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Supplemental: Directed evolution of *Anabaena variabilis* phenylalanine ammonia-lyase (PAL) identifies mutants with enhanced activities

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Methods

Microbial strains, plasmids, and growth conditions

All *Escherichia coli* strains were cultured in lysogeny broth (LB) (VWR International, Randor, PA) at 37 °C with rotary shaking at 250 rpm. All media was solidified using 1.5 % (w/v) agar (Teknova Inc, Hollister, CA). Minimal media (MM) conditions are described in the "Optimization of growth-coupled enrichment" section below. *E. coli* DH5α was used as a host for the construction of the expression vectors and cultured as above only supplemented with chloramphenicol (25 μg/mL) (RPI Corp). Initial expression in MM was performed in *E. coli* MG1655(DE3)$^{\Delta endA, \Delta recA}$ and later moved to *E. coli* MG1655^{rph+} for final experiments.

All cloning was performed in *E. coli* DH5α with reagents from New England Biolabs, Inc (Ipswich, MA). Preliminary expression experiments were conducted using the inducible pACYC-Duet1_*Av*PAL*, constructed by using the surrounding sites for restriction endonucleases NcoI and XhoI. For subsequent experiments requiring constitutive expression, the plasmid pBAV1k was used to express *Av*PAL*.

Enzyme activity assays

The activity of all *Av*PAL constructs was measured by production of *t*CA over time. Cultures were sonicated on ice using a Sonifier SFX 150 (Branson Ultrasonics, Danbury, CT) (2 s on; 10 s off; 4 min; 55 %), and debris was separated from the lysate by centrifuging at 10,000 × g for 10 min. Ten microliters of lysate were then mixed with 190 μL of pre-warmed 50 mM phe (Tokyo Chemical Industry, Portland, OR) in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) in a 96-well F-bottom UVStar (Greiner Bio-One, Kremsmünster, Austria) microtiter plate. Absorbance at 290 nm was measured every 30 s at 37 °C using a SpectraMax M3 (Molecular Devices) plate reader.

Each construct included a N-term His-tag used for immobilized metal affinity chromatography (IMAC) purification. Briefly, overnight cell cultures were sonicated in 3 mL Equilibration buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, 15 % (w/v) glycerol, pH 8.0). The lysate was loaded onto a prepared column with 2 mL TALON Metal Affinity Resin (Clontech Laboratories, Inc., Mountain View, CA). After being washed twice with 5 column volumes (CV) of Equilibration buffer, pure protein was then eluted off the column with 2.5 mL of Elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 500 mM imidazole, 15 % (w/v) glycerol, pH 8.0), collecting 0.5 CV fractions until dry. Elution fractions showing clean protein bands on an SDS-PAGE were then dialyzed and concentrated in Storage buffer (20 % (v/v) glycerol in PBS, pH 7.4) using a 10K MWCO Microsep Advance Centrifugal Device (Pall Corporation, Port Washington, NY) as directed. Purified protein extracts were aliquoted and stored at -20 °C, replacing lysate in subsequent characterization and activity assays. Protein concentration was measured by Bradford method using bovine serum albumin (BSA) as the standard.

*Av*PAL library creation

Random mutagenesis libraries were created using two rounds of error prone PCR, with the amplicon of the first reaction serving as the template DNA for the second. Each reaction contained 1× Standard *Taq* reaction buffer (New England Biolabs, Inc.), 5 mM $MgCl_2$, 0.15 mM $MnCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 0.4 μM each primer, 0.4 ng/μL template DNA, and 0.05 U/ml *Taq* DNA polymerase. The reactions were amplified using the following PCR cycle conditions: 95 °C denaturation, 1 min; 16 cycles of 95 °C denaturation, 30 s; 46 °C annealing, 45 s; and 68 °C extension, 2 min, followed by 68 °C extension for 5 min. The

target vector, pBAV1k was amplified separately using *Phusion* PCR, and the two were combined using Gibson assembly. The reaction was purified with a E.Z.N.A. Cycle Pure Kit (Omega) before being transformed by electroporation into *E. coli* MG1655^{rph+}.

Optimization of growth-coupled enrichment

Growth was measured by seeding cultures at OD_{600} 0.05 and monitoring cell density over time. Initial experiments used a base nitrogen-deficient minimal media (MM^{N-}) (33.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 μM $FeSO_4$, 0.4 % (v/v) glycerol, 10 μg/mL thiamine, 20 μM IPTG, 12.5 μg/mL chloramphenicol, pH 7.4) that was supplemented with 9.35 mM phe ($MM^{phe,init}$) or 9.35 mM NH_4Cl ($MM^{full,init}$). Variable conditions were changed across the parameters outlined in Figure 2, as well as moving to a more favorable strain for growth in minimal media. This resulted in a final $MM^{N-,opt}$ (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 1× Trace Metals (Teknova, Inc.), 0.2 % (v/v) glucose, 10 μg/mL thiamine, 12.5 μg/mL chloramphenicol, pH 7.4) supplemented with 30mM phe ($MM^{phe,opt}$) or 9.35 mM NH_4Cl ($MM^{full,opt}$). To enrich the active population of the *Av*PAL library, cells were subcultured into fresh $MM^{phe,opt}$ once they reached OD_{600} 0.2.

Flow cytometry

Plasmids, both with a pBAV1k backbone, expressing sfGFP or *Av*PAL* were mixed in a 1000:1 or 10:1 ratio as a mock mutant library and transformed by electroporation into *E. coli* MG1655^{rph+}. Cells were recovered for 1 h before being washed and seeded in 5 mL of selective media as prepared above. Cell density was measured over time until reaching OD_{600} 0.2, when the cells were subcultured to OD_{600} 0.05 for the next round of enrichment. Cells were also plated at each subculture for PCR amplification to confirm the presence of either sfGFP or *Av*PAL*. Cells at each point of subculture were also diluted to OD_{600} 0.05 for flow cytometry analysis. A minimum of 10,000 events were collected using a blue laser on an Attune NxT flow cytometer (Life Technologies, Carlsbad, CA). Fluorescence of sfGFP was detected on the BL1-H channel with 488 nm excitation, and loss of fluorescence was revealed as a measure of active *Av*PAL* enrichment.

Enzyme kinetics

*Av*PAL* and selected mutants were purified as described above. The activity of 0.1 μg of protein was measured by the production of *t*CA over 10 min by recording the absorbance of the reaction mix at 290 nm. Phe was added at varying concentrations from 35 μM to 17.5 mM in PBS, pH 7.4 (PBS) at 37 °C to begin the reaction. A Michaelis-Menten curve was fit in GraphPad Prism software using the initial rate at each phe concentration.

pH profile

The optimal pH of *Av*PAL* and selected mutants was determined by performing the enzyme activity described above. A 35 mM phe solution was buffered across a pH range (2 to 10) using phosphate-citrate buffer, prepared by varying concentrations of Na_2HPO_4 and citric acid. Total 0.2 μg protein was used to carry out the activity reaction in 200 μL at 37 °C.

Temperature stability

The effect of temperature on the stability of *Av*PAL* and selected mutants was determined by incubating the protein in PBS, pH 7.4 at temperatures ranging from 37 °C to 80 °C for 1 hour followed by measuring the enzyme activity. Each enzyme reaction was carried out using 1 μg of PAL protein and 35 mM phe as substrate in a total reaction volume of 200 μL at 37 °C.

Proteolytic stability

The proteolytic stability was evaluated by subjecting *Av*PAL* and selected mutants to a catalytic amount of trypsin as previously described[1]. Briefly, 100 μg/mL *Av*PAL enzyme was subjected to trypsin (40 μg/mL) (MilliporeSigma, Burlington, MA) in PBS at 37 °C. Enzyme activity of 1 μg of protein was then measured as described above.

References

1 L. Wang, A. Gamez, H. Archer, E. E. Abola, C. N. Sarkissian, P. Fitzpatrick, D. Wendt, Y. Zhang, M. Vellard, J. Bliesath, S. M. Bell, J. F. Lemontt, C. R. Scriver and R. C. Stevens, *Journal of Molecular Biology*, 2008, **380**, 623–635.

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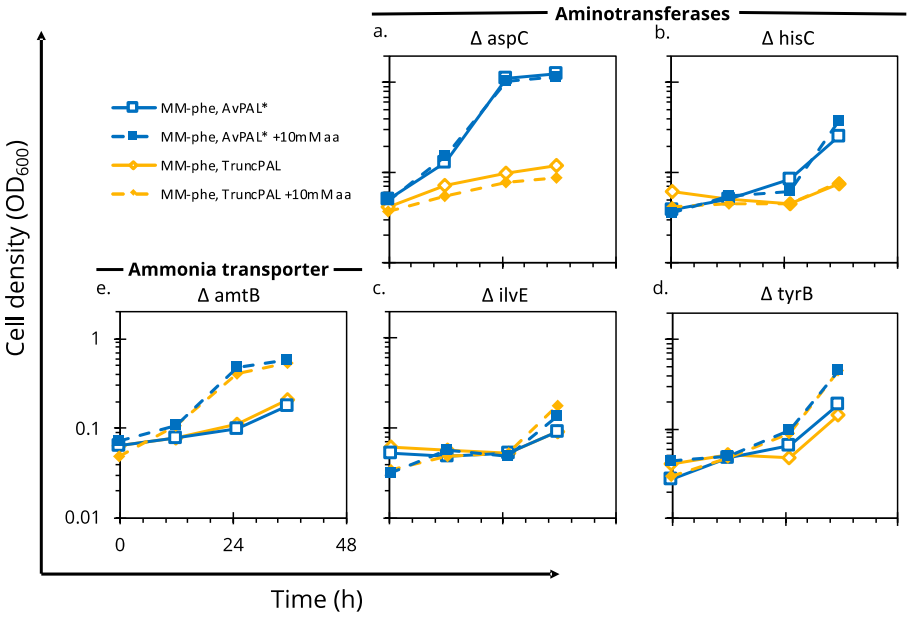

Figure S1. Growth of *E. coli* after gene deletions intended to lower basal growth on $MM^{phe,init}$. (a.-d.) Select aminotransferases with reported promiscuous activity on phenylalanine were deleted in an attempt to reduce the level of basal growth seen by wild-type *E. coli* on $MM^{phe,init}$. Each deletion strain showed no changes in growth whether or not expressing *Av*PAL*. (e.) The ammonia transporter AmtB was also deleted in an attempt to minimize cross-feeding of nitrogen between cells but had no benefit.

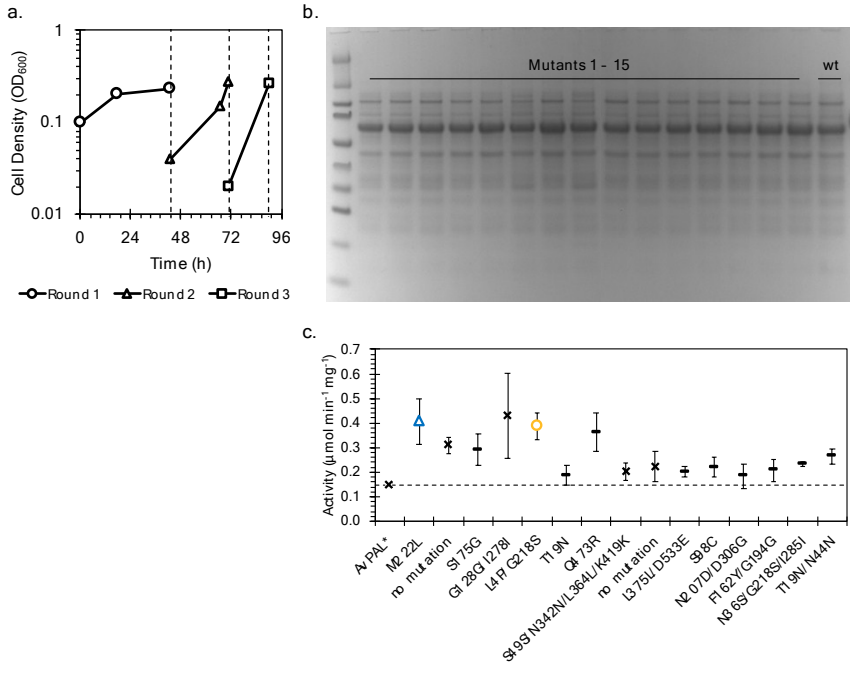

Figure S2. Identification of *Av*PAL* mutants by growth-coupled enrichment. (a.) The growth profiles of *E. coli* $MG1655^{rph+}$ cells expressing the *Av*PAL* mutant library grown in $MM^{phe,opt}$ over three rounds. (b.) SDS-PAGE gels of the lysate of fifteen randomly picked colonies from round 3 of the enrichment. Two gels have been cropped for concision with no image enhancement. (c.) Crude cell lysate activity of the selected mutants normalized to total protein.

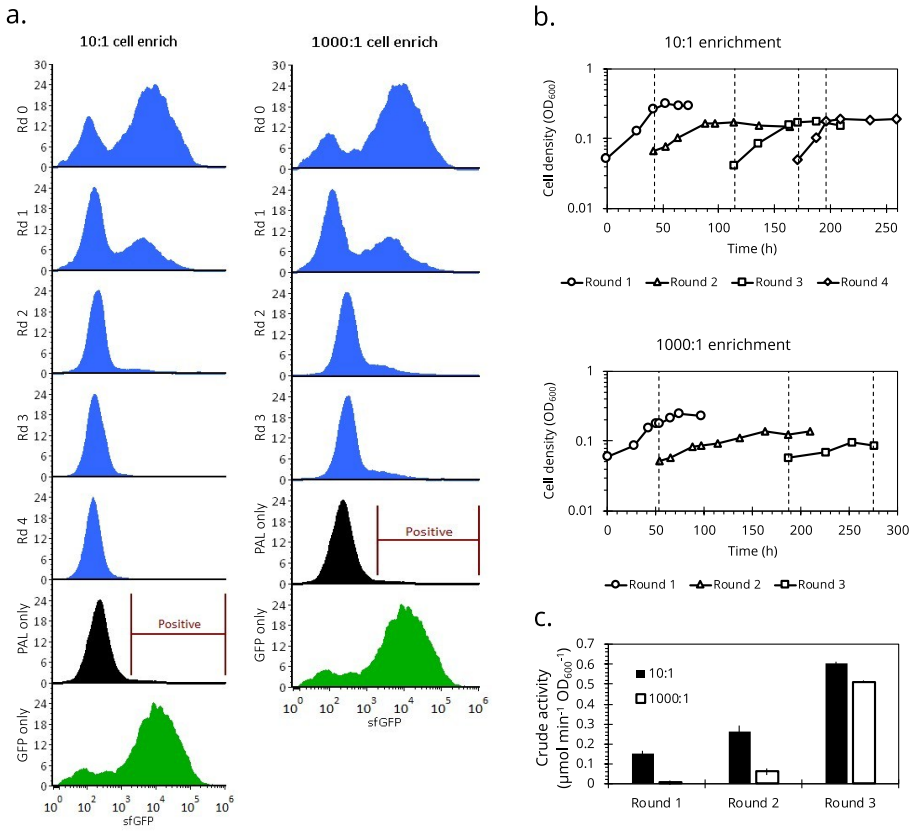

Figure S3. Validating enrichment with a mock library. After transforming a plasmid mix of *Av*PAL* and sfGFP in 1:10 or 1:1000 ratio, we were able to observe (a.) the loss of fluorescence, and (b.) the enrichment of cells expressing *Av*PAL* over sfGFP, over rounds of subculturing in MM^{phe} selective media. This was confirmed by (c.) an observed increase in *Av*PAL* activity on a per cell basis.

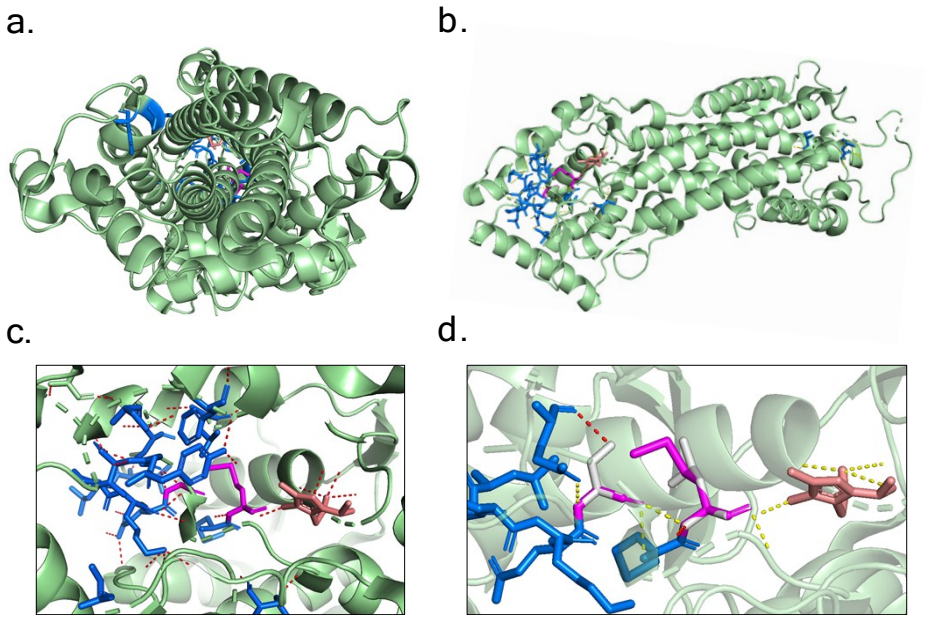

Figure S4. Crystal structure analysis of *Av*PAL* monomer with active site residues (blue), MIO-adduct (orange), and residues 218 and 222 (pink) highlighted. (a.) A top view looking down into the active site. (b.) Side-view of the monomer. (c.) Close up of the wildtype *Av*PAL* active site with predicted intra-residue hydrogen bonding. (d). Comparison of the wildtype and mutant active sites with residues 218 (left) and 222 (right) highlighted. Mutant residues G218S and M222L (gray) have altered intra-residue hydrogen bonding (red, dotted) compared to wildtype (yellow, dotted).