Directed Evolution of Enzymes

Concept, Methods, and Selected Applications in Catalysis

MacMillan Group Meeting

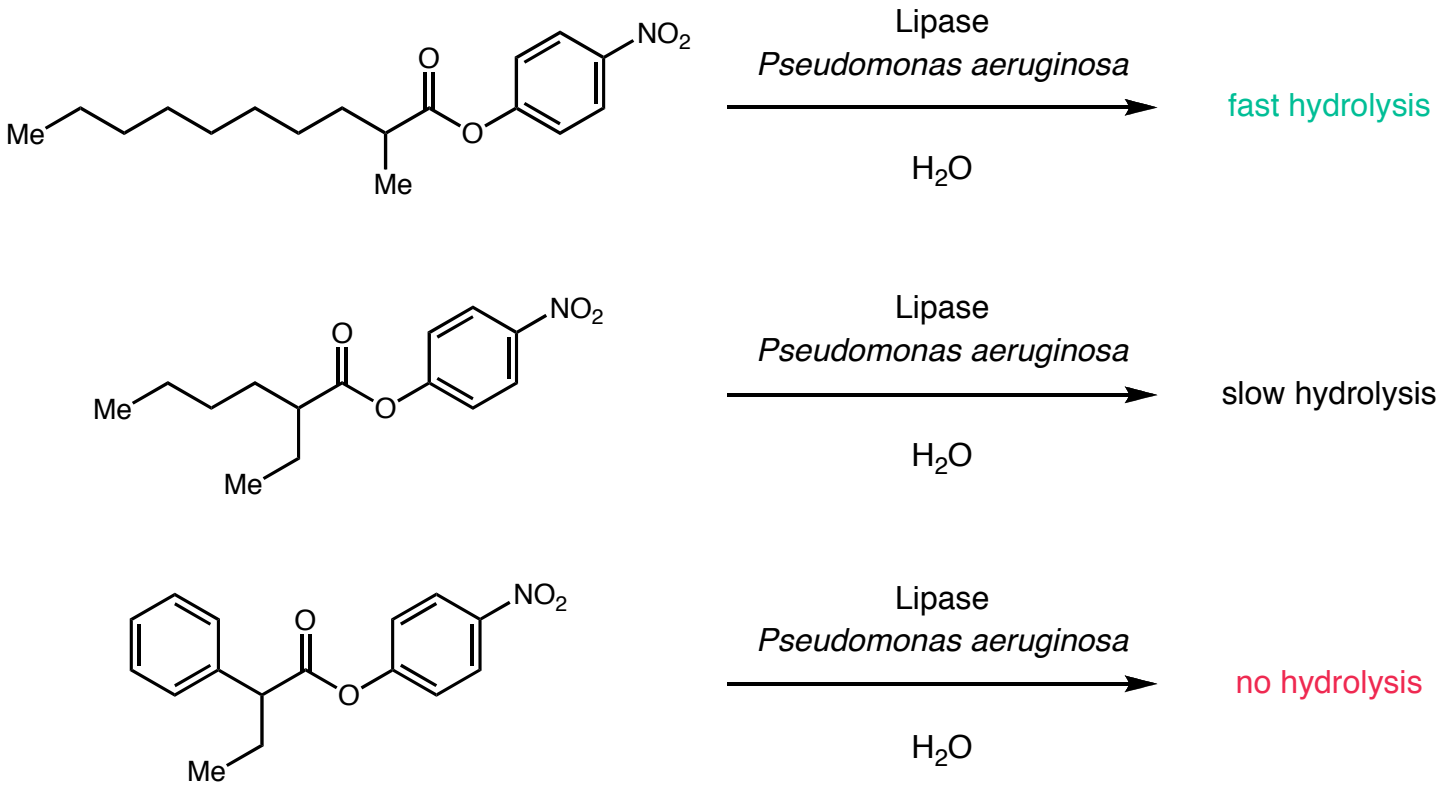
December 17, 2008

Sebastian Rendler

Enzymes — Tailor-made Biocatalysts

Evolutionary process leads to high substrate specificity

- High substrate selectivity = lacking generality



- Only a minor number of enzymes shows a desirable substrate promiscuity

Bornscheuer, U. T. & Kazlauskas, R. J. *Angew. Chem. Int. Ed.* **2004**, *43*, 6032.

Enzymes — Tailor-made Biocatalysts

Significant restrictions of natural enzymes

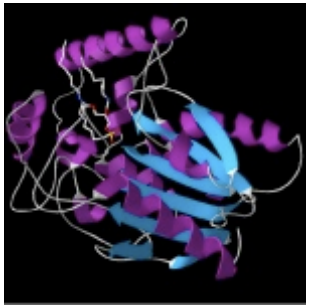
- **Substrate specificity**: Limited tolerance to electronically or sterically modified substrates
- Limited **solvent variability**: Water as almost exclusive solvent
- **Temperature**: Lacking stability at elevated temperatures due to denaturation
- **Enantioselectivity**: Satisfying values only for selected substrates

How to Expand the Synthetic Utility of Biocatalysts?

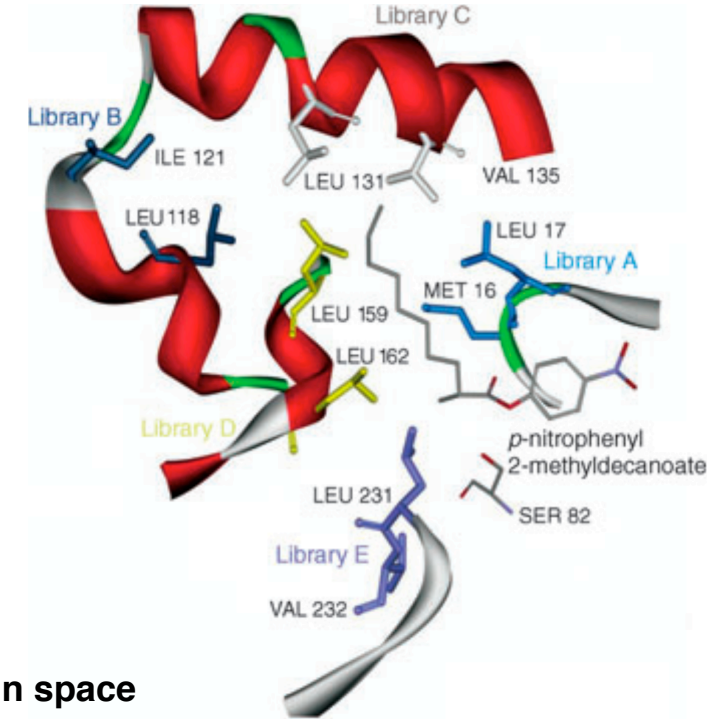
A closer look into the nature of enzymes

■ Enzymes are proteins (polypeptides) posessing complex three-dimensional structures

- e.g., molecular weight for *Pseudomonas aeruginosa* Lipase (PAL): ~29 kDa
- specific substrate-protein interaction in catalytically active binding pocket
- Limited degree of flexibility according to Koshland's "induced-fit" model



Zoom



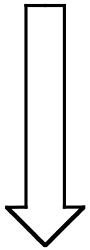
■ Chemical modification to increase substrate range?

- may require structural data
- non-trivial endeavor to address selected sites
- may be required for individually for every substrate/property
- **almost indefinite number of possible variations in protein space**

How to Expand the Synthetic Utility of Biocatalysts

Learning from nature

One substrate–One enzyme principle is the result of an evolutionary process



Can we use an evolutionary approach to engineer enzymes on a lab scale?

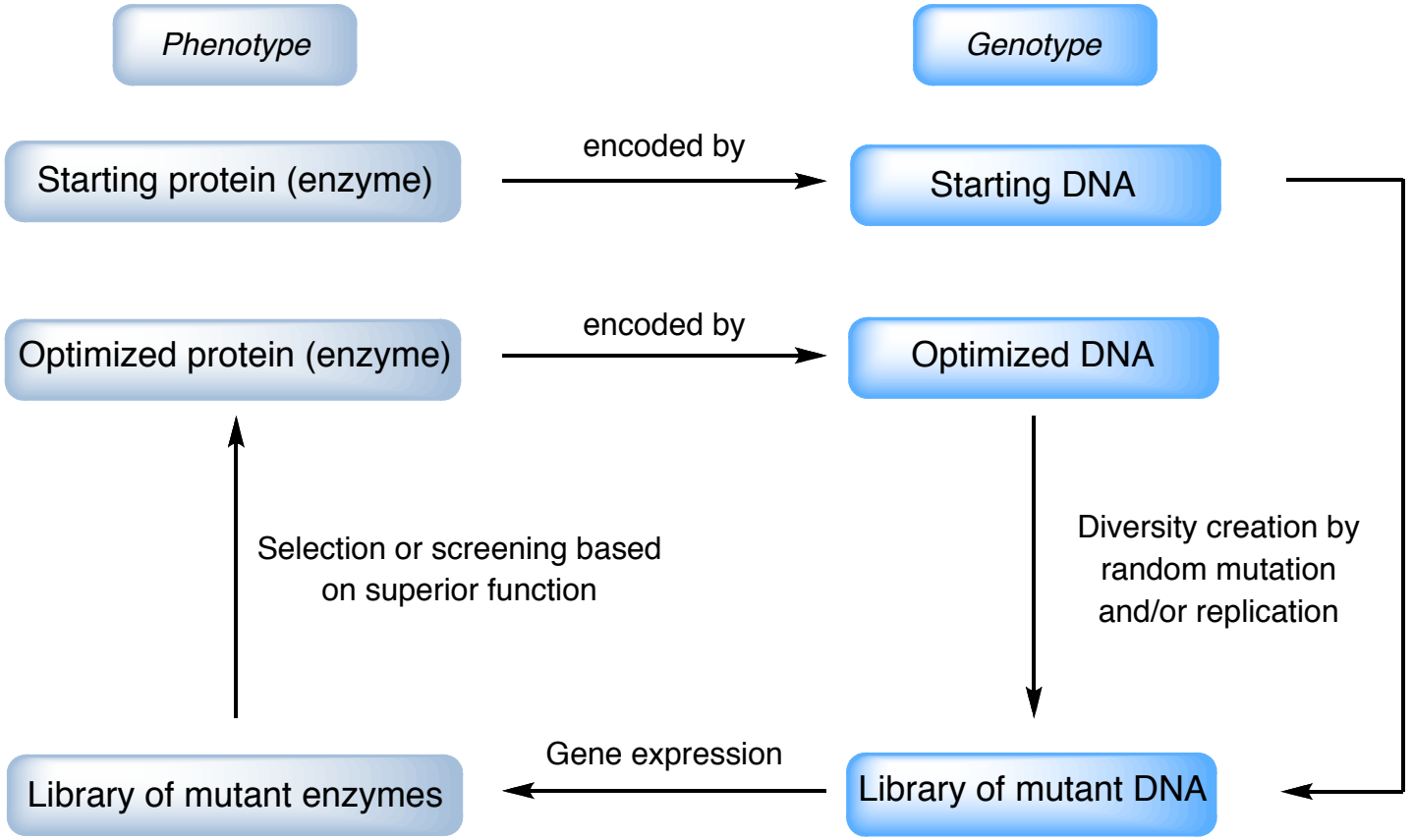
This requires....

- accelerate the evolutionary process from ~mio years to weeks
- availability of suitable experimental techniques
- establish a generally applicable concept

Directed Evolution

Definition

- General blueprint for an evolutionary process of an enzyme



adapted from: Hilvert D. *et al. Annu. Rev. Biophys.* **2008**, *37*, 153.

A Historical View on Directed Evolution

■ Timeline

1967

S. Spiegelman *et al.* report an *in vitro* darwinian experiment using self-replicating RNA (*PNAS* **1967**, *58*, 217)

1980's

rational mutagenesis approaches to engineer enzymes show only limited success

1990

J. R. Knowles *et al.* report the first true *random* mutagenesis by using the full sequence space (*PNAS* **1990**, *87*, 696)

1997

M. T. Reetz & K.-E. Jaeger *et al.* use directed evolution to improve enantioselectivity of an enzymatic resolution (*ACIEE* **1997**, *36*, 2830)

1960 ——————————————————————————→ **2000**

1971

M. Eigen reports a theory of evolution at the molecular level (*Naturwissenschaften* **1971**, *58*, 465)

1986

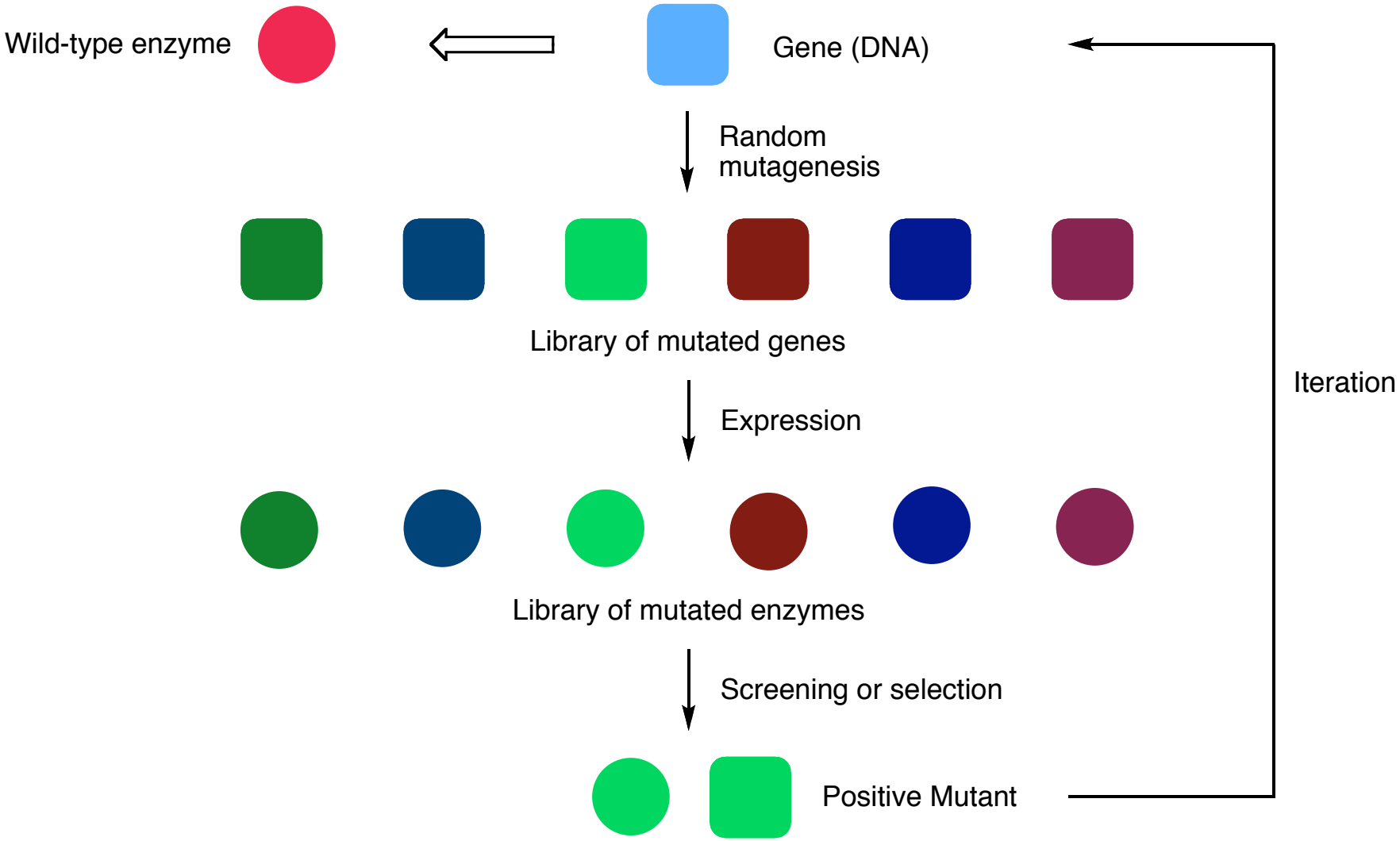
Researchers at Synergen (Boulder/CO) succeed in the first directed evolution using an *iterative rational* mutagenesis approach (*PNAS* **1986**, *83*, 576)

1993

F. H. Arnold *et al.* report the first *iterative random* mutagenesis and thus realize the evolutionary concept (*PNAS* **1993**, *90*, 217)

Part I — An Introduction into Methods and Concepts

Flow diagram for a directed evolution process

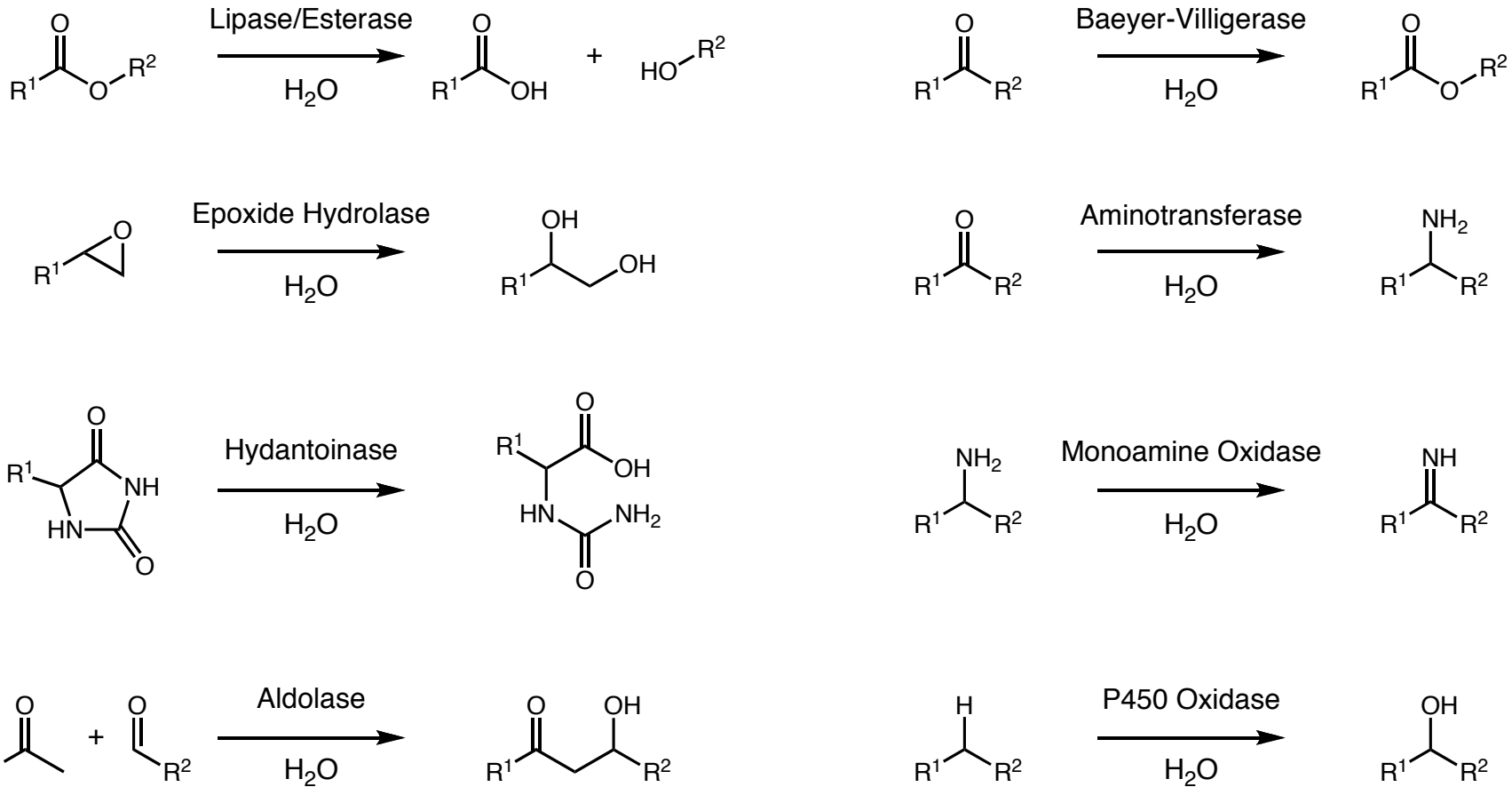


Reetz, M. T. in *Advances in Catalysis, Vol. 49* (Eds.: Knüpffer, H.; Gates, B. C.), Elsevier, San Diego, **2006**, 1.

Starting with the Wild-type Enzyme: Limitation by Reaction Types

A selection of most frequent applications of enzymes

■ Typical enzyme-catalyzed transformations

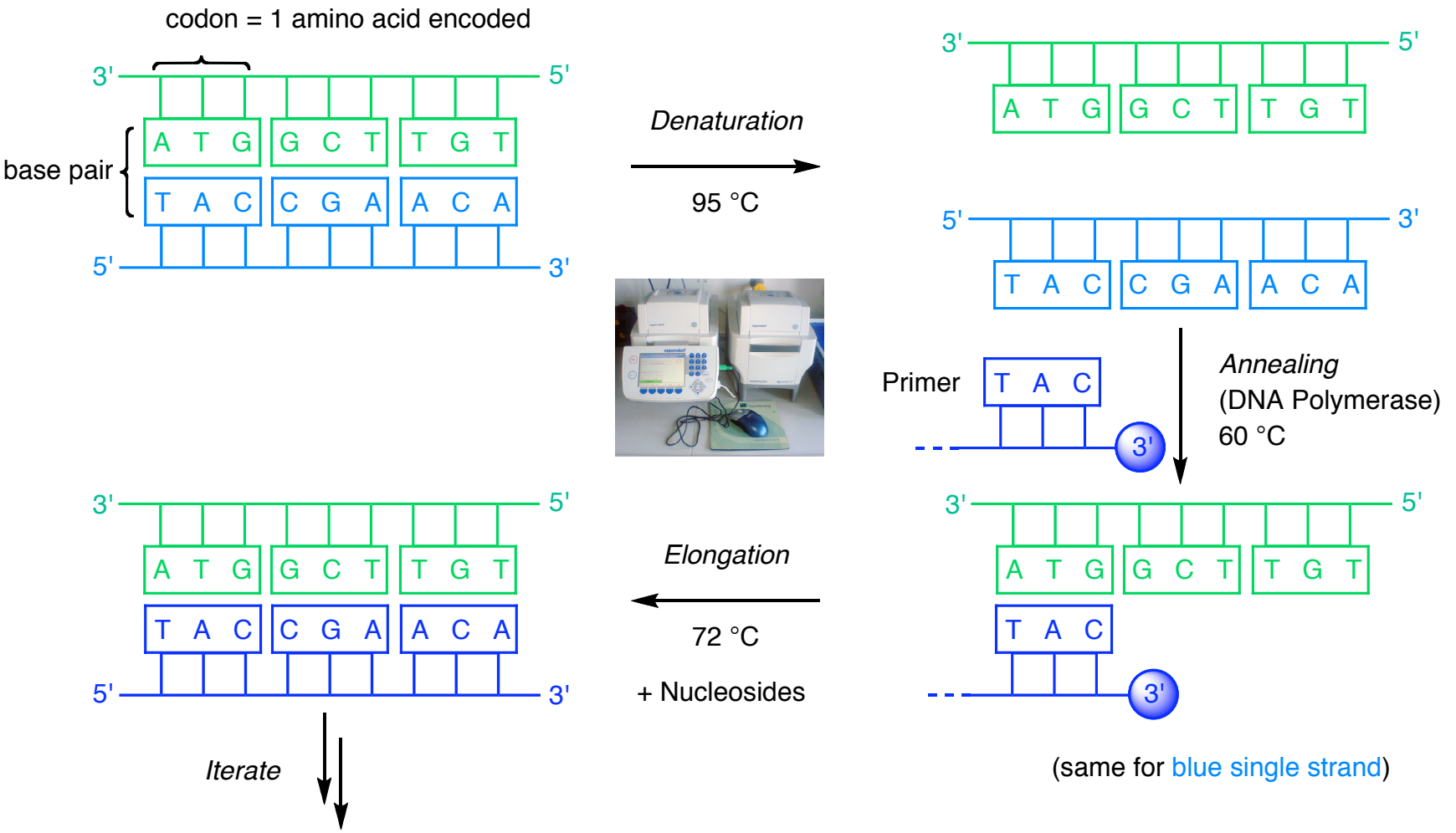


Asymmetric Organic Synthesis with Enzymes (Eds.: Gotor, V.; Ignacio A.; Garcia-Urdiales, E.), Wiley-VCH, Weinheim, **2008**.

DNA Replication: Polymerase Chain Reaction (PCR)

Fully automated routine technique

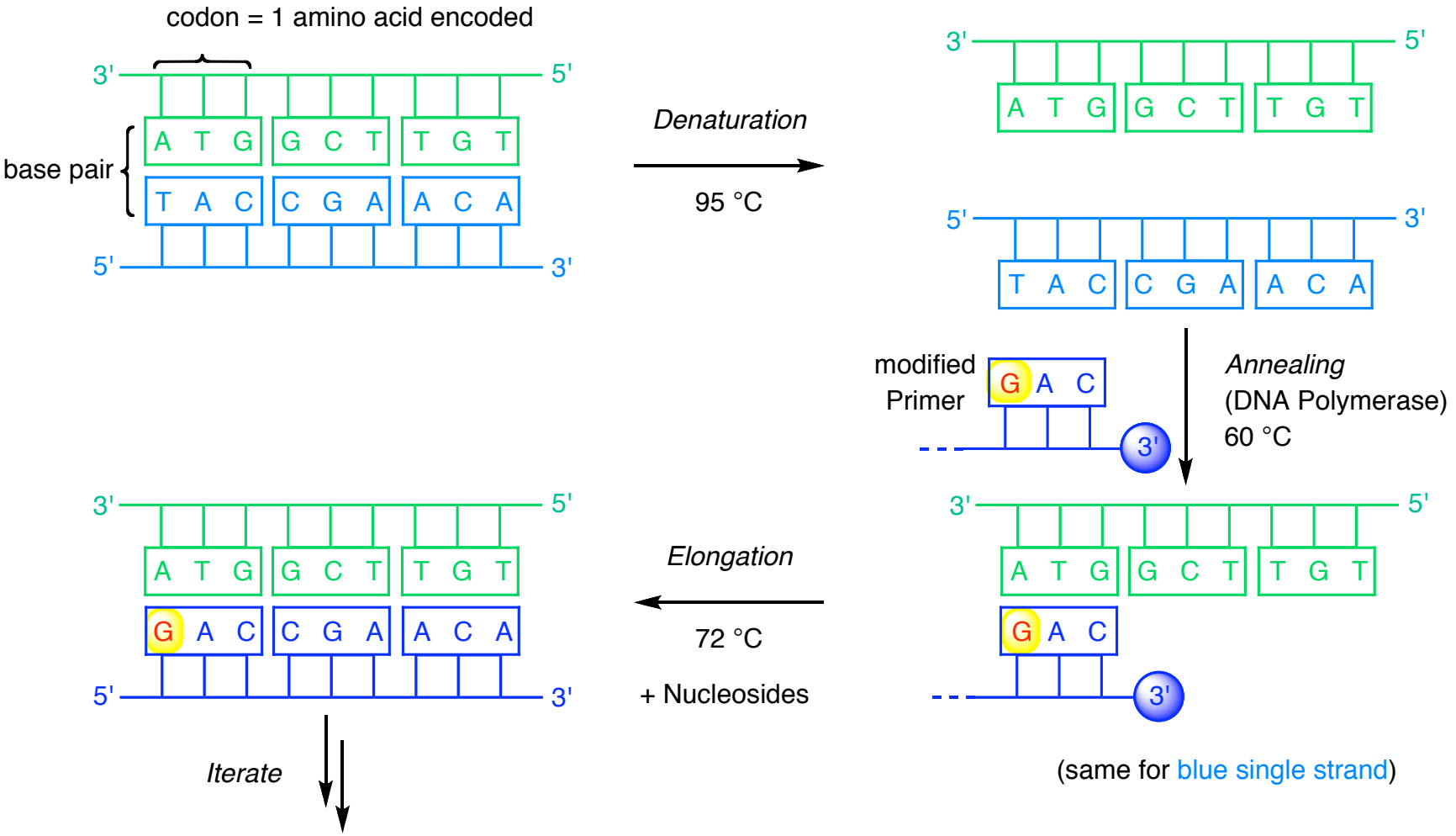
A brief description of the basics



Site-directed Mutagenesis

Non-recombinative methods

■ Modified primers facilitate selective introduction of a single-point mutation



Non-recombinative Mutagenesis Methods

More diversity

Saturation mutagenesis: Randomize a selected position

- Several mutated primers encoding all amino acids are used in a PCR-like process
- Can be useful to optimize a selected position previously identifed as a 'hot spot'

(Combinatorial) Cassette mutagenesis: Randomize a selected region

- Several mutated oligonucleotide sequences used to mutate a region previously identified
- 'hot region' usually close to binding site

Non-recombinative Mutagenesis Methods

High-througput methods

ep-PCR ('error-prone'-PCR): A 'sloppy' PCR variant

- Changing the experimental parameters (increased $MgCl_2$ concentration or addition of $MnCl_2$) leads to the incorportion of 2-3 'wrong' bases per replicated DNA strand
- Takes advantage of the complete sequence space in a fully statistical process
- Useful if no structural data available; can lead to the identification of hot spots

Bacterial mutator strains: Using artificially enhanced natural mutation during replication

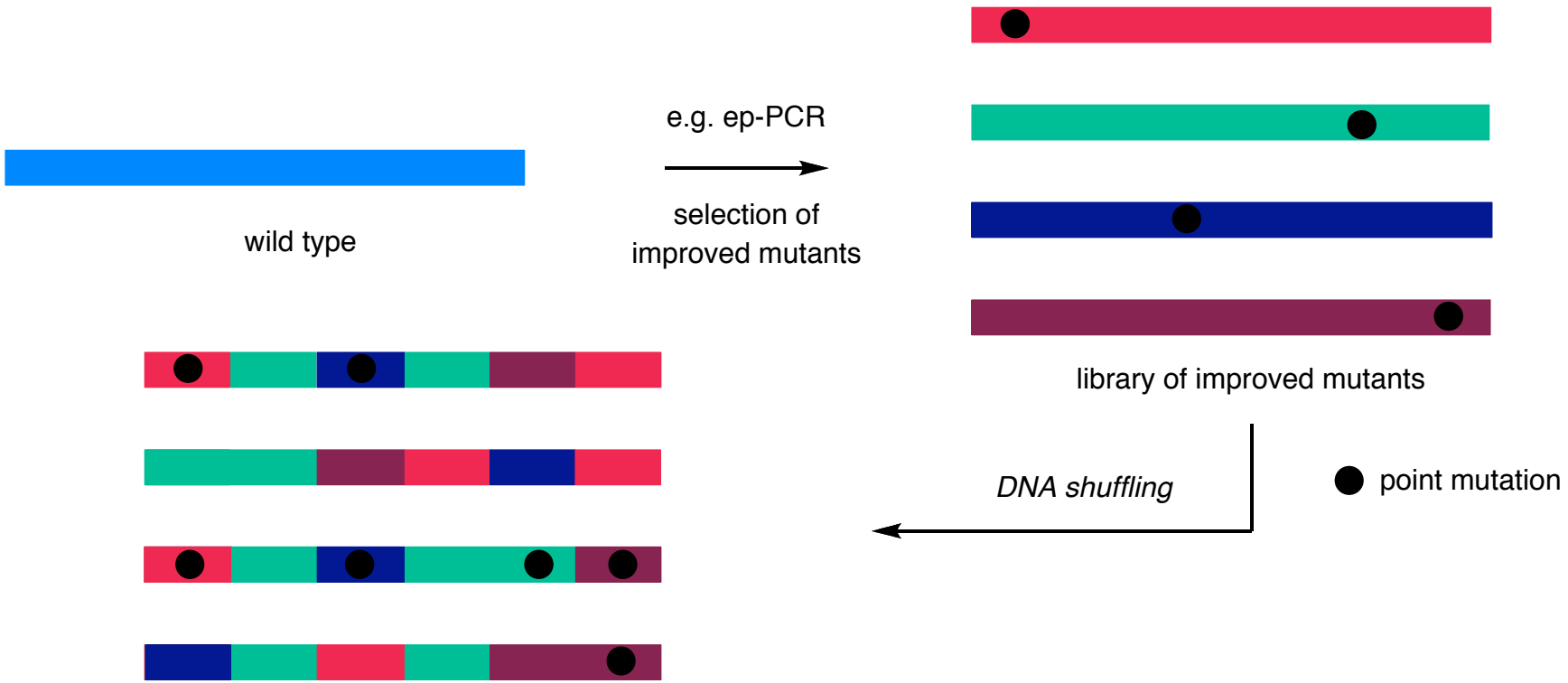
- Natural mutation rate in *E. coli* ~0.0025/1000 base pairs in 30 generations
- Caused by deffects in repair mechanisms
- Commercially available engineered bacterial strain XL1-Red causes 0.5/1000 base pair mutations

Recombinative Mutagenesis Methods

Rapid diversity creation

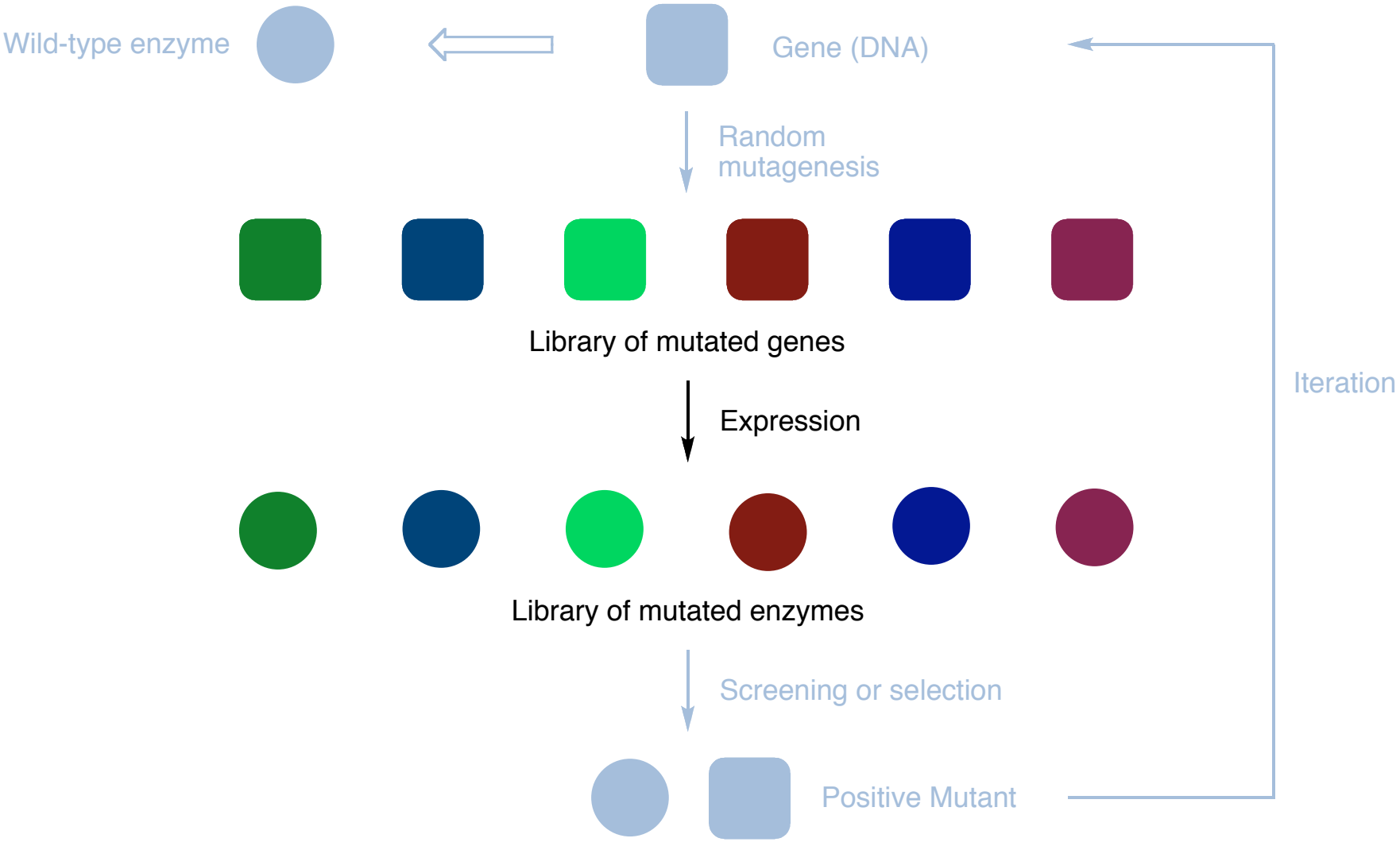
DNA shuffling: Cut & paste

- PCR-like recombination of small DNA fragments upon digestion to smaller oligomer units
- Recombinative method secures high mutation rate
- Particularly useful to amplify the desired properties of several mutated DNA strains in a second round



Part I — An Introduction into Methods and Concepts

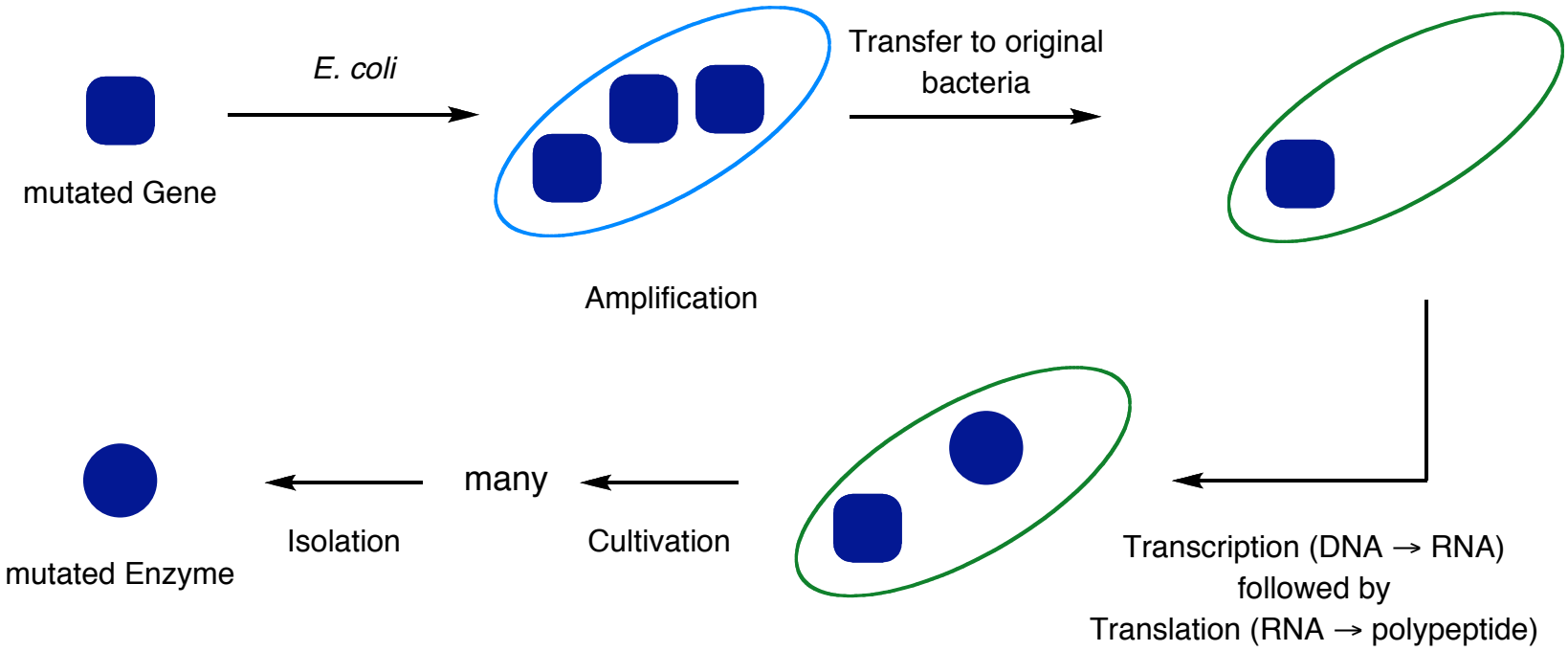
Flow diagram for a directed evolution process



Reetz, M. T. in *Advances in Catalysis, Vol. 49* (Eds.: Knüpffer, H.; Gates, B. C.), Elsevier, San Diego, **2006**, 1.

Gene Expression

- Mutated genes are introduced into bacterial hosts (*E. coli*) for amplification, transformed to the original bacteria which are cultivated



- (Automated) Picking of monoclonal colonies followed by testing for activity after isolation of the isolated enzyme

- Active mutants can (but do not need to) be subjected to sequencing

Reetz, M. T. in *Advances in Catalysis, Vol. 49* (Eds.: Knüpffer, H.; Gates, B. C.), Elsevier, San Diego, **2006**, 1.

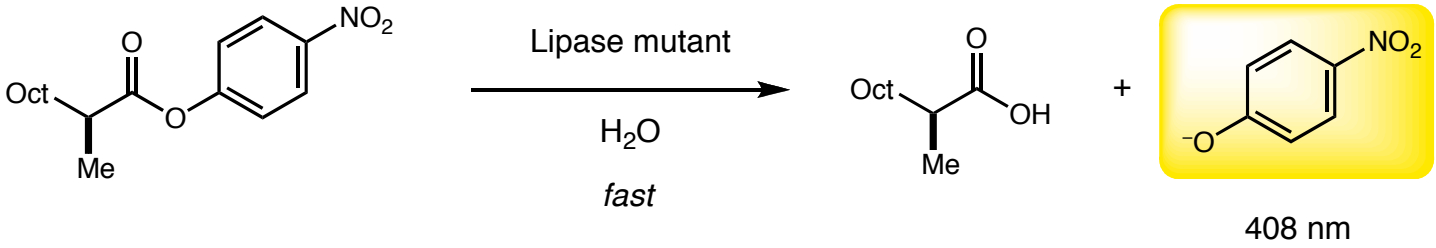
Screening vs. Selection

Rapid identification of active mutants

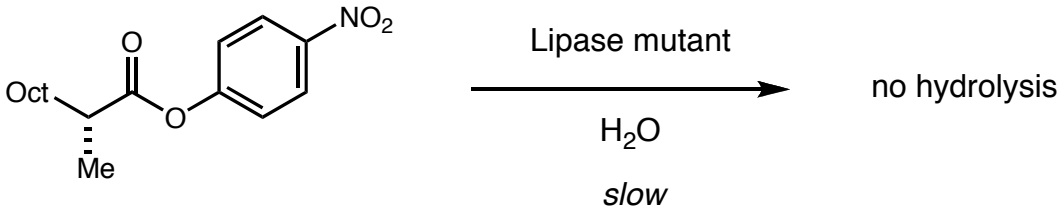
■ Screening: In vitro evaluation of all mutants

- 1st law of directed evolution: "You get what you screen for"
- preferably using high-throughput assays on 96- or 384-microtiter plates (>1000 mutants per day)
- e.g., UV/Vis-based time-resolved enantioselectivity/activity assay for a lipase mutant

in well A



in well B



- other methodes include: pH, fluorescence quenching, reporter enzyme systems, MS, NMR, IR, GC, HPLC

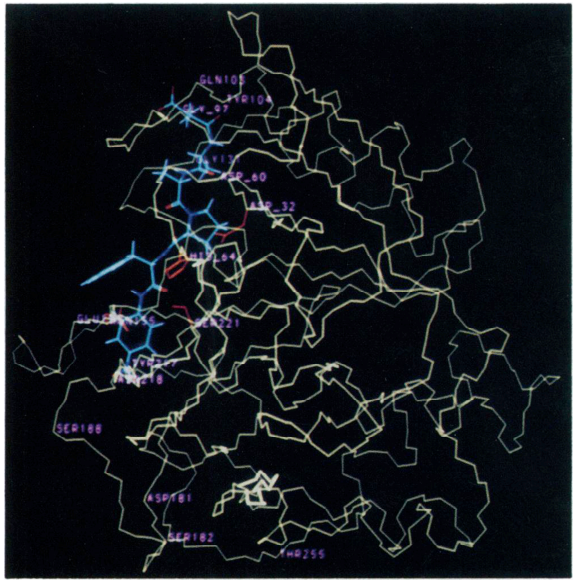
■ Selection: In vivo evaluation of all mutants, only active mutants are observed in assay

Reetz, M. T. in *Advances in Catalysis, Vol. 49* (Eds.: Knüpffer, H.; Gates, B. C.), Elsevier, San Diego, **2006**, 1.
Reymond, J.-L. *et al. Chem. Commun.* **2009**, advance view Oct. 17, 2008 (DOI: 10.1039/b813732c).

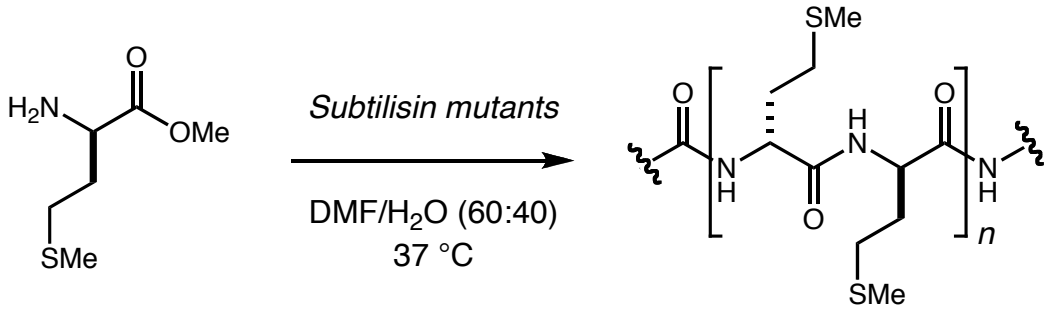
Part II — Selected Applications of Directed Enzyme Evolution

From solvent and temperature stability to enantioselective catalysis

■ Early examples: Arnold's original report on the improvement of solvent stability



Subtilisin E mutant PC 3



Relative activity: PC 3 mutant/wild type = 256/1

- PC 3 mutant contains 10 point mutations
- all beneficial mutations relatively close to binding site

■ Applied approach: Random followed by site-directed mutagenesis

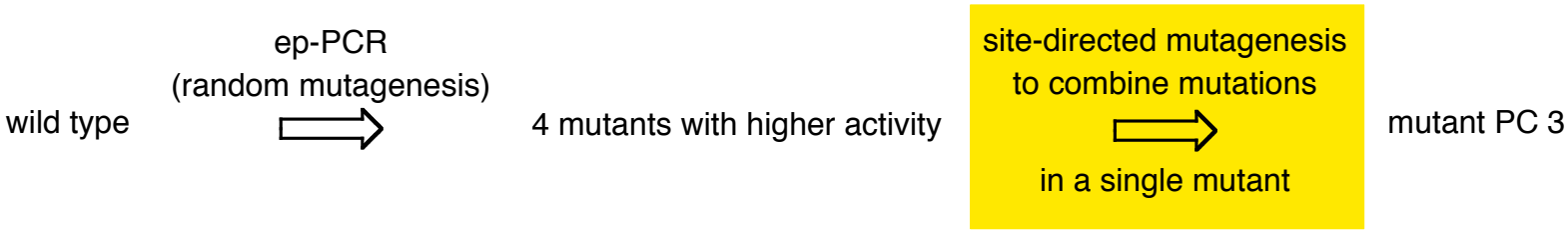
wild type → ep-PCR (random mutagenesis) ⇒ 4 mutants with higher activity → site-directed mutagenesis to combine mutations in a single mutant ⇒ mutant PC 3

Arnold, F. H. *et al. Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5618.

Arnold's Initial Approach to Directed Evolution

Engineering solvent stability

- Applied approach: random followed by site-directed mutagenesis



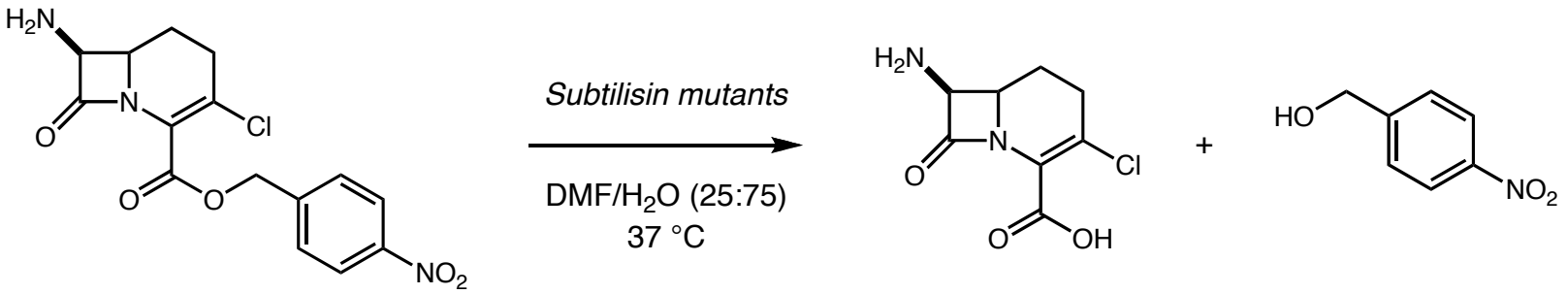
- Second step is less efficient because it does not take advantage of the mutants obtained in the initial mutation cycle, rather follows a manual procedure

Arnold, F. H. *et al. Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5618.

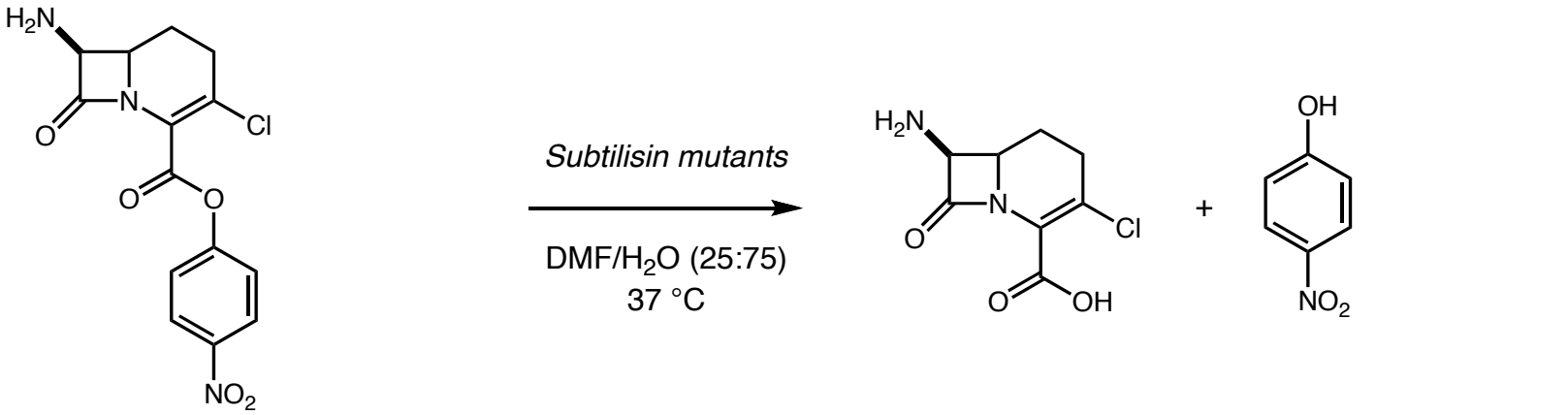
An Improved Approach Using DNA Shuffling

Engineering the hydrolytic activity of a lipase

- *para*-Nitrobenzyl esterase: A problem from process research



- Model reaction suitable for high-throughput screening



Arnold, F. H. *et al. Proc. Natl. Acad. Sci. USA* **1997**, *94*, 7997.

Arnold, F. H. *et al. J. Mol. Biol.* **1997**, *272*, 336.

DNA Shuffling: Stemmer, W. P. C. *Nature* **1994**, *370*, 389.

An Improved Approach Using DNA Shuffling

Engineering the hydrolytic activity of a lipase

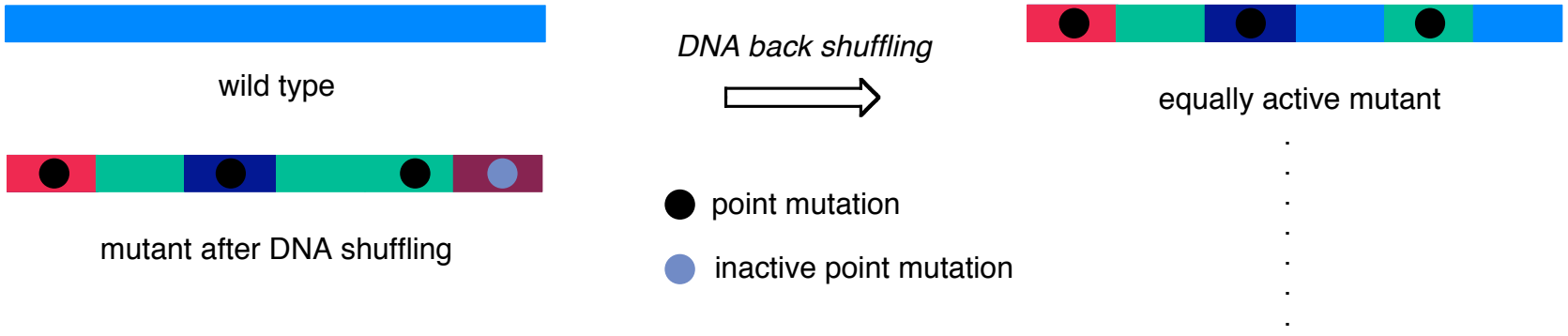
■ Recombinative DNA shuffling with active mutants leads to new mutants showing additive effects

wild type → ep-PCR (random mutagenesis), 4 sequential rounds → 4 mutants with higher activity → DNA shuffling, 2 sequential rounds → active mutant

Activity increase up to a factor of **~20**

Activity increase up to a factor of **~6**
(or 120 relative to wild type)

■ Back shuffling versus wild-type eliminates inactive point mutations



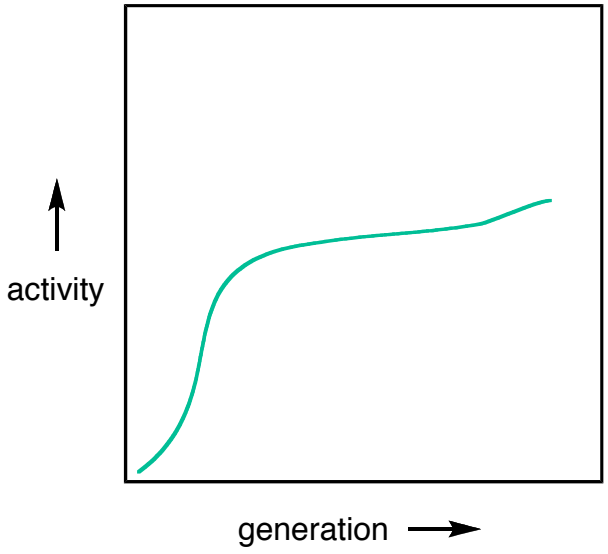
Arnold, F. H. *et al. Proc. Natl. Acad. Sci. USA* **1997**, *94*, 7997.

An Improved Approach Using DNA Shuffling

Engineering the hydrolytic activity of a lipase

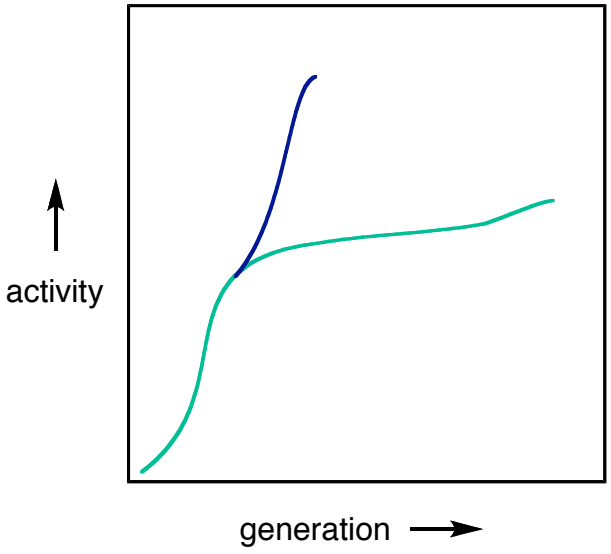
■ Recombinative methods can accelerate directed evolution

• typical acitivity curve using non-recombinative methods



random mutagenesis (ep-PCR)

• typical acitivity curve using both methods



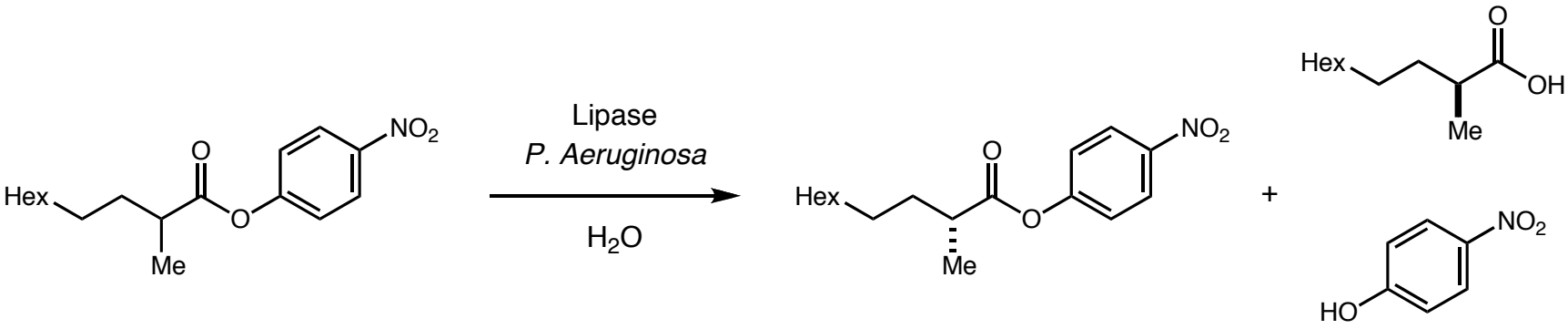
random mutagenesis (ep-PCR)

DNA shuffling

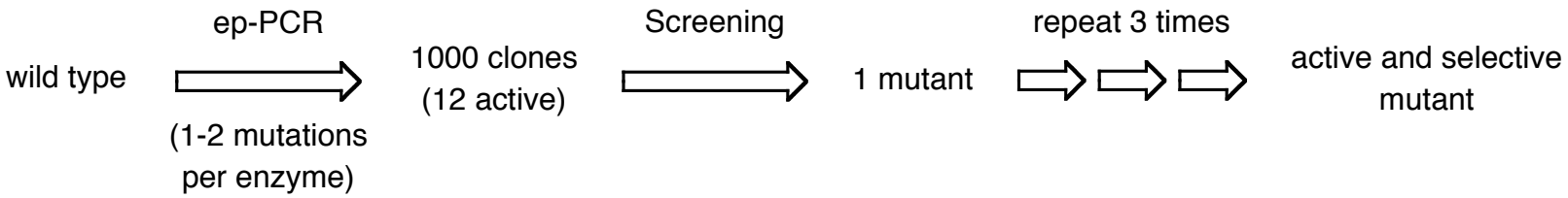
Directed Evolution of Enantioselective Enzymes

Introduction of the concept

■ Hydrolytic kinetic resolution using a lipase



■ Applied concept: Iterative random mutagenesis based on ep-PCR

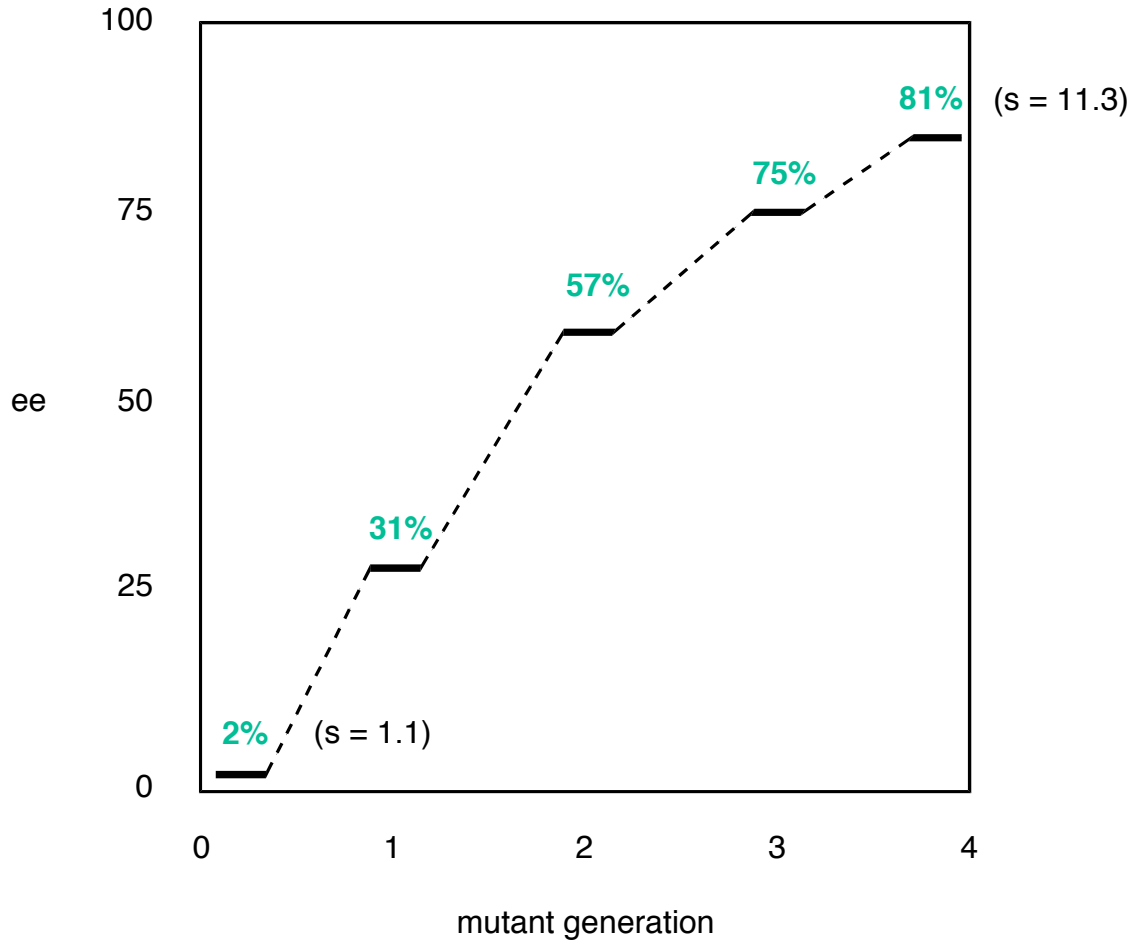


Reetz, M. T. & Jaeger, K.-E. *et al. Angew. Chem. Int. Ed.* **1997**, *36*, 2830.

Directed Evolution of Enantioselective Enzymes

Introduction of the concept

- From almost unselective wild types to moderate selectivity

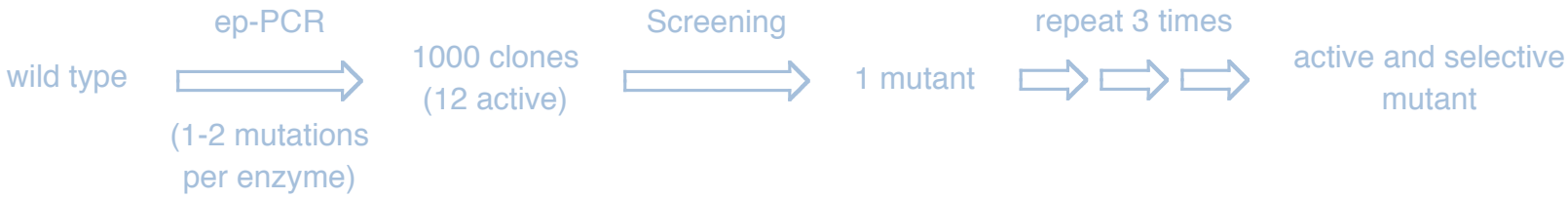


Reetz, M. T. & Jaeger, K.-E. *et al. Angew. Chem. Int. Ed.* **1997**, *36*, 2830.

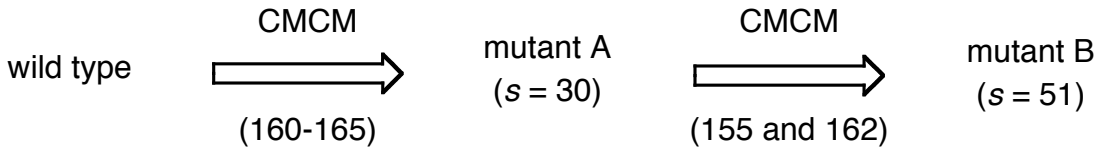
Enantioselective Enzymes: A Rational Approach

Hot spot identifcation

- Selective mutants showed frequent mutations at two amino acid positions 155 and 162



- Combinatorial cassette mutagenesis at one of the hot regions close to binding site (160-165)



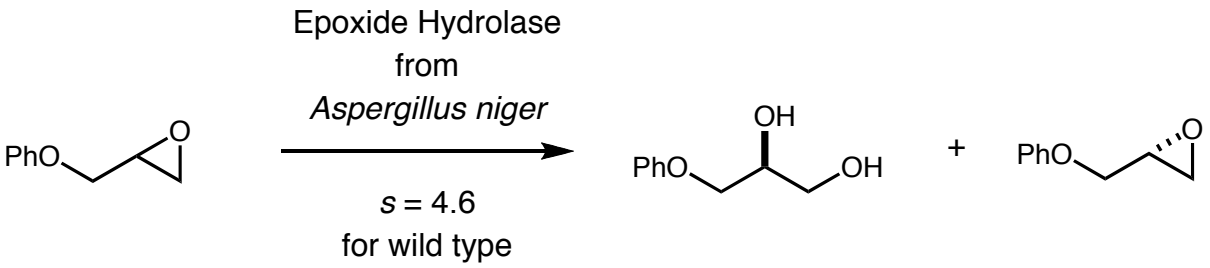
- Rational selection of hot regions based on previously gained information and structural insight

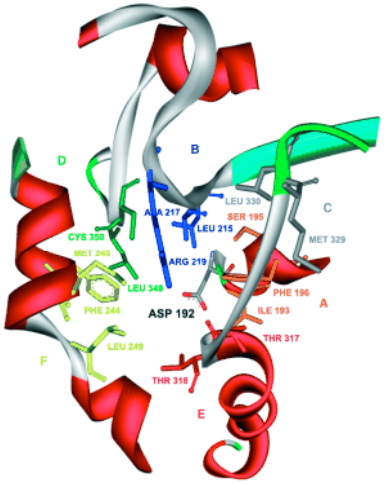
Reetz, M. T. & Jaeger, K.-E. *et al. Angew. Chem. Int. Ed.* **2001**, *40*, 3589.

Improved Efficiency by Focused Library Development

Reetz' CASTing approach

■ Hydrolytic kinetic resolution of epoxides: Epoxide hydrolase from *Aspergillus niger*

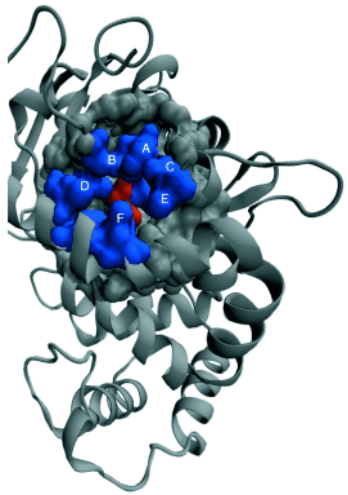




■ '**C**ombinatorial **a**ctive **s**ite saturation **t**est'

structural information ⇒ select promising positions close to binding pocket ⇒ conduct saturation mutgenesis simultaneously at two positions

⇒ select best mutant and repeat saturation mutagenesis for two other previously identified positions ⇒⇒ iterate last step



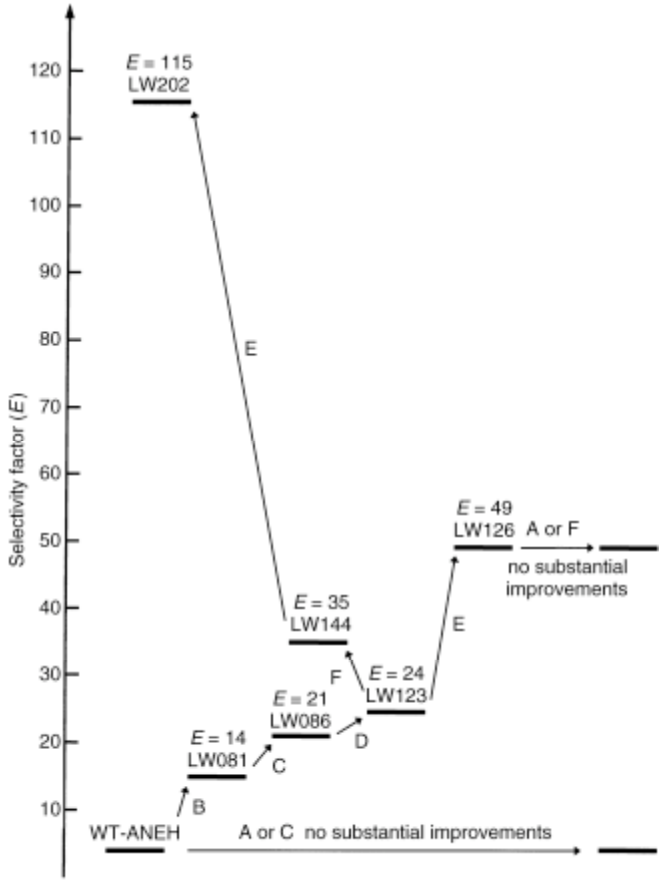
- requires structural information (X-ray) as a starting point
- reduces number of mutant in screen
- considers cooperative effects because two positions are randomized at the same time

Reetz, M. T. *et al. Angew. Chem. Int. Ed.* **2006**, *45*, 1236.

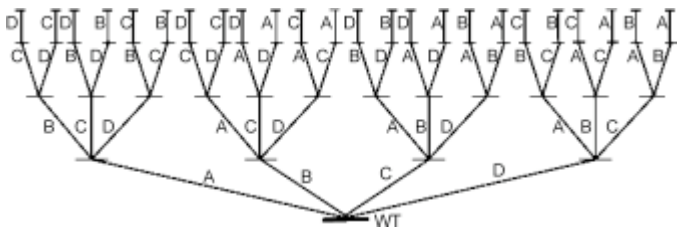
Improved Efficiency by Focused Library Development

Reetz' CASTing approach

■ Additivity of positive mutations shows only minor depenency on the order



- Five iterations of CASTing furnish a catalyst with *s* = 115



- Result of sequence A→B→C→D→E→F comparable to result of sequence A→B→C→D→F→E*
- Rational input dramatically reduces the required number of surveyed mutants

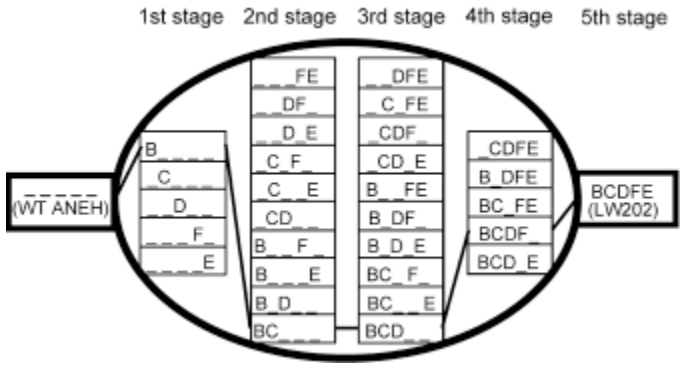
* A-F are different pairs of amino acids selected for randomization

Reetz, M. T. *et al. Angew. Chem. Int. Ed.* **2006**, *45*, 1236.

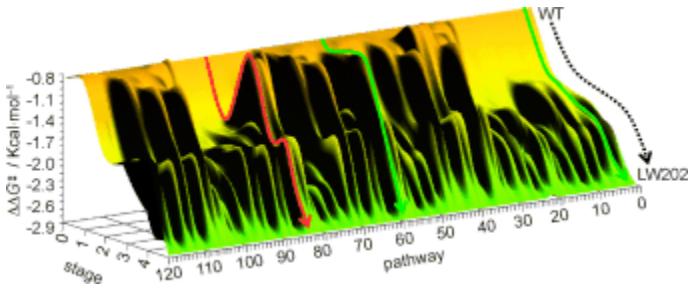
Improved Efficiency by Focused Library Development

Reetz' CASTing approach

■ Experimental and theoretical data reveal many ways to make a 'good' enzyme



- For 5 iterations 5! = 120 pathways to obtain desired mutant are possible

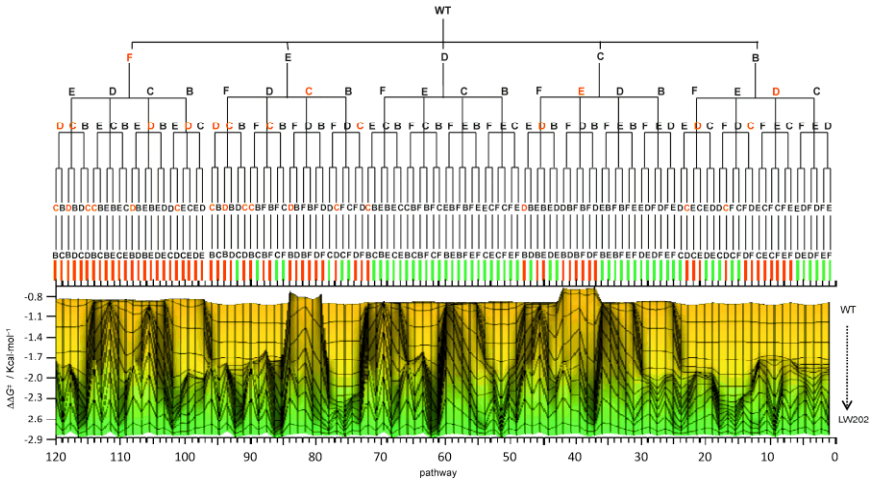


- Analysis of all 120 pathways reveals that many follow an energetically favorable pathway

- $s \sim \Delta\Delta G^{\ddagger}_{(R-S)}$ ⇨ every path having negative $\Delta\Delta G^{\ddagger}$ is favorable

favorable

unfavorable



- 55 of 120 pathways (46%) are favorable

⇨ high probability to find an active mutant

⇨ if not, one single step backwards is implied

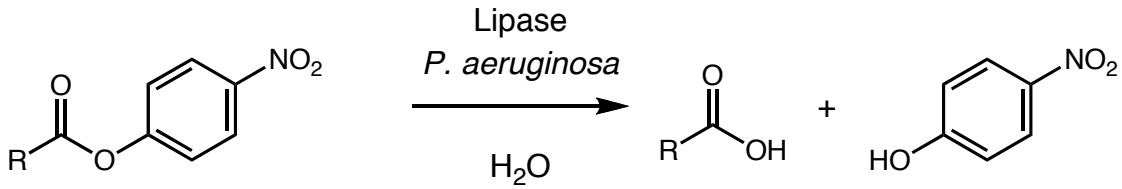
Reetz, M. T. *et al. ChemBioChem.* **2008**, *45*, 1236.

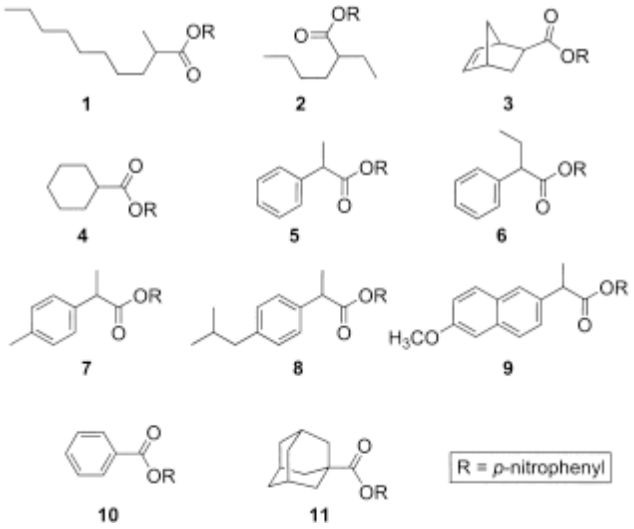
CASTing for... Substrate Tolerance

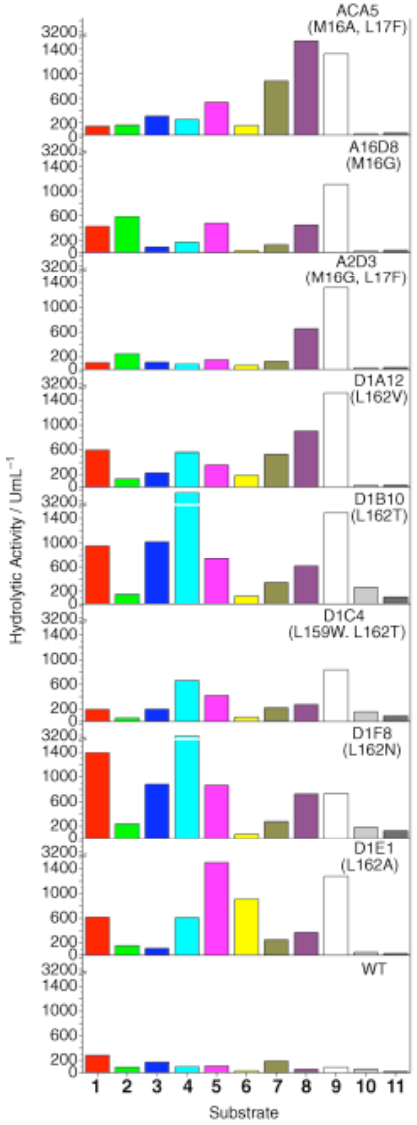
Rational approach to directed evolution

Expanding substrate scope of lipase-catalyzed ester hydrolysis

- Positions selected for iterative saturation mutagenesis selected based on X-ray data







Reetz, M. T. *et al. Angew. Chem. Int. Ed.* **2005**, *44*, 4192.

CASTing for... Thermostability

Rational approach to directed evolution

- X-ray struture data provide B values that quantify the flexibility of an atom ("smeering")

amino acid positions having highest B values are selected for saturation mutagenesis ⇒ conduct saturation mutagenesis simultaneously at two of the selected positions ⇒ screen for mutant encoding the enzyme with highest temperature stability

⇒⇒ iterate last two steps by randomizing other selected positions

Result: Temperature range for the hydrolytic activity of a Lipase from *B. subtilis* can be extended from 50 °C to 80 °C

Reetz, M. T. *et al. Angew. Chem. Int. Ed.* **2006**, *45*, 7745.

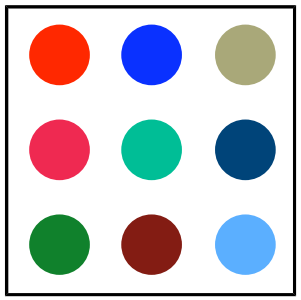
The Number Problem in Saturation Mutagenesis

Consequences of statistical considerations

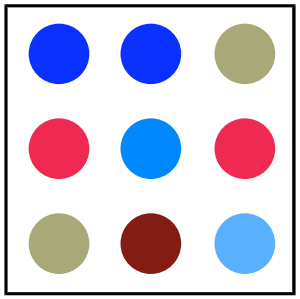
Required 'oversampling' in activity screens

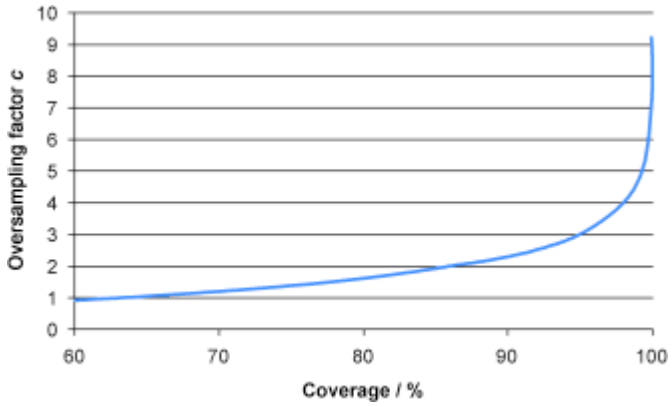
- In order to secure a high coverage of a generated library the actual number of enzyme variants to be screened is significantly higher than the number of mutants

ideal plate



real plate





Reetz, M. T. *et al. ChemBioChem* **2008**, *9*, 1797.

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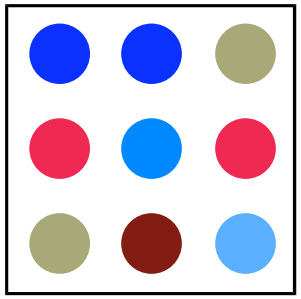
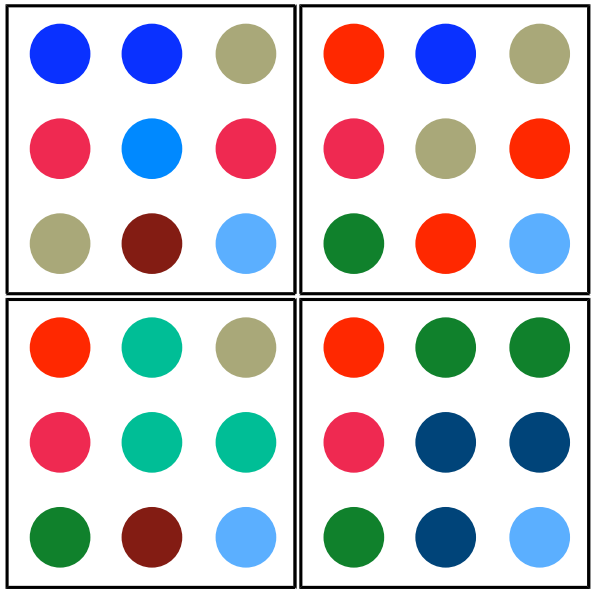


required screen size



Reetz, M. T. *et al. ChemBioChem* **2008**, *9*, 1797.

Reducing the Amino Acid Space

Encoding only 12 amino acids greatly reduces the screening effort

- A streamlined saturation mutagenesis approach based on encoding less amino acids

Surveying 20 AA		**Surveying 12 AA**	
No. of AA to be randomized	*No. of mutants to be screened at 95% library coverage*	*No. of AA to be randomized*	*No. of mutants to be screened at 95% library coverage*
1	94	1	34
2	3,066	2	430
3	98,163	3	5,175
4	3,141,251	4	62,118
5	100,520,093	5	745,433

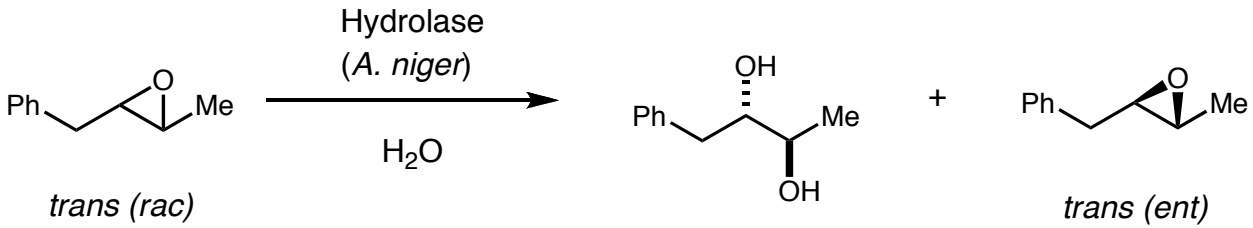
- Using a 'balanced mix of polar, nonpolar, aromatic, aliphatic, negatively, and positively charged amino acids while excluding most cases of structurally similar amino acids'

Reetz, M. T. *et al. ChemBioChem* **2008**, *9*, 1797.

Reducing the Amino Acid Space

Application in hydrolytic kinetic resolution

- High quality of obtained library: From a non-binding substrate to an enantioselective process



trans (rac) *trans (ent)*

- Saturation mutagenesis of 3 positions based on 20 amino acids (*15% coverage*)

wild type (does not accept substrate) → CASTing (sat. Mut.) → 5000 mutants → 38 active → best mutant: TOF = 17, *s* = 101

- Saturation mutagenesis of 3 positions based on 12 amino acids (*95% coverage*)

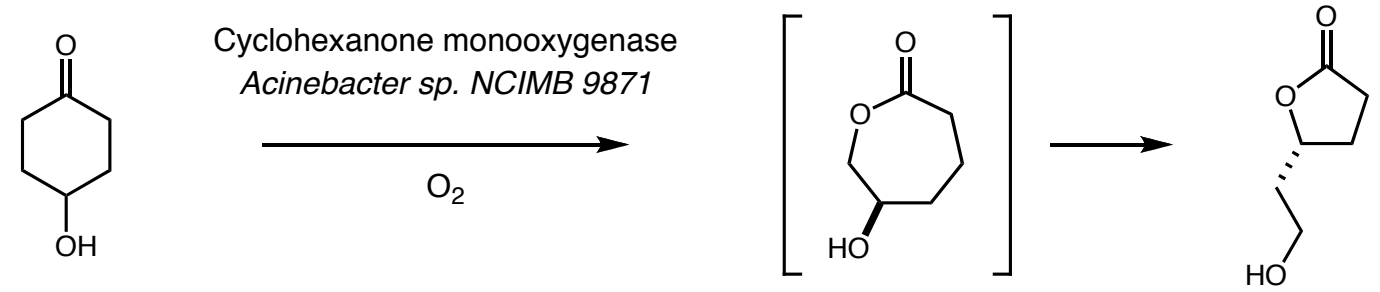
wild type (does not accept substrate) → CASTing (sat. Mut.) → 5000 mutants → 511 active → best mutant: TOF = 55, *s* > 200

Reetz, M. T. *et al. ChemBioChem* **2008**, *9*, 1797.

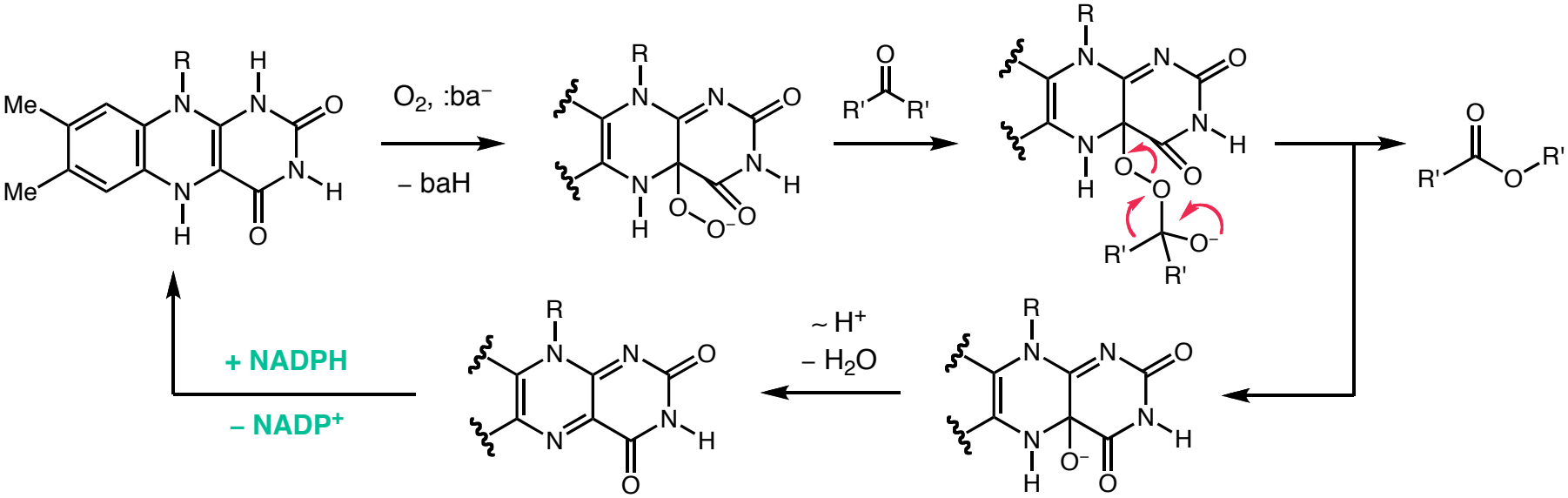
Enantioselective Baeyer-Villiger Oxidation

An example of whole cell catalysis

- Desymmetrization of cyclic ketones by engineered enzymes



- Whole cells contain additional co-factor NADPH responsible for flavin co-factor regeneration

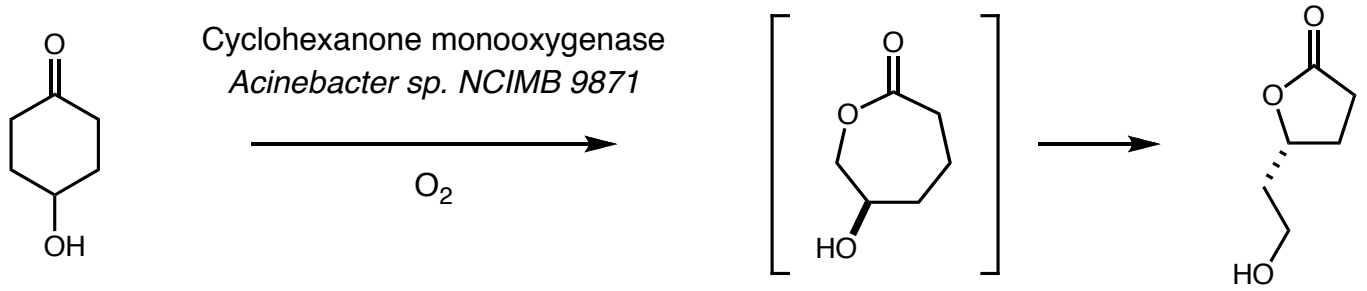


Reetz, M. T. & Kayser, M. M. *et al. Angew. Chem. Int. Ed.* **2004**, *43*, 4075.

Enantioselective Baeyer-Villiger Oxidation

An example of whole cell catalysis

■ Desymmetrization of cyclic ketones by engineered enzymes



■ Strategy: Iterative random mutagenesis by ep-PCR

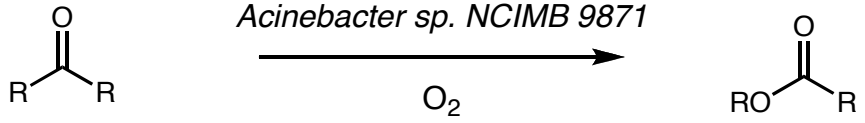
wild type (9% ee) ⇒ 1st round of ep-PCR ⇒ 10,000 mutants ⇒ 24 *R*-selective & 12 *S*-selective ⇒ selected mutant shows 1 single point mutation and 40% *ee* (*R*) ⇒ 2nd round of ep-PCR ⇒ best mutant shows 4 single point mutations and 90% ee (*R*)

Reetz, M. T. & Kayser, M. M. *et al. Angew. Chem. Int. Ed.* **2004**, *43*, 4075.

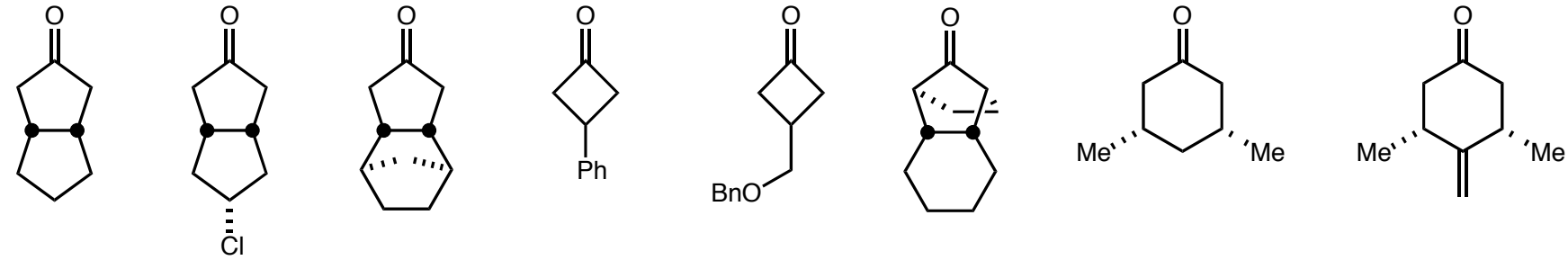
Enantioselective Baeyer-Villiger Oxidation

An example of whole cell catalysis

- Substrate promiscuity by generating a mutant library



- Different mutants show different activity and selectivity for a range different substrates



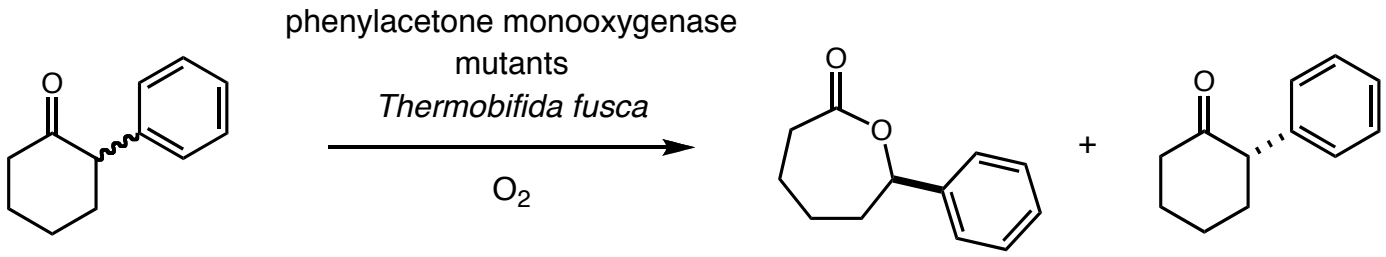
1-K2-F5	1-K2-F5	1-C2-B7	1-K6-G2	1-K2-F5	1-K6-G2	1-K2-F5 1-H7-F4	1-K2-F5 1-H7-F4
94% ee	99% ee	92% ee	96% ee	83% ee	94% ee	>99% ee	94% ee

Mihovilovic, M. D. & Reetz, M. T. *et al. Org. Lett.* **2006**, *8*, 1221.

Enantioselective Baeyer-Villiger Oxidation

Restricting the amino acid space

A rational approach to generate an active catalyst



Saturation mutagenesis using a dramatically restricted amino acid space

wild type
(*s* = 1.2 for *S*)

⇒

selection of amino acids being used for saturation mutagenesis based on sequence comparison with related enzyme

⇒

simultaneous saturation mutagenesis at **4 vicinal positions** using reduced set of encoded amino acids

⇒

2587 mutants
(@ 95% coverage)
best mutant:
s = 48 (*R* selective)

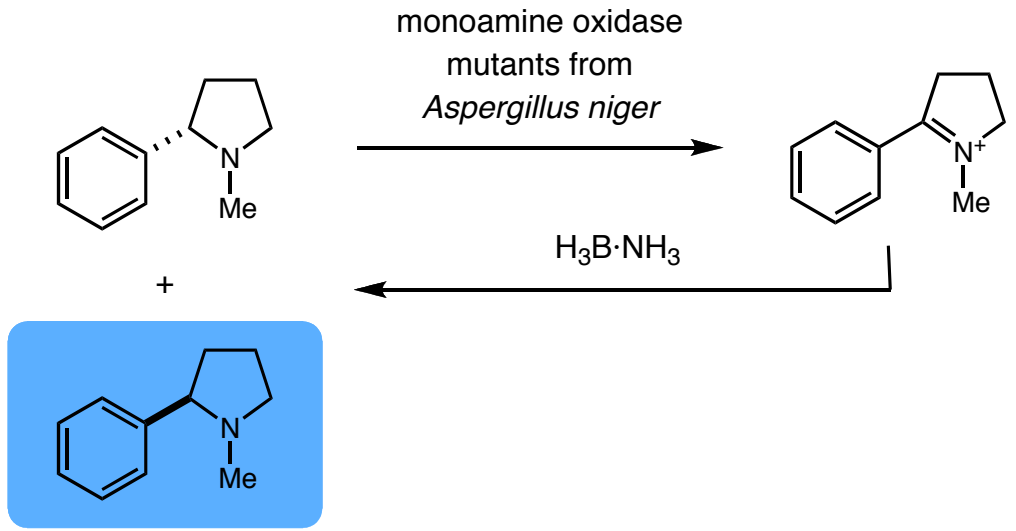
- > 3 mio. mutants needed if all 20 amino acids would have been considered
- no X-ray data avaliable → relevant positions and amino acids identified by comparison with sequence of *Acinebacter* active site that is known to be a Baeyer-Villigerase

Reetz, M. T. *et al. Chem. Commun.* **2008**, 5499.

Amine Oxidases: Deracemizations of Tertiary Amines

Application of mutator strains as an alternative to ep-PCR

■ Directed evolution coupled in a tandem sequence



■ Strategy: Iterative random mutagenesis using bacterial mutator strains

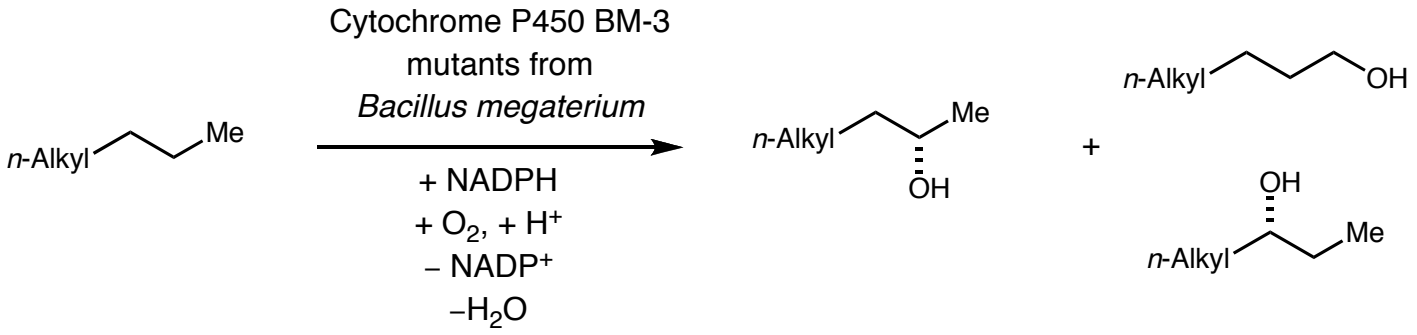
wild type (*low* activity) ⇒ Iterative random mutagenesis using XL1 mutator strains ⇒ screening indictates mutant bearing 5 point mutations as most active ⇒ s = 134 (*R* selective)

Turner, N. J. *et al. J. Am. Chem. Soc.* **2006**, *128*, 2224.
see also: Turner, N. J. *et al. Angew. Chem. Int. Ed.* **2002**, *41*, 3177.
see also: Turner, N. J. *et al. Angew. Chem. Int. Ed.* **2003**, *42*, 4807.

Engineered P450 Enzymes: Enantioselective C–H Oxidation

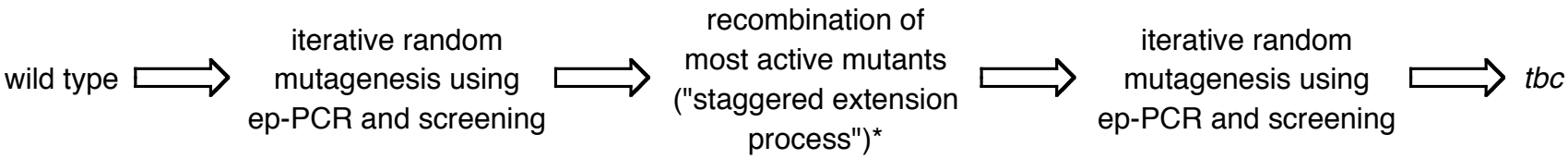
Remarkable regioselectivity for linear alkanes

■ Directed evolution to devolop enantio- and regioselective C–H hydroxylation



- natural substrates: Fatty acids (C-12 to C-18): e.g. myristic acid (54% ω-1, 25% ω-2, 20% ω-3)
- alkanes are as such toxic substrates limiting the activity of enzymes

■ Strategy



* a PCR-like variant of DNA shuffling

Arnold, F. H. *et al. J. Am. Chem. Soc.* **2003**, *125*, 13442.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2003**, *42*, 3299.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2007**, *46*, 8414.

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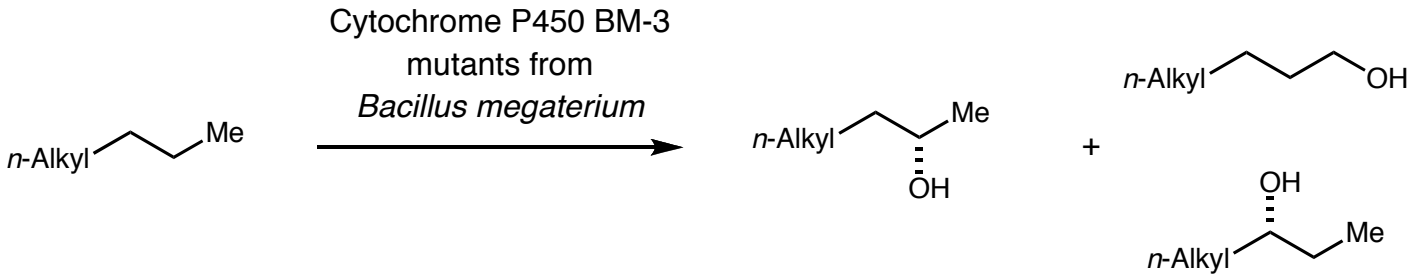

■ Strategy

wild type ⇨ iterative random mutagenesis using ep-PCR and screening ⇨ recombination of most active mutants ("staggered extension process")* ⇨ iterative random mutagenesis using ep-PCR and screening

⇨ best mutant 139-3: **~40-fold** increase in activity (*vs* wild type) ⇨ recombination of most active mutants ("staggered extension process")* ⇨ iterative random mutagenesis using ep-PCR and screening ⇨ *tbc*

* a PCR-like variant of DNA shuffling

Arnold, F. H. *et al. J. Am. Chem. Soc.* **2003**, *125*, 13442.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2003**, *42*, 3299.
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⇨ best mutant 139-3: **~40-fold** increase in activity (*vs* wild type) ⇨ recombination of most active mutants ("staggered extension process")* ⇨ iterative random mutagenesis using ep-PCR and screening ⇨

Site-directed saturation mutagenesis ⇨ recombination of most active mutants by DNA shuffling ⇨ mutant 1-12G shows **~480-fold** increase in activity (*vs* wild type)

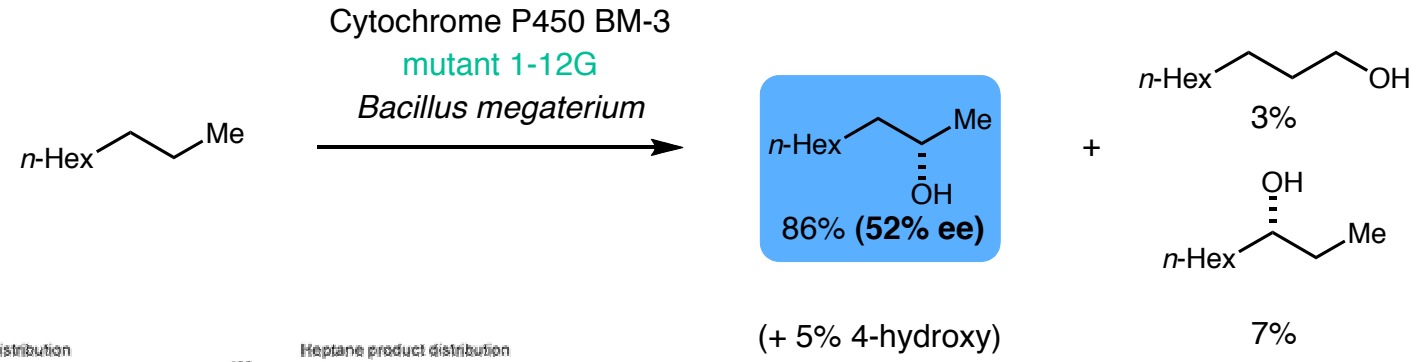
* a PCR-like variant of DNA shuffling

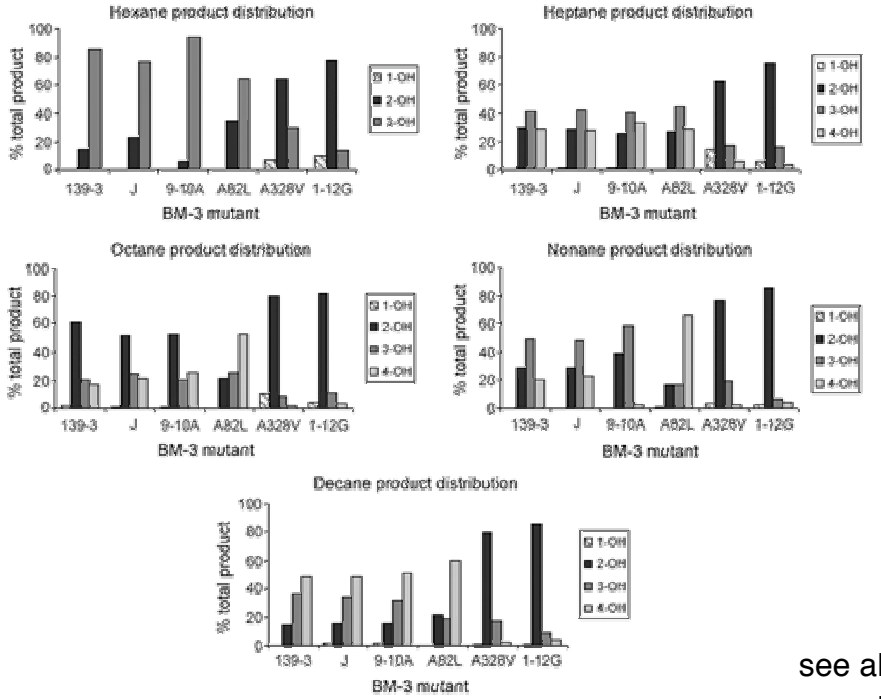
Arnold, F. H. *et al. J. Am. Chem. Soc.* **2003**, *125*, 13442.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2003**, *42*, 3299.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2007**, *46*, 8414.

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Remarkable regioselectivity for linear alkanes

■ Directed evolution to devolop enantio- and regioselective C–H hydroxylation





■ Switch from terminal hydroxylation preference (wild-type) to internal positions

Arnold, F. H. *et al. J. Am. Chem. Soc.* **2003**, *125*, 13442.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2003**, *42*, 3299.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2007**, *46*, 8414.

Engineered P450 Enzymes: Enantioselective C–H Oxidation

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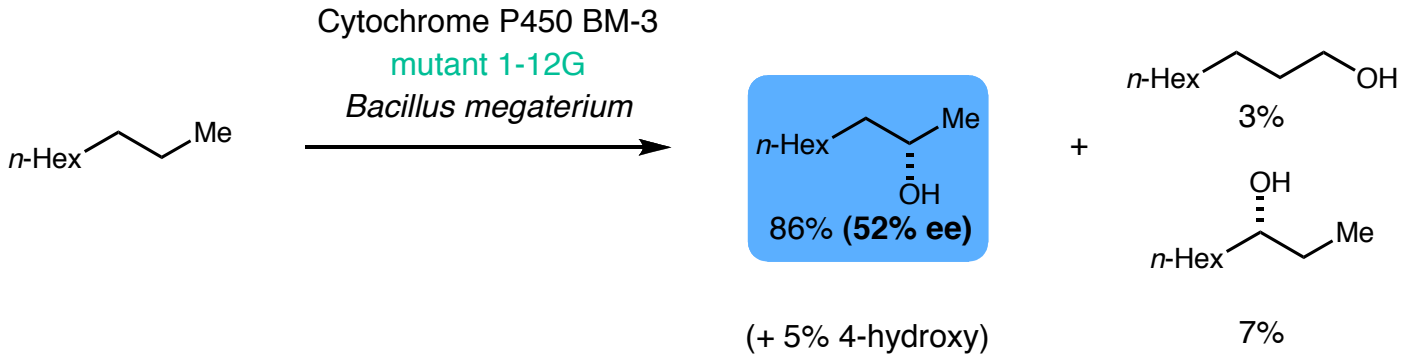


- Several linear alkanes (C-3 to C-9) as well as ethers are accepted
- Activity-based directed evolution, not ee-driven iterative process

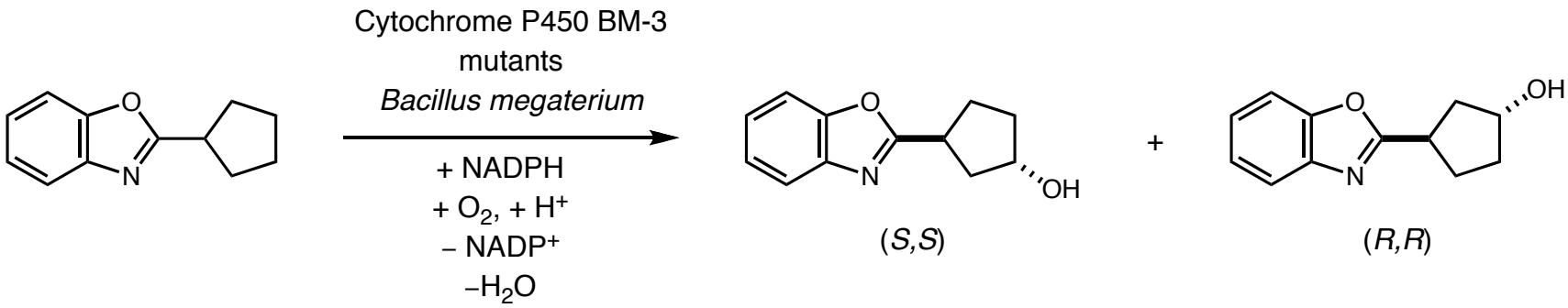
  → screening for enantioselectivity could furnish highly enatioselective enzyme

Arnold, F. H. *et al. J. Am. Chem. Soc.* **2003**, *125*, 13442.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2003**, *42*, 3299.
see also: Arnold, F. H. *et al. Angew. Chem. Int. Ed.* **2007**, *46*, 8414.

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Regio-, diastereo- and enantioselective hydroxylations of cyclopentane derivatives

- Same mutant library facilitates enantioselective hydroxylation of functionalized cycloalkanes



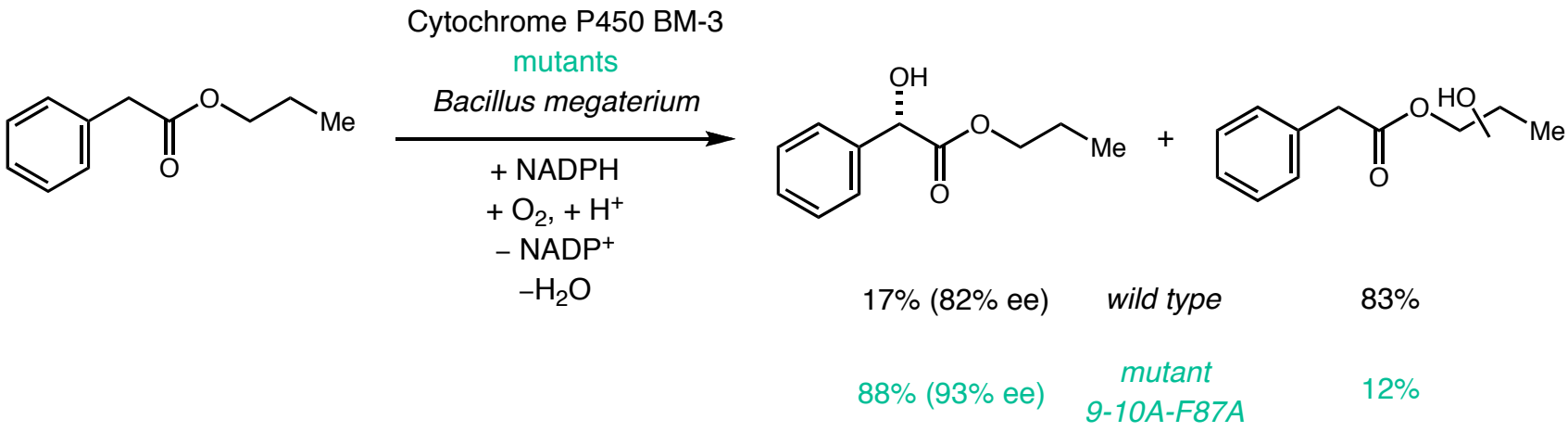
wild type	25% ee (*R,R*), 20% (80% 2-hydroxy), TON 2.6
mutant B	88% ee (*S,S*), 96:4 dr, 90%, TON 215
mutant 1-12G	88% ee (*R,R*), 98:2 dr, 95%, TON 213

de Raadt, A. & Arnold, F. H. *et al. Chem. Commun.* **2005**, 2597.

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Hydroxylation of aryl acetic acids

- Mutant library also facilitates enantioselective hydroxylation of aryl acetic acids

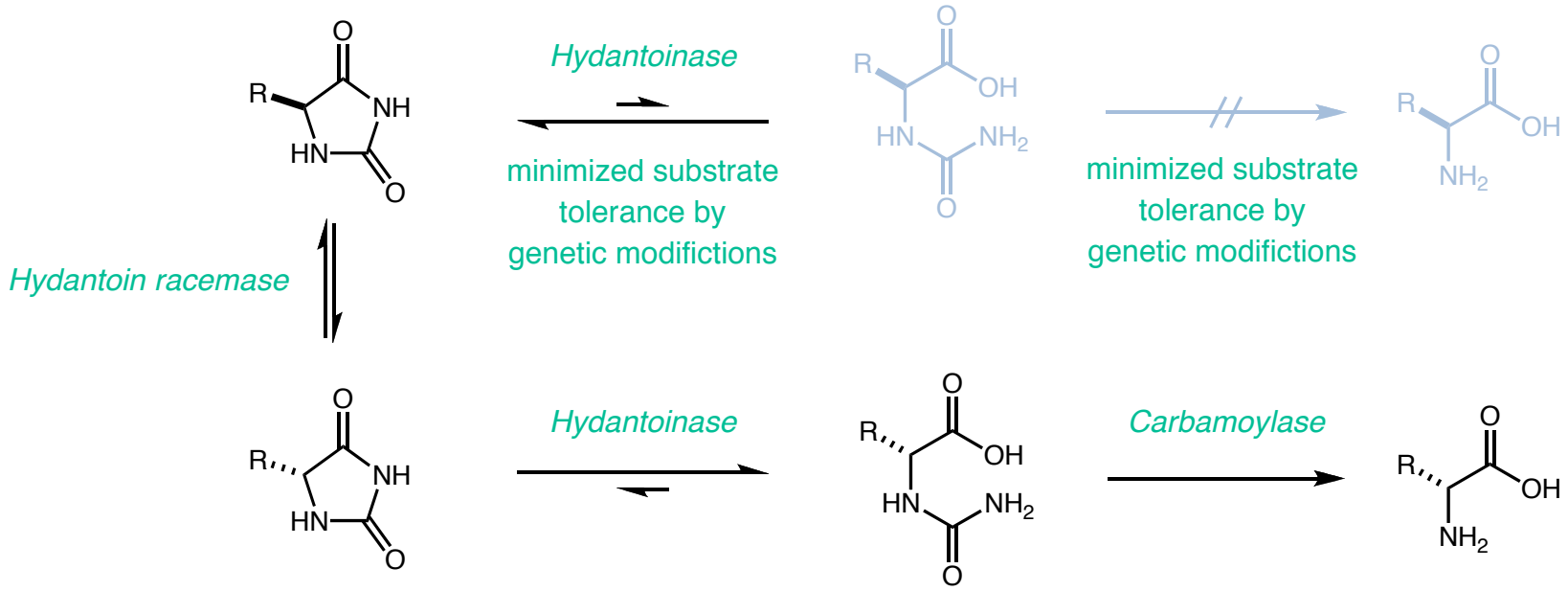


Baeckvall, J. E. & Arnold, F. H. *et al. J. Am. Chem. Soc.* **2006**, *128*, 6058.
for alkene epoxidation, see: Arnold, F. H. *et al. Chem. Eur. J.* **2006**, *12*, 1216.

Degussa Synthesis of Enantiopure D-Amino Acids

An Industrial Scale Application of Engineered Enzymes: 'White Biotechnology'

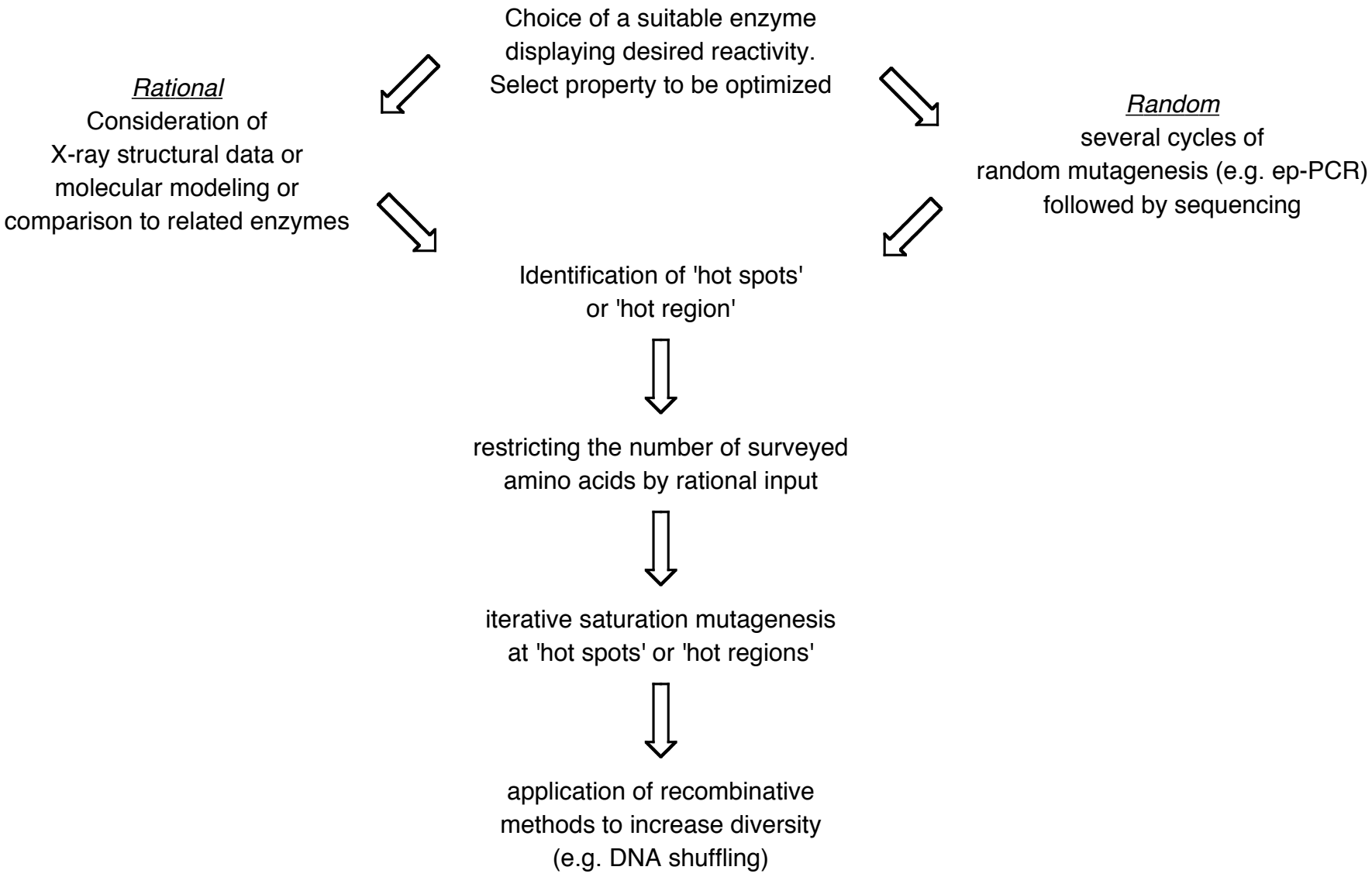
■ Triple enzymatic dynamic kinetic resolution process based on engineered enzymes



- *E. coli* host carries genetically modified hydantoinase and carbamoylase from *Arthrobacter crystallopoietes* DSM20117
- Degussa utilizes white biotechnology to produce a wide range of natural and unnatural AA's
- e.g., D-aminobutyric acid, D-serine, D-methionine, D-tryptophan, D-phenylalanine

May, O. *et al. Org. Proc. Res. Dev.* **2002**, *6*, 452.
May, O. *et al.,* Int. Patent WO 2004/042047 A1.
Trauthwein, G. *et al.,* Ger. Patent DE 102 44 347 A1.

Summary — Streamlining Directed Evolution of Enzymes



GOAL: Minimize number of surveyed mutants (= time, material), maximize the synthetic utility

Scope and Limitations of Directed Evolution of Enzymes

Scope

- Recent years show a considerable extension of **substrate scope** due to more sophisticated approaches
- Obtainable **enantioselectivities** are for most examples highly competitive
- Other important properties like **temperature stability** and **solvent stability** are adjustable
- So far, **typical reactivity** modes of enzymes have been explored, such as hydrolysis, oxidation, reduction

Advantages

- A routinely conducted **tailor-made catalyst production** for each substrate seems within reach
- One substrate — one catalyst; yet, protein space allows to develop **specific catalysts** for each substrate

Limitations

- Number of known synthetically interesting **enzyme wild types** appears limited
- **Structural information** is an urgent need but typically not available
- **Reactivity modes** of these enzymes cover only parts of the organic chemistry repertoire

Perspective — New Directions

Addressing current limitations

■ Overcoming limitations in reaction space

- Design of hybrid organometallic/bioorganic catalysts and engineering them by directed evolution

  Landmark paper: Wilson, M. E., Whitesides, G. M. *J. Am. Chem. Soc.* **1978**, *100*, 306.

  Review: Ward, T. R. *et al. Chem. Commun.* **2008**, 4239.

- *De novo* design (*in silico followed* by *in vitro*) of artificial enzymes incorporating new reactivity patterns

  Baker, D.; Röthlisberger, D. ; Houk, K. D. ; Barbas III, C. F.; Hilvert, D. *et al. Science* **2008**, *19*, 1387.

  Highlighted by: Ward, T. R. *Angew. Chem. Int. Ed.* **2008**, *47*, 7802.

- Expanding the reactivity scope by incorporation of new functionalized unnatural amino acids ("Expanding the Genetic Code")

  Recent example: Schultz, P. G. *et al. Proc. Natl. Acad. Sci. USA* **2008**, *105*, 17688.

  Review: Schultz, P. G *et al. Angew. Chem. Int. Ed.* **2005**, *44*, 1987.

General References

■ General reviews: Directed evolution of enzymes focusing on enantioselective catalysis

- *Asymmetric Organic Synthesis with Enzymes* (Eds.: Gotor, G.; Alfonso, I.; Garcia-Urdiales, E.), Wiley-VCH, Weinheim, **2008**.

- Reetz, M. T., "Directed Evolution as a Means to Engineer Enantioselective Enzymes" in *Asymmetric Organic Synthesis with Enzymes* (Eds.: Gotor, G.; Alfonso, I.; Garcia-Urdiales, E.), Wiley-VCH, Weinheim, **2008**, pp. 21-62.

- Reetz, M. T., "Directed Evolution of Enzymes for Organic Synthesis", in *Advances in Catalysis, Vol. 49* (Eds.: Gates, B. C..; Knözinger, H), Elsevier, San Diego, **2006**, pp. 1-69.

- Taylor, S. V.; Kast, P.; Hilvert, D. "Investigating and Engineering Enzymes by Genetic Selection", *Angew. Chem. Int. Ed.* **2001**, *40*, 3310-3335.

- Reetz, M. T.; Jaeger, K.-E., "Superior Biocatalysts by Directed Evolution", *Top. Curr. Chem.* **1999**, *200*, 32-57.

■ Further reading on high-throughput screening Methods (not covered)

- Reymond, J.-L.; Fluxa, V. S.; Maillard, N., "Enzyme Assays", *Chem. Commun.* **2009**, DOI: 10.1039/b813732c.

- *Enzyme Assays — High-Throughput Screening, Genetic Selection and Fingerprinting* (Ed.: Reymond, J.-L.), Wiley-VCH, Weinheim, **2005**.

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■ Short reviews & accounts

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- Koltermann, A.; Kettling, U., "Principles and Methods of Evolutionary Biotechnology", *Biophys. Chem.* **1997**, *66*, 159-177.
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- Zhao, H.; Arnold F. H., "Combinatorial Protein Design: Strategies for Screening Protein Libraries", *Curr. Opin. Struct. Biol.* **1997**, *7*, 480-485.
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