Biodegradation of fluorinated environmental pollutants under aerobic conditions
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Genes and proteins involved in the degradation of 4-fluorophenol by *Arthrobacter* sp. strain IF1


The cloning and sequencing was done by M.I.M.Ferreira during a research stay at the Environmental Molecular Biology Laboratory, RIKEN, Wako, Japan.

The mass spectrometry analysis was done by H.P. Permentier, University of Groningen.
ABSTRACT

Arthrobacter sp. strain IF1 is able to grow on 4-fluorophenol (4-FP) as a sole source of carbon and energy. We report the cloning and sequencing of two gene clusters that each harbor a monooxygenase with high sequence similarity to the oxygenase component of 4-nitrophenol-, 4-chlorophenol- and 4-hydroxyphenyl acetate monooxygenase systems. One cluster also contained a gene for a flavin reductase. The monooxygenase (FpdA2) and reductase (FpdB) were purified from E. coli cells expressing the corresponding genes. The expression of the FpdA2 was very good but the (purified) protein was unstable. We characterized the inactivated enzyme by MALDI-MS/MS and the results suggested that FpdA2 underwent truncation by the activity of a protease. FpdA2 and FpdB together catalyzed NADH-dependent hydroxylation and dehalogenation of para-substituted phenols. The results indicate that strain IF1 transforms 4-FP to hydroquinone by a two-component monooxygenase system of which one component provides reduced FAD at the expense of NADH and the other catalyzes para-hydroxylation of 4-FP and other 4-substituted phenols.

INTRODUCTION

Halogenated phenols are used as building blocks in the synthesis of pharmaceuticals, agrochemicals, and performance materials. As a result of improper waste disposal or open applications, they frequently occur as pollutants in water and soil. Some microorganisms have evolved pathways that allow biodegradation of these compounds (10, 20, 28, 52). Most studies on such pathways have been done with bacteria that degrade and dehalogenate chlorinated phenols. Since the 1990s, the industrial use of fluorinated compounds has been growing (24, 49).

The cleavage of the carbon-fluorine bond in organofluorine compounds is especially interesting in view of its kinetic stability and high bond energy. However, pathways for the biodegradation of fluorinated compounds and the enzymes catalyzing defluorination have been scarcely examined, although some routes are known (37).

Defluorination of fluoroaromatics can occur prior to ring cleavage, e.g. via oxygenases that defluorinate 2-fluorobenzoate (12, 39) or fluorobenzene (9). In other cases, defluorination occurs after ring cleavage via the formation of fluorinated muconolactones (45), which can be produced from 4-fluorobenzoate (21) and fluorobenzene (9) via 4-fluorocatechol. Bacterial and fungal phenol hydroxylases can convert fluorophenols to
fluorocatechols or fluoropyrogallols, which are metabolized to fluoromuconic acids by ring-cleavage dioxygenases (6, 7). Defluorination of 4-fluorophenol prior to ring cleavage has recently been described by us in a strain of *Arthrobacter*, but the enzymatic basis of defluorination was not solved in detail (14).

For chlorinated phenols, two main metabolic routes have been described. Pathways in which the chlorophenol is oxidized to a substituted catechol, in some cases with partial dehalogenation, followed by *ortho*-cleavage of the aromatic ring and post ring-cleavage dehalogenation, occur in bacteria that degrade mono- and dichlorophenols (20, 23, 52, 53). On the other hand, routes in which the substituted phenol is converted via hydroquinone (or a substituted hydroquinone) to maleylacetate are also known, mainly in organisms that grow on polyhalophenols (31, 33, 34, 41, 48, 55). The further aerobic metabolism of hydroquinone may proceed via direct ring fission (8, 35) or via hydroxylation to hydroxyhydroquinone (1,2,4-trihydroxybenzene) (13), which can undergo ring fission by an intradiol dioxygenase (27, 32, 36, 38). Genes for this latter hydroquinone degradation route have been cloned from *Cupriavidus necator* (formerly *Ralstonia eutropha*) strain JMP134 and *Ralstonia pickettii* DTP0602 which both grow on 2,4,6-trichlorophenol (22, 33, 34) and from a strain of *Sphingobium chlorophenolicum* that can grow on pentachlorophenol (10, 40).

Here, we report the characterization of two 4-fluorophenol (4-FP) catabolic gene clusters from *Arthrobacter* sp. strain IF1, an organism that was isolated on basis of its capability to utilize 4-fluorophenol as carbon source for growth (14). We describe the two-component flavin monooxygenase genes that are involved in the initial steps of 4-FP degradation. The activities of the encoded monooxygenase and its associated reductase are also described.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Arthrobacter* sp. strain IF1 was grown in Luria-Bertani (LB) medium or in a synthetic medium (14) at 30°C. *Escherichia coli* BL21(DE3) (Stratagene) was grown in LB medium and when necessary 1 mM of IPTG and 100 µg/ml of ampicillin were added.

**Cloning and sequencing of monooxygenase genes.** The cloning and sequencing of the fluorophenol catabolic gene clusters was done by M. I. M. Ferreira in the laboratory of T. Kudo. DNA isolation and cloning were done as described by Sambrook et al. (44). Genomic DNA was isolated from strain IF1 as previously described (16), and plasmid DNA
with the Large-Construct Isolation Kit from Qiagen. The degenerate primer set and the PCR procedures that were used for the cloning have been described by Ferreira et al. (15).

Genomic DNA was separately digested by \textit{Apa}I and \textit{Bam}HI, and putative monooxygenase sequences were detected by Southern blotting with a DIG-hybridization system as described (15, 25), using a probe obtained by labeling of PCR products that were obtained by amplification from genomic DNA as mentioned above. A band of 5 kb was detected with genomic DNA that was treated with \textit{Apa}I and fragments of this size were cloned into pBluescriptII KS+ (Stratagene) to give library A. Fragments of 9 kb were detected with \textit{Bam}HI-restricted DNA and cloned into pHSG397 (Takara) to give library B. For screening, the libraries were transformed into \textit{E. coli} cells and transformants were inoculated in several Falcon tubes containing 2 ml of LB and chloramphenicol (pHSG397) or ampicillin (pBluescriptII KS+). After overnight growth at 30°C, DNA was isolated and screened for the presence of the 4-FP monooxygenase gene by PCR with the primers used earlier for preparation of the probe. Positive cultures were plated and colonies were screened again by PCR. DNA was isolated from the positive clones, subcloned into pUC19 (Takara), and used for sequencing.

Dideoxy sequencing was done using an ABI PRISM BigDye Ready Reaction kit and ABI Model 3700 sequencer and sequences were analyzed as described (25).

**Sequence analysis comparison and structural model.** The amino acid sequence of FpdA2 was initially compared to those in the databases using the BLASTp program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequences, which showed high similarity, were aligned using the CLUSTAL W program (www.ebi.ac.uk/clustalW).

A predicted structure was obtained using the Swiss-Model server (http://swissmodel.expasy.org) (3), with 4-chlorophenol monooxygenase (CPMO) of \textit{Burkholderia cepacia} AC1100 (PDB 3HWC, unpublished) as the template (61% sequence identity).

**Expression of \textit{fpd} genes in \textit{E. coli}.** The nucleotide sequences of \textit{fpdA1}, \textit{fpdA2} and \textit{fpdB} were amplified with PCR primers (sequences mentioned above) and cloned in pET17b (Novagen) as translational fusions in the \textit{Nde}I restriction site of the vector. \textit{E. coli} BL21(DE3) was used for expression.

**Purification of 4-fluorophenol monooxygenase (FpdA2).** The 4-FP monooxygenase (FpdA2) was purified from \textit{E. coli} BL21(DE3)(pETfpdA2). Cells were grown in LB medium containing ampicillin until the OD$_{600}$ reached 0.5. IPTG was then added (0.5 mM) and the culture was incubated overnight at 20-22°C with shaking. Cells were harvested by
centrifugation, washed twice with TEMG buffer (50 mM Tris-\(\text{SO}_4\), pH 7.5, 0.5 mM EDTA, 1 mM \(\beta\)-mercaptoethanol, 5% glycerol), resuspended in the same buffer, and disrupted by sonication. After centrifugation (40,000 \(\times\) g, 60 min), the extract was loaded on a DEAE Sepharose column (60 ml bed volume) pre-equilibrated with TEMG buffer. FpdA2 was eluted with a linear gradient of 0–0.5 M (\(\text{NH}_4\)\(_2\)\(\text{SO}_4\) in TEMG, concentrated by ultrafiltration (Amicon YM-30 membrane), and separated on a hydroxyapatite column (50 ml) using 10-400 mM potassium phosphate buffer (pH 7.0) containing 1 mM \(\beta\)-mercaptoethanol and 5% glycerol. FpdA2 was concentrated by ultrafiltration and stored at -20°C.

**Purification of flavin reductase (FpdB).** Flavin reductase was purified from *E. coli* BL21(DE3)(pETfpdB), cultivated, induced and lysed as described above for FpdA2. Cell-free extract was fractionated on a DEAE Sepharose column, after which FpdB protein was concentrated and dialyzed against 1.5 M (\(\text{NH}_4\)\(_2\)\(\text{SO}_4\) in TEMG buffer, which caused precipitation. The protein pellet was dissolved in 4 ml TEMG buffer (pH 7.5) and fractionated on a Superdex 200 column (320 ml bed volume) using TEMG buffer containing 0.15 M NaCl. FpdB was concentrated by ultrafiltration and stored at -20°C.

**Analysis of protein by mass spectrometry.** Selected protein bands (A, B and C) from SDS-PAGE (Fig. 4A, lane 1) were excised, destained and digested with trypsin (Progema, Madison, WI, USA). After washing twice with 25 mM ammonium bicarbonate and 50% acetonitrile, gel pieces were dried in a Speed-Vac. For tryptic digestion, dried gel pieces were swollen in 10 ng/\(\mu\)l trypsin solution that was prepared in 100 mM \(\text{NH}_4\)HCO\(_3\) and incubated at 37°C for 12 to 15 h. Peptides were recovered by adding a mixture of 75% acetonitrile and 25% of 5% formic acid in water. Samples from digested proteins were prepared for MS by mixing 0.5 \(\mu\)l of the sample with 0.5 \(\mu\)l matrix solution (5 mg/ml \(\alpha\)-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetate) and spotted on a stainless steel 192-well target plate. They were allowed to air dry at room temperature, and analyzed on a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) MALDI-TOF/TOF mass spectrometer. For MS spectra, 1500 laser shots were acquired, and subsequently precursors from the resulting peptide spectra in the \(m/z\) range 840-4000 with a signal-to-noise threshold of 50 were automatically selected for analysis by MS/MS, with a maximum of 25 precursors per spot, excluding the most commonly observed peptide peaks of trypsin and keratin.

**Enzyme assays.** 4-Fluorophenol monooxygenase was measured at 25°C in incubations containing 50 mM phosphate buffer (pH 7.0), a suitable amount of
monooxygenase, 1 mM ascorbic acid, 10 µM FAD, 3 µg of reductase (FpdB), 180 U ml\(^{-1}\) catalase (Fluka), 2.5 mM NADH, and 400–600 µM of substrate. Reactions were started by adding NADH. Samples of 25 µl were taken with intervals of 5–25 min and quenched by addition of HPLC eluent (see below). The samples were centrifuged and the supernatants were analyzed by HPLC. One unit of enzyme activity corresponds to one µmole of 4-FP converted per min.

Flavin reductase activity was determined by following the oxidation of NADH at 340 nm (\(\varepsilon_{340} = 6.22\) mM\(^{-1}\) cm\(^{-1}\)). Reaction mixtures contained 50 mM phosphate buffer (pH 7.5), 300 µM NADH, and 100 µM FAD or FMN. The reaction was initiated by adding enzyme and initial rates were used for calculating kinetic parameters.

**Analytical methods.** Isocratic HPLC of 20 µl samples was carried out using a Lichrospher 100 RP8 reversed-phase column (250 mm × 4.6 mm, 5 µm particle size) in connection with Jasco PU-980 pumps, a Jasco MD-910 diode array detector, and a Jasco UV-2075 detector. The mobile phase (1 ml min\(^{-1}\)) was 70/30 (v/v) acetic acid/methanol containing 0.02 M ammonium acetate, pH 4.5.

For gas-chromatographic analysis, samples (300 µl) were extracted with an equal volume of ethyl acetate containing mesitylene as internal standard followed by analysis on a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector and a HP-5 column (30 m × 0.25 mm × 0.25 µm) (Agilent 19091J-413). Helium (1 ml min\(^{-1}\)) was the carrier gas and the temperature was 5 min at 50°C, followed by 15°C per min increase to 250°C.

Fluoride, chloride, bromide and nitrite were measured in 50 µl samples using a Dionex DX 120 ion chromatograph (Dionex, Sunnyvale, CA, USA) equipped with an Alltech A-2 anion column (100 × 4.6 mm, 7 µm) and an Alltech guard column (50 × 4 mm). The eluent was a mixture of NaHCO\(_3\) and Na\(_2\)CO\(_3\) in deionized water with a flow rate 1.2 ml min\(^{-1}\).

**RESULTS**

**Cloning of 4-FP monooxygenases genes.** In order to obtain the 4-FP degradation genes from strain IF1, we first used a PCR approach. Using degenerate primers, designed on basis of the alignment of six published gene sequences of two-component aromatic monooxygenases (17, 24, 30, 33, 43), a 731 bp PCR product was obtained with DNA of
strains IF1 as the template. Its nucleotide sequence revealed similarity with 4-nitrophenol-(42) and 4-chlorophenol-monooxygenase genes (38). Hybridization analysis of this labeled PCR fragment with genomic DNA restricted with ApaI, BamHI, EcoRI, HindIII or PstI gave two positive signals in each case, whereas four bands were obtained when the DNA was restricted with SalI. Since the probe contained only one SalI restriction site, this indicates that strain IF1 has two highly similar or identical copies of the 4-FP monooxygenase gene, with different flanking regions.

Based on the hybridization results, two DNA libraries were constructed and screened for the presence of the 4-FP monooxygenase through PCR, which yielded a positive clone from each library. The sequence of the insert of an ApaI clone consisted of 5,145 bp (cluster A). For a BamHI clone, an insert of 9,373 bp was found (cluster B). BLAST sequence similarity searches with the deduced amino acid sequences of the ORFs identified a number of homologs, allowing annotation (Fig. 1). Both clusters have a large segment that is similar to a p-nitrophenol catabolism gene cluster (accession number EF052871) of Arthrobacter sp. JS443 (42). The putative genes involved in 4-fluorophenol degradation were designated *fpd*.

![Fig. 1. Organization of the ORFs in the *fpd* gene regions of *Arthrobacter* sp. strain IF1. The size and direction of each ORF is represented by the open arrows. ORFs that are expected to be involved in 4-fluorophenol metabolism are indicated with *fdp*. Regions of high similarity between cluster A and cluster B are indicated in black lines. Regions of high similarity between the clusters indicated and 4-nitrophenol (42) region are indicated with a dashed pattern. Regions of lower similarity between cluster B and the 4-NP gene region are indicated with a dotted pattern.](image-url)
**FIG. 2.** Partial sequence alignment of 4-fluorophenol monooxygenase (FpdA2) with 4-nitrophenol monooxygenase (accession no. ABL75143, 4NPMO), 4-chlorophenol mono-oxygenase (PDB 3HWC) and 4-hydroxyphenylacetate 3-monooxygenase (PDB 2YYG). Residues discussed in the text are highlighted and in bold font.

**Sequence and structural alignment of FpdA2.** Each cluster contained a putative monooxygenase gene, and these were designated fpdA1 and fpdA2 for cluster A and B, respectively. They are 93% identical at the DNA sequence level and 98.9% identical at the deduced amino acid sequence level. The closest homologs of which the function is established are the hydroxylase proteins of the two-component 4-nitrophenol monoxygenase.
monooxygenases (4NPMO) from strains *Arthrobacter* sp. JS443 (99% amino acid sequence identity, (42)) and *Rhodococcus opacus* SAO101 (72% identity, (30)), the hydroxylase component of 4-chlorophenol monooxygenase (3HWC) from *Arthrobacter chlorophenolicus* A6 (96% identity, (38)) and 4-hydroxyphenylacetate 3-hydroxylase (2YYG) by *Thermus thermophilus* HB8 (29). The sequences of these monooxygenase and the alignment is presented in Fig. 2. These monooxygenase systems consist of a reductase that reduces FAD at the expense of NADH and a hydroxylase that uses FADH\(_2\) and O\(_2\) to hydroxylate the substrate, and are classified as Class D flavoprotein monooxygenases (TC-FDM) (51).

**FIG. 3.** Modeling of FpdA2. Left: alignment of the predicted FpdA2 structure (pink, grey) with the crystal structures of 4HPAMO (2YYG, blue) and 4CPMO (3HWC) (green). 1, poorly aligned loop with L\(^{199}\); 2, loop with G\(^{443}\); 3, hydroxyphenylacetic acid in 2YYG; 4, flavin isoalloxazine ring. Right: overlay of the model structure of FpdA2 with the flavin and substrate of 4HPAMO (2YYG).

A predicted structure for FpdA2 was obtained using the Swiss-Model server with 4-chlorophenol monooxygenase (CPMO) of *Burkholderia cepacia* AC1100 (3HWC, unpublished) as the template (61% sequence identity). The resulting structure was compared to those of 4-hydroxyphenylacetate 3-monooxygenase (4HPAMO, 2YYG) from *Thermus thermophilus* HB8 (29) and CPMO (3HWC) (Fig. 3). In the tetrameric 4HPAMO, the part of the flavin-binding site that accommodates the AMP group of subunit A has contacts with subunit D, but the substrate-binding site is formed by residues from a single subunit. The active site regions around the substrate-binding site were reasonably well aligned, with the
major difference being longer loops surrounding the entrance to the active site in the 4HPAMO structure as compared to the other two enzymes (Fig. 3). These loops are S$^{197}$-TLLQ and F$^{441}$-FG (loop β5-β6, (29)). The latter region was predicted for the flavin-free form of FpdA2 due to the fact that the 4CPMO structure is that of the apo enzyme and the β5-β6 loop with F$^{447}$ clashes with the predicted FAD binding site. Another region where the proteins differed quite a lot is R$^{151}$-ARPPS.

In 4HPAMO with substrate-bound (4-hydroxyphenylacetate), clear interactions between substrate and enzyme are visible. The substrate is bound between L$^{143}$ and the main chain of helix 14. The position of L$^{143}$ is not conserved in FpdA2 and the most likely candidate for an interaction with the phenyl group is F$^{288}$. F$^{442}$ in the 4HPAMO structure has an edge-plane interaction with the aromatic moiety of the substrate. This is present as F$^{448}$ in the FpdA2 region that changes position upon FAD binding, and thereby contributes to the formation of the substrate-binding site. The proposed hydrogen bond donors (29) for the hydroxyl group of phenolic substrates are Y$^{104}$ and H$^{139}$, which are not conserved in FpdA2 and 4CPMO. Also, residue S$^{197}$ of 4HPAMO is missing in the latter two enzymes, in agreement with the observation that the S$^{197}$ interacts with the carboxylate of 4-hydroxyphenylacetate. The arginine that is proposed to stabilize the peroxyflavin is conserved in all three enzymes (R$^{100}$ in FpdA2).

The flavin-reductase gene. A putative reductase gene, termed fpdB, was only detected in cluster B. It is located upstream of fpdA2 on the opposite strand and the encoded protein has high similarity to the reductase component of a similar monoxygenases from *Arthrobacter* sp. JS443 ((42), 92% amino acid sequence identity), *R. opacus* SAO101 ((30), 47% identity) and *A. chlorophenolicus* A6 (86% identity). Analysis of FpdB with the Pfam database showed the presence in the N-terminus of a flavin reductase-like domain (Pfam01613), characteristic for proteins that provide reduced FAD to the hydroxylase component. The C-terminal segment of FpdB aligns weakly with the N-terminal segment of GntR-type transcriptional regulators (Pfam00392), indicating the presence of a C-terminal regulator domain.

Other genes involved in the degradation of 4-FP. Some other genes presumably involved in haloaromatic metabolism were detected (Fig. 1). In cluster A, the ORFs fpdD and fpdE encode proteins with sequence similarity to maleylacetate reductases that are involved in the degradation of p-nitrophenol (30, 42), and α/β-hydrolase fold family enzymes, respectively. The translated sequences from ORF5 and ORF6 in cluster A showed the highest similarity to proteins involved in conjugational plasmid transfer (pfam02534.12). In cluster
B, ORF \textit{fpdC} encodes a putative protein with high similarity to hydroxyquinol dioxygenases, e.g. the dioxygenase involved in 4-chlorophenol degradation (38), and ORF \textit{fpdX} encodes a putative periplasmic binding protein. An ORF designated \textit{fpdR} is present in front of the \textit{fpdA2} and may encode a transcriptional regulator because of the presence of a nucleotide-binding domain and a helix-turn-helix motif. It is similar to putative regulator genes encoded in \textit{p}-nitrophenol and 4-chlorophenol degradation gene clusters (38, 42). Finally, ORF \textit{fpdT2} with unknown function occurs at a similar position in the \textit{p}-nitrophenol gene cluster (42).

**Properties of 4-FP monooxygenase (FpdA2) and flavin reductase (FpdB).** To confirm the activity of the proteins encoded by the putative monooxygenase genes, \textit{fpdA2} and \textit{fpdB} were expressed in \textit{E. coli} BL21(DE3), yielding proteins with molecular masses of approximately 62 and 30 kDa. Purification of FpdA2 was achieved by a protocol that involved two chromatographic steps (Table 1 and Fig. 4A). Solutions of purified FpdA2 were colorless and showed no absorption in the region of 320-500 nm, which suggests that FpdA2 does not contain a flavin cofactor. The reductase component (FpdB) was also purified by column chromatography, after which only one band was detected by SDS-PAGE (Fig. 4B). Gel filtration chromatography indicated that FpdB behaves as an octamer. The FpdB protein used NADH to reduce either FAD or FMN (Table 2), but did not use NADPH or riboflavin as a substrate.

**FIG. 4.** (A) SDS-PAGE of purification steps of recombinant 4-fluorophenol monooxygenase from \textit{E. coli} BL21(DE3). Lane 1, inactivated 4-FPMO; lane 2, marker protein; lane 3, cell-free extract; lane 4, DEAE fraction; lane 5, hydroxyapatite fraction.

(B) SDS-PAGE of purification steps of recombinant flavin reductase from \textit{E. coli} Top10. Lane 1, marker protein; lane 2, cell-free extract; lane 3, DEAE fraction; lane 4, hydroxyapatite fraction.
In the presence of reductase, the activity of the purified hydroxylase was 160 nmol/min.mg protein. This would require the enzyme being present in strain IF1 at a level of at least 5-10 percent of the total cellular protein to allow the observed growth rate ($\mu = 0.1 \text{ h}^{-1}$), assuming a yield of about 50 mg cells per mmol fluorophenol consumed (14).

**Inactivation of purified 4-FPMO.** Purified 4-FPMO was active in fresh cell-free extract that was prepared by breaking the cells by sonication. However, purified 4-FPMO lost 40% of its activity when stored at -20°C for 24 h and became completely inactive within three days. Changing the concentration of EDTA in TEMG buffer did not cause an improvement of stability. A modified procedure in which cell-free extract was prepared in sonication buffer that was complemented with a commercially available protease inhibitor cocktail (one tablet of Mini Complete per 10 ml solution; Roche) did improve stability up to > 99%. The results suggest that FpdA2 is highly sensitive to small amounts of an *E. coli* protease that were not completely removed during purification, even though *E. coli* BL21(DE3), which is devoid of the major proteases like OmpT was used for expression.

**TABLE 1.** Purification of FpdA2 from *E. coli* BL21(DE3) pET17b.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total enzyme activity a (U)</th>
<th>Specific activity (U mg$^{-1}$)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>38</td>
<td>1350</td>
<td>9.5</td>
<td>0.007</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE Sepharose</td>
<td>15</td>
<td>250</td>
<td>7.5</td>
<td>0.030</td>
<td>79</td>
<td>4.3</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>7</td>
<td>38</td>
<td>6.0</td>
<td>0.160</td>
<td>63</td>
<td>23</td>
</tr>
</tbody>
</table>

*a* Reaction mixtures contained 400 $\mu$M of 4-FP, a suitable amount of FpdA2, and the components given under Materials and Methods.

An SDS-PAGE of the inactivated purified protein (prepared from enzyme stored at -20°C) showed three bands instead of the single prominent band that was present in the original purified enzyme. The three bands were designated as A, B and C (Fig. 4A, lane 1), and they indicate that part of the protein is truncated, accompanied by loss of activity.
TABLE 2. Kinetic parameters of FpdB $^a$.

<table>
<thead>
<tr>
<th>Fixed substrate</th>
<th>Varied substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD$^b$</td>
<td>NADH</td>
<td>13 ± 2</td>
<td>33 ± 4</td>
<td>2.5 × 10$^6$</td>
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<tr>
<td>FMN$^b$</td>
<td>NADH</td>
<td>122 ± 14</td>
<td>12 ± 2</td>
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<td>NADH$^b$</td>
<td>FMN</td>
<td>10 ± 1</td>
<td>63 ± 3</td>
<td>6.6 × 10$^6$</td>
</tr>
<tr>
<td>NADH$^b$</td>
<td>FAD</td>
<td>6 ± 1</td>
<td>23 ± 2</td>
<td>3.6 × 10$^6$</td>
</tr>
</tbody>
</table>

$^a$ Values refer to the varied substrate and are means of triplicate experiments with standard deviations.

$^b$ FAD and FMN concentrations were fixed at 100 µM, NADH at 300 µM.

**Analysis of the inactive protein.** To establish if truncation of FpdA2 occurs with purified enzyme, and if so, what the site of truncation is, we carried out a mass spectrometry analysis of tryptic peptides that were generated by trypsin treatment of protein present in excised gel pieces. The results of the mass spectrometry analysis are presented in Fig. 5 and Table 3. The reduction in mass of 6-15 kDa suggests that cleavage is close to the N- or the C-terminus of the enzyme. A peak of mass 1321.66 is present (weakly) in all bands, and it corresponds to peptide DVLGFGDTAELGK at D$^{478}$-K$^{490}$ (Fig. 6). This suggests that the cleavage (protein breakdown to form bands B and C) did not occur before K$^{490}$ if it is close to the C-terminus. A peak of mass 910.44 is clearly observed in band A and B but probably absent in band C. There is a peak at 909.40 Da which may obscure the 910.44 Da peak, but in both A and B, peak 910 is much more intense than peak 909. Therefore, we conclude that this peptide with mass 909.40 Da is absent in band C. The 910.44 peak corresponds to the peptide FQPEYAR found at the position F$^{506}$-R$^{512}$. The peptide S$^{513}$-A$^{535}$ was not seen in any of chromatograms of band A, band B and band C. Its mass is 2.39 kDa. There were 3 more peptides on the C-terminal side of the protein that were found in bands A and B: F$^{434}$-R$^{441}$ (970.50 Da), T$^{464}$-R$^{477}$ (1590.81 Da) and D$^{478}$-R$^{491}$ (1477.76 Da). If the 1321.66 Da peak mentioned above is wrongly assigned to peptide D$^{478}$-K$^{490}$ (which is possible since this is weak), we can conclude that the protein in band C is truncated after residue K$^{408}$, since the peak at 1168.59 Da (E$^{399}$-K$^{408}$) is clearly present in all bands.
**TABLE 3.** List of peptide sequences obtained from MALDI/TOF-TOF analysis and selected for the comparison of bands A, B and C. The highlighted peptides were observed in bands A and B only and absent in band C. Molecular masses of peptides with low intensity are presented under parenthesis. The highlighted peptides were found in band A and band B, but not in band C.

<table>
<thead>
<tr>
<th>A (mass)</th>
<th>B (mass)</th>
<th>C (mass)</th>
<th>Peptide sequence</th>
<th>Position</th>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1195.62)</td>
<td>1195.6470</td>
<td>-</td>
<td>TGKEYLESRL</td>
<td>3-12</td>
<td>1.20</td>
</tr>
<tr>
<td>909.4700</td>
<td>909.4676</td>
<td>909.4660</td>
<td>EYLESLR</td>
<td>6-12</td>
<td>0.91</td>
</tr>
<tr>
<td>1045.5103</td>
<td>1045.5114</td>
<td>(1045.52)</td>
<td>NYADFIFR</td>
<td>84-91</td>
<td>1.05</td>
</tr>
<tr>
<td>-</td>
<td>(1366.75)</td>
<td>-</td>
<td>NGRDLSGNIQR</td>
<td>127-138</td>
<td>1.37</td>
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<td>902.4705</td>
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<td>902.47</td>
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</tr>
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<td>1754.8494</td>
<td>1754.8428</td>
<td>1754.8337</td>
<td>DLVSPMFVDQYDR</td>
<td>147-161</td>
<td>1.76</td>
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<tr>
<td>1466.8270</td>
<td>1466.8336</td>
<td>1466.8300</td>
<td>NPVQSQLAELIR</td>
<td>322-334</td>
<td>1.47</td>
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<tr>
<td>954.5686</td>
<td>954.5662</td>
<td>954.5699</td>
<td>GIVIQPTAR</td>
<td>390-398</td>
<td>0.95</td>
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<tr>
<td>1168.5933</td>
<td>1168.5938</td>
<td>1168.6110</td>
<td>ELDHPYIGPK</td>
<td>399-408</td>
<td>1.17</td>
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<tr>
<td>970.5000</td>
<td>970.5060</td>
<td>-</td>
<td>FLTEFGTR</td>
<td>434-441</td>
<td>0.97</td>
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<tr>
<td>1590.8101</td>
<td>1590.8570</td>
<td>-</td>
<td>TEYQVDGPLTQLAR</td>
<td>464-477</td>
<td>1.59</td>
</tr>
<tr>
<td>(1321.64)</td>
<td>(1321.68)</td>
<td>(1321.56)</td>
<td>DVLGFGDTAELGK</td>
<td>478-490</td>
<td>1.32</td>
</tr>
<tr>
<td>(1477.7)</td>
<td>1477.7622</td>
<td>-</td>
<td>DVLGFGDTAELGKR</td>
<td>478-491</td>
<td>1.48</td>
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<tr>
<td>910.4478</td>
<td>910.4485</td>
<td>-</td>
<td>FQPEYAR</td>
<td>506-512</td>
<td>0.91</td>
</tr>
</tbody>
</table>

The molecular weights of bands A, B and C (Fig. 4A, lane 1) were calculated using a standard curve in which we plotted the log of the molecular mass of the proteins vs. the mobility of protein. This gave masses 61 kDa, 56 kDa and 50 kDa, respectively. The difference between bands A and C is 11 kDa, which for a C-terminal truncation would mean that the band C protein ends around residue R\textsuperscript{433}, which is consistent with the observed peptide masses which predict that the protein is cleaved between K\textsuperscript{399} and R\textsuperscript{441}, meaning that it must be between K\textsuperscript{408} and R\textsuperscript{441} in view of what is explained above.

There may be also a difference at the N-terminus. Peak 1195.6 Da (T\textsuperscript{3}-R\textsuperscript{12}) is present in B and weakly in A but absent in band C. The E\textsuperscript{6}-R\textsuperscript{12} peptide (909.6 Da) is present in all three bands, so only residues M\textsuperscript{1}-K\textsuperscript{5} may be absent in band C. There are no peptides that indicate a difference between bands A and B. A short truncation at the C-terminus is possible, but it can account at most for 2.4 kDa, which is less than the mass difference observed on gel. The difference between bands A/B, and C is likely due to a C-terminal truncation.
FIG. 5. MALDI/TOF spectra of (A) band A, (B) band B, and (C) band C of the inactive FpdA2 presented in lane 1 in figure 4A.
FIG. 6. Sequence of FpdA2 showing the peptides (highlighted) that are observed in bands A and B and absent in band C. Peptides that were not observed in band C (Fig. 4A, lane 1) indicate that truncation may have occurred in peptides T\(^3\)-R\(^{12}\) and/or L\(^{409}\)-R\(^{441}\). The underlined peptide was not detected in any of the bands.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate (µmol/min. mg(^{-1}))</th>
<th>Substrate degraded (^b) (µM)</th>
<th>Anion formed (^b) (µM)</th>
<th>Hydroquinone (^b) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Fluorophenol</td>
<td>46</td>
<td>344</td>
<td>312</td>
<td>145</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>39</td>
<td>292</td>
<td>279</td>
<td>92</td>
</tr>
<tr>
<td>4-Bromophenol</td>
<td>72</td>
<td>541</td>
<td>492</td>
<td>98</td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>40</td>
<td>303</td>
<td>280</td>
<td>150</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>64</td>
<td>130</td>
<td>-</td>
<td>10 (^c)</td>
</tr>
</tbody>
</table>

\(^a\) Reaction mixtures contained 600 µM of substrate, 0.15 mg ml\(^{-1}\) of 4-FPMO and components described in Materials and Methods. Aromatics were analyzed by HPLC or GC at different times, and anions by ion chromatography.

\(^b\) After 20 min incubation.

\(^c\) Hydroxyhydroquinone detected by GC.
Conversion of phenols by FpdA2 and FpdB. The purified FpdA2 in combination with FpdB transformed various substituted phenols with release of the respective anions (Table 4). No transformation was observed in the absence of NADH or FpdB. The highest transformation rate was observed with 4-bromophenol, whereas 4-chlorophenol, 4-fluorophenol, and 4-nitrophenol were converted at a lower rate. The disappearance of the \textit{para}-substituted phenols was accompanied by the release of more than 90\% of the substituent as halide or nitrite anions and formation of hydroquinone (Table 4).

The transformation of 4-FP into hydroquinone occurs in two steps. FpdA2 and FpdB transform 4-FP into benzoquinone, which is subsequently oxidized to hydroquinone chemically.

Slow conversion of hydroquinone was also observed, both in incubations where it was formed from a 4-substituted phenol and in incubations where it was added from the start, explaining that the amount of hydroquinone detected was lower than the amount of phenol converted. Some formation of trihydroxybenzene was seen by HPLC when hydroquinone was added (Table 4), but the instability of trihydroxybenzene, leading to brown products, made it impossible to establish mass balances and rates were too low to conclude that FpdA2 is responsible for hydroquinone metabolism. 4-Nitrocatechol was not formed from 4-nitrophenol or degraded by purified FpdA2 and FpdB.

DISCUSSION

The 4-FP degradation genes from \textit{Arthrobacter} sp. strain IF1 were detected on two different but related gene clusters. Cluster A harbors \textit{fpdA1DE}, which comprise a reduced FAD-dependent monooxygenase, a putative maleylacetate reductase and a hydrolase gene, while in cluster B \textit{fpdA2} encodes a similar monooxygenase, \textit{fpdB} a flavin reductase, and \textit{fpdC} a putative hydroxyquinol dioxygenase (Fig. 1). The sequences suggest that the 4-FP monooxygenases belong to the Class D flavoprotein monooxygenases, from which a 4-hydroxyphenylacetate 3-hydroxylase can be regarded as a prototype (17, 18, 43). Several enzymes of this class have been described to act on aromatic substrates such as 4-hydroxyphenylacetate (18, 43) or substituted phenols (24, 30, 33, 38, 48). Expression of the \textit{fpdA} genes, especially \textit{fpdA2}, is induced during growth on 4-FP, as indicated by Northern hybridization and RT-PCR, in agreement with a role in 4-fluorophenol metabolism (15). When 4-nitrophenol was the inducer, only the \textit{fpdA2} gene was well expressed, indicating that FpdA1 is not primarily involved in 4-nitrophenol metabolism in strain IF1 (15).
Comparison of the regions flanking the \textit{fpdA} genes led to the identification of a segment of about 290 nucleotides that is present upstream of the monooxygenase genes in both clusters and that probably was copied together with the monooxygenase gene in a duplication event. Since the sequence present in cluster A is more complete (larger) than the one found in cluster B, it is likely that \textit{fpdA2} of cluster B was copied from (a predecessor of) cluster A during the assembly of the clusters. Thus, cluster A may have served as the source of DNA segments for a new cluster that is involved in 4-fluorophenol transformation. The deduced amino acid sequences and gene organization in cluster B further indicate a close evolutionary relationship with a cluster involved in hydroxyquinol degradation in \textit{A. chlorophenolicus} A6 (38). An even more similar gene cluster was isolated by Perry and Zylstra from the 4-nitrophenol degrader \textit{Arthrobacter} sp. strain JS443 (42). Strain IF1 also grows on 4-nitrophenol (14), and 4-nitrophenol induced the expression of the \textit{fpdA2} gene.

Like the majority of the genes encoding a Class D flavoprotein monooxygenases (or TC-FDM family), \textit{fpdA2} has a reductase gene (\textit{fpdB}) in its proximity. This reductase has two distinct domains: the flavin reductase domain that is common to this family of enzymes, and a C-terminal region with a helix-turn-helix (HTH) motif, that has not been described for these proteins (22, 30, 33, 47), and is also present in the C-terminal region of the reductases encoded in the 4-chlorophenol and 4-nitrophenol gene clusters (38, 42). Based on sequence similarity to a putative regulator from \textit{Thermotoga maritima} (29\% identity) of which the three-dimensional structure was recently solved, it can be predicted that the C-terminal region of FpdB forms a domain entailing three $\alpha$-helices and a small $\beta$-sheet of two strands: a 2 stranded winged HTH, which is one of the two major structural HTH classes (2). HTH domains incorporated in enzymes may have a role in substrate recognition, localization, or feedback regulation of metabolic pathways (1, 2).

The purified FpdA2 appeared unstable and lost its activity when stored at -20°C. When the inactivated protein was loaded on SDS-PAGE, three bands of 61 kDa, 56 kDa and 50 kDa (A, B and C) were observed, which clearly indicates the degradation of the protein due to proteolytic activities. The presence of proteases in the host organism \textit{E. coli} BL21(DE3) could have caused some cleavage of 4-FPMO, which was prevented by adding protease inhibitors. It has been reported that wild-type cyclohexane monooxygenase (CHMO) after 24 h incubation in buffer had a residual activity of 20\%, whereas after incubation with protease inhibitor cocktail (PIC), the residual activity was about 60\%. On the contrary, the stability of the recombinant enzyme was not substantially affected by the presence of PIC (46).

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Analysis of the data obtained from mass spectrometric analysis of each band indicates that 4 different peptides situated at C-terminus were absent in band C, which had the lowest molecular weight, while all of them were present in bands A and B. The absence of these four peptides, (F$^{434}$-R$^{441}$, T$^{464}$-R$^{477}$, D$^{478}$-R$^{491}$ and F$^{506}$-R$^{512}$) indicated that protein truncation could have occurred around residue R$^{433}$, which would cause the release of a 11 kDa fragment of 4-FPMO, which also corresponds to the difference in masses between band A and band C proteins. The Rossmann sequence motif (GXGXXG/A) which serves as NAD binding domain (50), is situated at G$^{481}$-A$^{486}$ (Fig. 6), and truncation in peptide F$^{434}$-R$^{441}$ or T$^{464}$-R$^{477}$ would cause destruction of the binding site for NAD which might be the cause of the loss of activity. There are no typical protease sensitive sites in this region, such as the lysine-lysine pair that causes a similar C-terminal truncation during large-scale production of halohydrin dehalogenase in E. coli (C. Tarabiono, unpublished observation).

**FIG. 7.** Proposed catabolic pathway for 4-fluorophenol degradation. See text for details.

The FpdA2 and FpdB proteins, purified from E. coli transformants, were capable of catalyzing hydroxylation reactions with 4-substituted phenols and released the corresponding anions. The kinetic properties of FpdB (Table 2) are comparable to those of other flavin reductases. The $k_{cat}$ is lower than that of the reductase of a trichlorophenol monooxygenase (TcpX, (5)) but the kinetic parameters are similar to those of the reductase of a chlorophenol 4-monooxygenase system (TftD, (19)).
On the basis of genetic and biochemical information we propose a pathway for 4-FP degradation by Arthrobacter sp. strain IF1 that starts with monooxygenation at the para position, with release of fluoride (Fig. 7). Others and we observed the formation of hydroquinone during transformation of 4-fluorophenol (14), 4-chlorophenol (4), or 4-nitrophenol (27, 42). However, if one NADH is consumed per monooxygenase catalytic cycle with anion release, the aromatic product should be benzoquinone. Reduction of benzoquinone to hydroquinone is possible, and a distinct enzyme for this conversion has been proposed for trichlorophenol (5), penta-chlorophenol (11), and 4-nitrophenol (56) degradation pathways. Strain IF1 may have such a protein but it apparently is not required for formation of hydroquinone in vitro since we also detected this product with purified enzymes. Chemical reduction of a quinone by reduced nicotinamide cofactors was suggested for 2,6-dichloro-benzoquinone (11, 19) and 6-chlorohydroxybenzoquinone (5, 54) and we observed NADH oxidation with benzoquinone. Hydroquinone was slowly converted both by whole cells (14) and by mixtures of FpdA2 and FpdB, with some formation of hydroxyhydroquinone, but at this stage, we are not certain about the physiological significance. For the trichlorophenol (5, 54) and 4-nitrophenol (42) pathways, a hydrolysis reaction catalyzed by the initial monooxygenase was proposed, but we consider it impossible to convert benzoquinone to hydroxybenzoquinone this way because the stoichiometry does not fit.

The product undergoing ring fission in strain IF1 most likely is hydroxyquinol (trihydroxybenzene), either formed by hydroxylation of hydroquinone or by reduction of hydroxybenzoquinone (Fig. 7). The presence of a putative intradiol-cleavage type hydroxyquinol dioxygenase gene (fpdC) supports the possibility that hydroxyquinol is an intermediate in the degradation pathways for para-substituted phenols. The fpdC-encoded dioxygenase would produce maleylacetate, which likely can be converted into β-ketoadipate by the fpdD-encoded maleylacetate reductase (Fig. 7). Together with the work of others (38, 42), our results show that the initial steps of 4-nitrophenol, 4-chlorophenol, and 4-fluorophenol metabolism are highly similar.

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