44). Here, we showed that *P. fluorescens* ACB converts hydroquinone to 4-hydroxymuconic semialdehyde and that difluorinated hydroquinones are converted via difluorinated 4-hydroxymuconic semialdehydes to the corresponding difluoromaleylacetates. Formation of significant amounts of fluoride anion pointed at the activity of maleylacetate reductase (46, 47, 90).

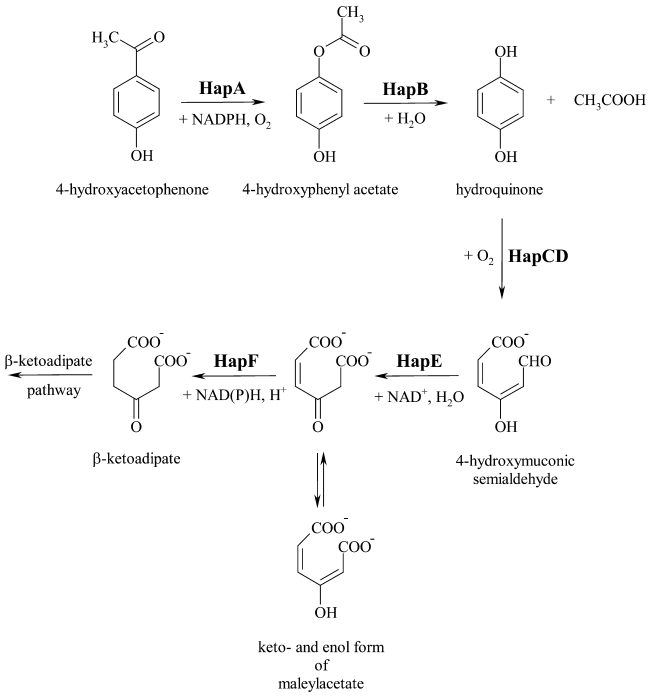

FIG. 3. Proposed degradation pathway of 4-hydroxyacetophenone by *P. fluorescens* ACB.

Cloning and sequence analysis of the genes involved in the catabolism of 4-hydroxyacetophenone revealed a gene cluster (*hapCDEFGHIBA*) involved in 4-hydroxyacetophenone degradation: 4-hydroxyacetophenone monooxygenase (HapA), 4-hydroxyphenyl acetate hydrolase (HapB), HQDO (HapD), 4-hydroxymuconic semialdehyde dehydrogenase (HapE), and maleylacetate reductase (HapF). Based on these results, we propose that *P. fluorescens* ACB degrades 4-hydroxyacetophenone to β-ketoadipate by the pathway depicted in Fig. 3.

In the *hapCDEFGHIBA* cluster, the function of *hapD* is linked to the conversion of hydroquinone to 4-hydroxymuconic semialdehyde. This reaction presumably is catalyzed by an iron(II)-dependent extradiol dioxygenase (93). These enzymes generally contain a His_2-carboxylate triad involved in binding the iron atom (49, 75, 78, 79, 82, 89). A BLASTP search for HapD sequence homologs in the microbial genome database revealed a small number of proteins (in *Pseudomonas aeruginosa* PA7, *Photorhabdus luminescens* subsp. *laumondii* TTO1, and seven *Burkholderia* genomes) that are highly similar in sequence (sequence identity of >59%). In the above-mentioned microbial genomes, the *hapD* gene is flanked by a *hapC*-like gene (Fig. 4). Biochemical studies presented in the accompanying paper (62) unambiguously showed that *hapC* is also required for the oxygenolytic ring fission of hydroquinone.

Sequence homology indicates that *hapI* encodes a [2Fe-2S] ferredoxin. In several related microorganisms, this electron transfer protein is required for the reductive reactivation of catechol 2,3-dioxygenases (27, 36, 37, 69, 84). This leads us to

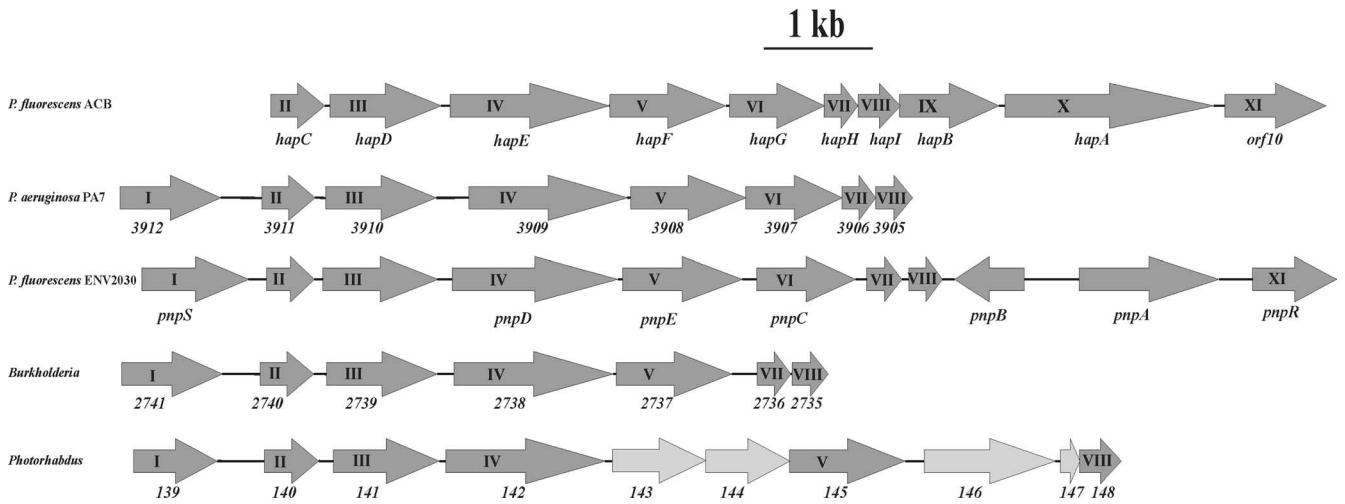

FIG. 4. Organization of the *hap* gene cluster and surrounding ORFs of a 14.8-kb DNA fragment of *P. fluorescens* ACB. For an explanation, see Table 2. The *hap* gene cluster is aligned with the *pnp* gene cluster from *P. fluorescens* ENV2030 (2, 96) and with gene clusters found in the genomes of *P. aeruginosa* PA7 (NC_009656 3912 corresponds to PSPA7_3912, e.g.), *Burkholderia cepacia* 18194 also called *Burkholderia* sp. strain 383 (NC_007511; chromosome 2; 2741 corresponds to Bcep18194_B2741, e.g.), and *Photorhabdus luminescens* subsp. *laumondii* TTO1 (NC_005126; gene number 139 corresponds to plu139, e.g.). Corresponding Roman numbers belong to comparable genes. The genes indicated with I and XI code for members of the family of LysR-type transcriptional regulators (15, 71, 74).

suggest that in *P. fluorescens* ACB, HapI might be involved in the reactivation of HapCD.

HapG has many structural properties in common with the iron(III)-dependent intradiol dioxygenases. This suggests that this enzyme is not involved in the catabolic pathway of 4-hydroxyacetophenone. HapG might be responsible for the observed activities of *P. fluorescens* ACB with catechols (60) and hydroxyhydroquinone. Why *hapG* is embedded in the *hap* cluster is not clear. However, there are more examples of the presence of a hydroxyhydroquinone dioxygenase in an organism where an HQDO serves as a key enzyme in the degradation pathway of aromatic compounds. Such a situation has been reported for the 4-nitrophenol pathways of *P. fluorescens* ENV2030 (96) and a *Moraxella* strain (77). A hydroxyhydroquinone dioxygenase is also present in the degradation pathway of 2,4-dinitrotoluene in *Burkholderia cepacia* R34, but another extradiol dioxygenase carries out the ring fission of 2,4,5-trihydroxytoluene (40). It seems that there is a progression in the organization of the pathway genes toward a compact region encoding the entire pathway. In that progression, remnants from previous assembly events (like *hapG*) persist. The presence of *hapG* in the *hap* gene cluster might indicate an intermediate point in the evolution of an optimal system for 4-hydroxyacetophenone degradation (40). Embedded in the *hap* gene cluster, *hapG* might be available to assist in other degradation pathways for the intradiol splitting of hydroxyhydroquinone or other catechols.

The *hap* gene cluster of *P. fluorescens* ACB shows similarities with the *pnp* gene cluster of *P. fluorescens* ENV2030 involved in 4-nitrophenol utilization (Fig. 4) (96). The *hapEFG* genes have the same size and orientation as the *pnpDEC* genes. Bang and Zylstra reported that *pnpD* (corresponding to *hapE*) codes for a 4-hydroxymuconic semialdehyde dehydrogenase showing homology with both eukaryotic and prokaryotic aldehyde dehydrogenases and that *pnpE* (corresponding to *hapF*) codes for a maleylacetate reductase (2). Furthermore, it was concluded that *pnpC* codes for the dioxygenase involved in the formation of 4-hydroxymuconic semialdehyde from hydroquinone (2). The *pnpC* gene corresponds to the *hapG* gene, and the amino acid sequence of the PnpC protein has a high level of sequence similarity with hydroxyhydroquinone dioxygenases (2, 96). Thus, the question arises whether PnpC is involved in the biodegradation of 4-nitrophenol in *P. fluorescens* ENV2030. From a gene cluster comparison (Fig. 4), we propose that an enzyme similar to HapCD is involved in the degradation of *p*-nitrophenol in *P. fluorescens* ENV2030.

It is striking that in the sequenced genome of *P. aeruginosa* PA7 an orthologous partial *hap* gene cluster is found (Table 2 and Fig. 4). This cluster contains, in the same orientation as in *P. fluorescens* ACB, all *hapCDEFGHI* orthologs. This observation suggests that *P. fluorescens* ACB has acquired the *hapAB* genes in a recent genetic event and has thereby become efficient in utilizing acetophenones as energy and carbon sources.

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