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A stereospecific solid-phase screening assay for colonies expressing both (*R*)- and (*S*)-selective ω-aminotransferases

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A novel solid-phase screening assay was developed for colonies expressing both (*R*)- and (*S*)-selective ω-aminotransferases. This high-throughput assay can be used to screen rapidly large variant libraries with enhanced substrate selectivity and enantioselectivities.

1. Introduction

Aminotransferases (ATAs) are pyridoxal phosphate-dependent enzymes (EC 2.6.1.x) that catalyse the reversible transfer of an amino group from an amine donor to a prochiral ketone substrate acceptor molecule. There is considerable interest in ATAs as biocatalysts in the production of enantiomerically pure chiral amino compounds. Such compounds are essential building blocks of intermediary metabolites and pharmaceutical drugs, highlighting the need for cost-effective and green approaches for their chemical syntheses [1]. Particular attention has been focused on the need to overcome problems in the use of ATAs in the synthesis of chiral amines; conversion of the ketone to the amine is hindered by the reaction equilibrium, non-enzymatic competitive substrate and/or product inhibition and a poor substrate tolerance profile. [2,3]. Equally important is the discovery of an effective assay required to rapidly screen variant ATAs with respect to substrate activity and enantioselectivity. Low-to-medium throughput screens are available; detecting the formation of amino acid complexes [4], monitoring conductivity

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with complementary UV spectrophotometric methods [5], an enzymatic cascade reaction [6,7] or by direct reaction with a colorimetric dye [8]. These liquid-phase assays are limited to microtitre plate-based systems reliant on automated liquid handling and colony picking which cannot effectively screen the large numbers of mutants which a directed evolution approach can often require.

An *in vitro* colony solid-phase screening assay is an attractive alternative because of its relative ease of operation and high-throughput nature that can be easily detected with the naked eye and no demand for specialized equipment [9,10]. Recently, we have published the application of using *ortho*-xylylenediamine dihydrochloride as the amino donor that generates a coloured precipitate via an aromatic isoindole formation. This potential application can be used as a solid-phase screen on a variety of ketone acceptors by shifting the equilibrium by *in situ* co-product removal [11]. Bornscheuer and co-workers [12] have developed a glycine oxidase-based assay for the substrate profiling of both (*R*)- and (*S*)-selective amine transaminases. Herein we have developed two stereo-complementary methodologies for detecting (*R*)- and (*S*)-selective ω-aminotranferases from a large repertoire of ATA variants expressed in the cytoplasm of *Escherichia coli* cells on a single agar plate.

2. Results and discussion

In the ATA reaction with pyruvate as the keto acceptor, the generation of an alanine co-product is channelled into a concomitant amino acid oxidase (AAO) affording hydrogen peroxide and regenerated pyruvate. Horseradish peroxidase (HRP) catalyses oxidation of 3,3′-diaminobenzidine (DAB) in the presence of H_2O_2 to form a localized reddish-brown polymerized quinone iminium precipitate (figure 1).

We sought to co-transform in separate, but analogous conditions an (*R*)-selective ATA ArR-ATA [13] and a known (*S*)-selective Cv-ATA [14] with D-AAO [15] on compatible vectors containing different origins of replications and different antibiotic markers leading to the overexpression of the proteins in *E. coli* (figure 2*a*) [16]. The presence of both genes in the cell is confirmed by 'colony PCR' and the simultaneous co-expression of the proteins is found in the electronic supplementary material. The freshly transformed cells were plated onto a Hybond-N membrane with the appropriate antibiotics and incubated at 30°C. After transferring membranes to IPTG induced plates at 20°C for 18 h, the membranes was subjected to repeated freeze–thawing cycles and screened for activity using HRP, colorimetric dye DAB and (*R*)-methylbenzylamine as the amine donor substrate. The co-expressed (*R*)-selective ArR-ATA and D-AAO was used as a positive control with red localized pigmentation displayed on the colonies within 10 min. No activity was observed with the co-expressed (*S*)-selective Cv-ATA and D-AAO after 1 h incubation at room temperature (figure 2*b*).

Our previously published liquid-phase assay for measuring the activity of ATAs uses either an L-AAO or a D-AAO, depending upon the ATA specificity [6]. However, expression of L-AAO in *E. coli* resulted in the accumulation of insoluble protein [17]. Another screening approach was required for (*S*)-selective ATAs by introducing an alanine racemase (AlaR) [18] co-expressed into the ATA/D-AAO gene expression system. The L-alanine produced by the aminotranferase can be converted into the D-enantiomer and is used as a substrate for D-AAO (figure 2). Unfortunately, it appeared that all three enzymes (ATA, AlaR and D-AAO) could not be expressed in a single cell presumably due to the toxicity in cellular metabolism. To circumvent these initial difficulties, a filtered sandwich screening technique [19] was applied via expressing AlaR on a second membrane as a single continuous layer or 'lawn' of cells. The membrane loaded with the bacterial colonies was laid on top of the expressed AlaR. L-alanine generated *in vitro* is secreted from the cytoplasm of the partially permeabilized cells and diffuses in to overexpressed AlaR cells. The resulting D-alanine transfers back into the cytoplasm of the active colonies producing the desired colour change from the hydrogen peroxide formation step (figure 3).

The screening systems developed above are ideally suited for use in a directed evolution project to aid in the efficient identification of variant enzymes from a large library of mutations.

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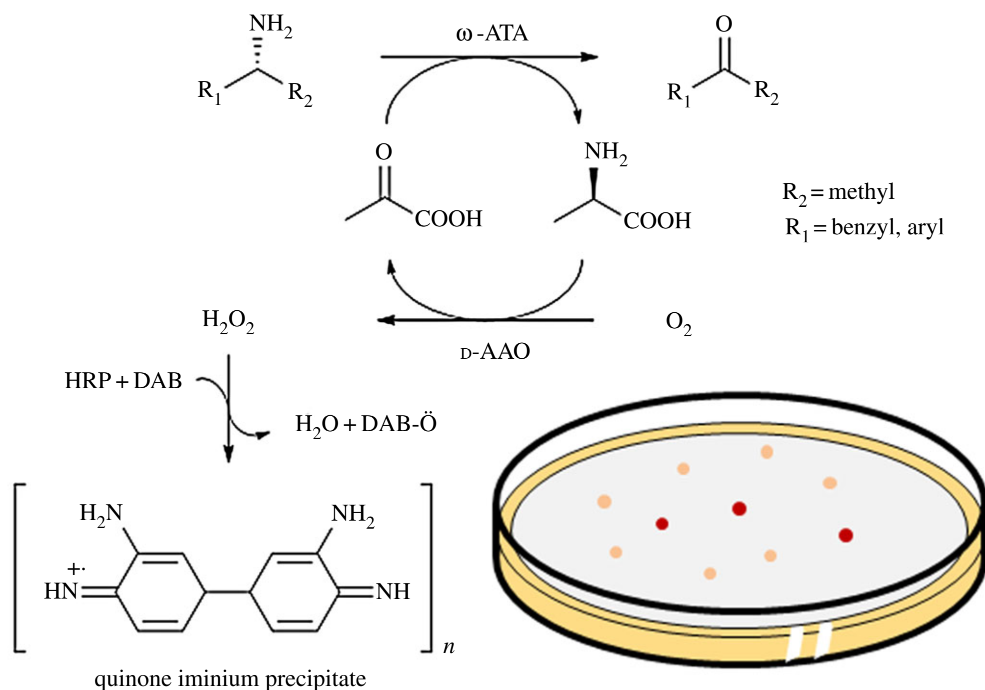

Figure 1. Schematic colorimetric assay screen for detecting D-alanine from the co-transformation of ATA and D-AAO in *E. coli* and plated on a membrane.

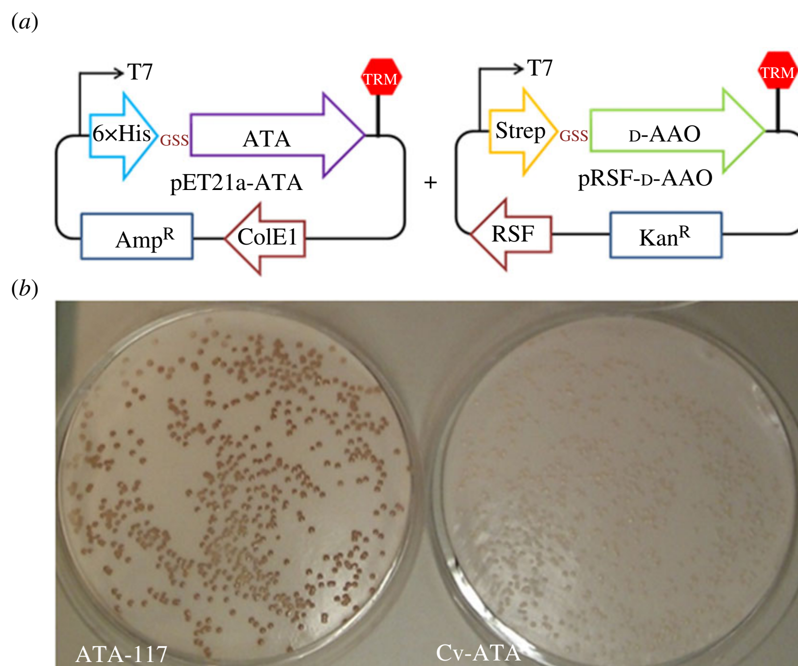

Figure 2. (*a*) Compatible vectors pET21a-ATA and D-AAO used in gene expression, (*b*) (*R*)-selective solid-phase screen able to detect colonies that use (*R*)-methylbenzylamine as a substrate. GSS, glycine–serine–serine; TRM, transcription terminator.

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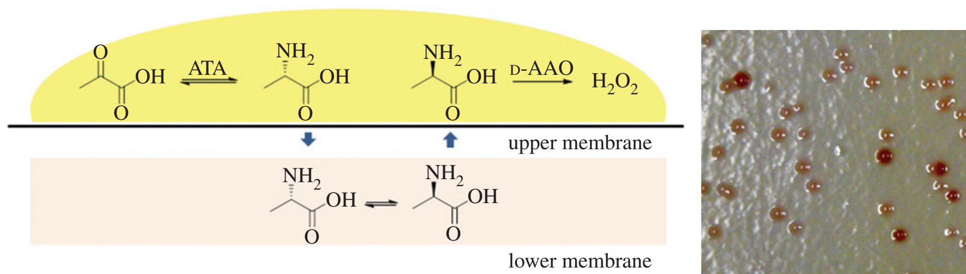

Figure 3. Schematic of (*S*)-selective ATA activity via addition of alanine racemase to convert L-alanine to D-alanine, allowing colour formation via D-AAO and HRP.

We have constructed and screened libraries of *ω*-ATA enzymes and used these above screens to help identify variants with increased activity. Most available ATAs possess narrow substrate specificities in terms of the substituent they can bind in their small binding pocket [20]. To address this, a site-directed mutagenesis library was designed at the binding pocket in (*R*)-selective ArR-ATA. The amino acid residue Phe116 was targeted and an NNS codon library was generated and screened against (*R*)-1-phenylbutylamine. ArR-ATA is able to accept either a methyl or ethyl group in its small binding pocket, by expanding the scope of this binding pocket to an eventual phenyl group would be considered industrially useful. Approximately, 1000 colonies were screened using the (*R*)-selective solid-phase screen, and 12 positive 'hits' were selected. Analysis of these mutants revealed that a tryptophan mutant Phe116Trp showed 50% increased activity with (*R*)-1-phenylbutylamine relative to the wild-type enzyme (see the electronic supplementary material).

An (*S*)-selective ATA (pLE1A17-ATA-50) kindly provided from c-LEcta metagenomic library gave 70–75% ee for (*S*)-(+)-1-methyl-3-phenylpropylamine from benzylacetone and L-alanine as the amine donor [21]. A random generated mutant library was screened using the sandwich screen solid-phase assay to identify colonies with greater activity towards (*S*)-(+)-1-methyl-3-phenylpropylamine. A colony was found containing a single point tyrosine mutation at a conserved residue Phe87 and gave the (*S*)-isomer in more than 98% ee.

3. Conclusion

We have developed a solid-phase screening protocol for colonies expressing both (*R*)- and (*S*)-selective ω-aminotransaminases. This high-throughput assay can be used to screen large variant libraries with enhanced substrate selectivity and enantioselectivity. Further development of this assay involving the incubation of these membranes for prolonged periods, such that, variants with increased thermostability can be rapidly identified from inherent residual activities, is currently under investigation.

Data accessibility. Cv-ATA DNA sequences: Genbank accession AAQ59697.1 (doi:10.1016/j.enzmictec.2007.05.011); ArR-ωATA DNA sequences: Genbank accession BAK39753.1 (doi:10.1007/s00253-011-3580-0). The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. S.C.W. developed the assay. N.J.T. designed the experiments. S.C.W., J.L.G. and I.S conducted the experiments. All authors interpreted the data, wrote the manuscript and have given approval to the final version of the manuscript.

Competing interests. The authors declare no competing financial interests.

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