Metabolic engineering
Engineering of cell factories for industrial biotechnology I

Lecture 9

Objectives

- Illustrate different approaches for
  - increasing productivity
  - creating new compounds

- You should know after the lectures 9 & 10
  - How to deal with repression/feedback inhibition
  - Change cofactor specificity of an enzyme (NADH/NADPH)
  - Consider cellular compartmentalization (natural and using scaffolds)
  - Be aware of the influence of precursor availability
  - Engineer single enzymes or complete pathways
  - How to exploit “evolution”
  - Shuffling of DNA sequences

- Examples cover
  - Biopolymers
  - Amino acids
  - Antibiotics
  - Isoprene
  - Ascorbic acid
  - lycopene

Manufacturing of amino acids

Increasing productivity

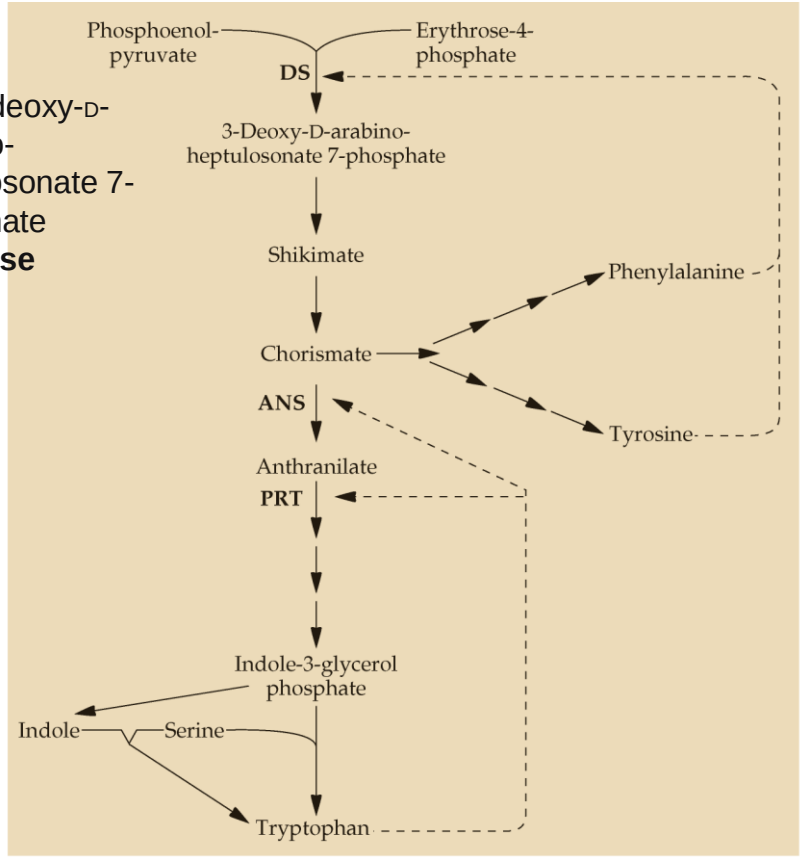
Derepressing metabolic pathways

Manufacturing of amino acids

- Amino acids are used extensively in the food industry as
  - flavor enhancers
  - antioxidants
  - nutritional supplements
  - agriculture as feed additives
  - medicine in infusion solutions for postoperative treatment
  - The chemical industry as starting materials for the manufacture of polymers and cosmetics.
- manufactured by fermentation, extraction from natural protein-rich resources and enzymatic synthesis from specific substrates
- It is estimated that more than 2.5 million tons of amino acids, worth more than $9 billion, were produced worldwide in 2008.
- L-Glutamic acid, which is used in the manufacture of the flavor enhancer monosodium glutamate, makes up around half of the total volume

Biosynthesis of aromatic amino acids – precursor and product formation

DS: 3-deoxy-D-arabino-heptulosonate 7-phosphate **synthase**



- Biosynthesis of the aromatic amino acids in all organisms begins with the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P)
- Proceeds to chorismate, from which the pathways to L-tryptophan, L-tyrosine, and L-phenylalanine branch
- Carbon flow up to chorismate is primarily controlled at the first reaction

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Biosynthetic pathways, regulations, and transport systems of aromatic amino acids in *Corynebacterium glutamicum*

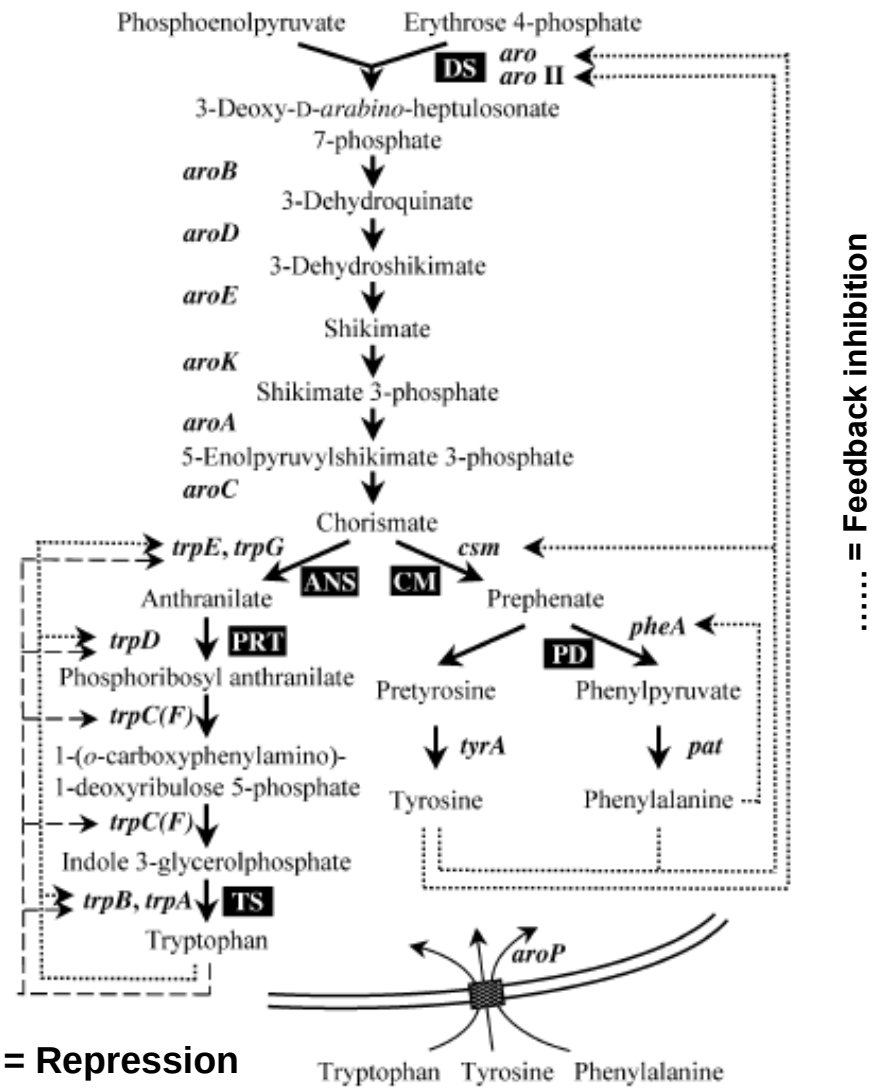
DS activity encoded by *aro* and *aroII* genes

- *Aro* encodes an L-tyrosine-sensitive type I DS
- *AroII* encodes a L-phenylalanine- and L-tyrosine-sensitive type II DS

Aro II forms a polypeptide complex with chorismate mutase (CM; *csm*)

- Aro is active alone
- **CM activity requires the presence of the AroII protein**

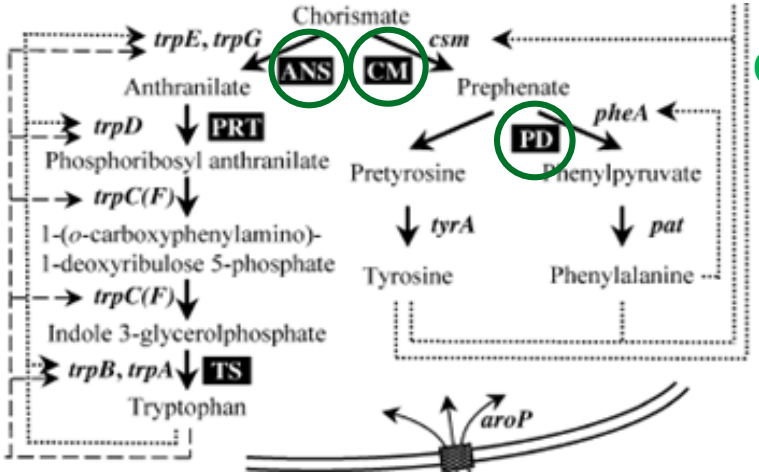
Due to this specific protein interaction, **simultaneous overproduction of the DS and CM proteins is necessary to achieve overexpression of CM activity**



Control of carbon flow in the branched pathways **by end-product inhibition** in *C. glutamicum*

- control of carbon flow in the branched pathways mainly through end-product inhibition at five enzymatic steps

- The concentrations of end-product(s) giving 50% inhibition
  - **0.0015 mM ANS**
  - 0.15 mM for PRT
  - 7.7 mM for TS
  - **0.0023 mM for CM**
  - **0.0025 mM for PD**



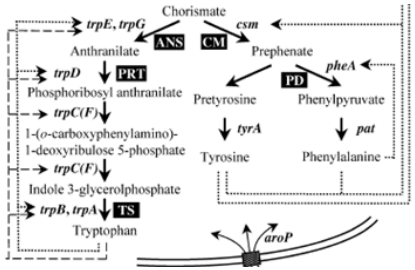
- Specific mutations causing desensitization of these key enzymes
  - Ser38Arg substitution leading to a desensitized ANS
  - Ala162Glu substitution leading to a desensitized PRT
  - Ser99Met substitution leading to a desensitized PD

Control of carbon flow in the branched pathways **by regulation of gene expression** in *C. glutamicum*

- Formation of all enzymes in the L-tryptophan branch is several-fold repressed by L-tryptophan through an attenuation control

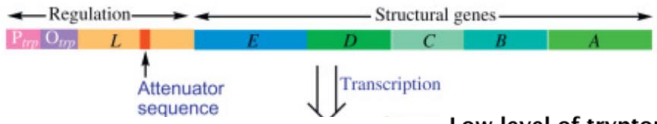
------ = Repression

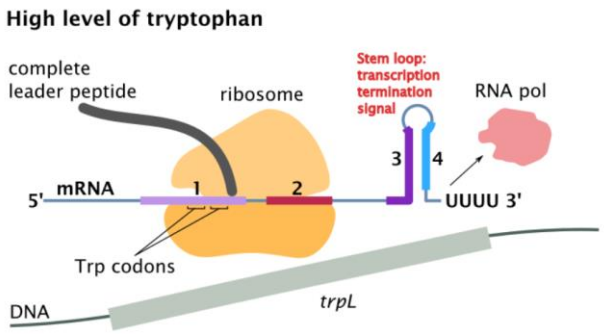
…… = Feedbck inhibition

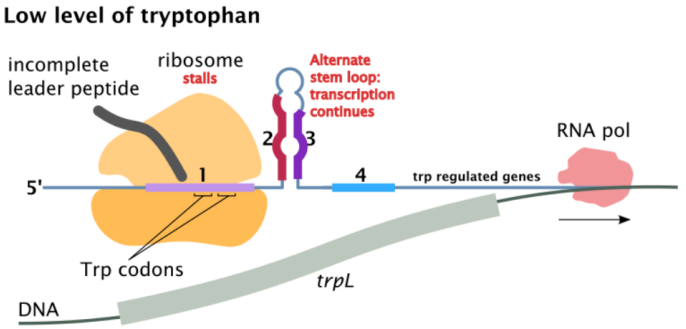


- A point mutation in attenuator region resulted in increased ANS and PRT activities

attenuation control:







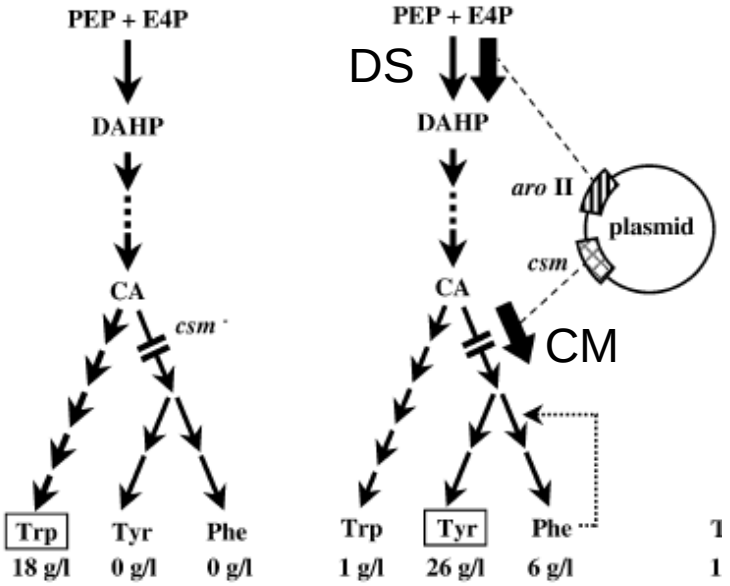
Strategies for identification of anthranilic acid synthetase with altered regulation

- Enzymes from different organisms have often altered activity profiles due to different types of regulation
- A library of chromosomal DNA was cloned into a *C*. *glutamicum- E. coli* shuttle vector and introduced into a mutant strain of C. *glutamicum* that produced no active anthranilic acid synthetase (ANS)
  - Mutant strain is unable to grow on minimal medium unless anthranilate is added
  - transformants can be selected by their ability to grow in the absence of anthranilate

- The vector carrying the anthranilic acid synthetase gene was then transferred to a wild-type strain of *C*. *glutamicum*

How can you test that the new ANS is not under control of feedback inhibition?

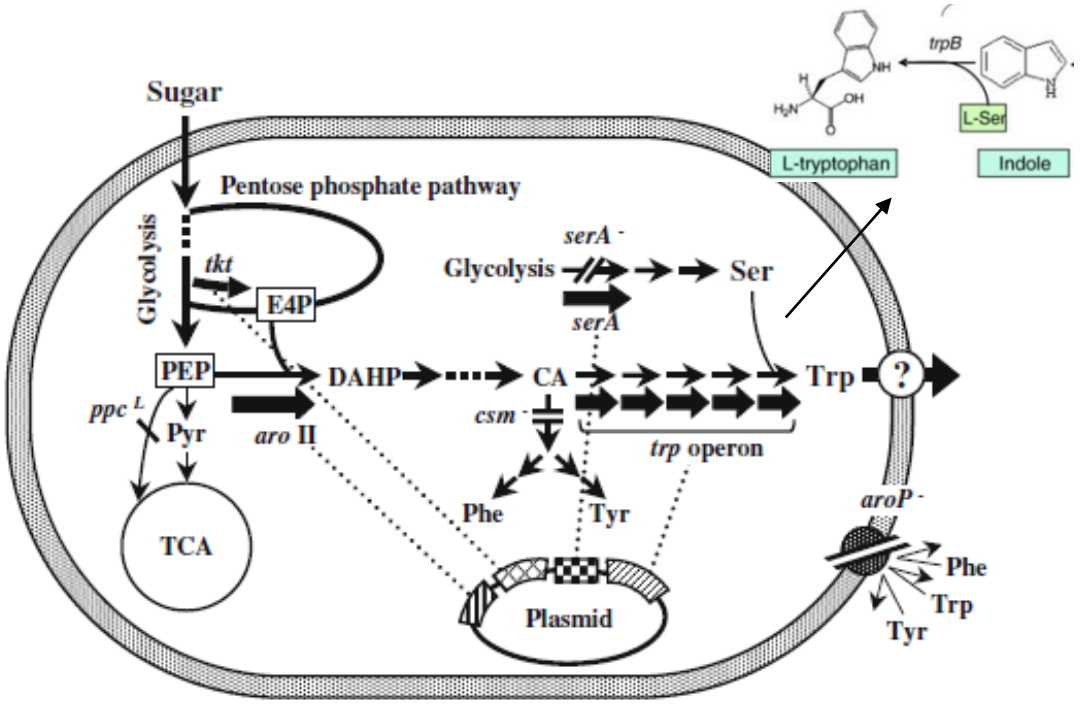
Outline of metabolic conversion from L-tryptophan to L-phenylalanine and L-tyrosine in *C. glutamicum*



- Simultaneous amplification of aro II encoding the desensitized type II-DS, together with csm encoding the branch-point enzyme CM, in an L-tryptophan-producing mutant of C. glutamicum, has led to a shift from 18 g/l of L-tryptophan to 26 g/l of L-tyrosine

Outline of metabolic engineering for L-tryptophan production demonstrated in *C. glutamicum*.

- Focus of engineering directed to precursor biosynthesis
  - PEP carboxylase and transketolase in central metabolism
  - Serine biosynthesis
- Serine is needed in 1:1 stoichiometry
- *serA* is overexpressed on plasmid and serves at the same time as selection marker



serA gene as selection marker to ensure plasmid stability in a strain lacking *serA!*

Metabolic engineering for L-tryptophan production

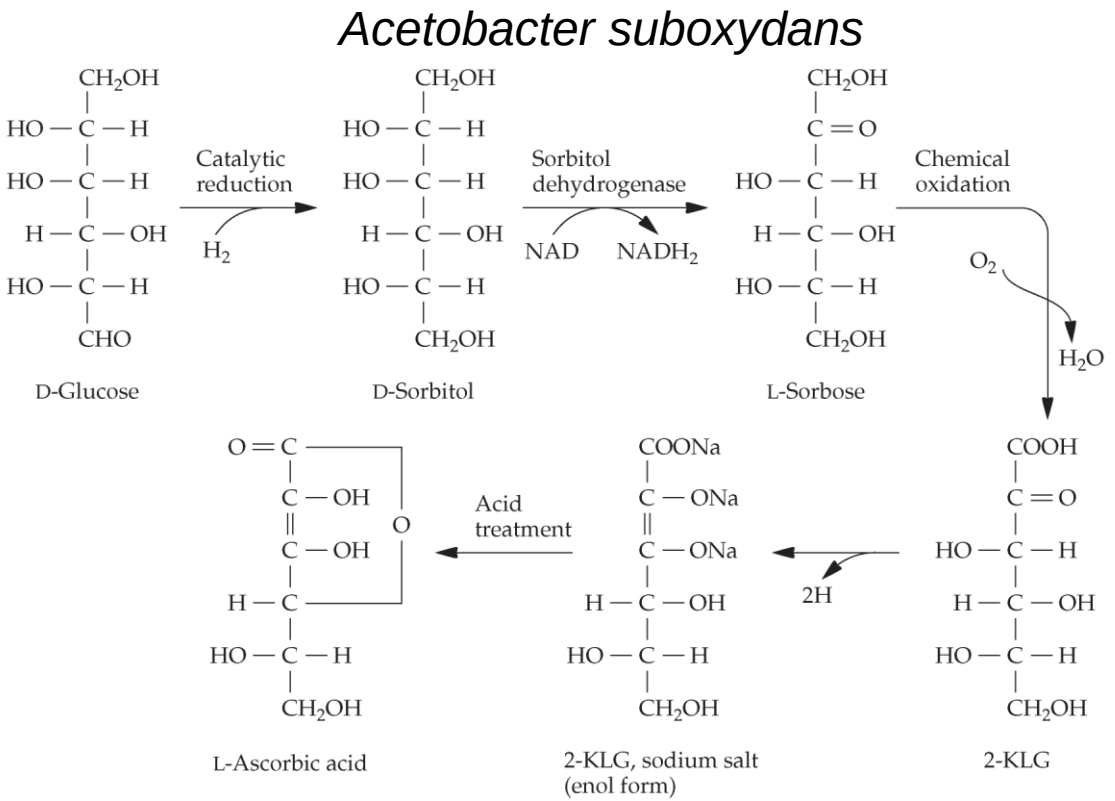
- Biosynthesis of L-tryptophan is strictly controlled at several steps in *C*. *glutamicum*
- Overproduction of L-tryptophan requires
  - genetic removal of all the metabolic controls existing both in the common pathway and in the L-tryptophan branch
  - amplification of DS, which initiates the common pathway, should be an important strategy to increase net carbon flow down the common pathway.
  - balanced supply of precursors to achieve efficient production, considering that biosynthesis of 1 mol of L-tryptophan from glucose requires 1 mol each of E4P and PEP as starting precursors
  - In addition, consumes 1 mol each of PEP, L-glutamine, phosphoribosyl-5-pyrophosphate, and L-serine on its biosynthetic pathways

Production of ascorbic acid

cellular compartmentalization

Changing co-factor specificity

Commercial synthesis of L-ascorbic acid



Except for the microbial conversion of D-sorbitol to L-sorbose, all the steps are chemical reactions. The microbial conversion is carried out by *Acetobacter suboxydans,* which produces the enzyme sorbitol dehydrogenase.

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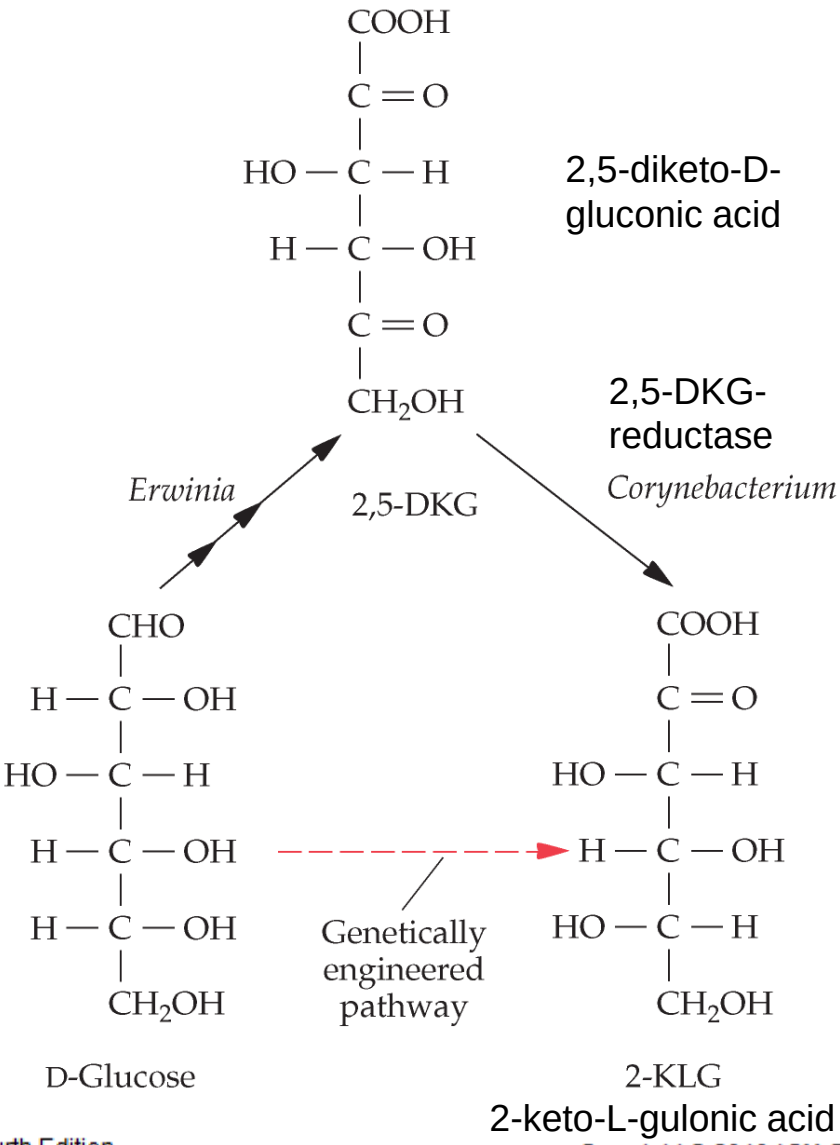
Alternatives for biological 2-KLG production

- *Erwinia herbicola* has a set of three enzymes that synthesize 2,5-DKG from D-glucose.
- *Corynebacterium* has an enzyme that converts 2,5-DKG to 2-KLG
- 2-KLG, which is the immediate precursor of L-ascorbic acid, can be produced from D-glucose either by cocultivating of these two microorganisms
- or by genetically engineering *Erwinia* to express the enzyme from *Corynebacterium* which converts 2,5-DKG to 2-KLG.



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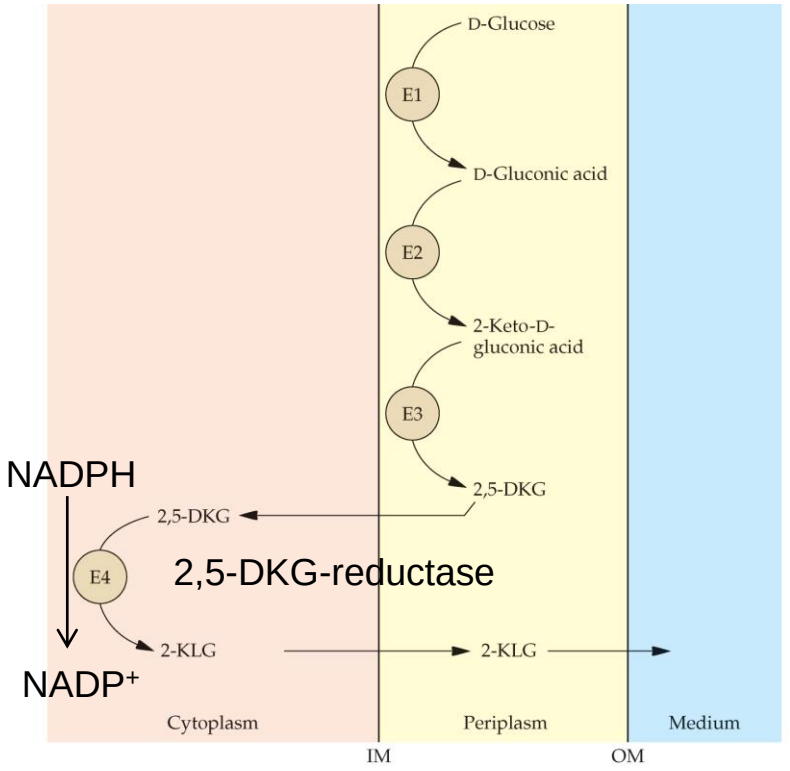
Conversion of D-glucose to 2-KLG by recombinant *Erwinia herbicola*

- The pathway is localized in cytoplasm and periplasm
- The co-factor NADPH is only present in the cytoplasm
- Introduced 2,5-DKG reductase must be localized to the cytoplasm



- IM (inner membranes) and OM (outer membranes)

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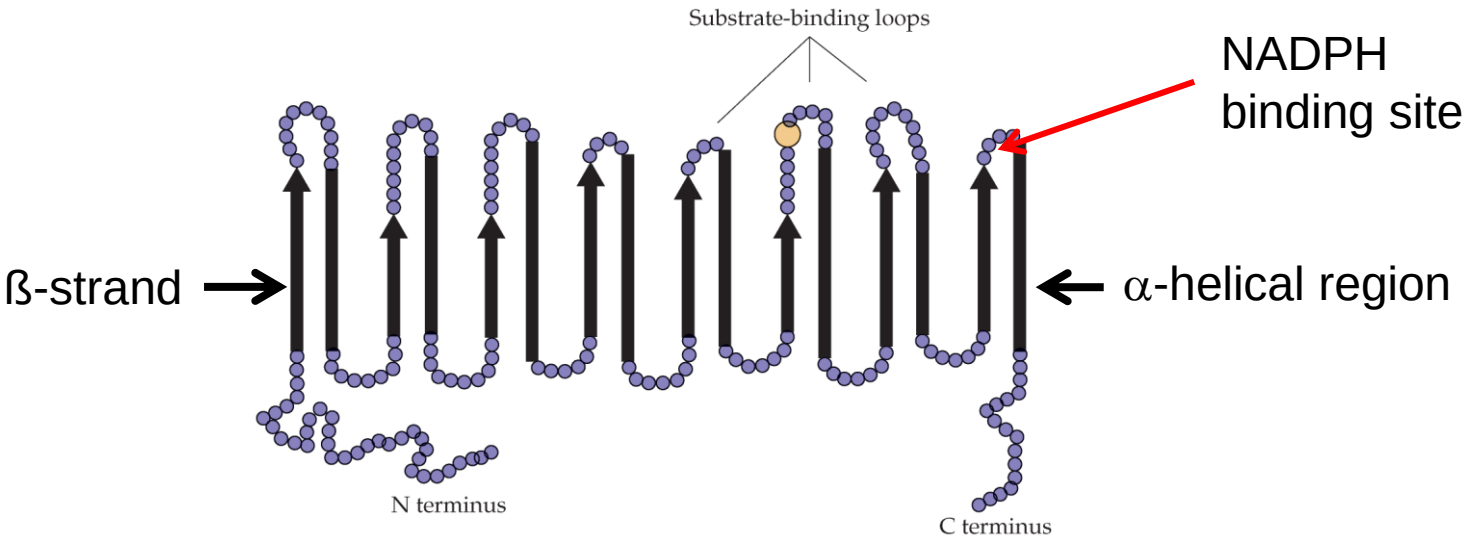
Protein engineering of 2-KLG reductase – activity and cofactor specificity



- Predicted structure of 2,5-DKG reductase
- The three loops that may be involved in substrate binding and NADPH binding site are indicated

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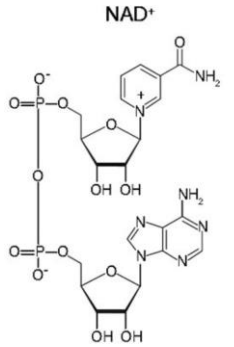
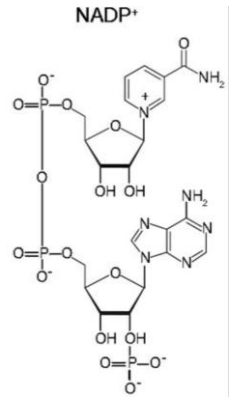
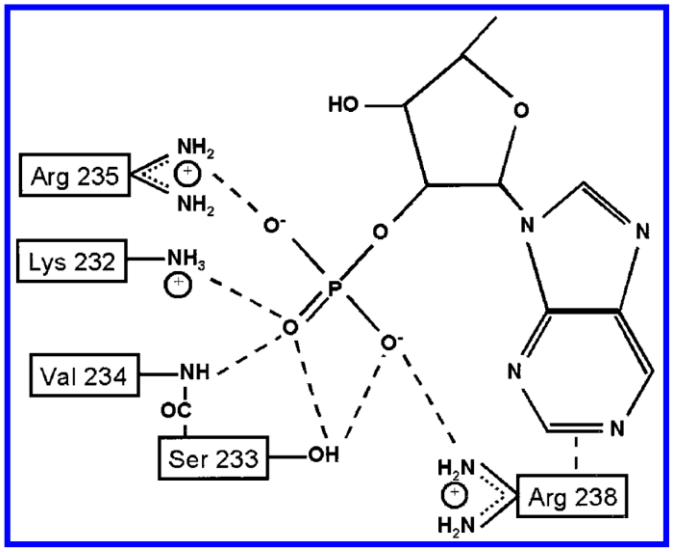
Changing the cofactor specificity of 2,5-DKG reductase from NADPH to NADH

- Cellular concentration of reduced NADH is usually about 10-fold greater than that of NADPH
- NAD(H) and NADP(H) differ only by a phosphate group attached to the 2’ position of the adenosine ribose.
- high degree of cofactor specificity is important for proper functioning in natural metabolic pathways, but in a modified or artificial metabolic pathway, this specificity may be undesirable





Changing the cofactor specificity of 2,5-DKG reductase from NADPH to NADH

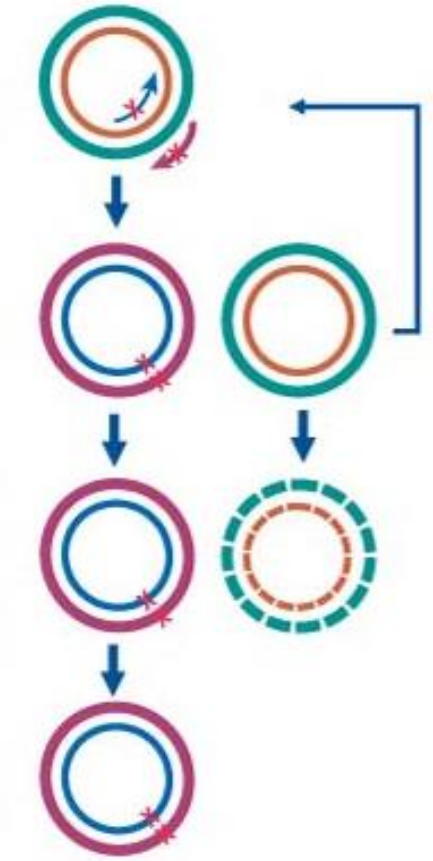


Arg: R
Lys: K
Val: V
Ser: S

Amino acids in the wild-type 2,5-DKG reductase that make noncovalent interactions with the 2'-phosphate group of NADPH.

Banta et al., Biochemistry 2002, 41, 6226-6236

Basic strategy for introducing point mutations



1. Mutant Strand Synthesis
Perform thermal cycling to:
- Denature DNA template
- Anneal mutagenic primers containing desired mutation
- Extend and incorporate primers with *high-fidelity* DNA polymerase

2. *Dpn*I Digestion of Template
Digest parental methylated and hemimethylated DNA with *Dpn* I

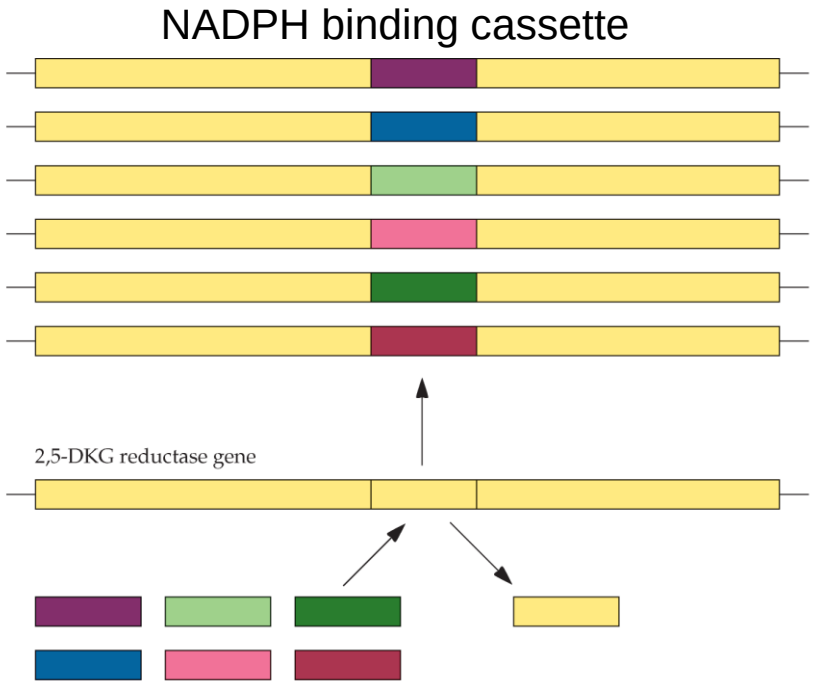
3. Transformation
Transform mutated molecule into competent cells for nick repair

Dpn I endonuclease (target sequence: 5´-Gm6ATC-3´)

Methylated in DNA isolated from E. coli but not methylated when PCR amplified

Changing the cofactor specificity of 2,5-DKG reductase from NADPH to NADH

- a 57-bp DNA fragment encoding the AA residues specific for cofactor recognition are removed
- Chemically synthesized 57-bp DNA fragments that contained an alteration in the DNA sequence that coded for one of the amino acids to be changed was inserted into the gene



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Changing the cofactor specificity of 2,5-DKG reductase from NADPH to NADH

	relative expression level	NADPH activity	NADH activity
wild type	**	***	
K232G/R235G	***	*	*
K232G/R235T	***	*	
K232G/R238E	***	**	
K232G/R238H	***	***	***
K232S/R235G	***		
K232S/R235T	***		
K232S/R238E	***		
K232S/R238H	***	***	**
R235G/R238E	***		
R235G/R238H	***	***	*
R235T/R238E	***		
R235T/R238H	***	***	*
K232G/R235G/R238E	***	**	
K232G/R235T/R238E	***		
K232G/R235G/R238H	***	**	***
K232G/R235T/R238H	***	***	***
K232S/R235G/R238E	***		
K232S/R235T/R238E	***		
K232S/R235G/R238H	***		
K232S/R235T/R238H	***	*	
K232R/V234F/R235N	***		
K232R/V234F/R235N/R238H	***	*	
S233T/R235S	***	***	*
S233T/R235S/R238H	***	***	**
V234T/R235F/R238T	*		

Arg: R
Lys: K
Val: V
Ser: S
Gly: G
Glu: E
His: H
Thr: T

Banta et al., Biochemistry 2002, 41, 6226-6236

A General Tool for Engineering the NAD/NADP Cofactor Preference of Oxidoreductases

Jackson K. B. Cahn,[†,§] Caroline A. Werlang,[†,⊥] Armin Baumschlager,[†,¶] Sabine Brinkmann-Chen,[†] Stephen L. Mayo,[‡] and Frances H. Arnold[*,†]

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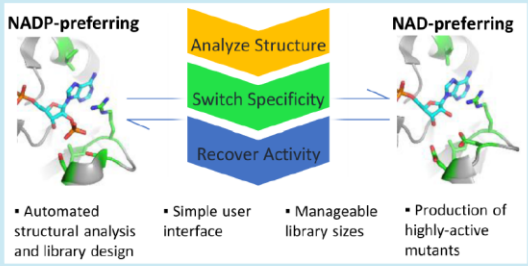
Supporting Information

ABSTRACT: The ability to control enzymatic nicotinamide cofactor utilization is critical for engineering efficient metabolic pathways. However, the complex interactions that determine cofactor-binding preference render this engineering particularly challenging. Physics-based models have been insufficiently accurate and blind directed evolution methods too inefficient to be widely adopted. Building on a comprehensive survey of previous studies and our own prior engineering successes, we present a structure-guided, semirational strategy for reversing enzymatic nicotinamide cofactor specificity. This heuristic-based approach leverages the diversity and sensitivity of catalytically productive cofactor binding geometries to limit the problem to an experimentally tractable scale. We demonstrate the efficacy of this strategy by inverting the cofactor specificity of four structurally diverse NADP-dependent enzymes: glyoxylate reductase, cinnamyl alcohol dehydrogenase, xylose reductase, and iron-containing alcohol dehydrogenase. The analytical components of this approach have been fully automated and are available in the form of an easy-to-use web tool: Cofactor Specificity Reversal−Structural Analysis and Library Design (CSR-SALAD).



KEYWORDS: *cofactor specificity, oxidoreductases, protein engineering, library design, semirational engineering*

Cahn et al., ACS Synth. Biol. 2017, 6, 326−333

Frances H. Arnold
The Nobel Prize in Chemistry 2018

Born: 25 July 1956, Pittsburgh, PA, USA

Affiliation at the time of the award: California Institute of Technology (Caltech), Pasadena, CA, USA

Prize motivation: "for the directed evolution of enzymes."

Prize share: 1/2

https://www.nobelprize.org/prizes/chemistry/2018/arnold/facts/

Engineering complete pathways *in vivo*

Creating diversity and selection

NATURE PROTOCOLS | PROTOCOL

Rapid editing and evolution of bacterial genomes using libraries of synthetic DNA

Ryan R Gallagher, Zhe Li, Aaron O Lewis & Farren J Isaacs

Affiliations | Contributions | Corresponding author

Nature Protocols **9**, 2301–2316 (2014) | doi:10.1038/nprot.2014.082
Published online 04 September 2014

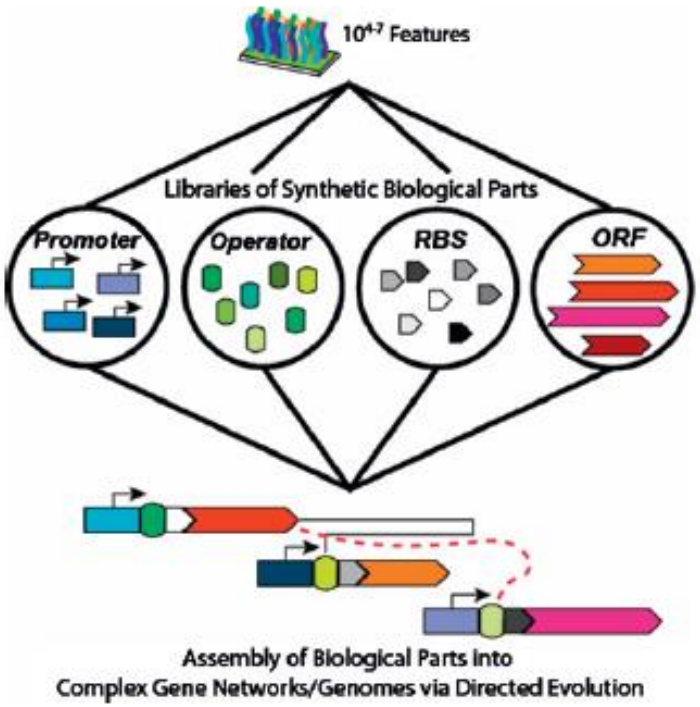
nature Vol 460|13 August 2009|doi:10.1038/nature08187

LETTERS

Programming cells by multiplex genome engineering and accelerated evolution

Harris H. Wang[1,2,3]*, Farren J. Isaacs[1]*, Peter A. Carr[4,5], Zachary Z. Sun[6], George Xu[6], Craig R. Forest[7] & George M. Church[1]

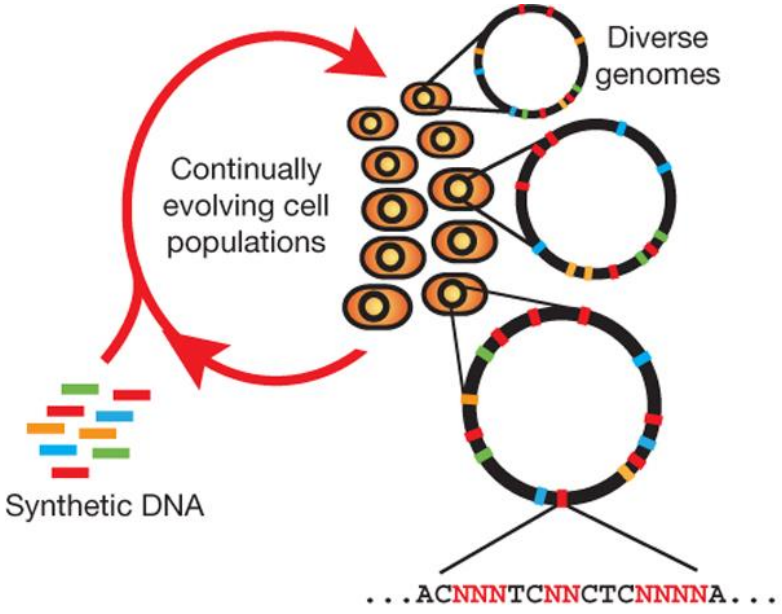
Generating diversity – random vs. directed mutation



- Genome vs. single gene
- Random:
  - Chemical, UV-mutagenesis
  - Error-prone PCR
  - SELECTION NEEDED!
- Directed
  - Point-mutations
- Random-directed
  - Degenerate oligos
  - MAGE

Multiplex automated genome engineering (MAGE)

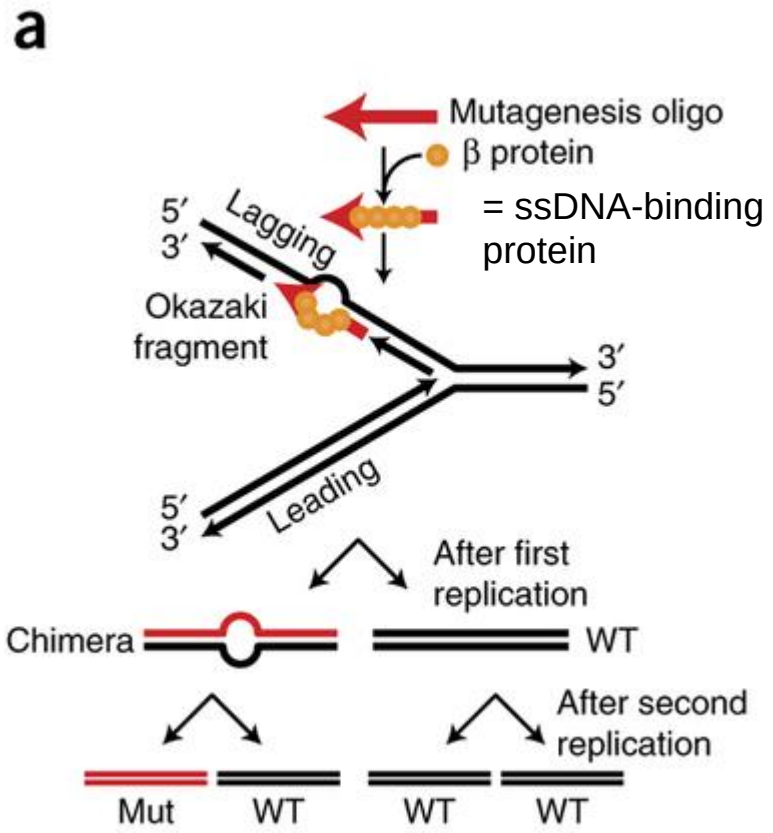
- MAGE enables the rapid and continuous generation of sequence diversity at many targeted chromosomal locations across a large population of cells through the repeated introduction of synthetic DNA.
- Each cell contains a different set of mutations, producing a heterogeneous population of rich diversity.
- Degenerate oligo pools that target specific genomic positions enable the generation of a diverse set of sequences at each chromosomal location.
- **Efficient screening and selection procedure must be available**



Wang *et al.* 2009, *Nature*, 13;460(7257):

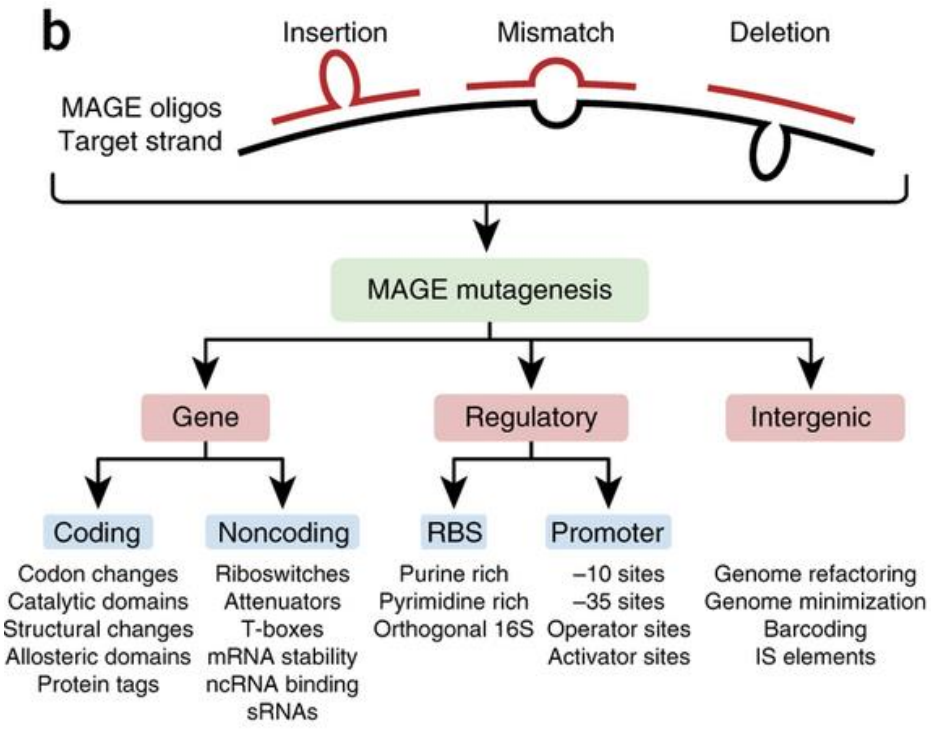
Multiplex automated genome engineering (MAGE)

- MAGE harnesses phage homologous recombination proteins to create targeted, rapid, scar-less modifications of bacterial chromosomes across many genomic loci
- The mutagenesis oligo is bound by β-protein and anneals to the lagging strand of the replication fork, between discontinuous Okazaki fragments.
- At the end of replication, one wild-type (WT) chromosome and one chimeric chromosome exist.
- During the second round of replication, two strands of the chimeric chromosome segregate into a fully WT and a fully mutant (mut) chromosome.

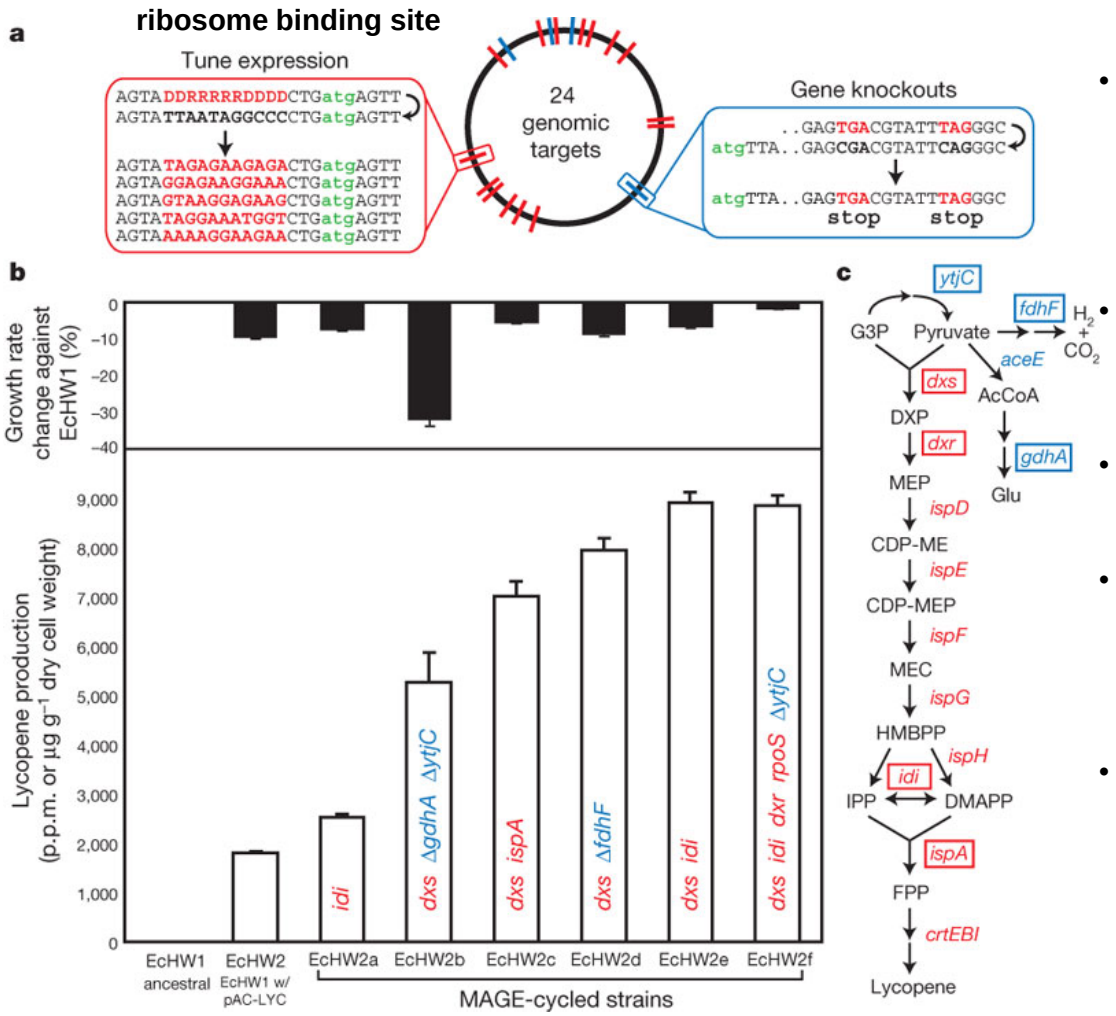


Multiplex automated genome engineering (MAGE)

- MAGE is capable of introducing insertion, deletion or mismatch mutations.
- By targeting coding, regulatory or intergenic regions, these mutations can be used to modify transcription rates, translation rates, mRNA stability, enzyme activity and so on.



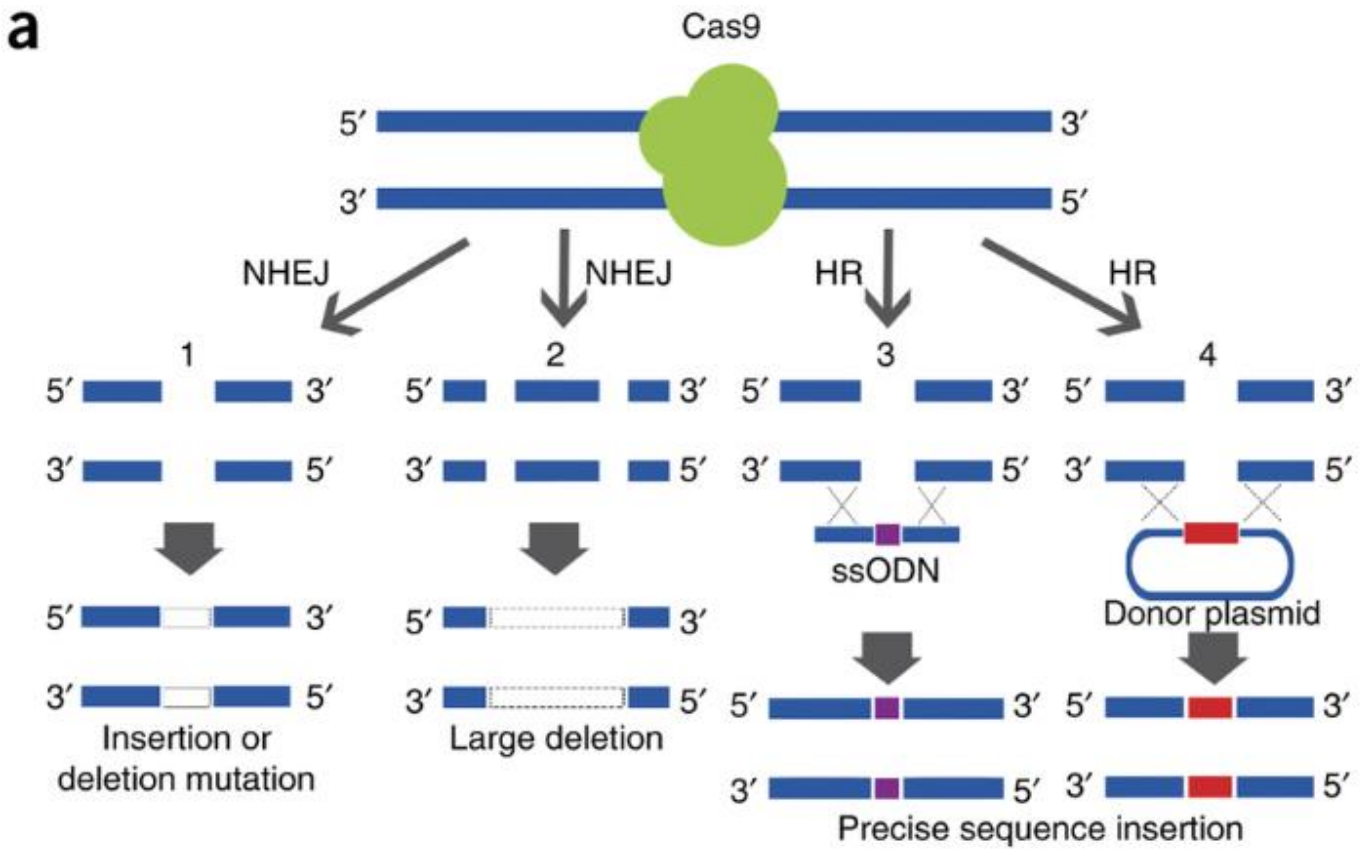
Optimization of the lycopene production using MAGE



- **Twenty endogenous genes documented to increase lycopene yield were targeted to tune translation.** For each of the 20 genes, 90-mer oligos **containing degenerate ribosome binding site (RBS)** were generated.
  Four genes from secondary pathways were targeted for inactivation by oligos that introduced two **nonsense mutations**.
- Black bars represent the growth rate of isolated variants relative to the ancestral EcHW1 strain.
- White bars represent lycopene production **Colour-coded labels in each white bar represent genetic modifications found by sequencing**.
- Modifications to the lycopene biosynthesis pathway of isolated variants EcHW2a–f with relevant genes highlighted by rectangular boxes. Blue labels represent knockout targets, red labels represent RBS tuning targets.

Wang *et al.* 2009, *Nature*, 13;460(7257):

Alternative: CRISPR/Cas9 based genome engineering



Shao et al., Nature Protocols 9:2493–2512 (2014)