

Supplementary Table 8

The evolvability of promiscuous protein functions

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Examples for variants exhibiting large improvements in promiscuous activities and small changes in native activity^a

Enzyme	Variant name	Type of native activity and fold change relative to the wt ^b	Type of promiscuous activity and fold change relative to the wt ^b	Mutations ^c	Reference
1. Bipheyl dioxygenase (KF707 and LB400)	pSHF1003	Oxygenation of chlorinated biphenyls 1.4-1.8	Oxygenation activity for toluene and benzene >100^d	Shuffled gene	1
2. Heart myoglobin	-	Binding of CO and O ₂ 0.82-0.98	Peroxidase activity 25	T39I,K45D F46L I107F	2
3. Cytochrome c peroxidase ^e	AN5	Oxidation of cytochrome c 0.95	Oxidation of guaiacol 300	V5I, R48H,T180A, D224V, S282D	3
4. Cytochrome P450 BM3	139-3	Hydroxylation of lauric and palmitic acid ~0.3^f	Hydroxylation of hexane 20^f	11 mutations ^g	4
5. Galactokinase (GalK)	-	Phosphorylation of D-galactose 0.8	Phosphorylation activity for various promiscuous D and L sugars 5.1-40.1^h	Y371H	5
6. N-acetylneuraminate (NAL) (<i>E. coli</i>) ⁱ	-	Glycolysis of Neu5Ac 0.3	Aldol condensation 19	L142R	6
7. Lipase	M2	Lipase ^j (triolein) 0.8	Phospholipase ^j (lecithin) 9.8	H15P K103R, N173S.	7
8. Cre recombinase ^k	Fre 17	Recombination of Lox P sites 1.15	Recombination of Lox H sites 220	S2P, V7L, N10D, H196Q, E262Q	8

9. Subtilisin ^l	GES208	Peptidase activity 0.4	Esterase activity 25	V143H, D181N	9
10. Muconate lactonizing enzyme (MLE II)	E323G	MLE activity (muconate) 0.07	OSBS activity (β elimination) $\sim 10^6$	E323G	10
11. PBP2X DD-transpeptidase	MutE	DD-peptidase activity 1.5	Lactamase activity (cefotaxime) $\sim 10^5$	G336A,F450L Q452H	11
12. β -Glucuronidase	variant ^p	Hydrolysis of pNP glucuronide, pNP galacturonide and pNP-glucoside 0.1-1^m	Hydrolysis of pNP-fucoside, oNP-galactoside-6-phosphate and pNP galactoside. 20-200^m	T509A,D531E, S557P, N566S	12
13. α -lytic protease ^l	M192A	Peptidase activity Ala at the P1 position 0.5	Peptidase activity Phe at P1 position $8.4 \cdot 10^5$ Leu $2.4 \cdot 10^4$	M192A	13
14. Triazine hydrolase	variant 12	Dechlorination ⁿ and deamination of atrazine and amino atrazine. 0.2-1.5	Dechlorination ^{m,n} and deamination of different triazines 13.3-1550	Shuffled gene (AtzA and TriA)	14
15. Stoeffel fragment of Taq polymerase I	SF2	Incorporation of dNTPs ~ 0.1	Incorporation of rNTPs 10^2-10^4	A597T, E615G	15
16. Stoeffel fragment of Taq polymerase I	SFM19	Incorporation of dNTPs 0.3-20	Incorporation of 2O-methyl ribonucleoside ^m $>10^4$	I614E,E615G	16
17. Glutathione transferase A1-1 ⁱ	GIMFhelix	Aromatic substitution of CDNB 0.09	Michael addition reaction hexenal and nonenal 94 - 304.	Grafting residues and structural elements from GST A4-4 ^o .	17
18. Human estrogen receptor α (hER α)	T7-18	Binding affinity to 17 β -Estradiol (E2) 0.3-0.6	Binding affinity to testosterone $7.6 \cdot 10^3$	E353Q,F461I, A569T	18

^a This Table summarizes the results of an extensive search of the directed evolution literature. In most cases, however, data are available only for the final variants of the evolutionary process that were often selected for maximum increase in the

promiscuous (evolved) activity, and lowest native activity. The examples listed here were taken from experiments in which there was no selection pressure for a decrease in the native activity, or, where data regarding the intermediates of the evolutionary process were available. Listed are variants that show the highest improvement in the promiscuous activity and the lowest change in the natural activity.

^b Comparison between the improved variants and the wt (wild-type) is based on the k_{cat}/K_M for enzymatic activities and binding constants for changes in binding activity.

^c Mutations are shown for variants generated by random mutagenesis and/or DNA shuffling of a single gene.

^d Catalytic activity of pSHF 1003 and the two parental wt proteins (KF707 and LB400) for toluene and benzene could not be detected. We therefore assumed that the improvement was >100fold.

^e First order rate constants of the oxidation of cytochrome C and guaiacol are used for comparison of the wt and the improved variant AN5.

^f Maximum turnover rates (mol substrate/min/mol enzyme) of the hydroxylation of lauric acid, palmitic acid and hexane are used for comparison of the wt and the improved variant 139-3.

^g See reference for specific mutations.

^h Phosphorylation activity for D-talose, 6-Amino-D-galactose, D-galacturonic acid, L-Altrose and L- glucose are not reported for the wt GalK, the maximal improvement shown in the table (40.1-fold) is based on the lower limit of detection (k_{cat}/K_M of $0.6 \text{ s}^{-1}\text{M}^{-1}$) for 6-Deoxy-D-galactose for the wt enzyme.

ⁱ Variants characterized in this work were generated by rational design.

^j Units activity per mg of enzyme of the lipase and phospholipase activity with tributyrin and lecithin are used for comparison of the wt and the improved variant M2.

^k This work involves 35 generation cycles, only one variant from generation 17 (Fre 17) is shown. Fre 17 variant was characterized in details for recombination activity in *E. coli* by the number of Kan resistance colonies.

^l Initial rates (μM product/100 μl of induced cell lysate/min) of the amidase and esterase activity are used for comparison of the wt and the improved variant GES208.

^m Comparison between the wt and the evolved variant are only estimated numbers and reflect the lowest activity changes.

ⁿ Initial rates (μM product/h) were measured on crude cell lysates containing $\sim 40\text{ng}$ of enzyme in 100 μl reaction.

^o GIMFhelix was created by grafting the GST A4-4 residues 12, 107, 108, 111 and the C terminal peptide (residues 208-222)

^p This variant is an intermediate in the directed evolution of β -glucuronidase towards β -galactosidase and was isolated after two rounds of evolution.

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