

A novel *meta*-cleavage product hydrolase from *Flavobacterium* sp. ATCC27551

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Abstract

The organophosphate degrading (*opd*) gene cluster of plasmid pPDL2 of *Flavobacterium* sp. ATCC27551 contains a novel open-reading frame, *orf243*. This was predicted to encode an α/β hydrolase distantly related to the *meta*-fission product (MFP) hydrolases such as XylF, PhnD, and CumD. By homology modeling Orf243 has most of the structural features of MFP hydrolases including the characteristic active site catalytic triad. The purified protein (designated MfhA) is a homotetramer and shows similar affinity for 2-hydroxy-6-oxohepta-2,4-dienoate (HOHD), 2-hydroxymuconic semialdehyde (HMSA), and 2-hydroxy-5-methylmuconic semialdehyde (HMMSA), the *meta*-fission products of 3-methyl catechol, catechol, and 4-methyl catechol. The unique catalytic properties of MfhA and the presence near its structural gene of *cis*-elements required for transposition suggest that *mfhA* has evolved towards encoding a common hydrolase that can act on *meta*-fission products containing either aldehyde or ketone groups.

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meta-Fission product (MFP) hydrolases catalyze a rare class of reaction, namely hydrolytic cleavage of a carbon–carbon bond [1]. Despite showing only weak primary sequence homology, these proteins have unique structural features typically found in enzymes that belong to the α/β hydrolase family [2]. They typically possess a catalytic triad comprising a serine, an aspartate, and a histidine residue, and they do not have any organic or inorganic compounds as cofactors [2].

MFP hydrolases involved in the degradation of monoaromatic compounds act primarily on the ketone group-containing compound 2-hydroxy-6-oxohepta-2,4-dienoate (HOHD), a *meta*-fission product of 3-methylcatechol. They show very low activity towards the aldehyde group-

containing compounds hydroxymuconic semialdehyde (HMSA) and 2-hydroxy-5-methylmuconic semialdehyde (HMMSA), *meta*-fission products (MFPs) of catechol and 4-methylcatechol [3]. HMSA and HMMSA are preferentially catabolized by hydroxymuconic semialdehyde dehydrogenase (HMSD) [3].

Hence two divergent pathways have evolved to catabolize *meta*-fission products [3]; a hydrolytic pathway mediated by a hydroxymuconic semialdehyde hydrolase (HMSH), and the 4-oxalocrotonate/dehydrogenase pathway, mediated by a hydroxymuconic semialdehyde dehydrogenase (HMSD). These two divergent pathways can be found in the same organism and are induced non-specifically by any of the monophenolic substrates [4]. However, the non-specific induction of these two pathway enzymes imposes a metabolic burden on the cell and raises the question as to why a high-affinity hydrolase for MFPs of different catechols has not evolved [3,4].

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We now report that the open-reading frame, *orf243*, encoded in the organophosphate degrading (*opd*) gene cluster of plasmid pPDL2 of *Flavobacterium* sp. ATCC27551 encodes an MFP hydrolase that shows high affinity for both ketone and aldehyde group-containing *meta*-fission products. We have consequently designated this open-reading frame *mfhA* (*meta*-fission hydrolase A).

Materials and methods

Bacterial strains and culture conditions. Cultures of *Escherichia coli* and *Pseudomonas aeruginosa* PAO1161 were grown in LB medium at 37 °C and 30 °C, respectively. When necessary, antibiotics such as ampicillin (100 µg/ml), chloramphenicol (30 µg/ml), and kanamycin (25 µg/ml) were added to the growth medium.

Construction of plasmids. To express MfhA with an N-terminal His-tag in *E. coli*, the structural gene, *mfhA*, was amplified from pSM2 [5] using a 5' primer 5'CGCGGATCCATGGCGCATGCCCGCGTTC3' with a *Bam*HI site (in bold type) and a 3' primer 5'GCCCTGCAGCTGTGACTAACCCGGCGCGG3'. The PCR product was cloned into pRSETA (Invitrogen) using *Bam*HI to give pSM12. To express *mfhA* in *P. aeruginosa* [6], it was cloned in a broad-host-range vector, pMMB206 [7]. The gene was amplified from pSM12 using the primer 5'ACAAGATC TAGGGAGACCACAACGGTTTCCC3' with a *Bgl*II restriction site (in bold type) and a T7 terminator primer. The PCR product was digested with *Bgl*II and cloned in pMMB206 digested with *Bam*HI. A recombinant plasmid with the correct insert orientation was designated pSM13. The plasmid pSM13 was mobilized into *P. aeruginosa* by following standard protocols [8] and the presence of plasmid in exconjugants was confirmed by the method of Kado and Liu [9].

Expression and purification of MfhA. Overexpression of *mfhA* in *E. coli* BL21(DE3) [10] or in *P. aeruginosa* was carried out in LB medium. One millimolar IPTG was added to a culture at an OD₆₀₀ of 0.5 and 2 h later induction of MfhA was monitored using 12.5% SDS-PAGE. The cell pellet was resuspended in 50 mM sodium phosphate buffer, pH 7.2, containing 0.5 M NaCl and lysed by a freeze-thaw method [11,14]. The soluble fraction containing the expressed protein was used for further purification. As MfhA contains N-terminal his-tag the protein was affinity purified using Hi-Trap chelating affinity column (Amersham Biosciences) following manufacturers' protocols. Active fractions were pooled and analyzed on 12.5% SDS-PAGE to assess purity and subunit molecular weight. The molecular mass of native MfhA was estimated by gel-filtration chromatography using Sephacryl S-200 (75 × 1.5 cm). Blue dextran (2000 kDa), Alcohol dehydrogenase (150 kDa), β-galactosidase (116 kDa), and albumin (67 kDa) were used as molecular size markers.

Enzyme assays. MfhA activity was determined at 28 °C in 100 mM sodium phosphate buffer (pH 7.5), using a 1 ml reaction volume containing appropriate concentrations of MFPs of catechol (HMSA), 3-methylcatechol (HOHD), and 4-methylcatechol (HMMSA). The reaction was initiated by addition of pure MfhA and the decrease in absorbance at wavelength 389 nm (HOHD), 376 nm (HMSA), and 382 nm (HMMSA) was recorded. The molar extinction coefficients used for *meta*-cleavage products HOHD, HMSA, and HMMSA were 11.9 mM⁻¹ cm⁻¹, 40.0 mM⁻¹ cm⁻¹, and 24.5 mM⁻¹ cm⁻¹, respectively [30]. The MFPs were prepared fresh daily using *E. coli* DH5α (pWWO-6000) and procedures described elsewhere [13].

Kinetic properties of MfhA. The steady-state kinetic properties of MfhA were determined using MFPs HOHD, HMSA, and HMMSA as substrates at concentrations over the range of 1–50 µM in 100 mM sodium phosphate buffer (pH 7.5) at 28 °C. The enzyme concentration was adjusted so that the reaction was linear for at least 2 min and six independent experiments were carried out at each concentration for each substrate. Kinetic parameters were calculated using Graphpad prism software (www.graphpad.com). One unit of enzyme activity was defined as the amount of enzyme required to convert 1 µmole of substrate per min. To estimate the optimum pH for MfhA, the enzyme activity was measured

over a pH range of 6.0–10.0 in 50 mM Na₂HPO₄ (pH 6–8) buffer or 50 mM Tris (pH 8–10). The molar extinction coefficient of MFPs at different pH was taken as described elsewhere [37]. The effect of temperature on enzyme activity was measured by varying the temperature from 5 to 85 °C in 50 mM sodium phosphate buffer (pH 7.5). The effect of metal ions on MfhA activity was determined using a 10 µM concentration of chloride, sulfate or nitrate salts of Fe²⁺, Ca²⁺, Cu²⁺, Mg²⁺, Mn²⁺, Co²⁺, Li²⁺, and Zn²⁺. The effects of chelating agents such as EDTA and group-specific inhibitors such as PMSF, DEPC, *N*-ethylmaleimide and, *p*-chloromercuribenzoate on MfhA activity were all assessed using a concentration of 10 µM.

Modeling the structure of MfhA. Threefold prediction methods, GenThreader [15], hybrid fold prediction [16], and 3DPSSM [17], were considered for structure prediction. Homology modeling was performed using MODELLER [18] starting from the best structural template. Models generated were validated using VERIFY3D [19] and the best model was selected. Structure-annotation was performed on the sequence alignment between query and template using the program JOY [20]. Interactions with possible ligands were simulated using the docking program, GRAMM [21,22], which predicts structure of the complex formed by using their atomic coordinates without any prior information as to their binding sites. Structural comparisons were performed after best fit using SUPER [23]. The electrostatic surface representations were projected using GRASP [24].

Results and discussion

Structural modeling of MfhA

Our previous studies suggested that *orf243* (*mfhA*), encoded in the organophosphate degrading (*opd*) gene cluster of plasmid pPDL2 of *Flavobacterium* sp. ATCC27551, encodes an α/β hydrolase somewhat distantly related to MFP hydrolases such as XylF, PhnD, and CumD [5]. A dendrogram for known MFP-hydrolases shows MfhA is very distantly related to the rest of the MFP-hydrolases (Fig. 1), creating a new subfamily, designated as subfamily IV based on the classification described previously [25].

Given this distant relationship to MFP-hydrolases we undertook structural modeling of MfhA to examine whether it has all the expected features of this protein family. All threefold prediction methods suggested an α/β hydrolase as the most probable fold with high confidence (90% probability). A *meta*-cleavage product hydrolase, CumD, from *Pseudomonas fluorescens* (PDB code: 1luo) was the best template, with the highest score for compatibility of structure (lowest e-value) according to 3DPSSM. CumD was therefore used as the template for modeling the putative hydrolase. Alignment of MfhA with the template sequence (see Fig. 2) confirmed our earlier predictions [5] that residues Ser58, Asp183, and His215 were likely candidates for the catalytic triad residues of the enzyme.

The structure of CumD was therefore used as the template for modeling and the resultant MfhA model (Fig. 3A), which was validated using the HARMONY webserver (Fig. 3B) [35], showed very good agreement with the 3D structure of CumD [26]. It has the distinctive core and lid domains characteristic of the MFP hydrolases [27] and the predicted catalytic triad residues, Ser58, Asp183, and His215 [5], are indeed located within the

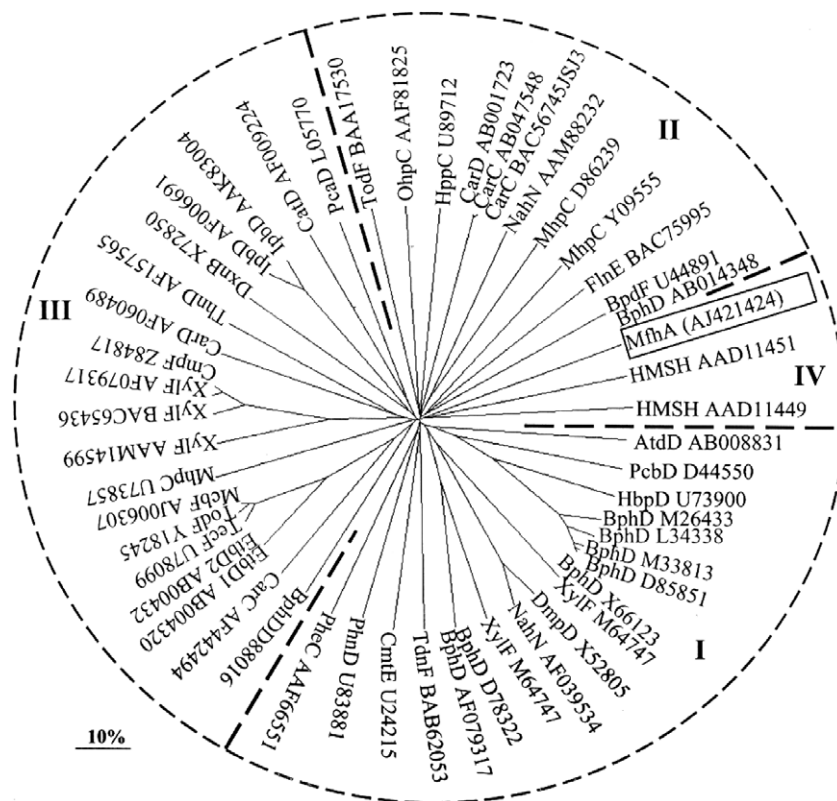


Fig. 1. Phylogenetic Tree of the *meta*-fission product hydrolases. Tree was constructed by the neighbor-joining method by aligning the sequences of 52 MFP-hydrolases. MFP-hydrolases were grouped into four subfamilies. Mfha is shown as part of subfamily IV and is shown in a box. Scale represents distance expressed as percentage of divergence. Bootstrap values are not shown. GenBank accession numbers for MFP-hydrolases are shown after the enzyme name.

potential substrate-binding pocket between those domains. As coordinates of the precise substrates of Mfha (HOHD, HMSA, and HMMSA) were not available we probed the interaction of the protein with coordinates of the related molecules catechol, 3-methylcatechol, and 4-methylcatechol, using GRAMM. In each instance, facile docking was observed in the vicinity of the putative active site and the *meta*-fission products of these molecules could be expected to behave similarly.

Despite high structural similarity between CumD and Mfha, the cavities at the substrate-binding site (interface between core and lid regions) are rather different when projected by surface representation (Fig. 4). The cavities in the Mfha model are more pronounced owing to several residue differences. For instance, the residue immediately after the active site near the glycine-rich loop is Phe in CumD but is replaced by a smaller residue (Ala) in Mfha. Perhaps, additional interactions are required between Phe and methyl groups of the substrate in CumD. Furthermore, the electrostatics distributions in the two proteins are remarkably different (Fig. 4) indicating that their interactions with other protein molecules could be distinct. Whereas CumD is highly negatively charged (total charge = -12) (Fig. 4A), Mfha is slightly positively charged (total charge = +1) (Fig. 4B) as measured using GRASP [24].

When compared to some other MFP hydrolases, apparently Mfha is truncated at the N-terminus (Fig. 2). As a consequence the first three β -strands seen in CumD are absent from Mfha. However, the absence of these three β -strands in this superfamily is not uncommon. We find that nearly one-fourth of the structural entries at 40% sequence identity cut-off in this superfamily, as recorded in PASS2 database [28], are shorter and can be aligned without the N-terminal peripheral beta-strands. These include nine PDB entries, 1ehya (epoxide hydrolase), 1ei9a (palmitoyl thioesterase), 1fj2a (acyl thioesterase), 1i6wa (lipase A), 1qj4A (hydroxynitrile lyase), 1auoA (carboxylesterase), 1brt (bromoperoxidase A2), 1c4x (BPHD), and 1c1v (triacylglycerol hydrolase).

Heterologous expression of Mfha

Overexpression of Mfha with an N-terminal His-tag was carried out using pSM12 in *E. coli* BL21(DE3). A 30 kDa band was observed only in IPTG-induced cultures but most of the expressed protein was found in the particulate fraction indicating that Mfha was accumulated in inclusion bodies (data not shown). Attempts were made to reconstitute active enzyme by solubilizing the inclusion bodies in the denaturing agents 8 M urea or 8 M sodium lauroylsarcosine followed by dialysis in progressively

	Region 1	Region2	Region3
liuo (29)		ILIHgSgPGvsAyanWrlTlTpaLskfyrViapdMVGFgftdrpenynysk	
MfhA (1)		Mmahar-----vherfs lsvr kmhfrvvtpiss-----dieaafawlg	
		bb aaaa a	
liuo (79)		AsWVdhIigiMdaleiekahIVGNsFGGgLAITATAlryserVdrMVLmGA	
MfhA (39)		sdgAkrfh-----ldTkrmiVaGNsagGylTLttGyrvkpkPrALVaLYG	
		aaaaaaaa bbbbbb aaaaaaaaaaaa bbbbbb	
		← lid domain begins	
liuo (129)		aGtrfdvteglNaVWgYtpsienMrnLLDifAydrslVt del Arlr----	
MfhA (84)		YGrlnaAwysqpnlfpeynktkiTreeAsags-dggvIsdsskrkGdadT	
		aaaa aaaaaaaaa aaaaa	Region4
		lid domain ends ->	
liuo (175)		-yeaSiqpgfgesFssMFpeprgrwIdalassdedIktLpneTLIIHGRe	
MfhA (133)		IyryYrqnglwpqevsgfpssiaeliagyepaknVtrEypPpTLLMHGtd	
		aaaaaaaa aaaaaa aaaa bbbbbb	
liuo (224)		DgVVplssSlrLGeLIdrA----qlhvFgrCGHwTQiEqtldrFnrlVveF	
MfhA (183)		DhDVpyeeSanMALgFekHGvpyvLktidrgGhgFggspdqIedayrsm	
		aaaaa bbbb aaaa aaaaaaaaaaa	
liuo (270)		Fnean	
MfhA (233)		Rdfi	

Fig. 2. Alignment between MfhA and the *P. fluorescens* CumD hydrolase (PDB code: liuo) employed as the template for modeling. The alignment has been structure-annotated using JOY [20] where upper case, lower case, blue text, and red text denote solvent-buried, solvent accessible, helical, and β -strands, respectively. Hydrogen bonds to main chain amide, main chain carbonyl are marked by bold and underline, respectively. Residues with positive-phi are marked in italics. The catalytic residues are indicated with yellow boxes and the lid region is marked in pale blue. Regions (1–4) of significant insertion or deletion are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

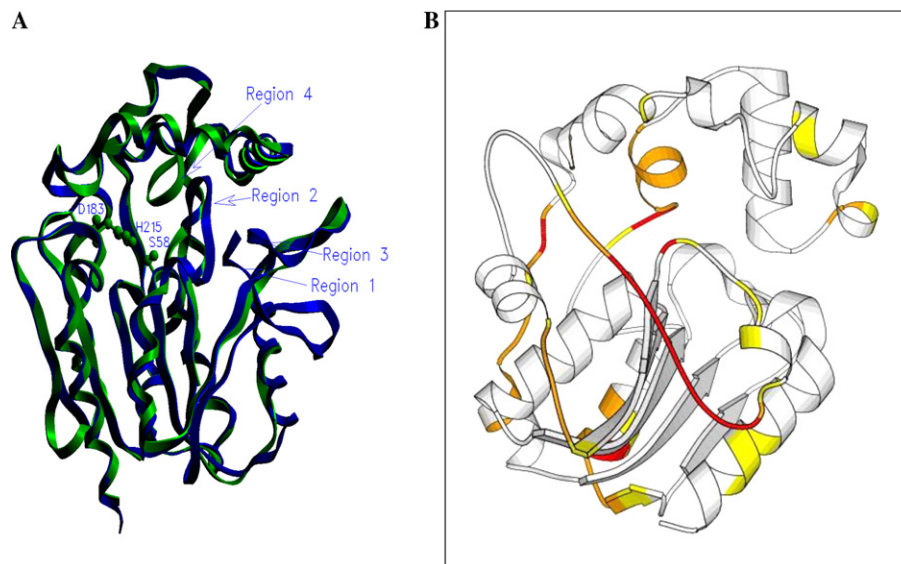


Fig. 3. (A) Overlay of the MfhA modeled structure with the known structure of the CumD meta-fission product hydrolase from *P. fluorescens* (PDB code: liuo). CumD is shown in blue and MfhA in green. Major regions of difference identified from the initial alignment (Fig. 2) are indicated as are the active site residues of MfhA (S58, D183, H215). (B) Structure validation of MfhA model using HARMONY webserver [35]. HARMONY validation scores are mapped on the structure using MOLSCRIPT [36]. Red and orange colored regions indicate possible local errors in the structure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

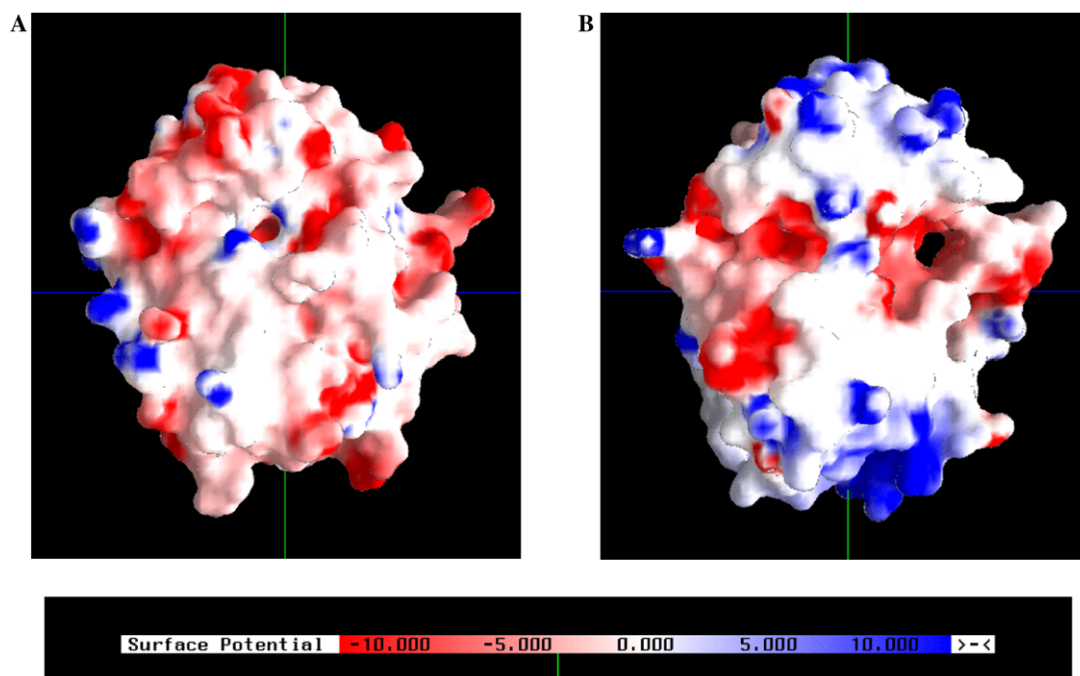


Fig. 4. Electrostatic surface representation of (A) CumD and (B) the MfhA model. View from the “top” surface of the proteins with the “lid” on the top. Figure generated using GRASP [24].

decreased concentrations of these agents [11]. Whilst some MfhA was then found to be soluble, suggesting successful refolding, this protein showed no catalytic activity when HOHD, HMSA, and HMMSA were used as substrates. Interestingly most of the MfhA encoded by the plasmid pSM13 in *P. aeruginosa* PAO1161 was not only found to be in soluble form but also showed activity when the above-mentioned *meta*-fission products were used as assay substrates.

Biochemical properties of MfhA

The molecular weight of MfhA was determined in denaturing and native conditions using SDS-PAGE and gel filtration chromatography, respectively. In denaturing conditions MfhA had a molecular weight of 27 kDa on SDS-PAGE [5] which was increased to 30 kDa by the N-terminal histidine tag used in this study. The molecular mass of MfhA was found to be ~120 kDa by gel-filtration chromatography (data not shown), indicating that it is a homotetramer, a structure previously reported for other MFP-Hydrolases [29–31]. MfhA purified from *P. aeruginosa* successfully hydrolyzed *meta*-fission products such as HOHD, HMSA, and HMMSA and it obeyed classic Michaelis-Menten kinetics for all of the substrates tested (data not shown). The apparent steady-state kinetic parameters were calculated from Lineweaver-Burk plots and are shown in Table 1. MfhA showed similar activities for HOHD and HMSA, and low activity towards HMMSA.

The hydrolase activity had a pH optimum of 8.5 and when assessed over a temperature range of 5–85 °C using HOHD as substrate, it showed a maximum activity at

75 °C, after which activity declined rapidly. At this temperature optimum MfhA lost just 21% of its activity after incubation for 2 h. MfhA activity was unaffected by divalent metal ions like Fe²⁺, Ca²⁺, Cu²⁺, Mg²⁺, Mn²⁺, Co²⁺, Li²⁺, and Zn²⁺ or by the chelating agent EDTA. However, activity was strongly inhibited by group-specific agents such as DEPC (82%) and PMSF (75%) suggesting the presence of serine and histidine as part of the catalytic residues. This is also in agreement with our earlier predictions that the residues Ser58, Asp183, and His215 could constitute the catalytic triad [5]. Consistent with the absence of cysteine residues in the deduced primary sequence, the thiol group modifying agents such as *N*-ethylmaleimide (7%) and *p*-chloromercuribenzoate (5%) showed no significant effect on enzyme activity. The enzyme showed no significant loss in activity when stored for 15 days at 4 °C or –20 °C in the presence of 20% glycerol, but after 30 days storage lost 20% activity at –20 °C, and 55% activity at 4 °C.

MFP-hydrolases are normally substrate specific, showing high activity towards ketone group-containing HOHD and low activity towards aldehyde group-containing HMSA and HMMSA [3,32]. By contrast MfhA shows similar affinities to both aldehyde and ketone group-containing MFPs; the K_m s for HOHD, HMSA, and HMMSA being 4.8, 5.7, and 7.4 μM, respectively (Table 1). As expected MfhA showed high activity with HOHD as a substrate but surprisingly also with HMSA as substrate. However, despite showing a high affinity towards HMMSA, MfhA had a relatively low activity with this substrate (Table 1). Nevertheless, the activity of MfhA towards HMMSA was considerably higher than those reported for other MFP

Table 1
Comparison of kinetic MFP-hydrolases (involved in mono-aromatic compounds degradation) purified from various bacterial strains

Enzyme	Substrate	K_m (μM)	V_{\max}^h	K_{cat} (s^{-1})	$K_{\text{cat}}/K_m \times 10^{-5}$ ($\text{M}^{-1} \text{s}^{-1}$)	Ref.
XylF ^a	HOHD	36	100	—	—	[12]
	HMSA	6.7	6.9	—	—	
	HMMSA	10	3.2	—	—	
HOHD ^b	HOHD	6.7	100	—	—	[29]
	HMSA	ND	7	—	—	
	HMMSA	ND	3	—	—	
HOHD ^c	HOHD	27	100	—	—	[29]
	HMSA	ND	3	—	—	
	HMMSA	ND	1	—	—	
EtbD1 ^d	HOHD	4.5	100	—	—	[37]
	HMSA	20	24.4	—	—	
	HMMSA	ND	ND	—	—	
TodF ^e	HOHD	17 (0.6)	—	35 (0.5)	20	[30]
	HMSA	18 (0.5)	—	2.2 (0.03)	1.2	
	HMMSA	64 (2)	—	2.1 (0.03)	0.3	
CumD ^f	HOHD	9.0 (0.9)	—	18 (1.8)	19	[38]
MfhA ^g	HOHD	4.8 (0.05)	100	57 (0.06)	119	This work
	HMSA	5.7 (0.3)	73	42 (0.3)	74	
	HMMSA	7.4 (0.4)	28	16 (0.12)	27	

^a From *Pseudomonas putida* mt-2.

^b From *Pseudomonas putida* NCIB 10015.

^c From *Pseudomonas putida* NCIB 9865.

^d From *Rhodococcus* sp. RHA1.

^e From *Pseudomonas putida* F1 (values taken from [30]).

^f From *Pseudomonas fluorescens* IP01.

^g At least six data points for the measurement of each substrate were collected. The turnover numbers for one subunit (K_{cat}) were calculated. Numerical values in parentheses indicate standard errors calculated from curve fitting.

^h Activities are expressed in percentage of specific activities on preferred substrate HOHD. ND, not detected.

hydrolases (Table 1). MFP-hydrolases showing low activity towards HMMSA are not uncommon in the literature. In fact Henderson and Bugg have elegantly demonstrated that MFP-hydrolases catalyze an enol–keto tautomerization of the substrate prior to hydrolysis [33]. In case of HMMSA, methyl substitution at position C5 provides stability to the enol-tautomer and thus it serves as poor substrate for *meta*-fission product hydrolases [33].

Like other MFP hydrolases, and the HOHD hydrolases from *Pseudomonas putida* NCIB 9865 and NCIB 10015 [29], MfhA shows maximum activity at pH 8.5. However, the temperature optimum of MfhA of 75 °C is higher than that for other MFP hydrolases [25,30].

Significance of MfhA in the evolution of *meta*-fission hydrolases

To ensure further degradation, *meta*-fission products are either hydrolyzed or dehydrogenated [3]. Degradation of aldehyde group-containing MFPs by the 4-oxalocrotonate pathway is considered to be an energy-yielding pathway due to the generation of reduced coenzyme NADH [34]. However, for the generation of the common metabolic intermediate 2-oxopent 4-enoate via the 4-oxalocrotonate pathway, the MFPs HMSA and HMMSA need to pass through three committed enzymatic steps [3,34]. Hence of the two potential degradation pathways the hydrolytic

pathway appears to be the simpler, as it requires only one committed enzymatic step. In view of the catabolic divergence and non-specific induction of the enzymes involved in degradation of MFPs it has been proposed that it should be advantageous to have a unique hydrolase with an extended substrate range [3]. Considering its unique catalytic properties, MfhA is potentially an MFP-hydrolase with the capability to degrade both aldehyde and ketone group-containing MFPs by channeling them into a simple hydrolytic pathway. Almost all MFP-hydrolases are encoded within a *meta*-pathway operon. By contrast, *mfhA* is part of the plasmid-encoded *opd* gene cluster of *Flavobacterium* sp. ATCC27551, a gene cluster that has features characteristic of horizontal transfer [5]. Though there is no experimental evidence, considering the presence of novel direct and indirect repeats flanking *mfhA*, it is tempting to speculate that *mfhA* was recruited to the plasmid pPLD2 through horizontal gene transfer, thereby diversifying the catabolic efficiency of its host.

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