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Identification, Cloning and Biochemical Characterization of *Pseudomonas putida* A (ATCC 12633) Monooxygenase Enzyme necessary for the Metabolism of Tetradecyltrimethylammonium Bromide

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Abstract This study presents the first report of the purification and characterization of a monooxygenase enzyme from *Pseudomonas putida* A (ATCC 12633) that is responsible for the oxidation of physiologically relevant quaternary ammonium compounds, the tetradecyltrimethylammonium bromide. The degradation of tetradecyltrimethylammonium bromide by *P. putida* A (ATCC 12633) is initiated by *N*-dealkylation and catalysed by tetradecyltrimethylammonium monooxygenase (TTABMO), resulting in the formation of tetradecylalkanal and trimethylamine. Based on sequence analysis, the gene for TTABMO (*ttbmo*) corresponded to an ORF named PP2033 in the genome of *P. putida* KT2440. Mutation in *ttabmo* blocked the utilization of tetradecyltrimethylammonium bromide by *Pseudomonas putida* A (ATCC 12633) as carbon and nitrogen sources. The enzyme can be highly overexpressed in *P. putida* Δ*ttabmo*-T7 in active form and purified as a hexahistidine fusion protein. Like the native enzyme, the his-TTABMO was found to be a monomer with molecular mass of 40 kDa, the isoelectric point 7.3, that catalyses the breakdown of tetradecyltrimethylammonium bromide and utilized NADPH and FAD as cofactor. The biochemical properties and the analysis of the respective protein sequence revealed that TTABMO represents a typical flavoprotein monooxygenase, which is member of a flavoprotein family that is distinct from Baeyer–Villiger monooxygenases.

Keywords Monooxygenase enzyme · *Pseudomonas putida* · Quaternary ammonium compounds · Tetradecyltrimethylammonium

Introduction

Quaternary ammonium compounds (QACs) are molecules with at least one hydrophobic long alkyl chain attached to a positively charged nitrogen atom. Among the QACs, the generic term “cetrimide” refers to mixtures of *n*-alkyltrimethyl ammonium bromide in

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which the *n*-alkyl group is between 8 and 18 carbons long, whereas Cetrimide USP is tetradecyltrimethylammonium bromide (TTAB) [1]. QACs have a wide range of commercial and consumer applications, such as detergents, antistatics, wetting and softening agents, biocides, germicides, deodorizers and emulsifiers [1, 2]. After usage, the residual products are discharged to sewage treatment plants or surface waters and, finally, to coastal waters, in which the biodegradability of QACs is limited by their antimicrobial activity [3, 4] and persist in natural and engineered biological systems, where they can be toxic for life [5]. It has been shown that QAC's biodegradation may occur through different pathways [4] being one of them the *N*-dealkylation which involves an O_2– and NADPH-dependent reaction catalysed by a monooxygenase activity with the production of trimethylamine (TMA) and the corresponding alkanal [3, 6]. *Pseudomonas putida* A (ATCC 12633) is capable of growth with TTAB as the sole source of carbon, nitrogen and energy. In this bacterium, the O_2-dependent breakdown of TTAB to tetradecylalkanal and TMA occurs in the presence of NAD(P)H, catalysed by tetradecyltrimethylammonium monooxygenase (TTABMO) activity [7]. The next step involved the oxidation of tetradecylalkanal to tetradecanoic acid, which would be further metabolized via β oxidation. The second product, TMA, is metabolized to NH_3 through oxidation by trimethylamine monooxygenase activity [8]. Despite recent advances in our understanding of the pathway proposed for TTAB metabolism in *P. putida* A (ATCC 12633) that provides evidence of total mineralization of this QAC [9], no information is available concerning the nature of the proteins involved or the genetic machinery they employ. In this study, we reported the amplification, cloning, functional expression and characterization of a monooxygenase enzyme of *P. putida* A ATCC 12633 that is required in the catabolic pathway of TTAB, specifically in the *N*-dealkylation of TTAB to tetradecylalkanal and TMA.

Material and Methods

Bacterial Strains, Plasmids and Culture Media

Table 1 summarizes the strains and plasmids used in this study. The strains were grown aerobically at 30 °C, with shaking either in Luria-Bertani (LB) medium or in basal salt medium (HPi-BSM) [10] supplemented with 20 mM glucose and either 18.7 mM NH_4Cl or 0.15 mM (50 mg/L) TTAB as the carbon and nitrogen sources, respectively. The growth was measured as attenuance at 660 nm (D_{660}) with a spectrophotometer (Beckman DU 640). *Escherichia coli* DH5α [11] was grown in LB medium at 37 °C. The antibiotics used were ampicillin (Ap) at 100–400 μg/mL, gentamicin (Gm) at 100–200 μg/mL, streptomycin (Sm) at 100–500 μg/mL and kanamycin (Km) at 50 μg/mL.

Steps to Obtain a Pure *P. putida* A (ATCC 12633) Protein Spot to Perform MALDI-TOF

Cells *P. putida* A (ATCC 12633) grown in HPi-BSM with 50 mg/mL TTAB to D_{660} 0.4 were removed by centrifugation at 10,000*g* for 10 min at 4 °C, washed and resuspended with HPi-BSM. Then, cells were disrupted by sonication at 20,000 Hz using a Vibra cell ultrasonic processor for 10 cycles, with 10 s for each cycle, and after centrifugation (16,000*g* for 10 min at 4 °C), the soluble fraction was collected and resolved by 7.5 % non-denaturing polyacrylamide gel electrophoresis (N-PAGE). To detect the in-gel TTABMO activity, the gels were incubated for 1 h in reaction mixture containing 1 mM TTAB, 0.5 mM NADPH, 14 mM phosphate buffer (pH 7.4) plus 0.01 % *2′7′*dichlorofluorescein to detect the fatty aldehyde formed under UV light [12]. The band with enzymatic activity was removed and treated with 60 % glycerol and 4 %

Table 1 Strains and plasmids

Strains and plasmids	Characteric(s)	Source or reference
Strains		
E. coli		
DH5α	Host strain for cloning vectors	[11]
P. putida		
A (ATCC 12633)	Wild type	[32]
Δ*ttabmo*	ATCC 12633derivative, *ttabmo::gm.*	This study
Δ*ttabmo*-T7	Δ*ttabmo* with T7 polymerase under Lac Z control	This study
KT2440	Wild type	www.pseudomonas.com
Plasmids		
pCR2.1TOPO	PCR product cloning vector, Amp^R	Invitrogen
mini-CTX1	Suicide Vector Te^r. 4469-bp EcoRI–HindIII fragment containing T7 pol gene under Lac Z control.	[33]
pASL01	pCR2.1TOPO-*ttabmo*	This study
pASL02	pCR2.1TOPO- *aacC1*	This study
pASL03	pCR2.1TOPO- *ttabmo::gm*	This study
pKNG101	Suicide Vector	[16]
pASL04	pKNG101- *ttabmo::gm*	This study
pBBRmcs-4	Cloning vector Amp^R	[15]
pASL05	pBBRmcs-4 containing *ttabmo*	This study
pET1.6	Expression vector, Amp^R	
pASL06	pET1.6-*ttabmo*	This study

ampholytes for isoelectric focusing (IEF; first dimension) on N-PAGE (7.5 % polyacrylamide gels and 1.8 % 3–10 ampholytes carrier-generated pH gradients) (Sigma-Aldrich Co.). After N-PAGE, a part of the gel was stained with Coomassie Blue G-250 to identify the protein position in the gel and another part was stained to detect TTABMO activity through fatty aldehyde formed [12]. The band with enzymatic activity was resuspended in Laemmli solution [13], boiled for 3 min and subsequently submitted to electrophoresis on 12 % SDS-PAGE. The protein bands were visualized by staining with Coomassie Blue R-250. After the bands of interest were localized, these were excised from the gel and sent to analysis.

Cloning of ORFs Potentially Encoding Protein with Putative TTABMO Activity and Generation of a Knockout Mutant in *P. putida* A (ATCC 12633)

Recombinant DNA techniques were performed according to standard protocols [14]. A 1,800-bp DNA fragment containing *orf pp2033* was amplified from *P. putida* KT 2440 genome using the primers *pp2033-Sal*I-FW and *pp2033-Bam*HI-RW (Table 2). The PCR product was cloned into the pCR®2.1-TOPO (Promega Corp.) to yield pASL01. Next, a 600-bp *Sma*I fragment containing the *aacC1* gene, which encodes Gm resistance, was PCR-amplified from pBBR1MCS-5 [15] using the primers UpGm and DownGm shown in Table 2. This fragment was ligated to pASL01, which had been previously digested with the same enzymes, producing pASL03 (Table 1). Using *Sal*I and *Bam*HI, the *pp2033::aacC1* fragment was subcloned into the pKNG101 suicide vector [16] to obtain pASL04 (Table 1). Finally, pASL04 was used for allelic exchange, in which it was electroporated into *P. putida* A (ATCC 12633).

Table 2 Oligonucleotides used in this study

Oligonucleotide name	Sequence (5′-3′)[a], relevant characteristics
*ttabmo-Nde*I-FW	TTGCCTGATGGTCCCATATGACGGC, *Nde*I restriction site overlapping the start codon of *ttabmo*
*ttabmo-BamH*I-RW	GCGGATCCCCCTGGCACAGGAGATGTAT, *Bam*HI restriction site before the stop codon of *ttabmo*
*pp2033-Sal*I-FW	ACGCGTCGACGAACTGGATGCGATGGAACT, located at start of the *pp2033* gene in the genome sequence of *P. putida* KT2440
*pp2033-BamH*I-RW	CGCGGATCCCCCTGGCACAGGAGATGTAT, located after the stop of the *pp2033* gene in the genome sequence of *P. putida* KT2440
UpGm:	TCCCCCGGGGGGTCGATGTTTGATGT, flanking the *aacC1*gene from pBBR1MCS-5
DownGm:	TCCCCCGGGATCTCGGCTTGAACGAA, flanking the *aacC1*gene from pBBR1MCS-5

Merodiploids were selected from cells grown in LB medium plus Sm (100 μg/mL) and Gm (120 μg/mL) (first crossover) and streaked onto LB plates containing 10 % sucrose and Gm (120 μg/mL) to score for loss of the pASL04 suicide vector, which contains a sacB gene. Gm-resistant colonies that were able to grow in the presence of sucrose were purified [16]. The correct insertion of the mutant allele of *pp2033* into the chromosome was verified by PCR analysis (results not shown). The resulting knockout *P. putida* Δ*ttabmo* was confirmed by PCR amplification of *aacC1* from the genome. To complement, the original 1.8-Kb fragment was subcloned in pBBRmcs-4 plasmid [15] using *Sal*I and *Bam*HI. The new construct, termed pASL05, was transferred into *P. putida* Δ*ttabmo* by electroporation.

Expression and Purification of Recombinant TTABMO

For protein expression, *ttabmo* was amplified from *P. putida* A (ATCC 12633) genome using specific oligonucleotides *ttabmo-Nde*I-FW and *ttabmo-Bam*HI-RW (Table 2). The *ttabmo* fragment was cloned into pCR®2.1-TOPO, sequenced and subcloned into pET1.6 using *Nde*I and *Bam*HI, resulting in the recombinant plasmid pASL06, which enables over expression under control of the T7 promoter. pASL06 was introduced by electroporation into an expression strain of *P. putida* Δ*ttabmo*-T7 that was created by the integration of mini-CTX-T7 at the *attB* site of *P. putida* Δ*ttabmo* [17]. *P. putida* Δ*ttabmo*-T7 harbouring pASL06 was grown in LB medium with 400 μg/mL of Ap until the D_{660} reached 0.6–0.7. After overnight induction at 16 °C with 400 μM of isopropyl-β-D-1-thiogalactoside (IPTG), cells were harvested, resuspended in 20 mM Tris–HCl, pH 7.4, disrupted by sonication, centrifuged at 16,000*g* for 10 min at 4 °C and the supernatant was applied to Ni-NTA agarose (Promega Corp.) that had been equilibrated with 50 mM Tris–HCl, pH 8.0 and 500 mM NaCl. The recombinant protein his-TTABMO was eluted with the same buffered solution containing 300 mM imidazole. The imidazole was removed by dialysis against 50 mM Tris–HCl, pH 8.0, and the purified enzyme was evaluated by 12 % SDS-PAGE.

In Vitro TTABMO Activity Assay

TTABMO activity was assayed at 30 °C in a 1-mL reaction mixture containing 1 mM TTAB plus 0.5 mM NADPH, 14 mM phosphate buffer (pH 7.4) and 0.1 mg protein/mL. After 30 min of incubation at 30 °C, the reaction was stopped by the addition of 0.5 mL of 37.5 % (*v*/*v*) TCA. After centrifugation at 12,000*g* for 10 min, aliquots of 1 μL and 100 μL of the supernatant were

used to detect the TMA product by fluorescence, as described [18]. Boiled cell extracts were used as a blank. The units of enzyme activity were expressed as nanomoles of TMA/minute/milligram protein. Protein concentration was determined using the method described by Bradford [19].

In Vivo Cross-Linking with Formaldehyde

Cross-linking assay was realized as described Ferreira et al. [20] with modifications. Briefly, cells of *P. putida* Δ*ttabmo*-T7 were grown in LB with 400 μg mL^{-1} of Ap until the culture reached a D_{660} of 0.5. After 3 h of induction with IPTG 400 μM, formaldehyde 1 % was added and cross-linking was allowed to proceed for 20 min at 30 °C. To stop the reaction, glycine 0.5 M was added and, after 5 min at room temperature, bacterial cells were harvested and resuspended in 20 mM Tris–HCl, pH 7.4. Cell-free extracts were obtained by sonic disruption, and recombinant protein was purified using Ni-NTA agarose as described above. As control, bovine serum albumin (Sigma-Aldrich Co.) was successfully cross-linked.

Spectroscopic Studies

UV-visible absorbance spectra were recorded in 1 mL quartz cuvettes with a spectrometer (Beckman DU 640). The reduced form of the enzyme was obtained by anaerobically reducing the enzyme in a stoppered cuvette. The hydroperoxyflavin form of the enzyme was created by introducing air into the cuvette, the spectrum was read, and the absorbance of NADPH and reduced enzyme were subtracted to obtain the spectrum of the hydroperoxyflavin [21].

Nucleotide Sequence Accession Number

The nucleotide sequence of the *ttabmo* gene from *P. putida* A (ATCC 12633) has been deposited in the GenBank database under the accession number JN797595.

Results and Discussion

Identification of a Protein with TTABMO Activity in *P. putida* A (ATCC 12633)

When the crude extract of *P. putida* A (ATCC 12633) containing the TTABMO activity was resolved by 7.5 % N-PAGE, a single band with enzymatic activity was detected (Fig. 1a, lane 2). This band was cut and submitted to IEF, with a resulting isoelectric point of 7.3 (Fig. 1, lane 3). The subsequent electrophoresis of this band via 12 % SDS-PAGE resulted in the revealing of three proteins, with molecular masses of approximately 65, 55 and 40 kDa (Fig. 1, lane 6). MALDI-TOF analysis of the spots showed that the 40-kDa polypeptide has 98 % sequence identity to the hypothetical protein PP2033 (NP_744183) of *P. putida* KT 2440 [22]; the 65-kDa polypeptide with GroEL, a general stress protein belonging to the family of heat-shock proteins [23], while the polypeptide of 55 kDa was not identified.

The *P. putida* A (ATCC 12633) *orf pp2033* homologue (*ttabmo*) was cloned, and a mutant strain called *P. putida* Δ*ttabmo* was obtained by insertion of gene which encodes for Gm resistance as described in the "Material and Methods". The *P. putida* Δ*ttabmo* strain grew at a rate similar to that of the wild-type strain in HPi-BSM with glucose and NH_4Cl (with doubling times of 2.3 and 2 h, respectively) or TMA (doubling times were 15 and 10 h, respectively) as the carbon and nitrogen sources, but was unable to utilize TTAB as a growth substrate. The mutant strain complemented with the plasmids pASL05 (Table 1) grew at a rate similar to that

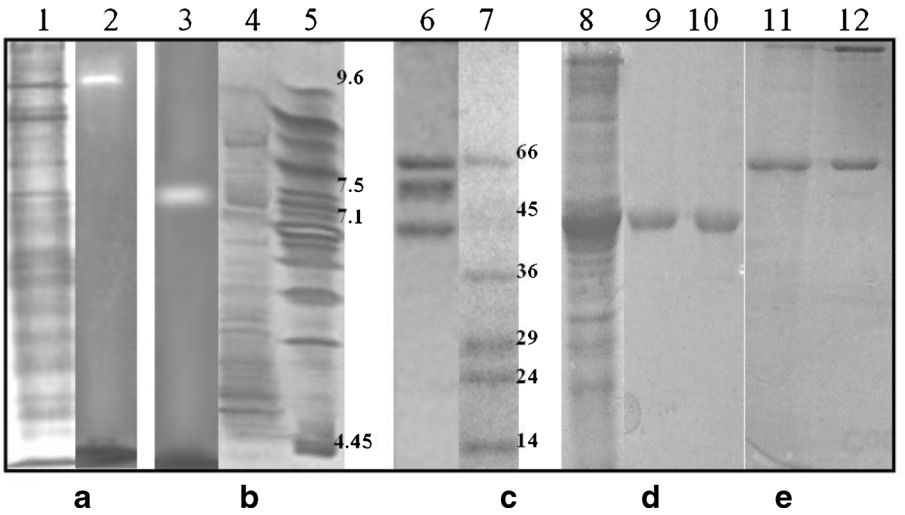

Fig. 1 Purification of native and recombinant his-TTABMO from *P. putida* A (ATCC 12633). **a** N-PAGE of native TTABMO. Detection with: Coomassie Blue G-250 (*lane 1*); 2′,7′dichlorofluorescein (*lane 2*). **b** IEF of band with TTABMO activity extracted from N-PAGE. Detection with: 2′,7′dichlorofluorescein staining (*lane 3*); Coomassie Blue R-250 (*lane 4*), IEF standards (BioRad) (*lane 5*). **c** SDS-PAGE of band with TTABMO activity extracted from IEF staining with Coomassie Blue G-250 (*lane 6*), molecular weight standards (BioRad) (*lane 7*). **d** SDS-PAGE (12 %) of the his-TTABMO induced with IPTG. Staining with Coomassie BlueG-250: Crude extracts (*lane 8*); his-TTABMO purified (*lane 9*); in vivo cross-linking of his-TTABMO (*lane 10*). **e** BSA (*lane 11*); cross-linked BSA (*lane 12*)

of the wild type in a basal salt medium with TTAB (doubling times were 12 and 9 h, respectively), and the TTABMO activity detected was 60 % of the activity detected in the wild-type strain (0.96 nmol TMA/min/mg protein and 1.6 nmol TMA/min/mg protein, respectively). Knockout of the homologous gene in strain *P. putida* A ATCC 12633 and complementation of the mutant confirmed that the gene indeed is necessary for TTAB oxidation.

Expression and Biochemical Characterization of the Recombinant TTABMO Activity

The *ttabmo* gene from *P. putida* A (ATCC 12633) was subcloned into vector pET1.6 to generate C-terminal his-TTABMO. Recombinant expression was performed in *P. putida* Δ*ttabmo*-T7 (Table 1), and the enzyme was purified by Ni-NTA resin as a soluble protein with a molecular weight of 40 kDa (Fig. 1, lane 9). The corresponding translated protein, TTABMO, contain 453 amino acid residues. Analysis with SignalP (http://www.cbs.dtu.dk/services/SignalP/) showed absence of signal peptide and possible cut sites found towards the N-terminus are of low probability, indicating that the native protein is probably not an integral membrane protein.

Purified his-TTABMO showed an isoelectric point of 7.3, the optimum pH for TTAB oxidation was 7.8 and TTAB oxidation was not detected in the absence of molecular oxygen or NADPH. When the enzyme was chemically cross-linked with formaldehyde and subsequently analysed by 12 % SDS–PAGE, a protein band of 40 kDa was detected, result that reflect the fact that the molecular architecture of his-TTABMO is monomeric in solution (Fig. 1, lane 10). Purified his-TTABMO activity with NADPH was 3-fold higher than with NADH (128.6 nmol TMA/min/mg protein and 43.5 nmol TMA/min/mg protein, respectively). Assays saturation of his-TTABMO by TTAB showed an apparent $K_{0.5}$ and n_{app} (apparent Hill coefficient) values for TTAB was 3.98×10^{-4} M and 1.9, respectively (Fig. 2). The apparent Km value for NADPH was

0.15 mM (not shown). These results are similar to obtained with native enzyme [7, 8]. The activity of purified his-TTABMO against different QACs under identical experimental conditions, such as tetrabuthylammonium bromide, hexadecyltrimethylammonium chloride, benzyltetradecyldimethylammonium chloride and benzylhexadecyldimethylammonium chloride, revealed that only hexadecyltrimethylammonium chloride was used as substrate at a considerable rate (72±6 nmol TMA min^{-1} (n=3)).

Figure 3 (trace B) shows the optical absorption spectra of purified his-TTABMO with absorption peaks at 372 and 452 nm, typical of oxidized flavoproteins [24]. The flavin content of his-TTABMO was 21 nmol/mg protein thus, using a molecular weight of 40 kDa, the FAD content is 0.87 mol/mol. A most ubiquitous group of monooxygenases is formed by the flavin-dependent monooxygenases, enzymes that employ an organic cofactor for oxygenation reactions [25]. In the called external flavoprotein monooxygenases (FPMOs), the reduced form of the flavin obtained with electrons from the NADH or NADPH reacts with molecular oxygen and produces the intermediate hydroperoxyflavin that is the species which can oxygenate the substrate [25, 26]. Mixing the his-TTABMO with an equimolar amount of aerated NADPH (in the absence of substrate TTAB) leads to the formation of a hydroperoxyflavin intermediate, characterized by the typical absorbance spectrum with a peak at≈380 nm [27] and citations included (Fig. 3, trace C), that indicate the reduction of FAD-containing his-TTABMO by NADPH. Over 3 min, the spectrum slowly reacquires the characteristic 442 nm peak of the oxidized flavin (Fig. 3, trace D). This result clearly indicates that his-TTABMO is a FPMOs that utilizes NADPH to reduce FAD and obtain the hydroperoxyflavin necessary to oxygenate the substrate.

Phylogeny of the *P. putida* TTABMO

The searches for conserved motifs by multiple alignments indicated that TTABMO of *P. putida* A (ATCC 12633) contains two dinucleotide-binding domains (Rossmann fold): the FAD

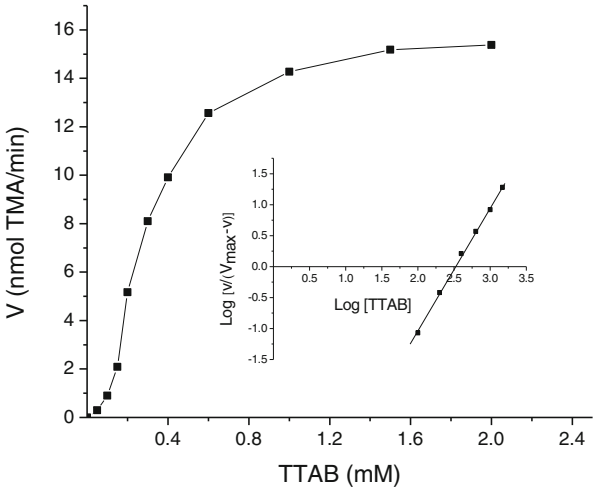

Fig. 2 Saturation curve of his-TTABMO by TTAB. The reaction mixture contained 14 mM phosphate buffer (pH 7.4), 0.5 mM NADPH and substrate as indicated. The reaction was initiated by addition of the enzyme (0.1 mg protein/mL), and the mixture was then incubated for 30 min. After the reaction was stopped with TCA, TMA concentrations were determined by the fluorescence assay. Data correspond to a representative experiment of three independently performed experiments, with enzyme extracts from three different preparations. *Inset* shows the Hill plot of the same data

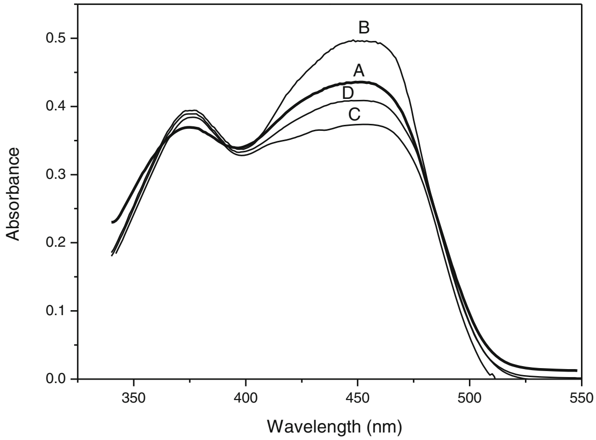

Fig. 3 Absorbance spectra of his-TTABMO. Free FAD (*trace A*); the fully oxidized form of the enzyme (*trace B*); the hydroperoxyflavin form produced by anaerobic reduction (*trace C*); slow decay of the hydroperoxyflavin intermediate after 3 min (*trace D*)

domain (GXGXXG) and the NADPH-binding domain (GXGXXA) (Fig 4). These domains are key characteristic sequence motifs commonly found in the FAD-containing proteins [28], including the external FPMOs [25]. External FPMOs can be grouped in six subclases (A–F) on the basis of amino acid sequence, tertiary structure and cofactor preference [25]. Also, the FPMOs can be described by the mechanism or by the type of target substrate that is oxygenated [29]. The central reaction in the FPMOs is always the same: formation of a peroxyflavin intermediate by reaction of reduced flavin with molecular oxygen. Depending on the type of monooxygenase, the flavin is reduced in the monooxygenase moiety itself, representing a tightly bound flavin cofactor (classes A and B); alternatively, the flavin acts as a coenzyme being reduced by an auxiliary flavin reductase component, and after which, the reduced flavin is bound by the monooxygenase component (classes C–F) [29]. FPMOs class B are single component enzymes, contain two Rossmann-fold motifs indicative for two binding domains for FAD and NADPH, respectively, and are able to oxidize both carbon atoms and heteroatoms [25]. Thus, the sequence analysis (Fig 4) in conjunction with the biochemical

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             ● ●  ●          ● ●   ●   ●              ● ●  ●
             * *  *          * *   *   :         . :  :* .  .
   ACJ37423 IIGAGVSGMI-----ETFQGPAFHSAQWD--HNVDWSGK--RVAVIGTGASAAQ-
   AAK54073 IIGAGESGMI-----ETFKGPMFHSAQWD--HNVDWSGK--RVGVIGTGASATQ-
 GAB75030.1 IIGAGFGGLA---GIEKFNGKIFHSSQWD--HQYDLTGK--TVASIGTGGSAIQ-
   P31513.5 IIGAGVSGLA-----NHFKGKCFHSRDYK--EPGVFNGK--RVLVVGLGNSGCDI
 AAZ55526.1 VVGAGFSGLY-----KDFAGNLYHTGNWPH-EPVDFSGQ--RVGVIGTGSSGIQ-
 ADF32068.2 IVGAGISGLG-----HQFKGTLVHPQHWP--EKLDYTGK--RVIVIGSGATAIT-
 EGL54178.1 ILGAGPSGMA-----EKFGGRILHAHDFR--DALEFKDK--TVLLVGSSYSAED-
   JN797595 ATGHGRTGAE-----INFIGLFDHVAAWILASAFADMGEPRHRVVMGVGTLA---
 np_7441833 ATGHGRTGAE-----INFIGLFDHVAAWILASAFADMGEPRHRVVMGVGTLA---
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Fig. 4 ClustalW analysis of the amino acid sequences of *P. putida* A (ATCC 12633) TTABMO (JN797595) and *P. putida* KT2440 PP2033 (NP_744183) using selected members of class B FPMOs: BVMOs: *Pseudomonas putida* JD1 (ACJ37423), *Pseudomonas fluorescens* (AAK54073), *Rhodococcus rhodochrous* (BAA24454), *Thermobifida fusca* YX (AAZ55526.1), *Acinetobacter radioresistens* (GAB75030.1), *Acinetobacter radioresistens* (ADF32068.2); FMOs: *Methylophaga aminisulfidivorans* (EGL54178.1), FMO3 from human (P31513). The *alignment* shows only partial sequences. Identical residues are marked with *asterisks*. Residues thought to be characteristic for the BVMO and dinucleotide-binding motifs are indicated by *black circles* at the top

properties of the purified enzyme suggests that TTABMO is a novel member of the FPMO class B. The FPMOs class B comprises three classes of FAD-dependent monooxygenases: the so-called flavin-containing monooxygenases (FMOs), the *N*-hydroxylating monooxygenases (NMOs), and the Baeyer–Villiger monooxygenases (BVMOs) [25]. All protein sequences of these monooxygenases contain two Rossmann-fold motifs. In addition, the BVMOs sequences contain other characteristic sequence motif, the FXGXXXHXXXW[P/D] [30] (Fig 4), although it has been showed that this motif can vary quite a bit [31]. Recently, based on a larger BVMO sequence alignment, a novel motif, the [A/G]GXWXXXX[F/Y]P[G/M]XXXD, was identified which is actually indicative of true type I BVMOs [31].

Sequence analysis of protein levels revealed that TTABMO of *P. putida* A (ATCC 12633) contains two Rossmann-fold motifs and the motif FXGXXXHXXXW[P/D] present in Type I BVMO (Fig 4). Although the new motif [A/G]GXWXXXX[F/Y]P[G/M]XXXD that plays a crucial role in catalysis allowing really discriminates between members of BVMOs and FMOs [31], is absent in TTABMO. This property led us to exclude that TTABMO is a member of the Type I BVMOs and suggest that this enzyme could be included as a member of the FMOs. However, all FMO sequences contain the FXGXXXHXXXF/Y motif [30], and this characteristic motif is absent in TTABMO of *P. putida* A (ATCC 12633) (Fig 4).

In conclusion, considering that the TTABMO of *P. putida* A (ATCC 12633) is encoded by a single gene, it has the characteristic of two dinucleotide-binding domains (Rossmann fold) (Fig 4); together with the above described biochemical properties (depend on NADPH as coenzyme, contain a tightly bound FAD cofactor, are able to oxidize heteroatoms C-N), we assigned the TTABMO to be a class B FPMO.

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