

Identification and characterization of a novel carboxylesterase (FpbH) that hydrolyzes aryloxyphenoxypropionate herbicides

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Abstract

Objective To identify and characterize a novel aryloxyphenoxypropionate (AOPP) herbicide-hydrolyzing carboxylesterase from *Aquamicrobium* sp. FPB-1.

Results A carboxylesterase gene, *fpbH*, was cloned from *Aquamicrobium* sp. FPB-1. The gene is 798 bp long and encodes a protein of 265 amino acids. FpbH is smaller than previously reported AOPP herbicide-hydrolyzing carboxylesterases and shares only 21–35% sequence identity with them. FpbH was expressed in *Escherichia coli* BL21(DE3) and the

product was purified by Ni–NTA affinity chromatography. The purified FpbH hydrolyzed a wide range of AOPP herbicides with catalytic efficiency in the order: haloxyfop-P-methyl > diclofop-methyl > fenoxaprop-P-ethyl > quizalofop-P-ethyl > fluazifop-P-butyl > cyhalofop-butyl. The optimal temperature and pH for FpbH activity were 37 °C and 7, respectively. **Conclusions** FpbH is a novel AOPP herbicide-hydrolyzing carboxylesterase; it is a good candidate for mechanistic study of AOPP herbicide-hydrolyzing carboxylesterases and for bioremediation of AOPP herbicide-contaminated environments.

Chenghong Wang and Jiguo Qiu have contributed equally to this study.

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Introduction

Aryloxyphenoxypropionate (AOPP) herbicides, that have the basic structure of an ester of 4-oxyphe-noxypropanoic acid (Fig. 1), are potent inhibitors of acetyl-CoA carboxylase (Nie et al. 2011). The most commonly used AOPP herbicides are fluazifop-P-butyl, cyhalofop-butyl, diclofop-methyl, fenoxaprop-P-ethyl, haloxyfop-P-methyl and quizalofop-P-ethyl (El-Metwally and Shalby 2007). AOPPs are effective herbicides for the control of annual and perennial grasses in broadleaf crop fields. However, the wide-spread use of AOPP herbicides has led to large

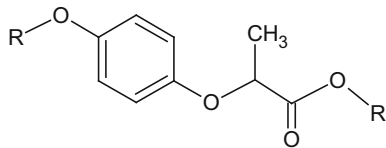


Fig. 1 The basic structure of aryloxyphenoxypropionate (AOPP) herbicides

amounts of them entering into the environment where they have high levels of chronic toxicity toward some aquatic organisms, disturb soil bacterial communities, and injure subsequent rotation crops (Aksoy et al. 2007; Dong et al. 2015; Liu et al. 2015; Trabelsi et al. 2015). Therefore, the behavior and degradation mechanisms of AOPP herbicides in the environment have received considerable attention.

Microorganisms are one of the most important factors in the fate of AOPP herbicides in the environment. Several AOPP-degrading strains have been isolated. Nie et al. (2011) isolated five cyhalofop-butyl-degrading strains from rice field soil. Liu et al. (2015) screened a fenoxaprop-P-ethyl-degrading strain from wheat field soil. Hou et al. (2011) and Dong et al. (2015), respectively, isolated the fenoxaprop-ethyl-degrading strains *Rhodococcus* sp. T1 and *Acinetobacter* sp. DL-2. In these strains, the initial degradation of AOPP herbicides involves the cleavage of the ester linkage, generating the corresponding acid. Four AOPP herbicide-hydrolyzing carboxylesterases have been reported: ChbH (332 amino acids with molecular mass 36 kDa) from *Pseudomonas azotoformans* QDZ-1 (Nie et al. 2011), Feh (380 amino acids with molecular mass 40 kDa) from *Rhodococcus* sp. T1 (Hou et al. 2011) and *R. ruber* JPL-2 (Liu et al. 2015), and AfeH (309 amino acids with molecular mass 34 kDa) from *Acinetobacter* sp. DL-2 (Dong et al. 2015).

In this study, we describe the degradation of AOPP herbicides by a bacterial strain *Aquamicrobium* sp. FPB-1, the cloning of a novel AOPP herbicide-hydrolyzing carboxylesterase gene *fpbH*, and the exogenous expression, purification and characterization of FpbH.

Materials and methods

Chemicals and bacterial growth medium

Fluazifop-P-butyl, fluazifop-P, cyhalofop-butyl, diclofop-methyl, fenoxaprop-P-ethyl, haloxyfop-P-methyl

and quizalofop-P-ethyl were obtained from Sigma-Aldrich and were of analytical grade. Lysogeny broth (LB) consisted of: 5 g NaCl, 10 g tryptone, and 5 g yeast extract per liter water, pH 7. The mineral salts medium (MSM) consisted of: 1.5 g K_2HPO_4 , 0.5 g KH_2PO_4 , 1 g $(NH_4)_2SO_4$, 1 g NaCl, and 0.2 g $MgSO_4 \cdot 7H_2O$ per liter water, pH 7. To make solid medium, 15 g of agar was added per liter. All media were sterilized by autoclaving at 121 °C for 30 min.

Growth and degradation experiments

Aquamicrobium sp. FPB-1 was isolated from a legume field in Jiangsu Province, China (Supplementary Fig. 1). It was grown in LB medium for 12 h, collected by centrifugation ($6000 \times g$, 10 min), washed twice with fresh MSM, and resuspended in 10 ml MSM as the stock culture. The density was adjusted to 480 mg cell dry weight l^{-1} , and then 1 ml stock culture was inoculated in 100 ml MSM supplemented with 10 mg of each respective substrate (fluazifop-P-butyl, cyhalofop-butyl, diclofop-methyl, fenoxaprop-P-ethyl, haloxyfop-P-methyl, or quizalofop-P-ethyl). Cultures were incubated at 30 °C with shaking at 150 rpm. Experiments were performed in parallel in three flasks, and negative controls inoculated with sterilized cells were carried out in the same conditions. Samples used for detecting cell growth and substrate degradation were collected at 5 h intervals. Bacterial growth was monitored by cell dry weight (Glazyrina et al. 2010), and the concentration of each substrate was determined by HPLC as described below.

Gene cloning and sequence analysis

Total DNA was extracted by the standard phenol/chloroform procedure. The fluazifop-P-butyl carboxylesterase gene was cloned by a shotgun method. A size-fractionated genomic library of strain FPB-1 was constructed according to the method described by Wang et al. (2009). The library was plated onto LB-agar containing 100 mg ampicillin l^{-1} and 100 mg fluazifop-P-butyl l^{-1} and incubated at 37 °C for approx. 12 h, and then incubated at 4 °C for about 24 h. Colonies that produced transparent halos were screened and further tested for their ability to degrade fluazifop-P-butyl. The DNA fragment inserted in a positive clone was sequenced using an automatic sequencer. Nucleotide and deduced amino acid

sequence analyses were performed using OMIGA 2.0. Sequence identity analysis was carried out using the BLAST programs at the NCBI (Marco et al. 2014; Arnold et al. 2006; Benkert et al. 2011).

Expression and purification of FpbH

The ORF without its translation stop codon was amplified by PCR with upstream primer 5'-GGGACCCATATGACGATGTTGGGTATGACG (*NdeI* site underlined) and downstream primer 5'-CTGAAAAGCTTTCGTGCATCGTGCTGCGCG (*HindIII* site underlined). Then, the amplified fragment was inserted into the *NdeI-HindIII* sites of expression vector pET-29a(+) to generate plasmid pET-fpbH, which was subsequently transformed into *Escherichia coli* BL21(DE3). Transformants were cultivated in 500 ml LB containing 50 mg kanamycin at 37 °C to 150 mg cell dry weight l⁻¹ and then induced with 0.5 mM IPTG for 24 h at 18 °C. Crude enzyme extracts of *E. coli* BL21 (DE3) were prepared by ultrasonication. The recombinant FpbH was purified with Ni²⁺-NTA resin (Qiagen), collected and dialyzed against 20 mM phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄ l⁻¹ at pH 7.5) overnight at 4 °C to remove imidazole. SDS-PAGE and the Bradford method were used to quantify the protein concentration using bovine serum albumin as a standard.

Enzymatic characteristics

The hydrolase activity of the purified FpbH was determined in 2 ml PBS containing 0.5 mM of each substrate and 0.5 µg purified FpbH. The mixture was incubated for 10 min at 30 °C; the reaction was terminated by adding 0.02 ml of 30% HNO₃ and cooling in liquid N₂. The disappearance of substrates was monitored by HPLC as described below. One unit of hydrolase activity was defined as the amount of enzyme that catalyzed the conversion of 1 µM substrate per min. The effects of temperature, pH, metal ions, and reagents on the enzyme activity were tested as described by Chen et al. (2016). Kinetic values were obtained from Lineweaver–Burk plots for different substrate concentrations in PBS at 30 °C.

Chemical analysis

The samples were initially extracted. Equal volumes of dichloromethane were added to the cultures and

then shaken for 1 h on a reciprocal shaker. The dichloromethane phase was dried by adding anhydrous Na₂SO₄, and then volatilized using a nitrogen stream at room temperature. The residues were dissolved in 200 µl methanol each and filtered through 0.22 µm millipore membranes. For HPLC analysis, a column of Kromasil 100-5-C₁₈ (internal diameter, 4.6 mm; length, 250 mm). The mobile phase was methanol/water (85:15, v/v) at 0.8 ml min⁻¹. Detection was at 230 nm, and the injection was 20 µl. The metabolites were further identified by MS/MS (Finnigan TSQ Quantum Ultra AM thermal triple quadrupole mass spectrometer) according to Dong et al. (2015). In MS/MS, the metabolites were separated, confirmed by standard MS, ionized by electrospray with a positive polarity, and scanned in the normal mass range from 200 m/z (mass to charge ratio) to 600 m/z. The capillary temperature was 350 °C, and the source voltage was 4 kV. The sheath gas was set at 35 arb. Characteristic fragment ions were detected using second-order MS.

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rRNA gene and *fpbH* gene from *Aquamicrobium* sp. strain FPB-1 have been deposited in the GenBank database with accession numbers KJ466199 and KJ466200 respectively.

Results and discussion

Degradation of aryloxyphenoxypropionate (AOPP) herbicides by strain FPB-1

Figure 2 shows the degradation of fluzifop-P-butyl and the growth of strain FPB-1. After 40 h of incubation, 96% fluzifop-P-butyl was degraded by strain FPB-1, in which time the cell dry weight increased from ~7 to ~67 mg l⁻¹. These results indicated that fluzifop-P-butyl could be degraded and utilized as the sole carbon source by strain FPB-1. Strain FPB-1 could also degrade and utilize diclofop-methyl (99%), haloxyfop-P-methyl (97%), quizalofop-P-ethyl (98.5%), fenoxaprop-P-ethyl (97%), and cyhalofop-butyl (96%) in the same conditions.

Members of the genus *Aquamicrobium* are widely distributed in the environment and are known for their abilities to degrade a wide variety of xenobiotic

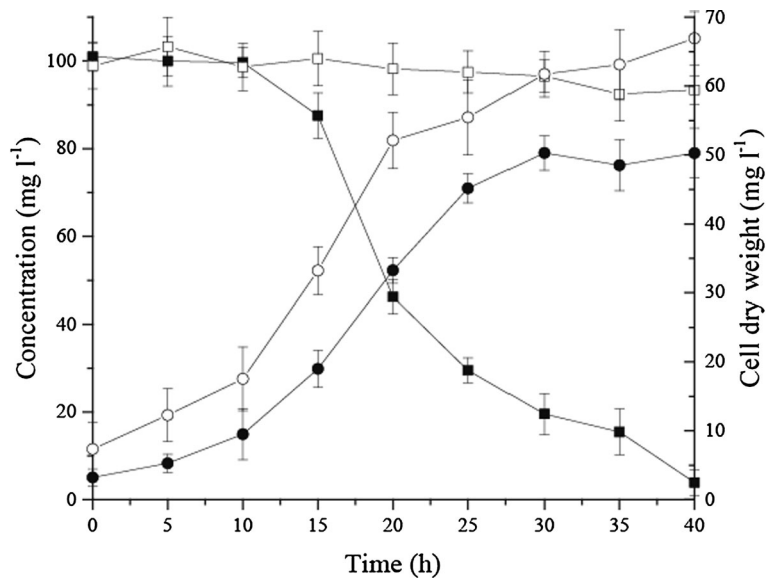


Fig. 2 Degradation and utilization of fluzazifop-P-butyl by *Aquamicrobium* sp. strain FPB-1. The cultures were incubated at 30 °C on a rotary shaker for 40 h. Symbols *opened square* and *filled square* indicate the concentration of fluzazifop-P-butyl in cultures inoculated with sterilized cells and living cells of strain

FPB-1, respectively. For the cultures inoculated with living cells, *filled circle* indicates the concentration of fluzazifop-P, and *opened circle* indicates the cell dry weight of strain FPB-1. All data were derived from three independent measurements, and the error bars indicate standard deviations

pollutants, such as thiophene-2-carboxylate, 2,4-dichlorophenol, 4-chloro-2-methylphenol, 4-chlorophenol and phenol (Bambauer et al. 1998; Fritsche et al. 1999; Kämpfer et al. 2009; Lipski and Kämpfer 2012; Jin et al. 2013). This is the first report of an *Aquamicrobium* strain that is capable of efficiently degrading AOPP herbicides.

Metabolite identification after fluzazifop-P-butyl degradation by strain FPB-1

A metabolite was detected by HPLC in samples taken after 36 h incubation during the degradation of fluzazifop-P-butyl by *Aquamicrobium* sp. strain FPB-1 (Fig. 3a). The metabolite had a retention time of 5.1 min, which was equal to that of authentic fluzazifop-P (Fig. 3b). Tandem mass spectrometry analysis showed a prominent deprotonated molecular ion at $m/z = 326$ $[M - H]^-$ (Fig. 3c) and a fragment ion peak at $m/z = 254$ (loss of a $-CH_3COOH$ group) (Fig. 3d), which were consistent with fluzazifop-P. Thus the metabolite was identified as fluzazifop-P. The fluzazifop-P generated at 40 h (~ 79 mg l⁻¹, ~ 0.24 mM) was approx. equivalent to the amount of fluzazifop-P-butyl (97 mg l⁻¹, ~ 0.25 mM) that had

disappeared from the medium (Fig. 2), suggesting that fluzazifop-P could not be further degraded. These results indicate that fluzazifop-P-butyl was converted to fluzazifop-P through hydrolysis of the ester bond.

Gene cloning and sequence analysis of *fpbH*

A positive clone that produced a transparent halo on assay plates, due to insoluble fluzazifop-P-butyl being transformed to soluble fluzazifop-P, was screened from approx. 40,000 transformants. The inserted fragment in the transformant was 2865 bp and contained five complete ORFs (named *orf1–orf4* and *fpbH* respectively) (Fig. 4). The five ORFs were then subcloned into the linear vector pMD18-T and used to transform *E. coli* DH5 α . *fpbH* was finally confirmed to be the target gene encoding the fluzazifop-P-butyl carboxylesterase (FpbH).

Sequence analysis indicated that *fpbH* consisted of 798 bp and encoded a protein of 265 amino acids with a deduced molecular mass of 28 kDa. The molecular mass of FpbH was smaller than those of the four previously reported AOPP herbicide-hydrolyzing carboxylesterases (each 309–380 amino acids, molecular masses 34–40 kDa). The deduced protein sequence

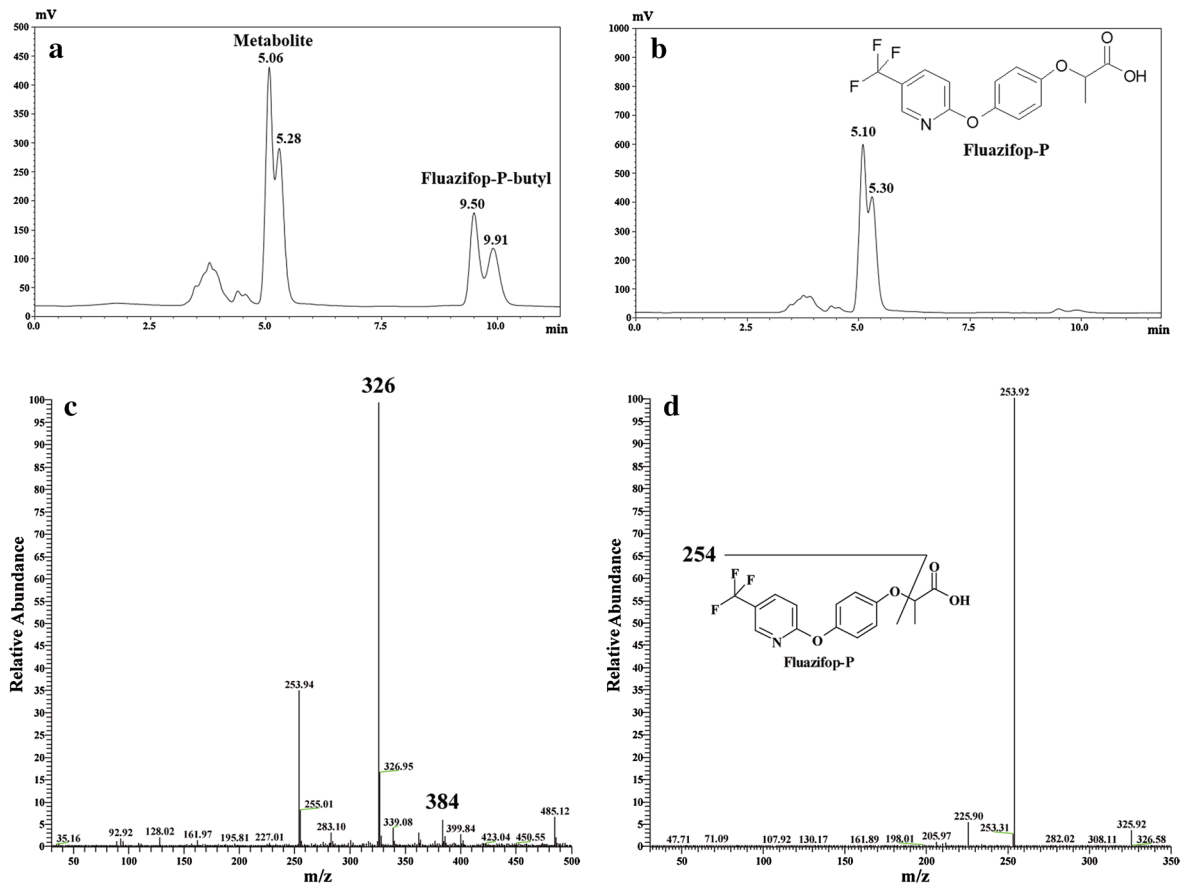


Fig. 3 HPLC and tandem mass spectrometry analysis of metabolite generated during fluazifop-P-butyl degradation by strain FPB-1. **a** HPLC spectrum of metabolite generated during fluazifop-P-butyl degradation. **b** HPLC spectrum of authentic

fluazifop-P standard. **c** MS/MS analysis of the transformation products. **d** The second-order MS analysis of m/z 326 $[M - H]^-$ characteristic ion peak, which was characterized as fluazifop-P

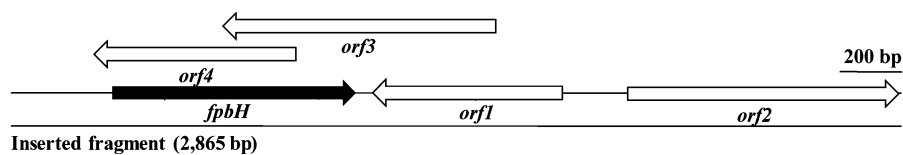


Fig. 4 Organization of the genes involved in fluazifop-P-butyl degradation by strain FPB-1. The arrows indicate the size and direction of transcription of genes. *orf1* encodes a putative

lysine transporter LysE, *orf2* encodes a putative porin, *orf3* and *orf4* encode putative hypothetical proteins, and *fpbH* encodes a putative alpha/beta hydrolase

was searched against the NCBI NR database using the BLASTP program. *Aquamicrobium* sp. strain FPB-1 FpbH showed 98–100% identities with some hypothetical or putative alpha/beta-hydrolases. However, FpbH shared only very low sequence identities, respectively 21, 29, 31 and 35%, with Feh, ChbH, Feh and AfeH, the four previously reported AOPP herbicide-hydrolyzing carboxylesterases. In a phylogenetic tree constructed based on FpbH and related

hydrolases, FpbH formed a subclade with the Fehs from *Rhodococcus* sp. JPL-2 and *Rhodococcus* sp. T1, and EstB from *Burkholderia gladioli* ATCC10248 (Fig. 5); however, the bootstrap value in this node was only 40%. ChbH and AfeH were not clustered with FpbH. The difference in molecular mass and the low sequence identities observed suggest that FpbH differs from the four previously reported AOPP herbicide-hydrolyzing carboxylesterases.

Multiple alignments of amino acid sequences of FpbH with related carboxylesterases showed that FpbH contains the catalytic triad Ser-Asp-His (Ser100, Asp213 and His239) (Fig. 6), which is highly conserved in alpha/beta-hydrolase-fold proteins (Bornscheuer 2002). A model of FpbH (Fig. 6) generated using the SWISS-MODEL server with an alpha/beta-hydrolase template (*Rhodopseudomonas palustris* CGA009 alpha/beta-hydrolase, PDB: 4psu.1.A) also indicates that FpbH contains this catalytic triad, and has the same arrangement of β -sheets and α -helices (β 1- β 2- β 3- α A- β 4- α B- β 5- α C- β 6- α D- β 7- α E- β 8- α F) as a typical alpha/beta-hydrolase (Bornscheuer 2002). These analyses further indicated that FpbH is an alpha/beta-hydrolase-fold protein. Most alpha/beta-hydrolase-fold proteins contain a highly conserved pentapeptide, G-X-S-X-G, which forms a tight turn in a β -strand- α -turn-helix motif (Wang et al. 2009). The G-X-S-X-G motif is present in ChbH from *Pseudomonas azotoformans* QDZ-1 and AfeH from *Acinetobacter* sp. DL-2. However, it is interesting to note that this G-X-S-X-G motif is not rigorous in FpbH (nor in Feh from *Rhodococcus* sp. T1 or *R. ruber* JPL-2), and is present as G-X-S-X-A.

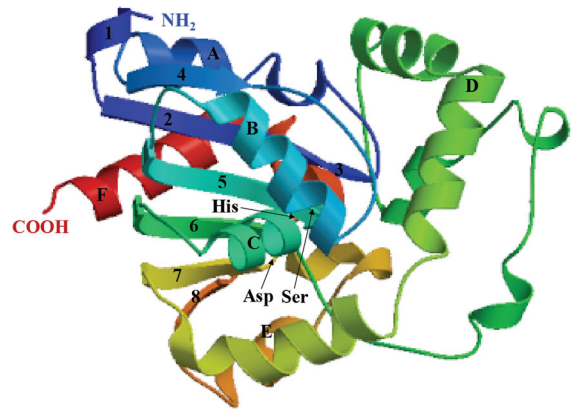


Fig. 6 Proposed model of FpbH generated by SWISS-MODEL using an alpha/beta-hydrolase template (PDB: 4psu.1.A). The β -sheets (1–8) are numbered sequentially and the α -helices are labelled from A to F. The positions of residues in the catalytic triad (Ser-Asp-His) are indicated

Gene expression and purification of His-tagged FpbH

Recombinant FpbH was induced in *E. coli* BL21 (DE3) and purified from the cell-free extract using Ni-nitrilotriacetic acid affinity chromatography. The

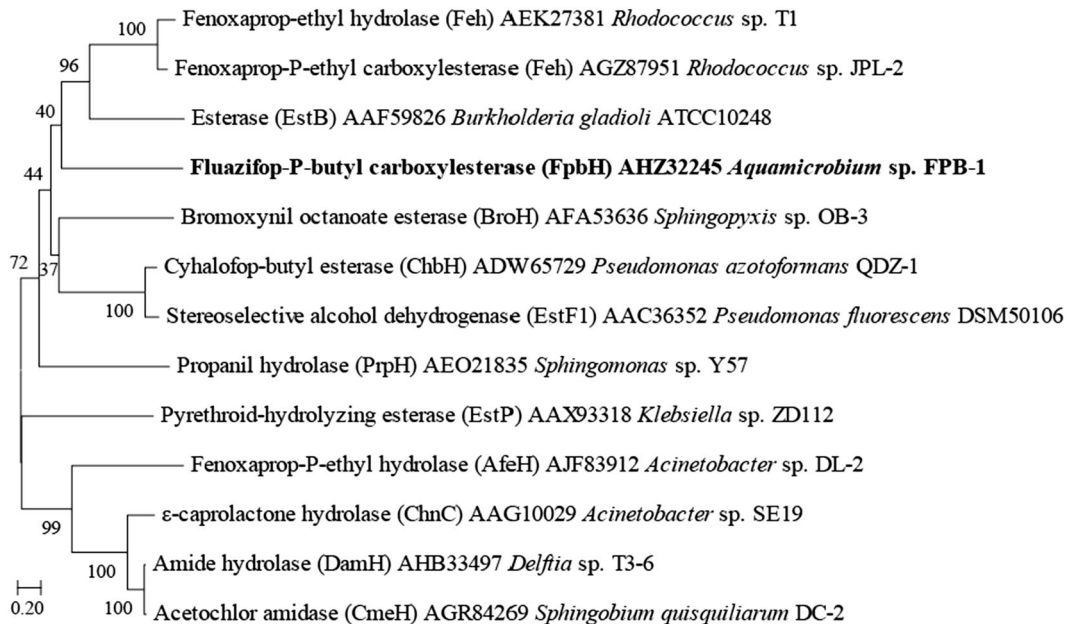


Fig. 5 Phylogenetic tree of FpbH and related proteins. The tree was constructed by the neighbor-joining method using MEGA 7.0. Bootstrap values obtained with 1000 resamplings are

indicated as percentages at all branches. The scale bar indicates 0.2 substitutions per protein position. The GenBank accession number for each protein is shown after the name

purified enzyme showed a single band on SDS-PAGE (Supplementary Fig. 2). The molecular mass of the denatured enzyme was ~ 28 kDa, in agreement with that deduced from its amino acid sequence. HPLC and tandem mass spectrometry analysis demonstrated that FpbH catalyzed the hydrolysis of fluazifop-P-butyl to fluazifop-P (Supplementary Fig. 3).

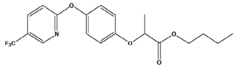
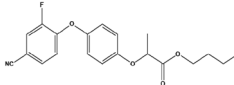
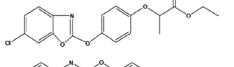
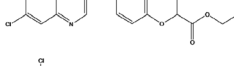
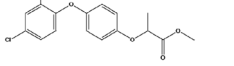
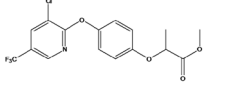
Characterization and kinetic analysis of FpbH

The effect of metal cations at 0.5 and 5 mM, as well as the metal chelating-agent EDTA on the activity of FpbH are shown in Supplementary Fig. 4a. The enzyme activity was not obviously affected by Li^+ or Fe^{2+} and Mg^{2+} . Fe^{3+} , Ca^{2+} and Zn^{2+} showed moderate inhibition and Ag^+ , Al^{3+} , Cu^{2+} , Pb^{2+} , Hg^{2+} , Ni^{2+} , Cr^{2+} , Mn^{2+} and Co^{2+} significantly inhibited the activity (5 mM had a much stronger inhibitory effect than 0.5 mM). The effects of Zn^{2+} , Cu^{2+} and Co^{2+} on the activity of FpbH are quite different to those on AfeH, whose activity was strongly inhibited by Zn^{2+} , slightly inhibited by Cu^{2+} and strongly stimulated by Co^{2+} (Dong et al. 2015). EDTA had little effect on the enzyme activity of FpbH, indicating that it has no requirement for metal ions, which is consistent with observations for ChbH (Nie et al. 2011), FeH (Liu et al. 2015), and

AfeH (Dong et al. 2015). The FpbH activity was detected at 10–60 °C and at pH values from 4 to 10 in the absence of added metal ions; the enzyme showed optimal activity at 37 °C (Supplementary Fig. 4b), which was lower than AfeH (50 °C) (Dong et al. 2015), and pH 7 (Supplementary Fig. 4c), which was lower than AfeH (pH 9) and Feh (pH 7.5) (Dong et al. 2015; Liu et al. 2015).

FpbH hydrolyzed all AOPP herbicides tested. The catalytic efficiency (k_{cat}/K_m) of FpbH for different AOPP herbicides was in the order: haloxyfop-P-methyl > diclofop-methyl > fenoxaprop-P-ethyl > quizalofop-P-ethyl > fluazifop-P-butyl > cyhalofop-butyl (Table 1). Thus, the alkyl chain length of the alcohol moiety strongly affected the biodegradability of the AOPP herbicide; the longer the alkyl chain, the poorer the degradation efficiency. It may be that the increase in length of the alkyl chain of the alcohol moiety increases steric hindrance of the enzyme-substrate interaction. The catalytic efficiency order of FpbH for different AOPP herbicides is different from that of AfeH (fenoxaprop-P-ethyl > haloxyfop-P-methyl > quizalofop-P-ethyl > cyhalofop-butyl), FeH (fenoxaprop-P-ethyl > quizalofop-P-ethyl > cyhalofop-butyl > fluazifop-P-butyl > haloxyfop-P-methyl > diclofop-methyl), and ChbH (quizalofop-P-ethyl \approx fenoxaprop-P-ethyl > cyhalofop-butyl \approx fluazifop-P-butyl > diclofop-meth

Table 1 Kinetic constants for hydrolysis of various herbicides by FpbH from *Aquamicrobium* sp. strain FPB-1

Substrate	Molecular structure	Specific activity ($\mu\text{M min}^{-1} \text{mg}^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)
Fluazifop-P-butyl		7.07 ± 0.17	4.46 ± 0.11	0.43 ± 0.01	10.37
Cyhalofop-butyl		6.62 ± 0.13	4.17 ± 0.08	0.45 ± 0.02	9.27
Fenoxaprop-P-ethyl		8.37 ± 0.35	5.28 ± 0.22	0.42 ± 0.04	12.57
Quizalofop-P-ethyl		7.95 ± 0.22	5.01 ± 0.14	0.39 ± 0.03	12.85
Diclofop-methyl		9.26 ± 0.20	5.92 ± 0.13	0.39 ± 0.04	15.18
Haloxyfop-P-methyl		9.99 ± 0.14	6.31 ± 0.09	0.32 ± 0.02	19.72

yl \approx haloxyfop-P-methyl) (Nie et al. 2011; Dong et al. 2015; Liu et al. 2015), which indicates that the chain length of the alcohol moiety and substitutions on the aromatic ring could affect the enzyme catalytic efficiencies to different extents. Additionally, the catalytic efficiencies (k_{cat}/K_m) of FpbH for different AOPP herbicides ($9.3\text{--}15\ \mu\text{M}^{-1}\ \text{s}^{-1}$) are higher than those of ChbH ($1.9\text{--}3.7\ \mu\text{M}^{-1}\ \text{s}^{-1}$) and FeH ($0.8\text{--}3.5\ \mu\text{M}^{-1}\ \text{s}^{-1}$). These data suggest that FpbH differs from previously reported AOPP herbicide carboxylesterases and could be a good candidate enzyme for biodegradation, especially when diclofop-methyl and/or haloxyfop-P-methyl are the dominant pollutants.

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Supplementary information Supplementary Fig. 1—Phylogenetic tree based on the 16S rRNA gene sequences of strain FPB-1 and related species.

Supplementary Fig. 2—SDS-PAGE of His-tagged FpbH.

Supplementary Fig. 3—HPLC and tandem mass spectrometry analysis of metabolite generated during fluzifop-P-butyl degradation by the purified FpbH.

Supplementary Fig. 4—Characterization of purified recombinant FpbH.

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