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## Isolation and Characterisation of a Halotolerant $\omega$ -Transaminase from a Triassic Period Salt Mine and Its Application to Biocatalysis

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**Abstract:** Chiral amines are valuable intermediates for the pharmaceutical industry, with up to 40% of current pharmaceuticals containing an amine functionality. However, their syntheses by conventional chemical methods often suffer from a number of key drawbacks. Transaminases (TAMs) are pyridoxal phosphate (PLP) dependent enzymes capable of transferring an amine group to a prochiral ketone, offering a green and economically viable alternative to chiral amine production. Despite some high profile successes, TAMs suffer from limited substrate scope and the ability to function under challenging conditions often required in reaction processes. Mining of untapped, extremophilic environments represents a viable approach in the search for novel enzymes. We report the cloning and expression of an (*S*)-selective  $\omega$ -TAM from a *Halomonas* sp. (Ad2-TAM), isolated from an extreme hypersaline environment formed during the Triassic era (circa 220 mya). Ad2-TAM exhibits an ability to convert a range of structurally diverse aldehyde and ketone substrates, with no decrease in conversion up to 1.5 M (8.8%) NaCl. The enzyme is also tolerant to the presence of organic cosolvents up to 30% and accepts a range of amino donors. These characteristics make Ad2-TAM a promising candidate for industrial applications, whilst also highlighting the value of extreme environments as a source of novel enzymes for the pharmaceutical industry as a whole.

### Introduction

Chiral amines are valuable and versatile building blocks for the pharmaceutical industry, with an estimated 40% of current pharmaceuticals containing an amine functionality.<sup>[1]</sup> Current chemical syntheses of chiral amines suffer from a number of drawbacks, including the need for toxic transition metal catalysts and volatile organic

solvents (VOCs), as well as insufficient stereoselectivity in a single step.<sup>[2]</sup> These drawbacks have, in part, driven the rise of biocatalysis for the production of optically active amines. Biocatalytic approaches initially involved hydrolases,<sup>[3]</sup> although other enzymes such as oxidases, lyases and transaminases (TAMs) have been applied for this purpose,<sup>[4]</sup> as well as dehydrogenases which have been reviewed recently.<sup>[5]</sup>

TAMs are pyridoxal phosphate (PLP) dependent enzymes capable of transferring an amine group to a prochiral ketone, resulting in the formation of optically active amines. Despite high profile successes of TAM use in the pharmaceutical industry, namely the production of the antidiabetic drug sitagliptin,<sup>[6]</sup> there is a growing need for new enzymes with improved capabilities. The application of TAMs is often hampered by a restricted substrate scope, lack of tolerance to harsh reaction conditions and an inability of enzymes to accept bulky substrates without considerable protein engineering.<sup>[6–8]</sup>

Approaches to discover or create new TAMs have involved both metagenomic approaches<sup>[9]</sup> and directed evolution of existing enzymes.<sup>[10,11]</sup> Another approach to overcome these challenges involves shifting the search for novel biocatalysts to previously untapped, extreme environments. The relatively understudied microbiomes of extreme environments represent a vast and exciting resource of novel organisms and biocatalysts. Extremophilic organisms are capable of functioning in conditions which render their mesophilic counterparts ineffective, such as high temperatures, extremes of pH and molar concentrations of salt. In this study we describe the isolation and characterisation of an  $\omega$ -TAM from a Triassic salt mine in Kilroot, Northern Ireland. The protein, annotated as adenosylmethionine-8-amino-7-oxononanoate aminotransferase (Ad2-TAM),<sup>[12]</sup> was identified, cloned and expressed from the genome of a bacterium from the genus *Halomonas*, given the designation CSM-2. CSM-2 was isolated from a thalassohaline environment formed over 200 million years ago and has remained relatively undisturbed ever since. This affords an opportunity to effectively take a step back along the evolutionary timeline and examine the microbes of the past. Such an insight could aid in the discovery of ancestral biocatalysts with potentially unique substrate ranges when compared to their modern cousins.

As well as tackling the issue of restricted substrate scope, biocatalysts from such an extreme hypersaline resource should prove capable of functioning under challenging reaction conditions often demanded by industrial processes.

## Experimental Section

### Organism isolation and identification

Organism CSM-2 was isolated by inoculating media containing peptone (5 g/L), halite from Kilroot salt mine (10%) and agar (1.5%) with 100  $\mu$ L undiluted brine collected from a brine stream in within the mine. Colonies were restreaked to ensure purity and colony PCR was carried out to amplify the 16S rRNA gene for identification, using 25  $\mu$ L DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, UK), 15  $\mu$ L PCR-grade water and 5  $\mu$ L each (2.5  $\mu$ M) of universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR conditions were set up with the following parameters: initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final single extension step at 72 °C for 10 min. Agarose gel electrophoresis of PCR products (1% gel, 90 V, 45 min) was used to determine size and purity of PCR amplicons. These were subsequently cleaned using a GeneJet PCR Purification kit (Thermo Fisher Scientific, UK) as per manufacturer's instructions and quantified using a Qubit<sup>®</sup> 2.0 Fluorometer (Invitrogen, UK). Concentration was adjusted to 10  $\mu$ g/mL and sent for sequencing with Eurofins Genomics using the 27F primer. The BLASTn function associated with the NCBI database was used to compare the resulting sequence to known sequences for identification.

### DNA extraction and PCR amplification of Ad2-TAm gene

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Genomic DNA was extracted from CSM-2 using GenElute™ Bacterial Genomic DNA Extraction kit (Sigma-Aldrich, UK) as per manufacturer's instructions. A sample of extracted DNA was sent to MR DNA Lab (Shallowater, TX, USA) for whole genome sequencing (WGS) using the Illumina MiSeq platform and the remainder retained for PCR. The assembled genome was annotated by uploading to the RAST server, and the Ad2-TAm gene was identified using the SEED Viewer tool and search term 'aminotransferase'. Forward (5'-TTTTTTGAATTCATGACCTCGCCTGTCTGG-3') and reverse (5'-TTTTTCTCGAGCTAATGTTGCCCTGAGCTTTC-3') primers were designed specific to the gene sequence (Invitrogen, UK) with restriction sites included for EcoRI and XhoI respectively (highlighted in bold). 2.5 µL (0.5 µM) of each primer along with 2 µL genomic DNA were added to 10 µL 5x Q5 reaction buffer, 1 µL (0.2 mM) dNTPs, 0.5 µL Q5 High-Fidelity Polymerase, 10 µL 5x Q5 high GC enhancer, and nuclease-free water (to 50 µL) (all New England Biolabs Inc., MA, USA). PCR parameters were as follows: initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min 30 s, followed by a final single extension step at 72 °C for 5 min. Amplicons were checked on a 1% agarose gel (90 V, 45 min) and bands of desired length were excised and DNA extracted using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) as per manufacturer's instructions.

### Preparation of pET28a+/Ad2-TAm vector

25 µL pET28a+ vector and Ad2-TAm were each cut using restriction enzymes EcoRI-HF (1 µL) and XhoI (1 µL) (both New England Biolabs Inc., MA, USA) in a double digest with 3µL CutSmart buffer (NEB) for 1 h at 37 °C. 1 µL Alkaline Calf Intestinal Phosphatase (CIP) (NEB) was subsequently added to the pET28a+ mix and incubated for a further 1 h at 37 °C. Each sample was purified using a GeneJet PCR Purification kit (Thermo Fisher Scientific, UK) as per manufacturer's instructions. Restricted pET28a+ (1 µL) and restricted Ad2-TAm gene (3 µL) were mixed with T4

DNA ligase buffer (1  $\mu$ L) and T4 DNA ligase (1  $\mu$ L) (both NEB), and adjusted to 10  $\mu$ L with ddH<sub>2</sub>O followed by overnight incubation at 16 °C.

### **Transformation and expression of pET28a+/Ad2-TAm in *Escherichia coli***

Following incubation, 2  $\mu$ L of ligation mix was added to 20  $\mu$ L One Shot<sup>®</sup> TOP10 Chemically Competent *Escherichia coli* cells and gently pipetted to mix. This was incubated on ice for 30 min, heat shocked at 42 °C for 1 min before returning to ice for a further 2 min. SOC medium was added (200  $\mu$ L; SOC = 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCL, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose per litre) and the mixture incubated at 37 °C with rotation for 1 h. The mixture was plated on nutrient agar containing 50  $\mu$ g/mL kanamycin sulphate and incubated overnight at 37 °C. Cells were also transformed using the same method with empty pET28a+ vector for use as a negative control. Colony PCR using 5  $\mu$ L RedTaq Polymerase (NEB), forward and reverse primers (0.5  $\mu$ L of each), and ddH<sub>2</sub>O (to 10  $\mu$ L) was carried out using the same gene-specific primers and PCR parameters as with the initial amplification. Amplicons were visualized on a 1% agarose gel (90 V, 45 min), sequenced by Eurofins Genomics, and aligned with the original gene sequence from WGS data using MEGA7 software. Individual colonies were picked and grown in 10 mL LB broth containing 50  $\mu$ g/mL kanamycin sulphate overnight at 37 °C. Plasmids were extracted using a QIAprep spin Miniprep kit (Qiagen, Hilden, Germany) following manufacturer's instructions. One Shot<sup>®</sup> BL21 DE3 Chemically Competent *E. coli* cells (20  $\mu$ L) were transformed with the recombinant plasmid (2  $\mu$ L) using the same protocol as before. Individual colonies were picked as before and used to inoculate 10 mL LB broth with 50  $\mu$ g/mL kanamycin sulphate, followed by incubation overnight at 37 °C with rotation. This primary culture was used to inoculate 1 L LB broth with 50  $\mu$ g/mL kanamycin sulphate, which was allowed to grow to early log phase (OD ~0.6) at 37 °C with rotation. This was induced using IPTG to a final concentration of 0.4 mM and incubated at 20 °C overnight with rotation. Following incubation, the culture was pelleted via centrifugation, freeze-thawed at -80 °C, suspended in 0.1 M potassium

phosphate buffer (pH 8.0), and sonicated on ice. This was further centrifuged to remove cell debris. For use as a cell-free extract, the supernatant (lysate) was decanted and lyophilized overnight. Alternatively, the lysate was clarified using 0.8  $\mu\text{M}$  filters and purified as described below.

### **His-tag-mediated purification of Ad2-TAm protein**

Clarified cell-free extract was purified by immobilized metal affinity chromatography (IMAC) using the N-terminal 6x His-tag encoded by the pET28a+ vector. An ÄKTA Prime Plus Liquid Chromatography System was used to load the clarate on a HisTrap™ HP 1 mL column (both GE Healthcare Life Sciences, UK) at a rate of 1 mL min<sup>-1</sup>. This was washed using buffer containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, made up to 1 L with dH<sub>2</sub>O and adjusted to pH 8.0 with NaOH. Purified protein was eluted in 1 mL fractions using buffer containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole made up to 1 L with dH<sub>2</sub>O and adjusted to pH 8.0 with NaOH.

### **Expression and phylogenetic analysis**

Recombinant Ad2-TAm protein and empty vector controls were analysed via SDS-PAGE using a NuPage® 4-12% Bis-Tris gel and NuPage® MES SDS running buffer, run at 200 V for 1 h. Recombinant Ad2-TAm was also analysed using Western Blot, with a gel set up as above and transferred to a nitrocellulose membrane at 30 V for 2 h in buffer containing, per/L, tris 3.48 g, glycine 14.42 g, methanol 200 mL, made up to 1 L with dH<sub>2</sub>O. The resulting membrane was blocked using a solution of 5% skimmed milk in PBS at 37 °C (60 rpm) for 1 h, rinsed thoroughly in PBS, and treated with 1 in 10,000 monoclonal anti-polyhistidine-peroxidase antibody solution (Sigma-Aldrich, UK) at room temperature (60 rpm) for 1 h. After rinsing with PBS,

this was developed using a solution containing 12 mg 3,3'-diaminobenzidine, 10  $\mu\text{L}$  30%  $\text{H}_2\text{O}_2$ , and 20 mL  $\text{dH}_2\text{O}$ .

#### **Colorimetric screening assay using *o*-XDA as amino donor**

Using a protocol adapted from Green et al (2014)<sup>[1]</sup>, *o*-XDA **1** (25 mM) and PLP (1 mM in potassium phosphate buffer, 100 mM, pH 8.0) and the substrate (final concentration of 5 mM from an initial 50 mM stock in DMSO) were added to a 96-well deep well plate to give a total volume of 180  $\mu\text{L}$ . The reaction was started by the addition of either 20  $\mu\text{L}$  rehydrated Ad2-TAm cell-free extract to a final working concentration of 20 mg/mL, or 20  $\mu\text{L}$  purified protein at a final working concentration of 0.5 mg/mL. A colour change from yellow to black denoted a positive result for TAm-catalyzed conversion.

#### **HPLC-based screening assay employing (*S*)-MBA as amino donor**

(*S*)-MBA **2** (25 mM) and PLP (1 mM in potassium phosphate buffer, 100 mM, pH 8.0), and the substrate (final concentration of 5 mM from an initial 50 mM stock in DMSO) were added to a 96-well deep well plate to give a total volume of 180  $\mu\text{L}$ . For assays employing sodium pyruvate and  $\alpha$ -ketoglutaric acid, stock concentrations of these substrates were prepared in water due to poor solubility in DMSO. These reaction mixes were then supplemented with an appropriate amount of DMSO. The reaction was initiated by the addition of 20  $\mu\text{L}$  rehydrated Ad2-TAm cell-free extract to a final working concentration of 20 mg/mL. Purified Ad2-TAm was also tested for ability to convert substrates using 20  $\mu\text{L}$  purified protein at a final working concentration of 0.5 mg/mL. After incubation for 16 h at 30  $^\circ\text{C}$  and 1200 rpm, the reaction was quenched with 800  $\mu\text{L}$  62.5% acetonitrile and denatured protein was removed by centrifugation. 200  $\mu\text{L}$  of supernatant was removed for analysis by HPLC using a Kinetex 5u C18 100A column (50 x 4.6 mm) and an Agilent 1260 Infinity model, with detection of acetophenone **4** coproduct at 240 nm. Product



formation in the assays employing purified protein was also detected using a diode array detector (DAD) at 10 nm intervals from wavelengths 210 nm to 270 nm. The concentration of acetophenone coproduct formation was determined over 8 min using a gradient of 5-50% organic solvent (where A = dH<sub>2</sub>O with 0.1% H<sub>3</sub>PO<sub>4</sub> and B = acetonitrile with 0.1% H<sub>3</sub>PO<sub>4</sub>). Acetophenone eluted at a retention time of 5.9 min. Assays using both Ad2-TAm and vector only controls were carried out in triplicate for each substrate, with an average taken for each and values for negative control subtracted from Ad2-TAm values.

### HPLC-based screening assay employing cinnamaldehyde as substrate

Amino donor (25 mM) and PLP (1 mM in potassium phosphate buffer, 100 mM, pH 8.0) and cinnamaldehyde **9** (final concentration of 5 mM from an initial 50 mM stock in DMSO) were added to a 96-well deep well plate to give a total volume of 180  $\mu$ L. The reaction was started by the addition of 20  $\mu$ L rehydrated Ad2-TAm cell-free extract to a final working concentration of 20 mg/mL. After incubation for 16 h at 30 °C and 1200 rpm, the reaction was quenched with 800  $\mu$ L 62.5% acetonitrile and denatured protein was removed by centrifugation. 200  $\mu$ L of supernatant was removed for analysis by HPLC using a Kinetex 5u C18 100A column (50 x 4.6 mm) with detection at 210 nm. The concentration of cinnamyl amine **20** product formation was determined over 8 min using a gradient of 5-50% organic solvent (where A = dH<sub>2</sub>O with 0.1% H<sub>3</sub>PO<sub>4</sub> and B = acetonitrile with 0.1% H<sub>3</sub>PO<sub>4</sub>). Cinnamyl amine eluted at a retention time of 2.8 min. Assays using both Ad2-TAm and vector only controls were carried out in triplicate for each amino donor, with an average taken for each and values for negative control subtracted from Ad2-TAm values.

### Evaluation of enzyme performance under varying reaction parameters

(*S*)-MBA **2** (25 mM) and PLP (1 mM in potassium phosphate buffer, 100 mM, pH 8.0 unless changed to assess effect of changing pH), and benzaldehyde (final

concentration of 5 mM from an initial 50 mM stock in DMSO) were added to a 96-well deep well plate to give a total volume of 180  $\mu$ L. The reaction was initiated by the addition of 20  $\mu$ L rehydrated Ad2-TAm cell-free extract to a final working concentration of 20 mg/mL. After incubation for 16 h at 30 °C (unless changed to assess effect of changing temperature) and 1200 rpm, the reaction was quenched with 800  $\mu$ L 62.5% acetonitrile and denatured protein was removed by centrifugation. 200  $\mu$ L of supernatant was removed for analysis by HPLC using a Kinetex 5u C18 100A column (50 x 4.6 mm) and an Agilent 1260 Infinity model, with detection of acetophenone 4 coproduct at 240 nm. The concentration of acetophenone coproduct formation was determined over 8 min using a gradient of 5-50% organic solvent (where A = dH<sub>2</sub>O with 0.1% H<sub>3</sub>PO<sub>4</sub> and B = acetonitrile with 0.1% H<sub>3</sub>PO<sub>4</sub>). Acetophenone eluted at a retention time of 5.9 min. Assays using both Ad2-TAm and vector only controls were carried out in triplicate for each substrate, with an average taken for each and values for negative control subtracted from Ad2-TAm values.

To assess the effect of temperature on enzyme performance, assays were set up as described above but incubated at different temperatures, ranging from 20-45 °C.

To assess the effect of changing pH on enzyme performance, assays were set up using a universal buffer<sup>[2]</sup> in place of phosphate buffer. This contained 25 mM citric acid, 25 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM Tris, 12.5 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> and 25 mM KCl, adjusted using NaOH, with pH values from pH 6.0 to 11.0 investigated.

To assess the effect of organic solvents on enzyme performance, more concentrated stock solutions of potassium phosphate buffer (pH 8.0) were prepared to reduce the aqueous volume in the reaction accordingly, with PLP concentrations also adjusted in buffer solutions accordingly. Once diluted by the required amount of organic solvent, the overall concentrations of potassium phosphate buffer (pH 8.0) and PLP in solution remained at 100 mM and 1 mM, as before. Concentrations of organic solvents investigated ranged from 0-30%.

To assess the effect of increasing salt concentration on enzyme performance, potassium phosphate buffer stock solutions were prepared containing the required amounts of NaCl and KCl respectively (warmed gently where necessary to dissolve fully). These were prepared so that the dilutive effect of other reaction constituents ensured the appropriate overall salt concentration in the assay. Buffer pH was

adjusted using small amounts of NaOH to account for the addition of salt. Salt concentrations assessed ranged from 0-2.5 M for both NaCl and KCl.

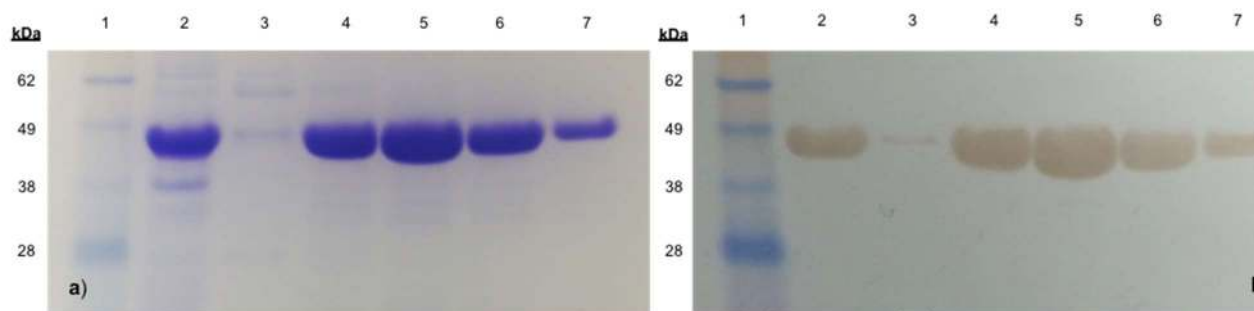
In assessing the effect of increased volume of reaction, (*S*)-MBA **2** (25 mM) and PLP (1 mM in potassium phosphate buffer, 100 mM, pH 8.0) and benzaldehyde **3** (final concentration of 5 mM from an initial 50 mM stock in DMSO) were added to a sterile 250 mL conical flask to give a total volume of 90 mL. The reaction was started by the addition of 10 mL rehydrated Ad2-TAm cell-free extract to a final working concentration of 20 mg/mL. After incubation for 16 h at 30 °C and 100 rpm, the reaction mixture was transferred to a sterile 1 L flask and the reaction quenched with 400 mL 62.5% acetonitrile. Denatured protein was removed by centrifugation. 200  $\mu$ L of supernatant was removed for analysis by HPLC using a Kinetex 5u C18 100A column (50 x 4.6 mm) with detection at 240 nm. The concentration of acetophenone product formation was determined over 8 min using a gradient of 0-50% organic solvent (where A = dH<sub>2</sub>O with 0.1% H<sub>3</sub>PO<sub>4</sub> and B = acetonitrile with 0.1% H<sub>3</sub>PO<sub>4</sub>) with subtraction of empty vector negative control. The acetophenone product eluted at a retention time of 5.9 min.

#### Determination of specific activity

(*S*)-MBA **2** (25 mM) and PLP (1 mM in universal buffer (as above) pH 9.0), and benzaldehyde **3** (final concentration of 5 mM from an initial 50 mM stock in DMSO) were added to a 1.5 mL microtube to give a total volume of 180  $\mu$ L. The reaction was initiated by the addition of 20  $\mu$ L rehydrated Ad2-TAm cell-free extract to a final working concentration of 1 mg/mL. Reactions were quenched with 800  $\mu$ L 62.5% acetonitrile at regular time intervals and denatured protein was removed by centrifugation. 200  $\mu$ L of supernatant was removed for analysis by HPLC using an XBridge<sup>®</sup> C18 3.5  $\mu$ m column (100 x 4.6 mm) and an Agilent 1260 Infinity model, with detection of acetophenone **4** coproduct at 240 nm. The concentration of acetophenone coproduct formation was determined over 10 min using a gradient of 5-50% organic solvent (where A = dH<sub>2</sub>O with 0.1% H<sub>3</sub>PO<sub>4</sub> and B = acetonitrile with 0.1% H<sub>3</sub>PO<sub>4</sub>). Acetophenone eluted at a retention time of 9.0 min. Assays using both

Ad2-TAm and vector only controls were carried out in triplicate, with an average taken for each and values for negative control subtracted from Ad2-TAm values. Ad2-TAm concentration in cell-free extract was determined using a combination of a Qubit<sup>®</sup> 2.0 Fluorometer (Invitrogen, UK) and SDS-PAGE densitometry.

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**Fig. 1.** SDS-PAGE **a)** and Western Blot **b)** of pre-purified clarate (well 2) and Ad2-TAm fractions, purified by His-tagged protein purification (wells 3-7), alongside SeeBlue® Plus2 Protein Standard (well 1).

## Results and Discussion

### Organism identification and gene selection

The organism, from which the TAM enzyme was cloned and expressed, was isolated from a brine sample from Kilroot salt mine and given the designation CSM-2 (This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NBYR000000000. The version described in this paper is version NBYR010000000). Using the Basic Local Alignment Search Tool nucleotide (BLASTn) function<sup>[13]</sup> of the National Center for Biotechnology Information (NCBI) database,<sup>[14]</sup> the organism was found to be a member of the genus *Halomonas*, with the closest neighbour deemed to be *Halomonas janggokensis* M24 (99% similarity).

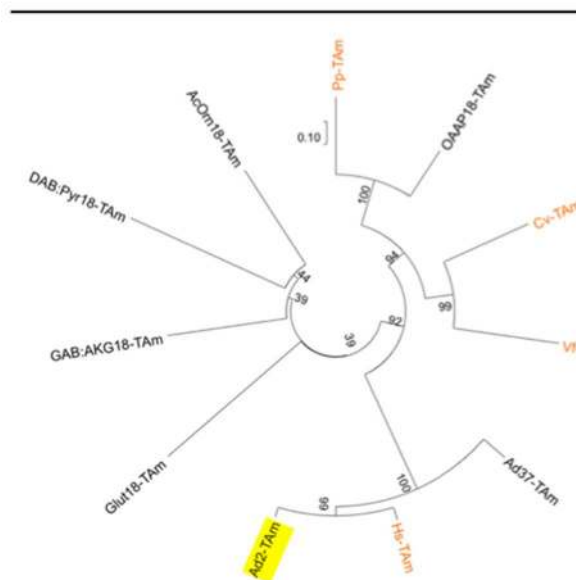
On uploading the assembled contigs from WGS data to the Rapid Annotations using Subsystems Technology (RAST) server,<sup>[12]</sup> the subsystems search tool revealed 29 results using the search term ‘aminotransferase’. The gene ‘adenosylmethionine-8-amino-7-oxononanoate aminotransferase’ was selected based on PLP fold type (Fold type I) and TAM class (Class III) known to be industrially relevant.<sup>[15]</sup> The gene was 1284 bp in length and the corresponding protein was given the designation Ad2-TAm.

## Purification and expression analysis

SDS-PAGE analysis following purification showed the formation of a 47 kDa protein (**Fig. 1A.**), commensurate with a gene length of 1284 bp. Analysis using Western Blotting further confirmed the presence of the desired protein, using the 6x His-tag encoded by the pET28a+ vector in combination with an N-terminal anti-polyhistidine-peroxidase antibody (**Fig. 1B.**).

## Phylogenetic analysis and alignment with other TAMs

A number of other TAMs were cloned and expressed from bacterial isolates using the same approach as with Ad2-TAM (**Supplementary Fig. 1**). These proteins were not expressed in soluble form and therefore did not possess any biocatalytic activity. Attempts to solubilise these proteins with urea followed by refolding in salt-containing buffer were unsuccessful. Varying expression conditions by reducing temperature and changing IPTG concentration was also tried without success. The amino acid sequences for these genes, relative to that of Ad2-TAM, are shown in **Fig. 2**, as well as a number of sequences from TAMs of known organisms: *Vibrio fluvialis* (Vf-TAM),<sup>[16]</sup> *Pseudomonas putida* (Pp-TAM),<sup>[17]</sup> *Chromobacterium violaceum* (Cv-TAM)<sup>[18]</sup> and *Halomonas* sp. (Hs-TAM).<sup>[19]</sup> A neighbour-joining tree was generated using MEGA 7,<sup>[20]</sup> with bootstrap values calculated based on 1000 replicates. Using protein BLAST (BLASTp) function<sup>[13]</sup> associated with the NCBI database, the closest neighbour to Ad2-TAM was determined to be a TAM from *Halomonas* sp., with a sequence similarity of 89%. Ad2-TAM shared relatively low homology with



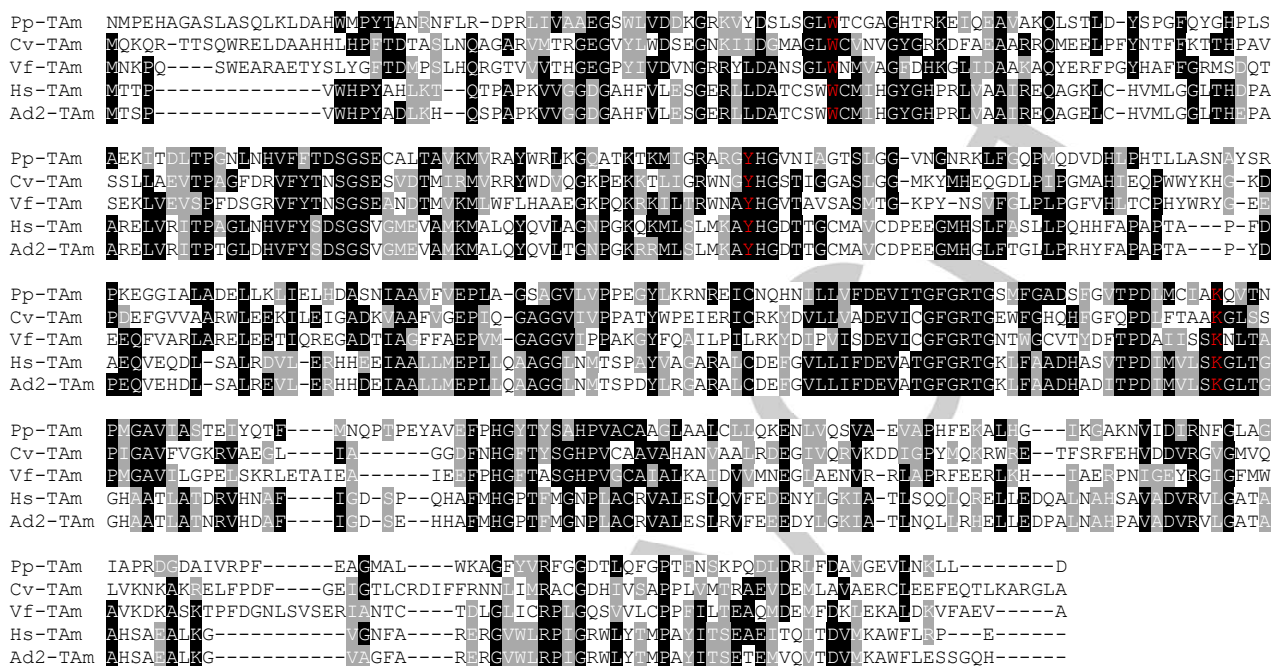
**Fig. 2.** Amino acid sequences of TAM genes from various genomes (including Ad2-TAM - highlighted in yellow) and a number of TAMs from identified species (Pp-TAM, Cv-TAM, Vf-TAM and Hs-TAM - shown in red) were used to create a neighbour-joining tree (bootstrap values calculated from 1000 replicates).

commonly employed TAMs (Vf-TAM - 28%, Pp-TAM - 28%, Cv-TAM - 30%).

Despite the low level of homology, many critical residues were conserved across all sequences (**Fig. 3**). These include a tryptophan residue at position 60 (W60), which is key for substrate specificity; a tyrosine residue at position 153 (Y153), which allows for hydrogen bonding of a hydroxyl group to the phosphate group of PLP; and a lysine residue at position 288 (K288), critical for Schiff base formation.<sup>[21]</sup>

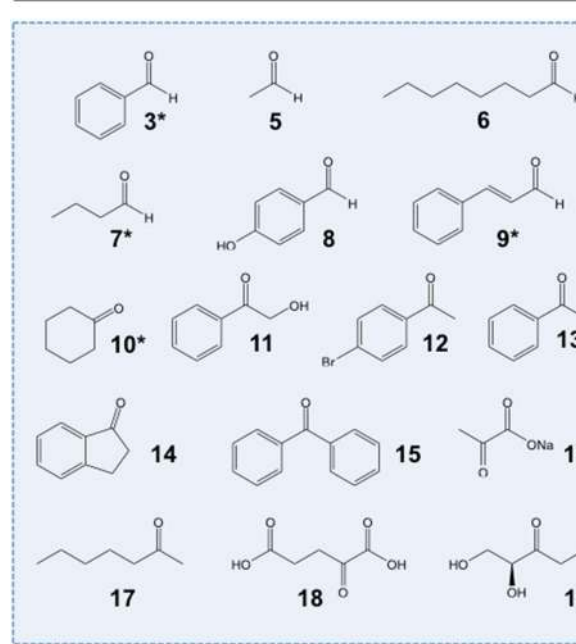
### Screening purified Ad2-TAM for ability to convert substrates

Purified Ad2-TAM was tested for the ability to convert substrates using a selection of compounds (shown with an asterisk in **Fig. 4**) and *o*-xylylenediamine (*o*-XDA) **1** as amino donor (**Scheme 1A**). Aldehydes, aromatic ketones, and a ketose were accepted by the purified enzyme, demonstrating the versatility of Ad2-TAM's substrate range (**Fig. 5**). In each case, the same imidazole-containing buffer used to elute Ad2-TAM from the purification column was used as a negative control (full triplicate results shown in **Supplementary Fig. 2**).



**Fig. 3.** Multiple alignment of amino acid sequence of Ad2-TAm with a number of TAm from identified species (Pp-TAm, Cv-TAm, TAm and Hs-TAm). Key residues for activity and substrate specificity are conserved across all sequences, including Trp60, Tyr153 and Lys288 (highlighted in red).

(*S*)-methylbenzylamine ((*S*)-MBA) **2** was also used as an amino donor in a HPLC-based assay (Scheme 1B.), employing benzaldehyde **3** as substrate. The resultant HPLC separation was observed over a range of wavelengths, with the more hydrophilic amino donor eluted early in the run with a retention time (RT) of 1.5 min, and the more hydrophobic acetophenone product (RT 5.9 min) and remaining benzaldehyde substrate (RT 5.1 min). This reaction proceeded with a 26% conversion.



### Screening Ad2-TAm cell-free extract for ability to convert substrates



Purification of the His-tagged protein allowed for characterisation and basic testing of the pure, expressed enzyme's ability to convert substrates. However, lack of stability associated with the purified protein led to difficulties in scale-up and increasing the enzyme concentration in the reaction. A number of approaches including lyophilisation and dialysis were attempted in order to overcome this issue, but led to precipitation of the protein on dialysis or resuspension. With the use of empty vector (pET28a+) controls, the cell-free extract was deemed to represent a more suitable approach to characterising the ability of Ad2-TAm to convert different substrates. It was possible to lyophilize and resuspend Ad2-TAm as a cell-free extract, allowing for greater enzyme loading in assays. This provided a more accurate reflection of the capabilities of Ad2-TAm as a biocatalyst.

### Colorimetric assay using *o*-XDA as amino donor

The colorimetric assay (**Scheme 1A.**) once again demonstrated the versatility of Ad2-TAm as a biocatalyst, with the enzyme able to accept a diverse range of substrates. As observed in **Fig. 7**, both aromatic and aliphatic aldehydes and ketones were aminated, whilst no colour change was observed with the vector only control in each case (**Supplementary Fig. 3**). This broad substrate acceptance is typical of Class III TAm enzymes,<sup>[9,22–24]</sup> and represents one of the key advantages of using this particular group for chiral amine synthesis.

This assay is useful as an initial visual screen for active TAm, but is limited by its high level of sensitivity, often producing positive results when no substrate is added to the reaction mixture, with PLP acting as amino acceptor. Despite this, there appears to be a broad correlation between the intensity of the black colour formed at 30 min (**Fig. 7**) and those substrates which were best converted at 16 h, as shown by the HPLC-based screening assay (**Table 1**). It is worth noting other colorimetric assays which have been developed recently for high-throughput screening of TAm may

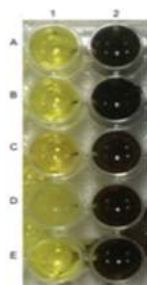
**Fig. 4** – Substrates screened with assay **A** (using (S)-M as an amino donor and HPLC detection of acetophenone at 240 nm) and **C** (using *o*-XDA as an amino donor and observing formation of coloured precipitate to denote TAm catalyzed substrate conversion). Those substrates used for testing of IMAC-purified Ad2-TAm are shown with asterisk.

circumvent such issues.<sup>[25]</sup>

### HPLC-based screening assay using (*S*)-MBA as amino donor

The same substrates used in the colorimetric assay were tested using a HPLC-based assay with (*S*)-MBA **2** as amino donor (**Scheme 1B**). Assays were carried out in triplicate, with results for vector only controls subtracted in each case. Acetophenone **4** product formation, detected at 240 nm, was used to determine conversion (%) for each substrate. Ad2-TAm performed well with many aldehyde substrates, with good conversion of both aliphatic and aromatic aldehydes (benzaldehyde **3** 55%, acetaldehyde **5** 52%). Increases in substrate acyl chain length led to a reduction in conversion (octanal **6** 22% vs. butyraldehyde **7** 49%), consistent with observations noted in the limited existing literature.<sup>[9]</sup> Similar results were observed with the addition of side groups to the benzyl ring of aromatic aldehydes (benzaldehyde 55% vs. 4-hydroxybenzaldehyde **8** and cinnamaldehyde **9**, 39% and 36% respectively). Aromatic ketones followed a similar pattern, with simpler structures showing higher levels of amination than their more complex counterparts (cyclohexanone **10** 49% vs. 2-hydroxyacetophenone **11** 39%, 4-bromoacetophenone **12** 12% and propiophenone **13** 4%). Bicyclic ketones such as the steroid building block 1-indanone **14** (7% conversion) and the biphenyl ketone benzophenone **15** (2% conversion) were poorly accepted by Ad2-TAm, most likely due to the steric constraint afforded by their bulky substituents. Aliphatic ketones showed poor conversion yields, (sodium pyruvate **16** 4%, 2-heptanone **17** 7%,  $\alpha$ -ketoglutaric acid **18** 6%), whilst Ad2-TAm displayed a reasonable ability to convert the ketose sugar L-erythrulose **19** (25% conversion). It is notable that Ad2-TAm exhibited greater conversion of aldehydes than ketone substrates, which can be attributed to the greater reactivity of aldehydes given the greater electrophilicity of their carbonyl

**Scheme 1. A.** *o*-XDA screening assay performed against multiple substrates. Reaction conditions: *o*-XDA 25 mM, substrate 5 mM, PLP 1 mM, potassium phosphate buffer, pH 8.0, 100 mM, enzyme as cell-free extract (20 mg/mL) or His-tagged purified protein (0.5 mg/mL), 16 h, 30 °C, 1200 rpm. **B.** (*S*)-MBA screening assay performed against multiple substrates. Reaction conditions: (*S*)-MBA 25 mM, substrate 5 mM, PLP 1 mM, potassium phosphate buffer, pH 8.0, 100 mM, enzyme as cell-free extract (20 mg/mL) or His-tagged purified protein (0.5 mg/mL), 16 h, 30 °C, 1200 rpm. **C.** Cinnamaldehyde screening assay performed using multiple amino donors. Reaction conditions: amino donor 25 mM, cinnamaldehyde 5 mM, PLP 1 mM, potassium phosphate buffer, pH 8.0, 100 mM, enzyme as cell-free extract (20 mg/mL) or His-tagged purified protein (0.5 mg/mL), 16 h, 30 °C, 1200 rpm.

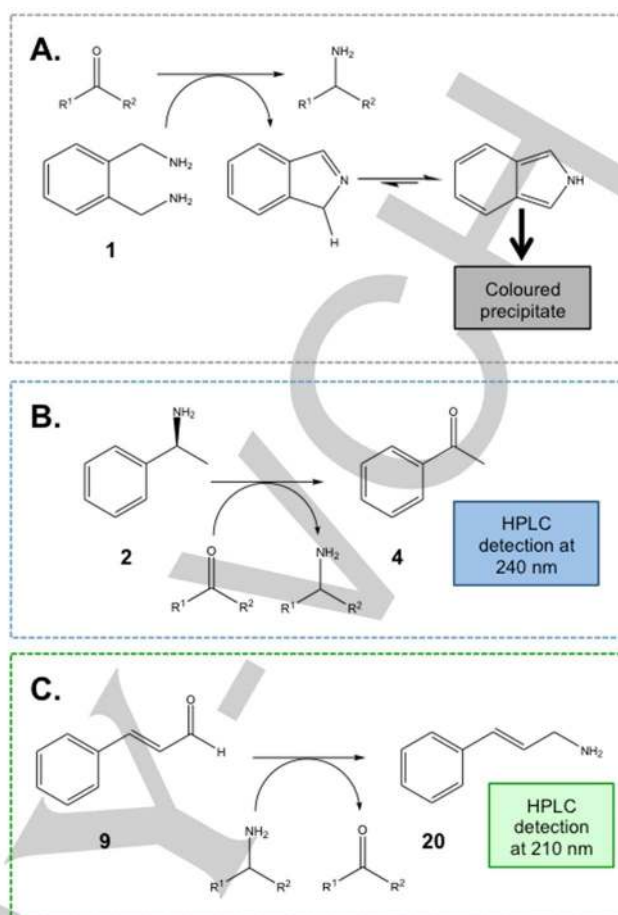


group vs. ketones.<sup>[22]</sup> Percentage conversion for all substrates tested are shown in **Table 1**. When no substrate was used in the reaction conversion values were <1%.

Specific activity was determined as 0.11 U/mg for **3**. One unit is defined as the amount of enzyme that produces 1  $\mu\text{mol}$  of acetophenone in 1 min. Although benzaldehyde may not be the cognate substrate for Ad2-TAM in nature, the relatively low value obtained highlights the potential need for directed evolution to improve specific activity of this enzyme towards certain substrates. Such site-specific mutations have proven extremely successful in improving the activity of TAMs by several orders of magnitude, with a growing number of examples in the current literature.<sup>[6,8,11]</sup>

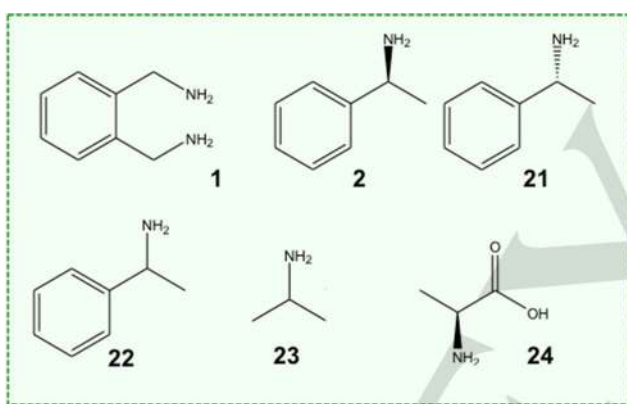
### PLC-based screening assay using cinnamaldehyde as substrate

The ability of Ad2-TAM to utilize a range of amino donors was examined with a HPLC-based assay using cinnamaldehyde **9** as substrate in each case (**Scheme 1C**). Assays for each amino



donor were carried out in triplicate, with values for vector only controls subtracted from Ad2-TAM to provide overall values. Formation of cinnamyl amine **20** product (detected at 210 nm) was used to determine relative ability of the enzyme to accept each amino donor. Of the compounds tested, *o*-XDA **1** proved to be the most effective amino donor (**Fig. 8A.**). As well as being readily accepted by the enzyme,

**Fig. 5.** Colorimetric assay employing a selection of substrates (5 mM) (A. 3, B. 7, C. 19, D. 9, E. 10). *o*-XDA (25 mM) was used as amino donor with all assays set up in potassium phosphate buffer, pH 8.0 (100 mM), containing PLP (1 mM). Reaction was started by addition of His-tagged purified Ad2-TAM to a final concentration of 0.5 mg/mL (wells in column 2) and allowed to proceed for 16 h at 30 °C and 1200 rpm. Elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole, pH 8.0) was used as a negative control (wells in column 1).



*o*-XDA also benefits from a favourable reaction equilibrium. The imine product of the initial deamination of *o*-XDA undergoes spontaneous tautomerization to form an aromatic isoindole. This effectively removes one of the products from the reaction mixture, allowing the equilibrium of the reaction to shift towards further product formation.<sup>[26]</sup> Recently this work has been developed further with the advent of ‘smart’ diamine donors, whose corresponding aminoaldehydes can dimerize or cyclize following transamination.<sup>[7]</sup> This has been shown to substantially reduce product inhibition, which can present significant problems for TAM-catalyzed reactions and have a negative impact on conversion yield.<sup>[27–29]</sup>

Varying the amino donor also reveals the enantioselectivity of Ad2-TAM. The relative ability of Ad2-TAM to accept (*S*)-MBA **2** (vs. *o*-XDA at 100%) as an amino donor was 89%. Comparing this value with its ability to accept the (*R*)-enantiomer of MBA **21** (17%) and a racemic mixture **22** of the two isomers (52%), clearly shows Ad2-TAM to be an (*S*)-selective enzyme (**Fig. 8A.**).

**Fig. 8A.** shows the smaller amino donors isopropylamine **23** (IPAm) and L-alanine **24** to be less readily accepted by Ad2-TAM as amino donors, although some conversion was still observed.

## Characterisation of the catalytic ability of Ad2-TAm using different reaction parameters

Using a HPLC-based screening assay with (*S*)-MBA as amino donor and benzaldehyde as amino acceptor, the catalytic ability of Ad2-TAm was determined over a range of temperatures and pH (Fig. 8B. and C respectively). As expected with a halotolerant bacterial enzyme, Ad2-TAm displayed a largely mesophilic profile in terms of a temperature optimum of 30 °C, as has been observed with other bacterial TAmS.<sup>[9]</sup> Despite optimum conversion at pH 9.0, Ad2-TAm continued to bring about a relatively high level of conversion from pH 8.0 to 11.0. This is consistent with limited published data on TAmS from halotolerant bacteria.<sup>[22]</sup> Many halotolerant organisms are also alkalotolerant, owing to the environments in which they are often found. High-salt environments are often alkaline in nature, such as soda lakes, resulting in the emergence of haloalkalotolerant microorganisms.

Ad2-TAm showed an ability to convert substrate in organic solvents, with 67% conversion in 10% dimethylformamide (DMF). Previous use of DMF as a solvent

Fig. 6. Amino donors screened with assay B (using cinnamaldehyde as amino acceptor and HPLC detection of cinnamyl amine at 210 nm).

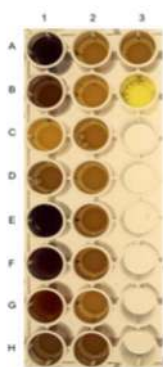


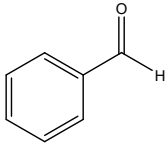
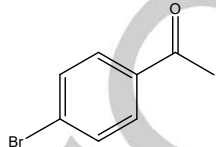

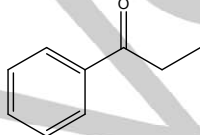
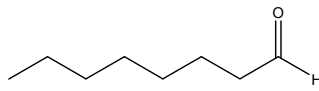
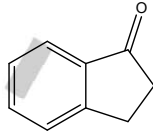
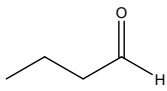
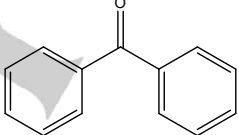
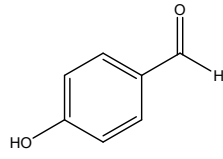
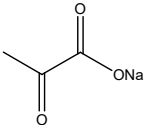
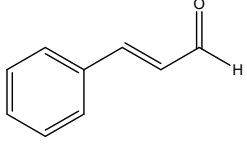
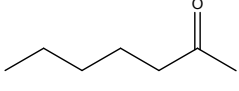
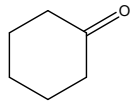
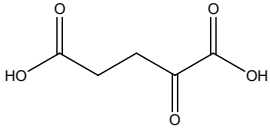
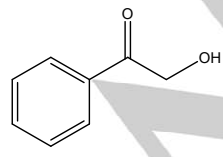
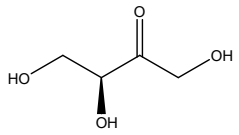
Fig. 7. Colorimetric assay employing a number of substrates (5 mM) (A1. 3, B1. 5, C1. 19, D1. 17, E1. 7, F1. 6, G1. 9, H1. 10, A2. 13, B2. 12, C2. 14, D2. 15, E2. 18, F2. 16, G2. 11, H2. 8). *o*-XDA (25 mM) was used as amino donor with all assays set up in potassium phosphate buffer, pH 8.0 (100 mM), containing PLP (1 mM). Reaction was started by addition of Ad2-TAm cell-free extract to a final concentration of 20 mg/mL and allowed to proceed for 30 min at 30 °C and 1200 rpm. DMSO in place of substrate was used as a control (well A3). Empty pET28a+ vector cloned into *E. coli* BL21 DE3 cells was used as an additional control with no substrate (well B3).

has been applied to TAm-catalyzed reactions in the

production of pharmaceuticals. Its addition as a co-solvent improved both the reaction process and substrate loading in the production of an intermediate for the pro-drug silodosin, used in the treatment of benign prostatic hypertrophy.<sup>[31]</sup> With Ad2-TAm, an increase above 10% concentration of DMF led to a dramatic reduction in conversion. However, increasing

the concentration of dimethyl sulphoxide (DMSO) from 10% to 20% led to an increase in conversion from 51% to 68%.<sup>[30]</sup>

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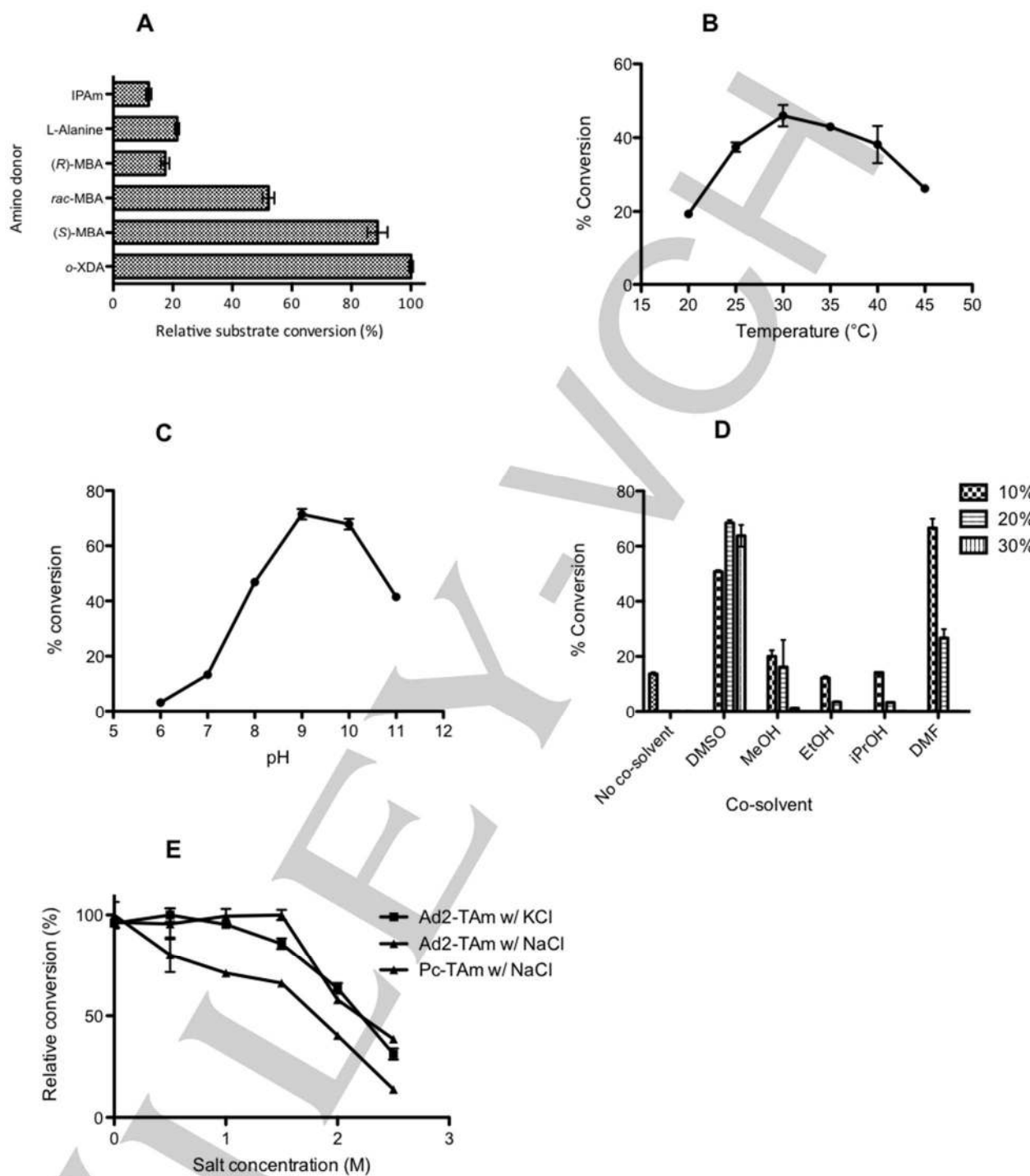
	Substrate	Conversion		Substrate	Conversion
3		55%	12		12%
5		52%	13		4%
6		22%	14		7%
7		49%	15		2%
8		39%	16		4%
9		36%	17		7%
10		49%	18		6%
11		39%	19		25%

**Table 1.** Overall conversion for a number of substrates under the following reaction conditions: (S)-MBA (25 mM), substrate (5 mM), P (1 mM), potassium phosphate buffer, pH 8.0 (100 mM). Reaction was started by addition of Ad2-TAm cell-free extract to a final concentration of 20 mg/mL and allowed to proceed for 16 h at 30 °C and 1200 rpm. Empty pET28a+ vector cloned into *E. coli* BL21 D cells was used as a negative control. All experiments were carried out in triplicate with empty vector control readings subtracted from cell-free extract readings. Conversions were obtained via detection of acetophenone formation at 240 nm and varied by  $\leq \pm 2\%$ , with the exception of acetaldehyde ( $\leq \pm 8\%$ ).

Subsequent decrease in conversion was only seen when DMSO was increased to 30%, although the enzyme continued to function well at this concentration. As many substrates for TAM-catalyzed reactions are poorly soluble,<sup>[6]</sup> a greater amount of substrate in solution may account for the increase in conversion at 20% DMSO. Nonetheless, substrate solubility can be an important limiting factor in TAM-catalyzed reactions,<sup>[6]</sup> and an enzyme capable of functioning at high concentrations of organic solvent could prove very valuable in chiral amine synthesis.

Ad2-TAm showed reasonable tolerance to a number of other organic solvents, showing  $>10\%$  conversion rates in methanol, ethanol and isopropanol (**Fig. 8D**). Conversion quickly deteriorated upon increases in concentration of these solvents above 10%. When no co-solvent was used in the reaction, an average conversion of only 14% was achieved.





**Fig. 8A.** The effect of different amino donors on the amination of cinnamaldehyde, shown as relative substrate conversion based on the detection of cinnamyl amine formation at 210 nm. B-E. The effect of various parameters on the amination of benzaldehyde using Ad2-TAm cell-free extract, using (S)-MBA as amino donor with acetophenone formation measured at 240 nm. Plotted values are the mean of triplicate measurements, with error bars representing  $\pm$  standard deviation in each case.

The effect of varying salt concentrations on conversion was also examined, with Ad2-TAm displaying a high level of tolerance to both NaCl and KCl (**Fig. 8E**). Acetophenone was formed with 54% conversion at 1.5M NaCl. Maximum

conversion with KCl was seen at 0.5 M, with 55% conversion, with >85% of this conversion still achieved at 1.5 M KCl.

Halotolerance of Ad2-TAM was assessed against the commercially available  $\omega$ -TAM from *Penicillium chrysogenum* (Pc-TAM), a mesophile (**Fig. 8E**). Ad2-TAM displayed much greater tolerance to increasing concentrations of salt than Pc-TAM, with maximum conversion observed at 1.5 M NaCl vs. only 66% relative conversion for Pc-TAM at this salinity (**Fig. 8E**). As NaCl concentration was increased further, Ad2-TAM continued to produce significantly greater conversions than the fungal enzyme. These observations are all the more impressive in view of the osmotic tolerance exhibited by fungi<sup>[32–36]</sup> such as *P. chrysogenum*. Moderately halophilic bacteria are adapted to high salt environments via a process known as ‘salting out’. Efflux pumps remove NaCl from within the cell, which is replaced by compatible solutes such as zwitterions or sugars in order to maintain the osmotic balance with outside the cell.<sup>[37–39]</sup> As such, their intracellular machinery does not possess the specific adaptations to high salinities exhibited by their haloarchaeal counterparts. These observations demonstrate the Ad2-TAM enzyme to be at the very least moderately halotolerant. The ability of this enzyme to function at increased salinity represents a promising addition to a currently scarce list of successes of TAMs from halotolerant organisms.

Using (*S*)-MBA **2** as amino donor and benzaldehyde **3** as substrate, the original 200  $\mu$ L reaction volume was increased to 100 mL, representing a 500 $\times$  increase in total reaction volume. A decrease from 55% to 30% conversion was observed on volume increase, suggesting further improvements would be needed to the process in order to apply Ad2-TAM as a biocatalyst on an industrial scale. Future work for upscale is ongoing, with details of product recovery, isolation and optical purity required before the suitability of Ad2-TAM can be fully assessed as a candidate for industrial applications. Increasing substrate loading beyond the 5 mM concentration used in this investigation is also a factor for further investigation. Despite these areas which require further work, the conversion seen in organic solvents, at high salt, and over a range of pH values, suggests Ad2-TAM has potential as a candidate for application in industrial processes.

## Conclusions

Limited substrate scope of current TAMs and their inability to tolerate challenging reaction conditions has driven the search for new enzymes. We have reported the cloning and expression of a novel biocatalyst from an extreme hypersaline environment, formed over 220 million years ago. Enzyme Ad2-TAM possessed a broad substrate range and an ability to function under challenging parameters. As well as accepting both aliphatic and aromatic ketones and aldehydes, Ad2-TAM also demonstrated an ability to convert substrate over a range of pH values, as well as in organic solvent and at elevated salinities. These characteristics highlight the potential benefits of employing enzymes from halotolerant organisms in biocatalysis, including a capacity to aminate substrates under conditions which are often too challenging for mesophilic enzymes. These observations also highlight Ad2-TAM as a potential biocatalyst for chiral amine synthesis in the pharmaceutical industry.

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