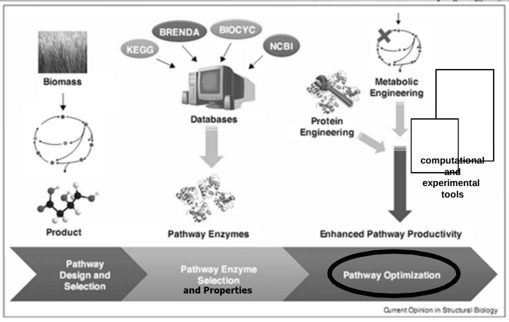
LOSCHMIDT LABORATORIES

5. Metabolic Engineering II

General workflow of ME project



Dhamankar , H. (2011) *Curr.Opin.Struct.Biol.*, 21:1

Outline

- Experimental (genetic) tools for ME
- Metabolic load (yield *vs.* viability of host)
- ME of biosynthetic (anabolic) pathways - examples
- ME of biodegradation (catabolic) pathways - examples
- Limitations and perspectives of ME
- Discussion

Experimental tools for ME

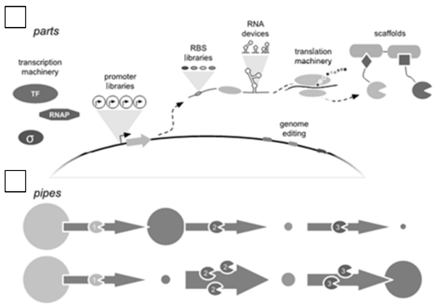
EXPERIMENTAL TOOLS ARE APPLIED HAND IN HAND WITH THEORETICAL TOOLS

- **experimentals tools = genetic tools (recombinant DNA technology)**
- **production of transgenic organisms**

engineering input on level of:

- **gene expression (DNA/RNA):** gene knockout, gene down/up-regulation, heterologous expression, codon optimization, chromosomal integration of gene(s)
- **protein:** protein engineering, proximity of enzymes (substrate channeling)
- **small molecules:** cofactor balancing

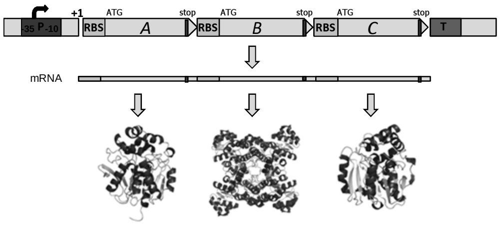
"Parts and pipes" in ME



Boyle, P.M. (2012) *Metabolic Engineering*, 14:223

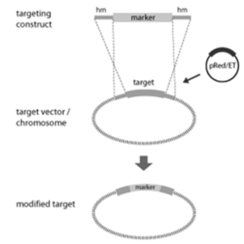
optimization of gene(s) expression

Bacterial operone



Gene knock-out

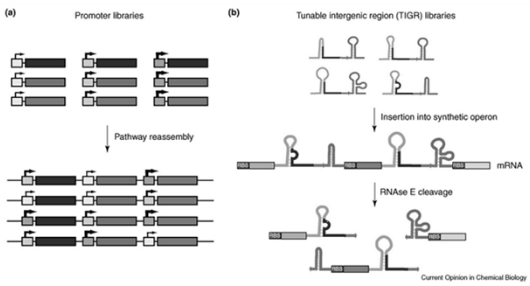
- gene(s) of interest is deleted from the chromosome – **permanent state**
- widely used technique based on **phage λ Red recombinase** (*E. coli*)
- analogous method used in yeast



www.genebridges.com

Gene down/up regulation

TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL LEVEL

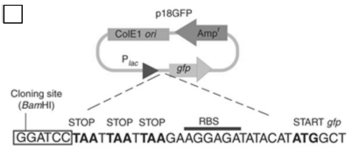


Santos, C.N.S. (2008) *Curr.Opin.Chem.Biol.*, 12:168

Gene down/up regulation

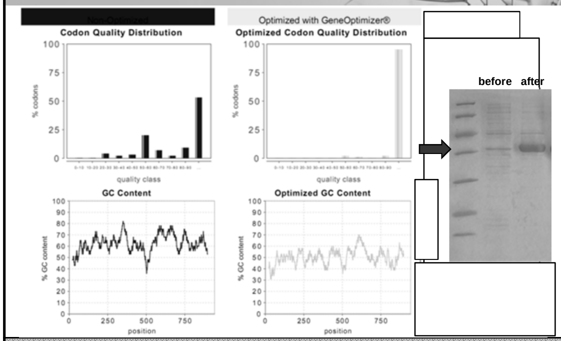
LEVEL of TRANSLATION

- **RNA interference** - homologous RNA sequences (gene knock-down) expression of one or more genes is reduced – **transient state**
- **engineering of ribosome binding sites** (RBS calculator[1]) Shine-Dalgarno sequence (consensus sequence AGGAGG)



[1]Salis, H.M. (2009) *Nature Biotechnology*, 27: 946

Codon optimization



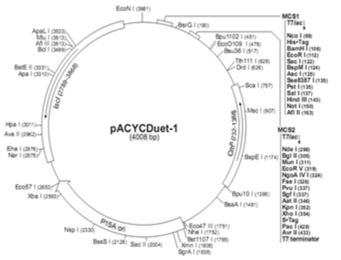
Expression from plasmids

HETEROLOGOUS GENE EXPRESSION FROM PLASMIDs

- **important characteristics of each plasmid**: **copy number**, origin of replication (ORI), **promoter**, selection marker, multi-cloning sites (MCS), tags or leading sequences
- **commercial vectors** (pBAD Invitrogen, pET Merck)
- **DUET vectors** (derivatives of pET) – suitable for heterologous expression of whole metabolic pathways.

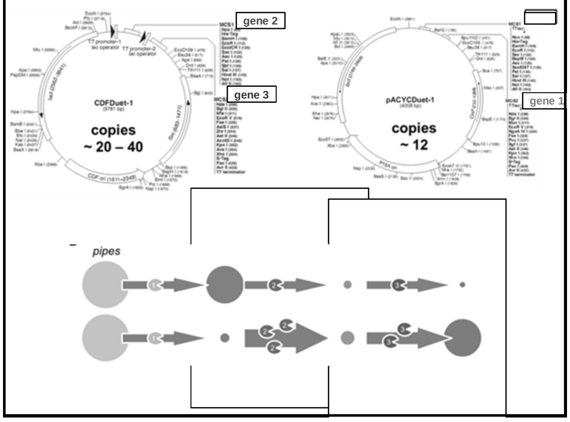
Expression from plasmids

DUET vector system (T7 promoter,variability in copy number)



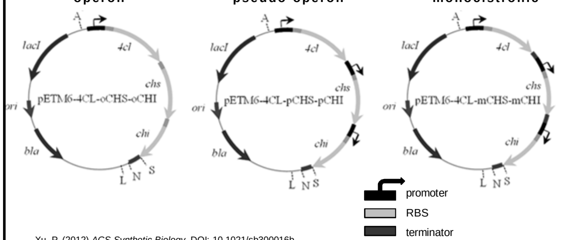
Vector	Promoter	Antibiotic resistance	Replicon (source) [copy number]
pETDuet-1	T7	Ampicillin or carbenicillin	ColE1 (pBR322) [~40]
pACYCDuet-1	T7	Chloramphenicol	P15A (pACYC184) [10–12]
pCDFDuet-1	T7	Streptomycin or spectinomycin	CloDF13 [20–40]
pRSFDuet-1	T7	Kanamycin	RSF1030 [>100]

www.merckmillipore.cz



Expression from plasmids

Gene order and configuration of genes in operone influence their expression.[1]



Xu, P. (2012) *ACS Synthetic Biology*, DOI: 10.1021/sb300016b

[1]Lim, H.N. (2011) *PNAS*, 108:10626

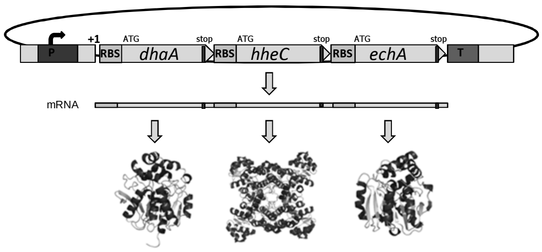
Expression from chromosome

HETEROLOGOUS GENE EXPRESSION FROM CHROMOSOME

- expression from chromosome is **advantageous** (higher stability, no antibiotic markers)
- **methods for integration:** homologous recombination (*recA, λ Red*), transposition (Tn5 and Tn7-based vectors)
- integration of **single genes** or whole synthetic **operones**
- subsequent duplication or **multiplication of insertions**

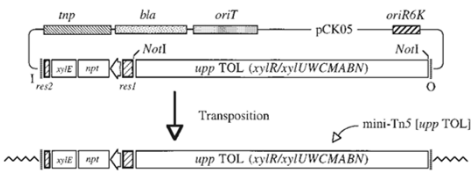
Expression from chromosome

SYNTHETIC OPERON DESIGN (software GeneDesigner 2.0)



Expression from chromosome

INSERTION OF DESIRED GENE(S) BY TN-5 BASED TRANSPOSITION (**non-specific**).



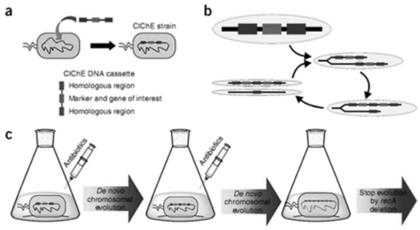
selection of positive clones on LB agar plates with respective antibiotic

de Lorenzo, V. (1990) *Journal of Bacteriology*, 172: 6568

Balancing gene expression VI

HETEROLOGOUS GENE EXPRESSION FROM CHROMOSOME

- multiplication of insertions: **Chemically Inducible Chromosomal Evolution (CIChE)**[1]



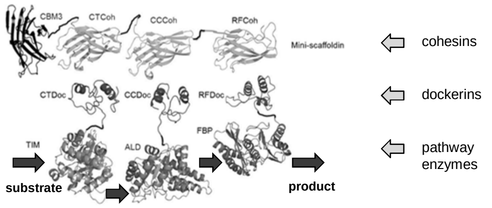
[1]Tyo, K.E.J. (2009) *Nature Biotechnology*, 27:760

protein level

Substrate channelling

Synthetic protein scaffolding made of bacterial dockerins and cohesins from cellulosome (*Clostridium*, *Bacteroides*).

- protein scaffolding used for increasing proximity of 3 glycolytic enzymes producing fructose-6-phosphate[1]



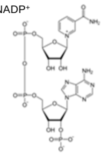
[1]You, C. (2012) *Angewandte Chemie*, 51:1

small molecules level

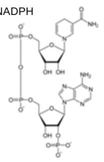
Cofactor balancing

- cofactors play a critical role especially in **redox reactions** (NAD(H), NADP(H))
- natural pathways (e.g. glycolysis) often employ **oxidoreductases**
- cofactor recycling and balancing is essential
- solution: enzyme mediated **cofactor recycling** through overexpression of NAD^+ kinase, transhydrogenases or dehydrogenases simultaneously with knock-outs of genes encoding enzymes from competing pathways

$NADP^+$



NADPH



time for break

metabolic load[1]

[1]Glick, B.R. (1995) *Biotechnology Advances*, 13:247

Metabolic load

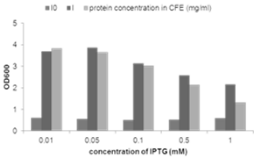
YIELD VS. VIABILITY OF CELL

1) **STATIC CONTROL:** static balancing of production of pathway enzymes - levels of enzymes remain unchanged throughout the whole cultivation (most of the standard techniques mentioned above)
2) **DYNAMIC CONTROL:** engineering of a dynamic response of host organism on **metabolic load and toxicity** of pathway components – levels of enzymes fluctuate during cultivation (challenge for future applications of ME)

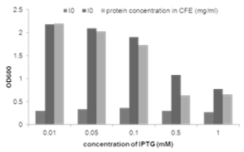
Metabolic load

- **overexpression of foreign proteins results in decrease of viability of host cell**

rich LB medium

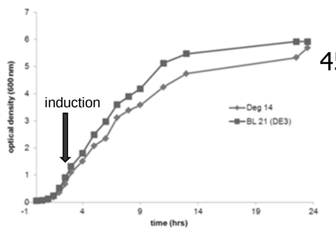


minimal medium



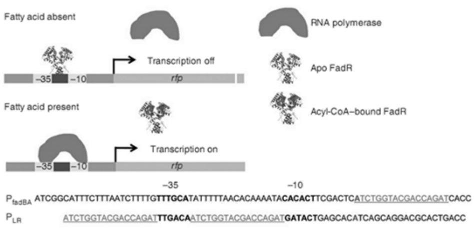
Metabolic load

- overexpression of foreign proteins results in decrease of viability of host cell



Dynamic control engineering

- **Dynamic Sensor-Regulator System (DSRS)**[1]
- Production of biodiesel from fatty acid ethyl ester in *E. coli*.



[1]Zhang, F. (2012) *Nature Biotechnology*, 30:354

ME of biosynthetic pathways

ME APPLIED IN ORDER TO IMPROVE (ESTABLISH) PRODUCTION OF:

- biofuels (ethanol, butanol, H_2, fatty acids derived esters)
- natural and non-natural alcohols
- natural and non-natural amino acids
- fatty acids
- peptides and proteins
- secondary metabolites: antibiotics, isoprenoids (artemisinin, taxol)
- oligo and polysacharides (biodegradable polymers)
- commodity chemicals (1,3-propanediol)
- and many others...

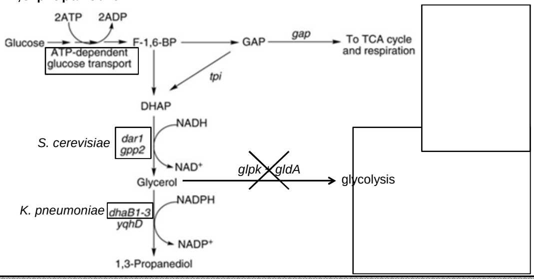
ME of biosynthetic pathways

Current limitations of biosynthesis using engineered organisms:

- missing standards
- low productivity (low activity of enzymes, side reactions, limits of host organisms)
- non-competitive economy of the biosynthetic proceses
- application of GMO (ethics)

ME of biosynthetic pathways

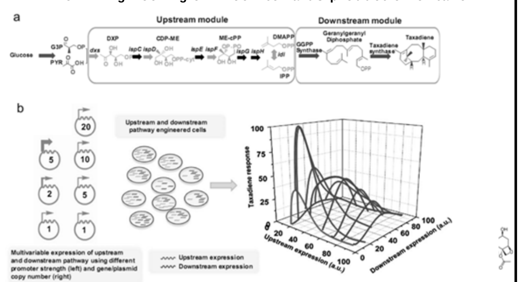
EXAMPLE No.1: Engineering of *E. coli* K12 towards production of 1,3-propanediol[1]



[1]Nakamura, C.E. (2003) *Current Opinion in Biotechnology*, 14:454

ME of biosynthetic pathways

EXAMPLE No.2: Engineering of *E. coli* towards production of taxol



[1]Ajikumar, P.K. (2010) *Science*, 330:70

ME of biodegradation pathways

- ME of biodegradation pathways for **biodegradation** of toxic compounds in industry, **biosensing** and *in situ* **bioremediation**.
- host organisms: **bacteria** (mostly improvement of natural strains isolated from contaminated sites) and **plants** (phytobioremediation)
- phenomena of toxicity and adaptation of bacteria (enzymes) towards anthropogenic substrates
- paraoxone, toluene, DCE, TCP, lindane

ME of biodegradation pathways

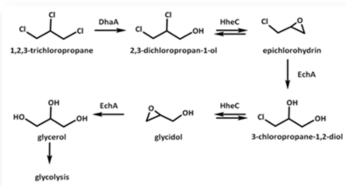
Current limitations of biodegradation using engineered organisms:

- **low competitivness** of engineered strains (different conditions in lab and in the environment)
- **decreased viability** of host organisms due to metabolic load and high toxicity of substrates and pathway intermediates
- application of **GMO** (ethics)
- limited number of "successful stories"
- → ME of biodegradation pathways is challenging

ME of biodegradation pathways

EXAMPLE: Synthetic pathway for biodegradation of 1,2,3-trichloropropane (TCP)

- TCP – anthropogenic compound, industrial use, emerging pollutant
- no natural strain capable of TCP utilization (lack of dehalogenating enzyme)



TCP pathway

Project workflow:

- 1989 – description of pathway for utilization of halogenated alcohols from *Agrobacterium radiobacter* AD1 (**HheC**, **EchA**)
- 1997 – description of haloalkane dehalogenase **DhaA** (*Rhodococcus sp.*)
- 1999 – heterologous expression of dhaAwt in *A.radiobacter* AD1[1]
- 2002 – heterologous expression of dhaAM2 in *A.radiobacter* AD1[2]
- **ultimate goal: bacterium utilizing TCP as a single carbon source**

PROBLEMS:

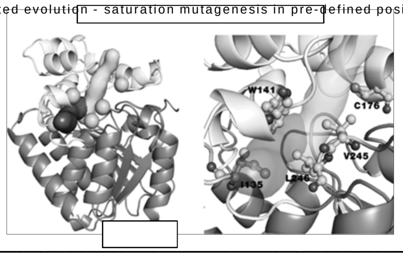
- low viability of constructs (TCP toxicity, low expression of enzymes)
- cumulation of toxic pathway intermediates
- low conversion of TCP to glycerol (3.6 mM/10 days)

[1,2]Bosma ,T. (1999 and 2002) *Applied Environmental Microbiology*, 65:4575 and 68:3582

TCP pathway

2009 - construction of DhaA31[1] (32-times improved activity with TCP)

- rational design - computer modelling for selection of hot spots
- directed evolution - saturation mutagenesis in pre-defined positions



[1]Pavlová, M. (2009) *Nature Chemical Biology*, 5:727

TCP pathway

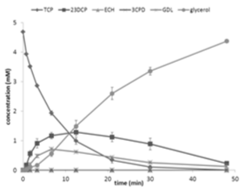
2009 – now: applied principles of metabolic engineering:

- gene synthesis and **codon optimization** for *E. coli*
- cloning in pET and DUET vectors, **overexpression**
- detailed characterization of pathway enzymes (**kinetic properties**)
- characterization and quantification of metabolites (**GC analysis**)
- **PROOF OF CONCEPT:** reconstruction of pathway ***in vitro***

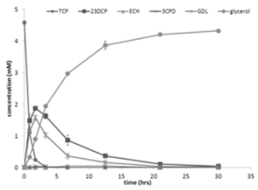
TCP pathway

In vitro **reconstruction of TCP pathway (soluble enzymes)**

DhaAwt, HheC, EchA
mixed in **ratio 1:1:1**



DhaA31, HheC, EchA
mixed in **ratio 1:1:1**



TCP pathway

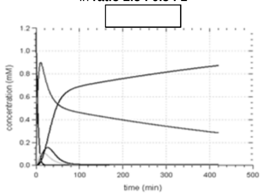
2009 – now: applied principles of metabolic engineering:

- **kinetic model** of the pathway
- reconstruction of pathway ***in vivo*** (*E. coli*)
- defined **toxicity** of TCP and pathway intermediates for cell
- **modular engineering** for balancing of gene expression (DUET vectors)
- **combinatorial approach:** construction of several variants of the pathway and selection of one with the most efficient conversion of TCP to glycerol

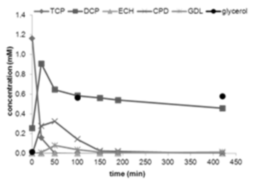
TCP pathway

In vivo **reconstruction of TCP pathway (*****E. coli*****, DUET vectors)**

reaction course **simulation**
DhaAwt, HheC, EchA
in **ratio 2.5 : 0.5 : 1**

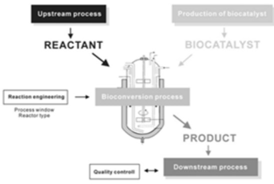


conversion of TCP by ***E. coli*** resting cells
DhaA31, HheC, EchA
in **ratio 2.5 : 0.5 : 1**



Current limitations of ME

- long way from lab scale (ml - L) to industry scale (10^3 - 10^5 L)
- costly processes (esp. product recovery and purification)
- low productivity of engineered pathways – requirement at least **100 g/L** for commodity chemicals (1,3-propanediol 135 g/L) or **1 g/L** for pharmaceuticals (taxadiene 1g/L)
- complexity of life



Perspectives of ME

- catalog of potentially useful **promiscuous activities** of known enzymes
- **screening** of new host organisms, pathways, enzymes (metagenome approach vs. sequencing and bioinformatics)
- construction of bacterial **chasses** with minimal genomes
- ***in silico* screening**
- ***de novo* design** of new enzymes (*in silico*) and gene synthesis
- engineering of ***in vitro* systems** (reduction of complexity)
- from the lab to the real applications: **decreasing the costs of the processes** (from gene synthesis to product purification)