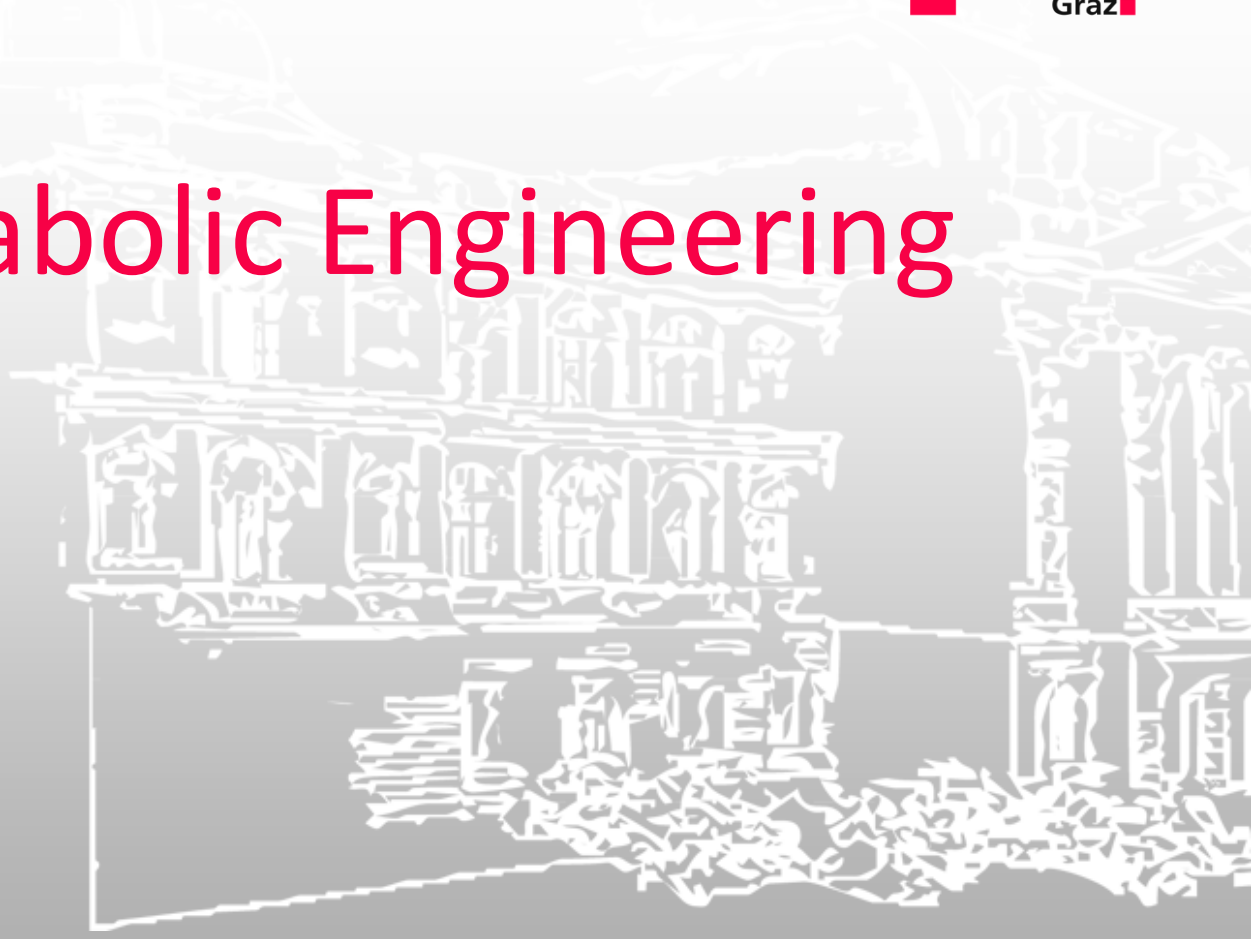
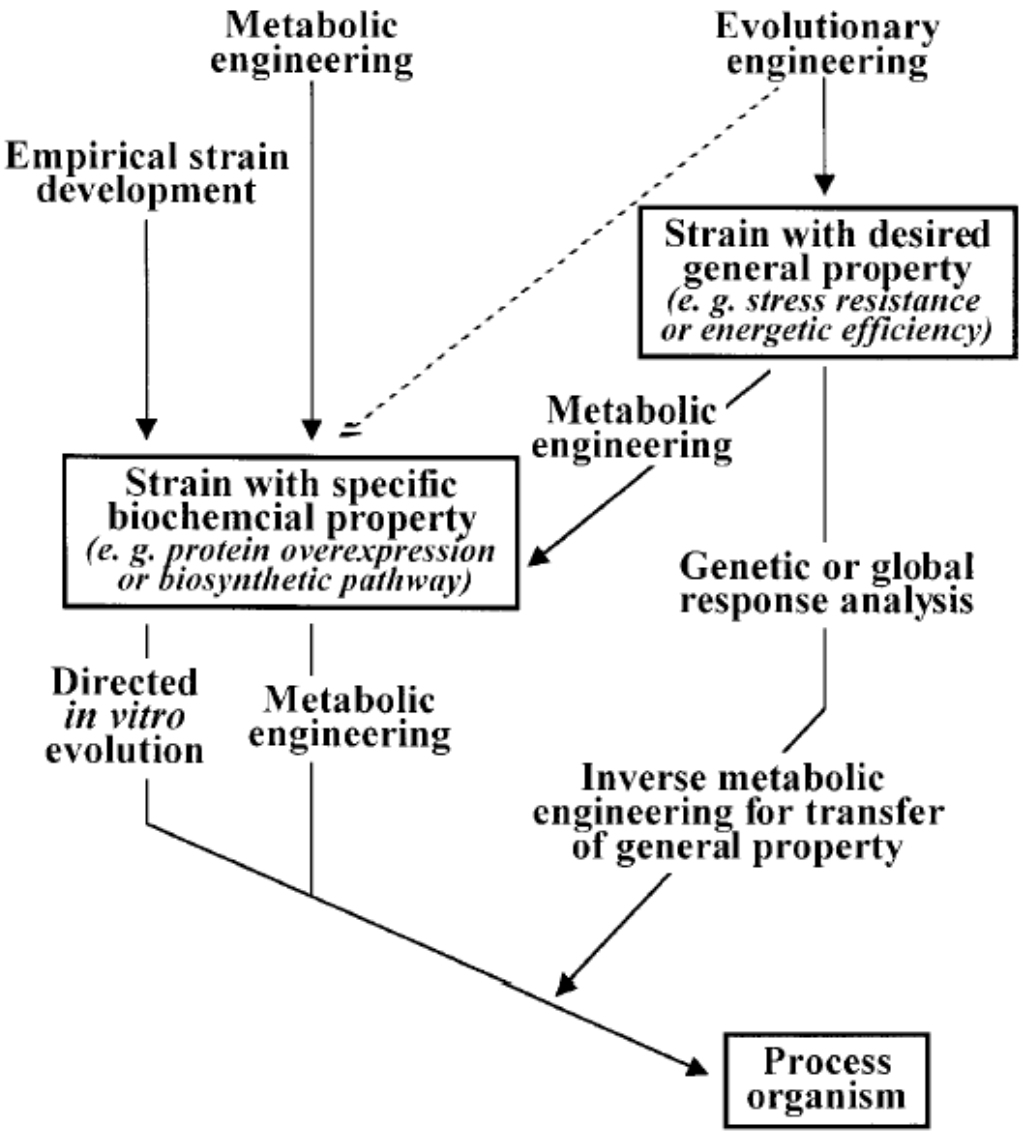
Metabolic Engineering



Strain Development



Strain Development

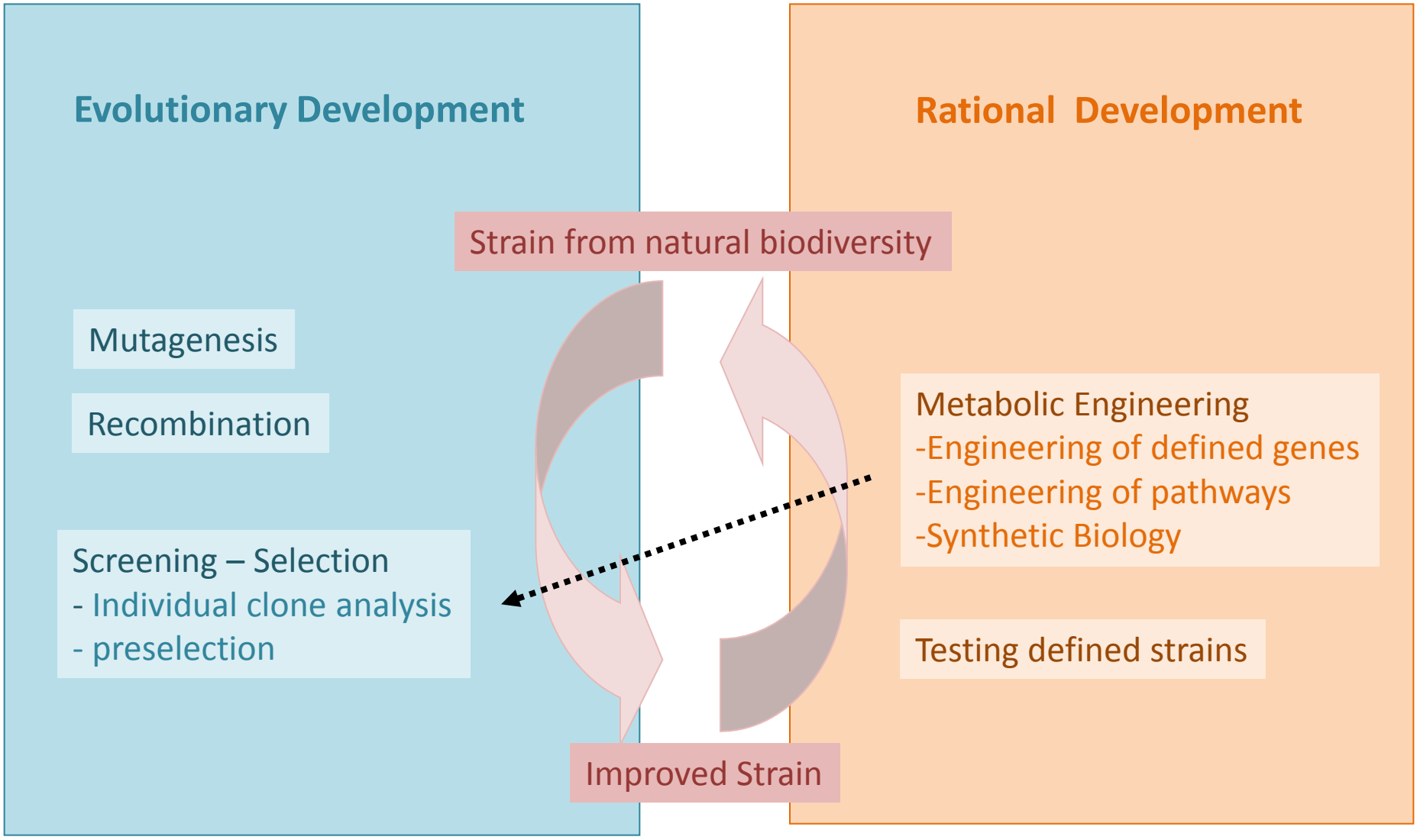


Table 1. Classification of mutations, their origins, and potential effects

Type of change	Length	Source of mutation	Effects[a]
Small local changes			
Substitution Insertion Deletion Duplication	1 bp 1 to several bp 1 to several bp 1 to several bp	Spontaneous mutagenesis Replication infidelities	Gene silencing Gene expression Cryptic gene activation Altered protein specificities
DNA rearrangements			
Inversion Duplication Insertion Deletion Excision	Several bp up to several kb	Homologous recombination Mobile genetic elements (i. e. IS elements, transposons)	Gene silencing Gene expression Cryptic gene activation Gene dosage Gene organization Gene mobilization Domain fusion Domain swapping
DNA acquisition		**Sexual Recombination** **Protoplast fusion**	
Horizontal DNA transfer	Several kb up to hundreds of kb	Transformation Conjugation Transduction (phage-mediated)	Increase of total genetic information content Gene silencing

[a] A particular source of mutation is not necessarily capable of causing all listed effects.

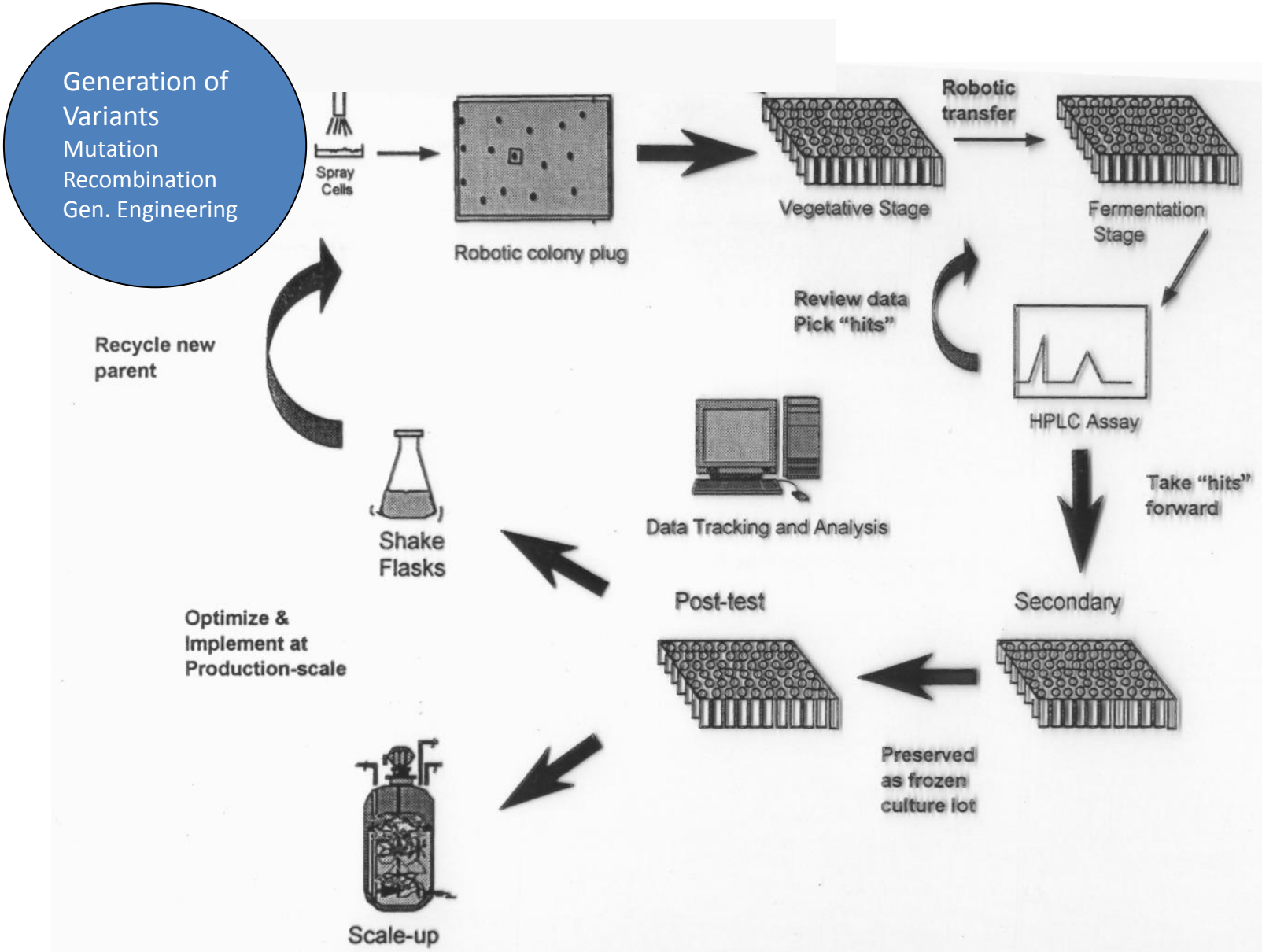


FIGURE 1 Schematic representation of an automated screening system.

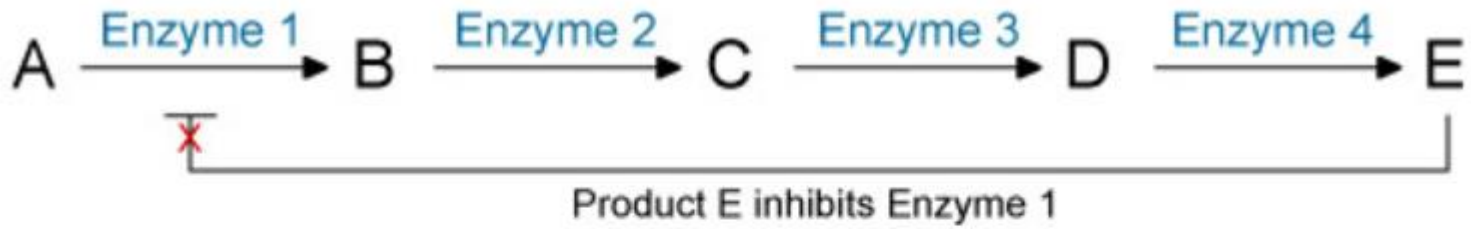
Rational approaches for strain improvement

Knowing about the molecular relationships in the metabolism

Regulation

Regulation of expression

Regulation of enzyme activity



https://wikispaces.psu.edu/download/attachments/48201949/image-8.jpg

Rational approaches for strain improvement

Knowing about the functional relationships of enzymes in the metabolism

Regulation

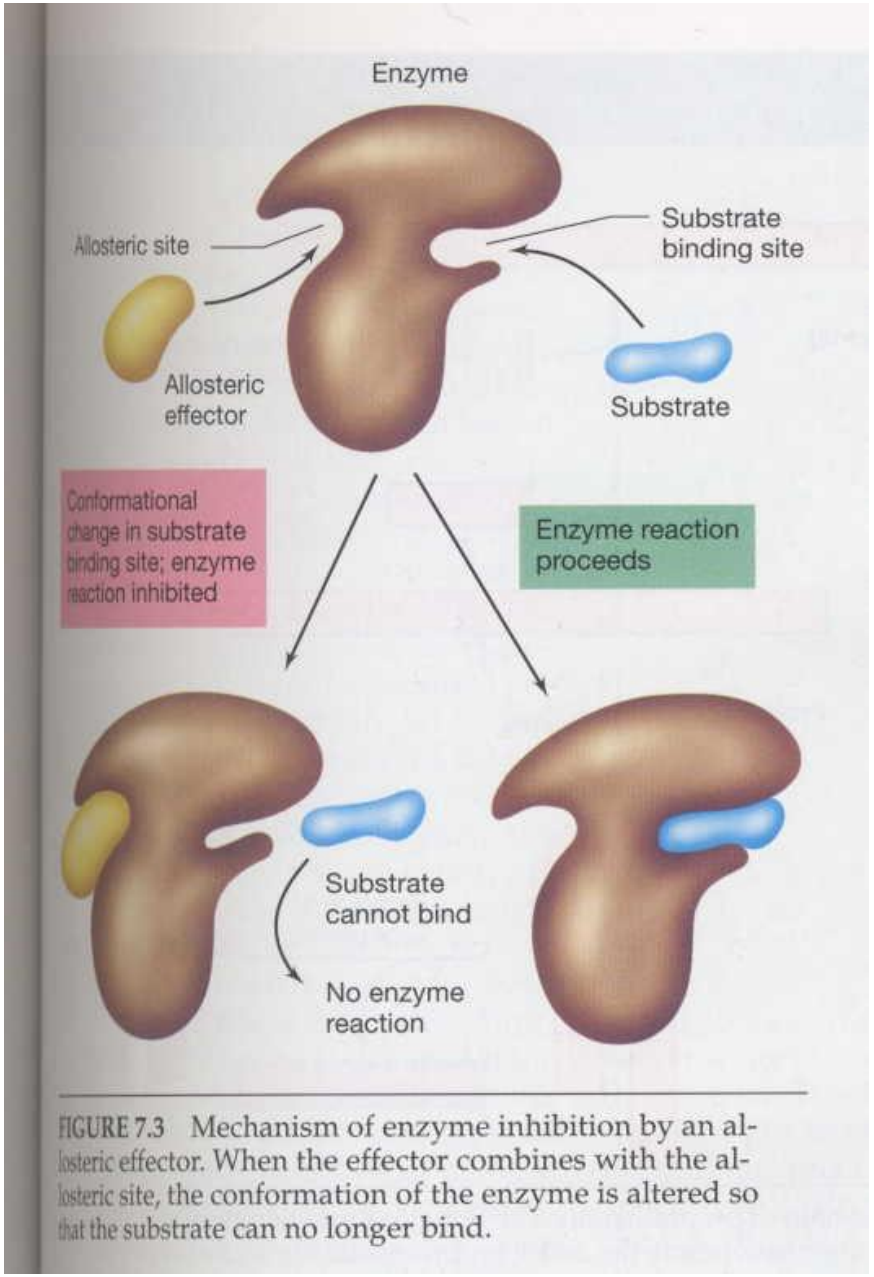
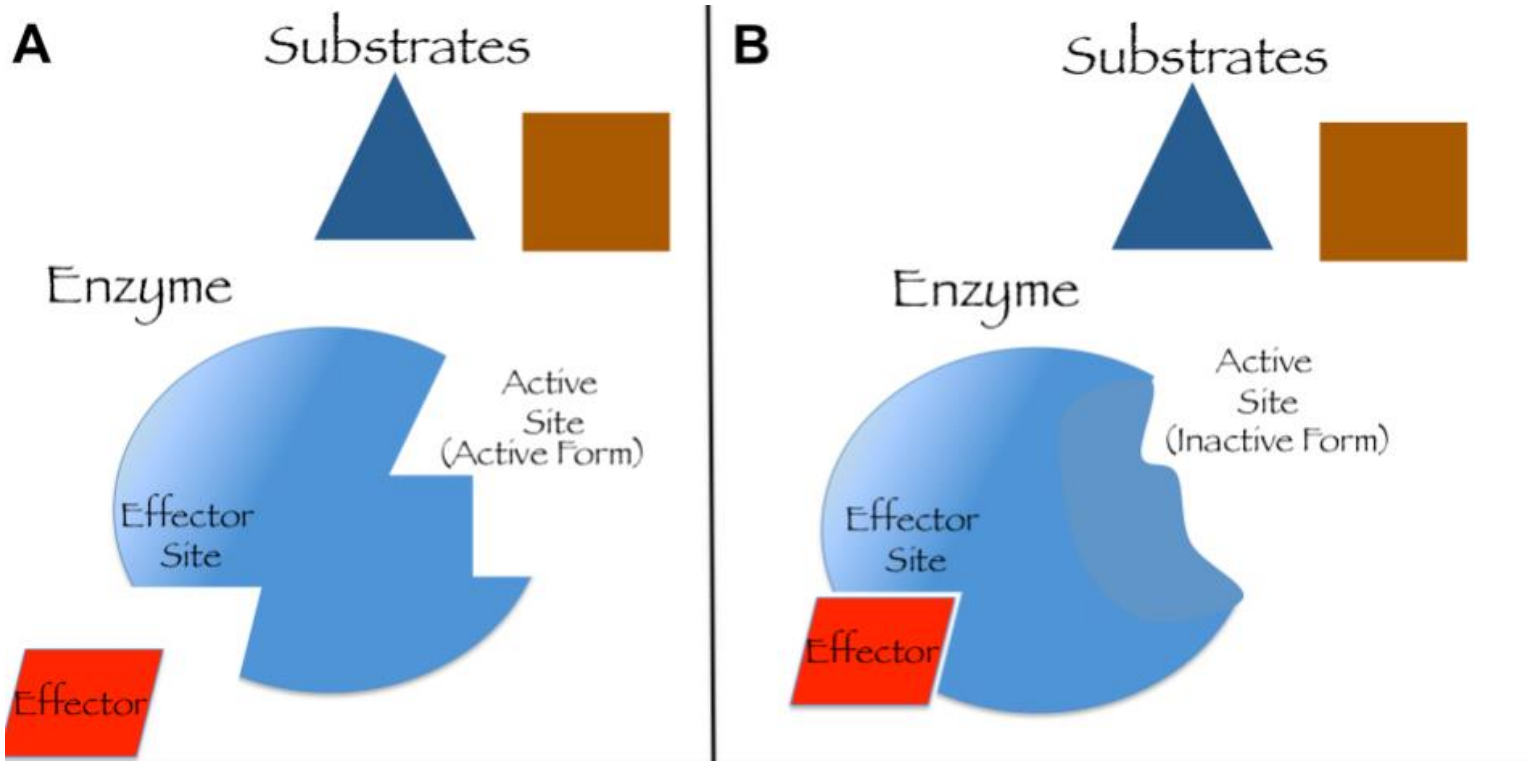


FIGURE 7.3 Mechanism of enzyme inhibition by an allosteric effector. When the effector combines with the allosteric site, the conformation of the enzyme is altered so that the substrate can no longer bind.

Rational approaches for strain improvement

Knowing about the functional relationships of enzymes in the metabolism

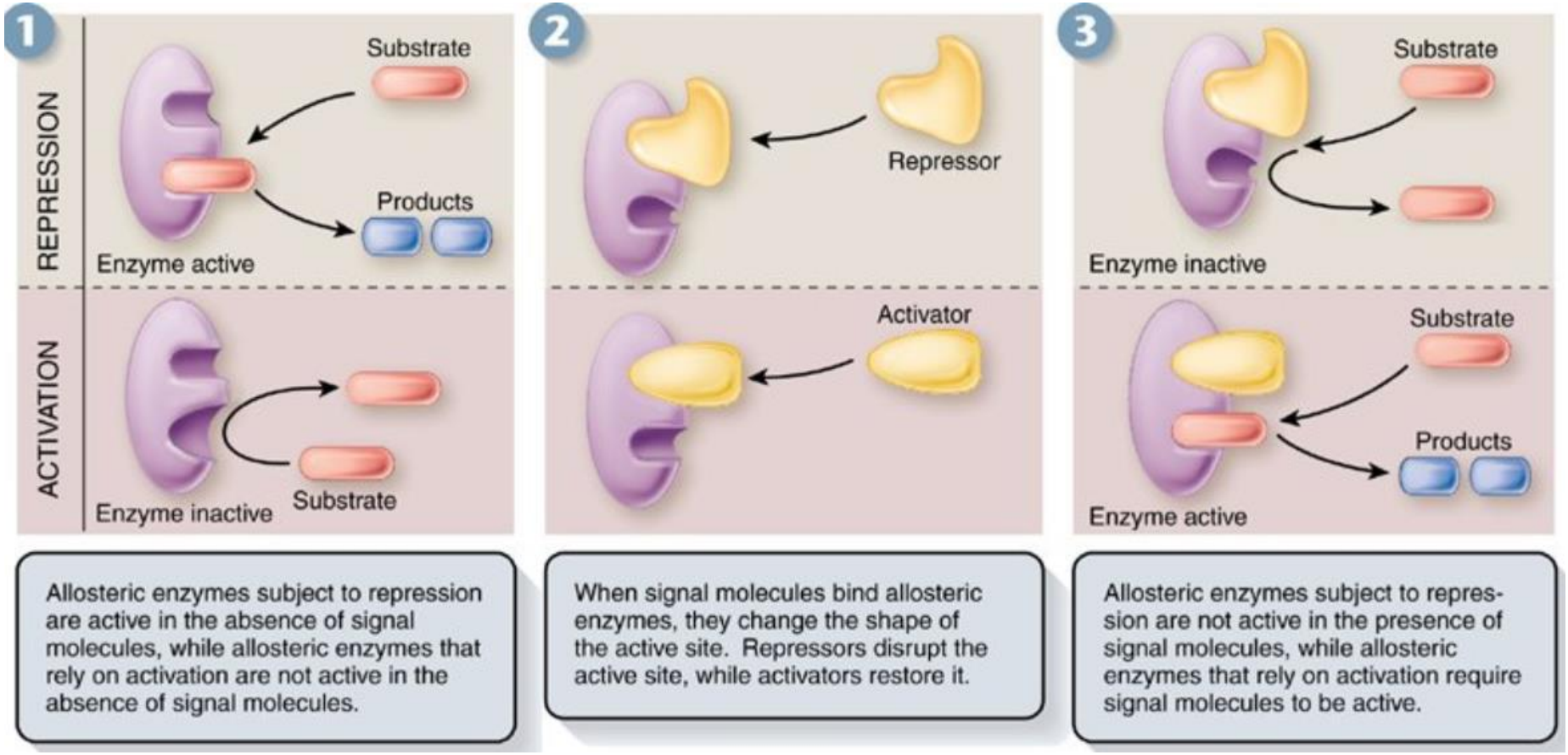
Regulation



Allosteric enzyme with active site and effector site. A) Effector unbound, Active site in active conformation – capable of processing substrate B) Effector bound, Active site in inactive conformation – incapable of processing substrate

https://downhousesoftware.wordpress.com/tag/allosteric/

Effector Actions may be Inhibitory (top) or Activating (bottom)



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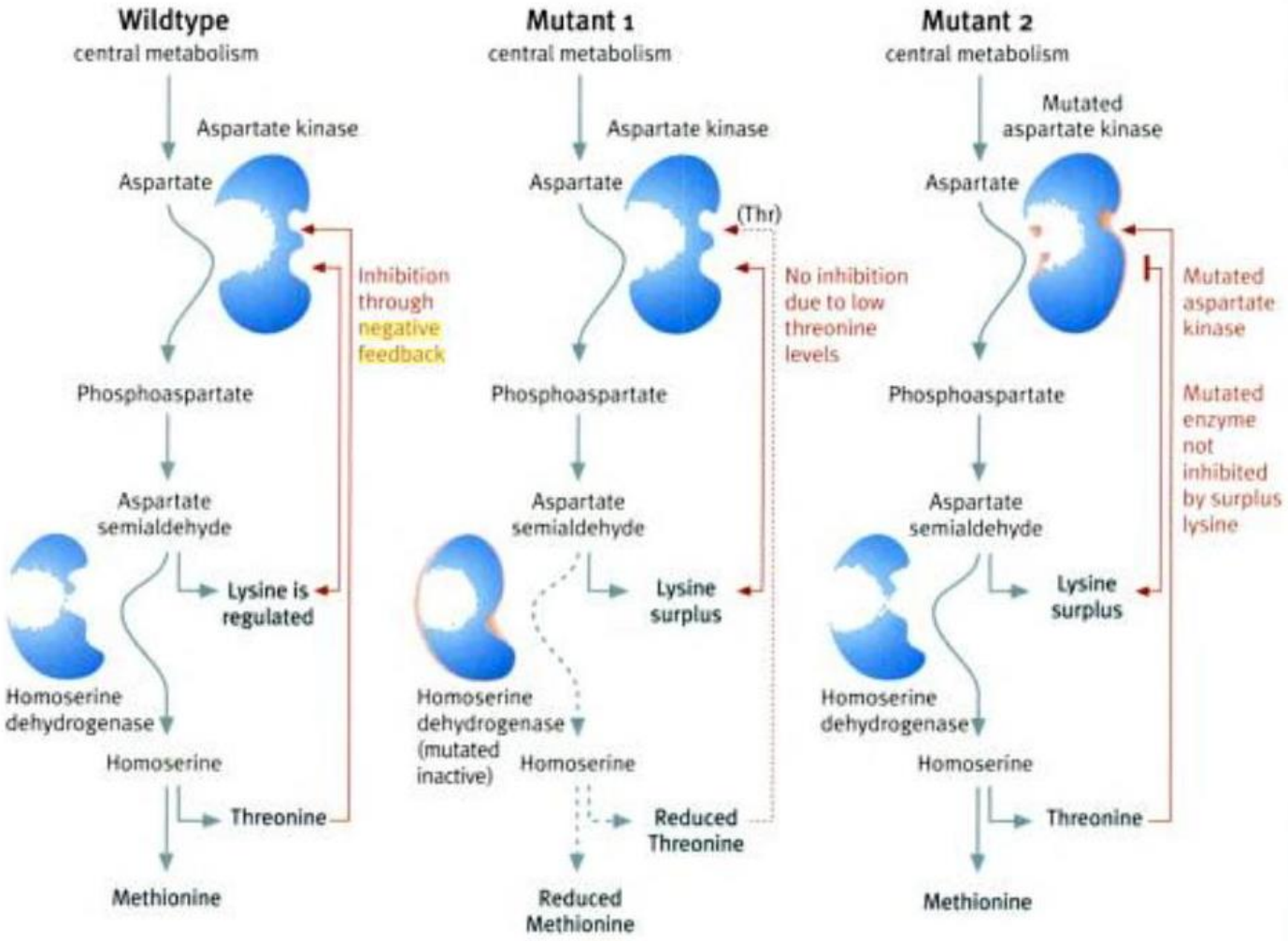
Deactivation of feedback regulation



Corynebacterium glutamicum

Fig. 4.21 Negative feedback during the synthesis of lysine in wildtype *Corynebacterium* (electron microscopic view, top) and two mutants. In the first mutant, the enzyme homoserine dehydrogenase has been inactivated. In the second mutant, aspartate kinase has been modified so that it cannot be inhibited even by excess production of lysine.

Reinhard Renneberg, Biotechnology for Beginners; Elsevier Spektrum, 2006

Screening – Rational Elements

Lysine antimetabolites	Threonine antimetabolites	Tryptophan antimetabolites
s-(2-aminoethyl)-l-cysteine	α-amino-β-hydroxy-valeric acid	5-methyl-tryptophan
4-oxalysine	β-hydroxy-lysine	4-methyl-tryptophan
L-lysine-hydroxamate	Norleucine	6-methyl-tryptophan
2,6-diamino-4-hexenoic acid	Aminohydroxy-valeric acid	5-fluoro-tryptophan
δ-hydroxy-lysine	Norvaline	DL-7-aza-tryptophan
α-chlorcaprolactam	N-2-Thienyl-methionine	2-azatryptophan
trans-4,5-dehydrolysine	2-amino-3-mehtyl-thiobutyric acid	
	2-amino-3-hydroxy-hexanoic acid	

Table 1: amino acid antimetabolites for selection of lysine, threonine or tryptophan overproducing strains

Mutants (phenotype)	Produced amino acid
Tyrosine$^-$	Phenylalanine
Phenylalanine$^-$	Tyrosine
Phe$^-$, Tyr$^-$	Tryptophan
Homoserine$^-$	Lysine
Leucine$^-$	Valine

Table 2: Secretion of amino acids by auxotrophic mutants

Antranikian, „Angewandte Mikrobiologie“, Springer 2006 (adapted)

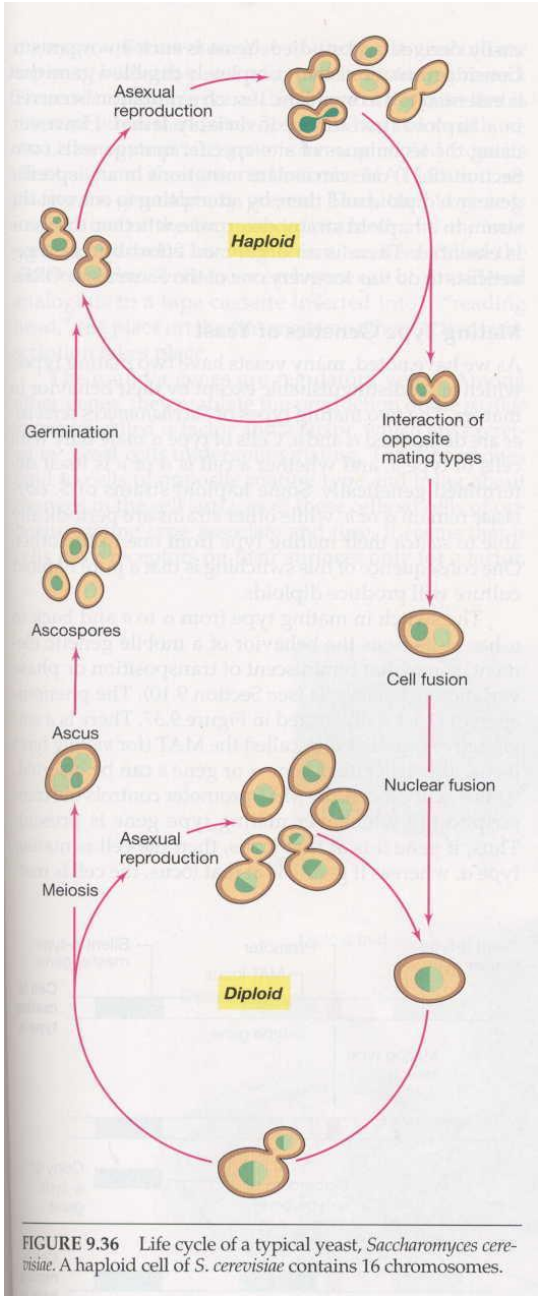


FIGURE 9.36 Life cycle of a typical yeast, *Saccharomyces cerevisiae*. A haploid cell of *S. cerevisiae* contains 16 chromosomes.

Recombination genetics

Specific/targeted crossing of organisms

Cell fusions

Gene transfer via parasexual mechanisms

Essential:
Screening - Selection

Metabolic Engineering

- Enhanced production of metabolites in homologous hosts
- Production of modified or new metabolites
- Modification of substrate utilization
- Metabolic pathway design for degradation of compounds (e.g. xenobiotics)
- Modification of cell properties for improved bioprocessing (e.g. growth, product recovery)

Metabolic Engineering

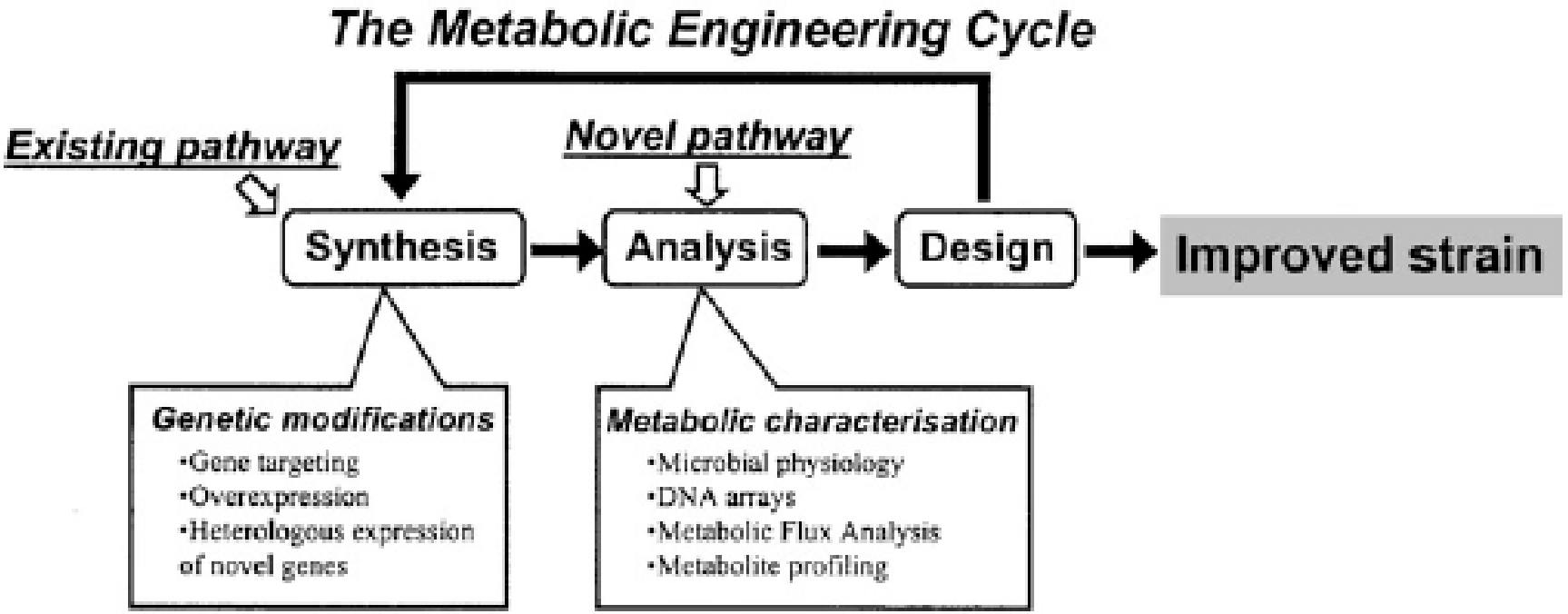
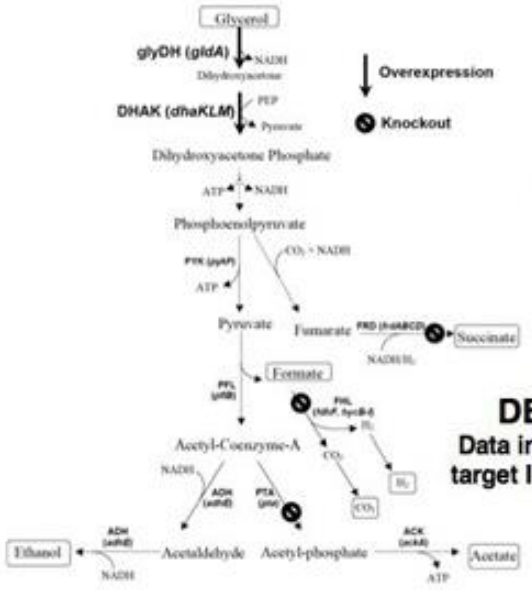


Fig. 1. Principles of metabolic engineering.

Metabolic Engineering (MetEng) and Metabolic Evolution (MetEvo)



Overexpression

Knockout

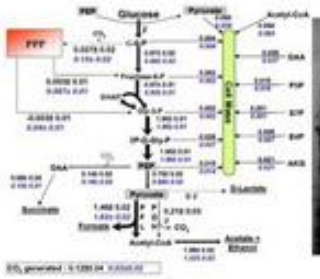
STRAIN CONSTRUCTION
Gene(s) "knockins" & "knockouts"
Mutagenesis & Selection

STRAIN CHARACTERIZATION
Tubes, Flask, Bioreactors

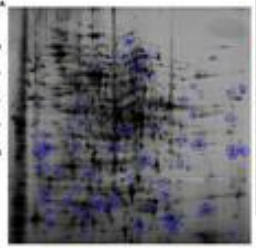
MetEng & MetEvo Cycles

DESIGN
Data integration & target Identification

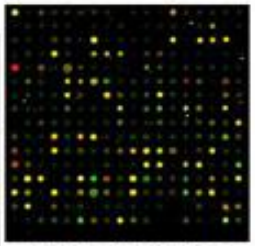
SYSTEMS ANALYSIS
Functional genomics tools



Fluxomics



Proteomics



Transcriptomics

Metrics: Concentration (g/L), Yield (g/g), Rate (g/L/h and g/gDW/h)

http://www.ruf.rice.edu/~metabol/metabolic_engineering.shtml

Analysis of metabolic networks

Essential approach: integral identification of influencing variables (parameters)

Metabolic Networks

Reaction network → involved biochemical reactions and reaction partners

Regulatory network → all regulatory interactions

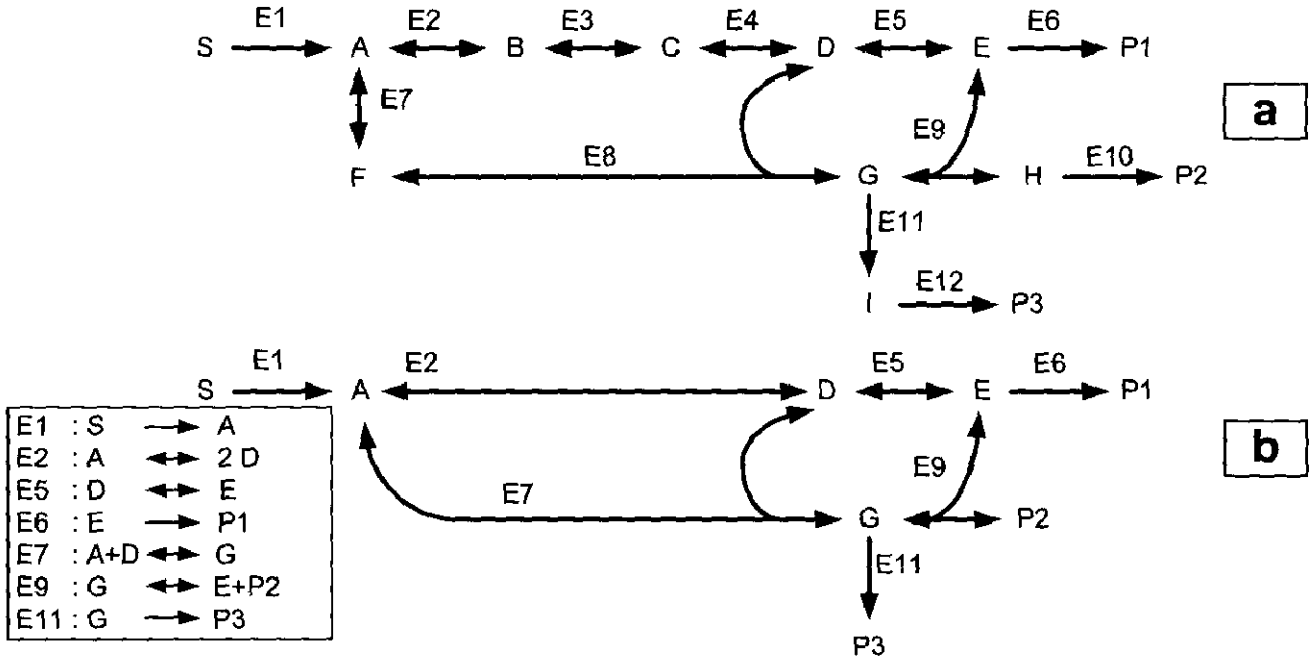
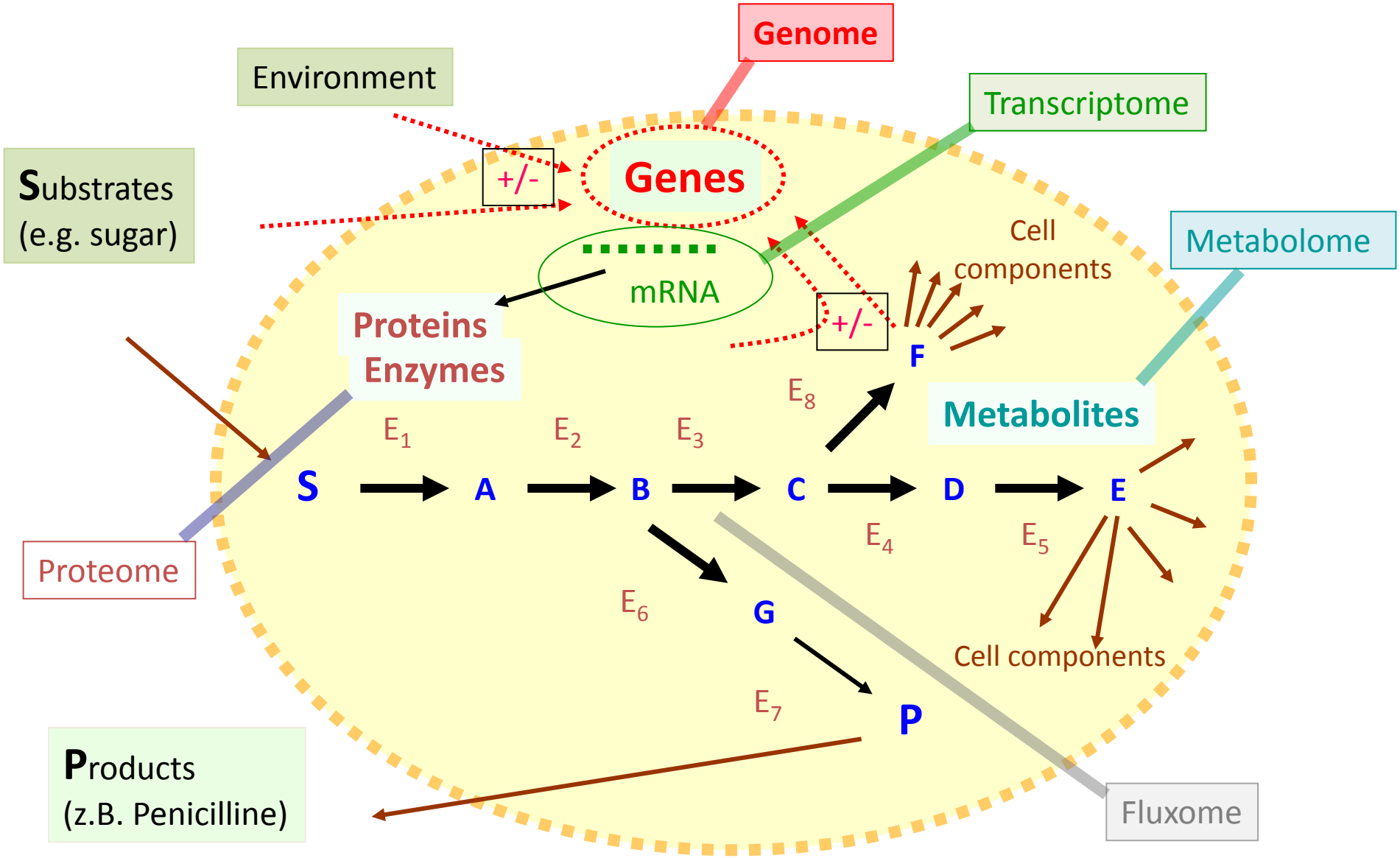


Fig. 12.3a, b. Example for a possible reaction network with 12 reactions (E1-E11)
a complete network
b simplified network in case of a steady state for all intermediates A to 1. The inserted box depicts the stoichiometric equations for b and the reversibility of the reactions

Cell function



Metabolome Analysis

Metabolism – Network

Metabolite pools – depending on state conditions
- Growth phase
- External conditions
  - chemical
  - physical
- genetic constellations

Metabolic flux - Flux Analyses
- Intracellular
- Transport by cell membrane systems

- Intracellular
- Extracellular

Sampling → Ultrafast Stopping of all activities

Metabolic Flux Analysis

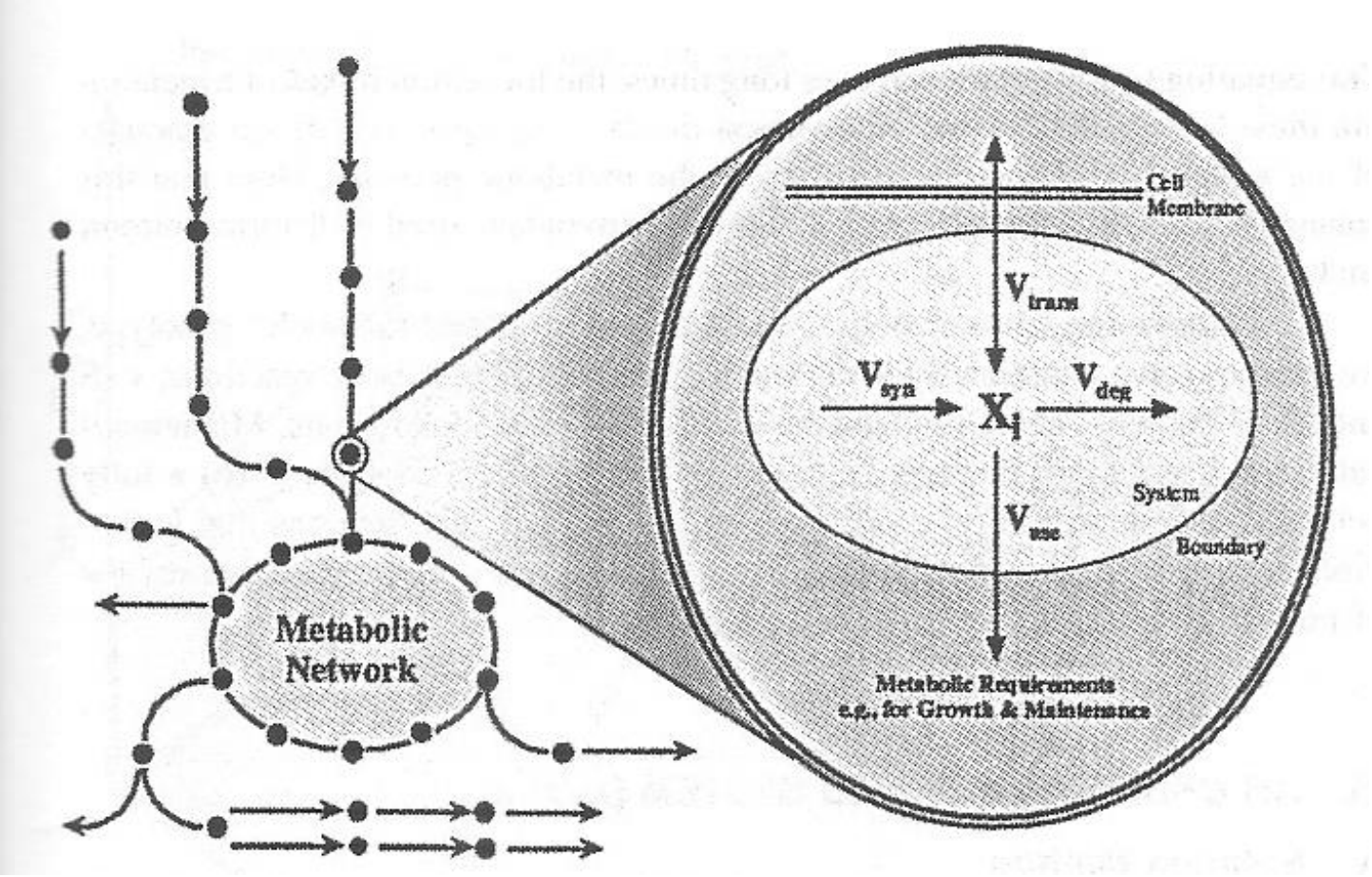


Figure 1 Flux balance models use material balances around each metabolite in a metabolic network. The concentration of each metabolite $\mathbf{X}_i$ is affected by various fluxes $\mathbf{V}_j$; $\mathbf{V}_{trans}$ is the uptake or secretion flux, while $\mathbf{V}_{use}$ is the flux required for growth and maintenance; $\mathbf{V}_{syn}$ and $\mathbf{V}_{deg}$ refer to the fluxes resulting from the metabolic synthesis and degradation of the metabolite.

Metabolic Flux Analysis

Dynamic mass balance for specific metabolite

$$\frac{dX_i}{dt} = v_{syn} - v_{deg} - (v_{use} +/- v_{trans}) \quad (1)$$

$$\frac{dX_i}{dt} = v_{syn} - v_{deg} - b_i \quad (2)$$

b_i = net transport

Metabolic network for n metabolites and m metabolic fluxes

$$\frac{dX}{dt} = S.v - b \quad (3)$$

X = n-dimensional vector

S = stoichiometric n x m matrix

b = vector of known metabolic demands

For steady state → $S.v = b$ (4)

28-5-15

Metabolic Flux Analysis

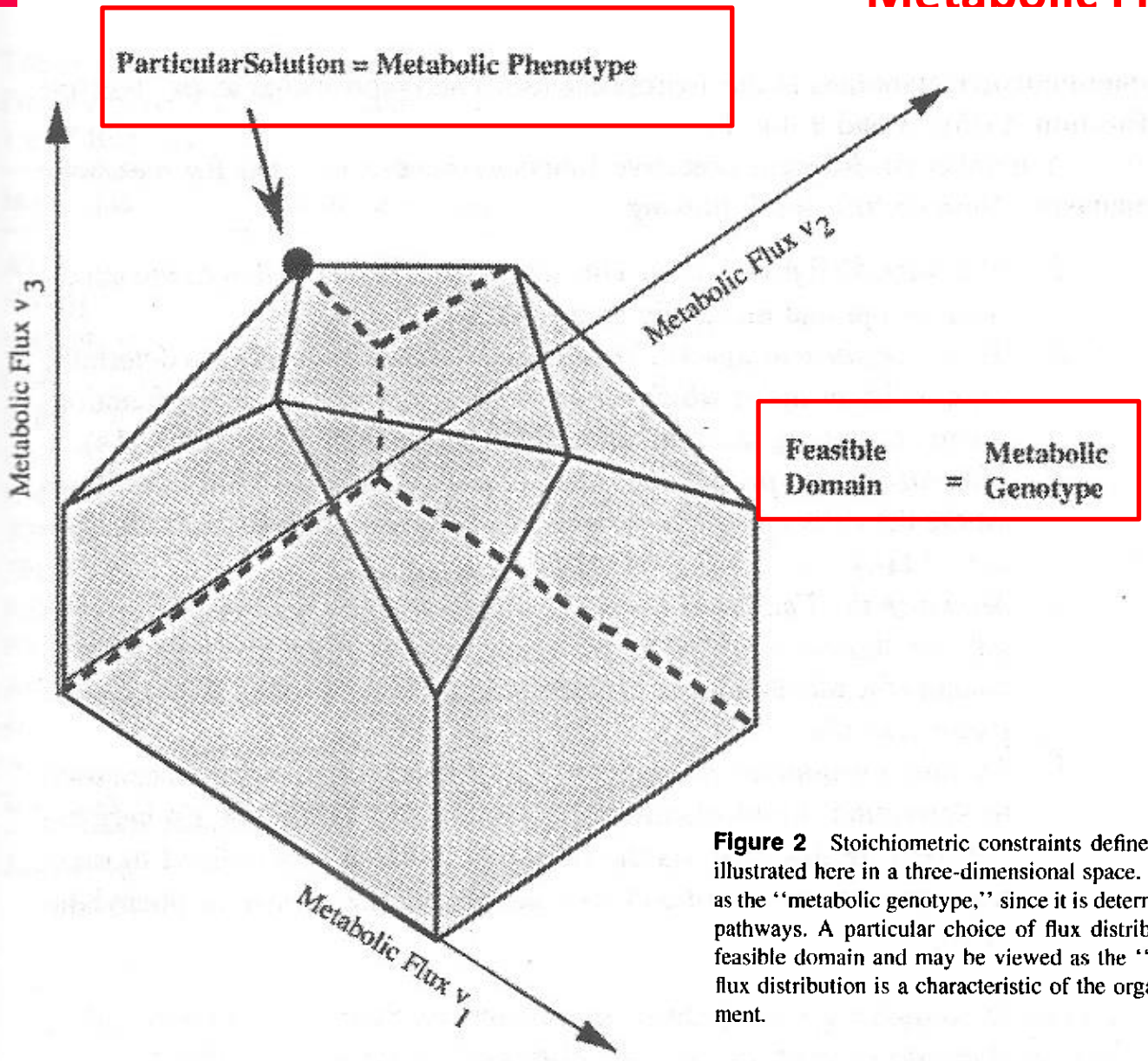


Figure 2 Stoichiometric constraints defined by equation 4 define a feasible domain, illustrated here in a three-dimensional space. The feasible domain is thus conceptualized as the ''metabolic genotype,'' since it is determined by the *m*-enzyme-catalyzed metabolic pathways. A particular choice of flux distribution is represented by a point within the feasible domain and may be viewed as the ''metabolic phenotype,'' since the particular flux distribution is a characteristic of the organism's response to that particular environment.

Metabolic Flux Analysis

A number of different objective functions have been used for metabolic analysis. These include the following:

1. **Minimize ATP production**. This objective is stated to determine conditions of optimal metabolic energy efficiency
2. **Minimize nutrient uptake**. This objective function is used to determine the conditions under which the cell will perform its metabolic functions while consuming the minimum amount of available nutrients
3. **Minimize redox production**. This objective function finds conditions where the cells operate to generate the minimum amount of redox Potential
4. **Minimize the Euclidean norm**. This objective has been applied to satisfy the strategy of a cell to minimize the sum of the flux values, or to channel the metabolites as efficiently as possible through the metabolic pathways
5. **Maximize metabolite production**. This objective function has been used to determine the biochemical production capabilities of *Escherichia coli*. In this analysis the objective function was defined to maximize the production of a chosen metabolite (i.e., lysine or phenylalanine).
6. **Maximize biomass and metabolite production**. By weighing these two conflicting objectives appropriately, one can explore the trade-off between cell growth and forced metabolite production in a producing strain

Metabolic Flux Analysis

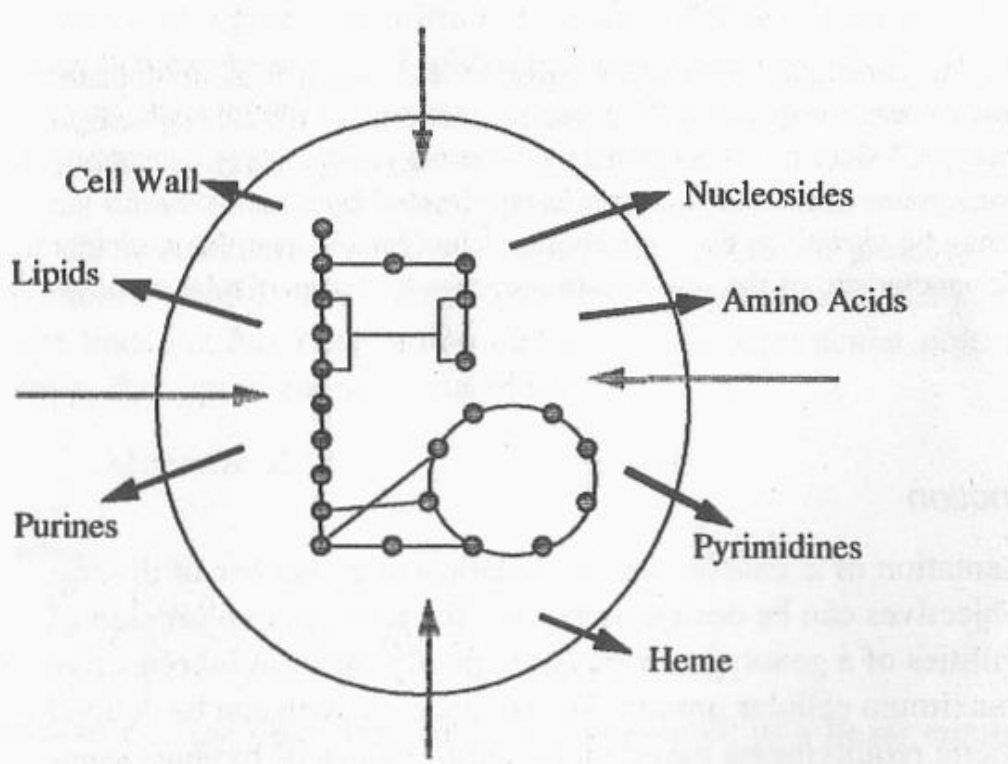


Figure 3 Definition of the maximal growth rate objective function.

Table 1 Metabolic Demands of Precursors and Cofactors Required for 1 g of Biomass

Metabolite	Demand (mmol)
ATP	41.2570
NADH	−3.5470
NADPH	18.2250
G6P	0.2050
F6P	0.0709
R5P	0.8977
E4P	0.3610
T3P	0.1290
3PG	1.4960
PEP	0.5191
PYR	2.8328
AcCoA	3.7478
OAA	1.7867
AKG	1.0789
SuccCoA	—

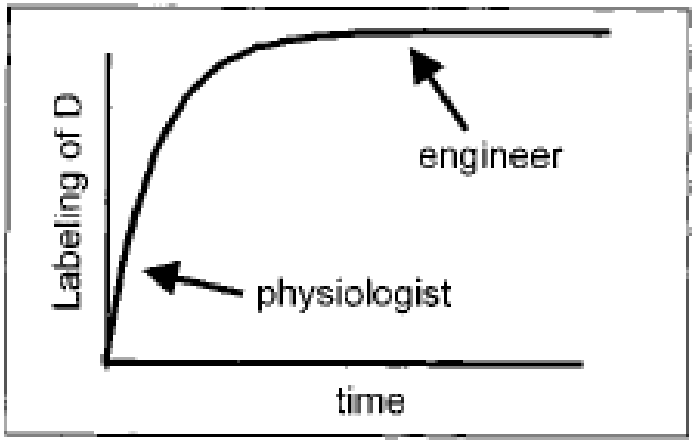
Metabolic Flux Analysis

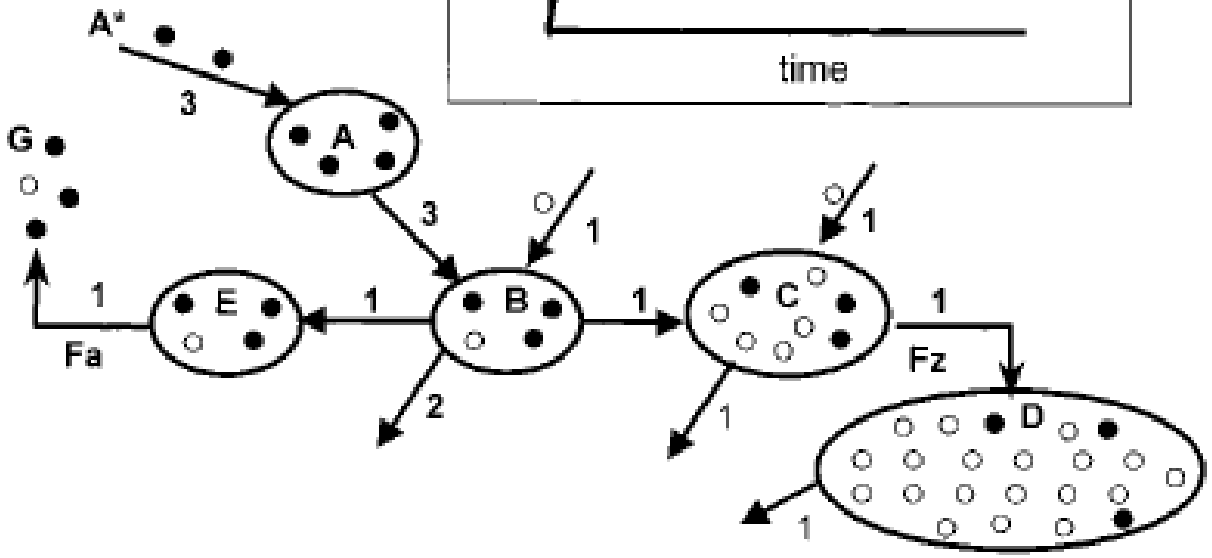
Methods for Flux Analysis

- Metabolite Pools (GC. LC, MS, NMR)

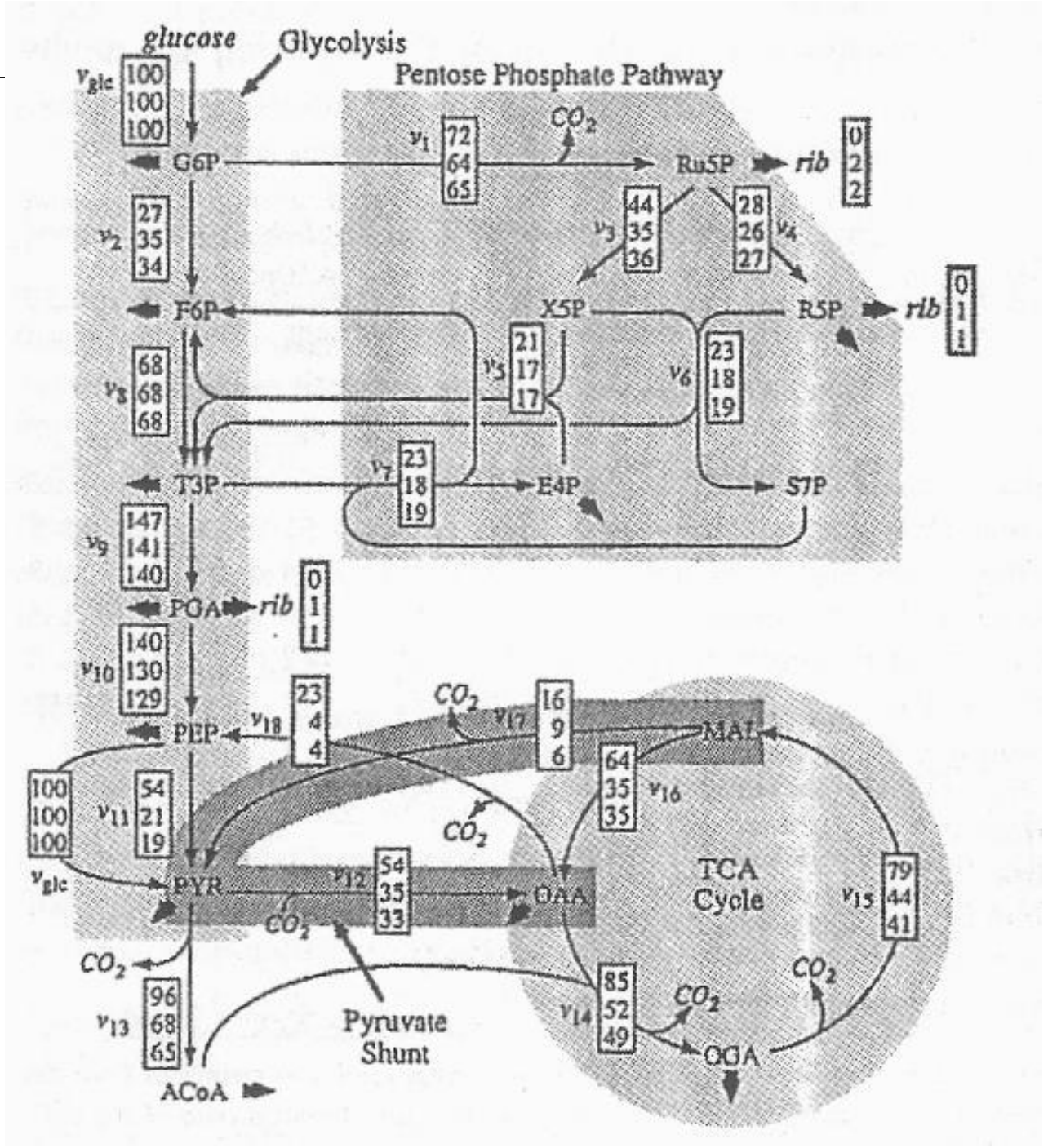
- **Isotope tracing** (^{13}C, ^{2}H, ^{18}O etc.)

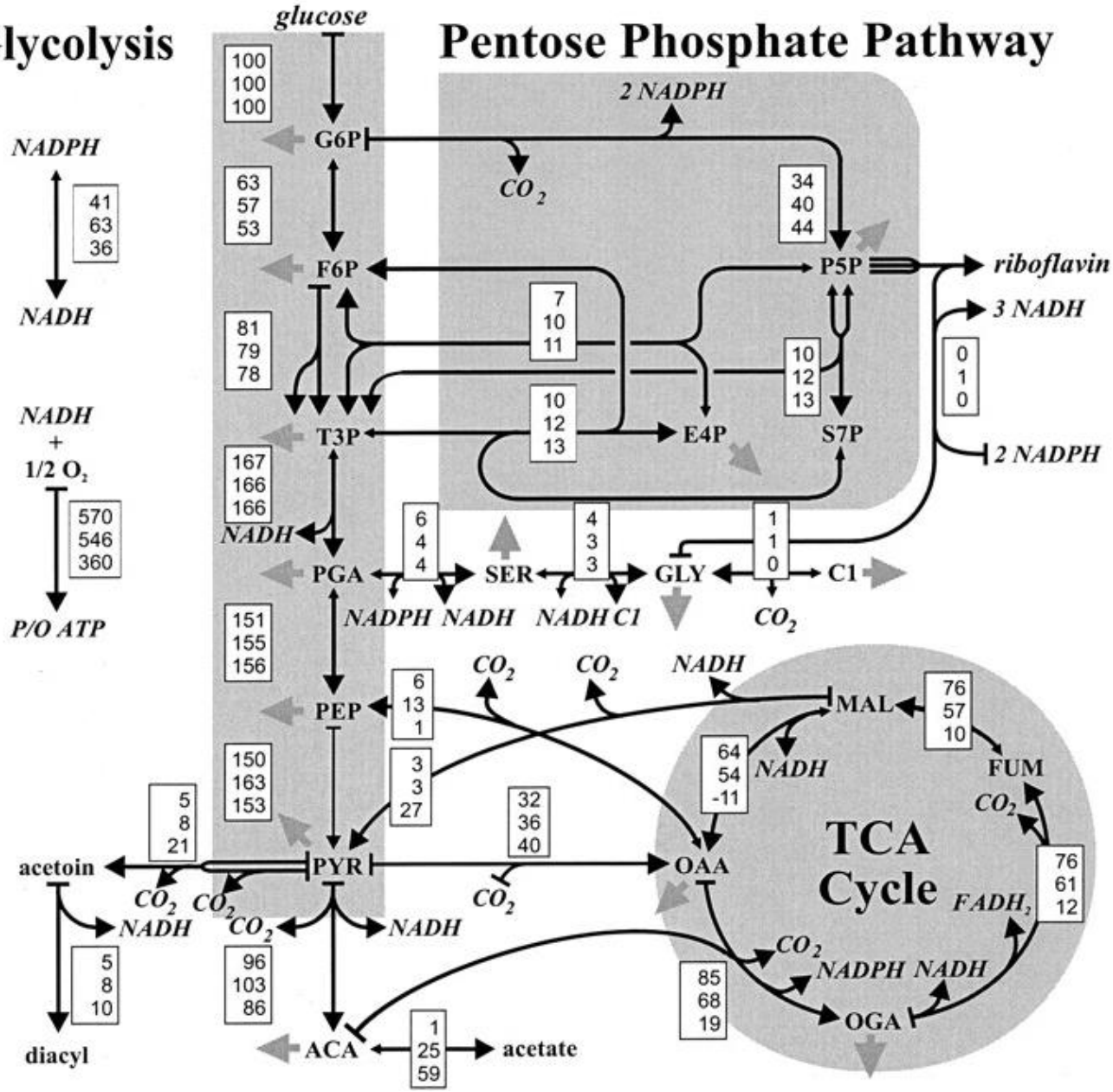
FIG. 1. Example metabolic tracer study from a physiologist's viewpoint. A labeled metabolite "A*" enters a pathway and travels through a number of compartments. Some compartments (A, B, C, E) are inaccessible for sampling and the isotopic enrichment cannot be directly measured. Numerals above flux arrows represent metabolite fluxes. A large pool of an end product, D, may be sampled but physical limitations prohibit continuing the experiment until labeling reaches steady state. Flow of tracer into D may represent a sink for metabolic physiologists and a steady-state system for metabolic engineers as shown in the inset graph. G represents a volatile end product such as CO_2.





FIux distribution in riboflavin-producing B. subtilis strain PRF. Numbers are normalized to glucose uptake and represent dilution rates of 0.11, 0.41, and 062 h^{-1} in glucose-limited chemostat.

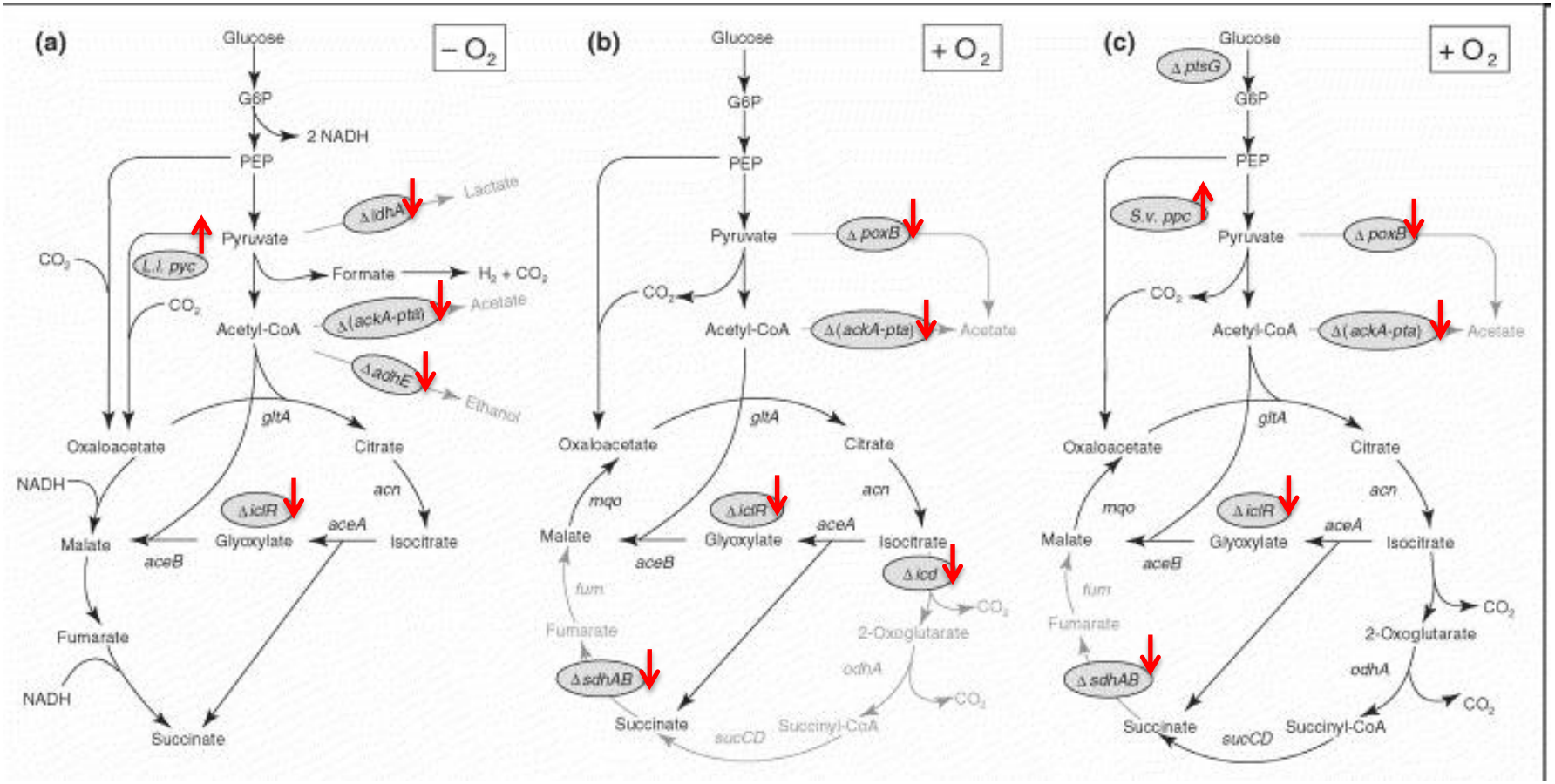




J. Bacteriol. December 2001 vol. 183 no. 24 7308-7317

Metabolic flux distribution in C-limited (top entry in the boxes), N-limited (middle), or P-limited (bottom) chemostat culture of B. subtilis at a D of 0.4 h−1. Fluxes are relative to the specific glucose consumption rate of each culture shown in Fig. 3. Large arrowheads indicate the primary direction of flux in a given reaction, and small arrowheads indicate that a reaction was considered reversible. Solid gray arrows indicate withdrawal of building blocks for biomass formation. For C-, N-, and P-limited cultures we recovered 97% ± 3%, 112% ± 7%, and 104% ± 7% %, respectively, of the consumed carbon in the determined products. The data for C-limited cultures were taken from Dauner et al. (9).

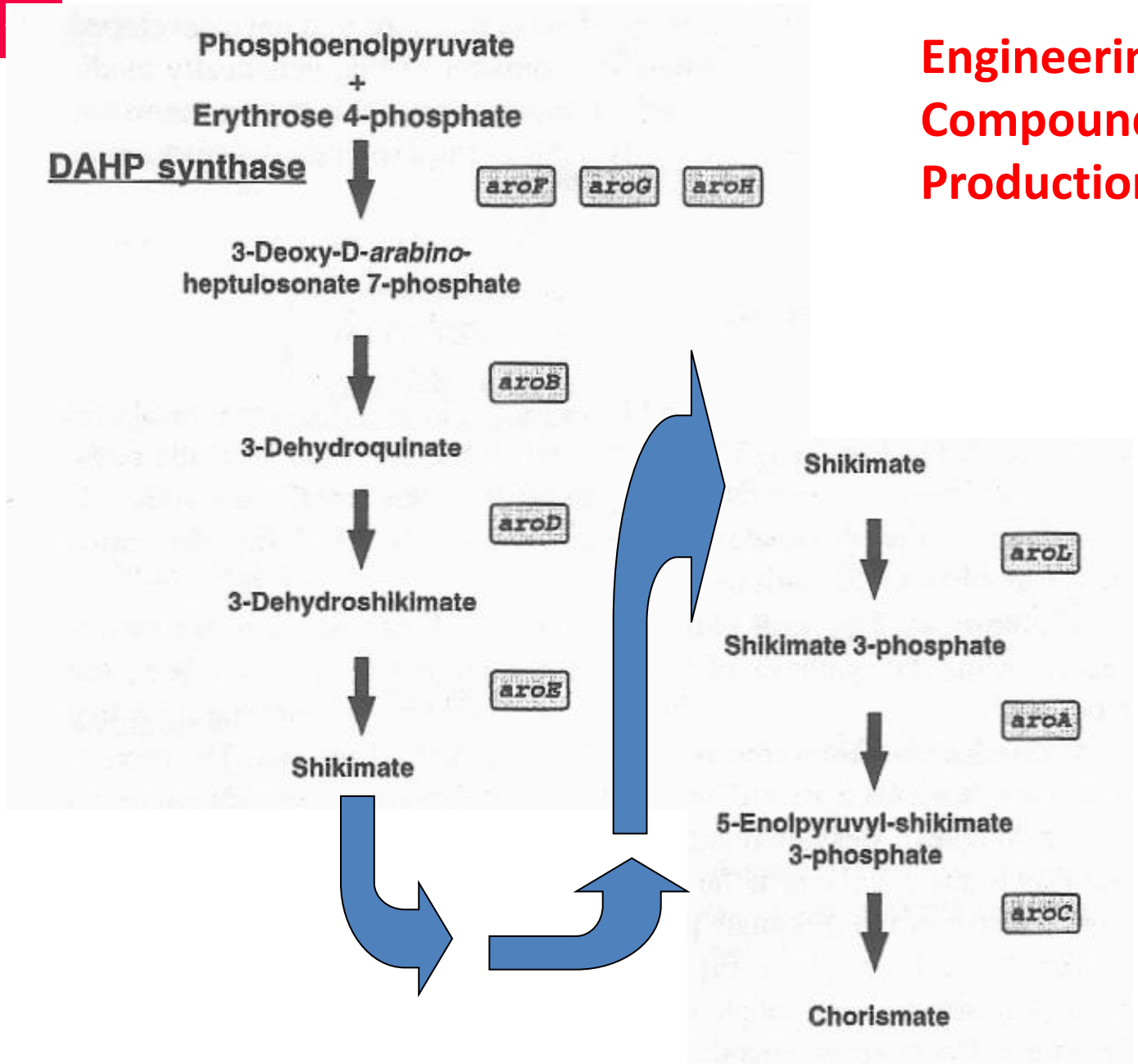
Metabolic Engineering for organic acid production



Metabolic engineering of E. coli strains for anaerobic or aerobic succinate production. Panel (a) shows the metabolism of a strain engineered for **anoxic succinate production** via the **reductive arm of the TCA cycle** and via the **glyoxylate cycle** [30••]. It contains deletions of the genes for lactate dehydrogenase (ldhA), acetate kinase and phosphotransacetylase (ackA-pta), aldehyde-alcohol dehydrogenase (adhE) and a repressor of the aceBAK operon (iclR), and it overexpresses the pyruvate carboxylase gene (pyc) from Lactococcus lactis [30••]. Deletion of iclR leads to induction of the glyoxylate cycle enzymes isocitrate lyase and malate synthase. Panel (b) shows a pathway for **aerobic succinate formation** exclusively via the **glyoxylate cycle** [31•]. The corresponding strain contains deletions of ackA-pta and poxB (pyruvate:quinone oxidoreductase) to avoid acetate formation, deletions of icd and sdhAB to block the TCA cycle, and of iclR to induce the aceBAK operon. The pathway shown in panel **(c) is similar to the one shown in (b)**, but the **icd gene** was **not deleted**, allowing succinate formation via both the glyoxylate cycle and the oxidative arm of the TCA cycle [32•]. The strain overexpresses the PEP carboxylase gene from Sorghum vulgare (S.v. ppc), which is resistant to feedback inhibition by malate. Next to the respective enzymatic reactions endogenous and heterologous gene names are given. Genetic changes are depicted in grey ovals and the resulting absence of enzyme reactions is highlighted in grey.

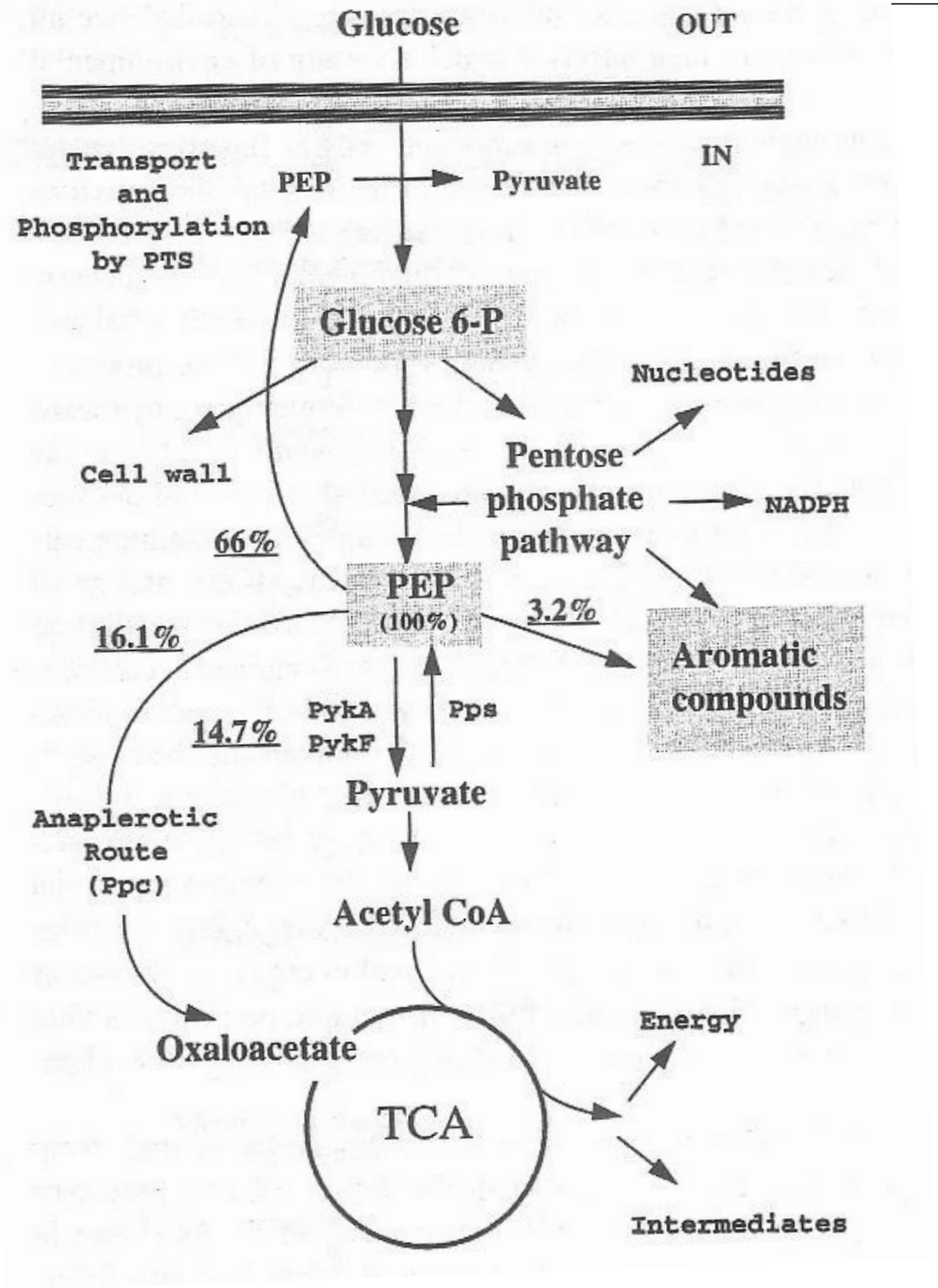
Current Opinion in Microbiology 2006, 9:268–274

Engineering Aromatic Compounds Production in *E.coli*

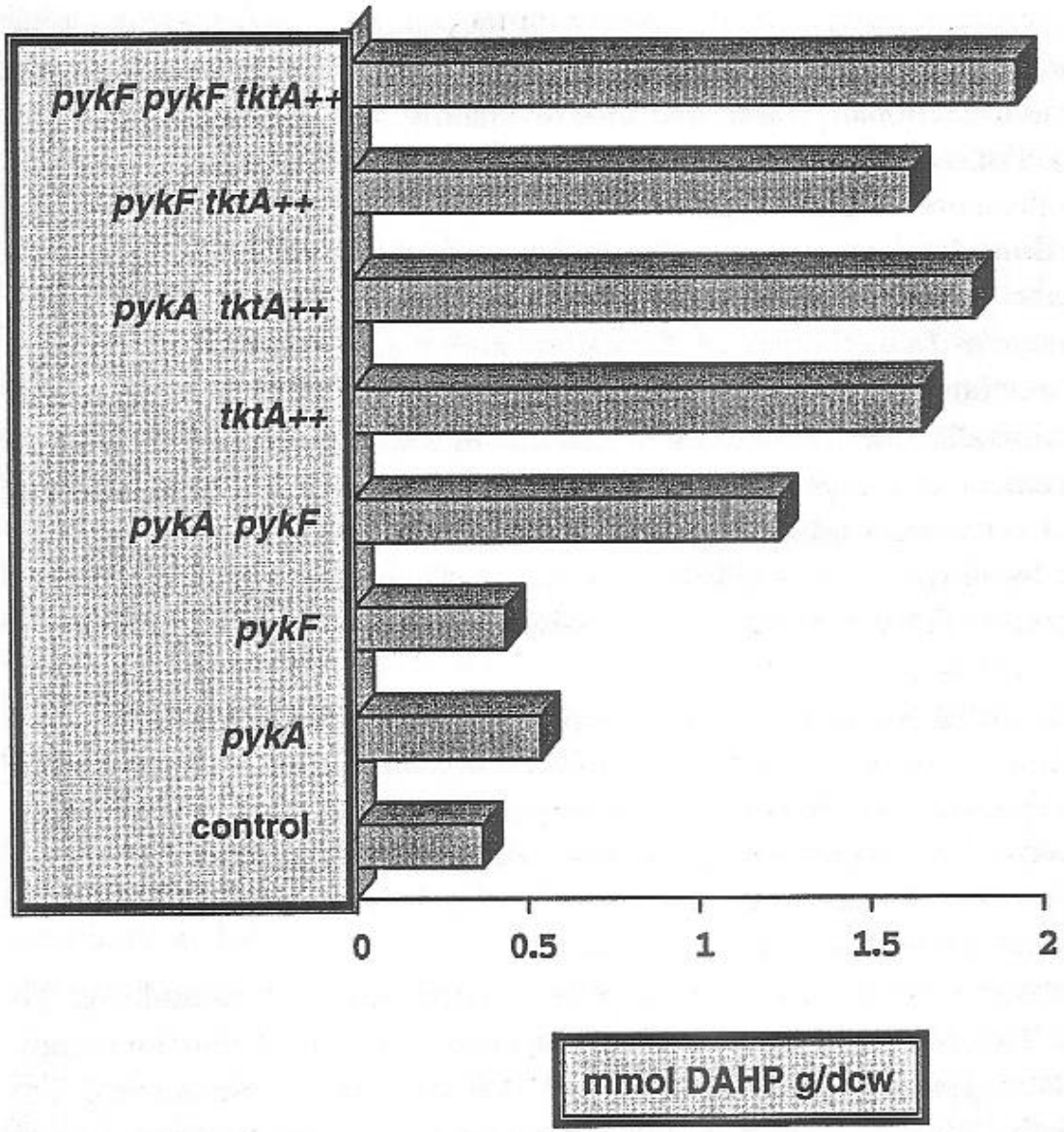


Engineering Aromatic Compounds Production in *E.coli*

Multiple metabolic pathways related to the formation and consumption of PEP



Engineering Aromatic Compounds Production in *E.coli*



DAHP production in strains of *E. coli* in which the central metabolic pathways related to formation of PEP and E4P have been manipulated. DAHP production is an indicator of carbon commitment to aromatic biosynthesis; pykA and pykF represent inactivation (by mutation) of the PykA and PykE isoenzymes of pyruvate kinase, respectively; tktA+ + indicates that the strain carried a p]asmid containing the *E. coli* tktA gene and thus bad elevated levels of transketolase activity; dcw, dry cell weight.

ß-Lactam Antibiotics

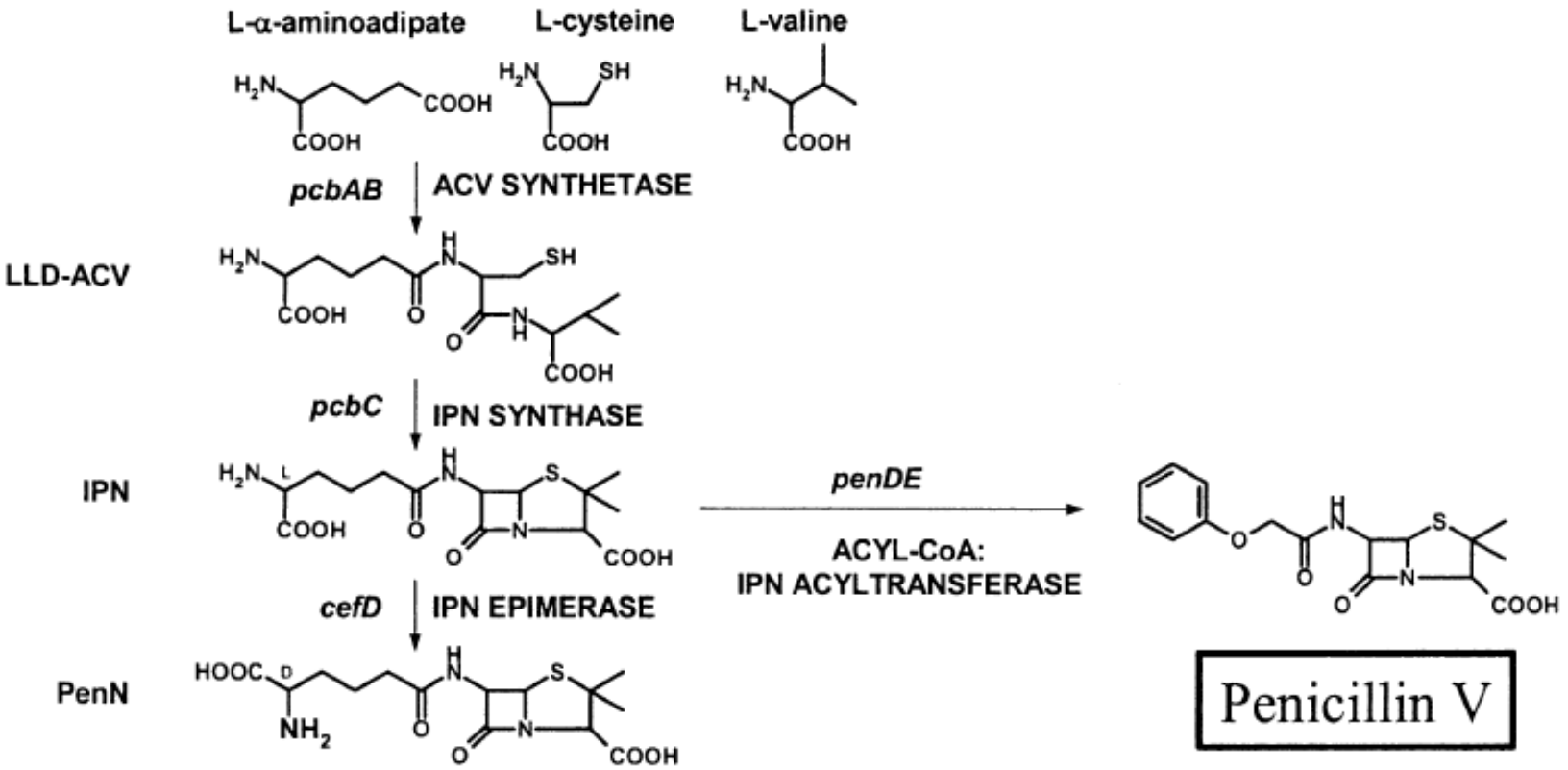
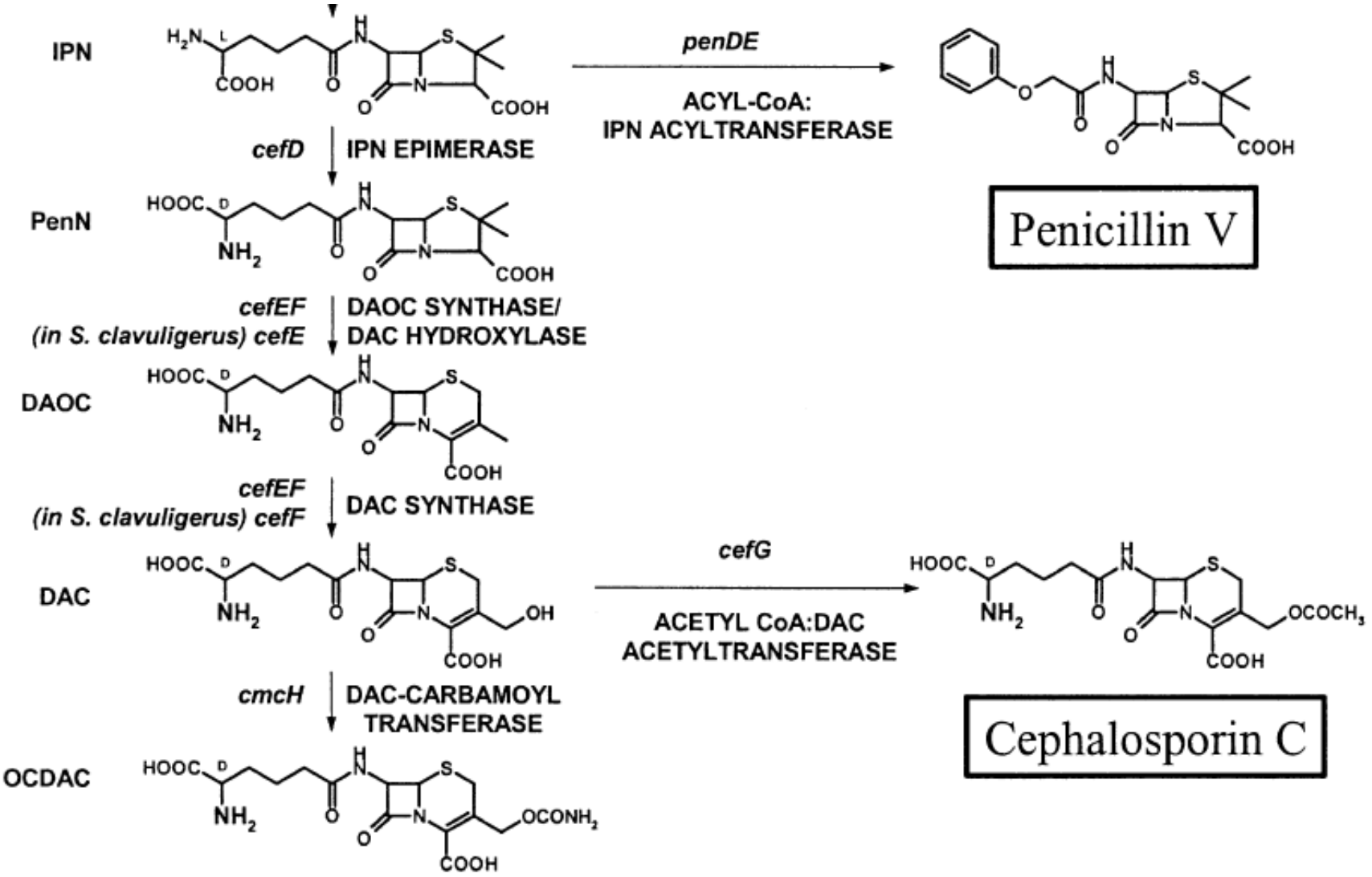


Fig. 2. Biosyntheses of the β-lactams penicillin V, cephalosporin C and cephamycin C. Abreviations: LLD-ACV, α-L-aminoadipyl-L-cysteinyl-D-valine; IPN, isopenicillin N; PenN, penicillin N; DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; OCDAC, O-carbamoyl-deacetylcephalosporin C; HOCDAC, 7-a-hydroxy-O-carbamoyl-deacetylcephalosporin C.

ß-Lactam Antibiotics



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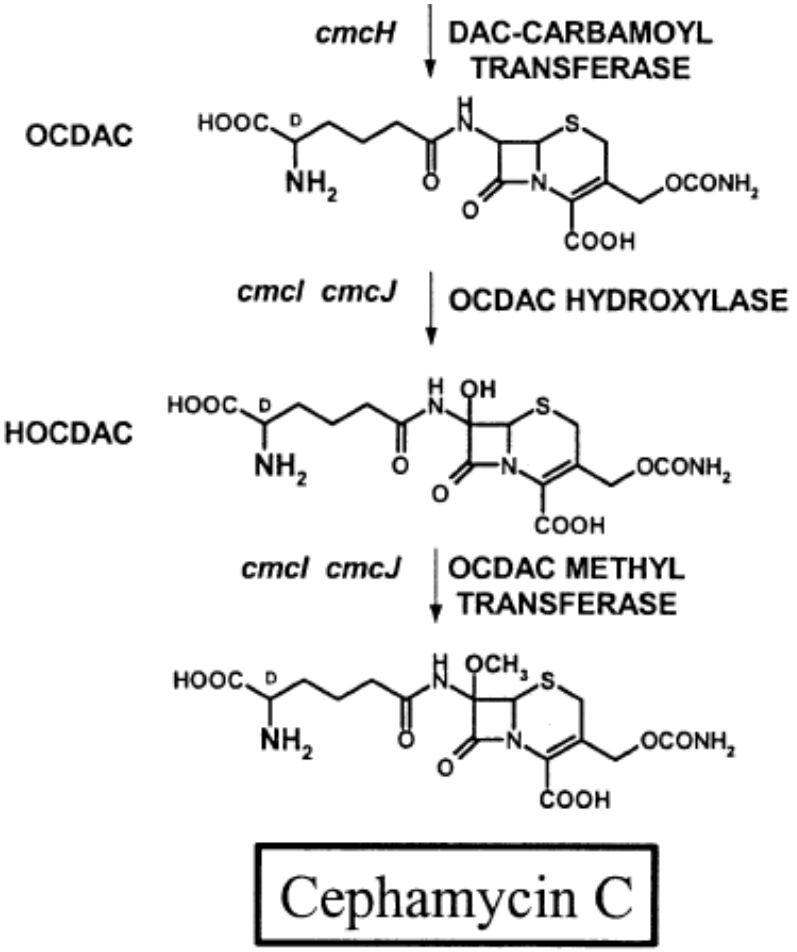
ß-Lactam Antibiotics



Cephalosporin C

Fig. 2. Biosyntheses of the β-lactams penicillin V, cephalosporin C and cephamycin C. Abreviations: LLD-ACV, α-L-aminoadipyl-L-cysteinyl-D-valine; IPN, isopenicillin N; PenN, penicillin N; DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; OCDAC, O-carbamoyl-deacetylcephalosporin C; HOCDAC, 7-a-hydroxy-O-carbamoyl-deacetylcephalosporin C.

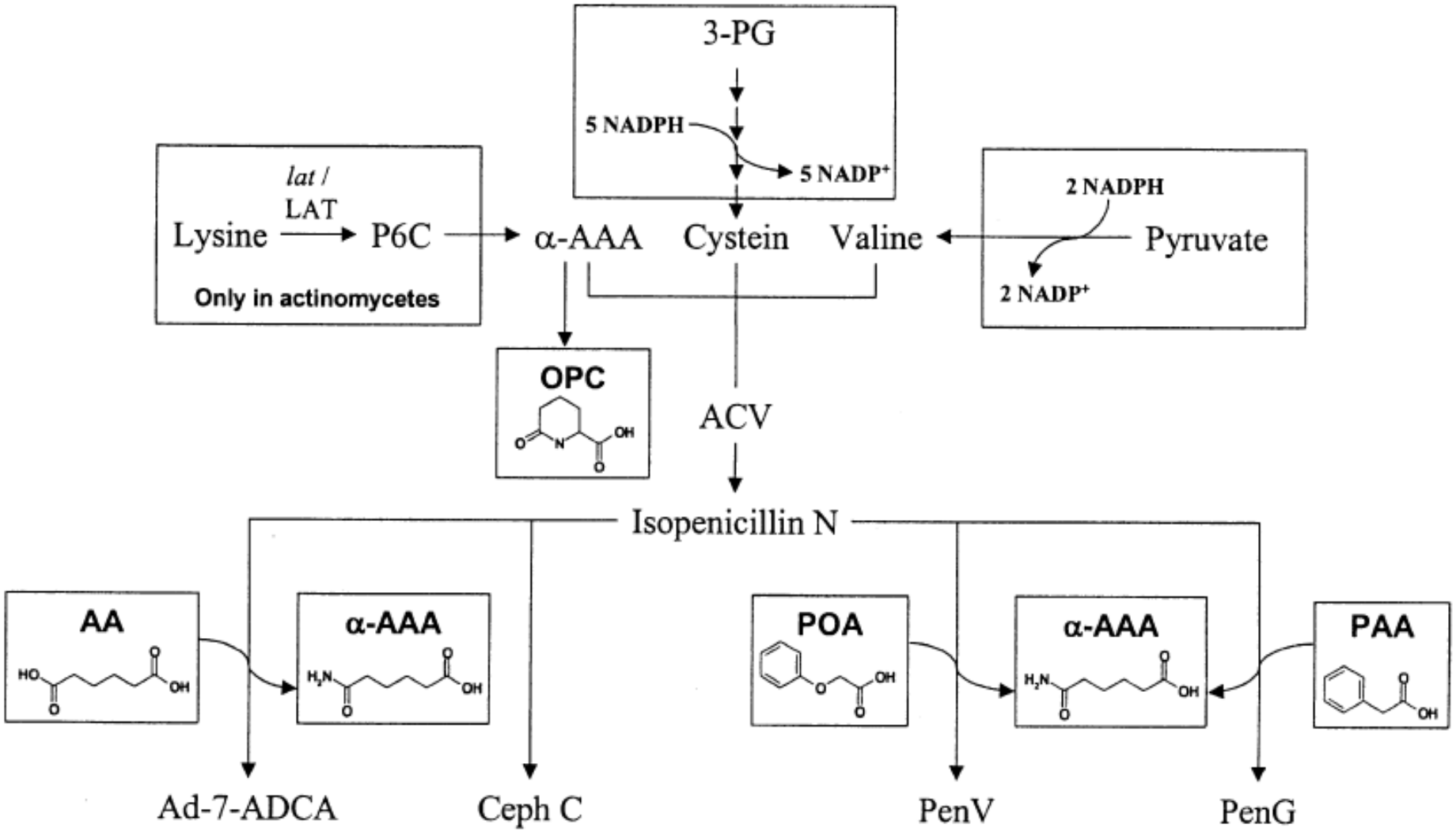


Fig. 6. Different precursors in the production of β-lactams.

ß-Lactam Antibiotics

Genes encoding the enzymes in the β-lactam biosynthesis pathways and kinetic properties of the enzymes

Protein	Organism	Gene	Gene size (bp)	Protein size(kD)	Reference	Km (μM)	pH opt	Reference
ACVS	*P. chr*	*pcbAB*	*11376*	426	Diez et al. (1990)			
			11328	424	Smith et al. (1990)	46 (Aad), 80 (Cys), 83 (Val)	8.4	Theilgaard et al. (1997)
	A. chr	*pcbAB*	*11136*	414.8	Gutiérrez et al. (1991)	170 (Aad), 26 (Cys), 340 (Val)	7.5	Banko et al. (1987), Baldwin et al. (1990)
						120 (Aad), 90 (Cys), 320 (Val)	8.3	Kallow et al. (1998)
	A. nid	*acvA*	*11310*	422.5	MacCabe et al. (1991)	nd	nd	Van Liempt et al. (1989)
	N. lac	*pcbAB*	*10947*	404.1	Coque et al. (1991)	nd	nd	Coque et al. (1996)
	S. cla	*pcbAB*	*nd*	nd	Tobin et al. (1991)	560 (Aad), 70 (Cys), 1140 (Val)		Jensen et al. (1988)
						630 (Aad), 120 (Cys), 300 (Val)	8.5	Zhang et al. (1992)
						630 (Aad), 430 (Cys), 380 (Val)		Kadima et al. (1995)
IPNS	*P. chr*	*pcbC*	*993*	38	Barredo et al. (1989)	130 (ACV)		
					Carr et al. (1986)		7.8	Ramos et al. (1985)
	A. chr	*pcbC*	*1014*	38.4	Samson et al. (1985)	170 (ACV)	nd	Baldwin et al. (1985a, b, 1987)
						300 (ACV)		Kupka et al., 1983; Pang et al., 1984
	A. nid	*pcbC*	*993*	37.5	Ramón et al. (1987)	nd	nd	
					Weigel et al. (1988)			
	N. lac	*pcbC*	*984*	37.5	Coque et al. (1991)	180 (ACV)	nd	Castro et al. (1988)
	S. cla	*pcbC*	*1316*	36.9	Leskiw et al. (1988)	320 (ACV)	7.0	Jensen et al. (1986)
IAT	*P. chr*	*penDE*	*1274*	39.9	Barredo et al. (1989)	4000 (IPN), 23 (IPN + PA-CoA)	8.0–8.5	Alvarez et al. (1987, 1993)
						9,3 (6-APA + PA-CoA)		
						6 (PA-CoA), 2000 (PenV)		
	A. nid	*acyA*	*1217*	39.2	Montenegro et al. (1990)	nd	nd	
IPNE	*A. chr*	*nd*	*nd*	nd		nd	nd	
	N. lac	*cefD*	*1194*	43.6	Coque et al. (1993)	270 (IPN)	7.0	Laiz et al. (1990)
	S. cla	*cefD*	*1194*	43.5	Kovacevic et al. (1989)	nd	nd	Usui and Yu (1989)
DAOCS/DACS	*A. chr*	*cefEF*	*996*	36.5	Samson et al. (1987)	29 (PenN),22 (2-oxo)/ 18 (DAOQ, 20(2-oxo)	7.5–7.8/7.0–7.5	Yeh et al. (1991)
DAOCS	*N. lac*	*cefE*	*942*	34.5	Coque et al. (1993)	52 (PenN), 3 (2-oxo)	(5–11)	Cortès et al. (1987)
	S. cla	*cefE*	*933*	34.5	Kovacevic et al. (1989)	35 (PenN), 22 (2-oxo)	7.4	Yeh et al. (1991)
DACS	*N. lac*	*cefF*	*933*	34.4	Coque et al. (1996)	nd	nd	
	S. cla	*cefF*	*954*	34.6	Kovacevic and Miller (1991)	25 (DAOC), 14 (2-oxo)	7.0–7.4	Yeh et al. (1991)
DAT	*A. chr*	*cefG*	*1332*	49.3	Gutiérrez et al. (1992)	nd	7.0–7.5	Fujisawa and Kanzaki (1975)
		cefG	*1299*	nd	Matsuda et al. (1992)			Felix et al. (1980)
		cefG	*1300*	41	Mathison et al. (1993)			
DACCT	*N. lac*	*cmcH*	1563	57.1	Coque et al. (1995b)	nd	nd	
	S. cla	*cmcH*	1566	nd	Alexander and Jensen (1998)	nd	nd	
P7	*N. lac*	*cmcI*	711	27	Coque et al. (1995a)	nd	nd	
	S. cla	*cmcI*	711	32	Xiao et al. (1991)	720 (CephC)	7.3–7.7	Xiao et al. (1991)
					Alexander and Jensen (1998)			
P8	*N. lac*	*cmcJ*	876	32	Coque et al. (1995a)	nd	nd	
	S. cla	*cmcJ*	933	nd	Alexander and Jensen (1998)	nd	nd	

Proteins: ACVS, α-L-aminoadipyl-L-cysteinyl-D-valine synthase; DACS, deacetylcephalosporin C synthase; DAOCS, deacetoxycephalosporin C synthase; DAT, acetyl-CoA: deacetylcephalosporin C acetyltransferase; IAT, acyl-CoA:IPN acyltranferase; IPNE, isopenicillin N epimerase; IPNS, isopenicillin N synthase. Organisms: *A. chr*, *Acremonium chrysogenum*; *A. nid*, *Aspergillus nidulans*; *N. lac*, *Norcardia lactamdurans*; *P. chr*, *Penicillium chrysogenum*; *S. cla*, *Streptomyces clavuligerus*. Affinity constants: Aad, L-α-aminoadipate; ACV, α-L-aminoadipyl-L-cysteinyl-D-valine; 6-APA, 6-aminopenicillanic acid; Cys, cysteine; DAOC deacetoxycephalosporin C; IPN, isopenicillin N; 2-oxo, 2-oxoglutarate; PenN, penicillin N; PenV, penicillin V; PA-CoA, phenylacetic acid-CoA; Val, valine.

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ß-Lactam Antibiotics

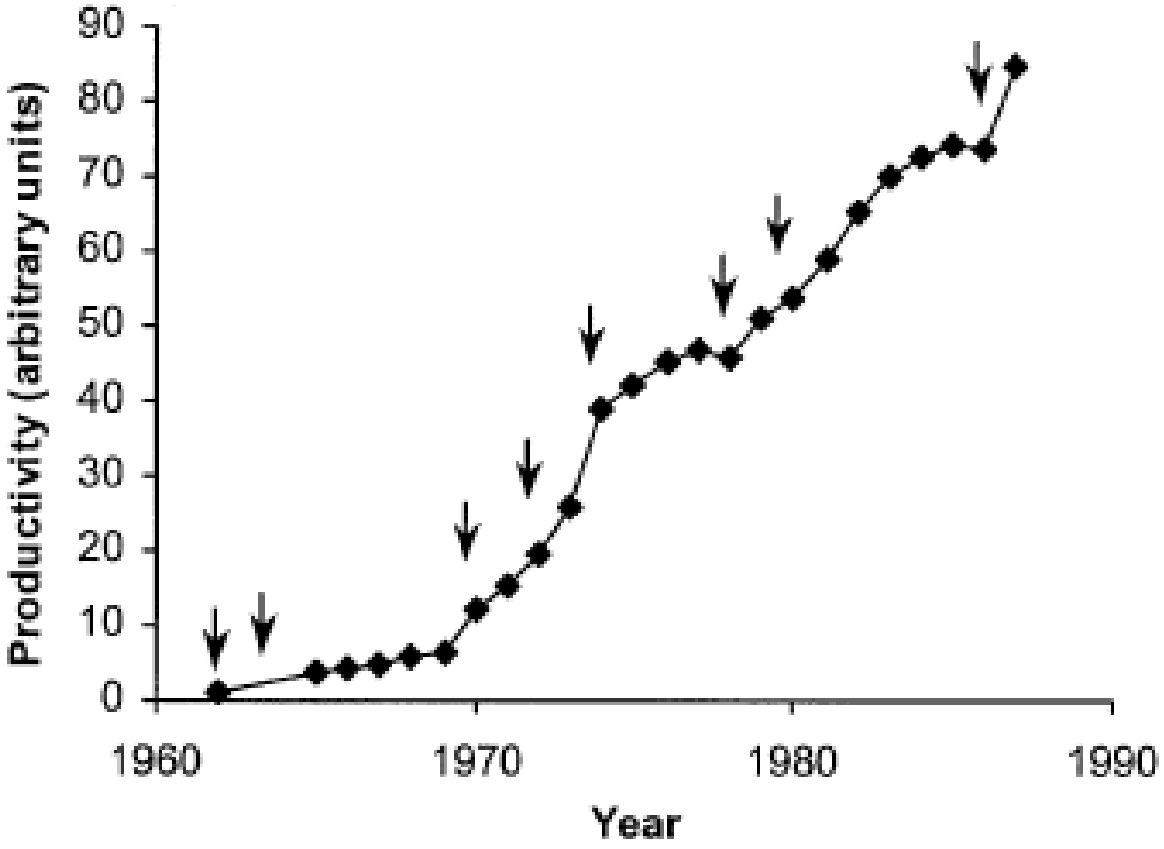


Fig. 3. Increase in productivity (output rate/unit volume, arbitary units) of pencillin G production by Gist Brocades, Delft, in the period between 1962 and 1987. The introduction of new productions strains is indicated with arrows. Based on Nielsen, 1997.

ß-Lactam Antibiotics

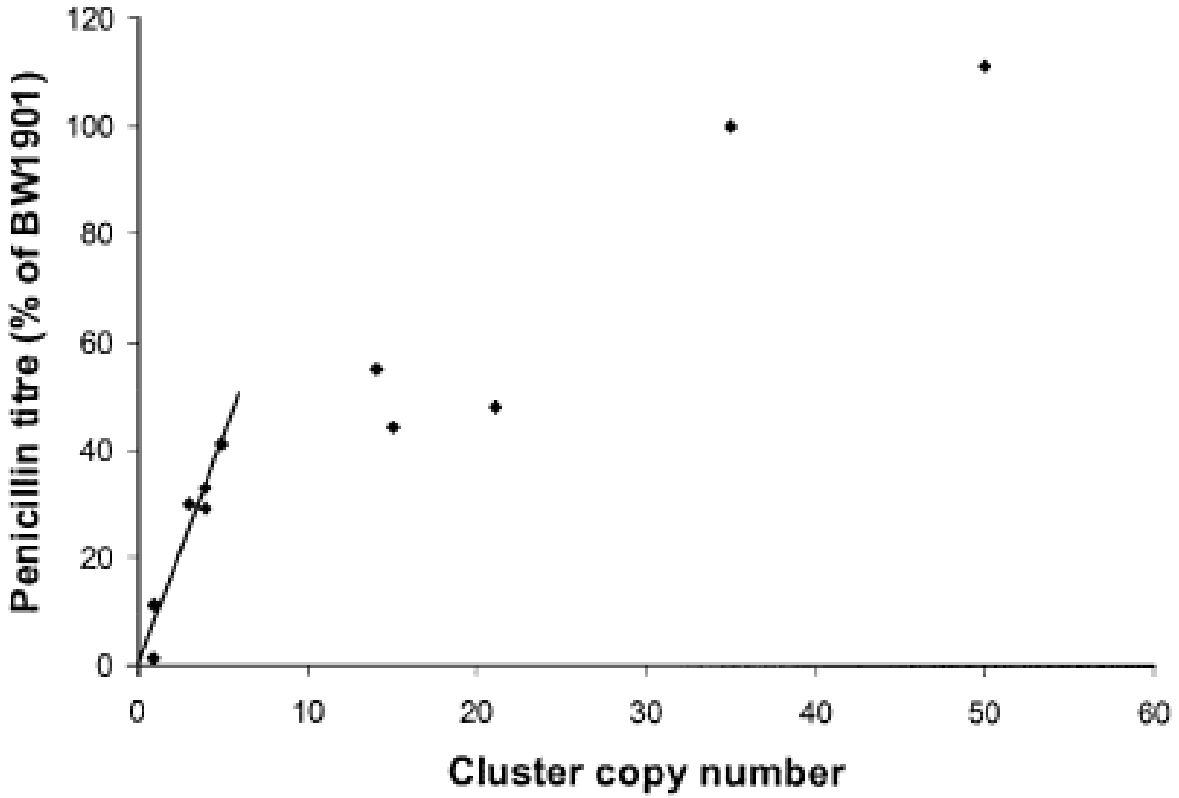


Fig. 4. Results of the SmithKline Beecham strain improvement series. Penicillin titre in percentage of the SmithKline Beecham strain BW 1901 versus penicillin cluster copy number. Modified from Newbert et al. (1997).

Production of
Cephalosporin products in
P. chrysogenum

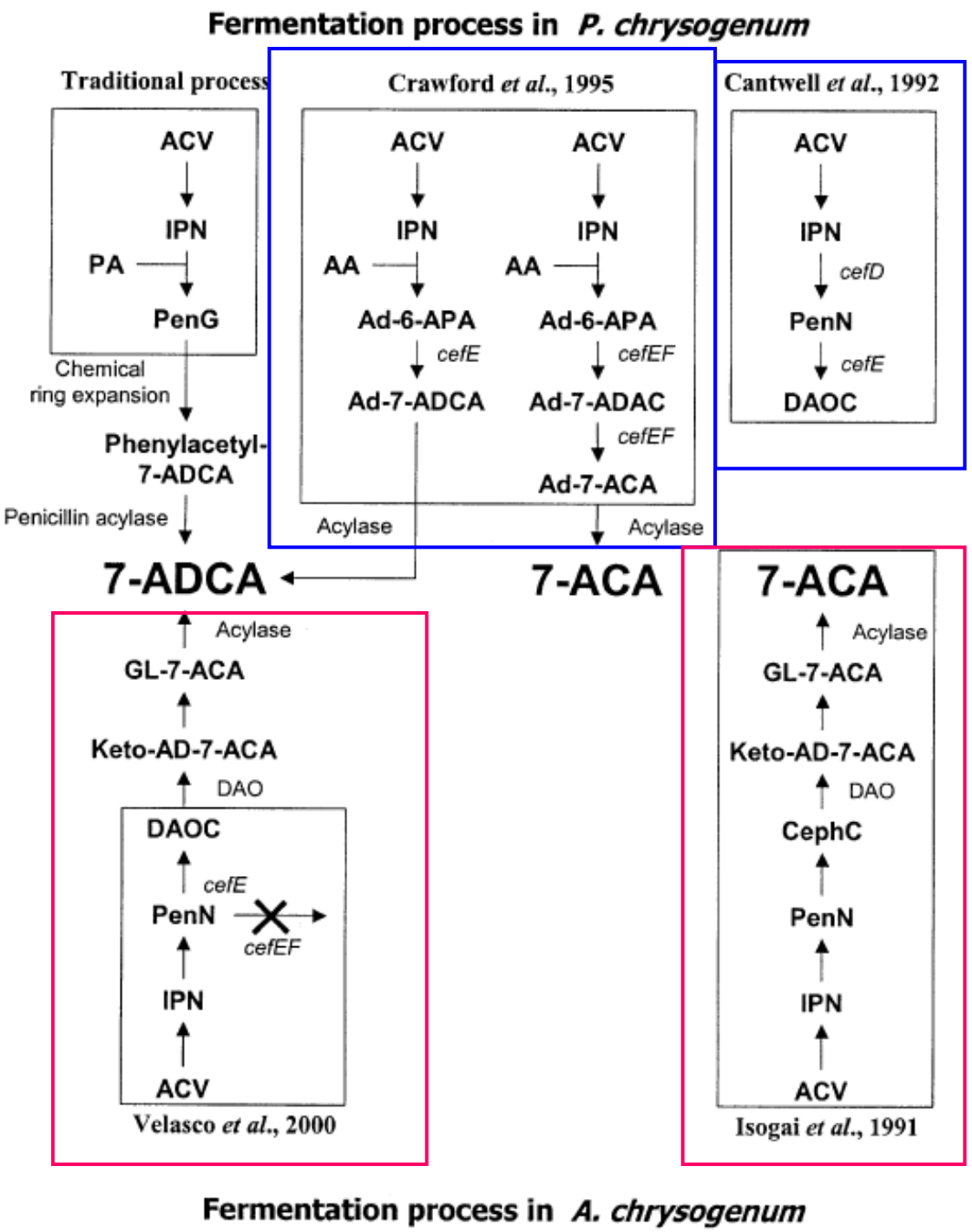


Fig. 5. Different strategies for producing cephalosporins directly from fermentation with the aims of developing bioprocesses for direct production of 7-ADCA and 7-ACA.

The traditional production process
7-ADCA is shown in the upper left
comer.

Modified from Velasco et al. (2000)

Production of Amino Acids

Table 1 World Production of Amino Acids

Production scale (tons/y)	Amino acid	Preferred production method	Main use
800,000	L-Glutamic acid	Fermentation	Flavor enhancer
350,000	L-Lysine	Fermentation	Feed additive
300,000	D,L-Methionine	Chemical synthesis	Feed additive
8000–100,000	L-Aspartate	Enzymatic catalysis	Aspartame
	L-Phenylalanine	Fermentation	Aspartame
	L-Threonine	Fermentation	Feed additive
	Glycine	Chemical synthesis	Food additive, sweetener
1000–8000	L-Tryptophan	Fermentation	Feed additive
	L-Arginine	Fermentation, extraction	Pharmaceuticals
	L-Cysteine	Reduction of cystine	Food additive, pharmaceuticals
100–1000	L-Alanine	Enzymatic catalysis	Flavor enhancer, pharmaceuticals
	L-Asparagine	Extraction	Pharmaceuticals
	L-Glutamine	Fermentation, extraction	Pharmaceuticals
	L-Histidine	Fermentation, extraction	Pharmaceuticals
	L-Isoleucine	Fermentation, extraction	Pharmaceuticals
	L-Leucine	Fermentation, extraction	Pharmaceuticals
	L-Methionine	Enzymatic catalysis	Pharmaceuticals
	L-Proline	Fermentation, extraction	Pharmaceuticals
	L-Serine	Fermentation, extraction	Pharmaceuticals, cosmetics
	L-Tyrosine	Extraction	Pharmaceuticals
	L-Valine	Enzymatic catalysis	Pharmaceuticals

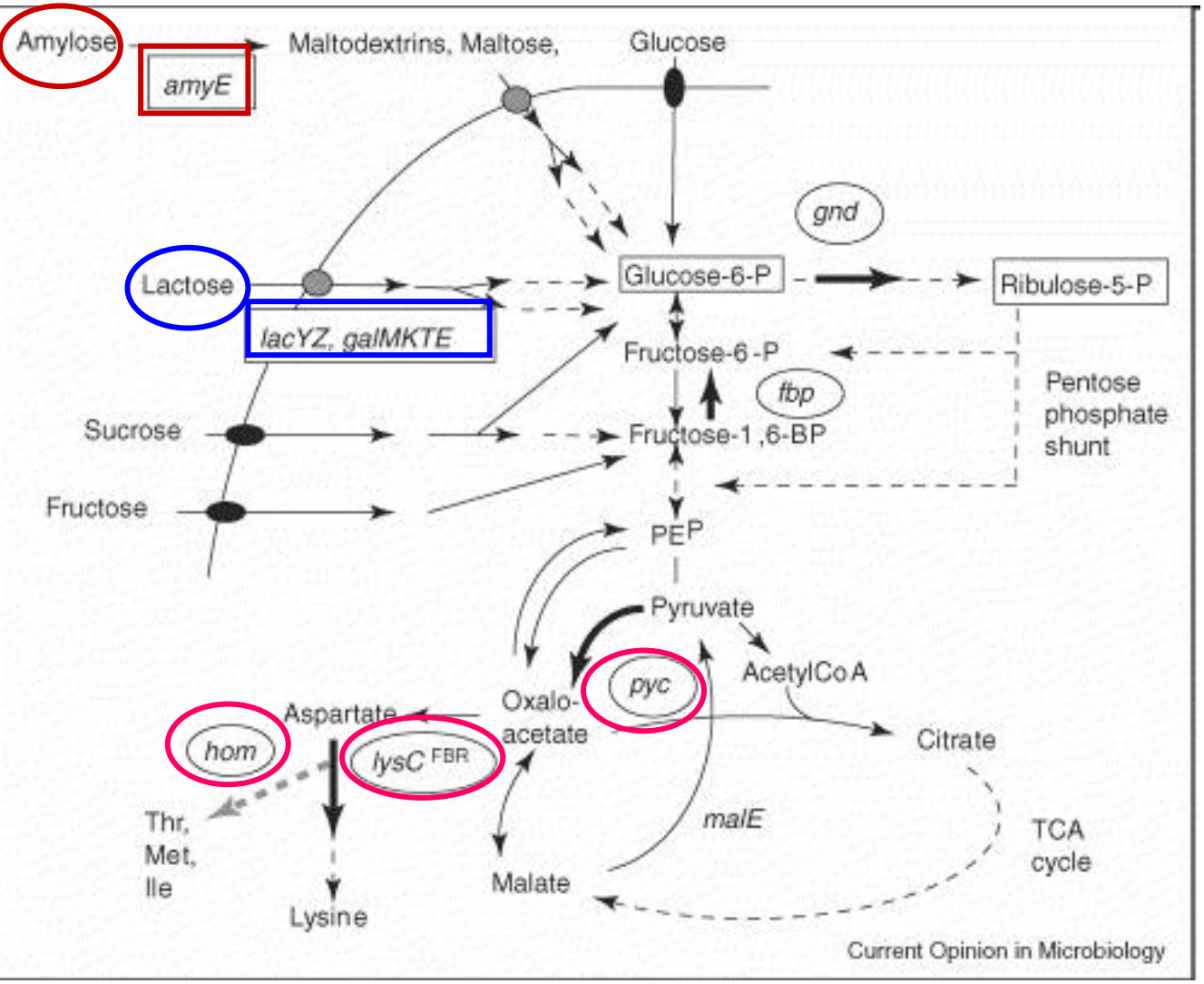
Source: Reprinted with permission from: Leuchtenberger W. Products of primary metabolism. Biotechnology 1996; 6:455–502.

Metabolic Engineering of Amino Acid Production

Table 2 Classical Development of an Amino Acid Producer: Development of an L-Arginine Producer of *C. glutamicum* by Subsequent Introduction of Mutations and the Known Characters of the Mutants

		Relative activity			
Strain	Phenotype selected	Acetylglutamokinase	Ornithine carbamyl-transferase	Feedback regulation of kinase	L-Arginine produced (g/L)
KY 10025	Wild-type strain	1.0	—	Sensitive	0
KY 10150	Isoleucine minus	—	1.0		0
DSS-8	D-Serine sensitive	19.9	11.5	Sensitive	1.5
KY 10479	D-Arginine resistant	19.1	—	Resistant	6.8
KY 10480	Arginine hydroxamate resistant	18.2	—	Resistant	16.6
KY 10508	Isoleucine plus	18.2	16.4	Resistant	19.9
KY 10577	Thiazole alanine resistant	18.7	19.3	Resistant	20–25

Metabolic Engineering of Amino Acid Production



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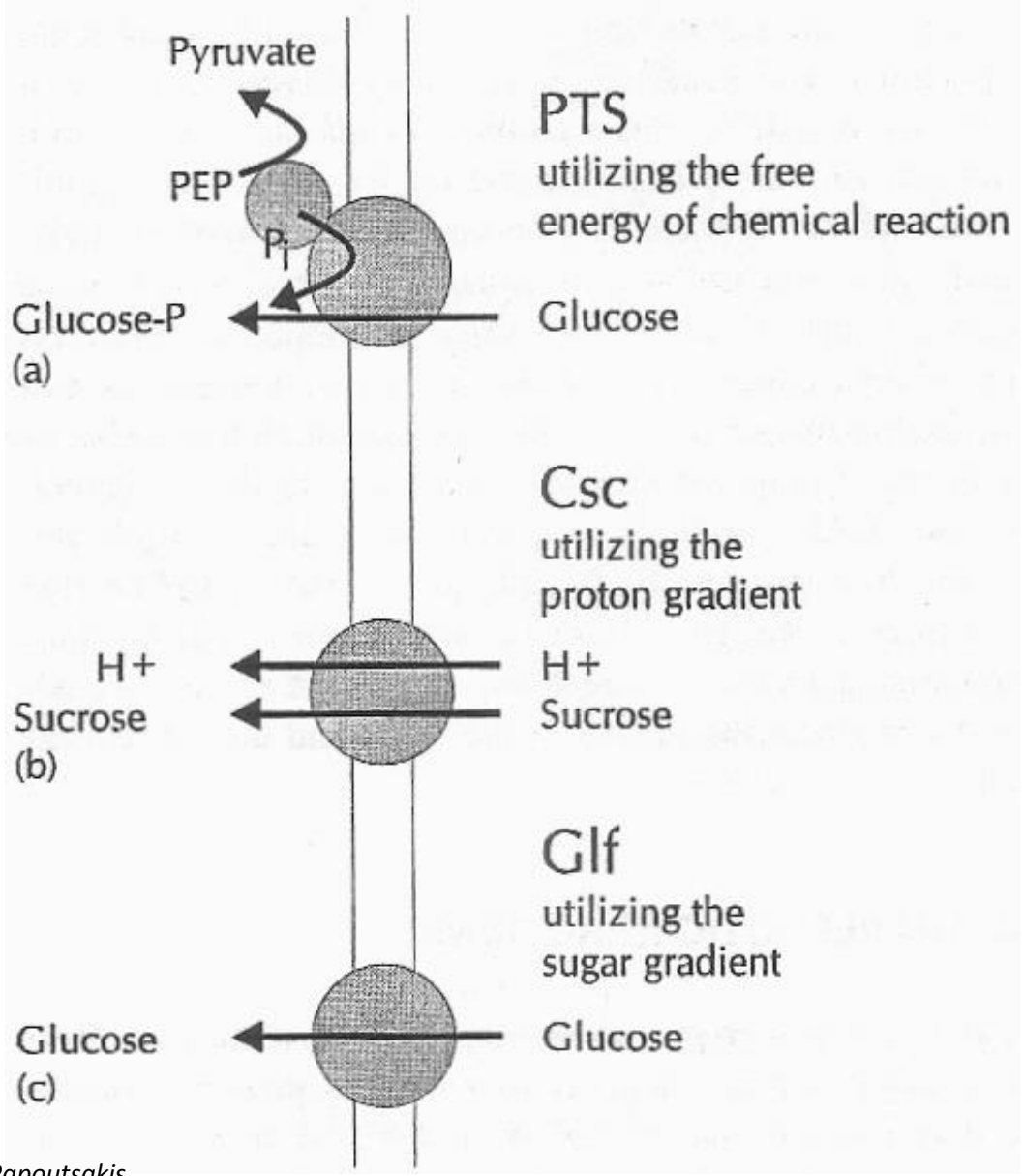
Metabolic engineering of C. glutamicum for lysine production. Aspartokinase, homoserine dehydrogenase and pyruvate carboxylase are well-known to be important for lysine production and mutant alleles of the respective genes lysC, hom and pyc were identified. Introduction of these alleles into C. glutamicum wild type enabled high-yield lysine production [36]. Additional introduction of a mutant gnd allele coding for increased lysine production on glucose [37] and overexpression of endogenous fbp increased lysine production on sucrose [38]. Expression of lacYZ from L. delbrueckii subsp. bulgaricus and of galMKTE from L. lactis subsp. cremoris enabled lysine production from whey [40]. Plasmid-borne expression of amyE from S. griseus enabled lysine production with amylose as carbon source [41]. Single or several enzymatic reactions are indicated by straight and interrupted lines, respectively. Bold arrows denote increased conversions and bold, grey, interrupted arrow the reduced conversion by homoserine dehydrogenase. Next to the respective enzymatic reactions, endogenous and heterologous gene names are given in circles and squares, respectively. Transport via phosphoenol-pyruvate-dependent transport systems is depicted in black ovals, transport via other systems in grey circles.

Engineering of Substrate Uptake

PTS: Phosphotransferase system
Csc: Sucrose permease
Glf: Glucose difusion facilitator

Different modes of sugar uptake and activation. Glucose uptake by the PTS, wher phosphoenol pyruvate (PEP) is required for translocation together with activation (**a**), sugar transport in symport with protons as in the case for Csc and GalP (**b**), and facilitated glucose diffusion by the facilitator Glf not requiring metabolic energy (**c**).



Taken from „Metabolic Engineering“, edited by Sang Yup Lee,E. Terry Papoutsakis

Engineering anaplerotic reactions

Anaplerotic reactions are those that form intermediates of the TCA

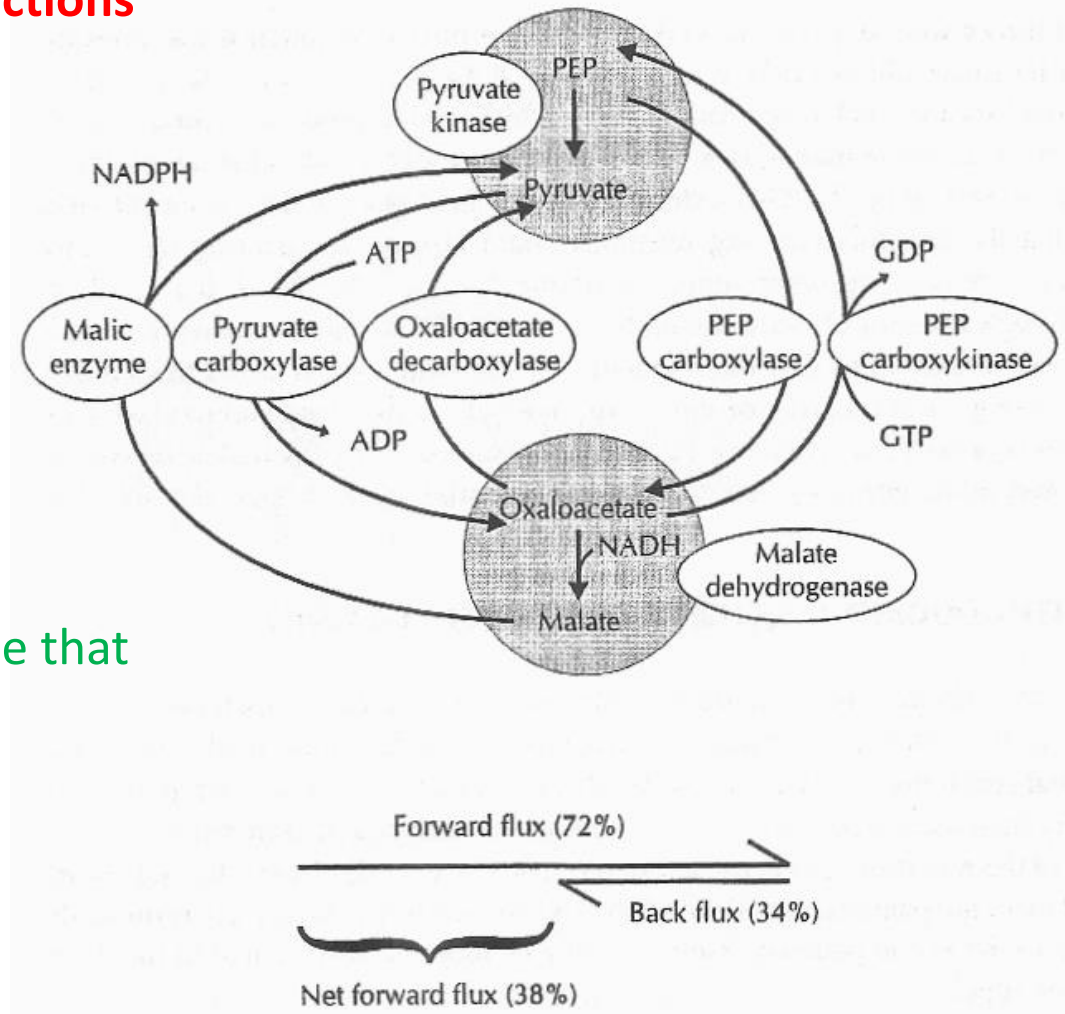


Figure 2 Interconversion of C-3 units to C-4 units. Enzymes of the anaplerotic reactions and adjacent reactions in *Corynebacterium glutamicum* that contribute to flux between C-3 units and C-4 units. The sum of forward flux (72%) and the sum of back flux (34%) are shown to result in a net forward flux (38%).

dapA Overexpression leads to growth limitation

Table 3 Molecularly Introduced Growth Limitation to Increase Product Excretion

Strain	*dapA* copies	Synthase activity[a]	Growth rate (1/h)	Intracellular alanine (mM)	Excretion rate[b]
13032	1	0.051	0.43	3	0
13032::*dapA*	2	0.072	0.37	6	0.25
13032 pKW3::*dapA*	6	0.250	0.36	8	2.7
13032 pJC23	20	0.630	0.22	9	3.8

[a] The specific synthase activity is in micromoles per minute per milligram of protein [µmol/(min · mg)].
[b] Lysine excretion rate in millimoles per minute per gram [mmol/(min · g)] dry weight.

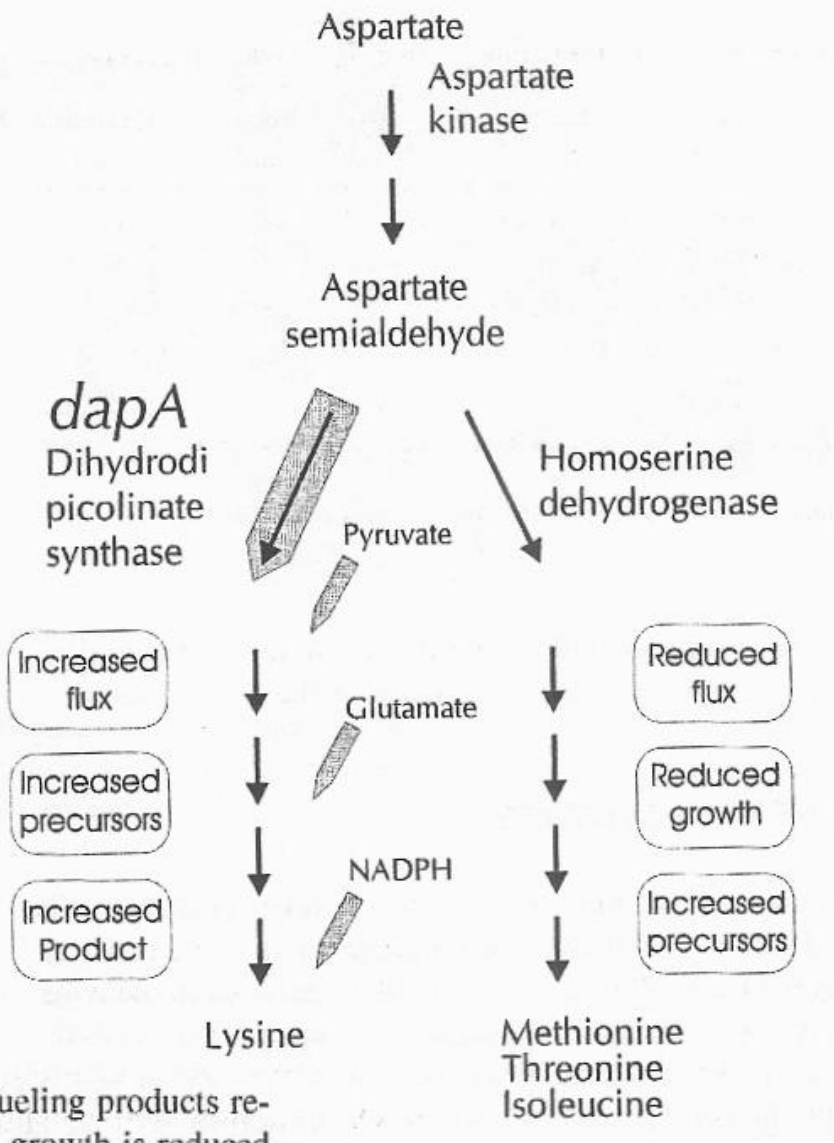


Figure 3 Growth limitation results in the increased availability of fueling products required for overproduction. As a consequence of *dapA* overexpression, growth is reduced and more pyruvate and further fueling products are available for L-lysine synthesis.

Engineering Branch Points

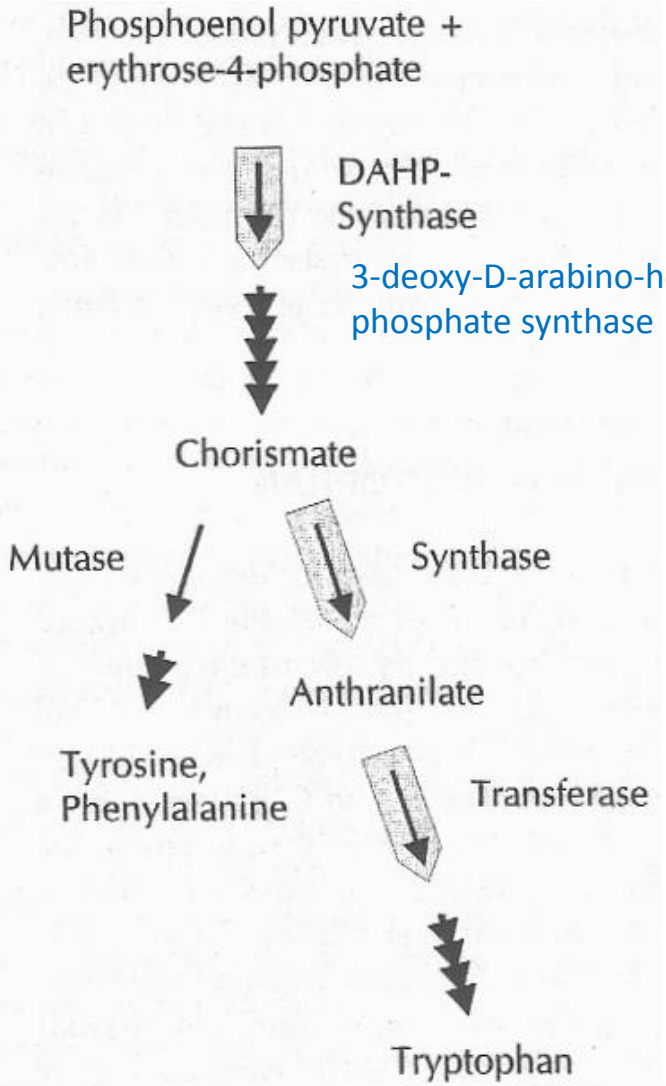


Figure 5 Engineering a branch point within amino acid synthesis. The relevant reactions (thick arrows) are shown to achieve chorismate formation and the entire conversion of this branch point metabolite to L-tryptophan.

Engineering Nitrogen Fluxes

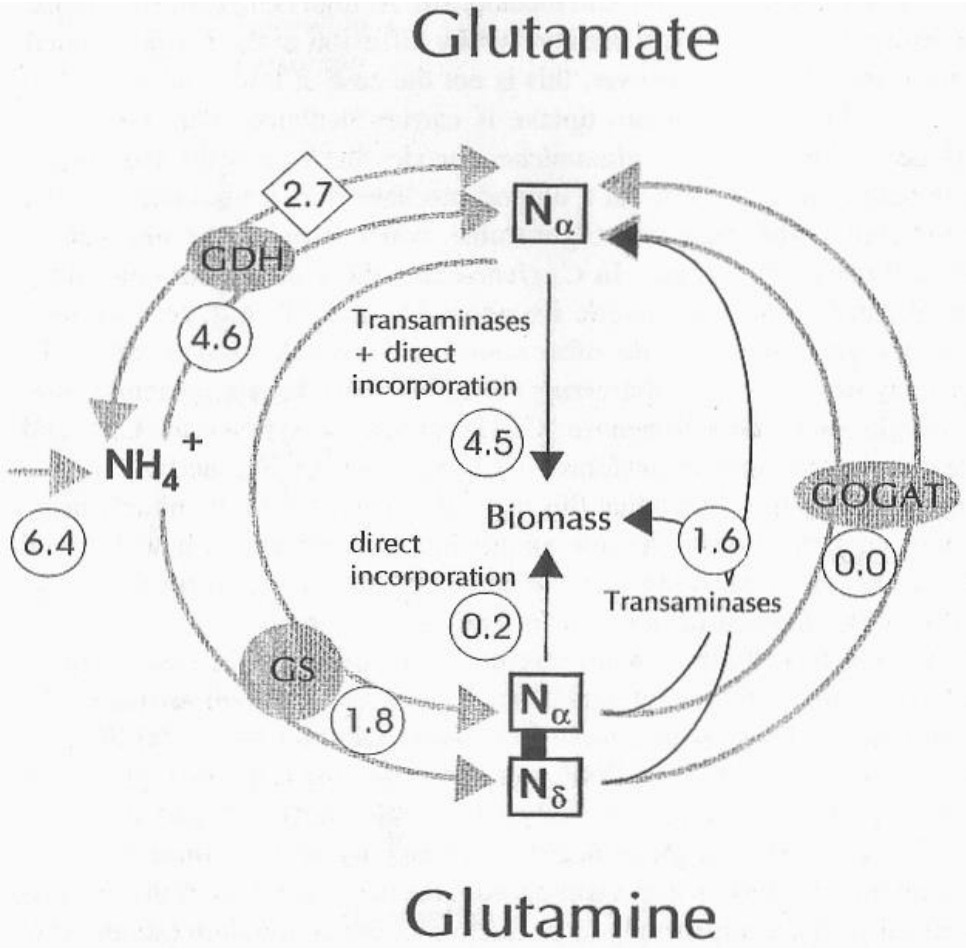
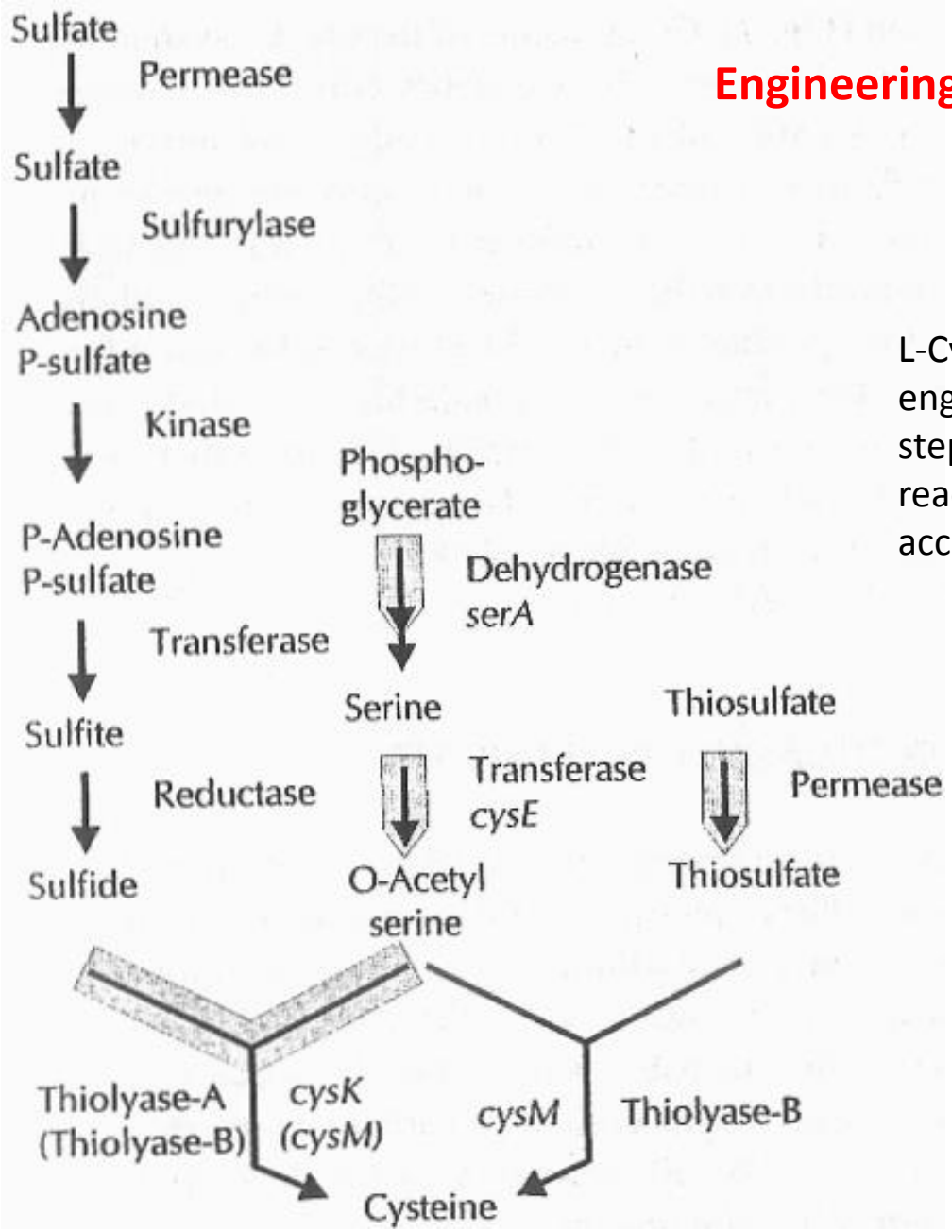


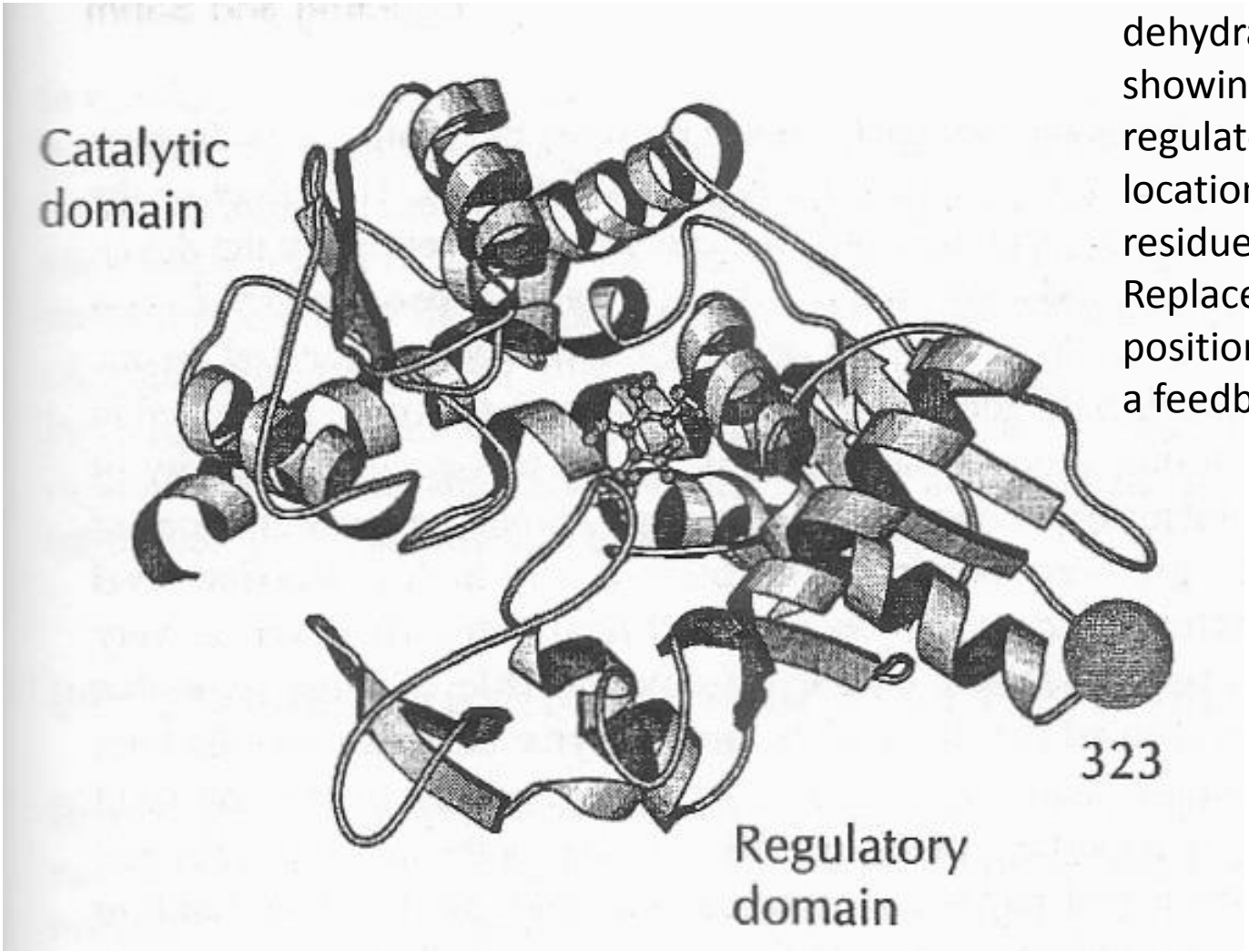
Figure 6 In vivo nitrogen fluxes as quantified by NMR spectroscopy. The fluxes via the glutamate dehydrogenase (GDH), glutamine synthetase (GS), and glutamate oxoglutarate aminotransferase (GOGAT) were quantified in *Corynebacterium glutamicum* grown in continuous culture at a dilution rate of 0.05 1/h. Numbers in circles give molar nitrogen net fluxes [µmol/(min · g) (dry weight)]; the exchange flux is given in the diamond.

Engineering Sulfur Metabolism



L-Cysteine synthesis requires the engineering of the sulfur incorporation step. Thick arrows mark the relvant reaction steps to obtain a high accumulation of L-cysteine.

Engineering of Enzyme Properties
→ Protein Engineering



Engineering enzyme activity by amino acid exchange.
Model of the Threonine dehydratase polypeptide showing the catalytic and regulatory domain, as well as location of the amino acid residue 323.
Replacement of valine in this position by alanine results in a feedback-resistant enzyme.

Concerted Flux increase

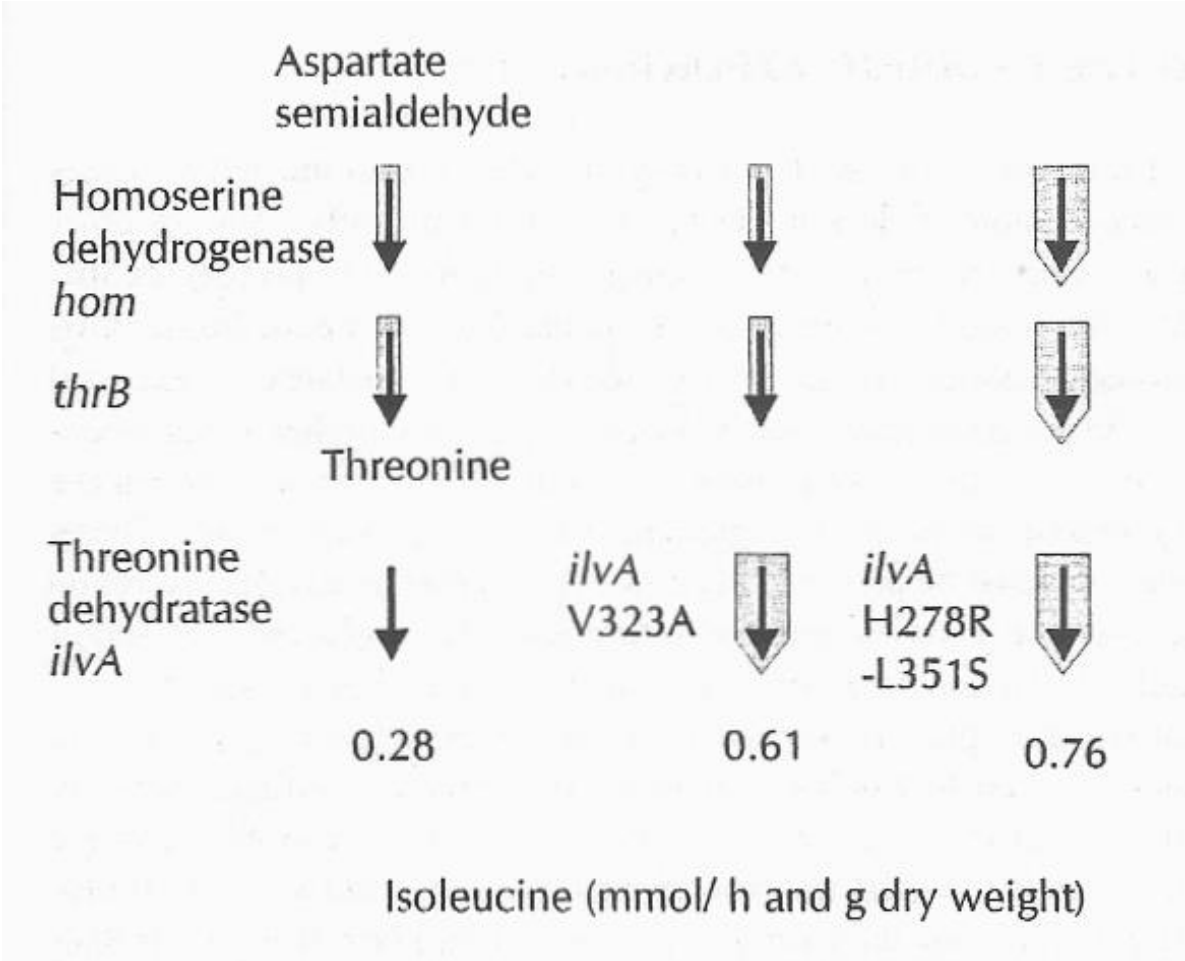


Figure 8 Concerted flux increase to achieve high L-isoleucine formation rates. The size of the arrows roughly corresponds to the respective expression levels of the genes. The most successful *ilvA* allele used at each step is given by the single-letter code for the amino acid replaced.

Engineering Transport Carrier

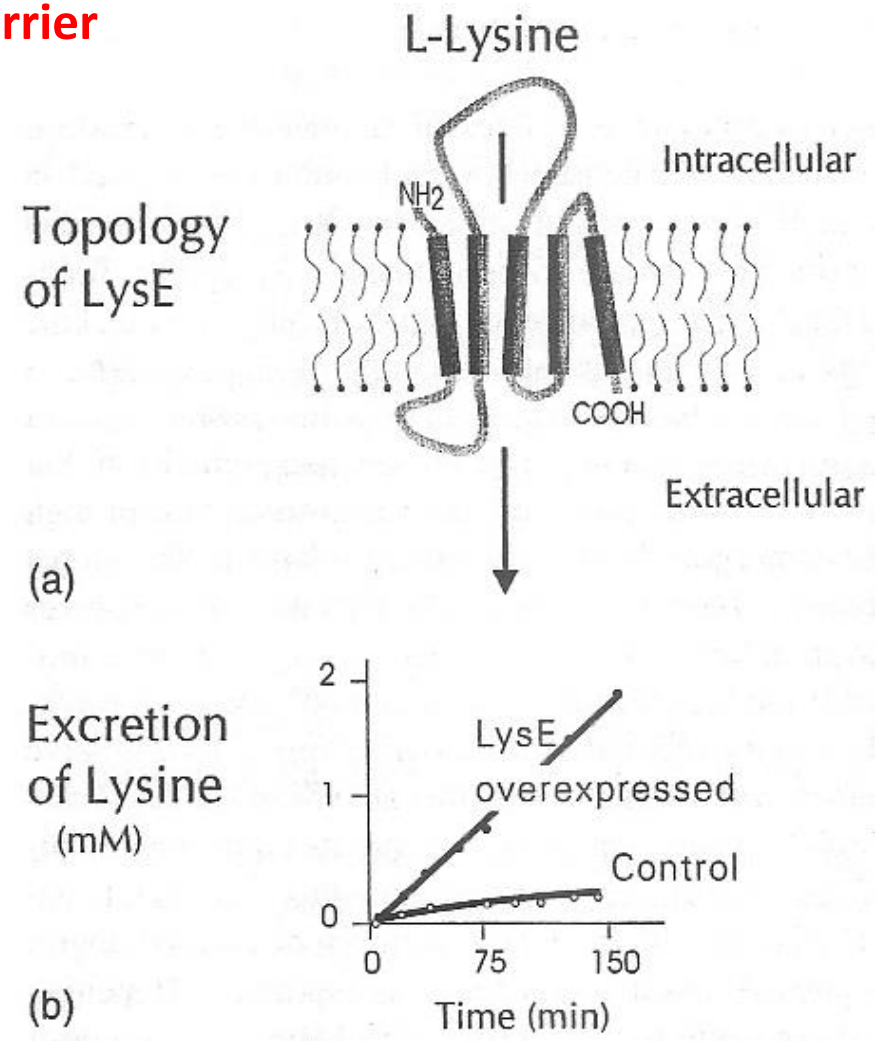


Figure 9 The L-lysine export carrier of *C. glutamicum*. (a) Model of the structure of this new type of carrier. (b) The increased excretion rate of L-lysine in response to overexpression of the carrier.

2.6.15