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Discovery and Characterization of a Novel Thermostable β-Amino Acid Transaminase from a *Meiothermus* Strain Isolated in an Icelandic Hot Spring

*Erica E. Ferrandi, Ivan Bassanini, Barbara Sechi, Marta Vanoni, Davide Tessaro, Sóley Ruth Guðbergsdóttir, Sergio Riva, Xu Peng, and Daniela Monti**

A *Meiothermus* strain capable of using β-phenylalanine for growth is isolated by culture enrichment of samples collected in hot environments and the genome is sequenced showing the presence of 22 putative transaminase (TA) sequences. On the basis of phylogenetic and sequence analysis, a TA termed Ms-TA2 is selected for further studies. The enzyme is successfully produced in *Escherichia coli* Rosetta(DE3) cells, with 70 mg of pure protein obtained from 1 L culture after purification by affinity chromatography. Ms-TA2 shows high activity toward (*S*)-β-phenylalanine and other (*S*)-β-amino acids, as well as a preference for α-ketoglutarate and aromatic aldehydes as amino acceptors. Moreover, Ms-TA2 is shown to be a thermostable enzyme by maintaining about 60% of the starting activity after 3 h incubation at 50 °C and showing a melting temperature of about 73 °C. Finally, a homology-based structural model of Ms-TA2 is built and key active site interactions for substrate and cofactor binding are analyzed.

1. Introduction

Pyridoxal-5'-phosphate (PLP)-dependent transaminases (TAs, EC 2.6.1.x) are enzymes that catalyze the transfer of an amino group from an amine donor substrate to a ketone or aldehyde acceptor substrate. These enzymes usually show an excellent stereoselectivity in the amination of prochiral substrates, as well as the ability to operate under environmentally friendly conditions. Therefore, they have been widely studied during the last decades as a green alternative technology to the use of transition metal catalysts in high-pressure hydrogenation reactions for the preparation of chiral amines in the pharmaceutical and fine chemical industries.[1–4] To further broaden TAs application, extensive research has been carried out to discover novel wild-type enzymes, as well as to improve natural biocatalysts by protein engineering.[2,5]

Among the different alternative approaches to search for new wild-type enzymes, the exploitation of bioinformatic tools to identify homologous sequences to known TAs has become by far the preferred method thanks both to the intrinsic easiness of application and to the vast sequence data available in public databases. Moreover, the study of sequence/structure–function relationships of TAs allowed to further extend enzyme discovery by combining simple sequence similarity analysis to the identification of key motifs and active site residues.[6,7] Interestingly, different TAs from extremophilic sources have been recently identified by sequence-based mining of genomes and metagenomes.[8–12]

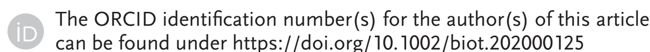
One of the limitations of the use of sequence similarity search by in silico database analysis is that, in most cases, the newly-discovered TAs are very similar to the enzymes used as templates, particularly in respect of the overall active site structure and substrate scope.[5] On the contrary, the use of activity-guided methods, such as enrichment cultures, as an alternative discovery approach may provide access to relatively diverse biocatalysts with useful functional properties. Indeed, the first examples of practically useful TAs, for example, the (*S*)-selective TAs from *Vibrio fluvialis* (Vf-TA),[13] *Achromobacter* (formerly *Alcaligenes*) *denitrificans* (Ad-TA),[14] *Mesorhizobium* sp. LUK (MLuk-TA),[15] and *Bacillus megaterium* (Bm-TA),[16] were discovered by screening microorganisms on the basis of their ability to grow in the presence of defined amines as sole nitrogen source. Remarkably, also the first (*R*)-selective TA from *Arthrobacter* sp. KNK168, subsequently engineered in the development of a biocatalyzed process for the preparation of the antidiabetic drug sitagliptin,[17] was discovered by culture enrichment in the presence of 3,4-dimethoxyamphetamine as sole nitrogen source.[18]

Dr. E. E. Ferrandi, Dr. I. Bassanini, B. Sechi, M. Vanoni, Dr. S. Riva, Dr. D. Monti
Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), CNR
Via Mario Bianco 9, Milan 20131, Italy
E-mail: daniela.monti@scitec.cnr.it

Dr. E. E. Ferrandi, Dr. S. R. Guðbergsdóttir, Prof. X. Peng
Department of Biology
University of Copenhagen
Ole Maaløes Vej 5, Copenhagen 2200, Denmark

Dr. I. Bassanini
Dipartimento di Scienze Farmaceutiche
Università degli Studi di Milano
Via Mangiagalli 25, Milan 20133, Italy

Dr. D. Tessaro
Dipartimento di Chimica, Materiali e Ingegneria Chimica
Politecnico di Milano
Via Mancinelli 7, Milan 20131, Italy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Although enrichment media have been further applied in more recent years to discover TAs,[5] this approach is still underused when considering samples from extreme environments as starting materials. In fact, to the best of our knowledge, there is just one report so far, related to the identification of a thermostable TA from the Antarctic bacterium *Albidovulum* sp. SLM16.[19] Enzymes from extremophiles may have a huge practical potential thanks to their higher robustness, a property that could make them more suitable for industrial applications than their mesophilic counterparts.[20,21]

In this work, we have exploited a culture enrichment approach to discover novel TAs, using different amines as selective medium and starting from samples collected in (hyper)thermophilic environments. Following the isolation of a *Meiothermus* strain capable to use β-phenylalanine (β-Phe) as a sole nitrogen source, a novel thermostable TA was identified and functionally characterized.

2. Experimental Section

2.1. Chemicals

Tryptone, yeast extract, glucose, glycerol, amino acceptors, and PLP were purchased from Sigma-Aldrich (St Louis, MO, USA). Racemic substituted β-Phe and enantiomerically pure (*R*)-β-Phe were synthesized according to published procedures.[22] Isopropyl-β-D-thiogalactopyranoside (IPTG) and agarose were obtained from VWR (Radnor, PA, USA). Gellan gum was from Alfa Aesar, Thermo Fischer Scientific, (Waltham, MA, USA). All other reagents were of analytical grade and commercially available.

2.2. Analytical Methods

HPLC analyses for the quantification of glutamic acid (*o*-phthaldialdehyde (OPA)-derivatized) were conducted using a Jasco 2080-PU pump equipped with a Jasco FP 920 detector and a Kinetex 5-µm EVO C18 100-Å 150 × 4.6-mm column (Phenomenex, Torrance, CA). L-Glutamic acid derivatization was achieved by diluting 20 x the reaction samples (50 µL) in 1 mM HCl and then adding 50 µL of OPA reagent (Sigma-Aldrich). The mobile phase consisted of MeOH/tetrahydrofuran/50 mM phosphoric acid (20:20:960), pH adjusted to 7.5 with NaOH (eluent A), and MeOH/H_2O (65:35) (eluent B). L-Glutamic acid-OPA derivative eluted at 9.6 min with the following gradient: $t = 0$ min, 80% A; $t = 17$ min, 70% A; $t = 32$ min, 20% A; $t = 37$ min, 20% A; $t = 47$ min, 80% A, flow rate 0.5 mL min^{-1}. Peaks were detected at 340 nm and calibration curves were prepared using standard solutions of L-glutamic acid-OPA derivative.

HPLC analyses for the quantification of acetophenone were conducted using a Shimadzu Prominence LC-20AD pump equipped with a SPD-20A UV–vis detector and a SHIM-Pack GWS C18 (150 mm × 4.6 mm, 5 µm) column (Shimadzu Corporation, Kyoto, JP). The mobile phase consisted of H_2O and trifluoroacetic acid (TFA) 0.05% (v/v) (eluent A) and acetonitrile and TFA 0.05% (v/v) (eluent B). Acetophenone eluted at 21.14 min with the following gradient: $t = 5$ min, 100% A; $t = 20$ min, 50% A; $t = 26$ min, 0% A; $t = 30$ min, 0% A; $t = 35$ min, 100% A, flow rate 0.5 mL min^{-1}. Peaks were detected at 254 nm and calibration curves were prepared using standard solutions of acetophenone.

CD spectra were recorded in the range 185–250 nm at 20 °C on a nitrogen flushed Jasco J-1100 spectropolarimeter (Easton,MD, USA) interfaced with a thermostatically controlled cell holder. Protein samples were diluted in degassed water (0.15 mg mL^{-1}) and analyzed in quartz cuvettes (0.1 cm path length). Apparent T_M variation of CD signal at 220 nm were estimated with the following temperature programs: 20 up to 65 °C at 5 °C min^{-1}, data pitch each 2 °C, hold 30 s; 65 up to 90 °C at 2.5 °C min^{-1}, data pitch each 0.5 °C, hold 30 s; 90 up to 95 °C at 5 °C min^{-1}, pitch data each 2 °C.

2.3. Culture Enrichments

Samples of grey clay from hot springs in the area of Hveragerði and Grændalur (Iceland) were used as source of microorganisms. Specifically, samples (Table S1, Supporting Information) isolated at temperatures ranging from 50 to 73 °C were inoculated in minimal medium A (50 mL, see Table S1, Supporting Information, for medium composition) supplemented with 100 mL L^{-1} of carbon source (100 mM glucose and 25 mM glycerol) and nitrogen source ((*rac*)-*sec*-butylamine, (*rac*)-β-Phe, or (*rac*)-α-methylbenzyl amine, Figure 1) stock solution prepared in 50 mM sodium phosphate buffer, pH 7.0.[23] Samples (Table S1, Supporting Information) collected at temperatures ranging from 80 to 90 °C were inoculated in minimal medium B (50 mL, Table S1, Supporting Information) supplemented with 100 mL L^{-1} of carbon source (100 mM glucose and 25 mM glycerol) and nitrogen source ((*rac*)-*sec*-butylamine, (*rac*)-β-Phe, or (*rac*)-α-methylbenzyl amine) stock solution prepared in water. Medium B was adjusted to pH 3.5 with 1 M H_2SO_4.

Inoculated flasks containing medium A were incubated at 55 °C under vigorous shaking while inoculated flasks containing medium B were incubated at 80 °C and 200 rpm.

After 3 or 4 transfers, cultures were plated on the corresponding medium A or B gellan gum plates prepared by supplementing a solution of medium A or B 2x with 4 mL L^{-1} of a solution containing 1 M $MgCl_2$ and 0.3 M $CaCl_2$, then diluting 1:1 with a solution of 1.4% (w/v) gellan gum in water. A pink bacterial strain was isolated on medium A gellan gum plates supplemented with (*rac*)-β-Phe and used for further study.

2.4. Genomic DNA Isolation, Sequencing, and Assembly

DNA extraction was performed on 5 mL overnight cultures of the isolated strain carried out on medium A (Table S1, Supporting Information) supplemented with 5 mL L^{-1} yeast extract solution in water (5% w/v). Genomic DNA (gDNA) was extracted using the E.Z.N.A. Bacterial DNA Isolation Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. The genome was sequenced by BaseClear (Leiden, The Netherlands) using Illumina HiSeq2500 system according to standard procedures. Illumina reads were assembled into contigs using SPAdes

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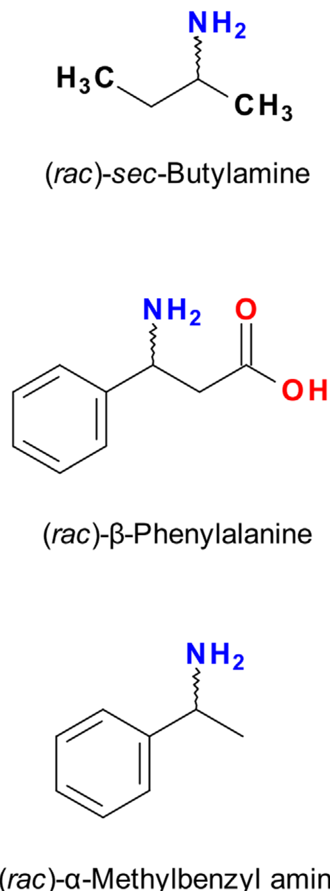

Figure 1. Chemical structure of the amines used for the enrichment cultures.

version 3.10[24] and linked together and placed into scaffolds using SSPACE version 2.3.[25] Gapped regions within scaffolds were (partially) closed using GapFiller version 1.10,[26] then assembly errors were corrected using Pilon version 1.21.[27] The genome was deposited in Genbank (Genbank ID: SESJ00000000).

The RNAmmer tool (http://www.cbs.dtu.dk/services/RNAmmer) was used to search the 16S rRNA sequence.

2.5. Bioinformatic and Protein Structure Analysis

The PGAP tool (https://www.ncbi.nlm.nih.gov/genome/annotation_prok) was used for genome annotation. Bioinformatic search for TA coding genes was carried out by aligning query sequences (Table S2, Supporting Information, entry 1–5) with database sequences using the program LAST (http://last.cbrc.jp/).[28] Phylogenetic trees were created by aligning the sequences reported in Table S2, Supporting Information, using the Clustal X program (selecting the options "exclude positions with gap" and "correction for multiple substitution"),[29] and visualized using the iTOL webserver (http://itol.embl.de/). Homology modeling was performed through the SWISS-MODEL server,[30] and refinement was done by using locPREFMD (http://feig.bch.msu.edu/web/services/locprefmd).[31] Among the 1120 templates collected in the BLAST and HHBlits on the SWISS-MODEL server, 6FYQ was chosen as it displays the best combination of QSQE (quaternary structure quality estimate) and GMQE (Global Mean Quality Estimation) scores that estimate the quality and accuracy of the tertiary structure of the resulting model. The quality of the model was subsequently assessed by using PROCHECK (http://servicesn.mbi.ucla.edu/PROCHECK/).[32] Molecular docking was performed using GOLD[33] and PyMOL[34] for results visualization. Ligand construction and optimization were performed using Discovery Studio package (version 19.1.0; BIOVIA, San Diego, CA), while conformational refinement was carried out using the Mercury module of the CCDC suite (https://www.ccdc.cam.ac.uk). The optimized conformer was then docked into the Ms-TA2 active site using the GOLD module of the Hermes CCDC-suite.

2.6. Ms-TA2 Cloning, Expression, and Purification

Ms-TA2 gene was amplified using genomic DNA as template and primers F1 (GAAGGAGATATACATATGGAACGCCCCTCGTCGGAAATC) and R1 (GTGATGGTGGTGATGATGATCGGCGGCTACCGCTCCCAAC) that include 18 nt (underlined) of overlap with the ends of the pETite vector (Lucigen, Wisconsin, USA) for the subsequent cloning in frame with C-term His-Tag sequence. PCR amplifications were carried out in 50 µL reaction mixtures containing gDNA (200 ng), primers (1 µM each), dNTPs (0.2 mM each), 4 U XtraTaq Pol and 5 µL of XtraTaq buffer (Genespin, Milan, Italy). PCR conditions were as follows: 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and 40 s, and then 72 °C for 10 min. Gene cloning in the pETite vector and transformation of pETiteMs-TA2 in *E. coli* Hi control 10G were carried out according to the Expresso T7 Cloning and Expression kit manual (Lucigen). pETiteMs-TA2 was subsequently purified using the E.Z.N.A. Plasmid mini kit II (Omega/VWR) and transformed in *E. coli* Rosetta(DE3) (Novagen). Protein expression was induced by addition of IPTG to cell cultures and the enzyme was purified from cell lysates by nickel-nitriloacetic acid (Ni-NTA) chromatography (for details, see Figure S5, Supporting Information). Purified Ms-TA2 was dialyzed against 0.1 M KP buffer, pH 9.0, at 4 °C for 24 h and stored at −80 °C. Protein content was measured using the Bio-Rad Protein Assay according to the method of Bradford and protein purity was verified by SDS-PAGE analysis (10% T, 2.6% C) according to the method of Laemmli. The molecular weight protein standard mixture from Bio-Rad (Karlsruhe, Germany) was used as reference. Gels were stained for protein detection with Coomassie Brilliant Blue.

2.7. Enzyme Activity and Thermostability Assays

Aspartate assay was carried out by incubating 10 µL of a solution of purified enzyme, 1 µL malic DH (2 U), 20 µL NADH (10 mM), 10 mM α-ketoglutarate, and 10 mM L-aspartate in 1 mL total volume of 0.1 M KP buffer, pH 9.0, and spectrophotometrically

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monitoring NADH oxidation at 340 nm ($\epsilon = 6.22$ mM^{-1} cm^{-1}) at rt on a Jasco V-530 UV–vis spectrophotometer (Easton, MD, USA). One unit of enzymatic activity was defined as the amount of enzyme that oxidizes 1 µmol of NADH per minute under the assay conditions described above.

To assay the enzyme thermostability, purified enzyme solutions (250 µL) were incubated at temperatures in the range of 20–80 °C for 3 h, then samples (10–50 µL) were added to the aspartate assay solution for residual activity determination.

2.8. Substrate Scope of Ms-TA2

To determine amino acceptor specificity of Ms-TA2, 0.5 mg of purified enzyme was added to a reaction mixture containing 0.1 M KP buffer, pH 9.0, 10 mM acceptor, 20 mM (*rac*)-β-Phe, 1 mM PLP in 0.5 mL final volume at 30 °C. Reactions on substrates **29**–**31** were carried out at 25 °C, pH 7.5. Conversions were determined after 24 h by HPLC analysis (see Section 2.2) estimating the amount of acetophenone formed by spontaneous decarboxylation of the β-keto acid produced by (*rac*)-β-Phe transamination. Amino donor specificity was evaluated in 0.5 mL reaction mixture containing 0.1 M KP buffer, pH 9.0, 10 mM donor, 10 mM α-ketoglutarate, 1 mM PLP, and 0.5 mg of purified enzyme at 30 °C. Conversions were determined after 24 h by estimating the formation of L-glutamate (after OPA derivatization) through HPLC analysis (see Section 2.2).

3. Results

3.1. Isolation of an Amine-Degrading Bacterium from "Hot" Environmental Samples

Environmental samples were collected in the high-temperature region around the triple volcano system Hengill-Hrómundartindur-Grændalur (Iceland), specifically in the area of Hveragerði and Grændalur (Table S1, Supporting Information), at temperatures ranging from 50 to 90 °C. Isolation from these "hot" environmental samples of a bacterial strain possessing transaminase activity was carried out by an enrichment procedure using different amines, that is, (*rac*)-*sec*-butylamine (*s*But), (*rac*)-β-Phe, or (*rac*)-α-methylbenzyl amine (MBA) (Figure 1), as the sole nitrogen source. The enrichment cultures were prepared by inoculating samples collected at temperatures ranging from 50 to 73 °C in medium A, pH 7.0, and allowing the cells to grow at 55 °C, or by inoculating samples collected at temperatures ranging from 80 to 90 °C in medium B, pH 3.5, and allowing the cells to grow at 80 °C.

Microscope inspection of the enrichment cultures permitted to observe bacterial growth only in the presence of β-Phe (samples Is4-4 or Is4-14) or MBA (samples Is4-11 or Is4-18), at either 55 °C (Is4-4 or Is4-11) or at 80 °C (Is4-14 or Is4-18) (Table S1, Supporting Information). When cells growth was observed, isolation of pure cultures was pursued by repeated transfer to fresh medium and streaking onto gellan gum medium plates supplemented with either β-Phe or MBA. A pure culture was obtained only for the sample Is4-4 inoculated in β-Phe-containing medium. Environmental conditions and a picture of the Is 4-4 sampling site are reported in Supporting Information (Table S1 and Figure S1, Supporting Information, respectively).

The isolated microorganism was a rod shaped bacterium (Figure S2A, Supporting Information) characterized by a pink pigmentation (Figure S2B,C, Supporting Information). The genomic DNA was isolated from a pure culture of the microorganism and sent for sequencing.

3.2. Genome Sequencing and Analysis

Genome sequencing generated 7 801 162 reads, which were assembled into 77 contigs, and subsequent genome annotation predicted 3356 genes, 3302 being protein-coding genes, 54 RNAs, and 158 pseudogenes (Table S3, Supporting Information).

For identifying the isolated microorganism, the 16S rRNA sequence (Figure S3, Supporting Information) was used as query for BLAST analysis. Different 16S rRNA genes, for example, those of Bacterium S119 (a thermophilic methanol-utilizing bacterium, Genbank ID: AY040675.1), of *Meiothermus silvanus* strain VI-R2 (Genbank ID: NR_07 4273.1) and of *M. silvanus* DSM 9946 (Genbank ID: CP002042.1), showed 99% sequence identity (100% query cover) to the 16S rRNA sequence of the isolated strain. A 100% identity (95% query cover) was shown with the 16S rRNA genes of *Meiothermus* sp. PNK-1 (Genbank ID: HM854861.1).

The characteristics of the novel microorganism are consistent with its belonging to the *Meiothermus* genus, whose species are described as Gram-negative, aerobic, rod-shaped bacteria, characterized by a pale red to bright red pigmentation and optimal growth temperatures between 50 and 60 °C.[35,36]

Very recently, the whole genome sequence of *Meiothermus* sp. PNK-1 was released in GenBank (NZ_QKOB01000001.1) and BLAST analysis showed that the isolated strain and *Meiothermus* sp. PNK-1 shares around 99% similarity. However, no other information on the *Meiothermus* sp PNK-1 strain are reported in the literature so far. On the basis of these data, the novel strain was named *Meiothermus* sp. PNK-Is4.

3.3. Identification of Novel TAs in *Meiothermus* sp. PNK-Is4 Genome

Since *Meiothermus* sp. PNK-Is4 was capable of growing in a medium supplemented with β-Phe, a multiple sequence alignment of the 22 ORFs annotated as "aminotransferases" by PGAP was first carried out using a set of previously described β-Phe-TAs as queries (Table S2, Supporting Information, entries 1–5). Two sequences (Ms-TA1 and Ms-TA2) were found showing some similarity with known β-Phe-TAs (Figure S4, Supporting Information). The gene sequences coding for Ms-TA1 and Ms-TA2 are 1479 bp and 1392 bp long, respectively, corresponding to proteins of 493 and 464 amino acids, respectively, that share less than 30% identity. According to the InterPro database (https://www.ebi.ac.uk/interpro), both proteins can be classified as class III amine transferases (IPR005814).

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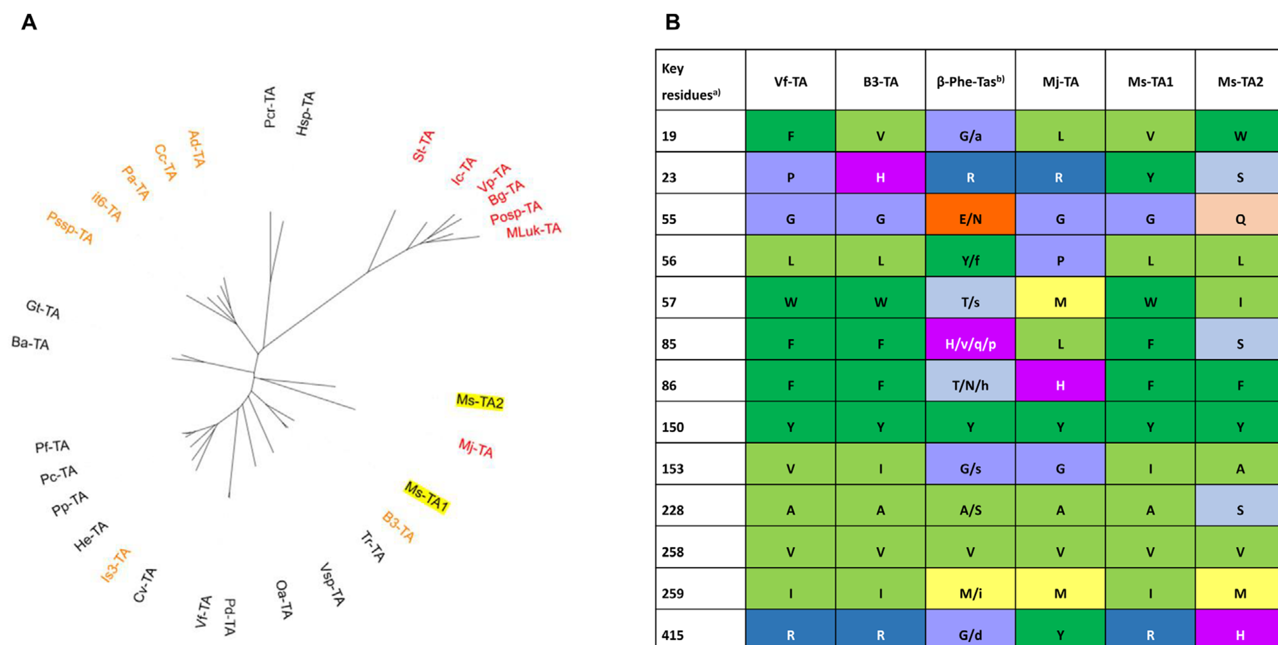

Key residues[a)]	Vf-TA	B3-TA	β-Phe-Tas[b)]	Mj-TA	Ms-TA1	Ms-TA2
19	F	V	G/a	L	V	W
23	P	H	R	R	Y	S
55	G	G	E/N	G	G	Q
56	L	L	Y/f	P	L	L
57	W	W	T/s	M	W	I
85	F	F	H/v/q/p	L	F	S
86	F	F	T/N/h	H	F	F
150	Y	Y	Y	Y	Y	Y
153	V	I	G/s	G	I	A
228	A	A	A/S	A	A	S
258	V	V	V	V	V	V
259	I	I	M/i	M	I	M
415	R	R	G/d	Y	R	H

Figure 2. A) Phylogenetic analysis. Ms-TA1 and Ms-TA2 are highlighted in yellow; TAs showing activity toward β-phenylalanine are shown in red, TAs showing activity toward β-alanine but not toward β-phenylalanine are shown in orange. For details about TAs sources, see Table S2, Supporting Information. B) Occurrence of key residues in selected TAs. The color code indicates the physicochemical properties of the residues. a) Numbering scheme is according to the Vf-TA sequence. b) β-Phe-TAs are the TAs shown in the red clade in Figure 2A and the corresponding residues are shown in uppercase letters or lowercase letters according to the degree of conservation (lowercase letters: the residue is present in only one of the five sequences, otherwise uppercase letter).

However, the similarity of the two new proteins with the TA sequences used for this analysis was too low to predict their possible substrate scope, the highest identity being just around 30% in both cases in the respect of the β-Phe-TA from *Mesorhizobium japonicum* (Mj-TA), a TA showing activity toward β-Phe as well as toward a broad range of aliphatic and aromatic amines.[37]

Therefore, a phylogenetic analysis (Figure 2A) was carried out including a broad set of functionally characterized (*S*)-selective TAs (Table S1, Supporting Information, entries 1–28).

As shown in Figure 2A, most of the known β-Phe-TAs form a distinct clade from other TAs that are active toward β-alanine (shown in red and orange in Figure 2A, respectively). Remarkably, Mj-TA, as well as Ms-TA1 and Ms-TA2, does not belong to the β-Phe-TAs clade.[37] However, Ms-TA2 results to be phylogenetically close to Mj-TA, while Ms-TA1 results to be phylogenetically close to B3-TA, a thermostable TA that is not active toward β-Phe.[12]

Additionally, a deeper sequence analysis was carried out by comparing the key residues of the new TA homologues and selected TAs with different substrate scope. This analysis was carried out on the basis of a possible classification (suggesting reaction and substrate specificity) of transaminases according to the TA fingerprint residues as recently suggested by Steffen-Munsberg et al.,[7] and included the well characterized Vf-TA, B3-TA, the TAs that belong to the above mentioned β-Phe-TAs clade, and Mj-TA (Figure 2B).

Ms-TA1 displays a fingerprint similar to those of B3-TA and Vf-TA, thus further indicating that this enzyme could be close to the so-called "high-activity" TAs.[7] On the other hand, Ms-TA2 shows a fingerprint very different from all the other TAs, the closest one being the Mj-TA fingerprint. It is noteworthy that neither the "flipping arginine" R415 conserved in most of the transaminases nor the arginine R23 that form a salt bridge with the carboxylic acid of β-Phe in most of the β-Phe-TAs are present in the Ms-TA2 fingerprint.

Hence, Ms-TA1 looked quite similar to the well described B3-TA, an enzyme not active toward β-Phe, whereas Ms-TA2 seemed to be closer to enzymes showing β-Phe-TA activity, such as Mj-TA, but with some interesting novelty potential. On the basis of this information, Ms-TA2 was selected for further studies.

3.4. Cloning, Recombinant Expression, and Functional Characterization of Ms-TA2

For recombinant expression, the gene coding for Ms-TA2 was cloned into the pETite vector in frame with a C-term His-Tag sequence. SDS-PAGE analysis showed that Ms-TA2 was successfully overexpressed in *E. coli* Rosetta(DE3) in soluble form, affording 70 mg L^{-1} of pure protein after protein purification by Ni-NTA chromatography (Figure S5A, Supporting Information).

The thermostability of Ms-TA2 was evaluated using a spectrophotometric assay based on the coupled reaction with a NADH-dependent malate dehydrogenase (Figure S6, Supporting Information). Thermal stability studies, performed by estimating the enzyme residual activity after 3 h incubation at different temperatures between 20 and 80 °C, showed that Ms-TA2 is a quite

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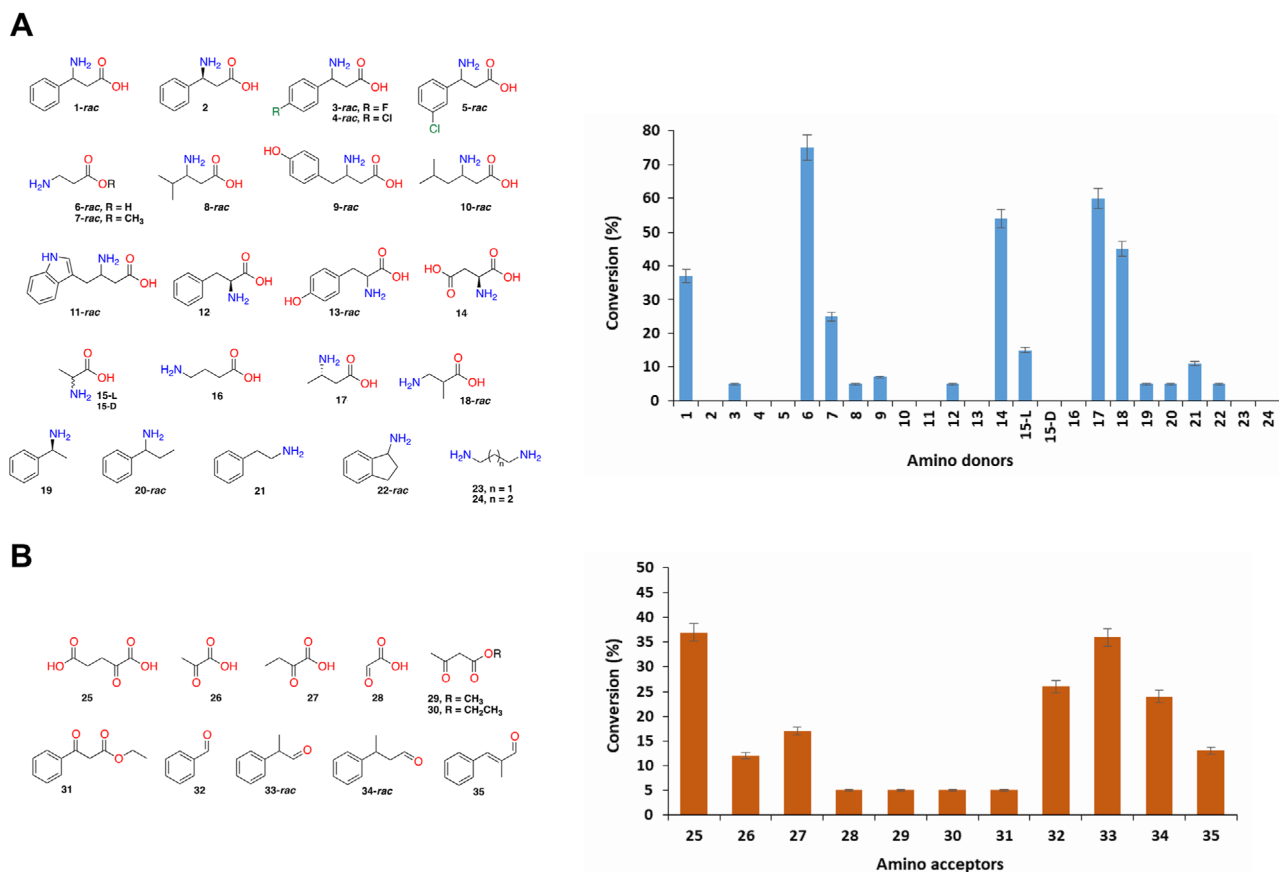

Figure 3. Substrate scope of Ms-TA2. For experimental details, see § 2.8 in Section 2. A) Amino donor spectrum of Ms-TA2. B) Amino acceptor spectrum of Ms-TA2.

thermostable enzyme maintaining about 60% of the starting activity at 50 °C (Figure S5B, Supporting Information). This result was consistent with the apparent melting temperature (T_M) of Ms-TA2 (73 °C ± 0.58) defined by monitoring thermal events at 220 nm in CD analysis.

To evaluate the substrate specificity of Ms-TA2, different amino acids, aliphatic and aromatic amines were investigated as potential donors (amino acceptor: α-ketoglutarate) (Figure 3A), while several keto acids, ketones, and aldehydes were considered as potential acceptors (amino donor: (*R*,*S*)-β-Phe) (Figure 3B).

As expected, Ms-TA2 showed a preference for β-amino acids, such as β-Phe (**1**), β-alanine (**6**), 3-β-aminobutyric acid (**17**), and β-amino *iso*-butyric acid (**18**) (Figure 3A). In the case of β-alanine, a lower conversion was observed with its methyl ester (**7**). Moreover, no conversion was observed in the presence of sterically hindered β-Phe derivatives, such as *p*-Cl-β-Phe (**4**) and *o*-Cl-β-Phe (**5**). As far as β-homo amino acids concern, a minor activity was detected only with β-homotyrosine (**9**), but resulted in low conversions. A similar result was obtained with some α-amino acids such as L-alanine (**15-L**) and L-phenylalanine (**12**), while significantly better conversions were obtained with L-aspartic acid (**14**). Among other aromatic amines, the best donor was 2-phenylethylamine (**21**), while no activity was detected in the presence of aliphatic diamines (**23**,**24**). In addition, Ms-TA2 resulted to be strictly *S*-selective, no conversion being observed in the presence of (*R*)-β-Phe (**2**) or D–alanine (**15-D**).

As far as the amino acceptor specificity concerns, Ms-TA2 showed good activity toward different α-keto acids and aromatic aldehydes (Figure 3B). In particular, the best amino acceptor among α-keto acids was α-ketoglutaric acid (**25**), while, in case of aromatic aldehydes, the best conversion was obtained with phenylpropionaldehyde (**33**). On the contrary, conversions were significantly lower with glyoxylic acid (**28**) and esters of β-keto acids (**29**–**31**).

3.5. Molecular Modelling and Docking Studies

A homology model of Ms-TA2 was built using as template the homodimeric transaminase from *Virgibacillus* sp. 21D (Vsp-TA) crystal structure (PDB: 6FYQ),[38] which contained two PLP molecules. Then, the aldimine formed by the reaction between (*S*)-β-Phe and the PLP cofactor (aldimine A, Figure 4B) was subjected to an in silico docking experiment to investigate its binding mode inside the active site. Figure 4 shows the lowest in energy and most statistically relevant docking pose (A) along with the non-covalent interactions established by aldimine A with the active site residues (B).

The binding site of the PLP cofactor looked very similar to that of homologous PLP-dependent enzymes.[38] Specifically, Gly111, Ala112, Leu303, and Thr304* (monomer B) established

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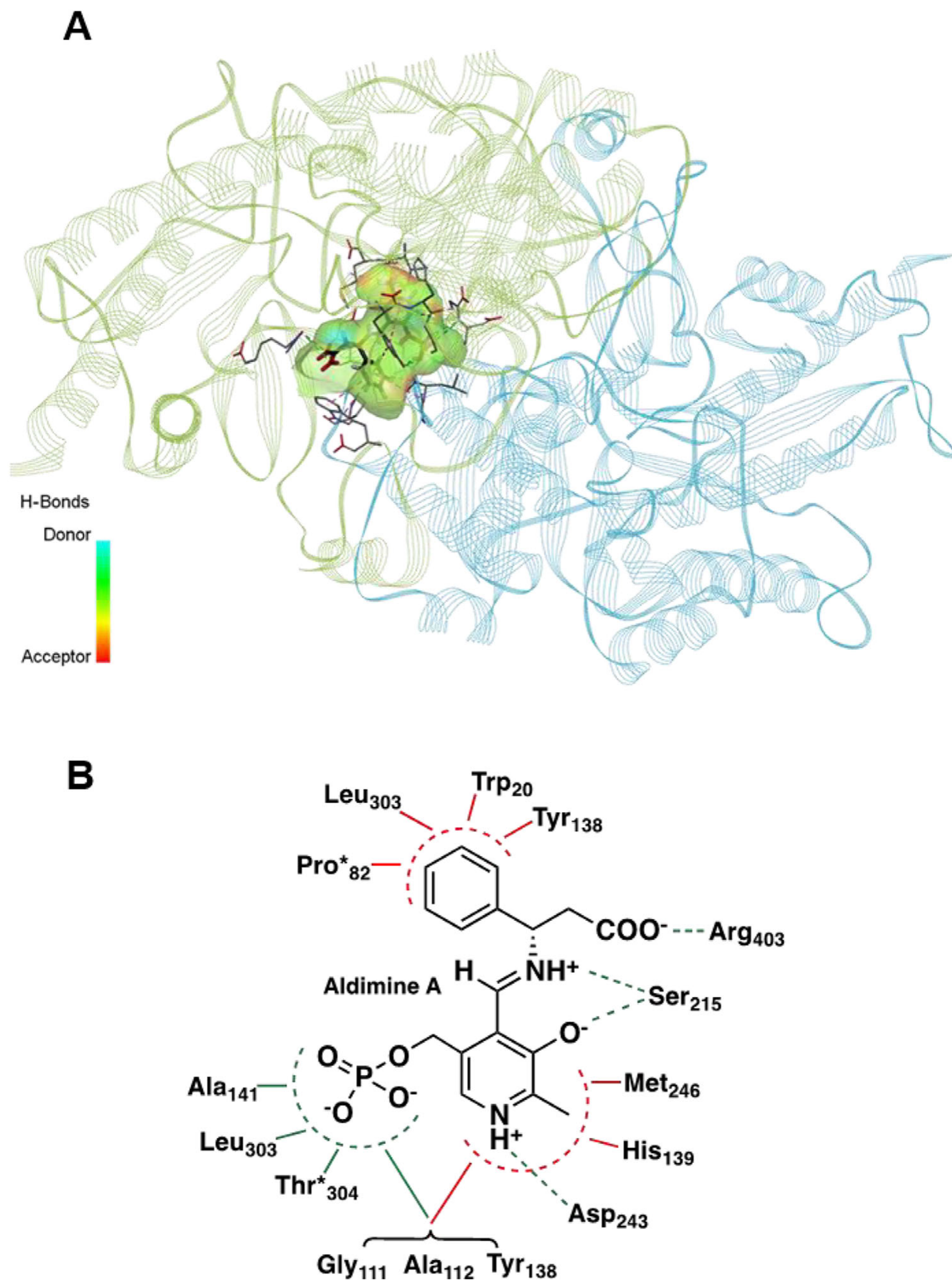

Figure 4. A) Docking of aldimine A into the homology-derived Ms-TA2 active site. B) Schematic representation of the non-covalent interactions established by aldimine A with the active site residues: green: hydrogen bonds or salt bridges, red: π–π stacking or π–alkyl interactions.

H-bonding interactions with the negatively-charged phosphoric group of PLP, while Asp243, His139, and Met246 interacted tightly with the pyridinium ring by establishing a salt bridge, $\pi-\pi$ and $\pi-$alkyl interactions, respectively. The role of Tyr138 appeared pivotal to keep aldimine A in the proper conformation inside the active site by acting as a "stapling" residue. In fact, while interacting with the PLP moiety by both forming a highly directional H-bond with the PLP phosphate group and a $\pi-\pi$ staking interaction, Tyr138 also kept in place the (*S*)-β-Phe aromatic ring by establishing another aromatic non-covalent bond. Ser215, similarly to Tyr138, interacted both with the PLP-moiety and the (*S*)-β-Phe skeleton by establishing a "three-pointed" triangular H-bond interaction acting both as a donor for the imine NH^+ group and as an acceptor for the phenate group of PLP.

The β-Phe side chain was docked into a hydrophobic binding pocket where the phenyl group interacted by $\pi-\pi$ staking and $\pi-$alkyl interactions with Trp20, Pro81*, Tyr138, and Leu303. Its negatively charged carboxylic acid formed instead a strong salt bridge with the cationic Arg403 residue (Figure 4B).

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4. Discussion

In this work, we applied culture-based methods in search of novel TAs from hot terrestrial environments. In fact, activity-guided methods, such as enrichment cultures of environmental samples, give in principle the possibility of uncovering enzymes with a superior novelty than those that can be found by homologous sequence searches.

To provide high diversity in the starting biological samples, the collection sites were carefully selected, with temperatures ranging from 50 to 90 °C, while pH varied from 2.0 to 8.0. Moreover, three structurally different amines were used as sole nitrogen sources.

Although microbial growth was observed in different cases, only one pure culture could be obtained, this fact being rather common when dealing with samples from extreme environments.[39,40] The isolated microorganism belongs to the *Meiothermus* genus[41] and, to the best of our knowledge, no TAs have been characterized so far from this genus, even if putative transaminases showing similarity to known TAs have been annotated in various *Meiothermus* genomes.[12]

Genome analysis of the isolated strain showed the presence of at least two potentially useful TA homologues. Ms-TA2 was selected for characterization, taking into consideration both sequence similarity and phylogenetic relationships with known TAs. In fact, according to sequence analysis, both Ms-TA1 and Ms-TA2 showed the highest sequence identity with B3-TA (64% and 41%, respectively), a TA showing no activity toward β-Phe.[12] However, according to the phylogenetic analysis, Ms-TA2, unlike Ms-TA1, resulted to be closer to Mj-TA,[37] an enzyme accepting β-Phe as substrate, and to other β-Phe-TAs.[9,23,42,43]

The functional characterization of Ms-TA2 confirmed the correctness of our predictions. In fact, the substrate scope of Ms-TA2 mostly resembled the one observed with other β-Phe-TAs that accept β-homo amino acids and other β-amino acids in addition to β-Phe, and usually show low or no activity toward aromatic amines.[9,23,43,44]

Conversely, Ms-TA2 showed high conversion using β-alanine as substrate, while most of the reported β-Phe-TAs are poorly active toward this compound. Instead, no activity was observed in the presence of sterically hindered β-Phe derivatives, e.g., the chloro derivatives **4** and **5**, which were in contrast accepted by other β-Phe-TAs.[9] Moreover, in agreement with its biological source, Ms-TA2 showed a quite good thermostability, which could be useful for practical synthetic applications.

In silico analysis of the Ms-TA2 model built using the homodimeric TA from the halophilic microorganism *Virgibacillus* sp. (Vsp-TA) provided insights into the possible interaction of substrate and cofactor with the active site residues. It is noteworthy that, although the low sequence similarity (34% identity at the amino acidic level), both Ms-TA2 and Vsp-TA are produced by extremophiles and show remarkable stability, Vsp-TA being stable in the presence of high concentration of salts,[38] whereas Ms-TA2 being a thermostable enzyme. However, although Vsp-TA accepted a wide range of (S)-aromatic amines, α-amino acids and aromatic aldehydes, previous studies did not assess its possible activity toward β-Phe.

The docking studies of the PLP-imine of (S)-β-Phe into the homology-derived active site of MS-TA2 suggested an overall

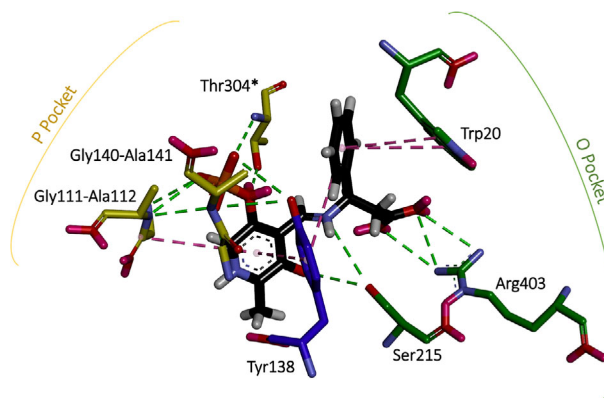

Figure 5. Definition of the P pocket (residues in yellow) and the O pocket (residues in green) established by the most relevant interactions of aldimine A (black) with the residues of the binding pocket. The stapling action of Tyr138 (blue) is also presented.

conservation of the binding pockets of class III TAs.[7] The amino acid residues identified as PLP-coordinating were in fact involved in the interaction with the cofactor and, as it was previously described,[23,38] both monomers of the homodimeric structure of MS-TA2 contributed to the generation of the active site located at the dimer interface. Moreover, similarly to other TAs, the negatively charged carboxylic acid of (S)-β-Phe formed a strong salt bridge with a cationic arginine residue (Arg403) and the pyridinium ion of PLP was kept in place by an interaction with an aspartic acid (Asp243) and by a frame of H-bonds, e.g., with Tyr138, Thr304*, and Ser215 residues, established with its phosphoric acid and hydroxyl group. According to the notation by Crismaru et al.,[23] a "P pocket" (pointing in the same direction as the PLP phosphate) and a "O pocket" (at the side of the hydroxyl substituent of the PLP) could be defined in our model to delineate substrate binding sites on the basis of two topologically fixed pockets. Specifically, the O pocket was defined by Trp20, Ser215, Arg403, while the P pocket by Gly111, Ala112, Gly140, Ala141, and Thr304*. Tyr138 acted as a stapling residue able to induce and stabilize a conformation of aldimine A, which guaranteed the placement of its different chemical groups inside the two pockets by coordinating both the aryl rings via $\pi-\pi$ stacking and forming an H-bond with PLP phosphate (Figure 5).

Interestingly, the binding pose of the PLP-aldimine of (S)-β-Phe in the active site of Ms-TA2 was characterized by a different conformation in the respect to that previously reported for the β-Phe-TA from *Variovorax paradoxus* (Vp-TA).[23] In fact, while in Vp-TA crystal structure the carboxylic acid and the PLP phosphate occupied a region of space pointing toward the same direction in the P pocket, in the Ms-TA2 model only the side chain of (S)-β-Phe was accommodated in the P pocket, while the carboxylic acid was instead bound in the O pocket thanks to previously described interactions with Arg403 and Tyr138.

Since the occurrence of conformational changes in TAs structures upon binding of substrates and cofactor has been widely described in the literature,[45,46] we are currently trying to solve the crystal structure of the enzyme to confirm and refine this preliminary in silico structural analysis, as well as to get more insights into the biocatalyst thermostability and substrate scope.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

amine transferases, biocatalysis, culture enrichment, thermostability, β-amino acids

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