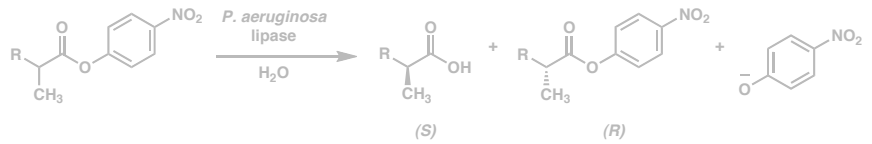
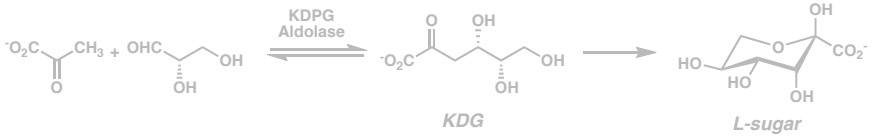


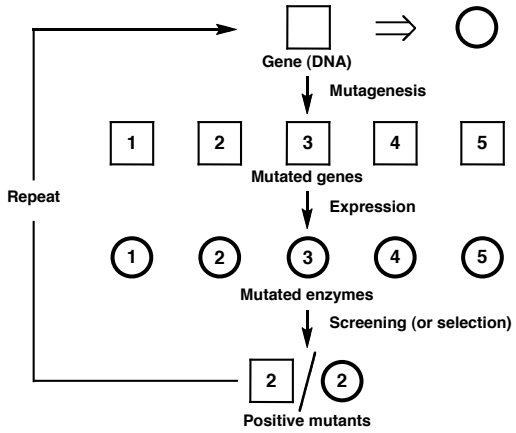
Directed Evolution of Enantioselective Enzymes

Narae Park
September 28th, 2009
147 Noyes



Directed Evolution of Enantioselective Enzymes

Introduction



***Reviews on directed evolution of enantioselective enzymes*:**

Reetz, *Angew. Chem. Int. Ed.* **2001**, 40, 284-310.
Reetz, *Proc. Natl. Acad. Sci.*, **2004**, 101, 5716-5722.
Reetz, *Appl. Microbiol. Biotechnol.* **2001**, 55, 519-530.
Reetz, *Curr. Opin. Biol.* **2000**, 4, 68-73.
Reetz, *J. Org. Chem.* **2009**, 74, 5767-5778.
Reetz, *Chem. Eur. J.* **2000**, 6, 407-412.

***Reviews on directed evolution*:**

Arnold, *Curr. Opin. Biotechnol.* **2000**, 11, 325-330.
Powell, *Angew. Chem. Int. Ed.* **2001**, 40, 3948-3959.
Hilvert, *Annu. Rev. Biophys.* **2008**, 37, 153-173.

Mutagenesis Methods
Non-recombinant Mutagenesis

Error prone PCR (epPCR):

Developed by Mullis in 1994.

Random mutations are induced over the entire gene.

Established by varying parameters (e.g., [$MgCl_2$] or [dNTPs]), using mutator strain *E. coli* (e.g., XL1-red) and/or using mutagenic polymerases.

$$N = \frac{19^M X!}{(X-M)!M!}$$

***N* = # of variants**
***M* = # of amino acid exchanges per enzyme molecule**
***X* = # of amino acids per enzyme molecule**

Disadvantage: Bias towards some amino acids over others due to degeneracy in codons.

M	*N*
1	5,700
2	16,190,850
3	30,557,530,900

***Calculated for a model enzyme with 300 amino acids**

Saturation mutagenesis:

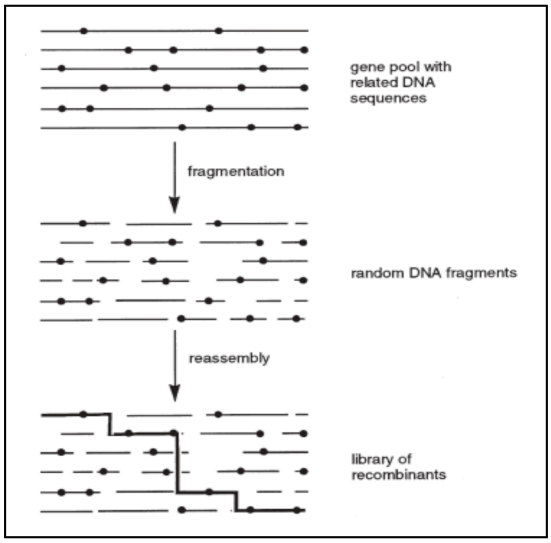
Introduction of all 20 amino acids at a target sequence.

Can be directed at one codon or a set of codons.

Arnold, *Chem. Eng. Sci.* **1996**, 51, 5091-5102.

Mutagenesis Methods
Recombinant Mutagenesis

***Schematic representation of DNA shuffling*:**



***DNA Recombination*:**

Process by which a strand of genetic material (e.g., DNA is broken and joined to a different DNA molecule.

***DNA Shuffling*:**

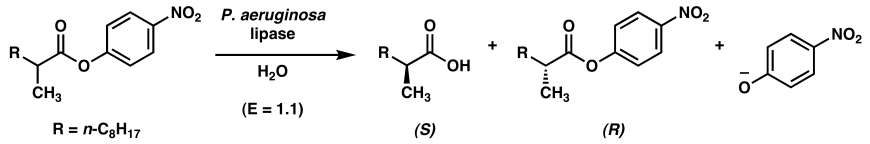
The idea is to generate diversity by recombination by combining beneficial mutations from related genes.

Advantage: a large sequence space

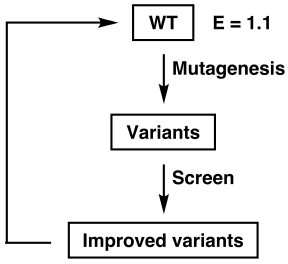
Stemmer, *Nature* **1994**, 370, 189-391.
Stemmer, *Nature* **1998**, 391, 288-291.
Reetz, *Top. Curr. Chem.* **1999**, 200, 31-57.
"Genetic recombination". Sep 27, 2009 <http://en.wikipedia.org>

Pseudomonas aeruginosa Lipase

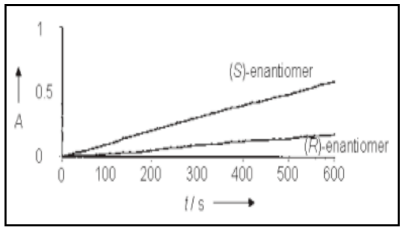
***P. aeruginosa* lipase (PAL):** **285 amino acids**
933 bp



Directed evolution of PAL:



Representative Screening Method:



Separate A_{410} (*p*-nitrophenolate anion) measurements of (*S*) and (*R*) enantiomers.

Reetz, *Angew. Chem. Int. Ed. Engl.* **1997**, 36, 2830-2832.

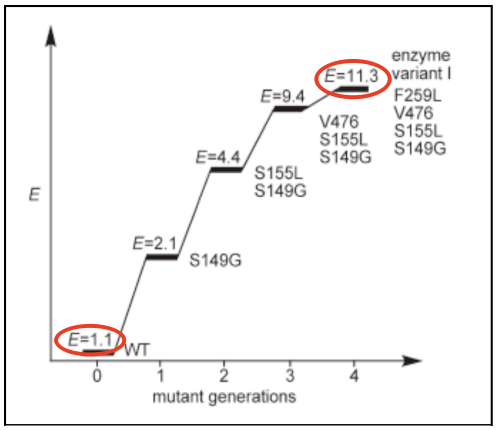
Pseudomonas aeruginoa Lipase

Error-prone PCR

Error Prone PCR

- **Introduced 1-2 bp mutations per lipase gene (up to 2 amino acid substitutions).**
- **A total of 4 rounds of epPCR.**

***Sequential increase of enantioselectivity in PAL in the course of the mutagenesis experiments*:**



Generation	# of clones tested	# of positive clones
1	1,000	12
2	2,200	10
3	2,400	1
4	2,000	6

*** Further rounds of mutations did not result in Improvement in enantioselectivity.**

$$E = \frac{\ln[(1-c)(1-ee(S))]}{\ln[(1-c)(1+ee(S))]}$$

Reetz, *Chem. Biol.* **2000**, 7, 709-718.
Reetz, *Angew. Chem. Int. Ed. Engl.* **1997**, 36, 2830-2832.

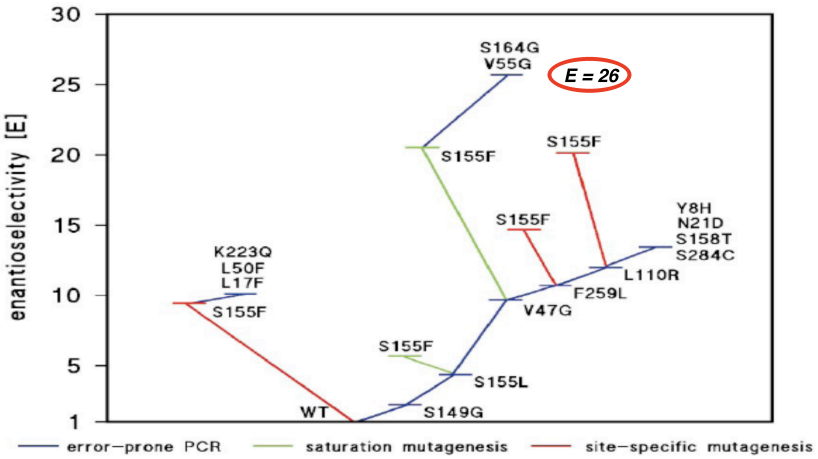
Pseudomonas aeruginosa Lipase

Saturation Mutagenesis

Saturation Mutagenesis:

A method used to introduce all 20 amino acids to "hot spots"
"Hot spots" for PAL has been identified by epPCR in previous studies.

***Evolution of PAL enantioselectivity*:**



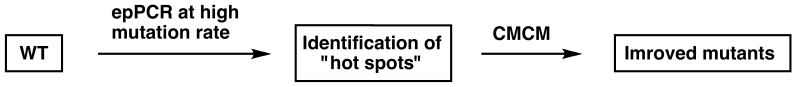
Reetz, *Chem. Biol.* **2000**, 7, 709-718.

Pseudomonas aeruginosa Lipase

Combinatorial Multiple Cassette Mutagenesis (CMCM)

***Combinatorial Multiple Cassette Mutagenesis (CMCM)*:**

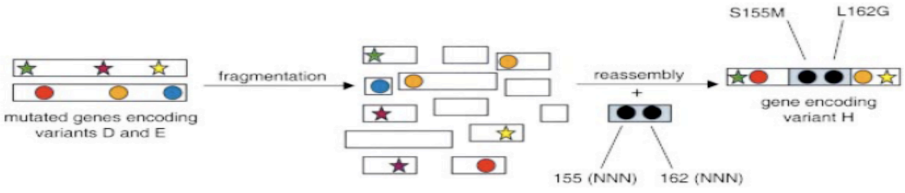
CMCM is a special type of DNA shuffling that allows for the generation of mutant-gene libraries in which the WT gene and cassettes composed of defined sequences are randomized.



Simplified CMCM 1 → Mutant G
Targeted amino acids 160-163 (epPCR)
Yielded mutant G (E160A, S161D, L162G; E = 30)

Simplified CMCM 2 → Mutants H and I
Targeted amino acids 155 and 162 (from previous studies)
Yielded mutants H (S155S, L162G; E = 34) and I (S155V, L162G; E = 30)

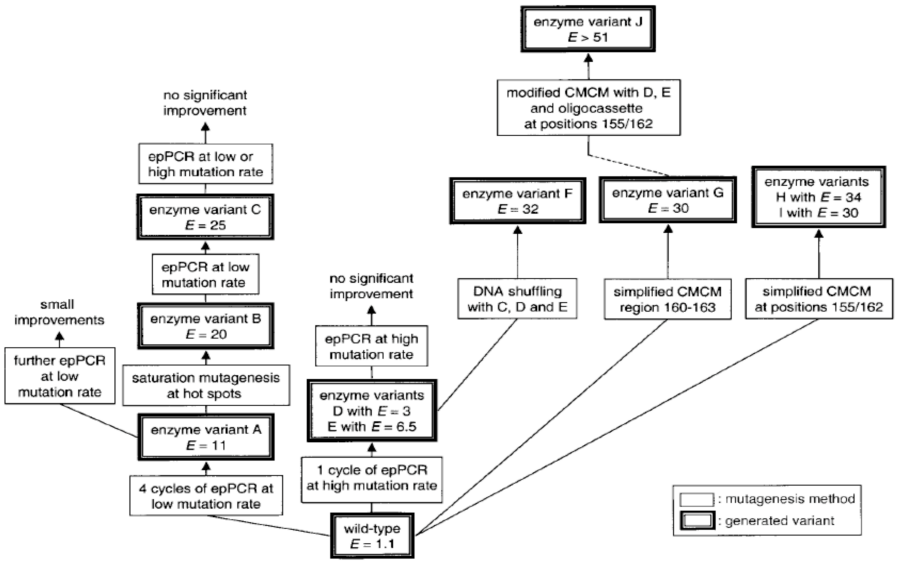
Modified CMCM → Mutant J
DNA shuffling with with mutants D, E (from epPCR) and oligocassette at positions at 155 and 162.
Yielded mutant J (D20N, S53P, S155M, L162G, T180I and T234S; E = 51)



Reetz, *Angew. Chem. Int. Ed.* **2001**, 40, 3589-3591.

Pseudomonas aeruginosa Lipase

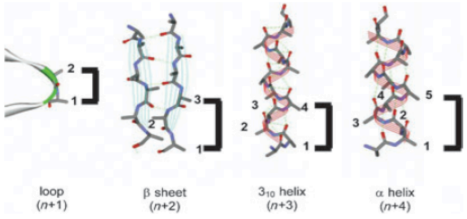
Summary

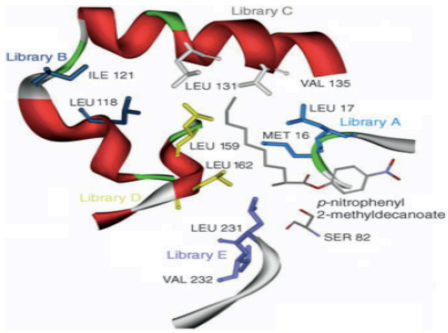


Reetz, *Angew. Chem. Int. Ed.* **2001**, 40, 3589-3591.

Pseudomonas aeruginosa Lipase

Expansion of Substrate Scope - CAST





Combinatorial Active-Site Saturation Test (CAST):

Randomization at several sets of two spatially close amino acid positions around the active site.

Requires 3D structure of the WT enzyme with a bound substrate.

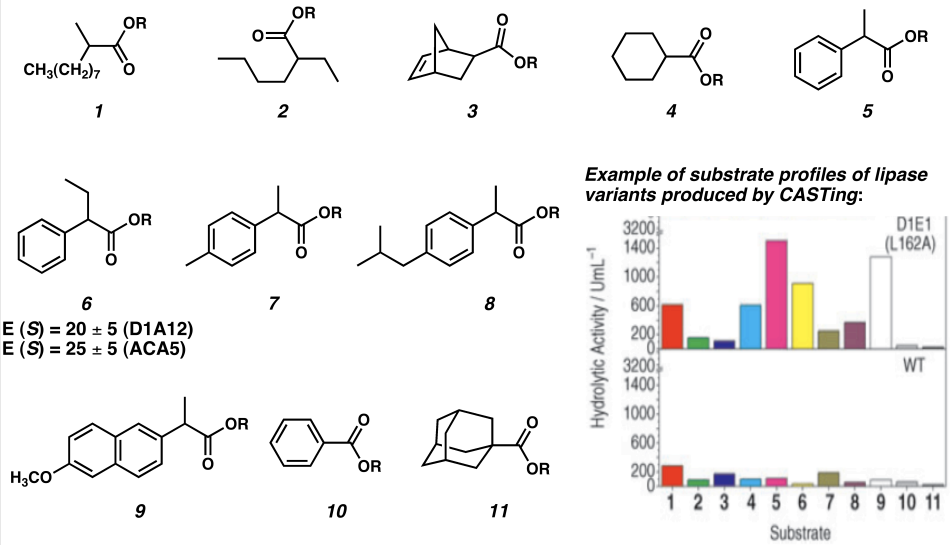
***Active mutants of PAL created by CASTing*:**

Mutant	Library	Mutations
ACA5	A	Met16Ala, Leu17Phe
A16D8	A	Met16Gly
A2D3	A	Met16Gly, Leu17Phe
D1A12	D	Leu162Val
D1B10	D	Leu162Thr
D1C4	D	Leu159Trp, Leu162Thr
D1F8	D	Leu162Asn
D1E1	D	Leu162Ala

Reetz, *Angew. Chem. Int. Ed.* **2005**, 44, 4192-4196.

P. aeruginosa Lipase

Expansion of Substrate Scope - CAST



E (*S*) = 20 ± 5 (D1A12)
E (*S*) = 25 ± 5 (ACA5)

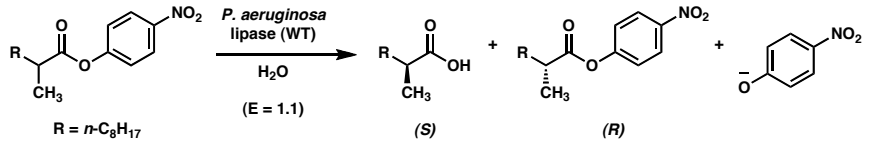
***Example of substrate profiles of lipase variants produced by CASTing*:**

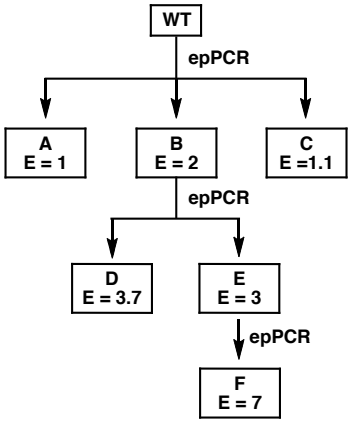
R = 4-nitrophenyl

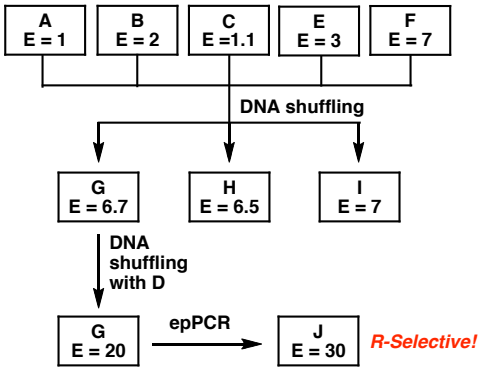
Reetz, *Angew. Chem. Int. Ed.* **2005**, 44, 4192-4196.

Pseudomonas aeruginosa Lipase

Reversal of Enantioselectivity





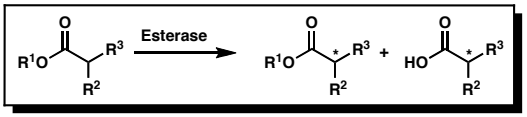


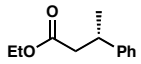
Reetz, *Chem. Comm.* **2001**, 2664-2665.

Pseudomonas fluorescens Esterases

Effects of Distant Mutations

Methyl(*R*)-3-bromo-2-methylpropanoate (MBMP)





Ethyl (S)-3-phenylbutanoate (EPB)

***Directed Evolution of PFE towards MBMP*:**

***E*=12**
(S) selective
WT
↓ Random mutagenesis using *E. coli* mutator strain
280 colonies
↓ Quick E
***E*=21** Identified Thr230Ile mutant
↓ Site saturation mutagenesis at Thr230
175 colonies
↓ Quick E
No improvement

Showed that distant mutations can influence enantioselectivity. (Thr230, Asp158 and Leu 181 are outside active site)

- **Saturation mutagenesis at positions 158 and 181 (identified in previous studies)**
- **PFE activity tested against 3-phenylbutyric acid resorufin ester (Quick E test)**

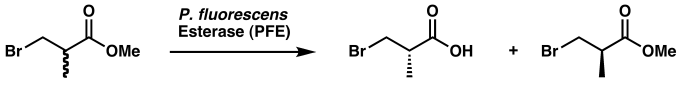
***E values toward EPB (R-selective)*:**

Mutation	E
WT	3.5
Asp158Leu	12
Asp158Phe	9
Leu181Ser	10
Leu181Thr	9
Asp158Phe, Leu181Ser	9
Asp158Phe, Leu181Thr	10
Asp158Leu, Leu181Ser	12
Asp158Leu, Leu181Thr	9

Kazlauskas, *Chem. Eur. J.* **2003**, 9, 1933-1939.

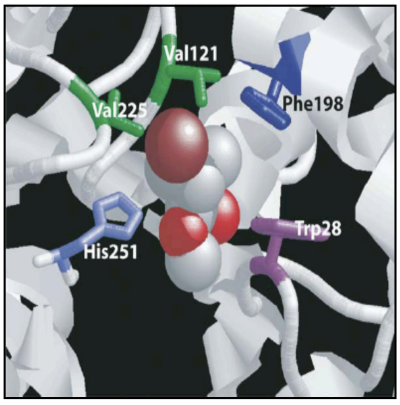
Pseudomonas fluorescens Esterases

Effects of Active Site Mutations



MBMP ***(S)*** ***(R)***

***Model of PFE active site with the R-MBMP bound*:**



Saturation mutagenesis at all 5 residues

This is 5-fold higher than WT!

Phe198, Val225 and His251 did not lead to mutants with enhanced enantioselectivity

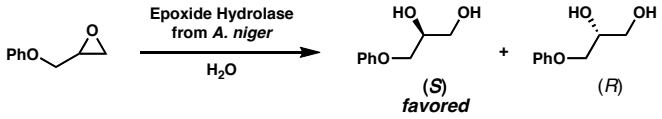
***Enantioselectivity of PFE toward MBMP*:**

Enzyme	E
WT	12 ± 2
Trp28Leu	58 ± 7
Trp28Phe	32 ± 1
Trp28Tyr	29 ± 1
Trp28Cys	ND
Val121Ser	61 ± 1
Val121Met	36 ± 1
Phe198Leu	10 ± 1
Phe198Trp	2.2 ± 0.5
Val225Ala	ND
Val225Ile	ND
Val225Ser	ND

Kazlauskas, *Chem. Biol.* **2005**, 12, 45-54.

Aspergillus niger Epoxide Hydrolases

Error-prone PCR



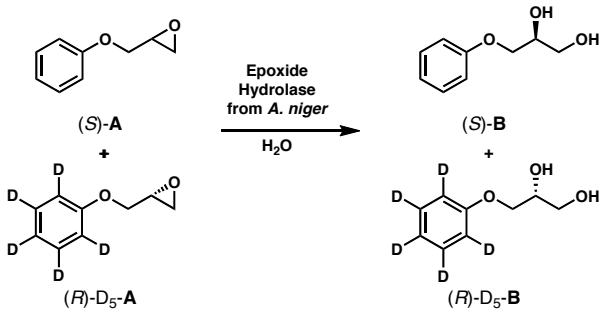
***Mutagenesis Method*:** **epPCR**

***Pretest*:** **Epoxide hydrolase activity was tested by reaction of an epoxide with 4-*p*-nitrobenzyl pyridine, causing a formation of a blue dye.**

ESI-MS screening method:

(*S*)-B & (*R*)-D_5-B should differ by 5 mass units.

Their respective ratios can be determined by ESI-MS.



Reetz, *Org. Lett.* **2004**, 6 (2), 177-180.
Schmid, *Anal. Chim. Acta* **1999**, 391, 345-351.

Aspergillus niger Epoxide Hydrolases

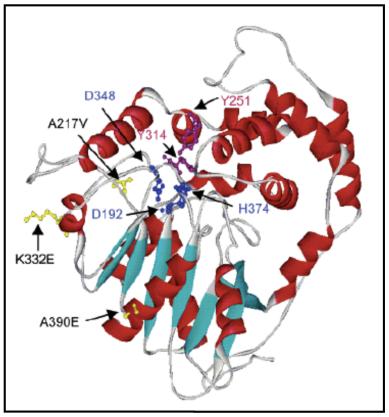
Error-prone PCR

After one round of epPCR:

Mutant	ee_p(*S*) (%)	conversion (%)	E	Amino acid exchanges
WT	56	33	4.6	N.A.
1	74	39	10.8	A217V, K332E, A390E
2	73	24	8.1	N.D.
3	69	35	7.7	N.D.
4	69	31	7.4	R219G
5	66	38	7.2	F84L M245L
6	64	38	6.8	N.D.
7	65	34	6.4	A327V
8	67	23	6.2	A327V
9	62	30	5.5	P222S

Molecular modeling of the WT enzyme suggests that the side chain of residue 217 is in van der Waals contact with the aromatic ring of the substrate.

The roles of remote substitutions are unclear.



Crystal structure of the wild type ANEH monomer

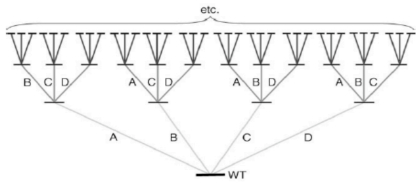
- **Blue - catalytic triad**
- **Purple - 2 tyrosines that activate the epoxide by hydrogen bonding**
- **Yellow - Amino acid substitutions**

Reetz, *Org. Lett.* **2004**, 6 (2), 177-180.

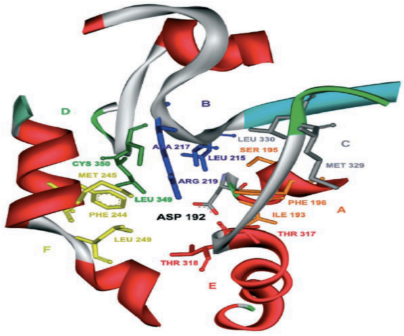
Aspergillus niger Epoxide Hydrolases

Improved Enantioselectivity – CAST

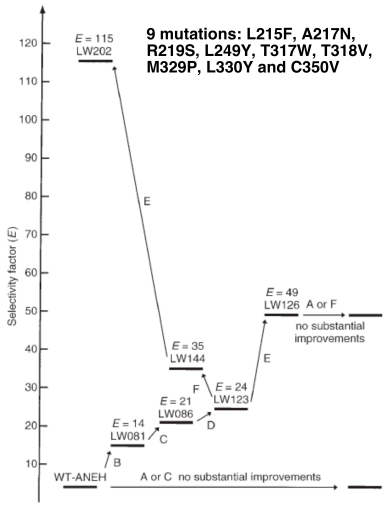
***Schematic illustration of interative CASTing*:**



***Defined radominzation sites*:**



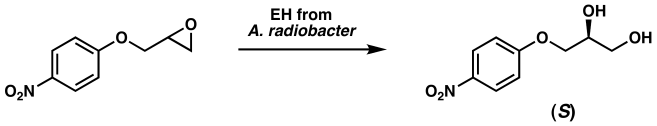
***Interative CASTing in the evolution of enantioselective ANEH*:**



Reetz, *Angew. Chem. Int. Ed.* **2006**, 45, 1236-1241.

Agrobacterium radiobacter Epoxide Hydrolase

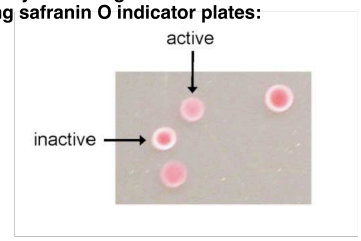
Error-prone PCR and DNA shuffling



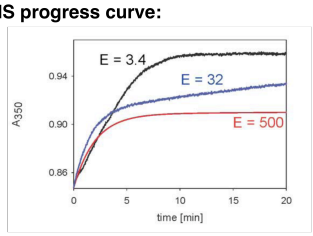
(*S*)

***1st Round Mutagenesis*:**	**Error-prone PCR: 5-7 bp substitutions per gene (2-3 amino acid substitutions per mutant)**
***2nd Round Mutagenesis*:**	**DNA shuffling (among mutants with enahanced selectivity)**
***Selection Method*:**	**Activity toward 1,2-epoxybutane using an agar plate assay with safranin O as an indicator.**
***Screening Method*:**	**UV/VIS progress curve monitoring A_{350} (pNPGE)**

Activity screening of colonies using safranin O indicator plates:



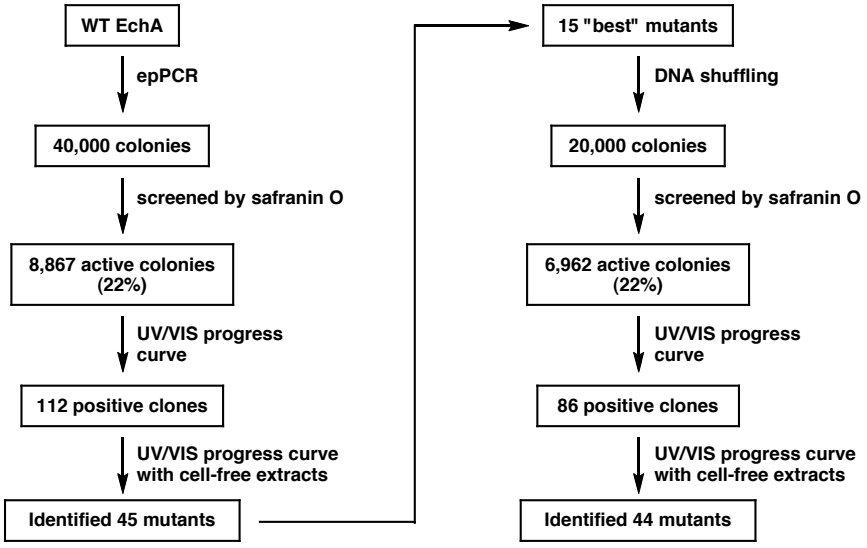
UV/VIS progress curve:



Janssen, *Chem. Biol.* **2004**, 11, 981-990.

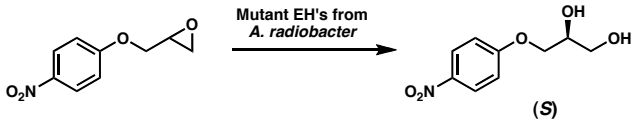
Agrobacterium radiobacter Epoxide Hydrolase

Error-prone PCR and DNA shuffling



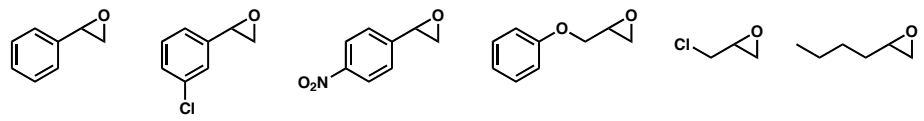
Janssen, *Chem. Bio.* **2004**, 11, 981-990.

Agrobacterium radiobacter Epoxide Hydrolase



Mutant	E	Mutations
WT	3.4	None
1	44	F108I, S217C, L277S
2	20	Y152F, E170G, E271V, D287E
3	22	F108I, S121C, D200E, Y215F
4	32	F108I, P205H, Y215H, E271V
5	26	F108I, V112I, D159E, E170G, S184A, Y215F
6	27	D131G, Q134L, Y152F, T227S, Y251C
7	19	A119G, Y152F, Y251C
8	21	Y152F, D159E, E271V

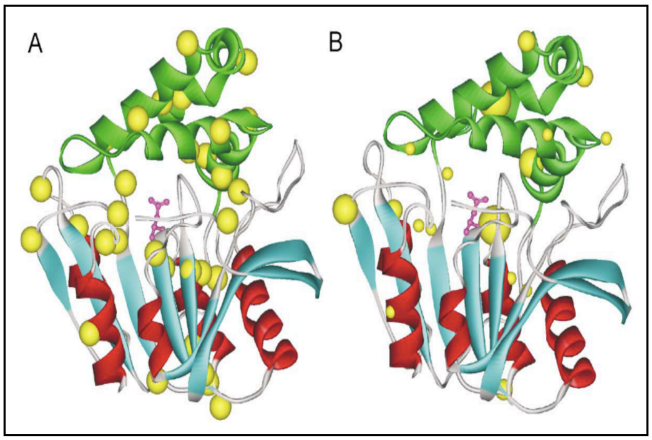
Substrate Scope:



Janssen, *Chem. Bio.* **2004**, 11, 981-990.

Agrobacterium radiobacter Epoxide Hydrolase

***Positions of the selected mutations in the X-ray structure of EchA*:**



(A) Initial mutations selected after epPCR

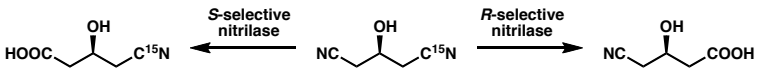
(B) Mutations selected after recombination by gene shuffling. The radius of the sphere is proportional to the frequency of the mutations in the final set of mutants.

Blue: main domain
Red: a/b-hydrolase fold
Green: cap domain
Purple: active site Asp
Yello: mutations (radius of the sphere ~ frequency of mutation

- **Sequence analysis showed that 16 of the 34 positions that were present in the EchA mutants after epPCR were present in the final 8 mutants.**
- **All recombinants contained at least one mutation of a residue that is part of the active site.**

Janssen, *Chem. Bio.* **2004**, 11, 981-990.

Nitrilase



^{15}N-(*S*)-**B**
m/z = 130

^{15}N-(*R*)-**A**

(*R*)-**B**
m/z = 129

Intermediate to the drug Lipitor

***GSSM library*:**

Nitrilase	100mM ee (%)	2.25M ee (%)	time (h)
WT	94.5	87.8	24
Ala55Gly	96.5	nd	>160
Ile60Glu	96.5	nd	>160
Ala190His	97.9	98.1	15
Ala190Ser	96.8	95.5	40
Ala190Thr	96.5	96.6	40
Asn111Ser	95.8	96.1	>160
Phe191Leu	97.9	nd	>160
Phe191Thr	97.9	nd	>160
Phe191Met	97.9	nd	>160
Phe191Val	97.9	nd	>160
Met199Glu	97.9	nd	>160
Met199Leu	97.9	95.4	160

***Gene Site Saturation Mutagenesis (GSSM)*:**

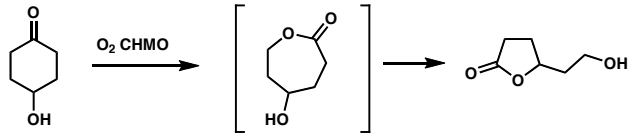
Each amino acid of a protein is replaced with each of the other 19 naturally occuring amino acids.

Advantage of the GSSM method is that introduction of ALL 20 amino acids at every position is possible. It is impossible to do so with epPCR due to degeneracy in codons.

Burk, *J. Am. Chem. Soc.* **2003**, 125, 11476-11477.

Cyclohexanone Monooxygenase

Baeyer-Villiger



***1st Round Mutagenesis*: epPCR at different rates**

***Screening Method*: High throughput GC assay**

***Altered CHMO mutants after first round of epPCR*:**

Mutant	AA exchange	R/S	ee (%)
WT	–	R	9
1	F432Y, K500R	R	34
2	L143F	R	40
3	F432I	R	49
4	L426P, A541V	R	54
5	L220Q, P428S, T433A	S	18
6	D41N, F505Y	S	46
7	K78E, R432S	S	78
8	F432S	S	79

Opposite to the usual S selectivity generally observed for 4-substituted cyclohexanone derivates

Reetz, *Angew. Chem. Int. Ed.* **2001**, 40, 284-310.
Reetz, *Angew. Chem. Int. Ed.* **2004**, 43, 4075-4078.

Cyclohexanone Monooxygenase

Baeyer-Villiger

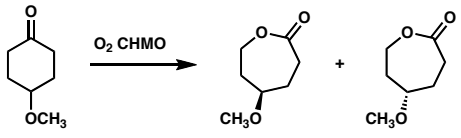
***Evolution toward R selectivity*:**

2nd round of epPCR with *R*-selective mutants

1,600 mutants screened

"Best" mutant (mutant 9): Evolved from mutant 2
90% ee
Mutations: L143F*, E292G, L435Q, T464A

***Oxidation of 4-methoxy cyclohexanone*:**



(*R*) (*S*)

***Enantioselectivities of CHMO toward 4-methoxy cyclohexanone*:**

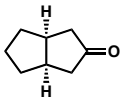
Mutant	*R*/*S*	ee
WT	*S*	78%
Mutant 9	*R*	25%
Mutant 8	*S*	98.6%

Enantiodivergent!

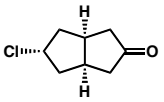
Reetz, *Angew. Chem. Int. Ed.* **2004**, 43, 4075-4078.

Cyclohexanone Monooxygenase

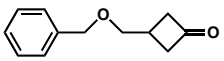
Bayer-Villiger – Expansion of Substrate Scope



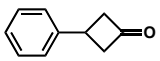
WT: 89% ee (–)
M4: 17% ee (–)
M8: 94% ee (–)



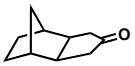
WT: > 99% ee (–)
M4: 60% ee (–)
M8: 99% ee (+)



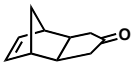
WT: 53% ee (+)
M4: 66% ee (+)
M8: 83% ee (–)



WT: 62% ee (–)
M4: 8% ee (–)
M8: 96% ee (–)



WT: n.d.
M4: 12% ee (+)
M8: 92% ee (+)

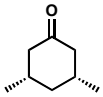


WT: n.d.
M4: 60% ee (+)
M8: 99% ee (–)

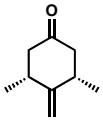
WT: 44/>99% ee (–/–)
M4: 94/>99% ee (–/–)
M8: 65/>99% ee (–/–)
*normal/abonormal

WT: 96% ee (–)
M4: 48% ee (–)
M8: 93% ee (–)

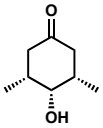
WT: 96% ee (–)
M4: 48% ee (–)
M8: 94% ee (–)



WT: >99% ee (–)
M4: >99% ee (–)
M8: >99% ee (–)



WT: 92% ee (+)
M4: >99% ee (+)
M8: >99% ee (+)

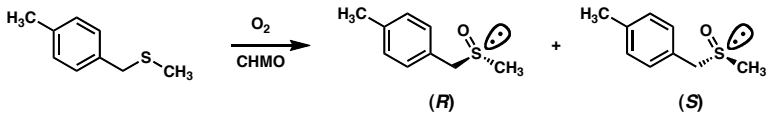


WT: 96% ee (–)
M4: >99% ee (–)
M8: >99% ee (–)

Reetz, *Org. Lett.* **2006**, 8, 1221-1224.

Cyclohexanone Monooxygenase

Oxidation of Thioesthers



Using the 1st round epPCR mutant libary from Bayer Villiger reaction:

Mutant	AA exchanges	Yield (%)	R/S	ee (%)	Sulfone yield (%)
WT	–	75	R	14	<1
1	D384H	75	R	98.9	7.9
2	F432S	55	R	98.7	20.0
3	K229I, L248P	77	S	98.1	5.6
4	Y132C, F246I V361A, T415A	52	S	99.7	26.6
5	F16L, F277S	84	S	95.2	5.6

Over-oxidation to achiral sulfone is a known process in other microbial oxidation of prochiral thioesters. However, it is only a minor process due to low rate of over-oxidation.

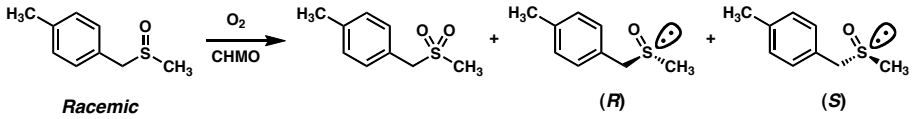
Previously identified as a highly enantioselective biocatalyst in asymmetric Bayer-Villiger reaction of 4-substituted cyclohexanones

Reetz, A*ngew. Chem. Int. Ed.* **2004**, 43, 4078-4081.

Cyclohexanone Monooxygenase

Oxidation of Thioesthers

***Oxidative Kinetic Resolution of Racemic Sulfoxide*:**



Racemic (***R***) (***S***)

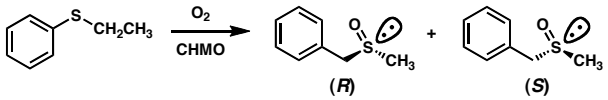
***Oxidation of racemic sulfoxide*:**

Mutant	Sulfone Yield	R/S	Sulfoxide ee
Mutant 1	43%	R	98.7%
Mutant 4	62%	S	98.9%

***Directed evolution to reduce sulfone formation*:**

- **epPCR using mutant 4**
- **Discovered a new mutant (with three new mutations) that leads to *99.8% ee* in favor of (*S*)-sulfoxide, with the amount of undesired sulfone being almost negligible (< 5%).**

***Testing Mutants 3 and 5 with a different substrate*:**



(*R*) (*S*)

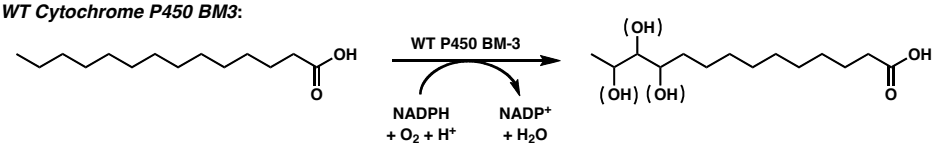
Mutant	R/S	ee
WT	R	47%
Mutant 3	S	98.9%
Mutant 5	R	88%

Reetz, *Angew. Chem. Int. Ed.* **2004**, 43, 4078-4081.

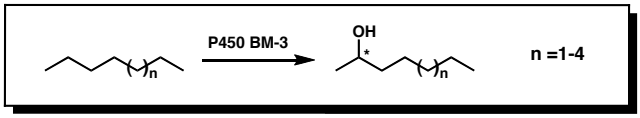
Bacillus megaterium Cytochrome P450 BM3

Hydroxylation

***WT Cytochrome P450 BM3*:**



***Desired Reactivity*:**



15 mutants (previously generated library)

→ **• DNA shuffling • Screen for increase in LADH consumption**

→ **Mutant J**

→ **• DNA shuffling • Screen for increase in LADH consumption**

→ **Mutant 9-10A**

→ **• epPCR • Screen for hydroxylation**

→ **0 mutants with enhanced activity**

Arnold, *J. Am. Chem. Soc.* **2003**, 125, 13442-13450.

Bacillus megaterium Cytochrome P450 BM3

Hydroxylation

15 mutants (previously generated library)

↓ • DNA shuffling • Screen for increase in LADH consumption

Mutant J

↓ • epPCR • Screen for increase in LADH consumption

Mutant 9-10A

↓ A328 mutations

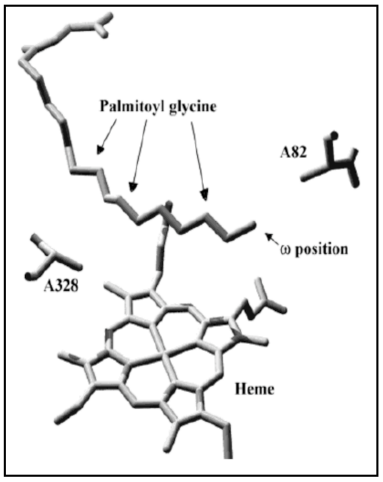
Mutant 9-10A-A328V

Not much change in hydroxylation activity, but more regioselectivity on C7-9 alkanes (70% *S*-2-octanol, 40% ee)

↓ DNA shuffling

Mutant 1-12G

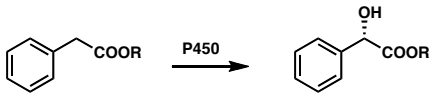
Generates R-2-alcohols (40-55% ee) of C7-10 alkanes



Arnold, *J. Am. Chem. Soc.* **2003**, 125, 13442-13450.

Bacillus megaterium Cytochrome P450 BM3

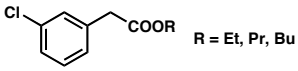
Hydroxylation



(*S*)

***Enzymatic Acitivity of Mutated Enzymes*:**

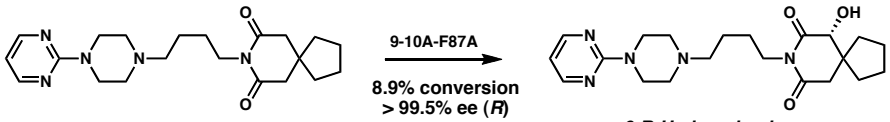
Enzyme	Substrate	TTN	% selectivity	% ee (*S*)
BM-3	Pr	63	17	82
	Bu	20	6	71
BM-3-F87A	Pr	623	99	85
	Bu	396	88	75
9-10A	Pr	77	41	85
	Bu	86	25	95
9-10A-F87A	Pr	1640	88	93
	Bu	660	76	89



R = Et, Pr, Bu

***Activity of Enzyme 9-10A-F87A toward m-chloro phenylacetic acid*:**

Substrate	TTN	% selectivity	% ee (*S*)
Et	86	25	95
Pr	1640	88	93
Bu	660	76	89



8.9% conversion
> 99.5% ee (*R*)

72% conversion with cofactor regeneration

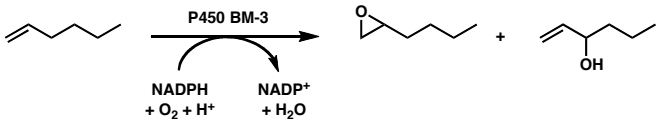
6-R-Hydroxybusiprone (anti-anxiety agent)

Arnold, *J. Am. Chem. Soc.* **2006**, 128, 6058-6059.

Bacillus megaterium Cytochrome P450 BM3

Epoxidation of Terminal Alkenes to (R)- and (S)-Epoxides

***Desired reaction*:**



***Selected from saturation mutagenesis at resiudes (11) closest to the heme*:**

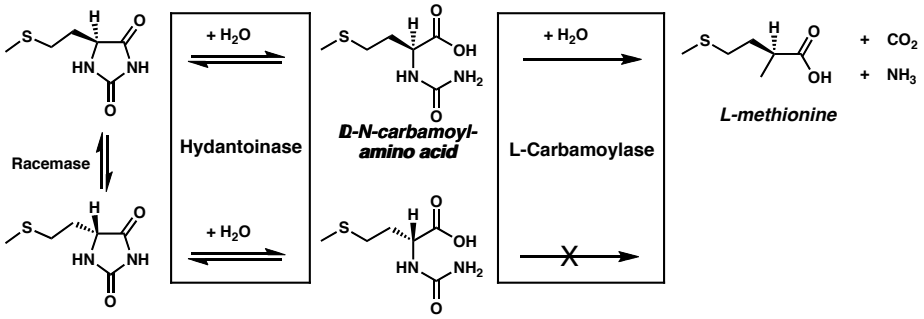
Mutation in 9-10A	TTN	Selectivity (%)	ee (%)
none	140	25	4 (*R*)
L75S	220	45	19 (*S*)
A82F	210	64	2 (R)
A82L	220	49	16 (*R*)
F87I	450	67	45 (*S*)
F87L	350	76	79 (*S*)
F87V	670	73	73 (S)
I263A	300	68	11 (*R*)
A328V	280	81	63 (*R*)

***DNA shuffling between the selected mutations*:**

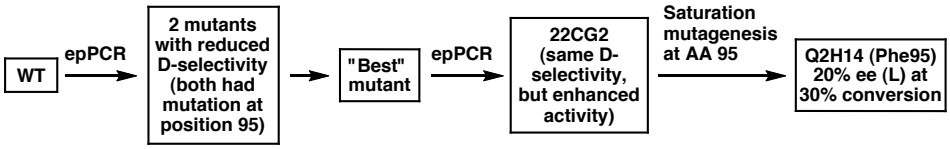
Variant	Substrate	TTN	Selectivity (%)	ee (%)
SH-44	1-pentene	1370	93	73 (*S*)
	1-hexene	1090	88	71 (*S*)
	1-heptene	500	85	65 (*S*)
	1-octene	200	84	55 (*S*)
RH-47	1-pentene	570	94	60 (*R*)
	1-hexene	610	93	60 (*R*)
	1-heptene	550	95	76 (*R*)
	1-octene	560	92	83 (*R*)

Arnold, *Chem. Eur. J.* **2006**, 12, 1216-1220.

Hydantoinases

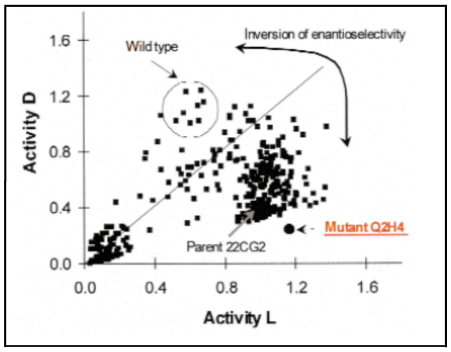


***Directed evolution of enantioselective hydantoinase*:**

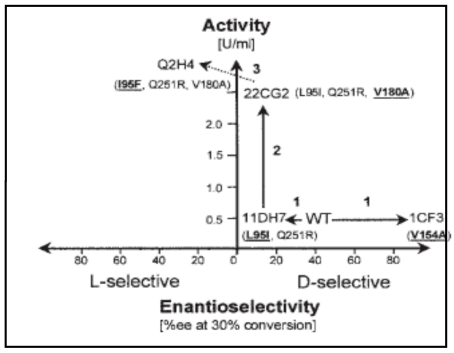


Arnold, *Nature Biotechnology* **2000**, 18, 317-320.

Hydantoinases



Screening was achieved by monitoring activities toward D and L-MTEH using pH indicator assay with cresol red (A_{580})



Schematic diagram of evolutionary progression of hydantoinase activity and enantioselectivity through two generations of epPCR and saturation mutagenesis.

L-Methionine production with a recombinant whole-cell catalyst:

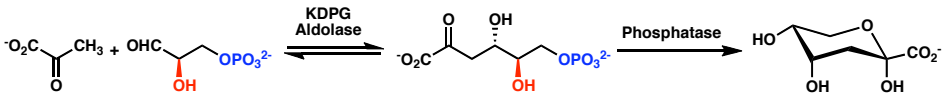
- **~91mM of L-Met produced from 100mM D,L-MTEH in 2 h (WT = ~66mM)**
- **>90% conversion achieved in less than 2 h (WT = 10 h)**
- **Accumulated D-C-Met intermediate concentration was reduced more than fourfold**

Arnold, *Nature Biotechnology* **2000**, 18, 317-320.

2-Keto-3-deoxy-6-phosphogluconate Aldolase

Synthesis of D & L-Sugars

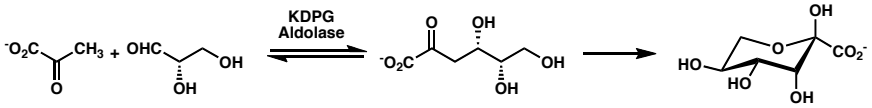
***WT 2-Keto-3-deoxy-6-phosphogluconate (KDPG) Aldolase*:**



Pyruvate ***Glyceraldehyde 3-phosphate (G3P)*** ***KDPG*** ***D-sugar***

Optimization for the acceptace of: **D and L aldehydes**
Non-phosphorylated aldehydes

***Example of desired substrate tolerance*:**

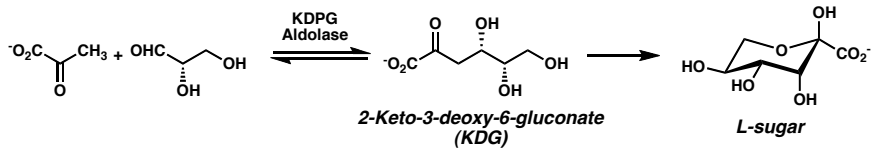


KDG ***L-sugar***

Toone, *J. Am. Chem. Soc.* **1996**, 118, 2117-2125.
Wong, *Chem. Biol.* **2000**, 7, 873-883.

2-Keto-3-deoxy-6-phosphogluconate Aldolase

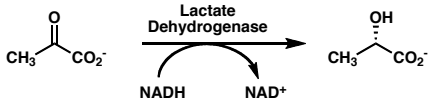
Synthesis of D & L-Sugars



2-Keto-3-deoxy-6-gluconate (KDG)

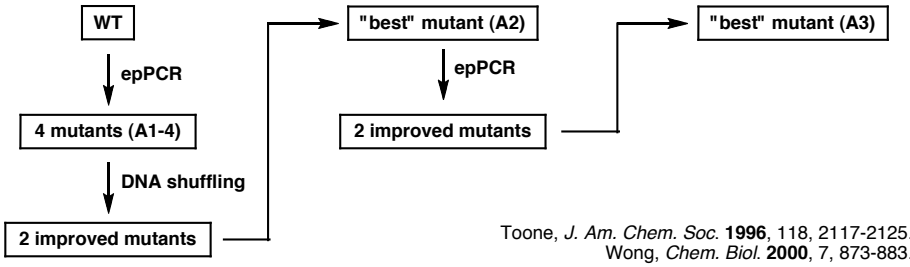
L-sugar

***KDG cleavage assay*:**



Consumption of NADH is monitored by UV/VIS at 340nm

***Directed Evolution of KDPG Aldolase*:**

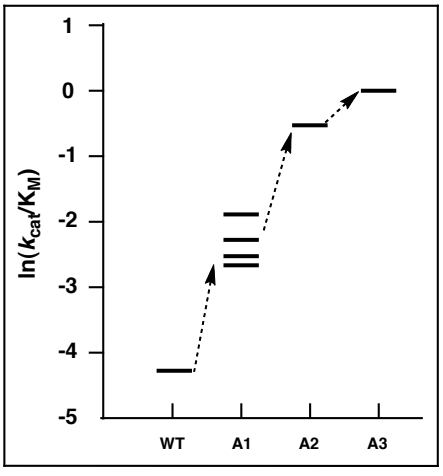


Toone, *J. Am. Chem. Soc.* **1996**, 118, 2117-2125.
Wong, *Chem. Biol.* **2000**, 7, 873-883.

2-Keto-3-deoxy-6-phosphogluconate Aldolase

Synthesis of D & L-Sugars

***Evolution of KDPG aldolase for KDG cleavage*:**



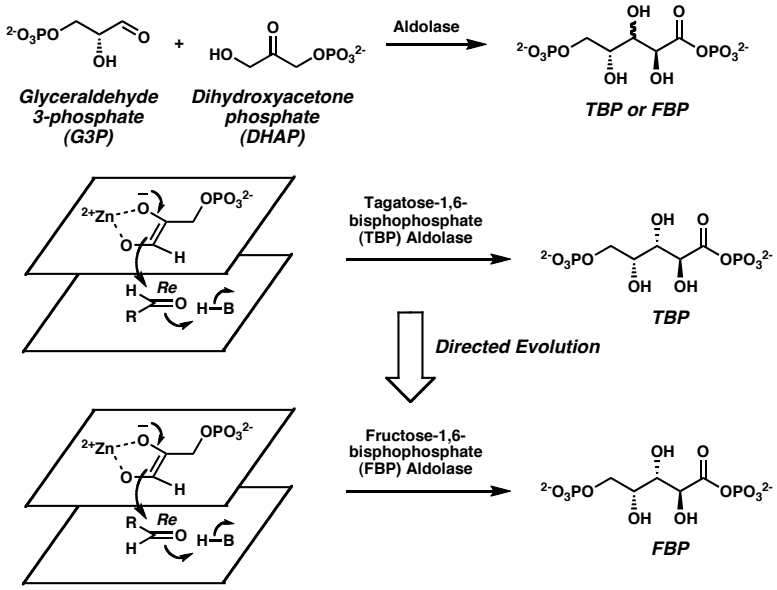
***Relative activity of KDPG aldolase variants for aldol addition reactions with pyruvate as the donor substrate*:**

Acceptor Substrate	WT	A3
D-Glyceraldehyde	100	181
L-Glyceraldehyde	too slow	26
D-Lactaldehyde	32	104
L-Lactaldehyde	–	–
DL-3--Fluoro-2-hydroxypropanal	45	95
D-Threose	–	–
D-Erythrose	–	–
DL-Glyceraldehyde-3-P	2.6×10^4	3.2×10^3
Glyoxylate	60	700
Glycoladehyde	10	23
Chloroacetaldehyde	too slow	19

Toone, *J. Am. Chem. Soc.* **1996**, 118, 2117-2125.
Wong, *Chem. Biol.* **2000**, 7, 873-883.

Tagatose-1,6-bisphosphate Aldolase

Evolved Diastereoselectivity

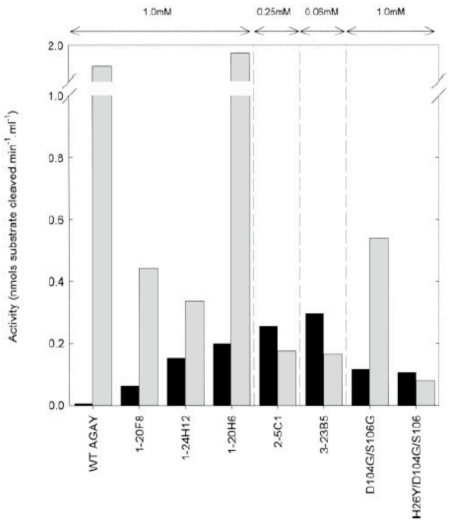


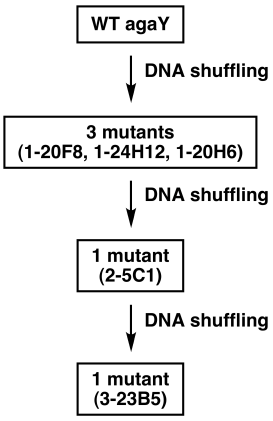
Berry, *Proc. Natl. Acad. Sci.* **2003**, 100, 3143-3148.

Tagatose-1,6-bisphosphate Aldolase

Evolved Diastereoselectivity

***Aldolase activities toward FBP (black) and TBP (gray)*:**





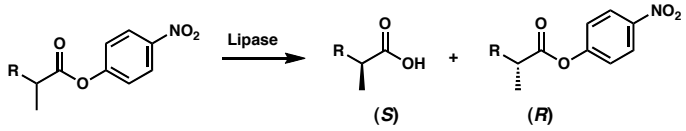
Residues identified from this study are either completely conserved (D104, S106 and P256) or semiconserved (H26) across all members of the FBP and TBP aldolases.

Berry, *Proc. Natl. Acad. Sci.* **2003**, 100, 3143-3148.

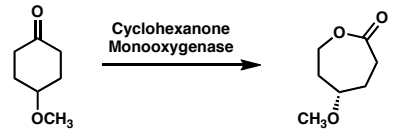
Directed Evolution of Enantioselective Enzymes

Summary

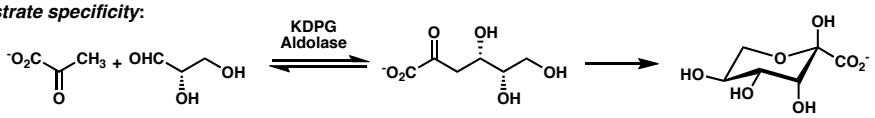
***Enhancement of enantioselectivity*:**



***Reversal of enantioselectivity*:**



***Modification of substrate specificity*:**



***Modification of diastereoselectivity*:**