Isolation and Characterisation of a Halotolerant ω-Transaminase from a Triassic Period Salt Mine and Its Application to Biocatalysis


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 (TAm) 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, TAm 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 ω-TAm from a Halomonas sp. (Ad2-TAm), isolated from an extreme hypersaline environment formed during the Triassic period (circa 220 mya). Ad2-TAm exhibits an ability to convert a range of structurally diverse aldehydes and ketones 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.[1] 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. [2] These drawbacks have, in part, driven the rise of biocatalysis for the production of optically active amines. Biocatalytic approaches initially involved hydrolases,[3] although other enzymes such as oxidases, lyases and transaminases (TAm) have been applied for this purpose,[4] as well as dehydrogenases which have been reviewed recently.[5]

TAm 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,[6] there is a growing need for new enzymes with improved capabilities. The application of TAm 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.[6–8]

Approaches to discover or create new TAm have involved both metagenomic approaches[9] and directed evolution of existing enzymes.[10,11] 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 ω-TAm from a bacterial isolate from a Triassic salt mine in Kilroot, Northern Ireland. The protein, annotated as adenosylmethylionine-8-amino-7-oxononanoate aminotransferase (Ad2-TAm),[12] 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.

**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 NBYR00000000. The version described in this paper is version NBYR01000000). Using the Basic Local Alignment Search Tool nucleotide (BLASTn) function\(^{13}\) of the National Center for Biotechnology Information (NCBI) database,\(^{14}\) the organism was found to be a member of the genus *Halomonas*, with the closest neighbour deemed to be *Halomonas jangokensis* M24 (99% similarity).

On uploading the assembled contigs from WGS data to the Rapid Annotations using Subsystems Technology (RAST) server,\(^{12}\) 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.\(^{15}\) 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 (Figure 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 (Figure 1B).

**Phylogenetic analysis and alignment with other TAs**

A number of other TAs were cloned and expressed from bacterial isolates using the same approach as with Ad2-TAm (Supplementary Figure 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 Figure 2, as well as a number of sequences from TAs of known organisms: *Vibrio fluvialis* (VF-TAm),\(^{16}\) *Pseudomonas putida* (Pp-TAm),\(^{17}\) *Chromobacterium violaceum* (Cv-TAm)\(^{18}\) and *Halomonas* sp. (Hs-TAm)\(^{19}\). A neighbour-joining tree was generated using MEGA 7,\(^{20}\) with bootstrap values calculated based on 1000 replicates. Using protein BLAST (BLASTp) function\(^{16}\) 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 commonly employed TAs (VF-TAm - 28%, Pp-TAm - 28%, Cv-TAm - 30%).

Despite the low level of homology, many critical residues were conserved across all sequences (Figure 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.\(^{21}\)

**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 Figure 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 (Figure 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 Figure 2).

Figure 2. Amino acid sequences of TAM genes from whole 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).

(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, compared with 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.

Figure 3. Multiple alignment of amino acid sequence of Ad2-TAm with a number of TAMs from identified species (Pp-TAm, Cv-TAm, Vf-TAm and Hs-TAm). Key residues for activity and substrate specificity are conserved across all sequences, including Trp60, Tyr153 and Lys288 (highlighted in red).
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 Figure 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 Figure 3). This broad substrate acceptance is typical of Class III TAM enzymes, 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 TAMs, 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 (Figure 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 TAMs may circumvent such issues.

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 a 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. 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...
Figure 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 NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0) was used as a negative control (wells in column 1).

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

Figure 7. Colorimetric assay employing a number of substrates (5 mM) (A, 1, B, 5, C, 1.9, D, 17, E, 1.7, F, 1.6, G, 1.9, H, 1.10, A2, 13, B, 12. C, 2.14, D, 2.15, E, 2, F, E, 16, G, 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 control (well A3). Empty pET28a vector cloned into E. coli BL21 DE3 cells was used as an additional control with no substrate (well B3).

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-IND anone 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%, α-ketoglutaric acid 18 6%), whilst Ad2-TAm displayed a reasonable ability to convert the ketose sugar L-erythrose 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 group vs. ketones.²² 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 μmol 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 Tambs by several orders of magnitude, with a growing number of examples in the current literature.⁶¹⁴

HPLC-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 (Figure 8A). As well as being readily accepted by the enzyme, 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.²⁶ Recently this work has been developed further with the advent of ‘smart’ diamine donors, whose corresponding aminodehydes can dimerize or cyclize following transamination.²⁷ 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.²⁷–²⁹

Varying the amino donor also reveals the enantiopreference 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 (Figure 8A.).

Figure 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.

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<th>Substrate</th>
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<td>3</td>
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Table 1. Overall conversion for a number of substrates under the following reaction conditions: (S)-MBA (25 mM), substrate (5 mM), PLP (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 DE3 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 < ±2%, with the exception of acetaldehyde (< ±8%).

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 (Figure 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.20 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.\textsuperscript{[22]} Many halotolerant organisms are also alkotolerant, 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.

\begin{figure}
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\caption{A 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 ± standard deviation in each case.}
\end{figure}
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 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.[32–36] 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%.[30]

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,[30] 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,[30] 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 (Figure 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.

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 (Figure 8E). Acetophenone was formed with 54% conversion at 1.5 M 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 α-TAm from Penicillium chrysogenum (Pc-TAm), a mesophile (Figure 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 (Figure 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[37–39] 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 zwitterionics or sugars in order to maintain the osmotic balance with outside the cell.[37–39] 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 TAm from halotolerant organisms.

Using (S)-MBA 2 as amino donor and benzaldehyde 3 as substrate, the original 200 μL reaction volume was increased to 100 mL, representing a 500× 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 TAm 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.

Conflict of Interest
The authors declare no conflict of interest.

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