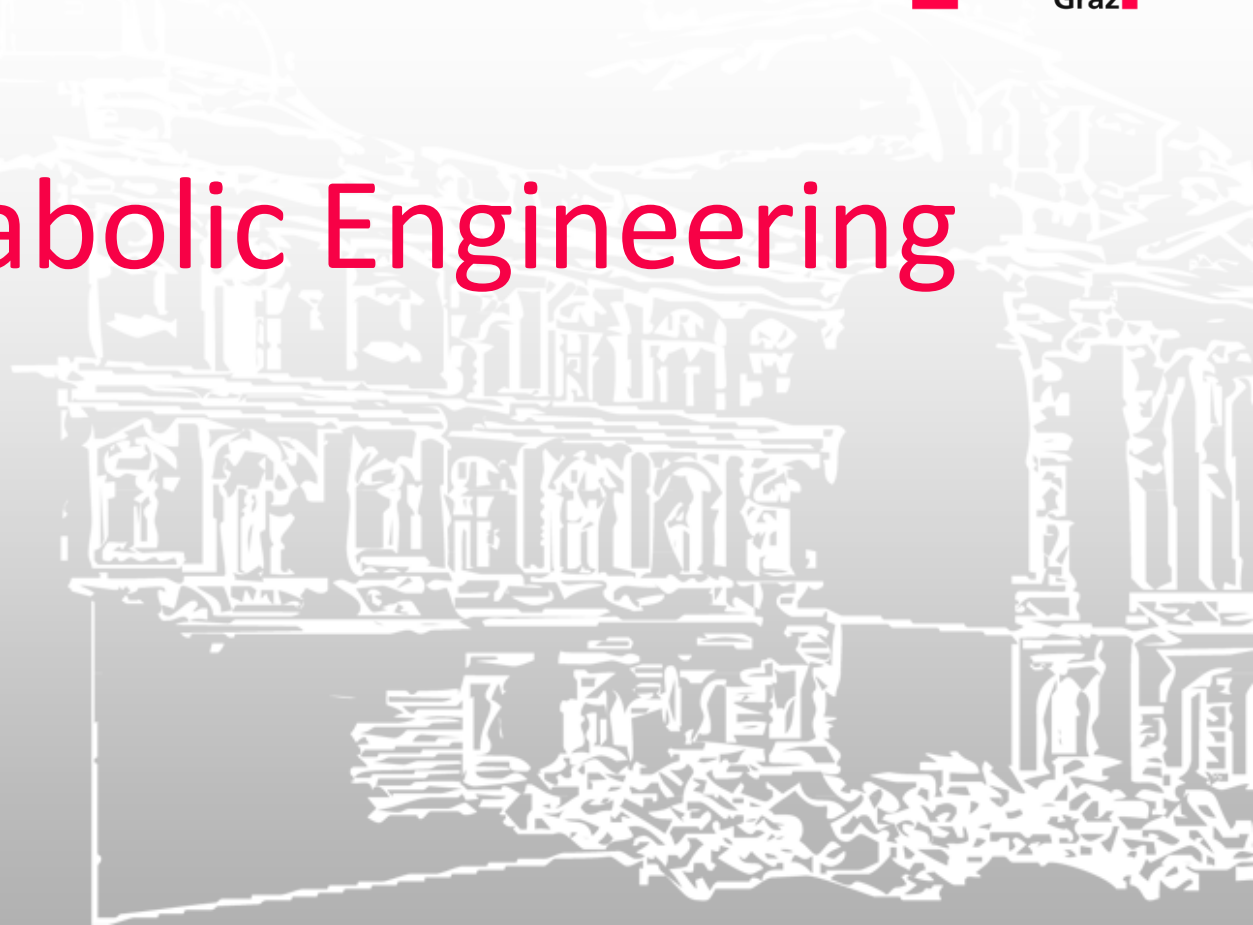
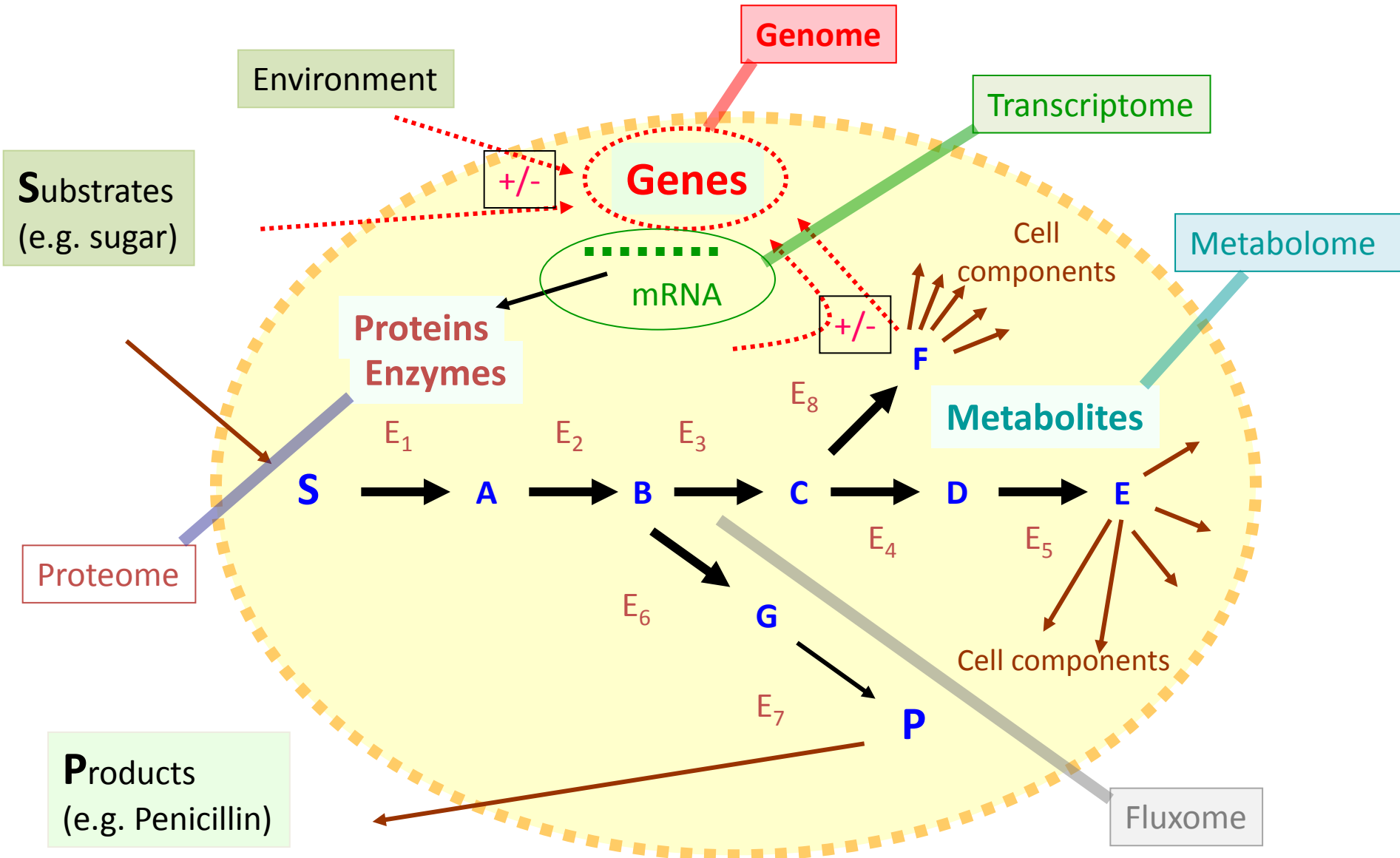


Metabolic Engineering



Cell function



Strain Development

Evolutionary Development

Mutagenesis

Recombination

Screening – Selection

- Individual clone analysis
- preselection

Rational Development

Metabolic Engineering

- Engineering of defined genes
- Engineering of pathways
- Synthetic Biology

Testing defined strains

Strain from natural biodiversity

Improved Strain

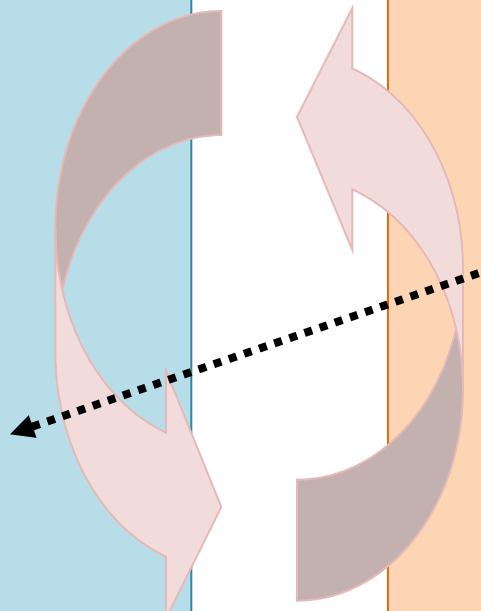


Table 1. Classification of mutations, their origins, and potential effects

Type of change	Length	Source of mutation	Effects ^a
Small local changes			
Substitution	1 bp	Spontaneous mutagenesis	Gene silencing
Insertion	1 to several bp	Replication infidelities	Gene expression
Deletion	1 to several bp		Cryptic gene activation
Duplication	1 to several bp		Altered protein specificities
DNA rearrangements			
Inversion	Several bp up to	Homologous	Gene silencing
Duplication	several kb	recombination	Gene expression
Insertion		Mobile genetic	Cryptic gene activation
Deletion		elements (i. e. IS elements,	Gene dosage
Excision		transposons)	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	Increase of total genetic information content
		Conjugation	Gene silencing
		Transduction (phage-mediated)	

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

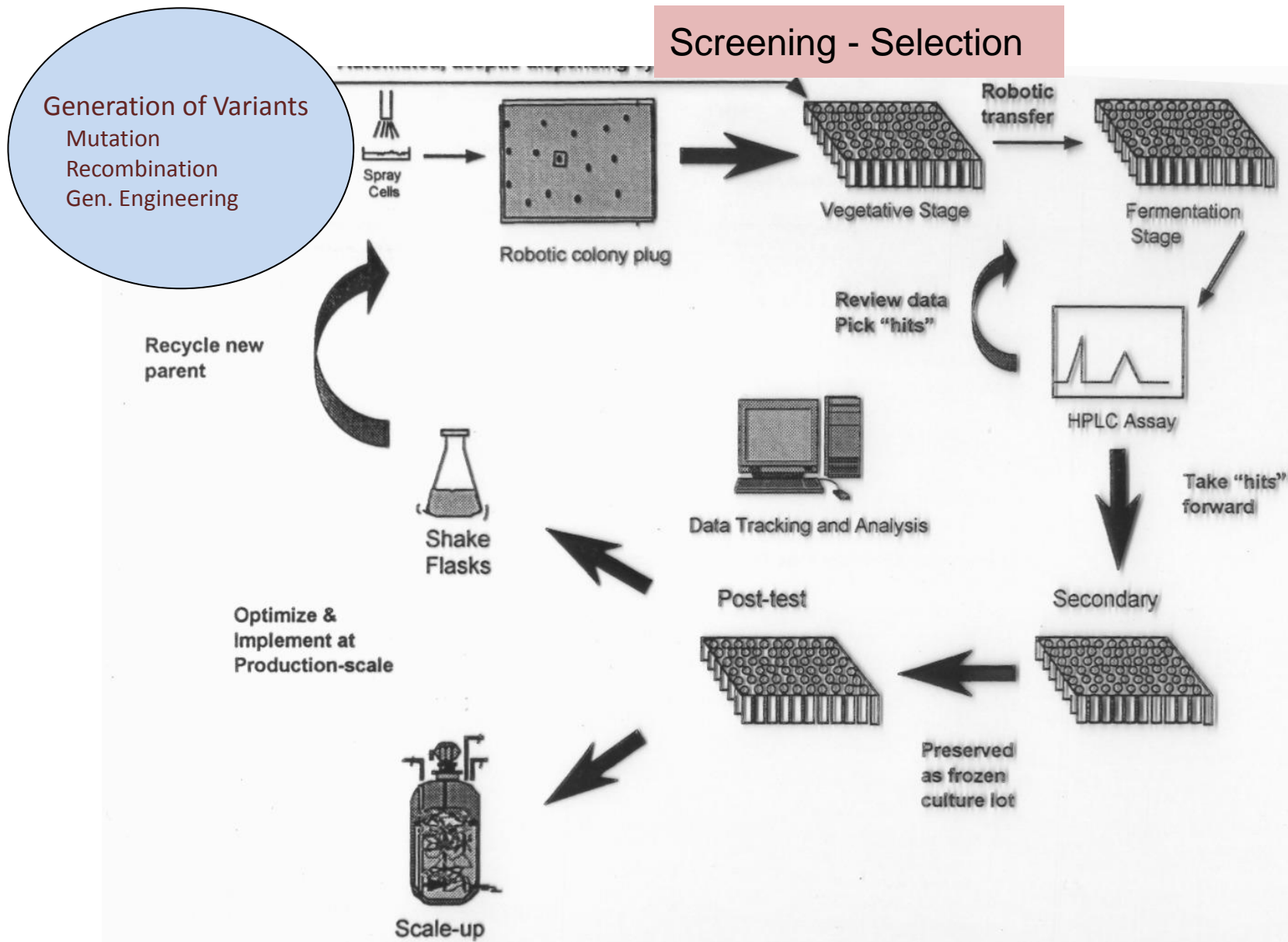


FIGURE 1 Schematic representation of an automated screening system.

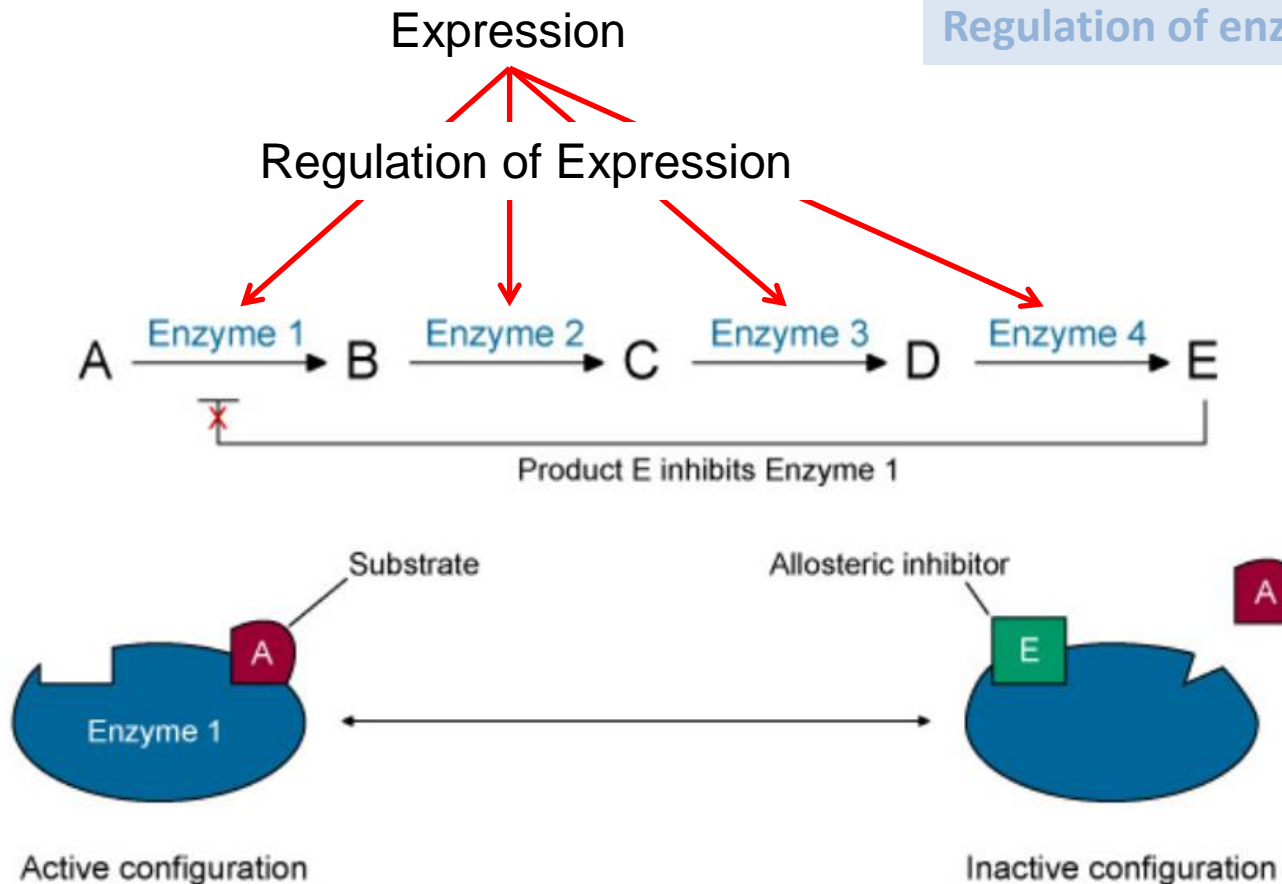
Rational approaches for strain improvement

Knowing about the molecular relationships in the metabolism

Regulation

Regulation of expression

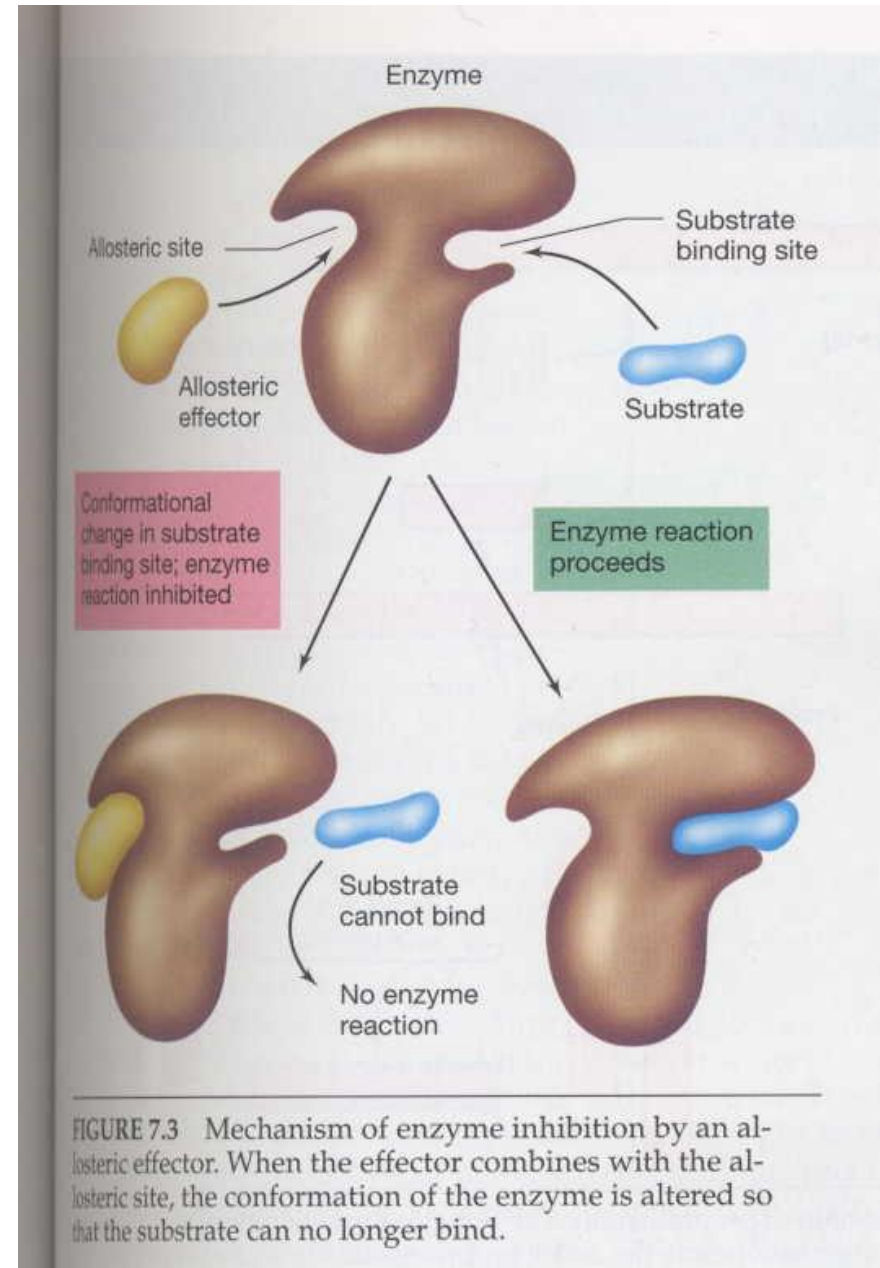
Regulation of enzyme activity



Rational approaches for strain improvement

Knowing about the functional relationships of enzymes in the metabolism

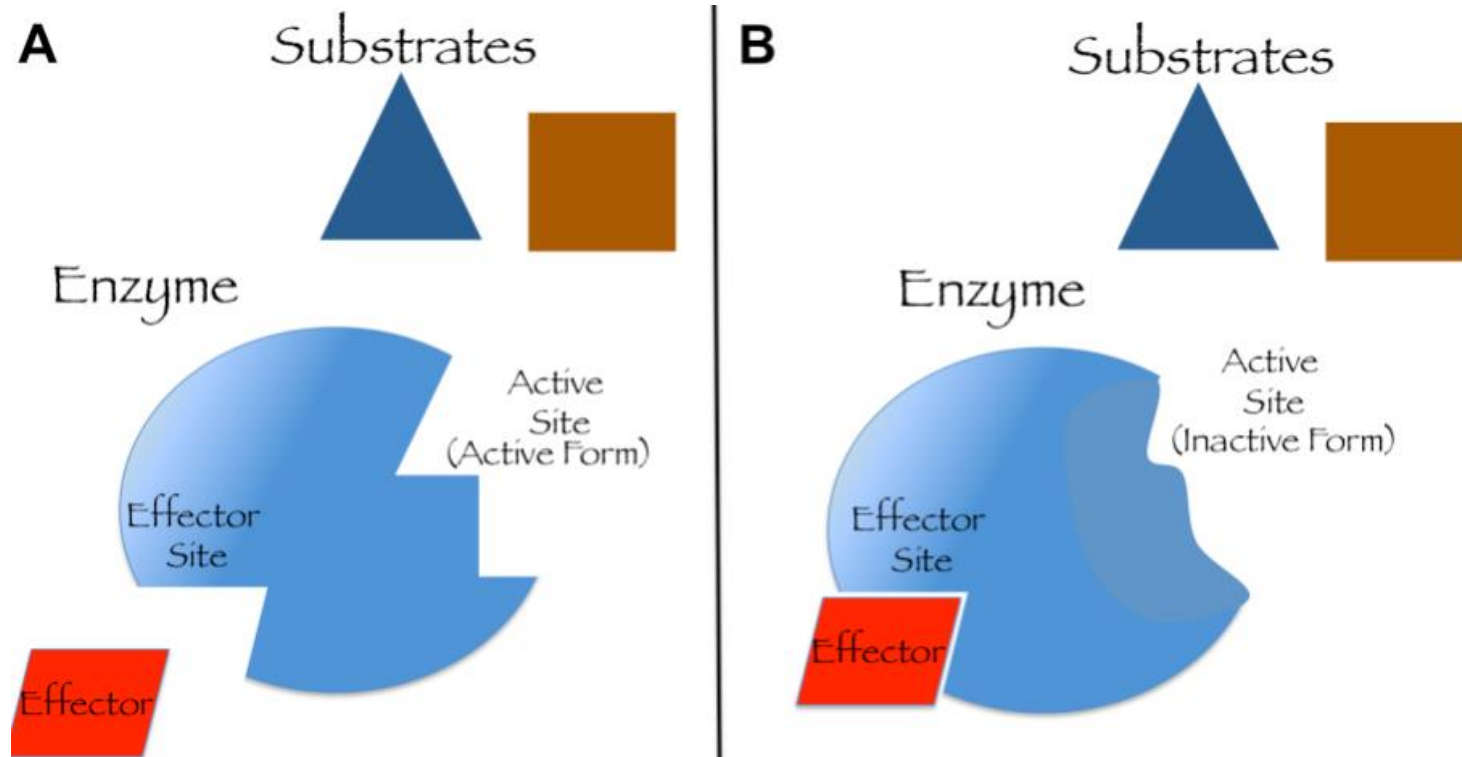
Regulation



Rational approaches for strain improvement

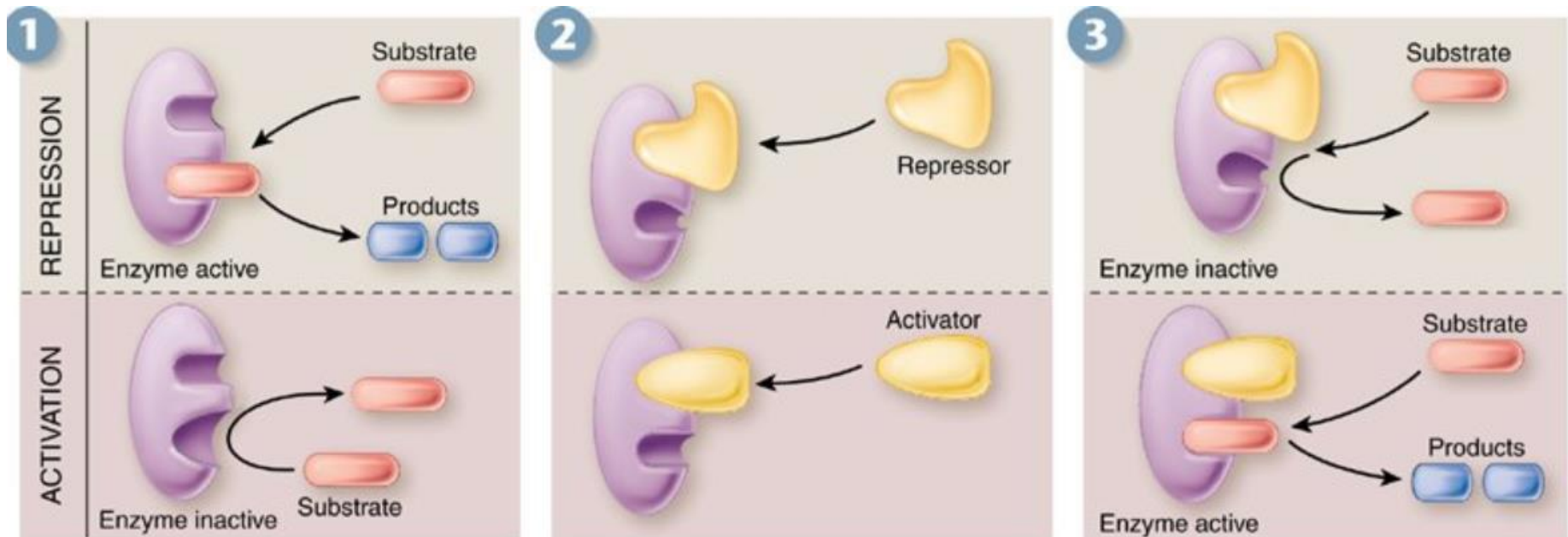
Knowing about the functional relationships of enzymes in the metabolism

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

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

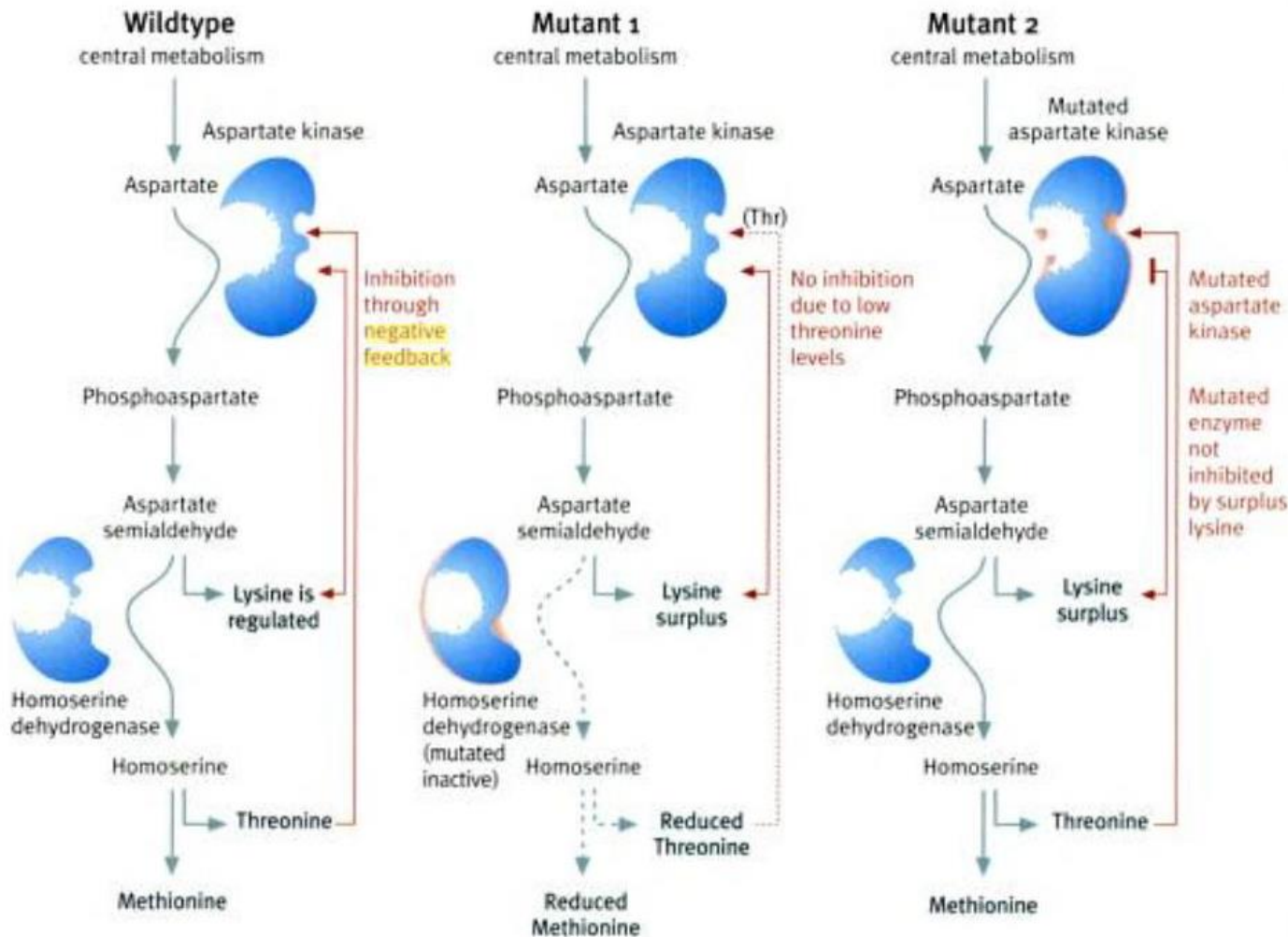


Allosteric enzymes subject to repression are active in the absence of signal molecules, while allosteric enzymes that rely on activation are not active in the absence of signal molecules.

When signal molecules bind allosteric enzymes, they change the shape of the active site. Repressors disrupt the active site, while activators restore it.

Allosteric enzymes subject to repression are not active in the presence of signal molecules, while allosteric enzymes that rely on activation require signal molecules to be active.

Deactivation of feedback regulation



Corynebacterium glutamicum

Fig. 4.21 Negative feedback during the **synthesis** of lysine in wildtype *Corynebacterium* (electron microscopical 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.

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-methyl-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

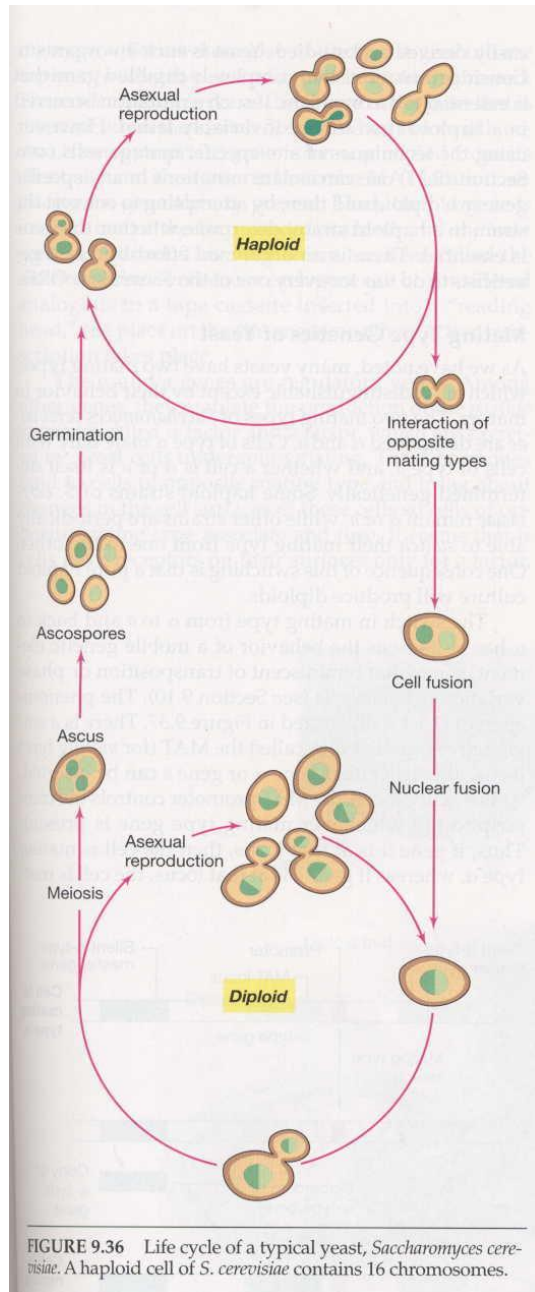


FIGURE 9.36 Life cycle of a typical yeast, *Saccharomyces cerevisiae*. A haploid cell of *S. cerevisiae* contains 16 chromosomes.

Recombination genetics

Specific/targeted crossing of organisms via sexual reproduction cycles

Cell fusion – fusion of somatic cells

Gene transfer via parasexual mechanisms

transduction

transformation

conjugation

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

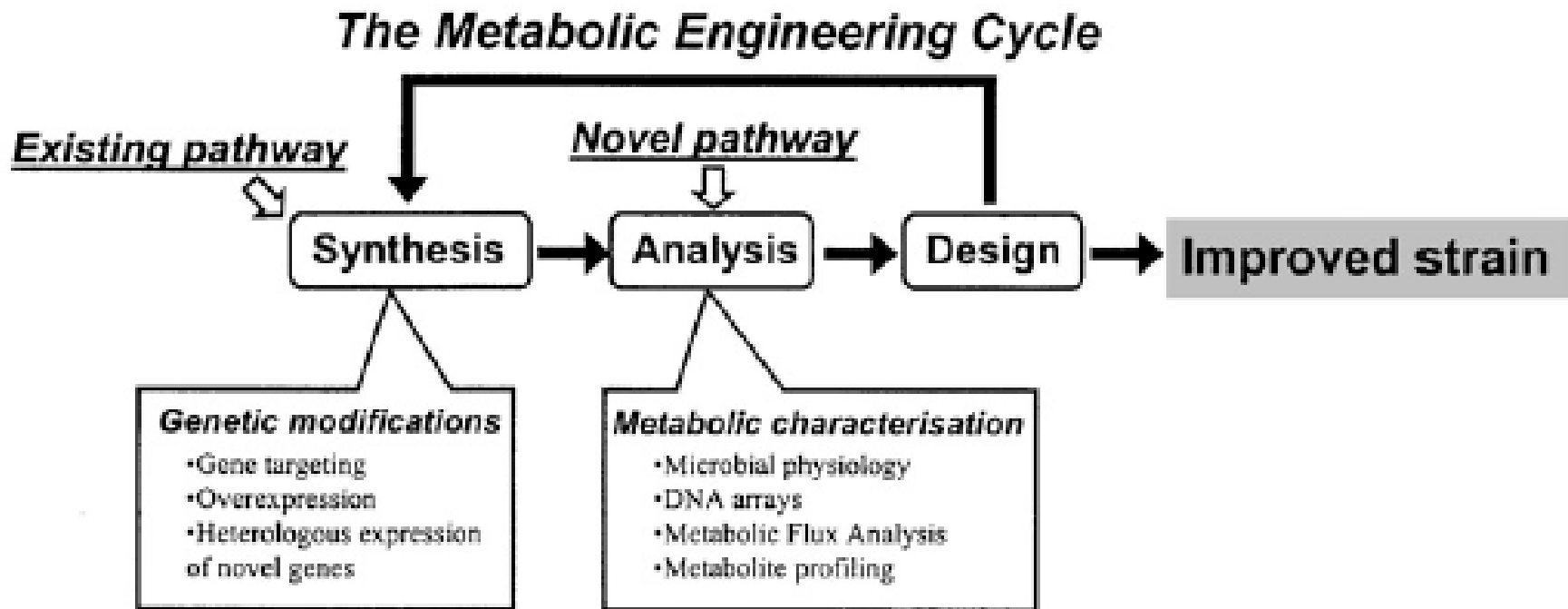
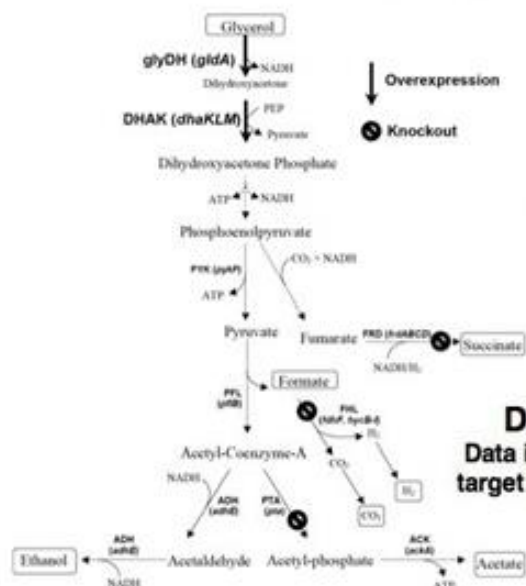


Fig. 1. Principles of metabolic engineering.

Metabolic Engineering (MetEng) and Metabolic Evolution (MetEvo)



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

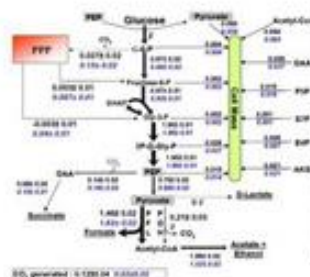
STRAIN CHARACTERIZATION
Tubes, Flask, Bioreactors



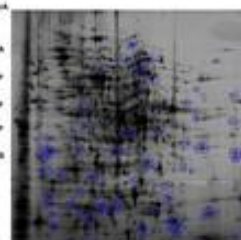
DESIGN
Data integration & target Identification

MetEng & MetEvo Cycles

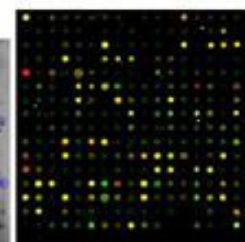
SYSTEMS ANALYSIS
Functional genomics tools



Fluxomics



Proteomics



Transcriptomics

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

Analysis of metabolic networks

Essential approach: integral identification of influencing variables (parameters)

Metabolic Networks

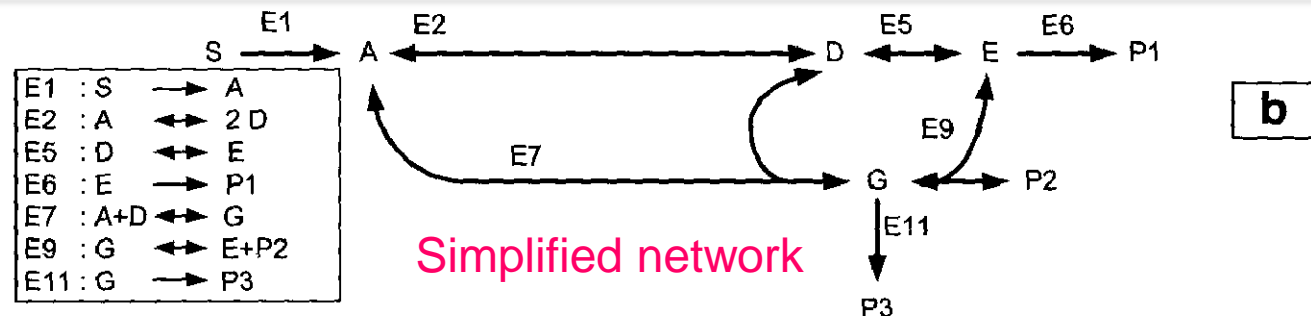
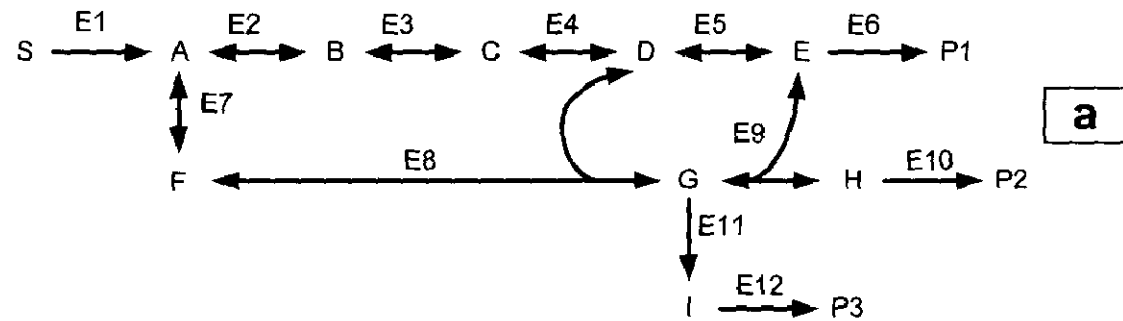
Reaction network → involved biochemical reactions and reaction partners

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



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

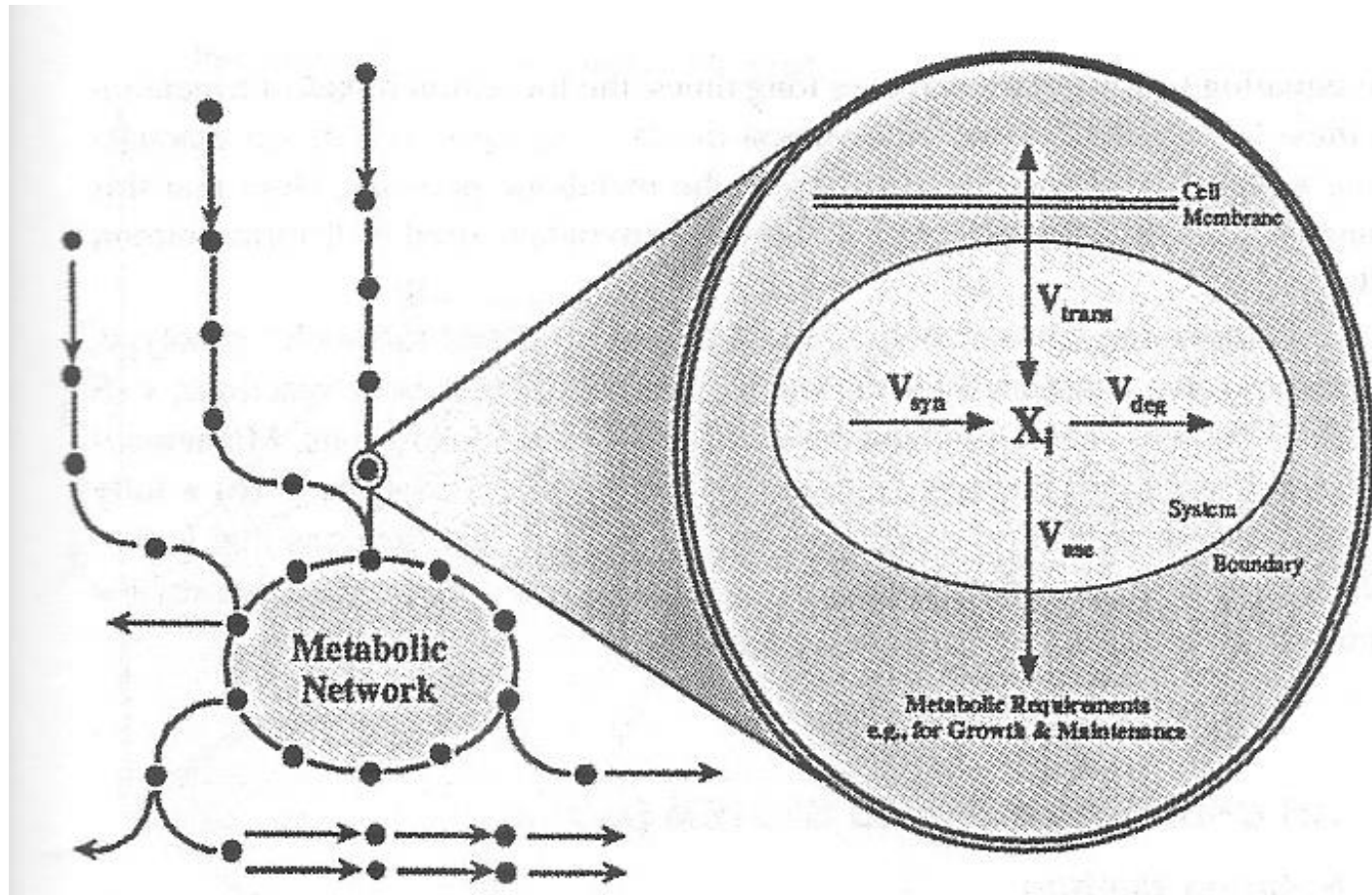


Figure 1 Flux balance models use material balances around each metabolite in a metabolic network. The concentration of each metabolite X_i is affected by various fluxes V_j ; V_{trans} is the uptake or secretion flux, while V_{use} is the flux required for growth and maintenance; V_{syn} and V_{deg}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

Metabolic Flux Analysis

Dynamic mass balance for specific metabolite

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

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

$b_i = \text{net transport}$

Metabolic network for n metabolites and m metabolic fluxes

$$\frac{dX}{dt} = S \cdot v - b$$

$X = n$ -dimensional vector (3)

$S = \text{stoichiometric } n \times m \text{ matrix}$

$b = \text{vector of known metabolic demands}$

For steady state



$$S \cdot v = b$$

(4)

Metabolic Flux Analysis

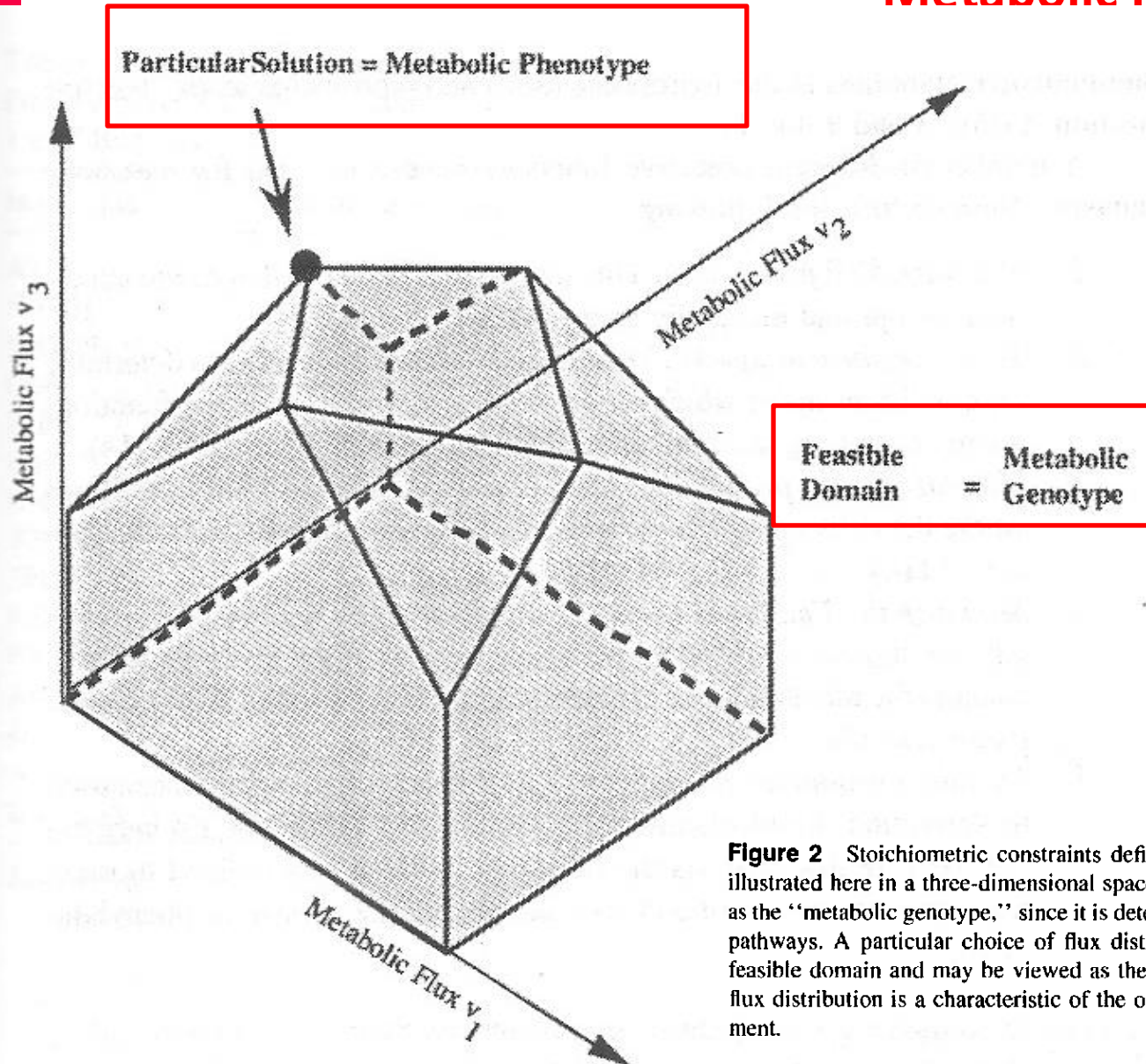


Figure 2 Stoichiometric constraints defined by equation 4 define a feasible domain, illustrated here in a three-dimensional space. The feasible domain is thus conceptualized as the “metabolic genotype,” since it is determined by the m -enzyme-catalyzed metabolic pathways. A particular choice of flux 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

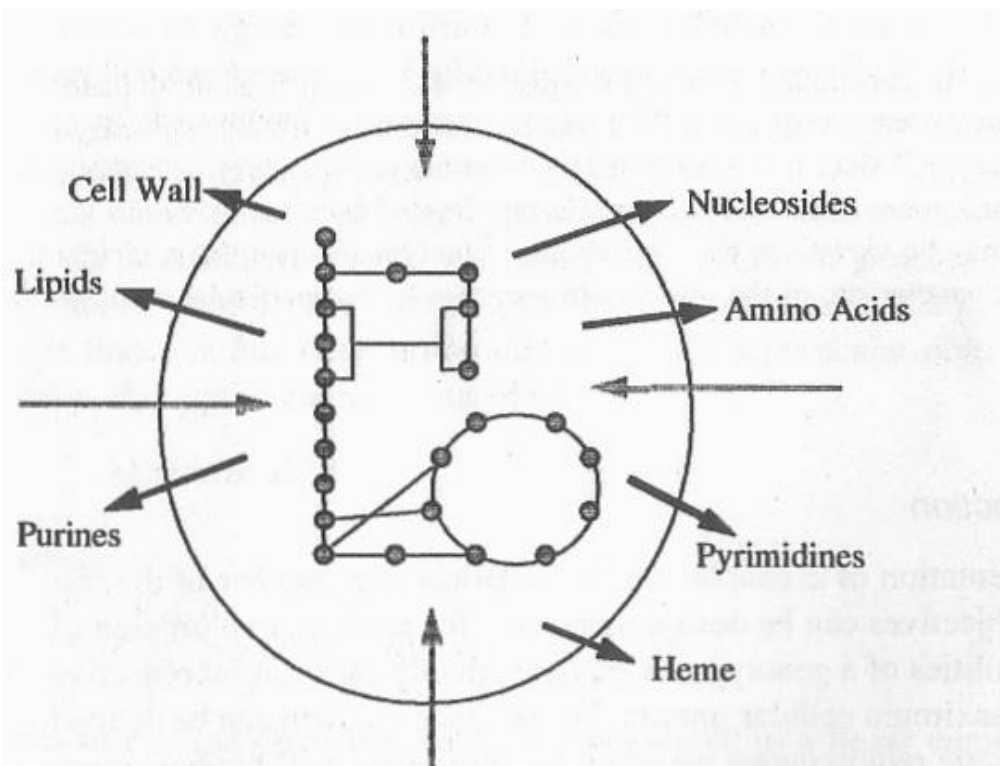


Figure 3 Definition of the maximal growth rate objective function.

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

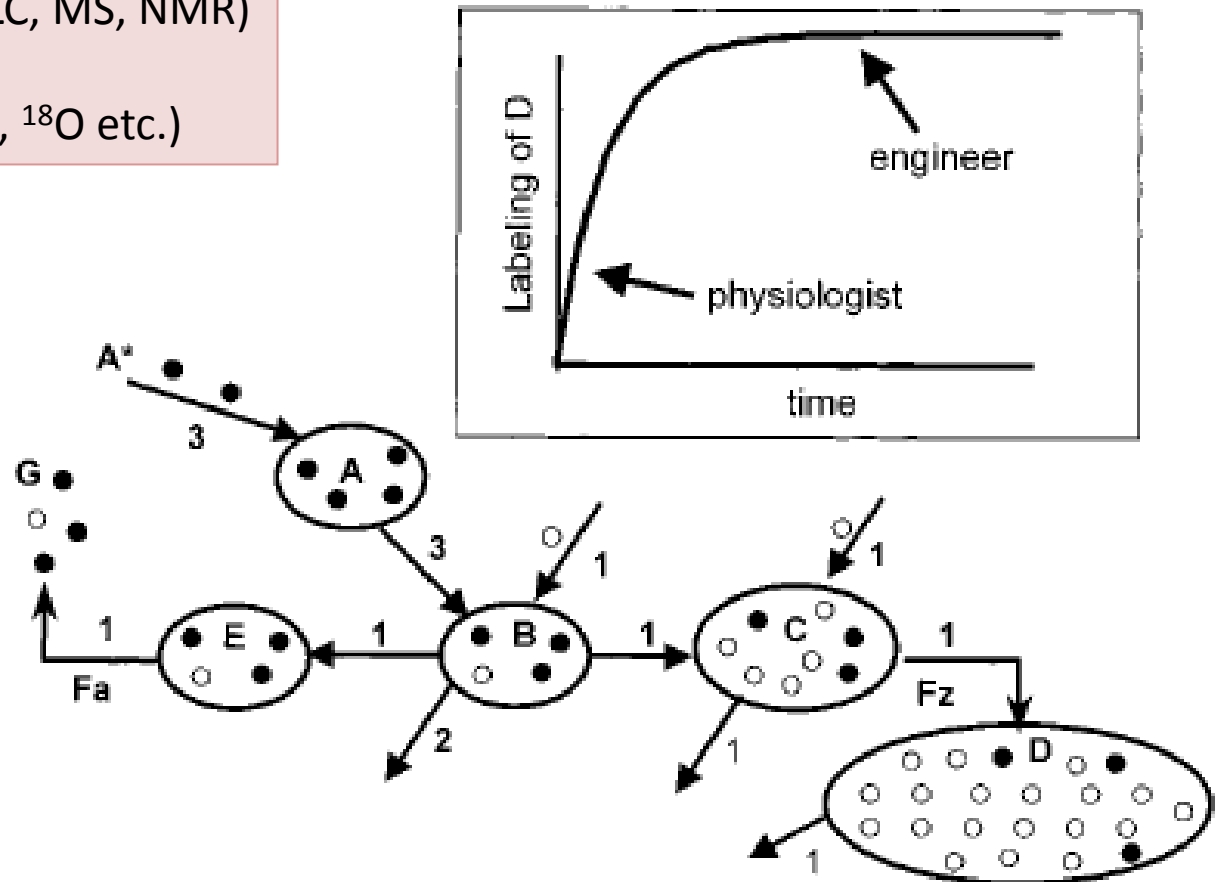
Metabolite	Demand (mmol)
ATP	41.2570
NADH	-3.5470
NADPH	18.2250
G6P	0.2050
F6P	0.0709
R5P	0.8977
E4P	0.3610
T3P	0.1290
3PG	1.4960
PEP	0.5191
PYR	2.8328
AcCoA	3.7478
OAA	1.7867
AKG	1.0789
SuccCoA	—

Metabolic Flux Analysis

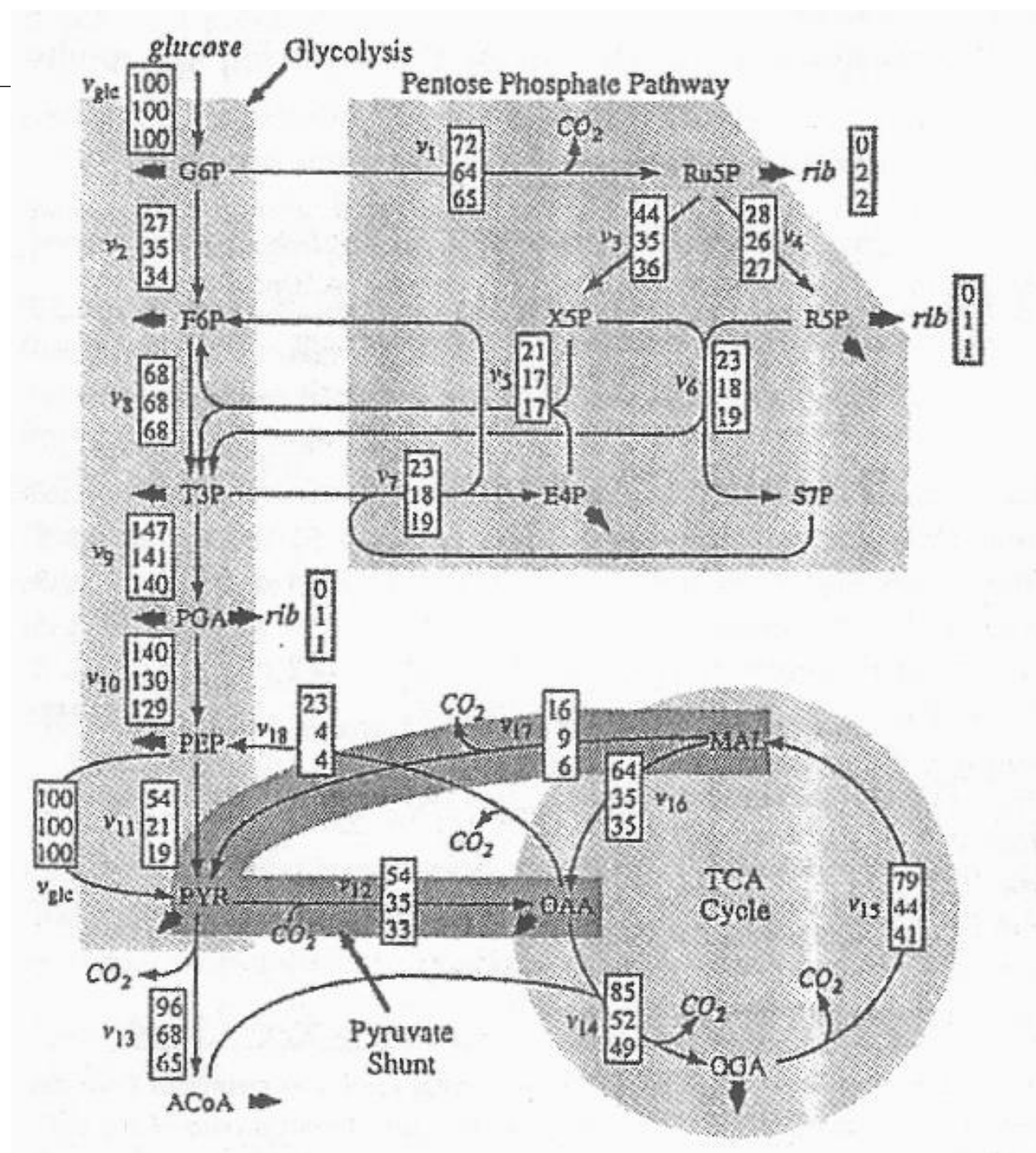
Methods for Flux Analysis

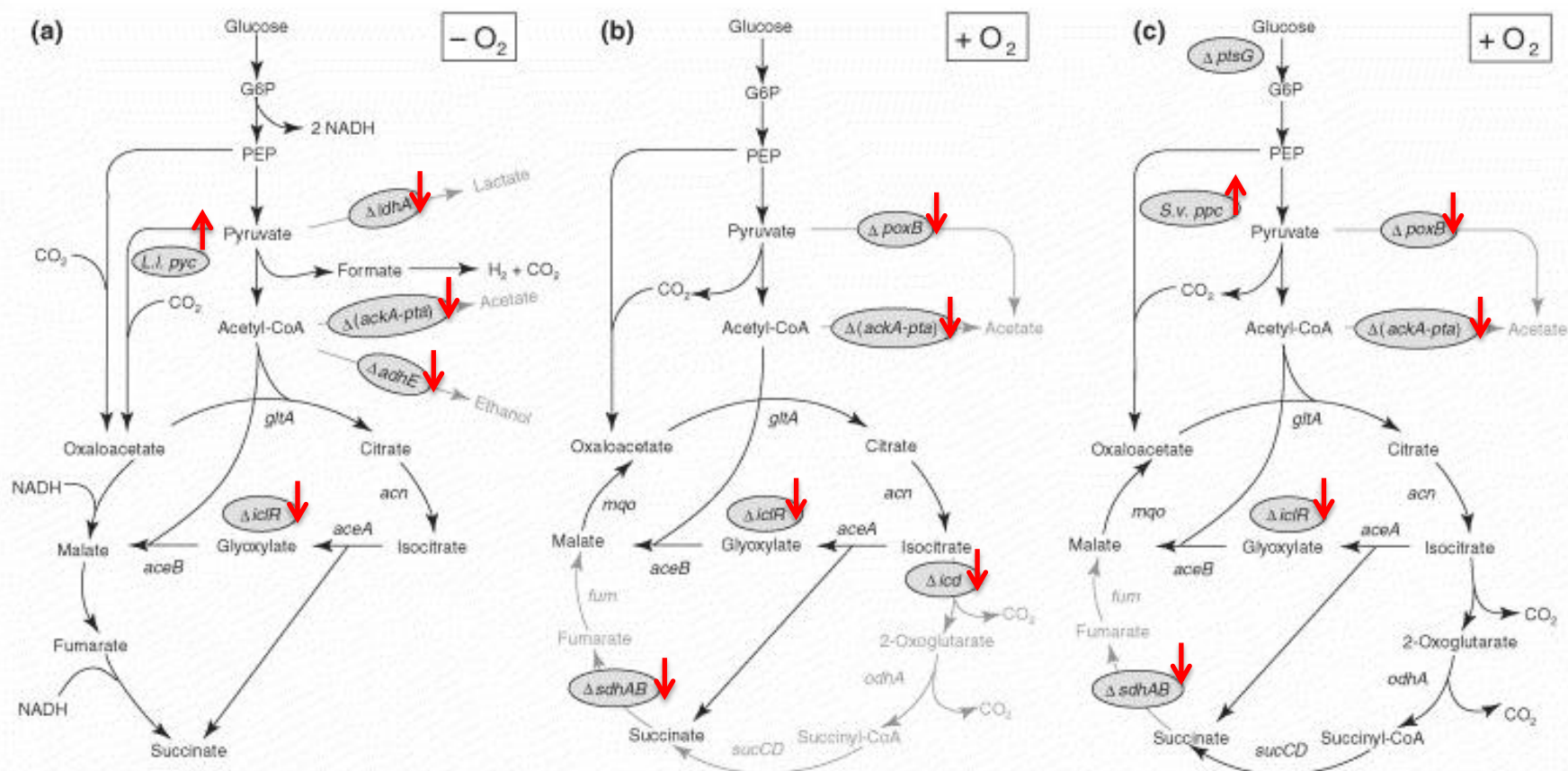
- Metabolite Pools (GC, LC, MS, NMR)
- **Isotope tracing** (^{13}C , ^2H , ^{18}O etc.)

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 .

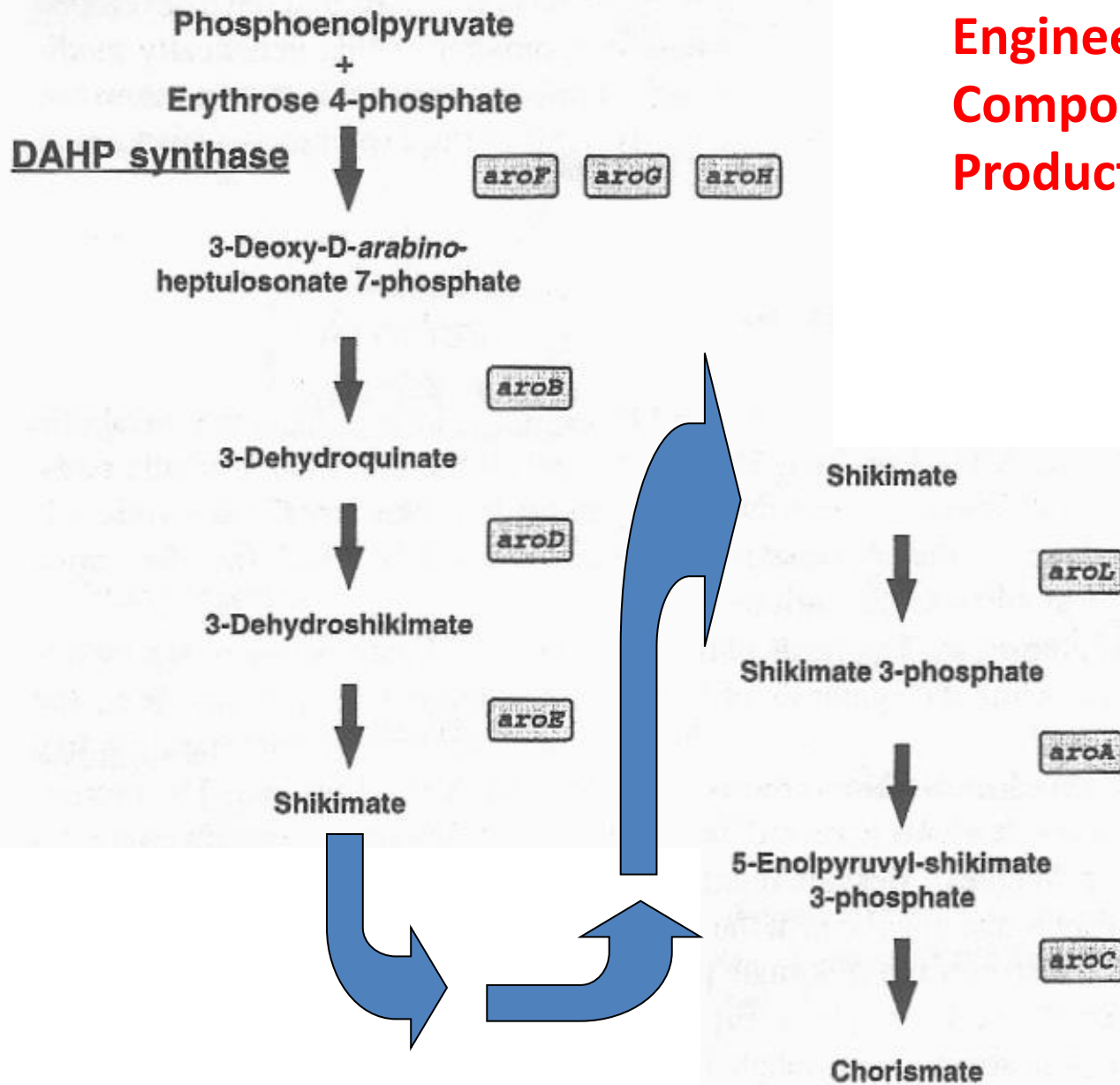


Flux distribution in riboflavin-producing *B. subtilis* strain PRF. Numbers are normalized to glucose uptake and represent dilution rates of 0.11, 0.41, and 062 h⁻¹ in glucose-limited chemostat.





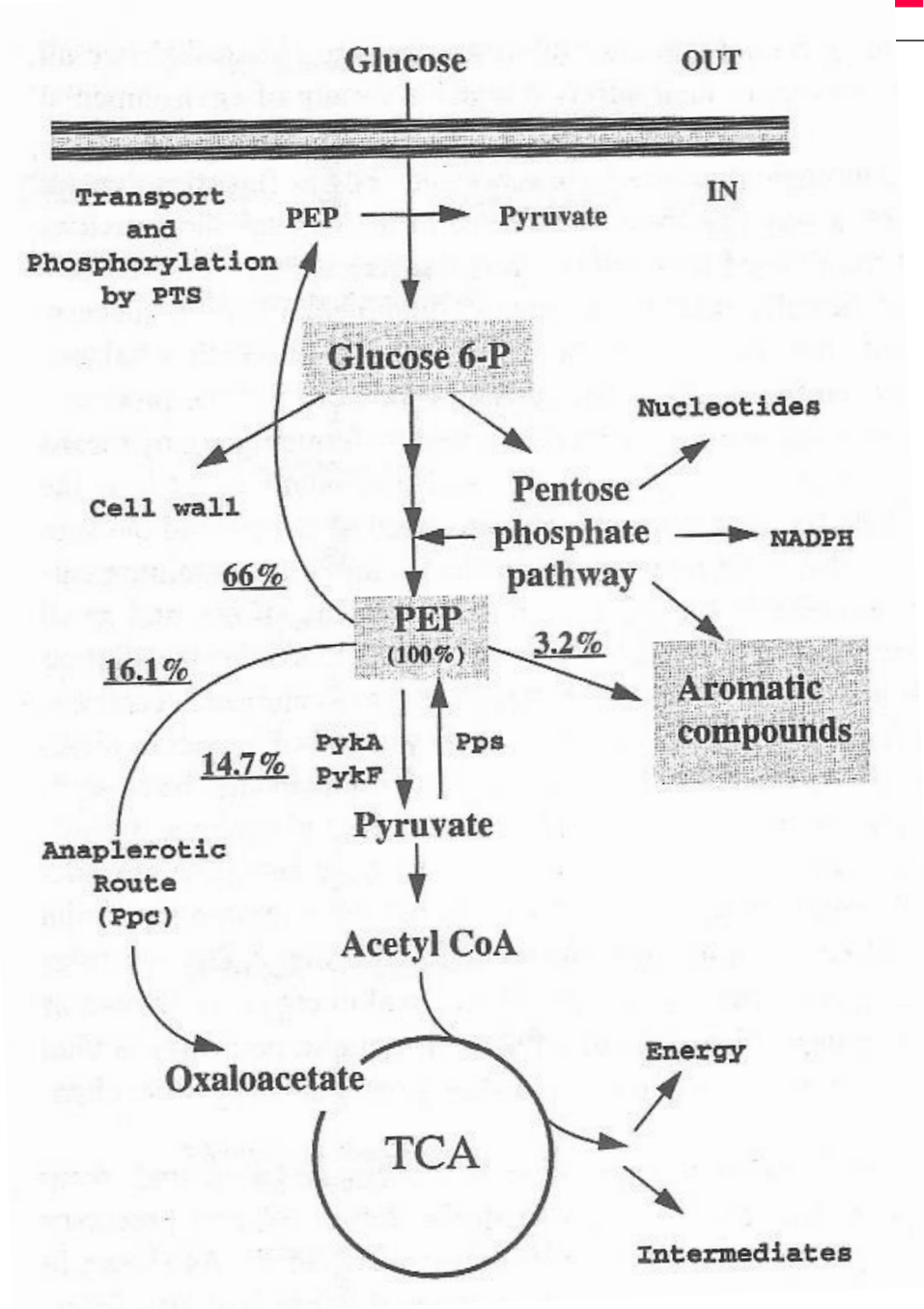
Metabolic engineering of *E. coli* strains for anaerobic or aerobic succinate production. Panel (a) shows the metabolism of a strain engineered for **anaerobic succinate production** via the **reductive arm of the TCA cycle** and via the **glyoxylate cycle** [30••]. It contains deletions of the genes 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.



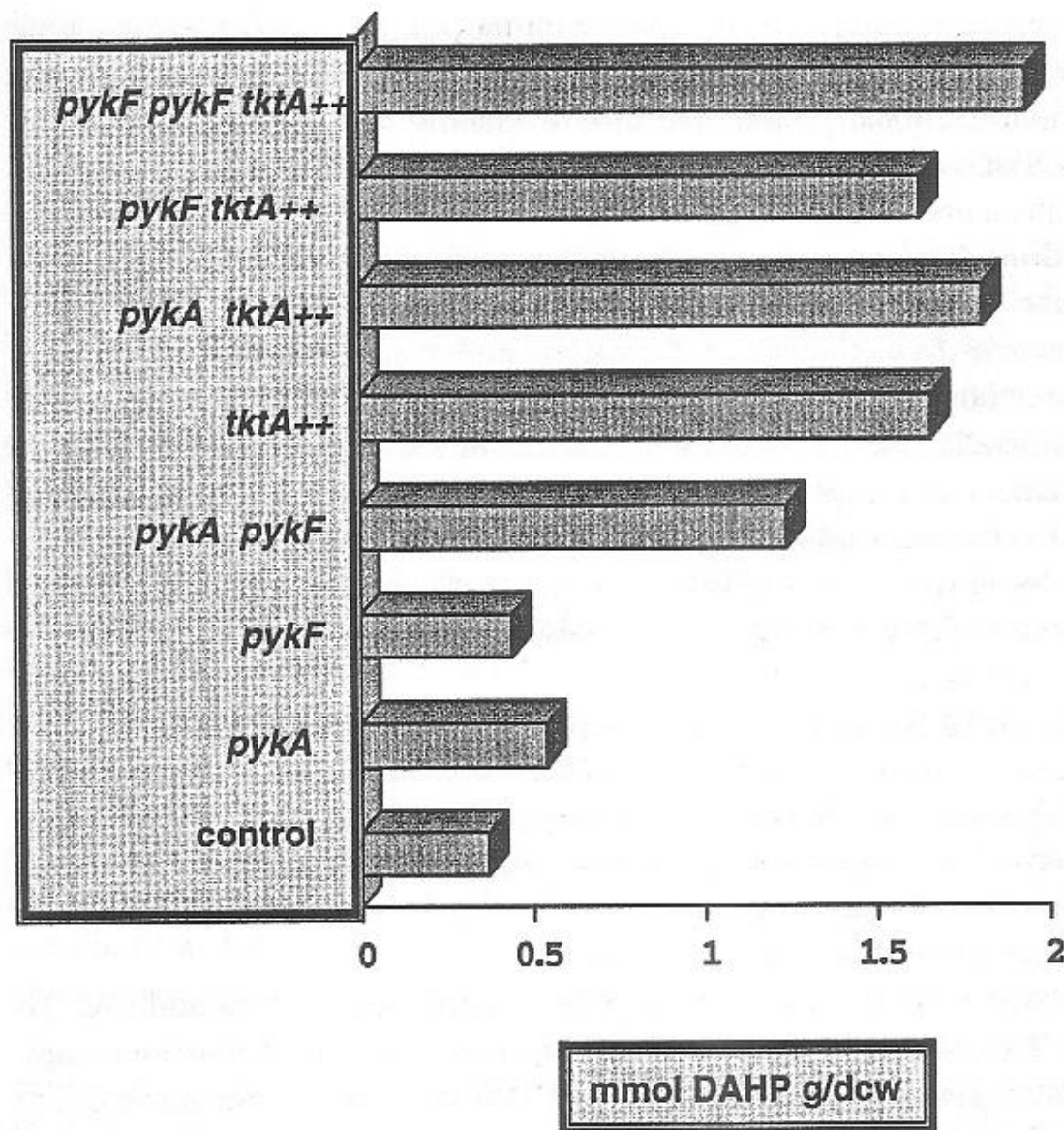
Engineering Aromatic Compounds Production in *E.coli*

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 plasmid containing the *E. coli tktA* gene and thus had elevated levels of transketolase activity; dcw, dry cell weight.

β-Lactam Antibiotics

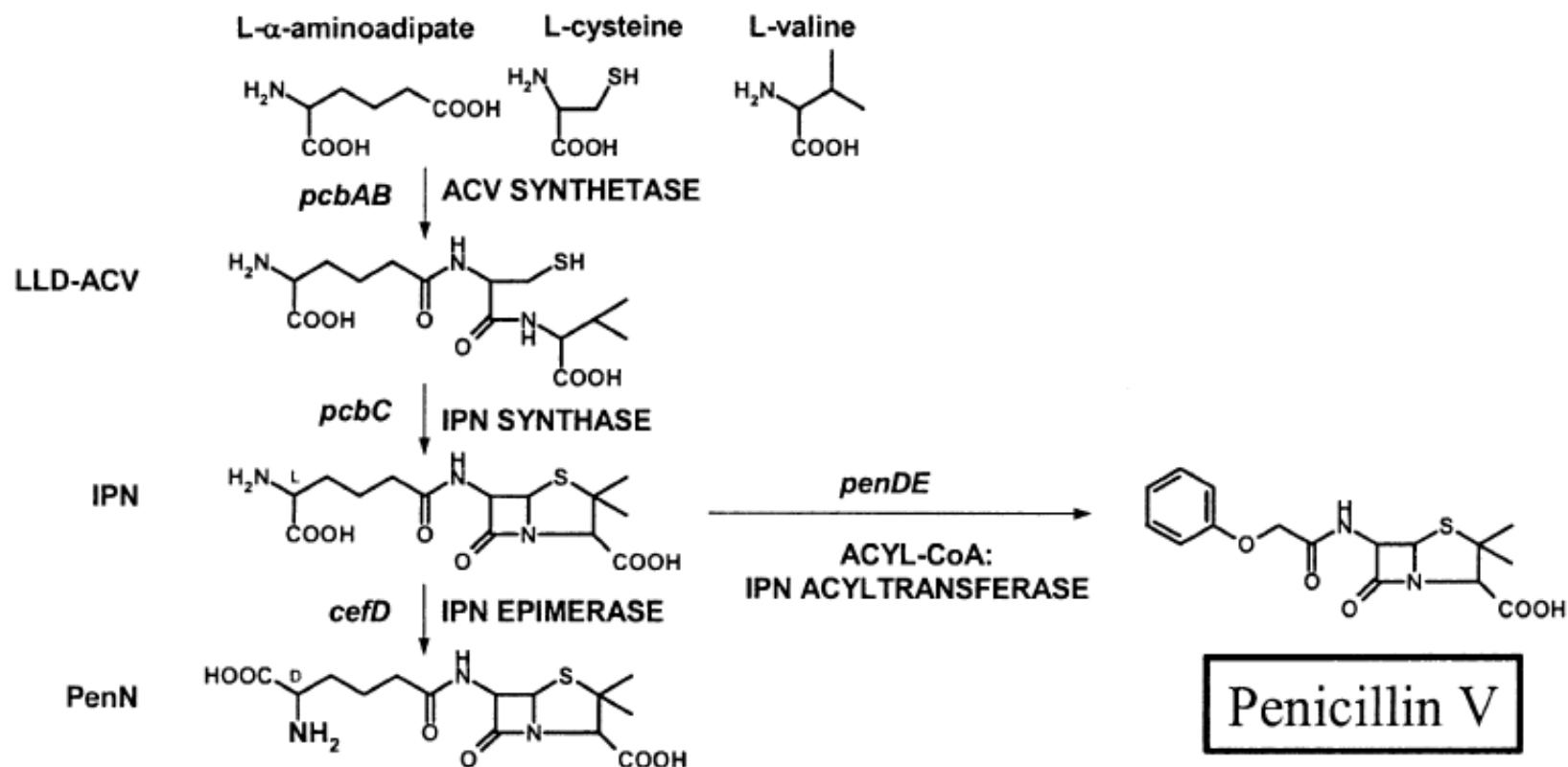


Fig. 2. Biosyntheses of the β-lactams penicillin V, cephalosporin C and cephamycin C. Abbreviations: LLD-ACV, α-L-aminoadipyl-L-cysteinyl-D-valine; IPN, isopenicillin N; PenN, penicillin N; DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; OCDAC, O-carbamoyl-deacetylcephalosporin C; HOCDAC,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

β-Lactam Antibiotics

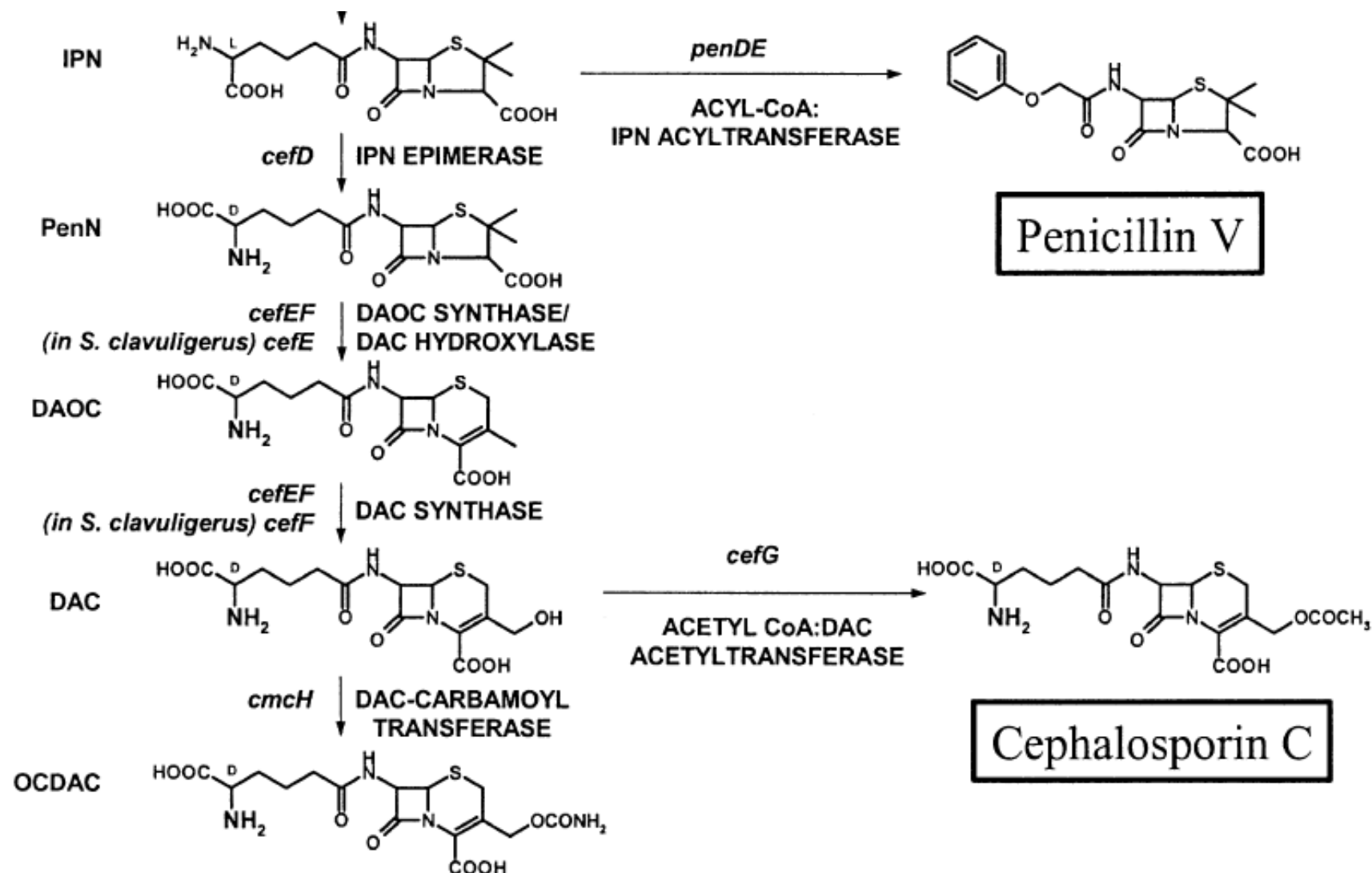


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β-Lactam Antibiotics

Cephalosporin C

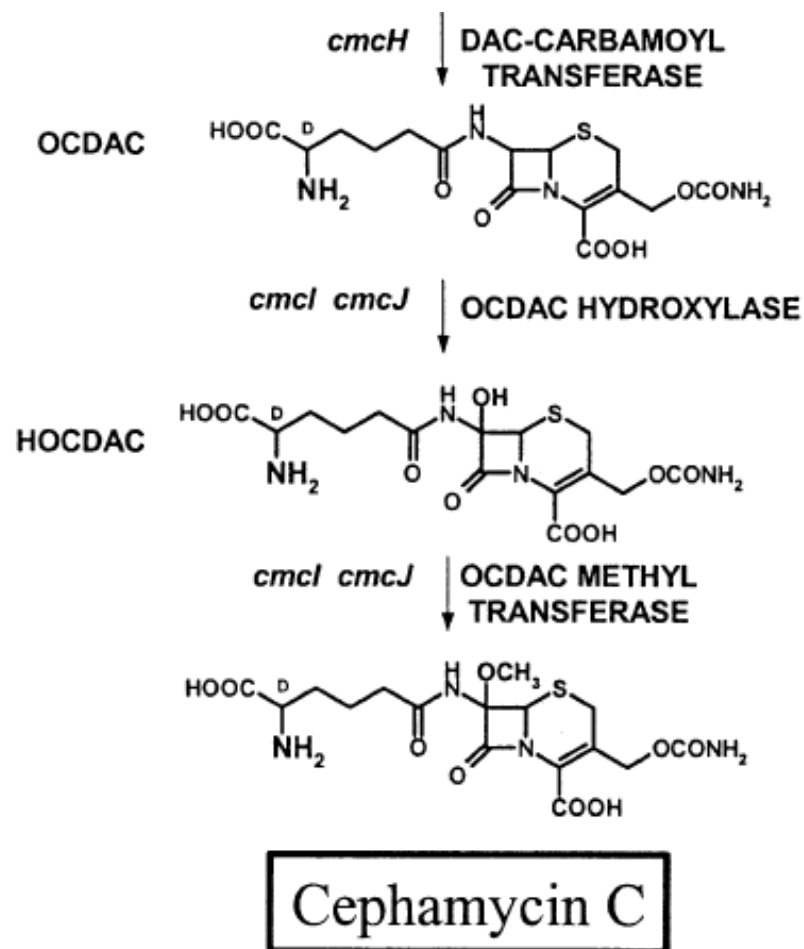


Fig. 2. Biosyntheses of the β-lactams penicillin V, cephalosporin C and cephamycin C. Abbreviations: LLD-ACV, α-L-aminoadipyl-L-cysteinyld-valine; IPN, isopenicillin N; PenN, penicillin N; DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; OCDAC, O-carbamoyl-deacetylcephalosporin C; HOCDAC,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

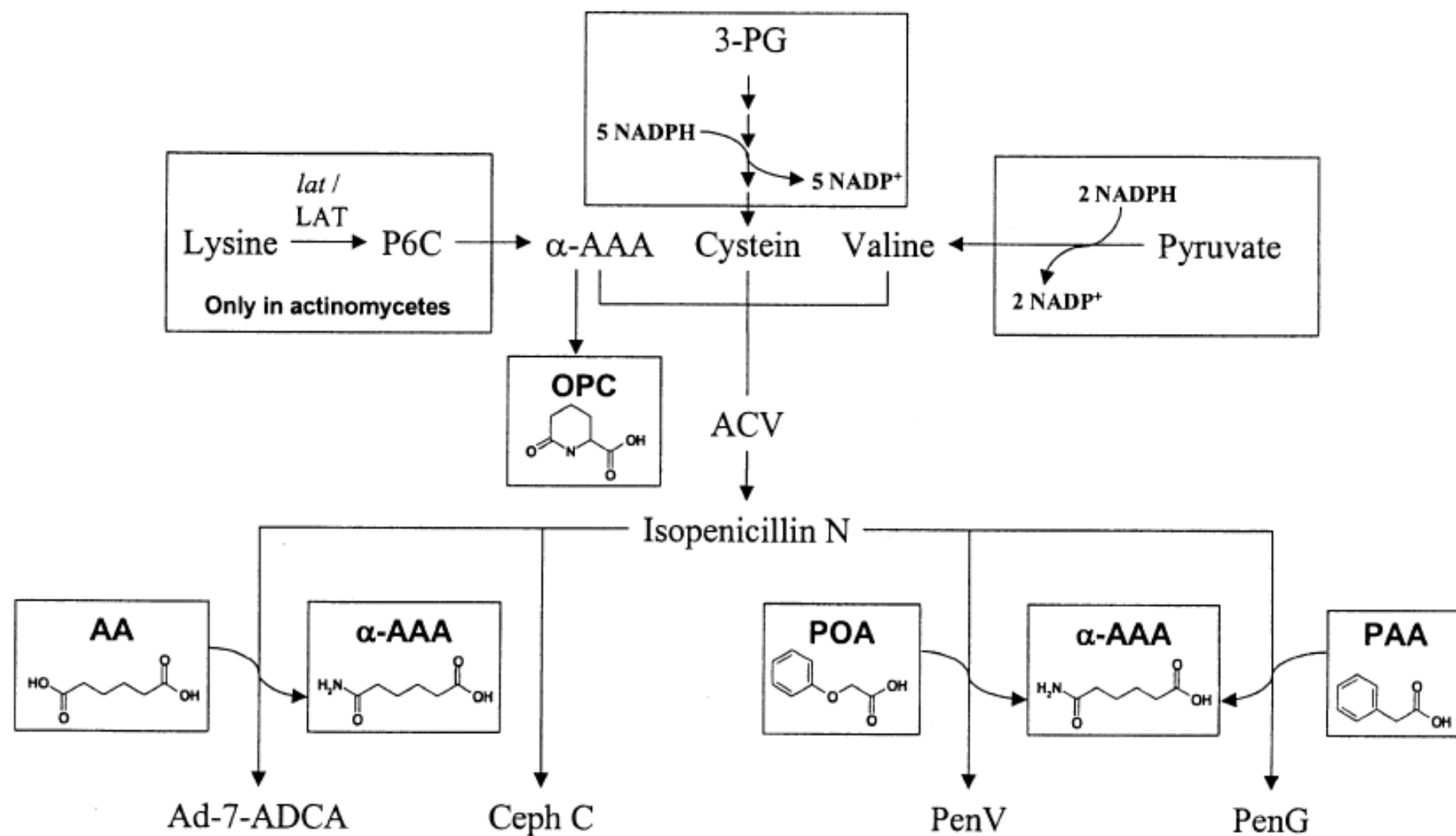


Fig. 6. Different precursors in the production of β -lactams.

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

Proteins: ACVS, α -L-aminoadipyl-L-cysteiny-D-valine synthase; DACS, deacetylcephalosporin C synthase; DAOCS, deacetoxycephalosporin C synthase; DAT, acetyl-CoA: deacetylcephalosporin C acetyltransferase; IAT, acyl-CoA:IPN acyltransferase; IPNE, isopenicillin N epimerase; IPNS, isopenicillin N synthase. Organisms: *A. chr*, *Acremonium chrysogenum*; *A. nid*, *Aspergillus nidulans*; *N. lac*, *Nocardia lactamdurans*; *P. chr*, *Penicillium chrysogenum*; *S. cla*, *Streptomyces clavuligerus*. Affinity constants: Aad, L- α -aminoadipate; ACV, α -L-aminoadipyl-L-cysteiny-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.

β -Lactam Antibiotics

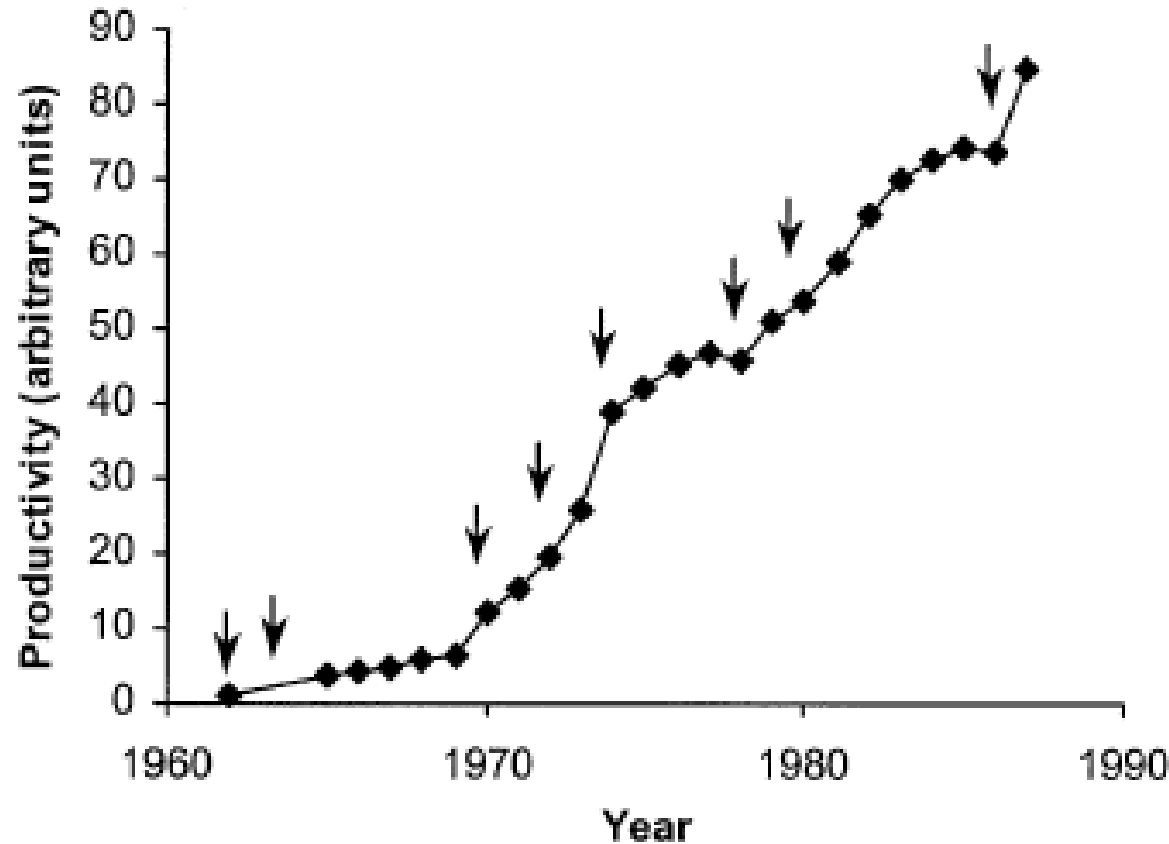


Fig. 3. Increase in productivity (output rate/unit volume, arbitrary units) of penicillin G production by Gist Brocades, Delft, in the period between 1962 and 1987. The introduction of new production strains is indicated with arrows. Based on [Nielsen, 1997](#).

β -Lactam Antibiotics

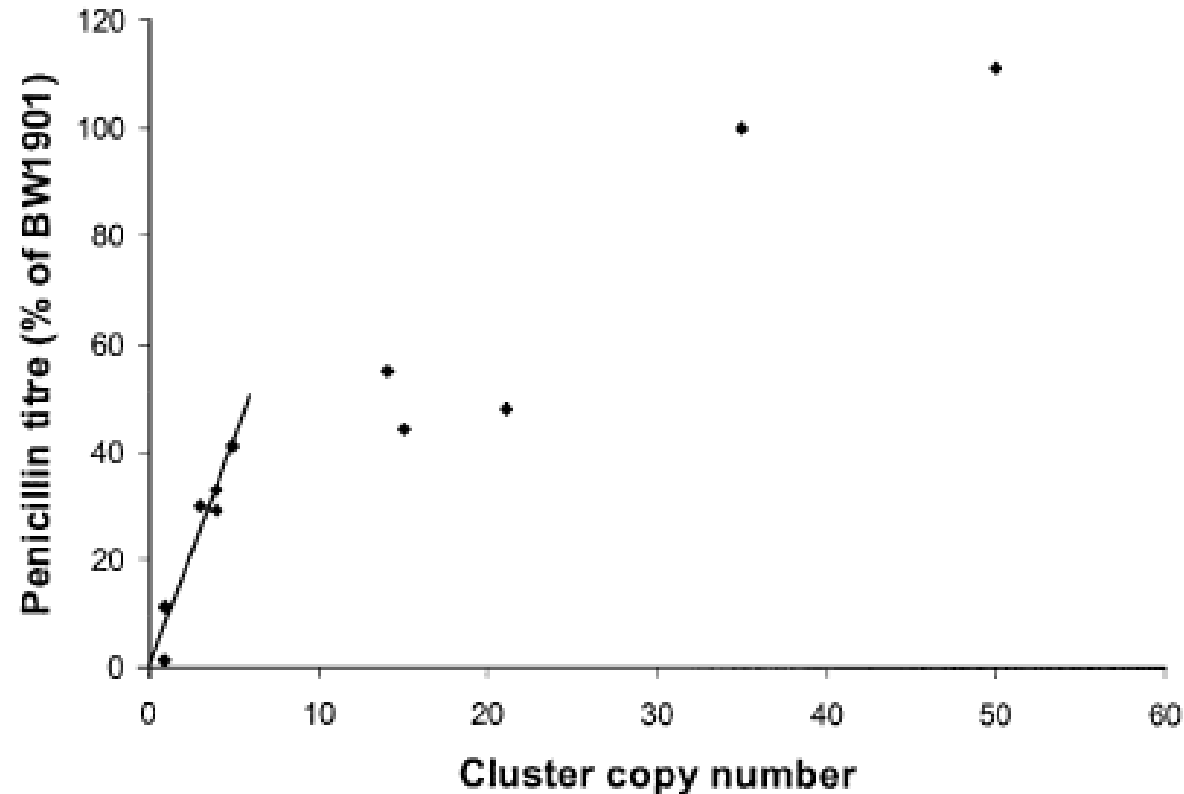


Fig. 4. Results of the SmithKline Beecham strain improvement series. Penicillin titre in percentage of the SmithKline Beecham strain BW 1901 versus penicillin cluster copy number. Modified from [Newbert et al. \(1997\)](#).

Production of Cephalosporin products in *P. chrysogenum*

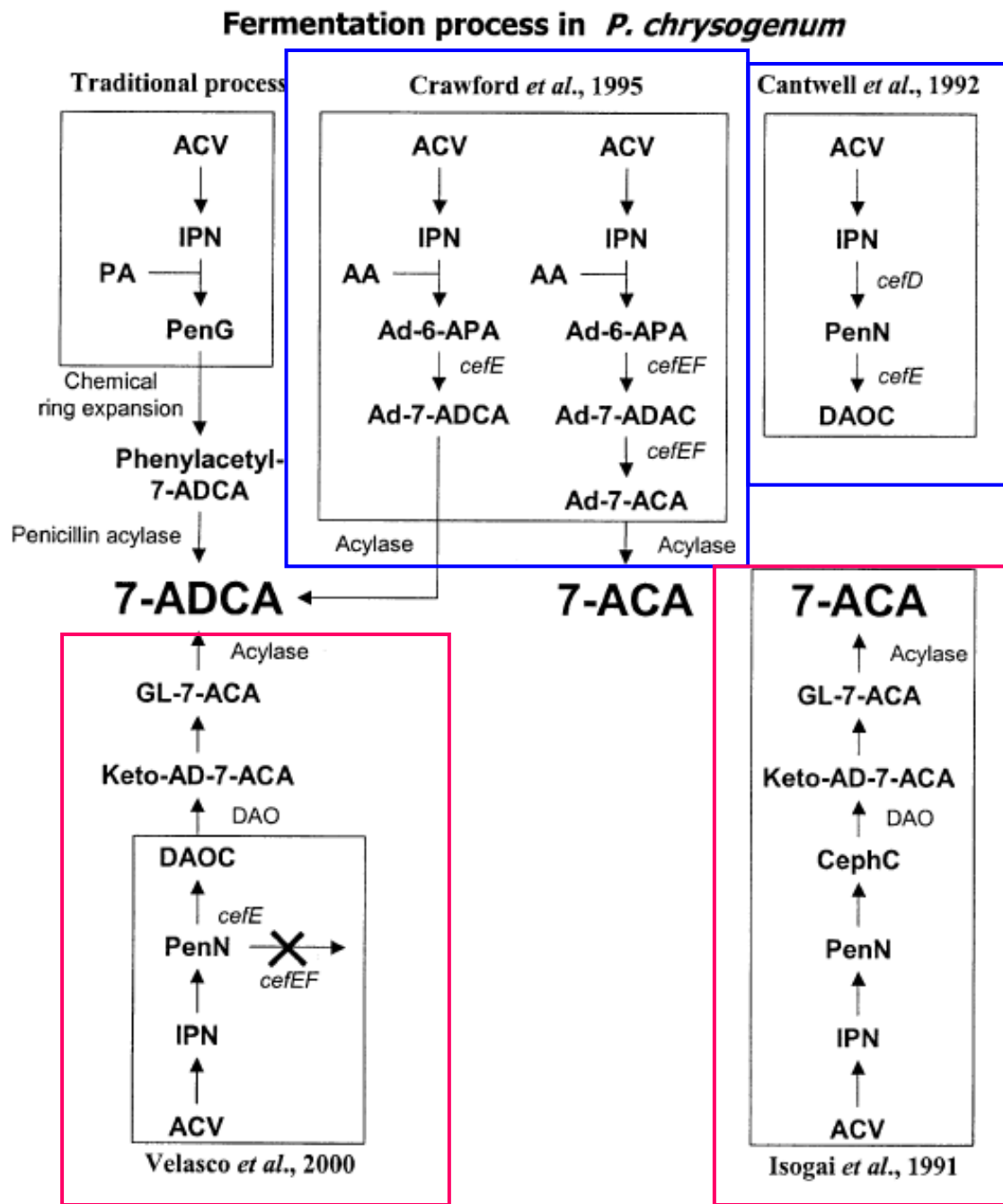


Fig. 5. Different strategies for producing cephalosporins directly from fermentation with the aims of developing bioprocesses for direct production of 7-ADCA and 7-ACA.

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

Modified from Velasco et al. (2000).

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	

Production of Amino Acids

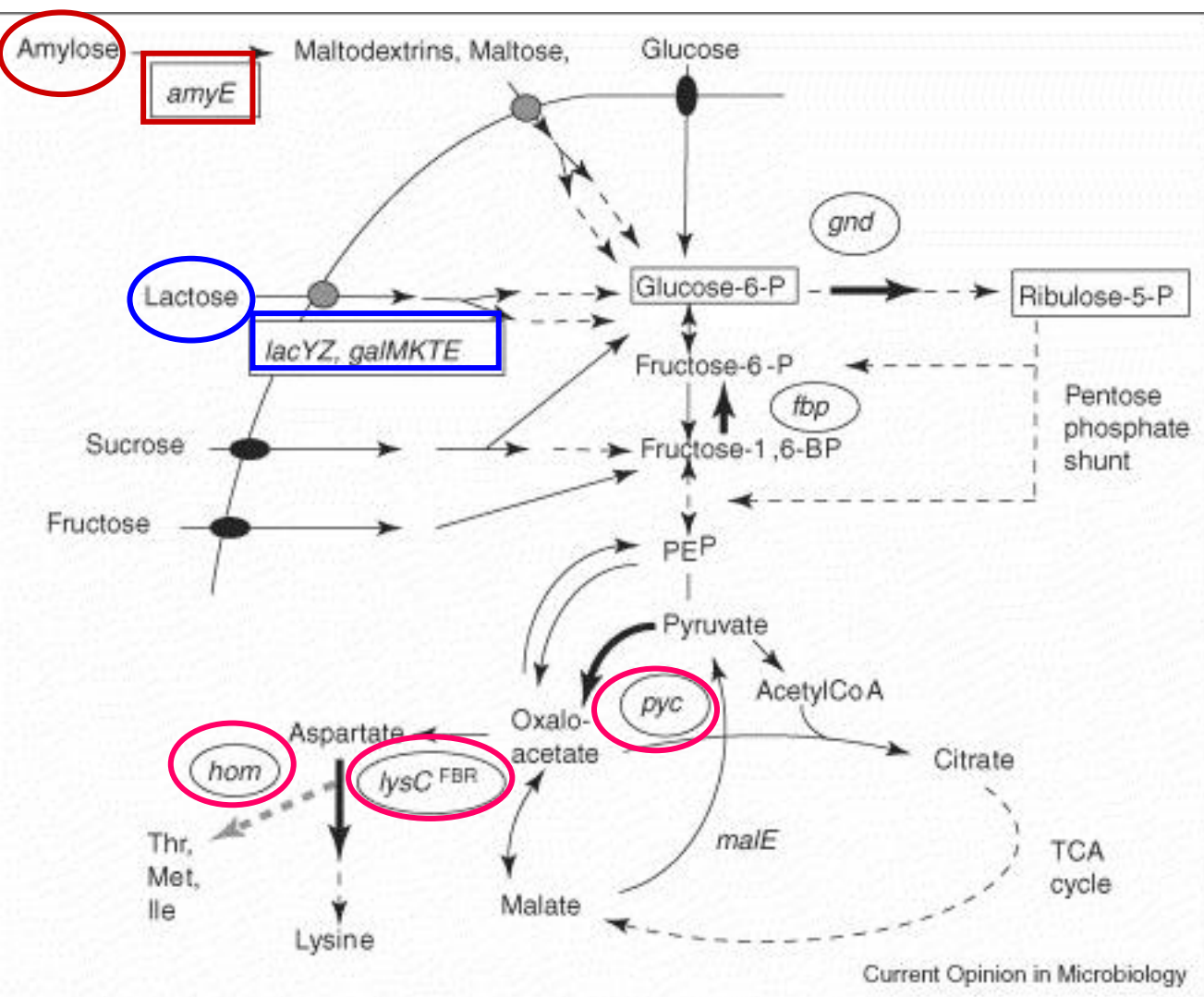
Source: Reprinted with permission from: Leuchtenberger W. Products of primary metabolism. Biotechnology 1996; 6:455–502.

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

Strain	Phenotype selected	Relative activity			L-Arginine produced (g/L)
		Acetylglutamokinase	Ornithine carbamyl-transferase	Feedback regulation of kinase	
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



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 phosphoenolpyruvate-dependent transport systems is depicted in black ovals, transport via other systems in grey circles.

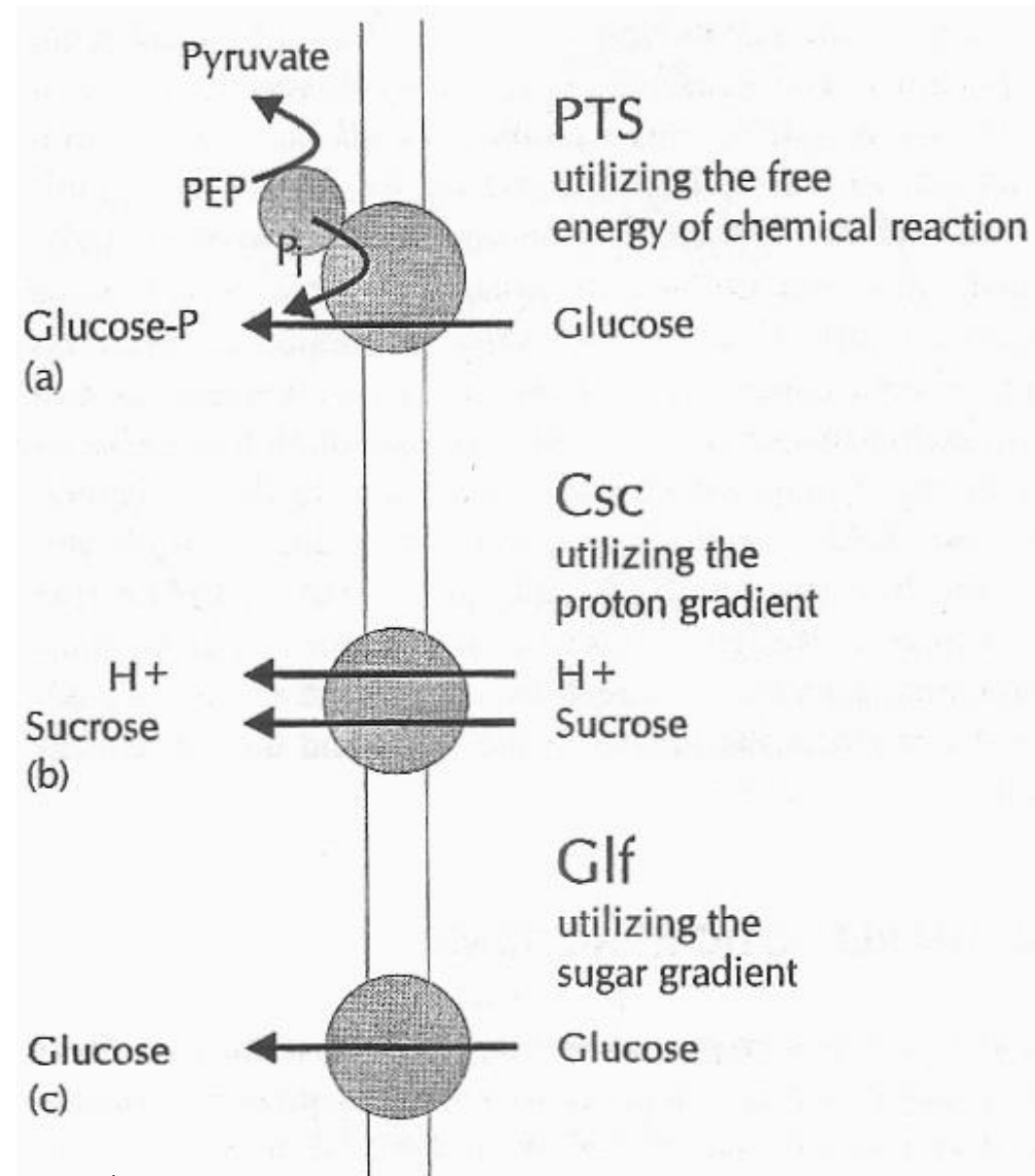
42 **Engineering of Substrate Uptake**

PTS: Phosphotransferase system

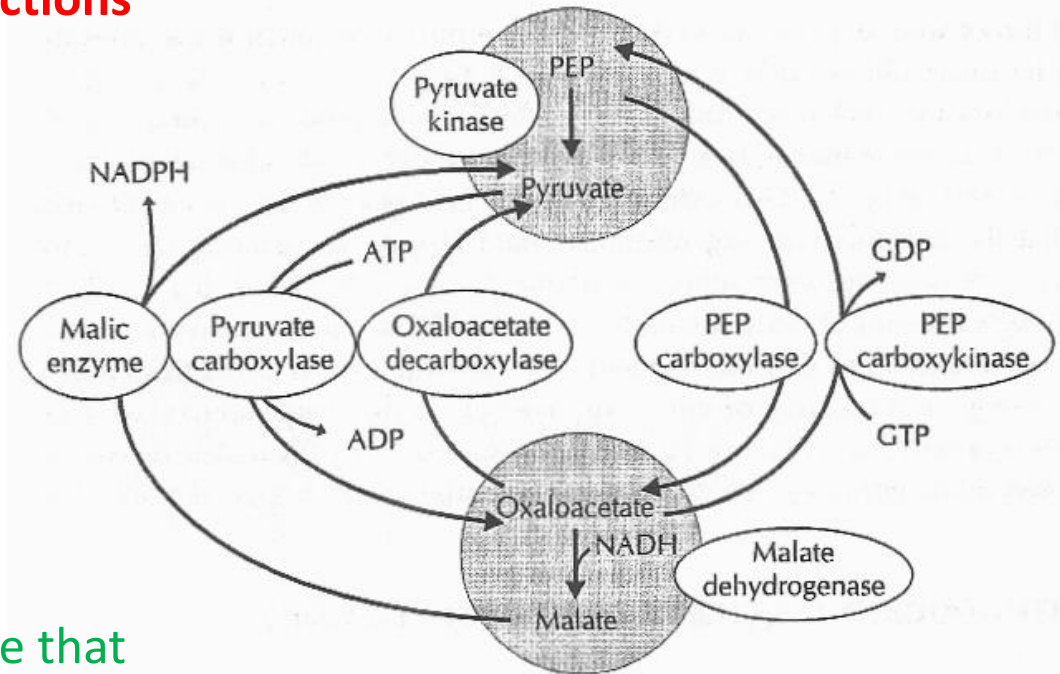
Csc: Sucrose permease

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).



Engineering anaplerotic reactions



Anaplerotic reactions are those that form intermediates of the TCA

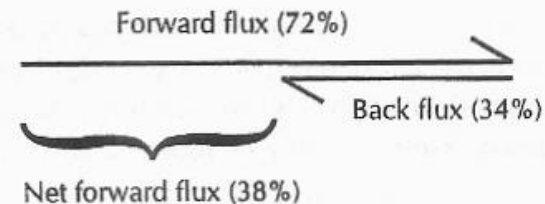


Figure 2 Interconversion of C-3 units to C-4 units. Enzymes of the anaplerotic reactions and adjacent reactions in *Corynebacterium glutamicum* that contribute to flux between C-3 units and C-4 units. The sum of forward flux (72%) and the sum of back flux (34%) are shown to result in a net forward flux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

dapA Overexpression leads to growth limitation

Table 3 Molecularly Introduced Growth Limitation to Increase Product Excretion

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

^a The specific synthase activity is in micromoles per minute per milligram of protein [$\mu\text{mol}/(\text{min} \cdot \text{mg})$].

^b Lysine excretion rate in millimoles per minute per gram [$\text{mmol}/(\text{min} \cdot \text{g})$] dry weight.

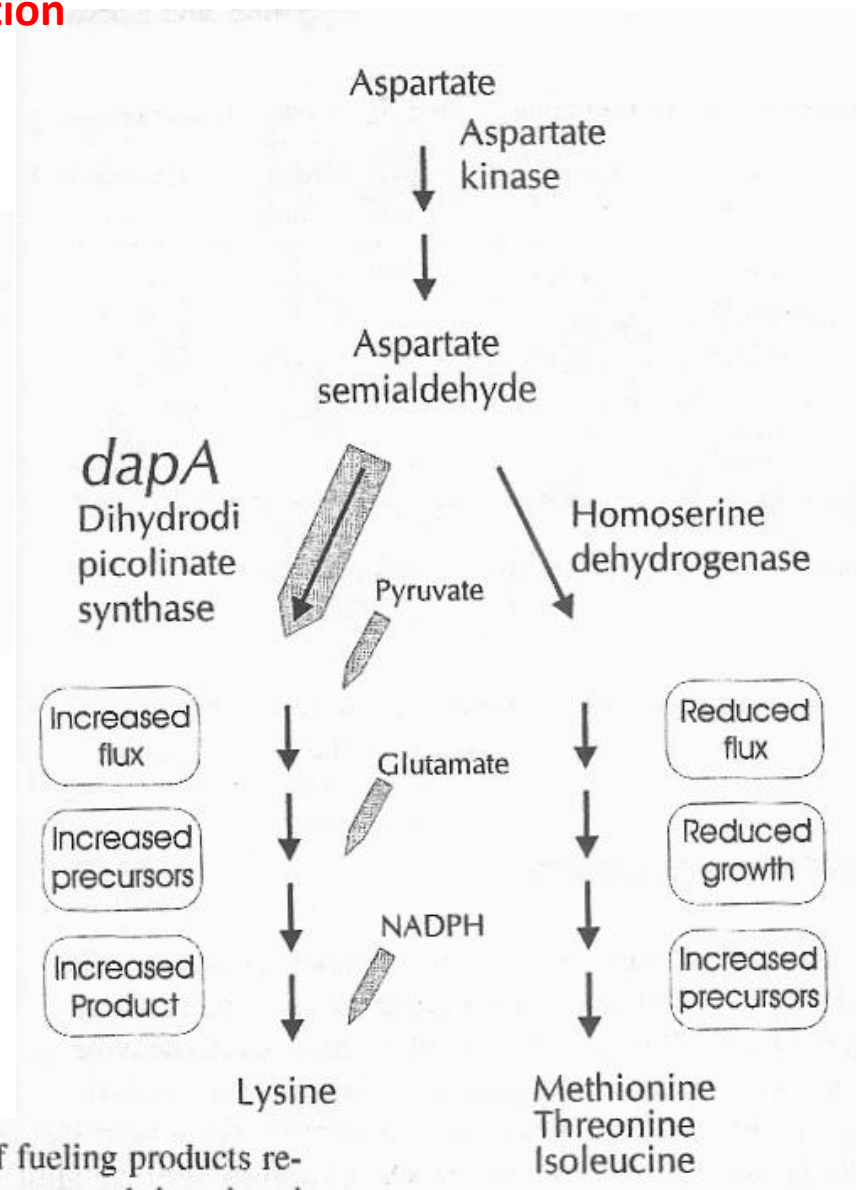


Figure 3 Growth limitation results in the increased availability of fueling products required for overproduction. As a consequence of *dapA* overexpression, growth is reduced and more pyruvate and further fueling products are available for L-lysine synthesis.

Engineering Branch Points

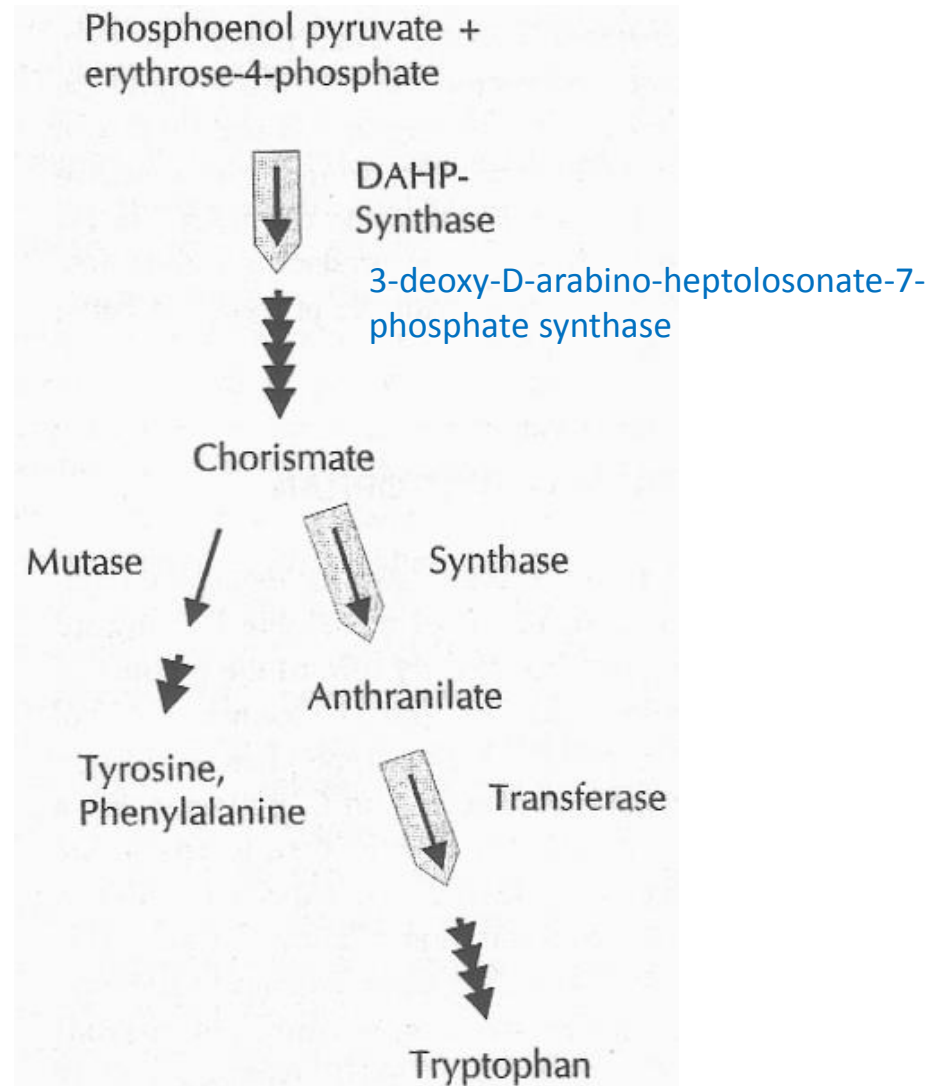


Figure 5 Engineering a branch point within amino acid synthesis. The relevant reactions (thick arrows) are shown to achieve chorismate formation and the entire conversion of this branch point metabolite to L-tryptophan.

Engineering Nitrogen Fluxes

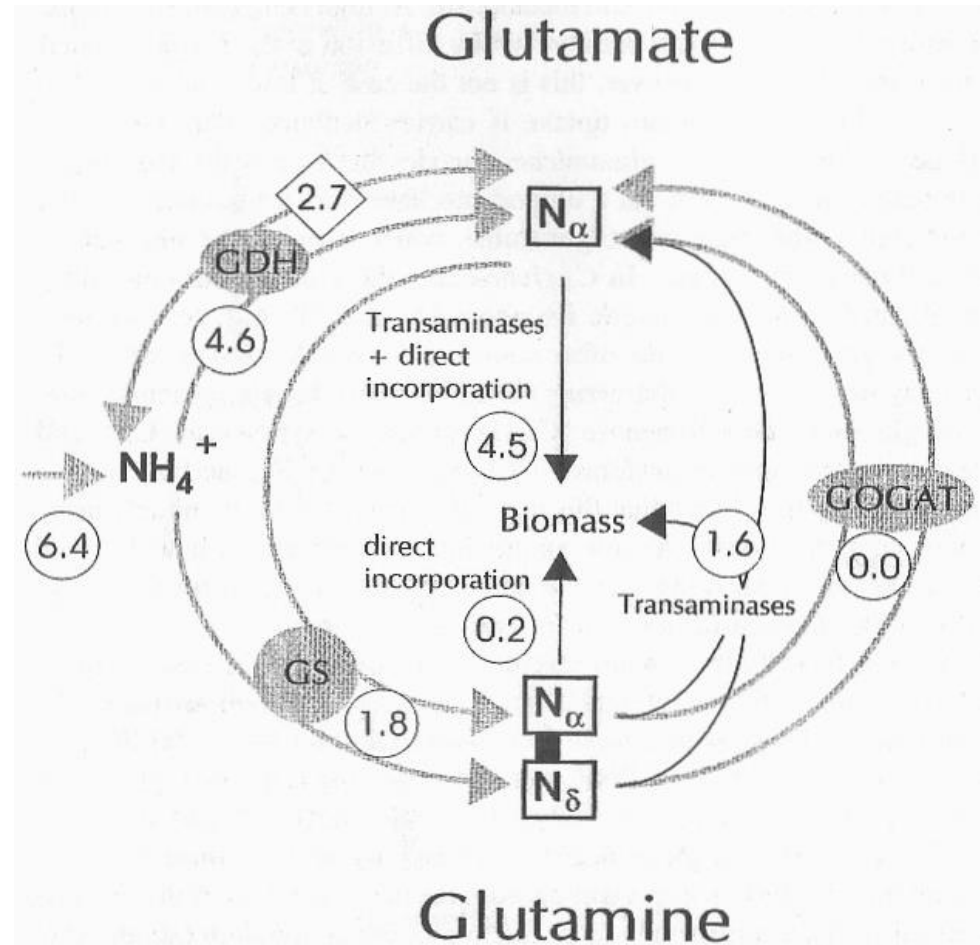
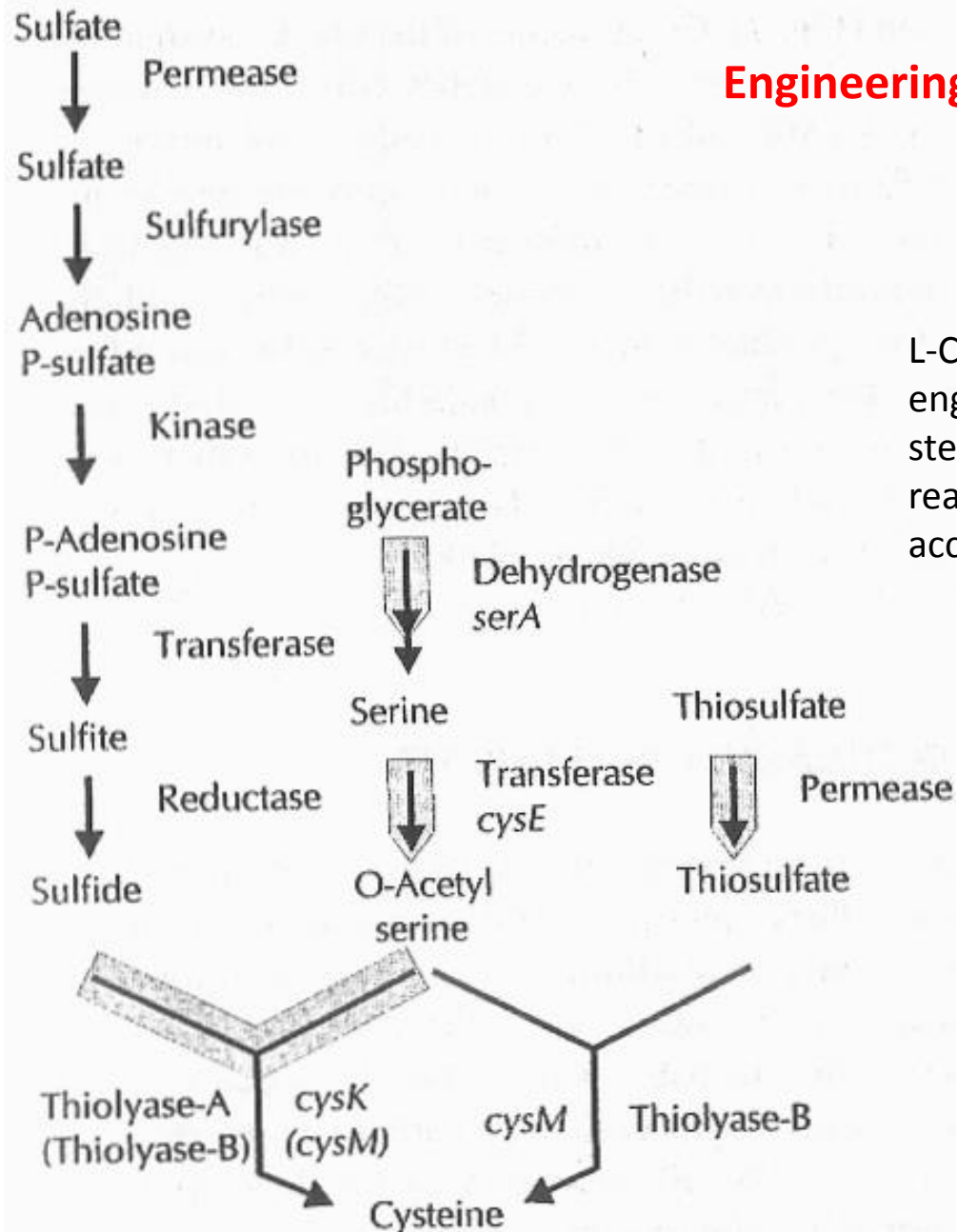


Figure 6 In vivo nitrogen fluxes as quantified by NMR spectroscopy. The fluxes via the glutamate dehydrogenase (GDH), glutamine synthetase (GS), and glutamate oxoglutarate aminotransferase (GOGAT) were quantified in *Corynebacterium glutamicum* grown in continuous culture at a dilution rate of 0.05 1/h.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

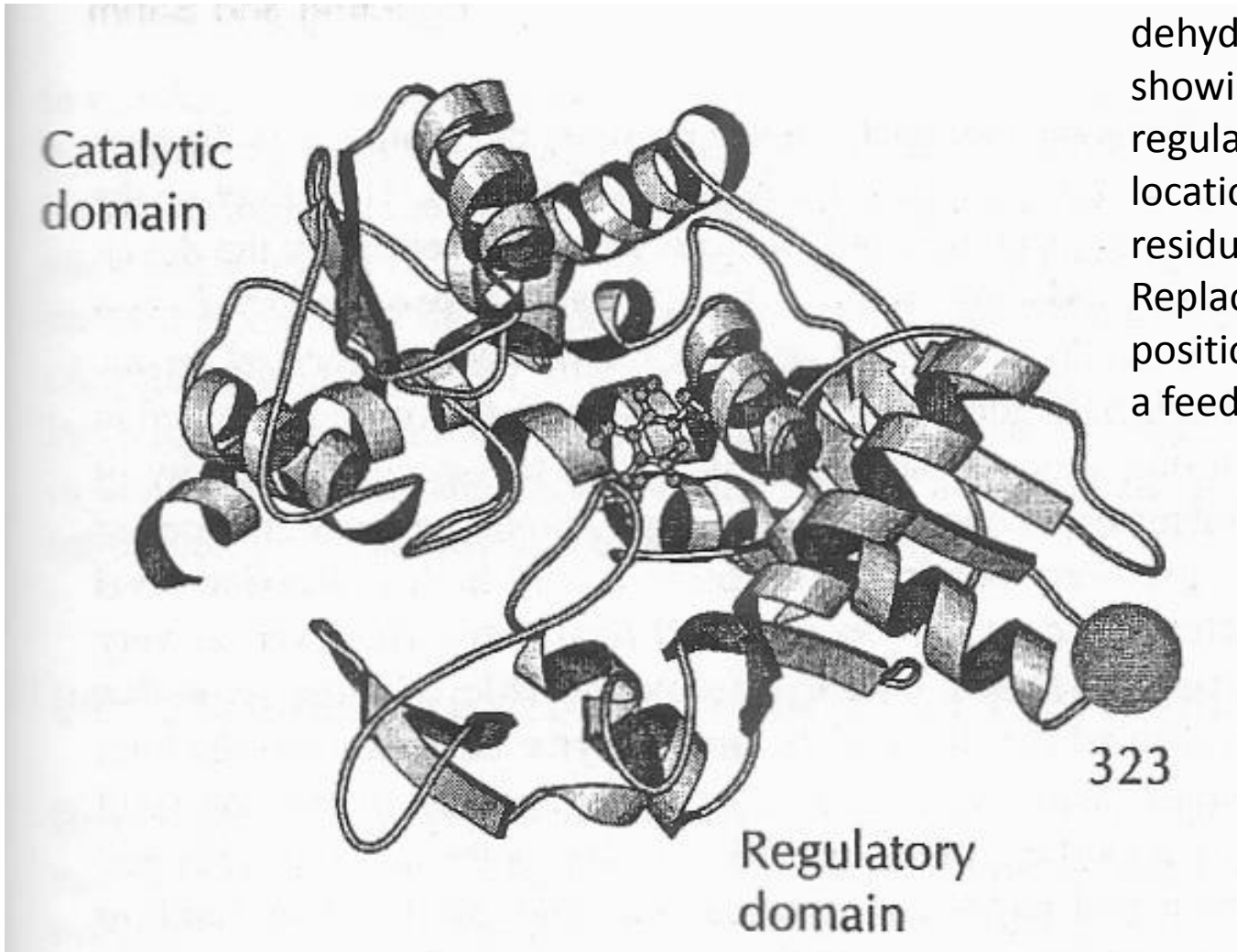
Engineering Sulfur Metabolism



L-Cysteine synthesis requires the engineering of the sulfur incorporation step. Thick arrows mark the relevant 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

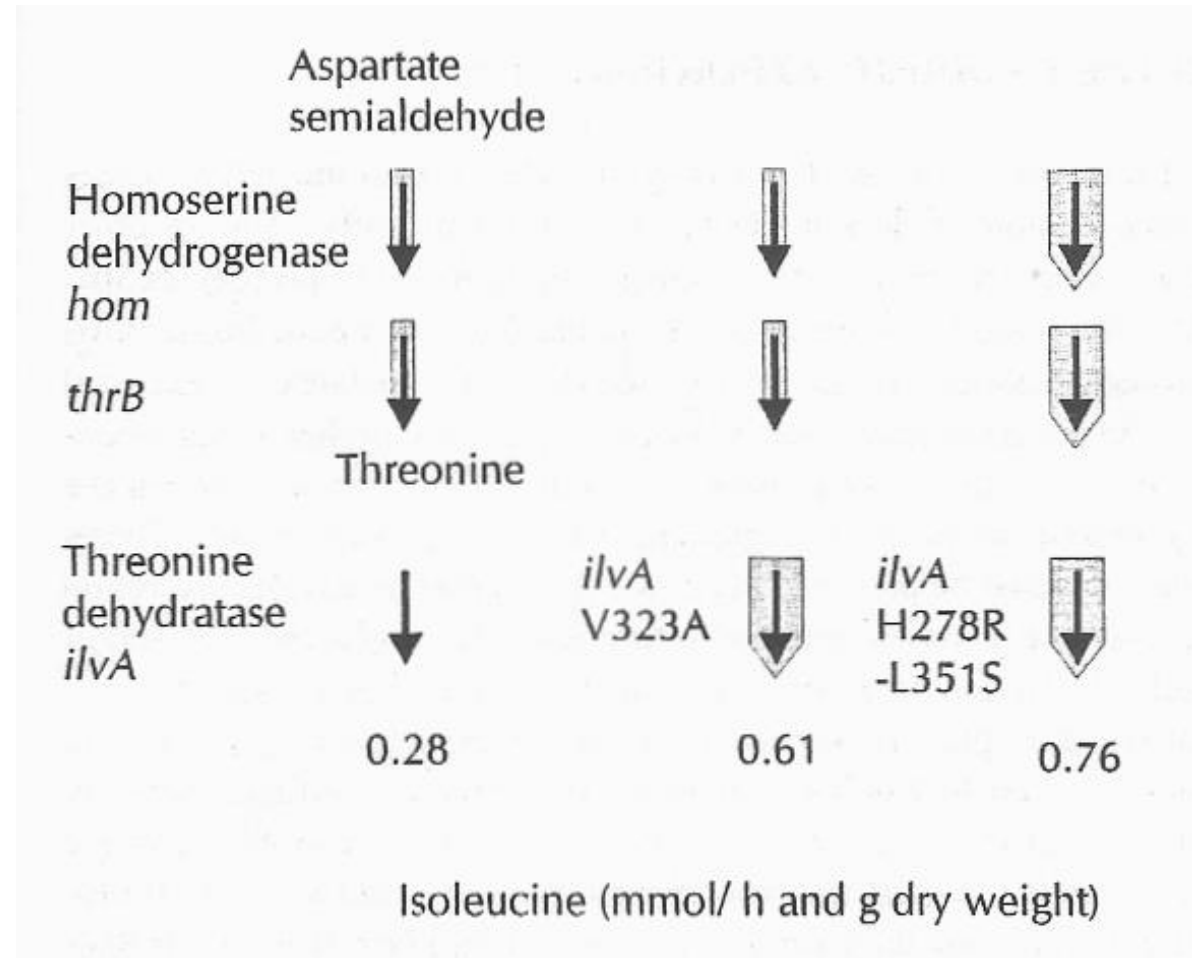


Figure 8 Concerted flux increase to achieve high L-isoleucine formation rates. The size of the arrows roughly corresponds to the respective expression levels of the genes. The most successful *ilvA* allele used at each step is given by the single-letter code for the amino acid replaced.

Engineering Transport Carrier

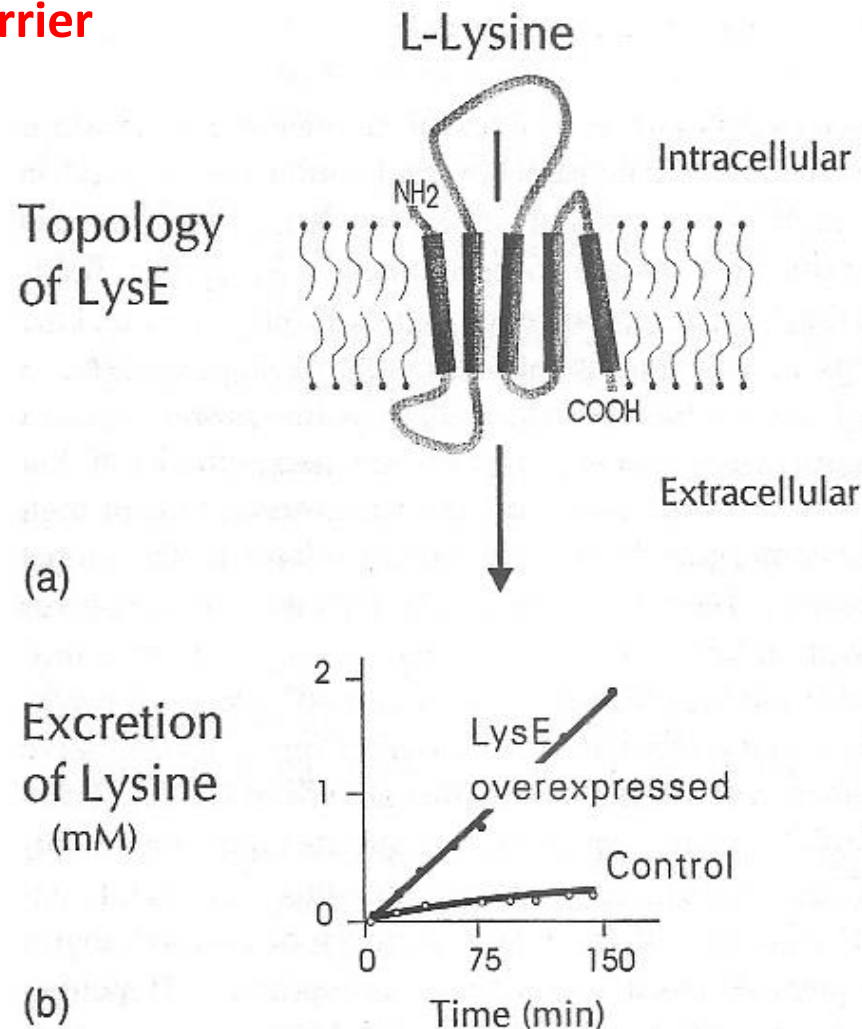


Figure 9 The L-lysine export carrier of *C. glutamicum*. (a) Model of the structure of this new type of carrier. (b) The increased excretion rate of L-lysine in response to overexpression of the carrier.