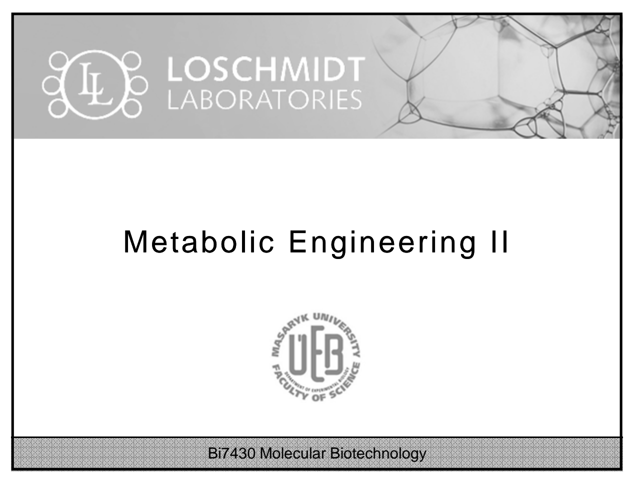
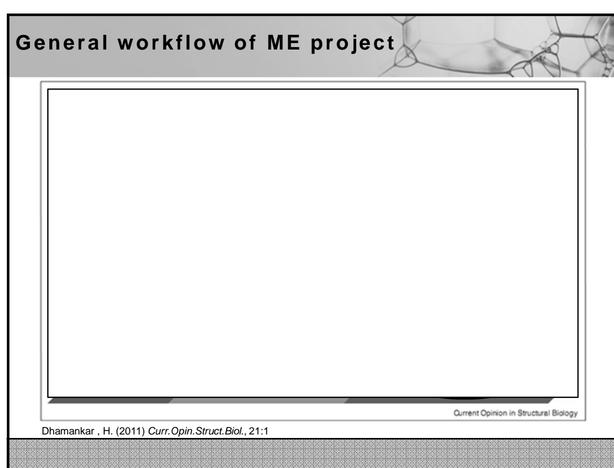
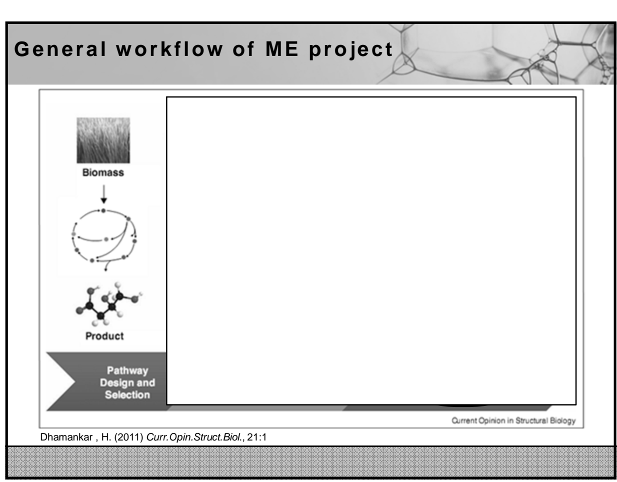
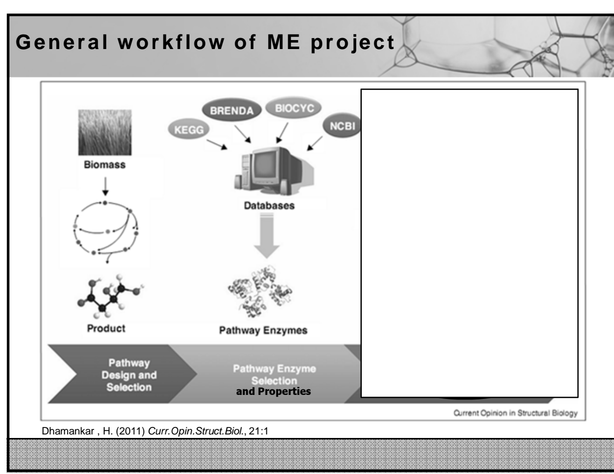
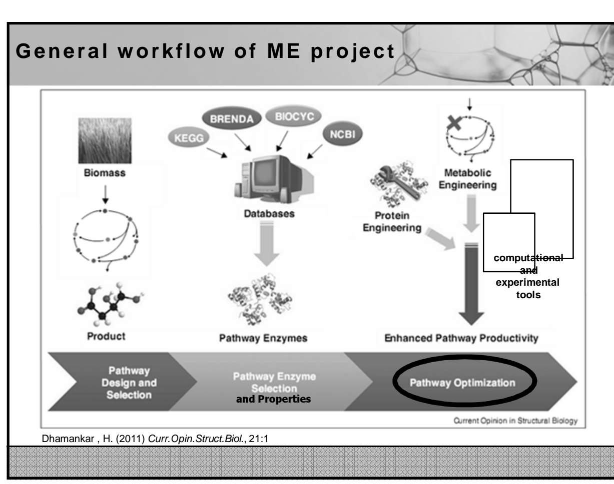
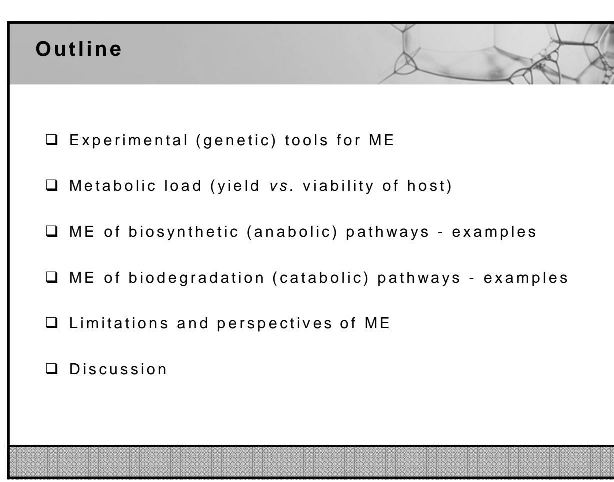

LOSCHMIDT
LABORATORIES
Metabolic Engineering II
Bi7430 Molecular Biotechnology


General workflow of ME project
Current Opinion in Structural Biology
Dhamankar , H. (2011) Curr.Opin.Struct.Biol., 21:1


General workflow of ME project
Biomass
Product
Pathway Design and Selection
Current Opinion in Structural Biology
Dhamankar , H. (2011) Curr.Opin.Struct.Biol., 21:1


General workflow of ME project
KEGG
BRENDA
BIOCYC
NCBI
Biomass
Databases
Product
Pathway Enzymes
Pathway Design and Selection
Pathway Enzyme Selection and Properties
Current Opinion in Structural Biology
Dhamankar , H. (2011) Curr.Opin.Struct.Biol., 21:1


General workflow of ME project
KEGG
BRENDA
BIOCYC
NCBI
Biomass
Databases
Metabolic Engineering
Protein Engineering
computational and experimental tools
Product
Pathway Enzymes
Enhanced Pathway Productivity
Pathway Design and Selection
Pathway Enzyme Selection and Properties
Pathway Optimization
Current Opinion in Structural Biology
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

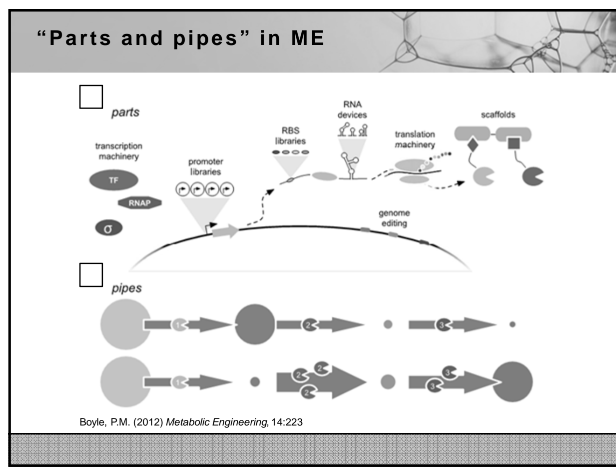
Experimental tools for ME

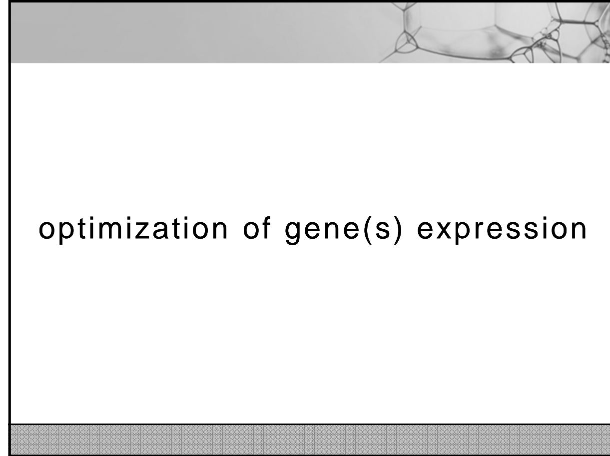
EXPERIMENTAL TOOLS ARE APPLIED HAND IN HAND WITH THEORETICAL TOOLS

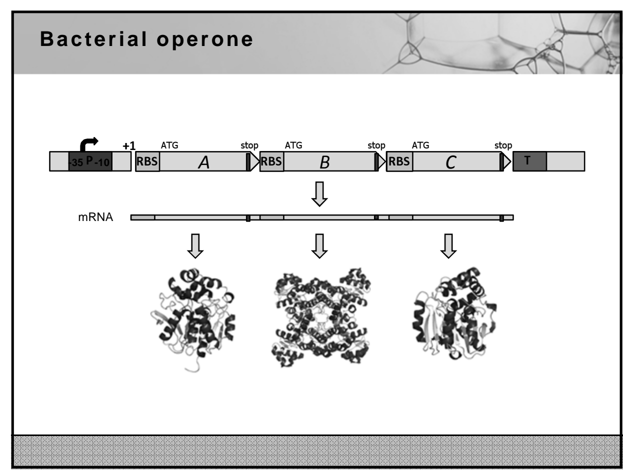
- **experimentals tools = genetic tools (recombinant DNA technology)**
- **production of transgenic organisms**

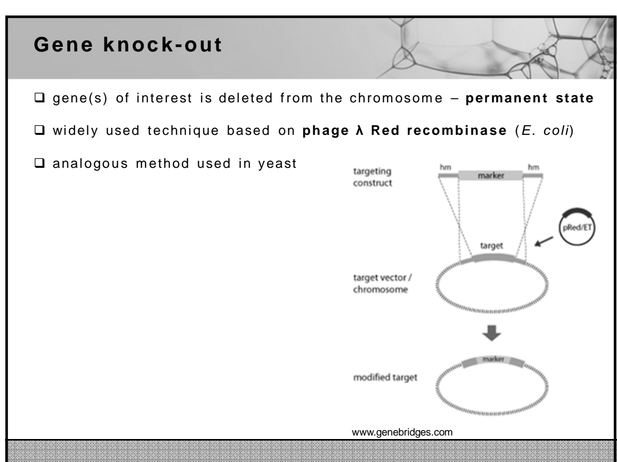
engineering input on level of:

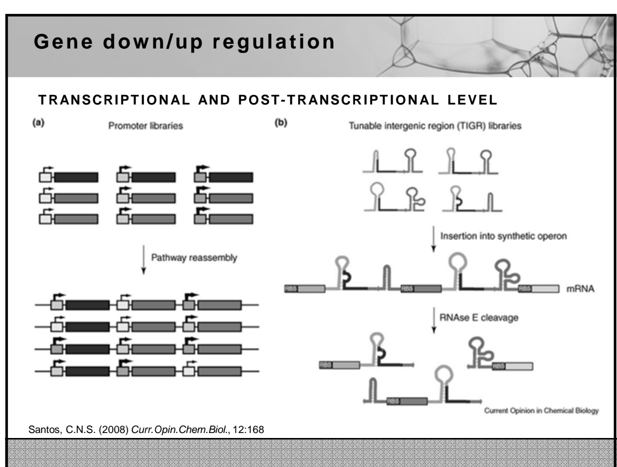
- **gene expression (DNA/RNA):** gene knockout, gene down/up-regulation, heterologous expression, codon optimization, chromosomal integration of gene(s)
- **protein:** protein engineering, proximity of enzymes (substrate channeling)
- **small molecules:** cofactor balancing

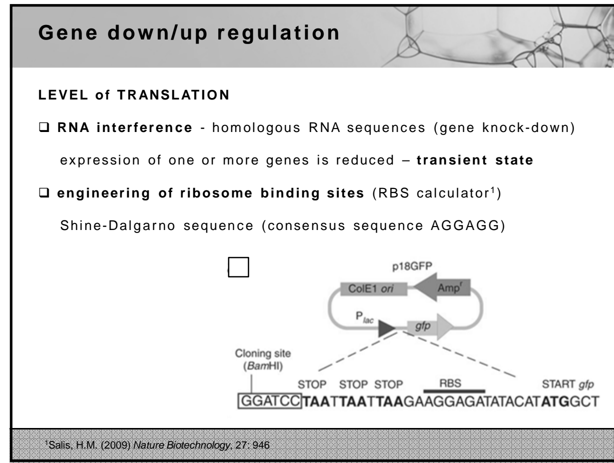


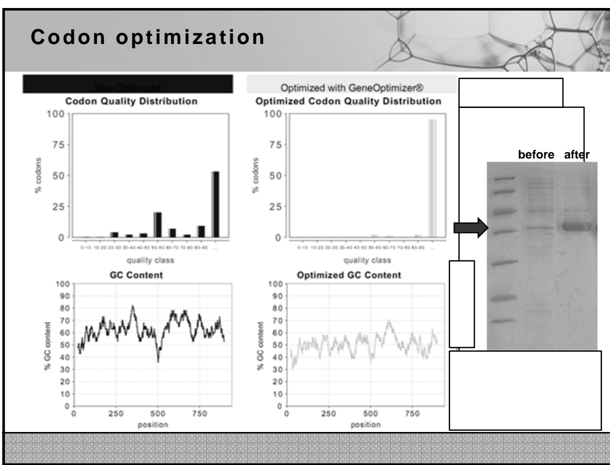


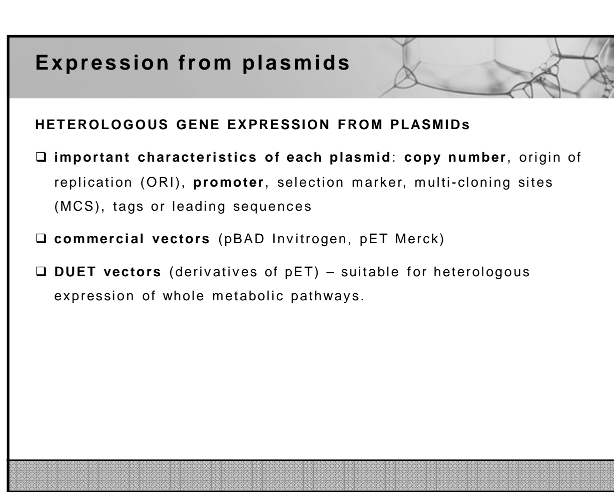

Bacterial operone
+1
ATG
stop
-35 P -10
RBS
A
B
C
T
mRNA

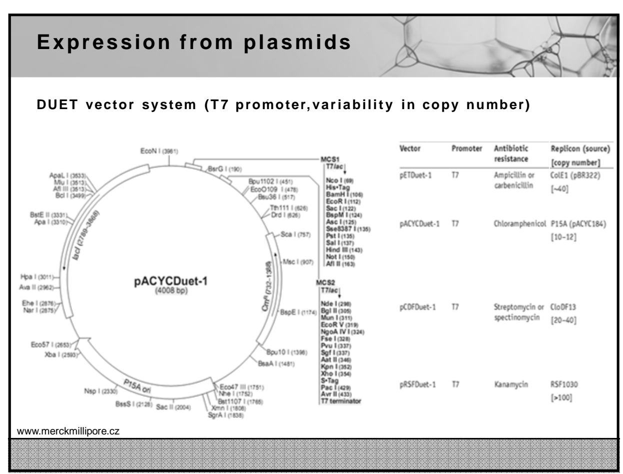

Gene knock-out
❑ gene(s) of interest is deleted from the chromosome – **permanent state**
❑ widely used technique based on **phage λ Red recombinase** (*E. coli*)
❑ analogous method used in yeast
targeting construct
hm
marker
hm
pRed/ET
target
target vector / chromosome
modified target
marker
www.genebridges.com


Gene down/up regulation
TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL LEVEL
(a)
Promoter libraries
(b)
Tunable intergenic region (TIGR) libraries
Pathway reassembly
Insertion into synthetic operon
mRNA
RNAse E cleavage
Current Opinion in Chemical Biology
Santos, C.N.S. (2008) Curr.Opin.Chem.Biol., 12:168

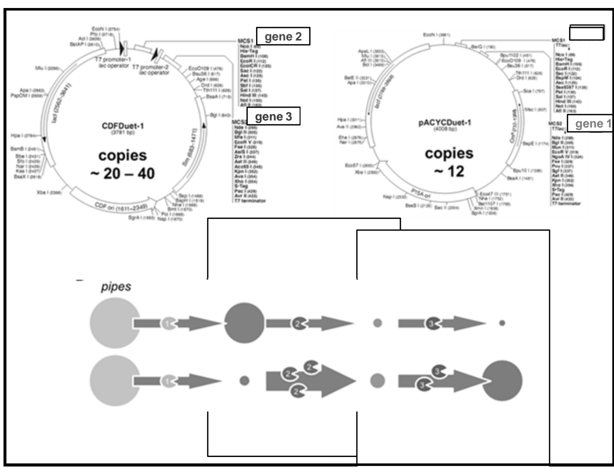

Gene down/up regulation
LEVEL of TRANSLATION
RNA interference - homologous RNA sequences (gene knock-down)
expression of one or more genes is reduced – transient state
engineering of ribosome binding sites (RBS calculator[1])
Shine-Dalgarno sequence (consensus sequence AGGAGG)
p18GFP
ColE1 ori
Amp
P lac
gfp
Cloning site (BamHI)
STOP STOP STOP
RBS
START gfp
GGATCCTAATTAATTAAGAAGGAGATATACATATGGCT
[1]Salis, H.M. (2009) Nature Biotechnology, 27: 946

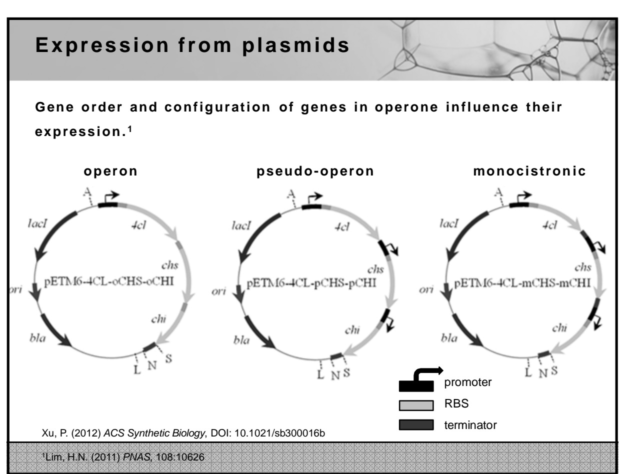

Codon optimization
Optimized with GeneOptimizer®
Codon Quality Distribution
Optimized Codon Quality Distribution
% codons
quality class
GC Content
Optimized GC Content
% GC content
position
before
after


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.



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]





Expression from chromosome

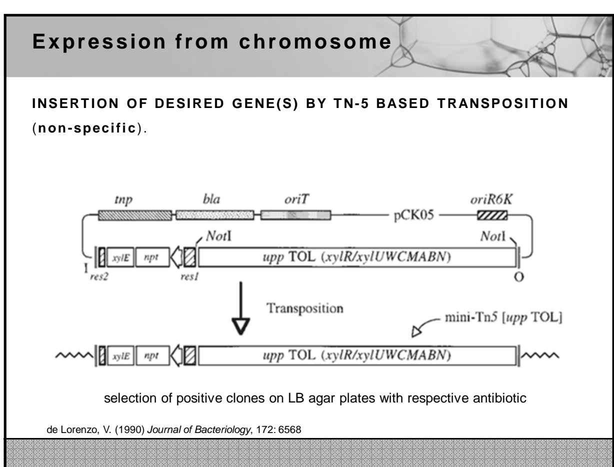
HETEROLOGOUS GENE EXPRESSION FROM CHROMOSOME

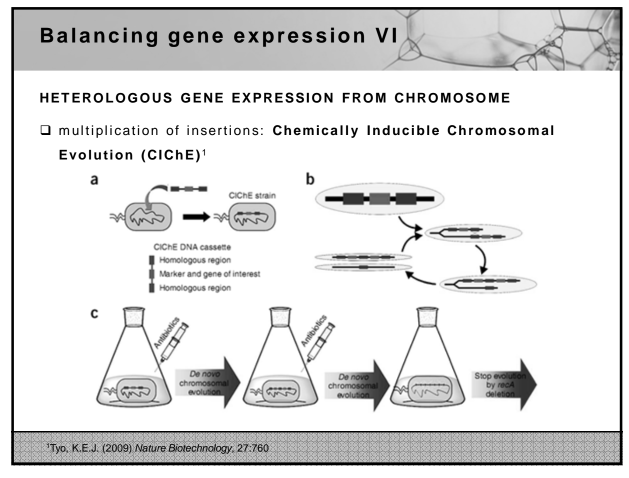
- expression from chromosome is **advantageous** (higher stability, no antibiotic markers)
- **methods for integration:** homologous recombination (*recA*, *λ Red*), transposition (Tn5 and Tn7-based vectors)
- integration of **single genes** or whole synthetic **operones**
- subsequent duplication or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

Expression from chromosome

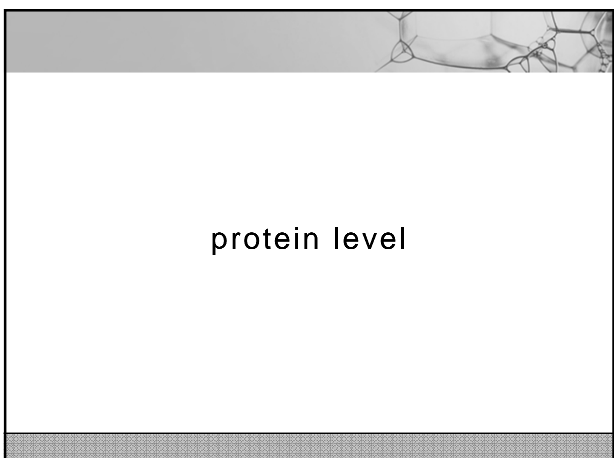
SYNTHETIC OPERON DESIGN (software GeneDesigner 2.0)

P
+1
ATG
stop
RBS *dhaA*
RBS *hheC*
RBS *echA*
T
mRNA

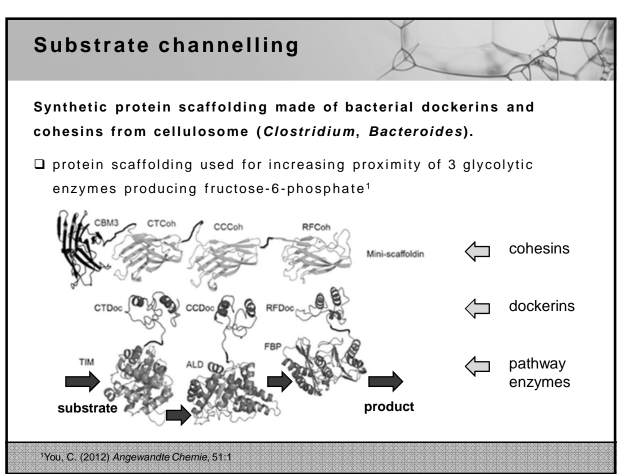




Balancing gene expression VI
HETEROLOGOUS GENE EXPRESSION FROM CHROMOSOME
❑ multiplication of insertions: Chemically Inducible Chromosomal Evolution (CIChE)1
a
b
CIChE strain
CIChE DNA cassette
Homologous region
Marker and gene of interest
Homologous region
c
Antibiotics
De novo chromosomal evolution
Antibiotics
De novo chromosomal evolution
Stop evolution by recA deletion
1Tyo, 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-phosphate1
CBM3
CTCoh
CCCoh
RFCoh
Mini-scaffoldin
CTDoc
CCDoc
RFDoc
TIM
ALD
FBP
substrate
product
cohesins
dockerins
pathway enzymes
1You, C. (2012) Angewandte Chemie, 51:1

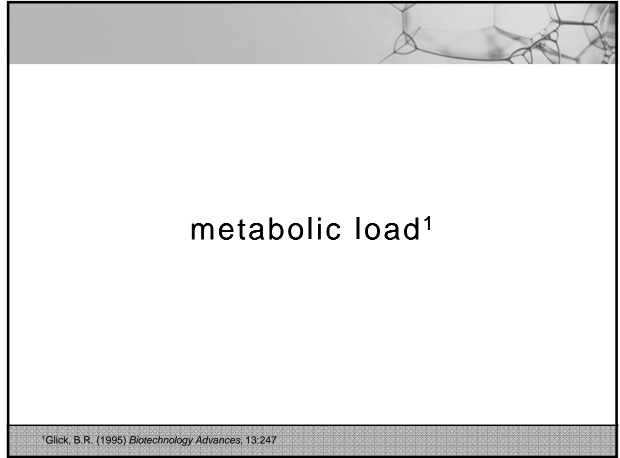
small molecules level

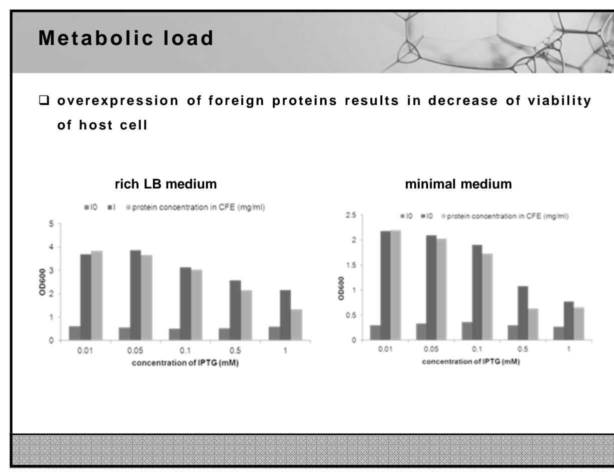
Cofactor balancing

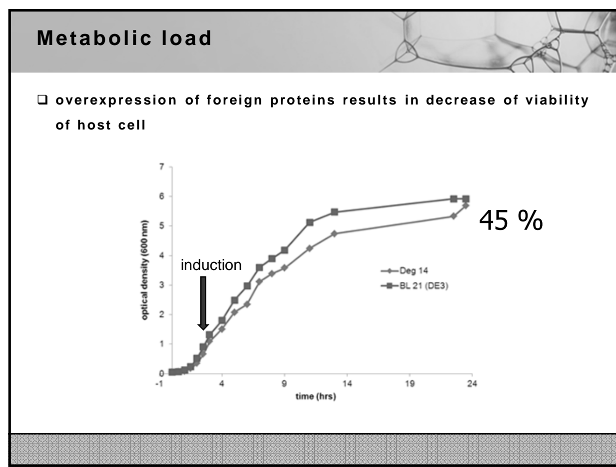
- cofactors play a critical role especially in **redox reactions** (NAD(H), NADP(H))
- natural pathways (e.g. glycolysis) often employ **oxidoreductases**
- cofactor recycling and balancing is essential
- solution: enzyme mediated **cofactor recycling** through overexpression of NAD^+ kinase, transhydrogenases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

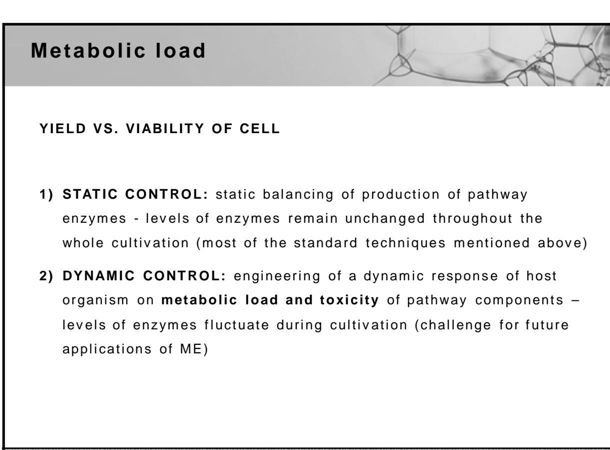
$NADP^+$

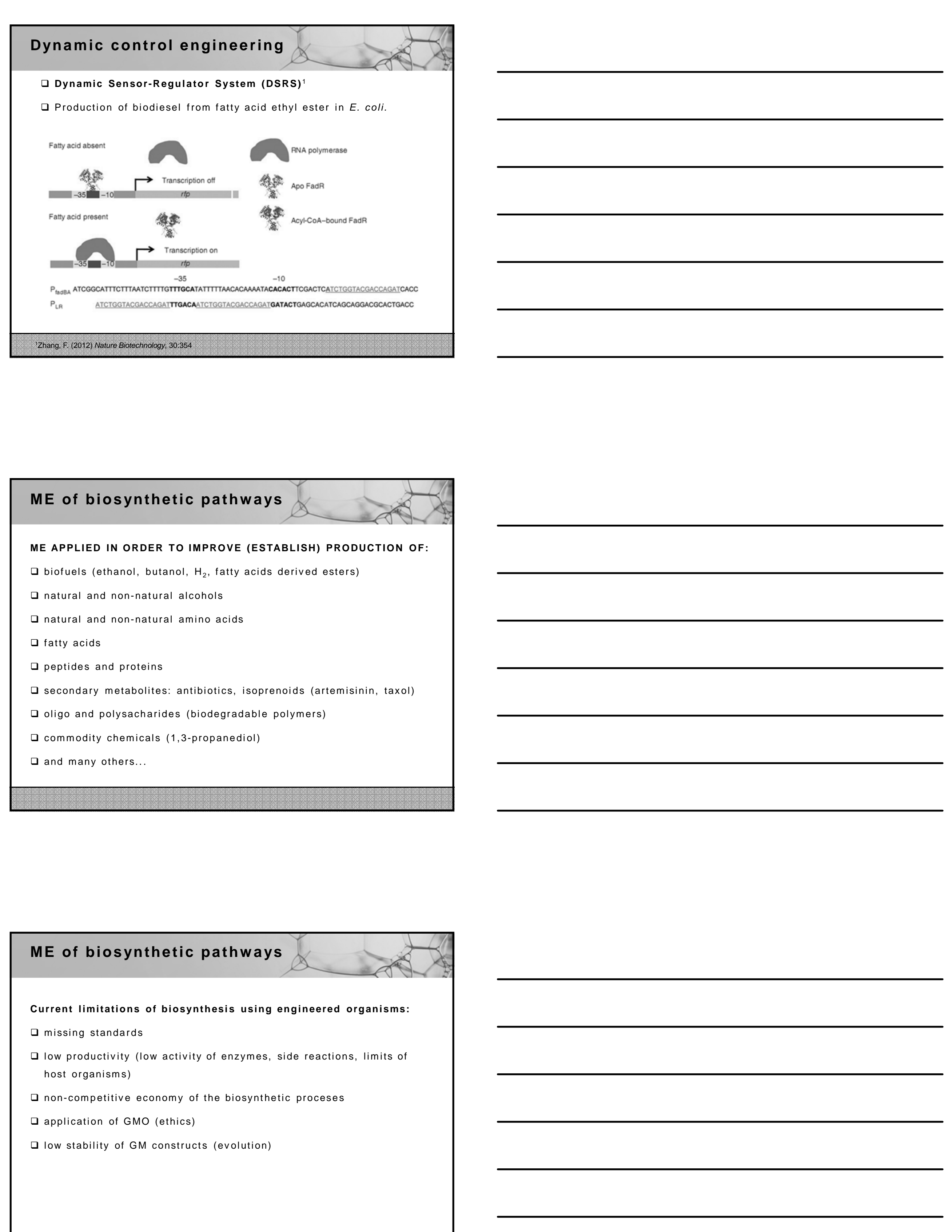
NADPH



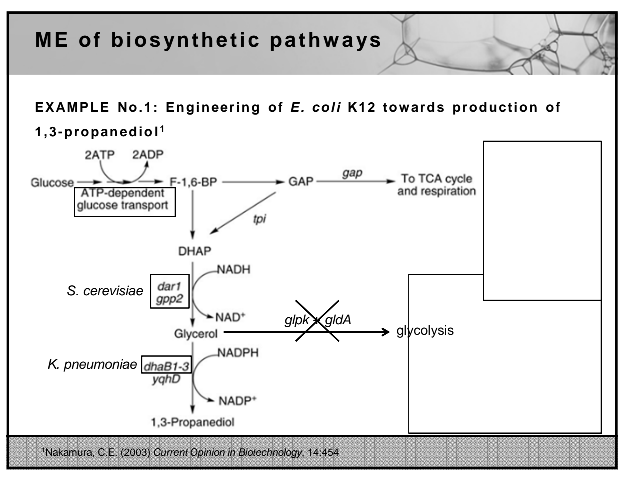

Metabolic load
overexpression of foreign proteins results in decrease of viability of host cell
rich LB medium
minimal medium
OD600
concentration of IPTG (mM)

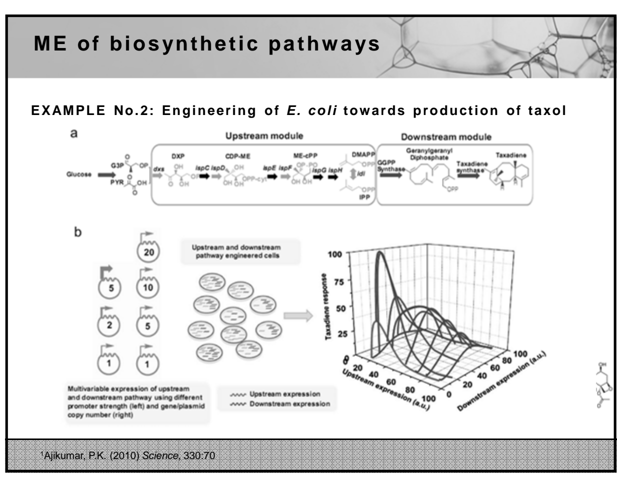

Metabolic load
overexpression of foreign proteins results in decrease of viability of host cell
induction
45 %
optical density (600 nm)
time (hrs)
Deg 14
BL 21 (DE3)

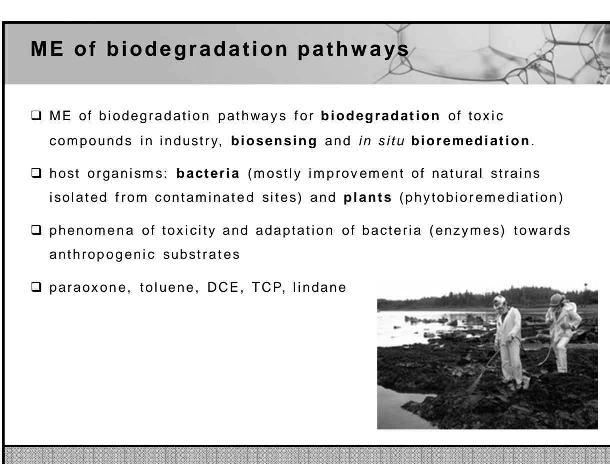

Metabolic load
YIELD VS. VIABILITY OF CELL
1) STATIC CONTROL: static balancing of production of pathway enzymes - levels of enzymes remain unchanged throughout the whole cultivation (most of the standard techniques mentioned above)
2) DYNAMIC CONTROL: engineering of a dynamic response of host organism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



Dynamic control engineering
Dynamic Sensor-Regulator System (DSRS)[1]
Production of biodiesel from fatty acid ethyl ester in E. coli.
Fatty acid absent
RNA polymerase
Transcription off
–35
–10
rfp
Apo FadR
Fatty acid present
Acyl-CoA–bound FadR
Transcription on
–35
–10
rfp
–35
–10
P fadBA ATCGGCATTTCTTTAATCTTTTGTTTGCATATTTTTAACACAAAATACACACTTCGACTCATCTGGTACGACCAGATCACC
P LR ATCTGGTACGACCAGATTTGACAATCTGGTACGACCAGATGATACTGAGCACATCAGCAGGACGCACTGACC
[1]Zhang, F. (2012) Nature Biotechnology, 30:354
ME of biosynthetic pathways
ME APPLIED IN ORDER TO IMPROVE (ESTABLISH) PRODUCTION OF:
biofuels (ethanol, butanol, H2, 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)
low stability of GM constructs (evolution)


ME of biosynthetic pathways
EXAMPLE No.1: Engineering of E. coli K12 towards production of 1,3-propanediol1
2ATP
2ADP
Glucose
ATP-dependent glucose transport
F-1,6-BP
GAP
gap
To TCA cycle and respiration
tpi
DHAP
NADH
S. cerevisiae
dar1
gpp2
NAD+
Glycerol
glpk
gldA
glycolysis
K. pneumoniae
dhaB1-3
yqhD
NADPH
NADP+
1,3-Propanediol
1Nakamura, C.E. (2003) Current Opinion in Biotechnology, 14:454


ME of biosynthetic pathways
EXAMPLE No.2: Engineering of E. coli towards production of taxol
a
Upstream module
Downstream module
Glucose
b
Upstream and downstream pathway engineered cells
Taxadiene response
Upstream expression (a.u.)
Downstream expression (a.u.)
Multivariable expression of upstream and downstream pathway using different promoter strength (left) and gene/plasmid copy number (right)
Upstream expression
Downstream expression
1Ajikumar, 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
- low stability of GM constructs

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)

1,2,3-trichloropropane → (DhaA) → 2,3-dichloropropan-1-ol ⇌ (HheC) ⇌ epichlorohydrin → (EchA) → 3-chloropropane-1,2-diol ⇌ (HheC) ⇌ glycidol → (EchA) → glycerol → glycolysis

TCP pathway

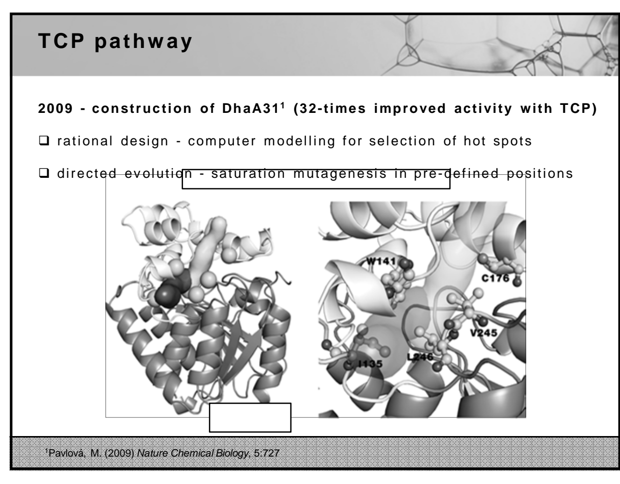
Project workflow:

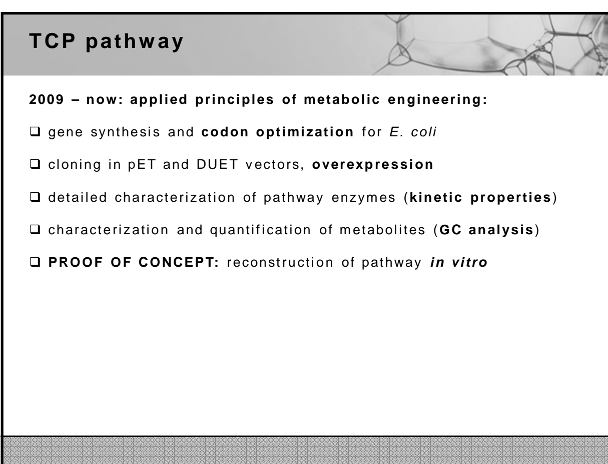
- 1989 – description of pathway for utilization of halogenated alcohols from *Agrobacterium radiobacter* AD1 (**HheC**, **EchA**)
- 1997 – description of haloalkane dehalogenase **DhaA** (*Rhodococcus sp.*)
- 1999 – heterologous expression of dhaAwt in *A.radiobacter* AD1[1]
- 2002 – heterologous expression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

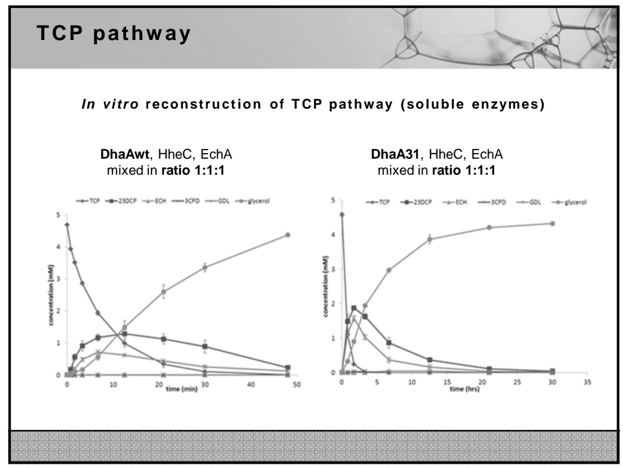
PROBLEMS:

- 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 DhaA31¹ (32-times improved activity with TCP)
❑ rational design - computer modelling for selection of hot spots
❑ directed evolution - saturation mutagenesis in pre-defined positions
W141
C176
V245
I135
L246
¹Pavlová, M. (2009) Nature Chemical Biology, 5:727

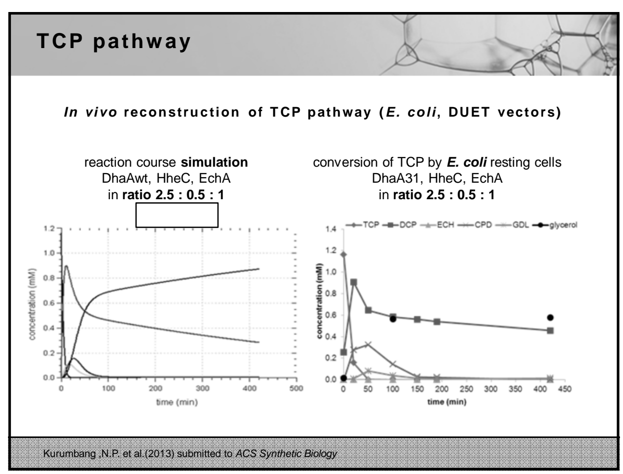

TCP pathway
2009 – now: applied principles of metabolic engineering:
❑ gene synthesis and codon optimization for E. coli
❑ cloning in pET and DUET vectors, overexpression
❑ detailed characterization of pathway enzymes (kinetic properties)
❑ characterization and quantification of metabolites (GC analysis)
❑ PROOF OF CONCEPT: reconstruction of pathway in vitro

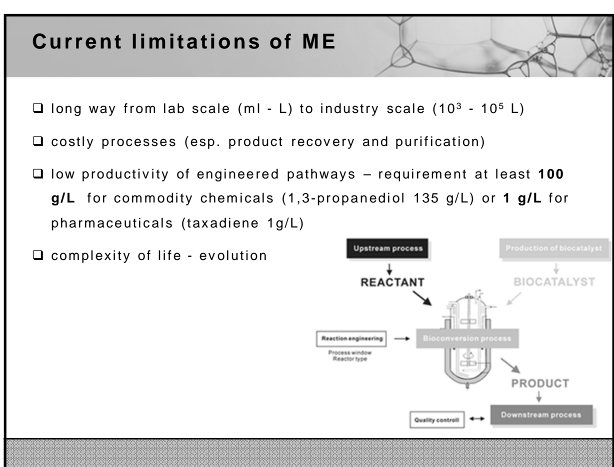

TCP pathway
In vitro reconstruction of TCP pathway (soluble enzymes)
DhaAwt, HheC, EchA
mixed in ratio 1:1:1
DhaA31, HheC, EchA
mixed in ratio 1:1:1
TCP
23DCP
ECH
3CPD
GDL
glycerol
concentration (mM)
time (min)
time (hrs)

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

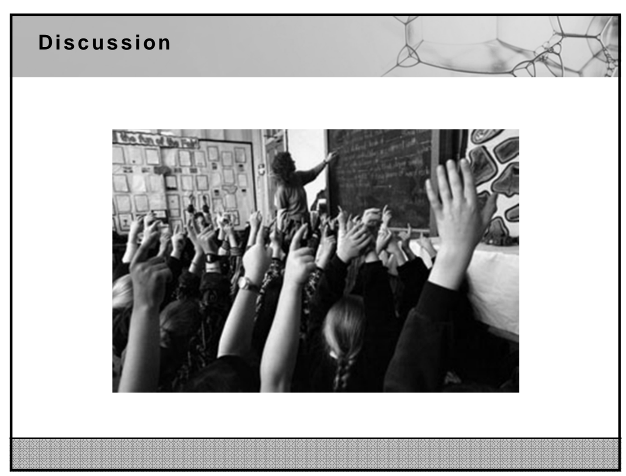


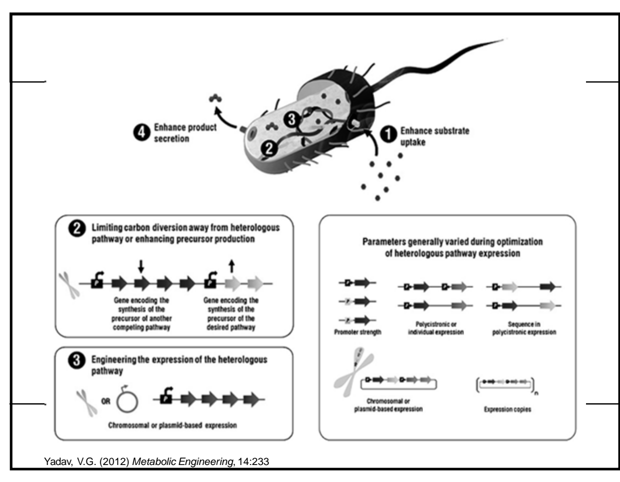


Perspectives of ME

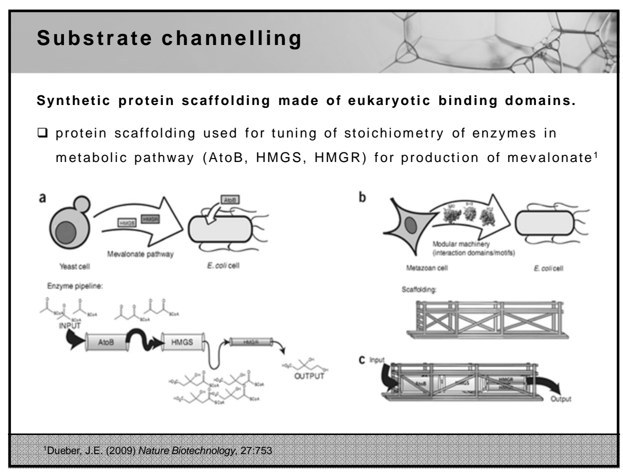
- catalog of potentially useful **promiscuous activities** of known enzymes
- **screening** of new host organisms, pathways, enzymes (metagenome approach vs. sequencing and bioinformatics)
- construction of bacterial **chasses** with minimal genomes
- ***in silico* screening**
- ***de novo* design** 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)

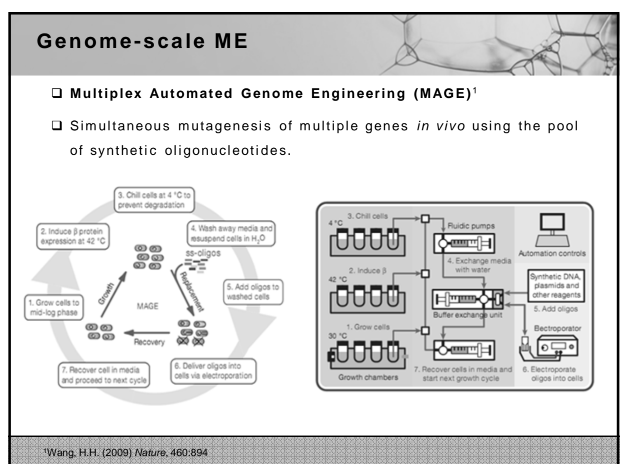
Discussion

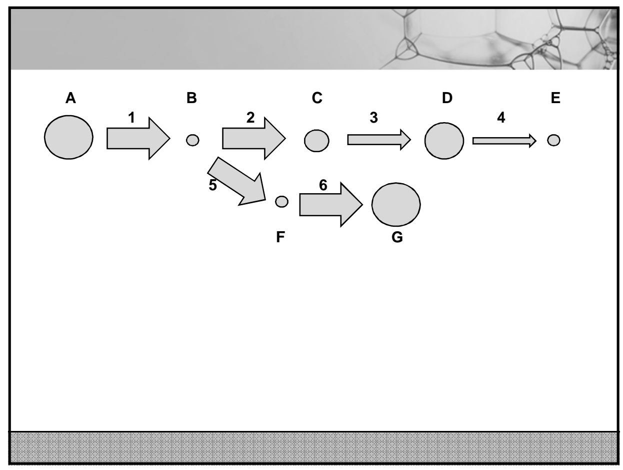


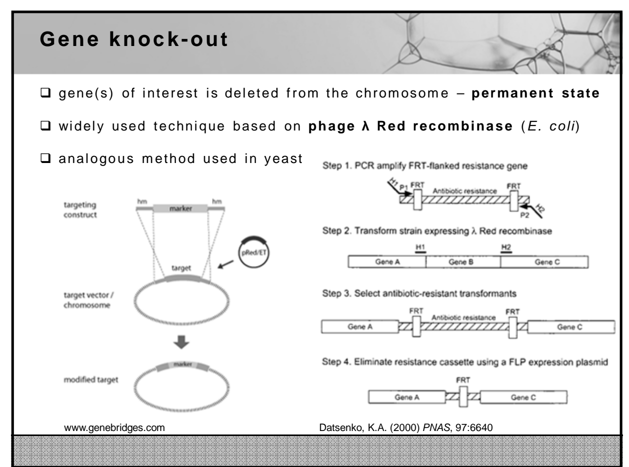


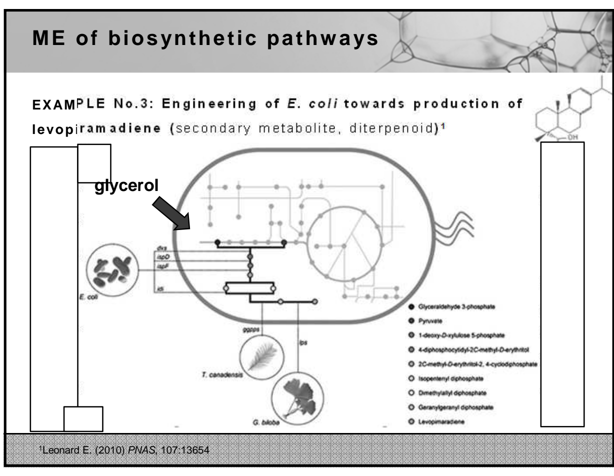
Yadav, V.G. (2012) *Metabolic Engineering*, 14:233


Substrate channelling
Synthetic protein scaffolding made of eukaryotic binding domains.
protein scaffolding used for tuning of stoichiometry of enzymes in metabolic pathway (AtoB, HMGS, HMGR) for production of mevalonate[1]
a
b
Mevalonate pathway
Yeast cell
E. coli cell
Modular machinery (interaction domains/motifs)
Metazoan cell
E. coli cell
Enzyme pipeline:
Scaffolding:
INPUT
AtoB
HMGS
OUTPUT
c
Input
Output
[1]Dueber, J.E. (2009) Nature Biotechnology, 27:753


Genome-scale ME
Multiplex Automated Genome Engineering (MAGE)[1]
Simultaneous mutagenesis of multiple genes in vivo using the pool of synthetic oligonucleotides.
3. Chill cells at 4 °C to prevent degradation
2. Induce β protein expression at 42 °C
4. Wash away media and resuspend cells in H_2O
ss-oligos
5. Add oligos to washed cells
1. Grow cells to mid-log phase
MAGE
Growth
Replacement
Recovery
7. Recover cell in media and proceed to next cycle
6. Deliver oligos into cells via electroporation
3. Chill cells
4 °C
Fluidic pumps
Automation controls
4. Exchange media with water
2. Induce β
42 °C
Synthetic DNA, plasmids and other reagents
Buffer exchange unit
5. Add oligos
1. Grow cells
30 °C
Electroporator
Growth chambers
7. Recover cells in media and start next growth cycle
6. Electroporate oligos into cells
[1]Wang, H.H. (2009) Nature, 460:894


A
B
C
D
E
1
2
3
4
5
6
F
G


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
Step 1. PCR amplify FRT-flanked resistance gene
Step 2. Transform strain expressing λ Red recombinase
Step 3. Select antibiotic-resistant transformants
Step 4. Eliminate resistance cassette using a FLP expression plasmid
targeting construct
target vector / chromosome
modified target
www.genebridges.com
Datsenko, K.A. (2000) PNAS, 97:6640


ME of biosynthetic pathways
EXAMPLE No.3: Engineering of E. coli towards production of levopiramadiene (secondary metabolite, diterpenoid)[1]
glycerol
E. coli
T. canadensis
G. biloba
Glyceraldehyde 3-phosphate
Pyruvate
1-deoxy-D-xylulose 5-phosphate
4-diphosphocytidyl-2C-methyl-D-erythritol
2C-methyl-D-erythritol-2, 4-cyclodiphosphate
Isopentenyl diphosphate
Dimethylallyl diphosphate
Geranylgeranyl diphosphate
Levopimaradiene
[1]Leonard E. (2010) PNAS, 107:13654