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-hydroxyacetophenones for their growth (17-19, 32, 34, 35). 4-Hydroxyacetophenone-grown cells of Pseudomonas putida ACB (19), and P. fluorescens ACB (60, 61). Whole cells converted 4-fluoroacetophenone to 4-fluorophenol. Prolonged incubations showed 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 degradation. The biochemical and genetic characterization of the degradation pathway revealed that hydroquinone is metabolized through 4-hydroxymuconic semialdehyde and maleylacetate to β-ketoacid. 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 (hapCDEGHIBA) of which at least four encode enzymes 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 hapE and hapB, three genes encoding a putative intradiol dioxygenase (hapG), a protein of the Yci1 family (hapH), and a [2Fe-2S] ferredoxin (hapF) 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.

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 (hapCDEGHIBA) of which at least four encode enzymes 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 hapE and hapB, three genes encoding a putative intradiol dioxygenase (hapG), a protein of the Yci1 family (hapH), and a [2Fe-2S] ferredoxin (hapF) 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.

ELUCIDATION OF THE 4-HYDROXYACETOPHENONE CATABOLIC PATHWAY IN PSEUDOMONAS FLUORESCENS ACB


Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands.

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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-hydroxyacetophenones for their growth (17-19, 32, 34, 35). 4-Hydroxyacetophenone-grown cells of Pseudomonas putida ACB (19), and 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. Phenylmethanesulfonyl 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
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 1H 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 difluoroquinones.** 2,3-Difluoro-, 2,5-difluoro-, and 3,5-difluoroquinones were prepared from the corresponding difluorinated 4-hydroxybenzoates by incubation with 4-hydroxybenzoate 1-hydroxylase from *Candida parapsilosis* CBS6804 (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 19F NMR. Next, the difluoroquinolones (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 19F 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 Bis-Tris [bis(2-hydroxyethyl)iminom-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 MgCl₂, and 1 mg of DNase, cells were disrupted three times through a precooled French press at 10,000 lb/in². 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-hydroxy-3-methyl-2,5-cyclohexadien-1-one by HPLC analysis of the reaction mixture (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 μl of 50 mM hydroquinone and 20 μl of desalted cell extract in 940 μl of 20 mM Bis-Tris, pH 7.0, containing 10% glycerol. One unit of HQDO activity was defined as the amount of enzyme that forms 1 μmol of semialdehyde product per minute. 4-Hydroxyquinone semialdehyde dehydrogenase activity was measured by the addition of 1 μM NAD⁺ to the above-produced semialdehyde and following the decrease of absorbance at 320 nm (77). 19F 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 D₂O 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 CFCl₃. The resonance of the 4-fluorobenzene internal standard was set at −114.2 ppm with respect to CFCl₃. The detection limit of an overnight 19F NMR measurement (60,000 scans) is 1 μM. The sample volume was 1.6 ml. 19F 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 Altima C₁₈ column (150 by 4.6 mm) running in 10% (vol/vol) acetonitrile and containing either 10% or 0% acetic acid. Products were detected with a Waters 990 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 20 mM Bis-Tris, pH 7.0, were incubated for 7 min at 25°C. Separation was achieved on a 150- by 2-mm Altima C₁₈ (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 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 10⁷ 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 sequence of 4-hydroxyphenyl acetate esterase and 4-hydroxyacetophenone mono-oxygenase, a PCR product was obtained that contained the esterase gene (hapE). This gene was used as a probe to screen the cosmid library, which yielded two positive clones (44). Sequencing of these cosmids 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-hydroxyxymuconic 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 fragmentation showed an m/z of 96.9, corresponding to the decarboxylation of 4-hydroxyxymuconic semialdehyde (77). Addition of Na⁺ to the incubation mixture resulted in a decrease of absorbance at 320 nm, most likely due to conversion of 4-hydroxyxymuconic 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.
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 19F 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. Furthermore, possible hydroxylation by a hydroquinone monooxygenase can be excluded because the chemical shift values of the newly formed 4-hydroxymuconic semialdehyde formation, the chemical shift of 1-hydroxylase from *P. fluorescens* or *P. parapsilosis* and products included because the chemical shift values of the newly formed 4-hydroxymuconic semialdehyde is based on the fact that 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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift(s) (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Difluoro-4-hydroxybenzoate</td>
<td>−143.9 (F2), −167.4 (F3)</td>
</tr>
<tr>
<td>2,3-Difluorohydroquinone</td>
<td>−163.8</td>
</tr>
<tr>
<td>2,3-Difluoro-4-hydroxymuconic</td>
<td></td>
</tr>
<tr>
<td>semialdehyde</td>
<td>−129.7 (F2), −154.4 (F3)</td>
</tr>
<tr>
<td><em>enol</em>-2,3-Difluoromaleylacetate</td>
<td>−130.3 (F2), −154.0 (F3)</td>
</tr>
<tr>
<td><em>keto</em>-2,3-Difluoromaleylacetate</td>
<td>−129.0 (F2), −142.1 (F3)</td>
</tr>
<tr>
<td>2,5-Difluoro-4-hydroxybenzoate</td>
<td>−121.4 (F2), −146.9 (F5)</td>
</tr>
<tr>
<td>2,5-Difluorohydroquinone</td>
<td>−144.6</td>
</tr>
<tr>
<td>2,5-Difluoro-4-hydroxymuconic</td>
<td></td>
</tr>
<tr>
<td>semialdehyde*</td>
<td>−110.7 (F2), −174.9 (F5)</td>
</tr>
<tr>
<td>3,5-Difluoro-4-hydroxybenzoate</td>
<td>−139.2</td>
</tr>
<tr>
<td>3,5-Difluorohydroquinone</td>
<td>−136.8</td>
</tr>
</tbody>
</table>

*Or enol-2,3-difluoromaleylacetate.*

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).

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.
Therefore, the chemical shift values of $-130.3$ and $-154.0$ ppm are assigned to enol-2,3-difluromaleylacetate. 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-difluromaleylacetate. 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-difluromaleylacetate. Halogenated maleylacetates generally are further degraded by reductive dehalogenation to $\beta$-keto-adipate (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-difluromaleylacetate (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 monoxygenase (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 monoxygenases (26, 58, 86) and shows the highest sequence identity with steroid monoxygenase from Rhodococcus rhodochrous (63) (Table 2).

![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-difluromaleylacetate; and 4, keto-2,3-difluromaleylacetate. The internal standard 4-fluorobenzoate (IS) and the formed fluoride anion (F$^-$) are also indicated.](http://jb.asm.org/)

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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) 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) with any other characterized protein.

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

<table>
<thead>
<tr>
<th>ORF</th>
<th>Frame</th>
<th>Position in sequence (nt)</th>
<th>Gene product</th>
<th>No. of residues</th>
<th>Mass (Da)</th>
<th>Function</th>
<th>Closest characterized/uncharacterized homolog(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hapC</td>
<td>+3</td>
<td>360–857</td>
<td>Hypothetical protein</td>
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<td>2020–3483</td>
<td>p-Cumar aldehyde dehydrogenase</td>
<td>487</td>
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<tr>
<td>hapF</td>
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<td>3492–4559</td>
<td>Maleylacetate reductase</td>
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<td>37,612</td>
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<td>4603–5478</td>
<td>Hydroxyquinone 1,2-dioxygenase</td>
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<td>32,159</td>
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<td>ZP_01296722, 76, P. putida</td>
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<tr>
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<td>5475–5786</td>
<td>Putative protein YciI family</td>
<td>103</td>
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<td></td>
<td>PCB1303, 17, P. putida</td>
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<td>13,866</td>
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<tr>
<td>hapB</td>
<td>+1</td>
<td>6169–7086</td>
<td>4-Hydroxyphenyl acetate esterase</td>
<td>305</td>
<td>33,262</td>
<td></td>
<td>AAC38151, 42, Pseudomonas sp. strain B11–1 R. eutropha IMP134</td>
</tr>
<tr>
<td>hapA</td>
<td>+2</td>
<td>7139–9061</td>
<td>4-Hydroxyacetophenone monoxygenase</td>
<td>640</td>
<td>71,957</td>
<td></td>
<td>J7158, 21, R. rhodochrous Mycobacterium sp. strain MCS</td>
</tr>
<tr>
<td>orf10</td>
<td>+3</td>
<td>9162–10094</td>
<td>Regulatory protein (LysR family)</td>
<td>310</td>
<td>35,222</td>
<td></td>
<td>P27131, 24, P. aeruginosa PA1</td>
</tr>
<tr>
<td>orf11</td>
<td>−3</td>
<td>10166–11626</td>
<td>Outer membrane channel protein</td>
<td>486</td>
<td>53,006</td>
<td></td>
<td>CAB72260, 55, P. putida</td>
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<tr>
<td>orf12</td>
<td>+2</td>
<td>11774–12382</td>
<td>Regulatory protein (TetR family)</td>
<td>202</td>
<td>22,221</td>
<td></td>
<td>Q9R9T9, 57, P. putida S12</td>
</tr>
<tr>
<td>orf13</td>
<td>+3</td>
<td>12450–13241</td>
<td>Regulatory protein (Ic1R family)</td>
<td>263</td>
<td>28,189</td>
<td></td>
<td>Q9R91U0, 57, P. putida S12</td>
</tr>
<tr>
<td>orf14</td>
<td>+1</td>
<td>13801–14736</td>
<td>Efflux pump (EmrA family)</td>
<td>311</td>
<td>34,623</td>
<td></td>
<td>P27303, 21, E. coli</td>
</tr>
</tbody>
</table>

* nt, nucleotides.
* Characterized homologs are shown in boldface.
* R. eutropha, *Kluyvera eutropha*; *P. syringae*, *Pseudomonas syringae*; *B. phymatum*, *Burkholderia phymatum*; *E. coli*, *Escherichia coli*; *C. salegins*, *Chromohalobacter salegins*. 
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*-cumin 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-ketoacetate, 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 difluormaleylacetates. Formation of significant amounts of fluoride anion pointed at the activity of maleylacetate reductase (46, 47, 90).

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 dehydrogenase (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-ketoacetate 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 intradiol dioxygenase (93). These enzymes generally contain a His₄-carboxylate tetrad 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. *laurentii* 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...
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(II)-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 pnp 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 hapC 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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