Korynebakterie, *Corynebacterium glutamicum*

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Rod *Corynebacterium*

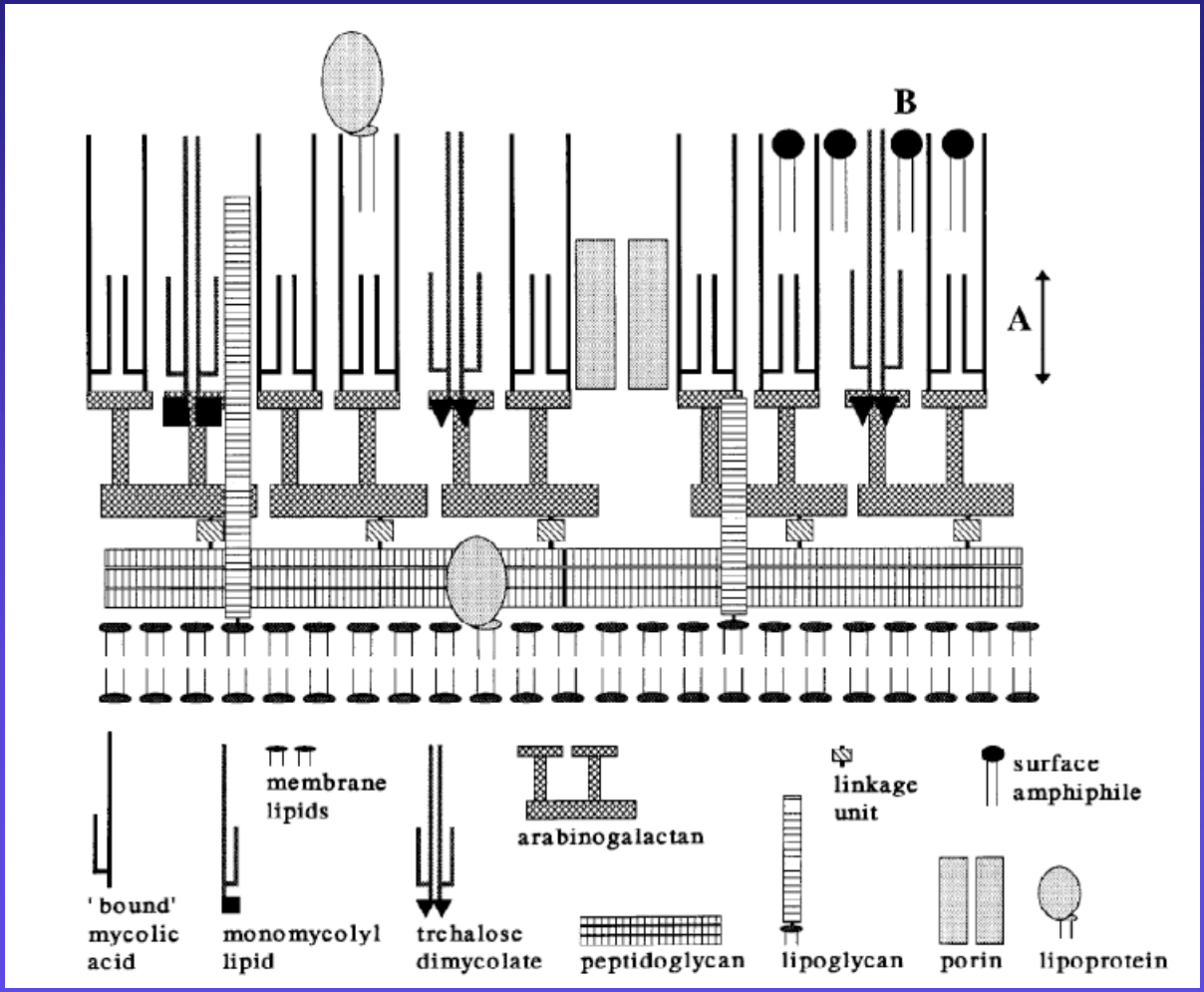
- Grampozitivní, aerobní či fakultativně anaerobní nesporulující, nepohyblivé bakterie
- Nepravidelná, někdy kyjovitě rozšířená tyčinka
- Aktinomycety (přítomnost kyseliny mykolové v buněčné stěně, homologie 16S rRNA)
- Příbuzné rody: *Mycobacterium*, *Rhodococcus*, *Nocardia*
- Patogenní druhy: *C. diphtheriae, C. jeikeium*
- Nepatogenní: *C. glutamicum* – producent AK

- Sekvence celého genomu známa: 20 kmenů

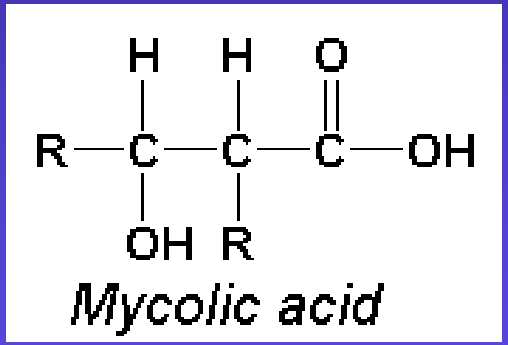
C. glutamicum, C. efficiens, C. diphtheriae, C. jeikeium

C. urealyticum, C. ulcerans, C. pseudotuberculosis

Buněčná stěna bakterií skupiny *Mycolata*
(aktinomycety obsahující kyseliny mykolové v buněčné stěně)

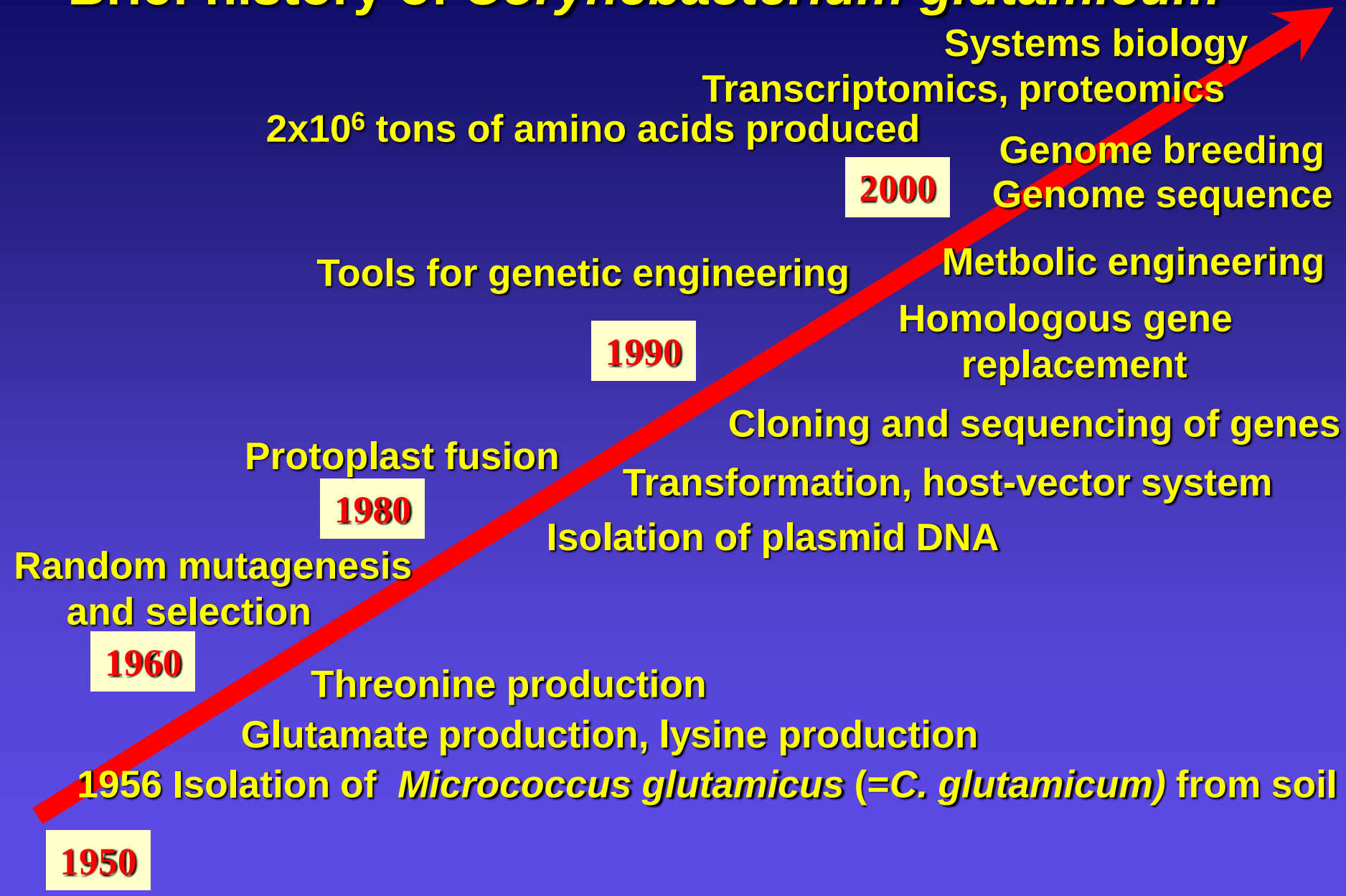


Mycobacterium
Corynebacterium
Rhodococcus
Nocardia



Rigidní BS
Rezistence
Změny hydrofobicity

Brief history of *Corynebacterium glutamicum*



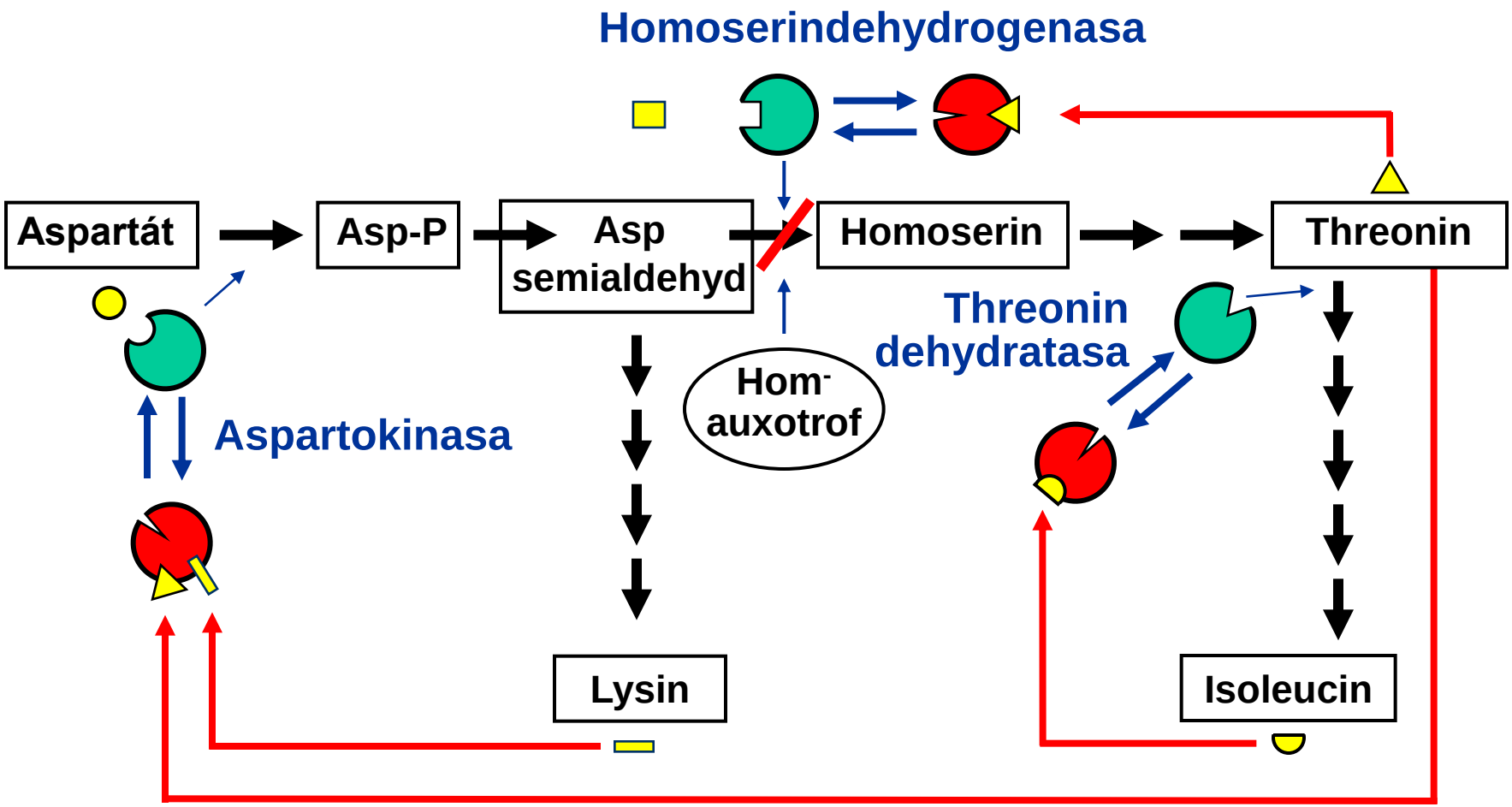
Výroba a použití L-aminokyselin

Aminokyselina	Roční světová produkce (t)	Způsob výroby	Použití
Glutamát	1 000 000	Fermentace	Ochucovadlo potravin
Methionin	350 000	Ch. syntéza	Terapie, krmiva
Lysin	250 000	Fermentace	Krmiva
Glycin	22 000	Ch. syntéza	Organické syntézy
Aspartát	7 000	Enzymaticky	Výroba aspartamu (sladidlo)
Threonin	4 000	Fermentace	Krmiva
Arginin	1 200	Fermentace	Infúze, terapie, kosmetika
Tryptofan	500	Fermentace	Infúze, terapie
Valin Leucin Isoleucin	500 500 400	Fermentace	Infúze, krmiva

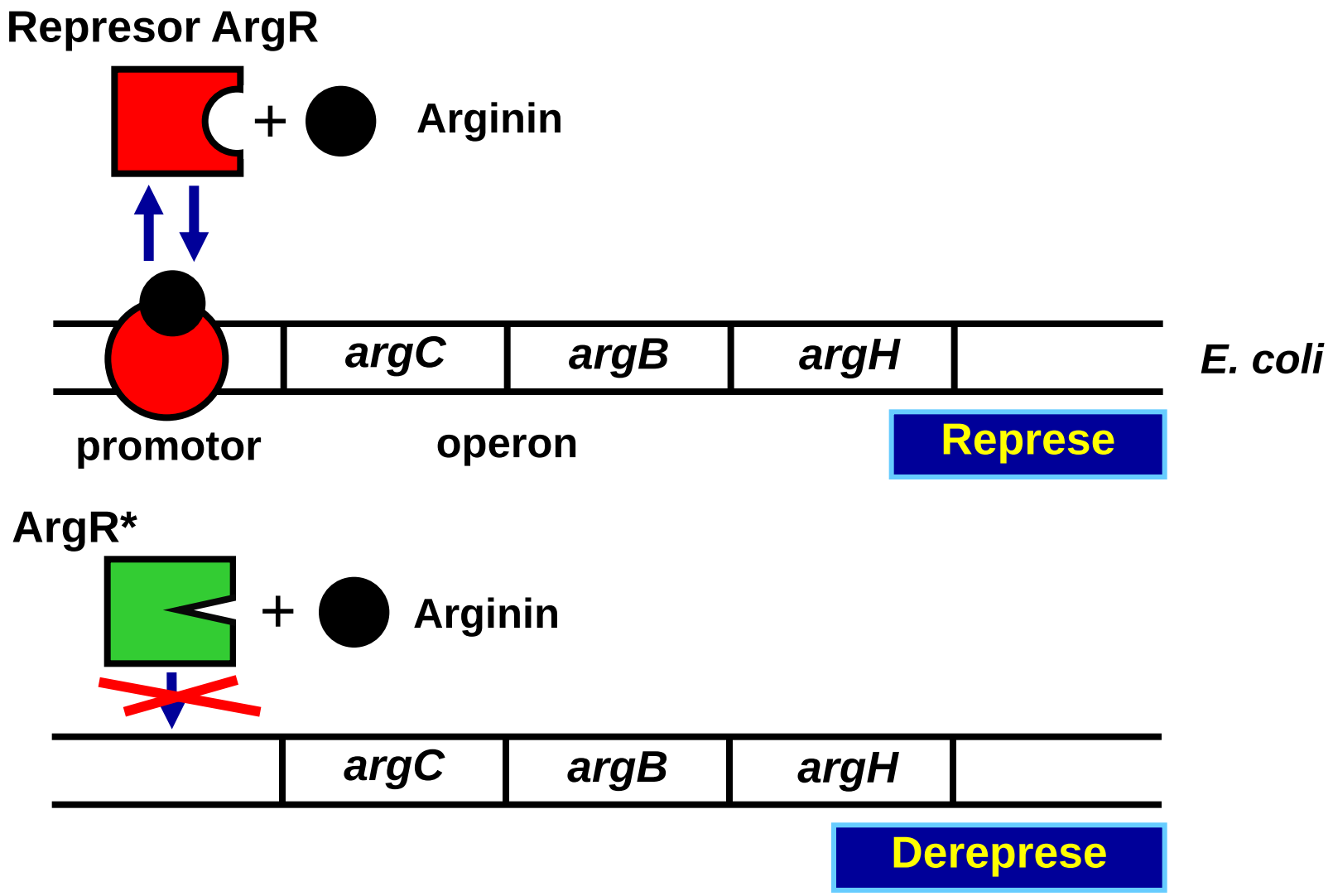
- Producenti aminokyselin: *E. coli*, *S. marcescens*, *C glutamicum*

Regulace biosyntézy aminokyselin

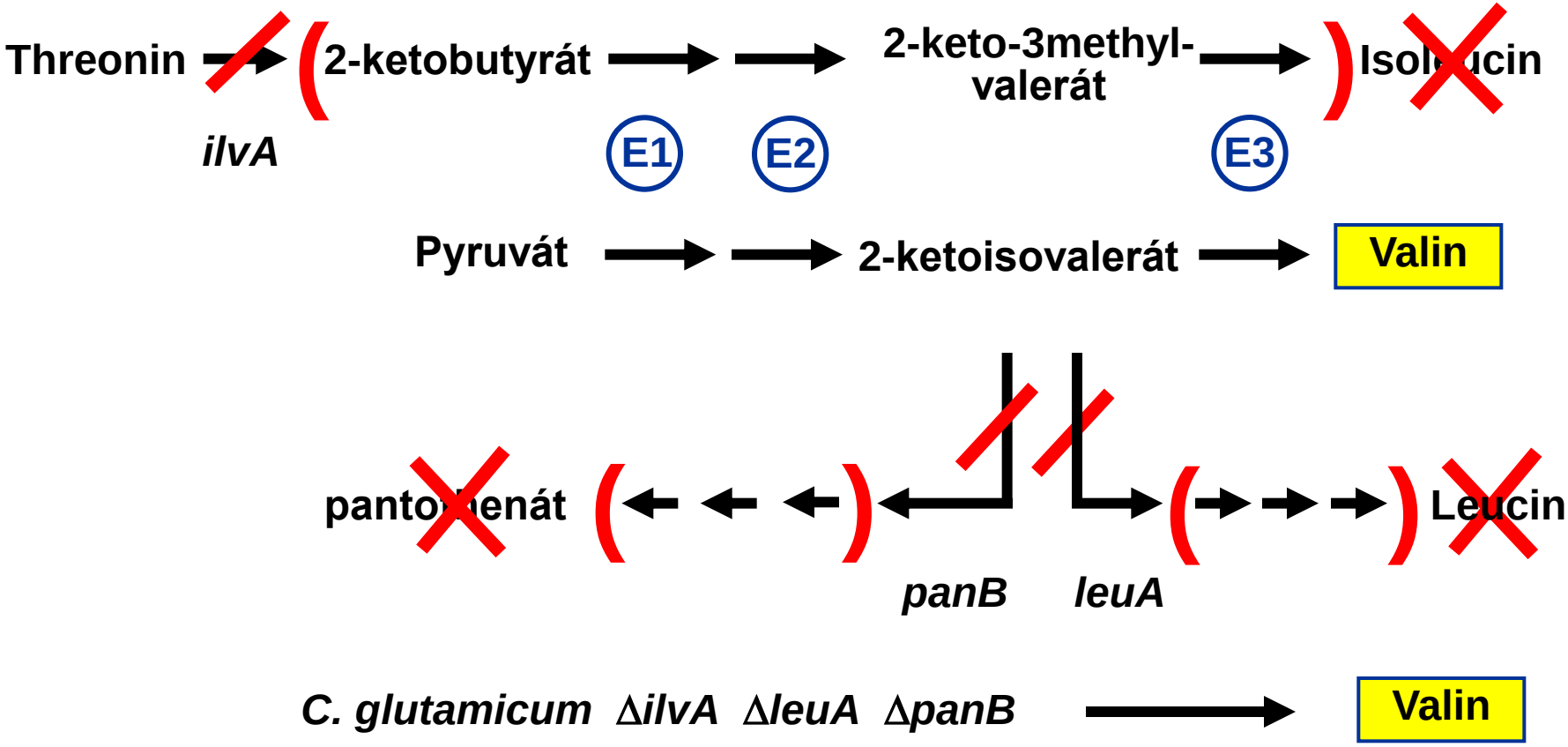
Regulační a auxotrofní mutanti



Regulační mutanti



Auxotrofní mutanti



Strategie genových manipulací
(metabolické inženýrství, design metabolismu)

- Klonování genů: kódujících enzymy rezistentní k inhibici
- dereprimovaných
- pro exkreci aminokyselin (*lysE*, *brnFE*)
- centrálního metabolismu (*pyc*)
- pro utilizaci jiných substrátů (lactosa, ara)
- Použití plazmidů s nízkým počtem kopií (3-5 kopií) - pNG2
- Integrace genů do chromosomu
- Exprese z cizorodých regulovatelných promotorů (P-lac)
- Modifikace přirozených promotorů cílenou mutagenezí
- Cílená inaktivace genů pro rozvětvené biosyntetické dráhy
- Cílená nebo náhodná mutageneze s použitím PCR
- *Genome breeding* – porovnávání genomů mutantů s WT
- Funkční genomika, analýza transkriptomu, sekvenování RNA
- Metabolomika, fluxomika + metabolické inženýrství

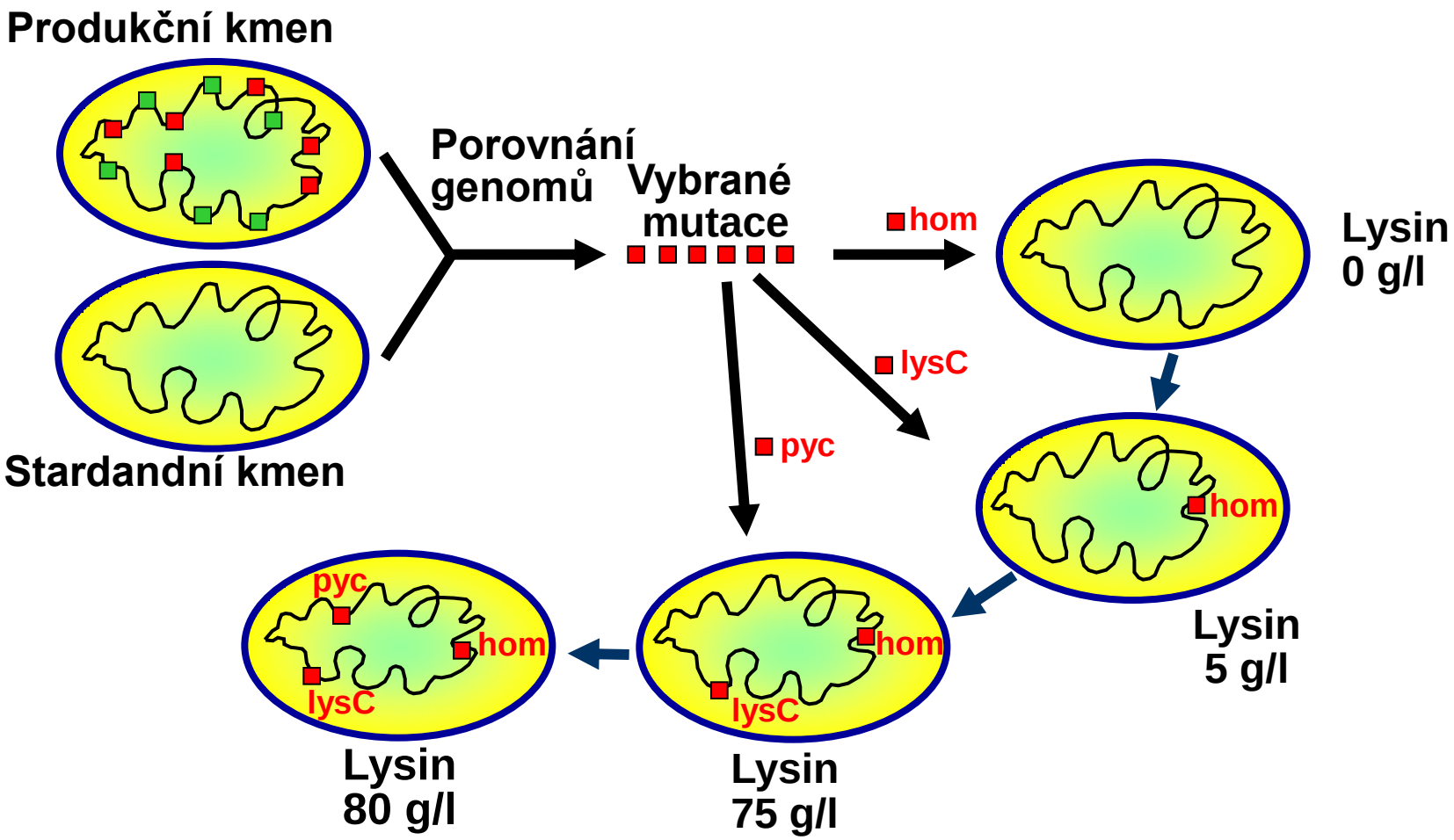
Producenti aminokyselin s geny klonovanými v plazmidu

- **Lysin:** *lysC*, *dapA*, *dapB*, *lysA* (*C. glutamicum*)
- **Isoleucin:** *hom*, *thrB*, *ilvA* (*C. glutamicum*)
- **Tryptofan:** trp operon (*C. glutamicum*)
- **Threonin:** *thrABC* (*E. coli*), *hom*, *thrB* (*C.glutamicum*)
- **Fenylalanin:** *aroF*, *pheA* (*E. coli*)
- **Histidin:** *his* operon (*S. marcescens*)

- **V mnohých případech vedlo klonování standardních genů k vysoké produkci aminokyselin**

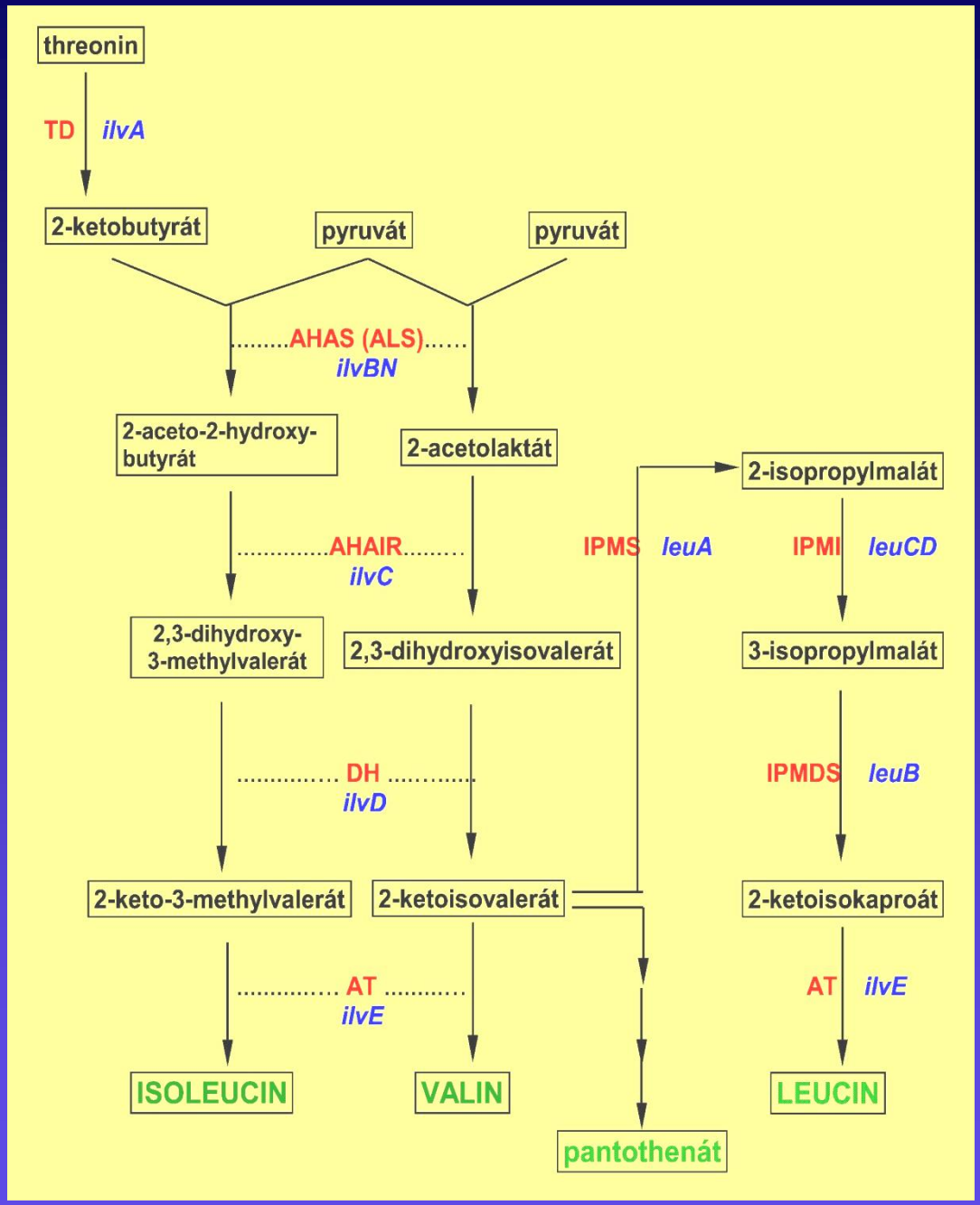
- **Problémy :** **Nízká exprese, nízká aktivita enzymů, nestabilita plazmidů, geny pro rezistenci, cizorodá DNA**

Konstrukce produkčních kmenů s minimálním počtem mutací



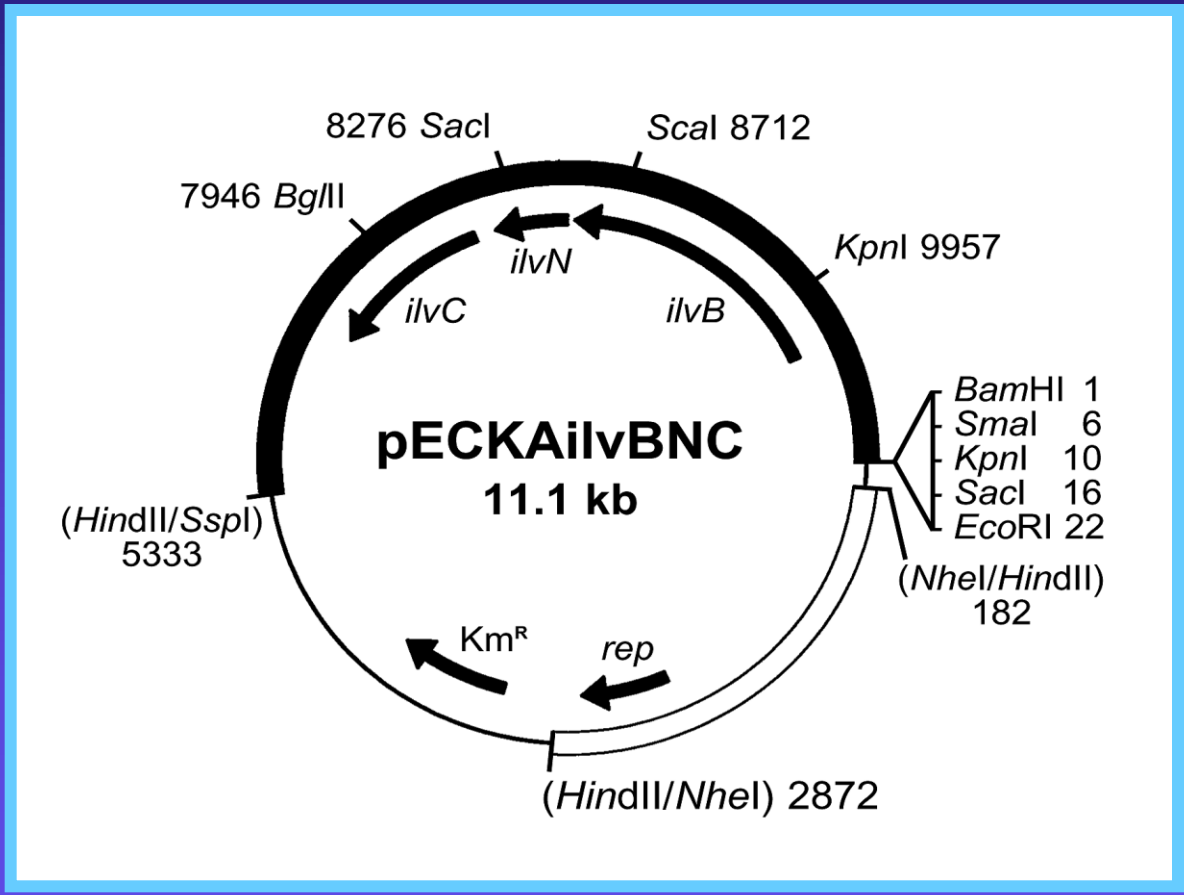
Biosyntéza isoleucinu, valinu a leucinu valinu v *C. glutamicum*

TD	- threonin dehydratasa
AHAS	- synthasa acetohydroxy kyselin (-acetolaktát synthasa)
AHAIR	- isomeroreduktasa acetohydroxykyselin
DH	- dehydratasa dihydroxy kyselin
AT	- aminotransferasa
IPMS	- isopropylmalát synthasa
IPMI	- isopropylmalát isomerasa
IPMDS	- isopropylmalát dehydrogenasa



Amplifikace biosyntetických genů klonováním v plazmidu

- Klonování fragmentu s operonem *ilvBNC* z *C. glutamicum*



- *C. glutamicum* (pECKAilvBNC) produkuje 30mM valin

Modulace exprese genů modifikací promotorů

- Aktivita přirozených promotorů genů může být modulována cílenou mutagenezí

- Výhody:

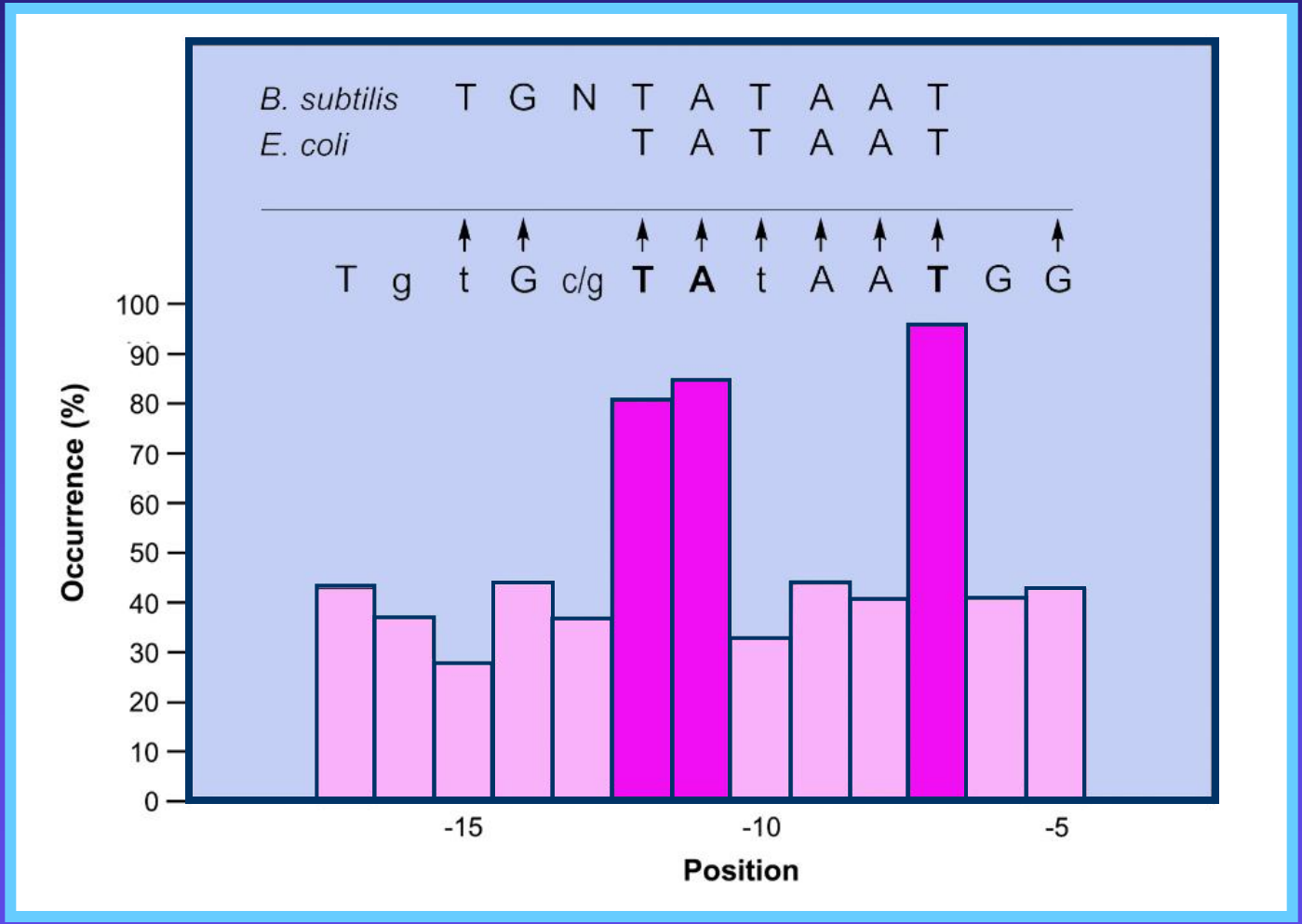
1. Obecná metoda
2. Zvýšení nebo snížení genové exprese
3. Relativně jednoduchá technika
4. Možnost předvídat
5. Přesná modulace aktivity
6. Konstitutivní promotory
7. Regulované promotory

- Zásahy do transkripčních signálů: delece, inzerce, mutace, výměny (na plazmidu nebo v chromosomu)

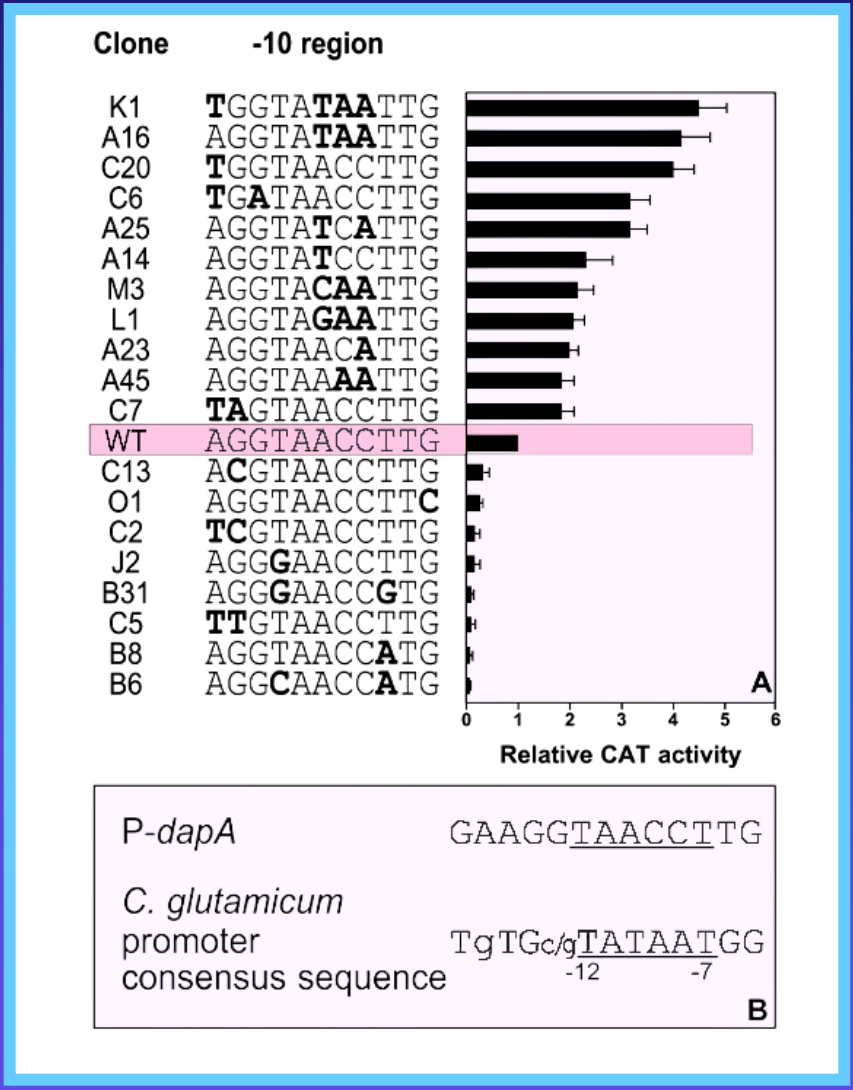
Metoda cílené mutageneze promotorů v chromosomu *C. glutamicum*

- Příprava fragmentu DNA (1,2 kb) nesoucího mutaci pomocí PCR
- Klonování fragmentu v plazmidu replikujícím se v *E. coli*
- Vnesení plazmidového konstruktu do buněk *C. glutamicum* transformací nebo konjugací
- Izolace integrantů podle selekčního znaku (rezistence)
- Izolace klonů po dvojité rekombinaci (vyštěpení sekvence vektoru) na základě přítomnosti podmíněně letálního genu *sacB*
- Ověření fenotypového projevu (auxotrofie, bradytrofie, produkce aminokyseliny)
- Amplifikace fragmentu s mutací (PCR), ověření sekvenováním
- Analýza aktivity promotoru v *promoter-probe* vektoru
- Sledování vlivu mutace promotoru na produkci aminokyseliny

Promotory *C. glutamicum*: 1. Statistická analýza 60 promotorů



Promotery *C. glutamicum*: 2. Mutační analýza



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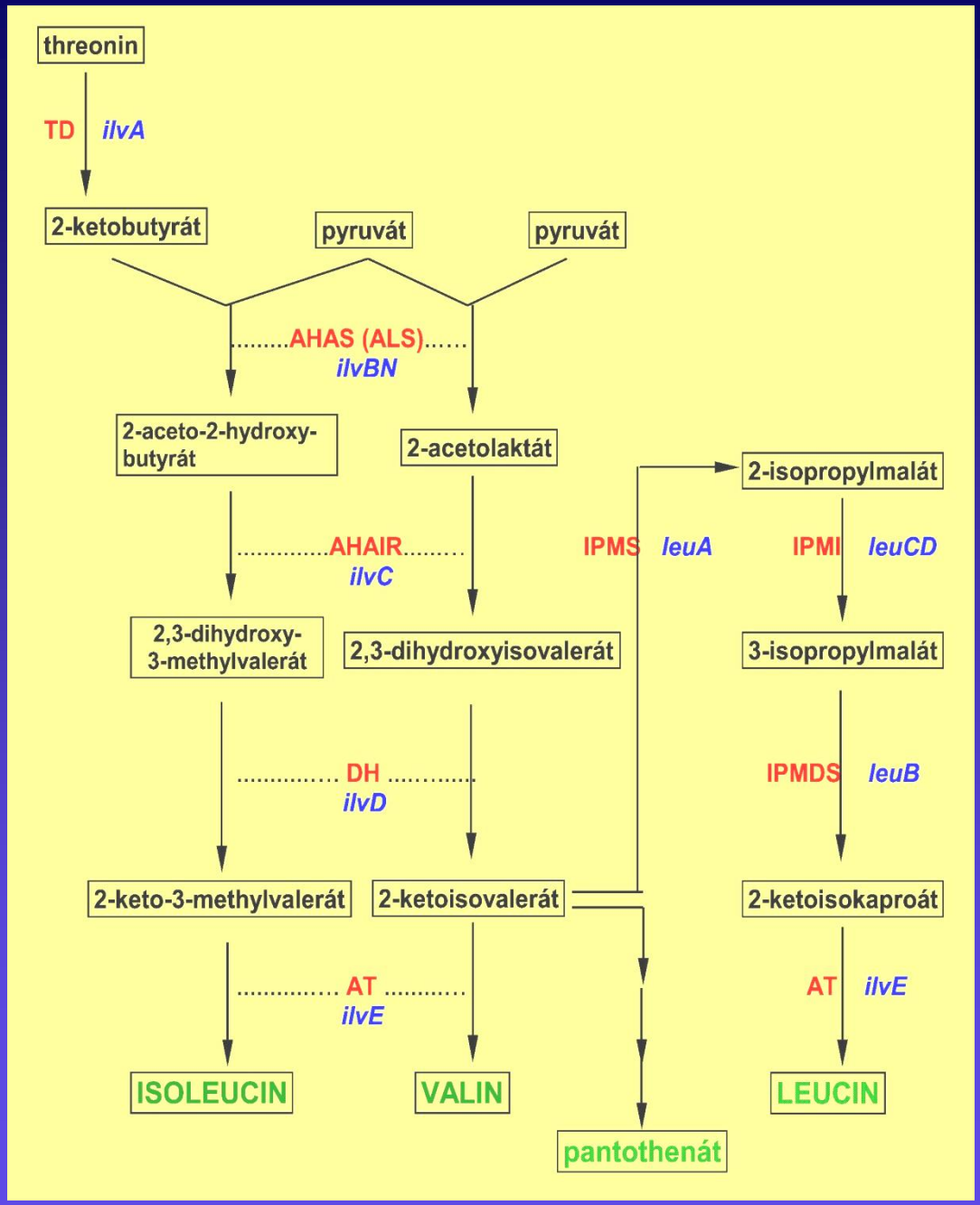


Delece genu *ilvA* (auxotrof ile^{-})
Mutace P-*ilvA* (bradytrof ile$^{\pm}$)

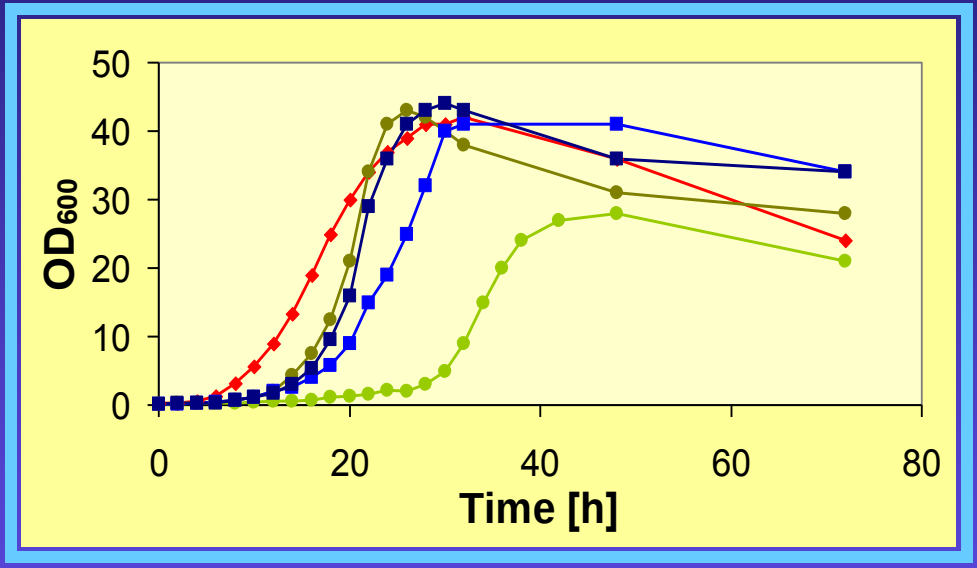
- Efekt Δ*ilvA*: produkce valinu
- Kmen Δ*ilvA* netvoří 2-ketobutyrát a isoleucin
- Do minimálního média musí být přidáván isoleucin
- Nahradí mutace *ilvA-leaky* deleci Δ*ilvA?*

	−10 +1	
CCTATGCCAAAGTAGGTGGAGAAGAT	TACACT AGTCCACC A TGAGTGAAAC	WT
	CACAGT	M1CG
	CACTGT	M1CTG
	TCCAGT	M2CG
	TCCAGA	M2CGA

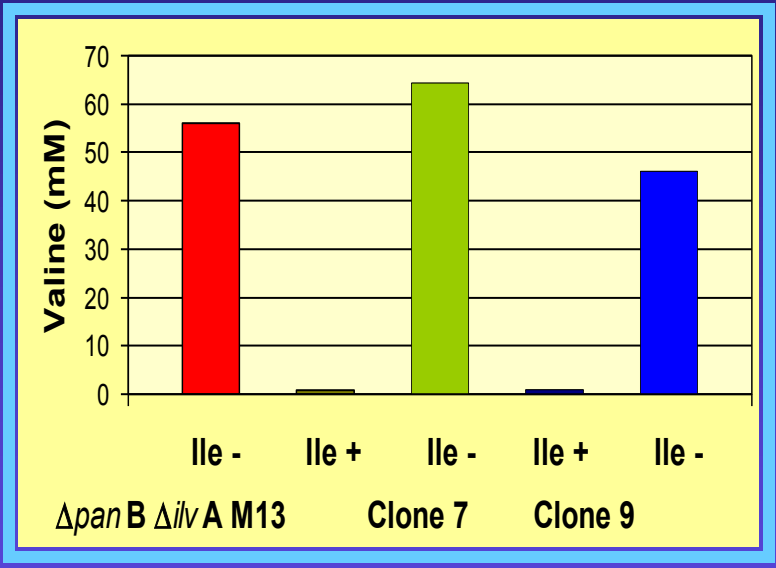
- Slabé mutantní promotory snižují transkripci *ilvA*
- *ilvA*-leaky mutace může nahradit Δ*ilvA* limitací růstu
- Kultivace v půdě bez isoleucinu, limitace růstu isoleucinem

Růst kmene s mutací P-ilvAM1CG a produkce valinu

Růst



Produkce valinu



- Slabý mutantní promotor snižuje transkripci *ilvA*
- Mutace *ilvA*M1CG zvyšuje produkci valinu
- Produkce valinu je silně závislá na rychlosti růstu
- Limitace růstu je významným faktorem produkce

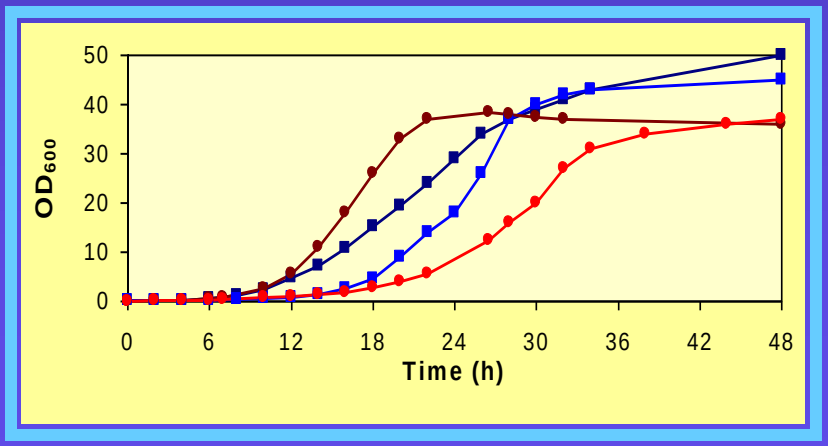
Mutace P-leuA (bradytrof leu±)

```
                                        -10      +1
CGAGTTGCTACCCACACCACAAAGTTGTTGTATGCTTCACCACATGACTTCGCG  WT
                                    GCATGCT             M2C
                                    TCAGGCT             M2TCG
                                    GTATGCA             M3A
                                    GAATGCA             M3AA
```

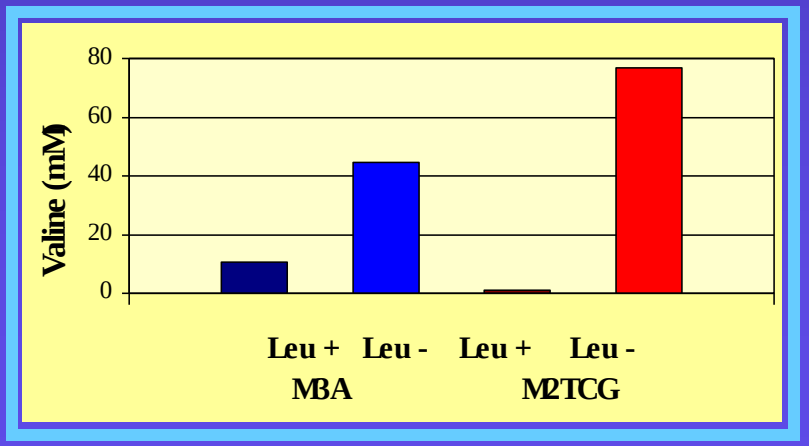
- Mutace M3A a M2TCG v chromosomu způsobují leu-*leaky* fenotyp

Kmeny *leuA*M3A a *leuA*M2TCG

Růst

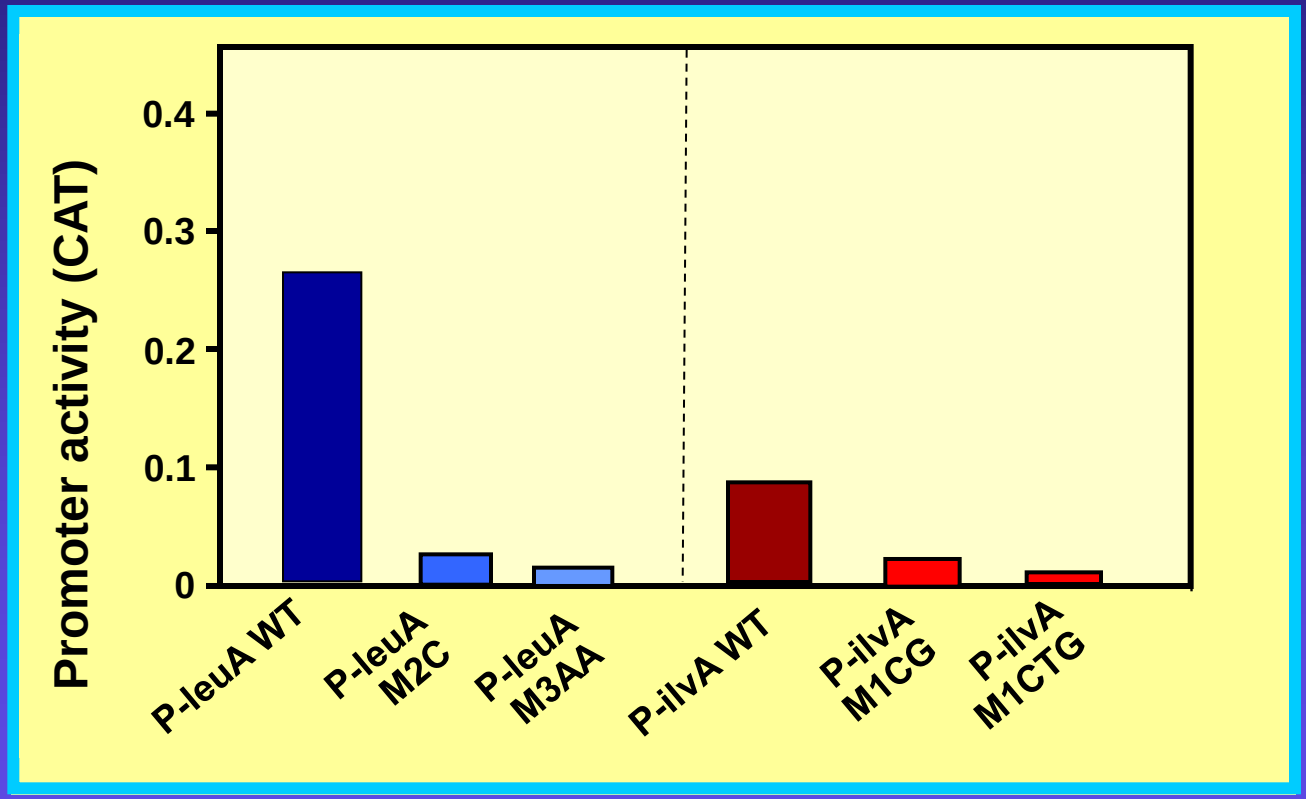


Produkce valinu



Aktivita mutantních promotorů P-*ilvA* and P-*leuA*

Byla změřena aktivita slabých mutantních promotorů P-*ilvA* and P-*leuA* klonovaných v *promoter-probe* vektoru pET2



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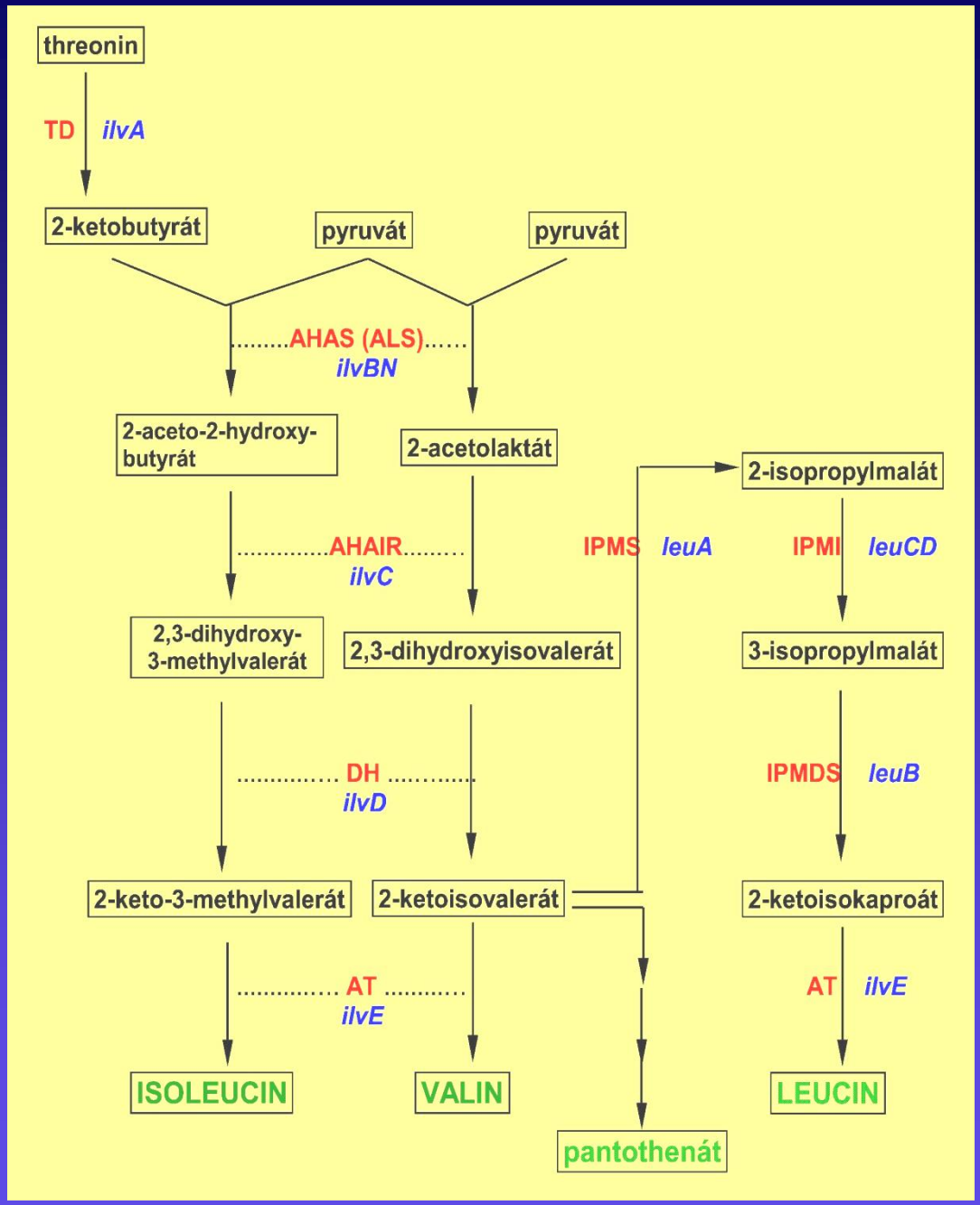


Up-mutations of promoters P-*ilvD* and P-*ilvE*

- Stronger promoters enhance transcription of *ilvD* and *ilvE*

P-*ilvD* modifications

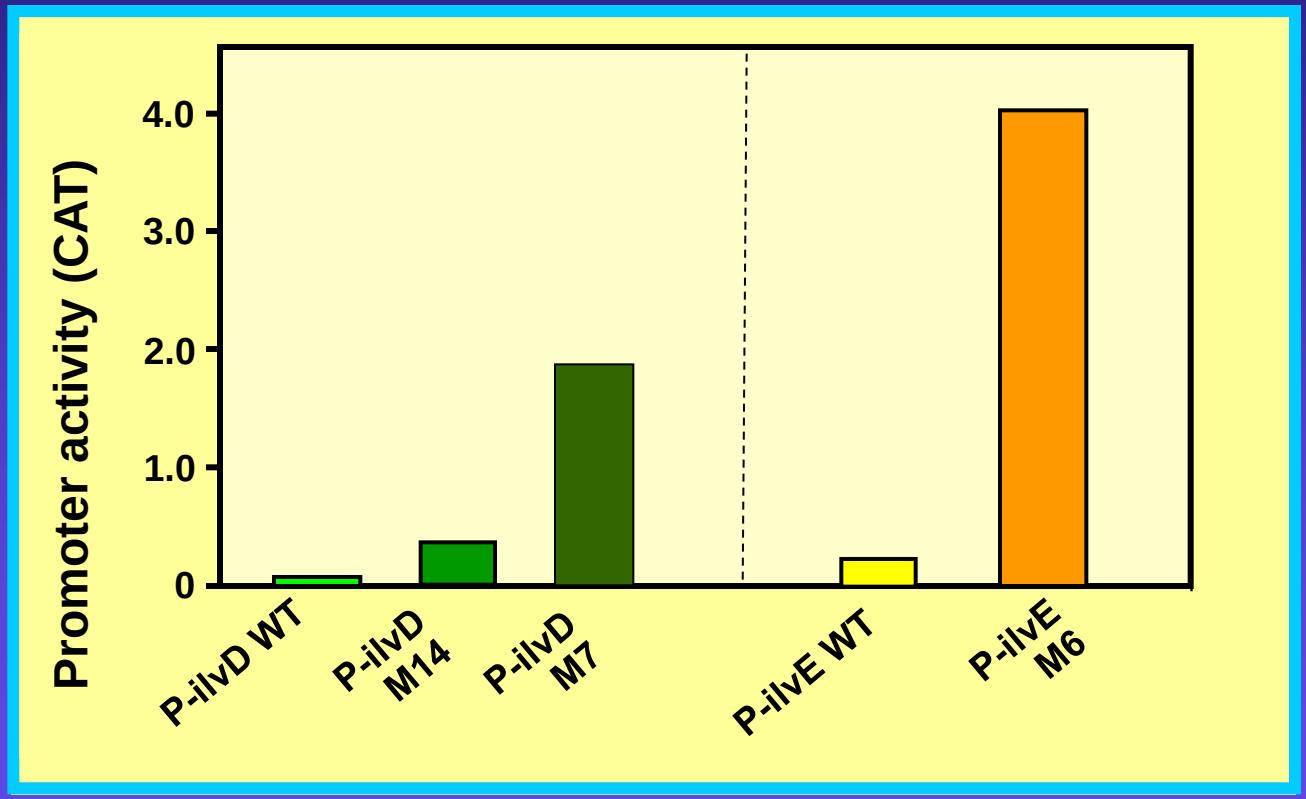
```
                                        -10        +1→TSP
CATCGTGATAACCCTGCGTCACAGCACTAGAGTGTAATAAGCCGTCCG     WT
                          TGTGCTATAGT   M7

                                              -10        +1→TSP
CATCGTGATAACCCTGCGTCACAGCACTAGAGTGTAATAAGCCGTCCG     WT
                        AAGCACTGTGGTATAAT   M14
```

P-*ilvE* modification

```
                                       -10        +1→TSP
TGTCAGGTGTATCTGTCAGGTAGCAGGTGTACCTTAAAATCCATG        WT
                          TGTGGTACCAT   M6C
```

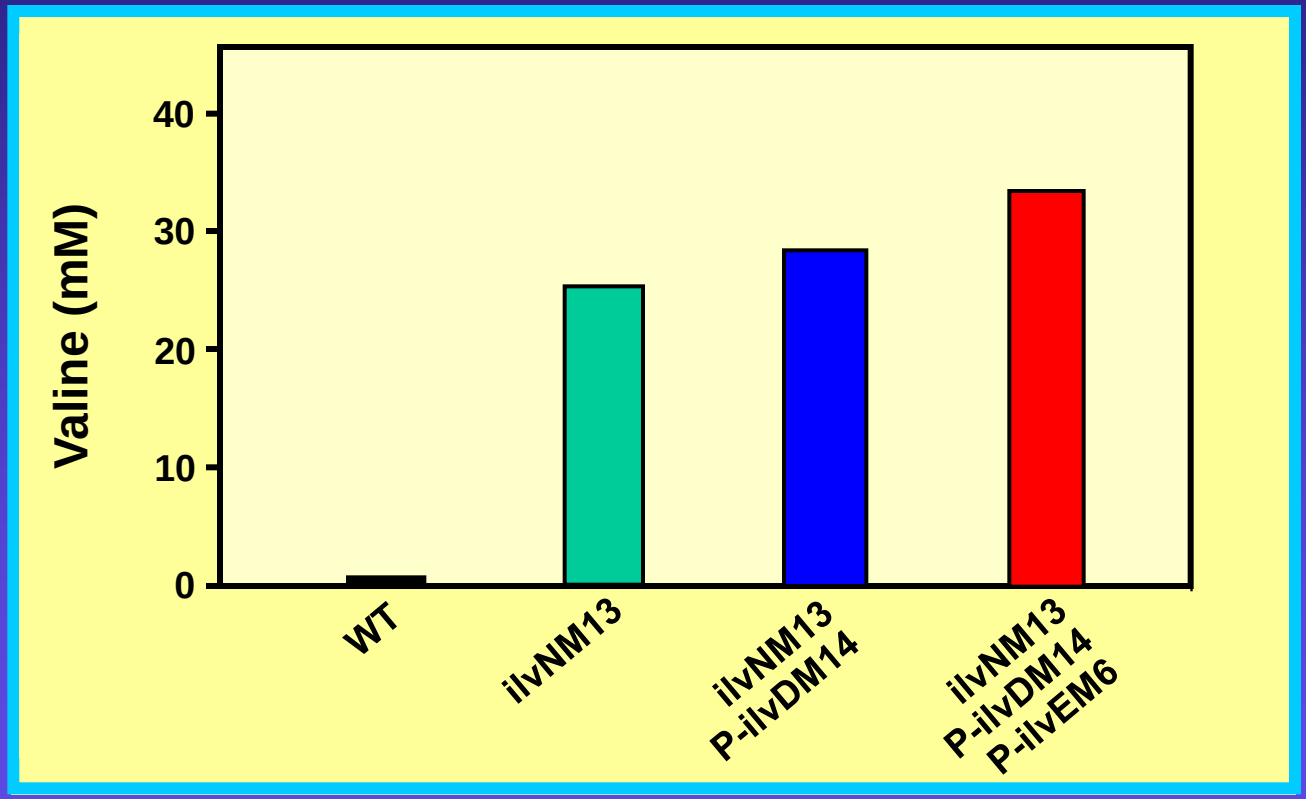
Aktivita mutantních promotorů P-*ilvD* and P-*ilvE*

Byla změřena aktivita silných promotorů P-*ilvD* and P-*ilvE* klonovaných v *promoter-probe* vektoru pET2



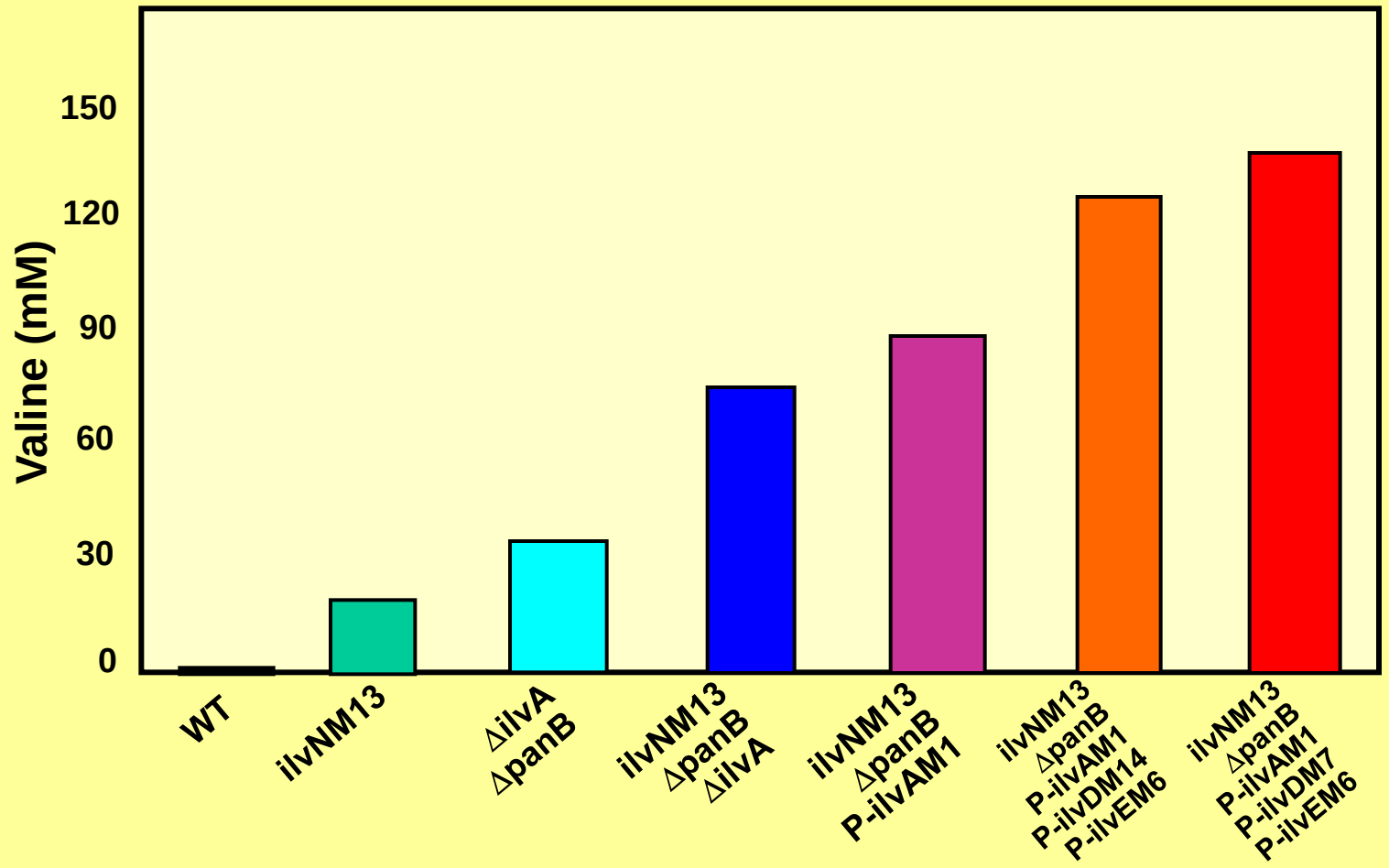
Valine production by strains with stronger promoters P-*ilvD* or P-*ilvE*

Up-mutations of the promoters P-*ilvD*M14 or P-*ilvE*M6 were introduced into *C. glutamicum* chromosome

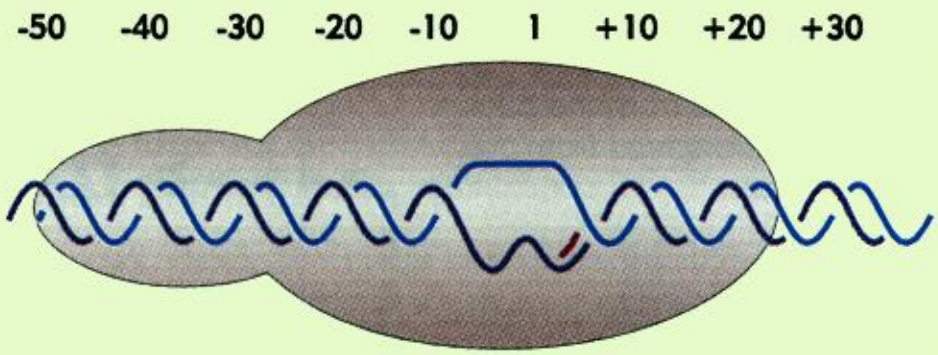
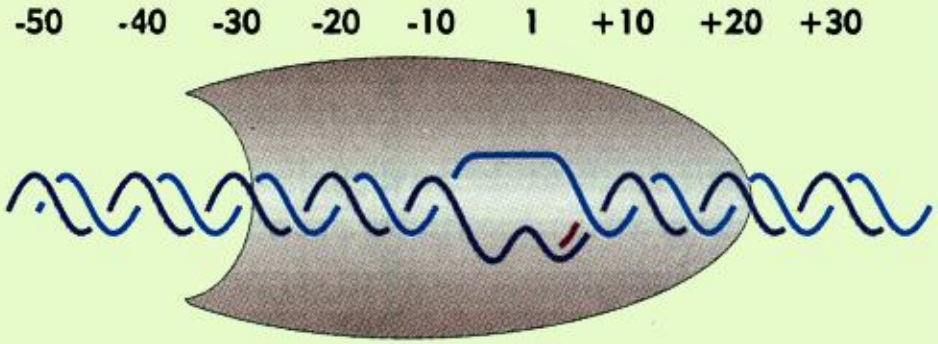
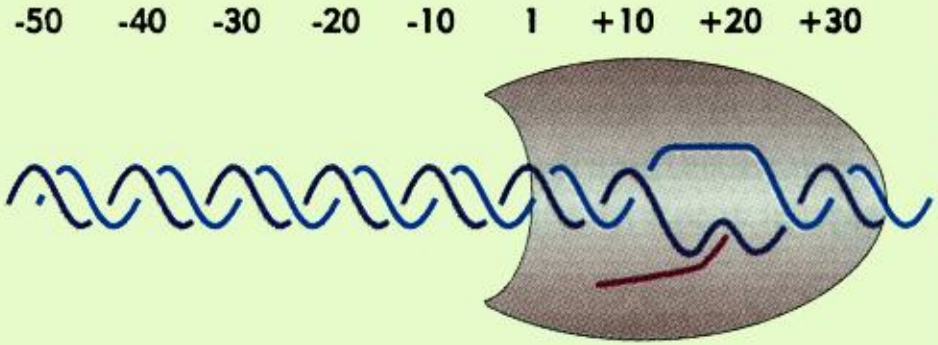


- P-*ilvD*M14 mutation increased valine production by 10%
- P-*ilvE*M6 mutation increased valine production by 30%

Valine production by *C. glutamicum* with accumulated mutations



Transcriptional initiation

Initiation complex	
Initial elongation complex	
General elongation complex	

Sigma subunits (factors)

- **Sigma subunit confers the holoenzyme the ability to recognize a specific binding site**
- **Sigma factor - a positive transcriptional regulator.**
- **Genes under control of a sigma form a regulon.**
- **Several sigma factors - several classes of promoters**
- **Sigma 70, sigma 54, ECF sigma factors**
- ***Escherichia coli*: 7 sigma factors**
- ***Mycobacterium tuberculosis*: 13 sigma factors**
- ***Bacillus subtilis*: 17 sigma factors**
- ***Streptomyces coelicolor*: 65 sigma factors**

Corynebacteria:

- ***C. glutamicum*: 7 sigma factors**
- ***C. efficiens*: 7 sigma factors**
- ***C. diphtheriae*: 9 sigma factors**
- ***Rhodococcus* sp. RHA1: 34 sigma factors**

Sigma factors in *E. coli*

Sigma 70 – primary sigma, vegetative (housekeeping)
TTGACA (16-18) TATAAT

Sigma S (38) – general stress, stationary phase
CTAt/c a/g c/gTTA (–14 to –4)

Sigma F (28) – flagellar
TAAAGTTT(11 bp) GCCGATAA

Sigma N (54) – nitrogen utilization
CTGGNA (6 bp) TTGCA

Sigma H (32) – heat shock
CTTGAA (13-17 bp) CCCCAT-T

Sigma E (24) – envelope stress response
GAACTT (16 bp) TCTGA

Sigma FecI – ECF

C. glutamicum sigma factors

SigA Housekeeping genes, exp. phase: 160 promoters[1]
SigB Transition phase, stresses: 13 promoters[2]
SigH Heat-shock and other stresses: 45 promoters[3]
SigM Oxidative stress: 4 promoters[4]
SigE Cell surface stress: 2 (in vitro transcription)

SigC, SigD, – promoters not yet described

[1]Pátek *et al.*, J. Biotechnol. 104:311-323 (2003); Pátek *et al.*, J. Biotechnol. 154:101-113 (2011)
[2]Larisch, *et al.*, BMC Genomics, 8:4 (2007); Ehira *et al.*, Appl. Environ. Microbiol. 74:5146-5152 (2008)
[3]Engels *et al.*, Mol. Microbiol. 52:285-302 (2004); Ehira *et al.*, J. Bacteriol. 191:2964-2972 (2009)
Busche *et al*. BMC Genomics (2012)
[4]Nakunst *et al.*, J. Bacteriol. 189:4696-4707 (2007)

Consensus sequences of *C. glutamicum* promoters

	-35		-10	promoters
SigA :	TTG^{a}/$_{c}$CA		TGnTAnAATnG	160
SigB :	TTGACA		TAnAAT	13
SigH :	GGAA	18-20	GTT	45
SigM? :	GGAA		GTT	(4)
SigE	GGAAC		GTT	(2-6)
SigC	??		??	
SigD	??		??	

Methods: Promoter (transcription) analysis 1

Transcriptional start point determination:
Primer extension PEX (reverse transcript 70 – 300 bp)
(= Reverse transcription + sequencing ALF sequencer)
5´RACE (rapid amplification of cDNA ends)

Promotor activity assays (reporter genes):
***cat* activity pET2+promoter (70-500 bp), cell disruption,**
CAT enzyme measurement in extract, acetyl-CoA

Fluorescence measurement: pEPR1+promoter (70-500 bp)
GFPuv, fluorescence exc. 385 nm, emission: 509 nm

In vitro transcription:
Detection of radioactively (^{32}P) labeled transcript
Isolated purified recombinant RNAP+sigma factor

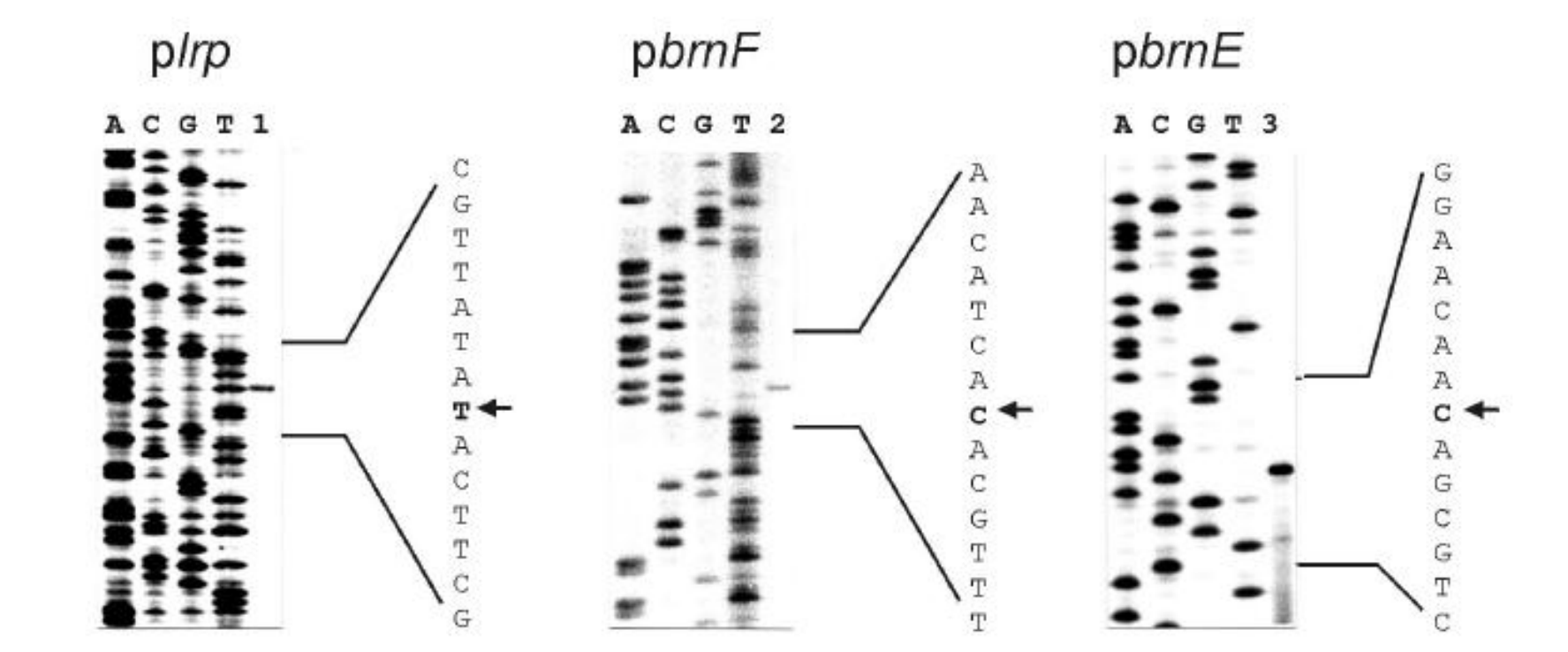
Methods: Promoter (transcription) analysis 2

RACE (rapid amplification of cDNA ends)
Northern (RNA-RNA) hybridization
RT PCR (reverse transcription + PCR)
Real-time RT PCR (qPCR) (real-time cycler)
Primer extension using non-radioactive in vitro transcript

Microarray transcriptome profiling
RNA sequencing (SQ of cDNA)
ROMA = runoff transcription + microarrays[1, 2]

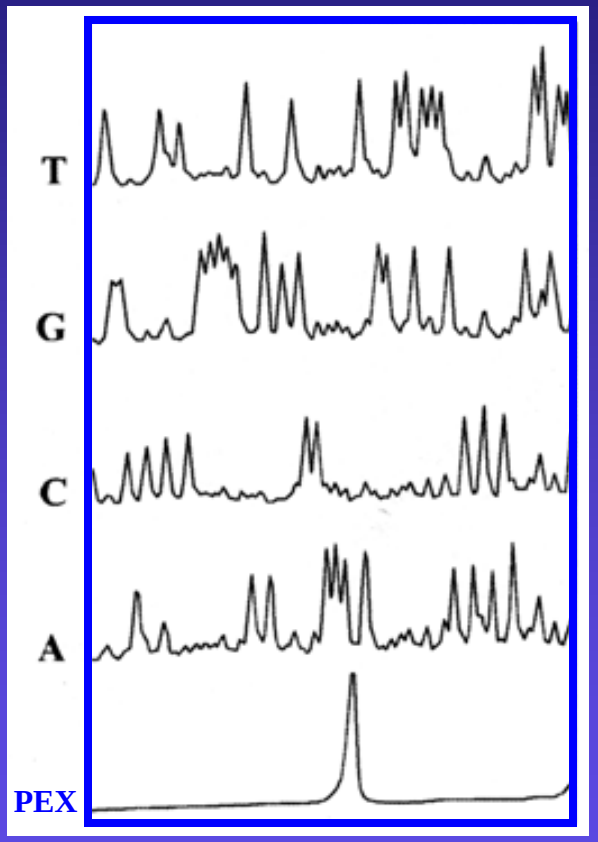
[1]Cao et al. J. Mol. Biol. 316:443-457 (2002)
[2]Keese et al. J. Bacteriol 192:1292-1298 (2010)

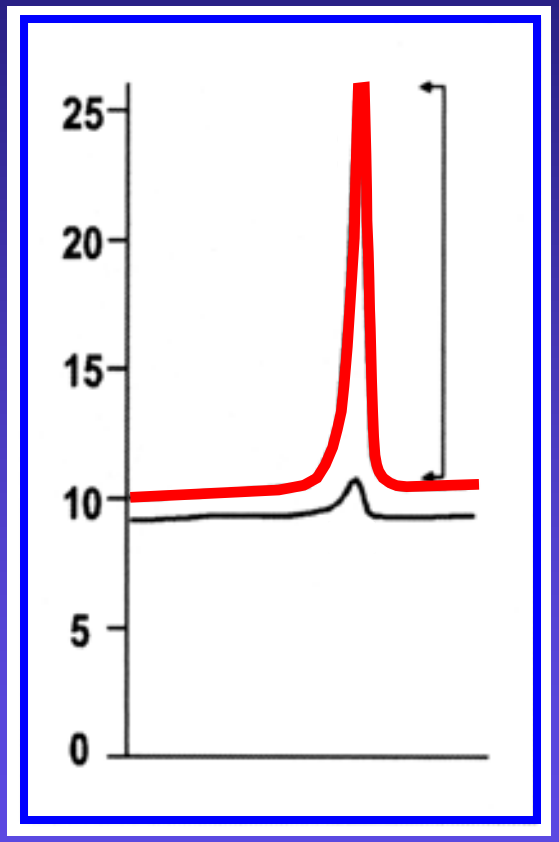
Primer extension analysis: radioactive



Primer extension analysis: non-radioactive Gene for chaperon *groES*

- Vegetative promoter regulated negatively by HrcA





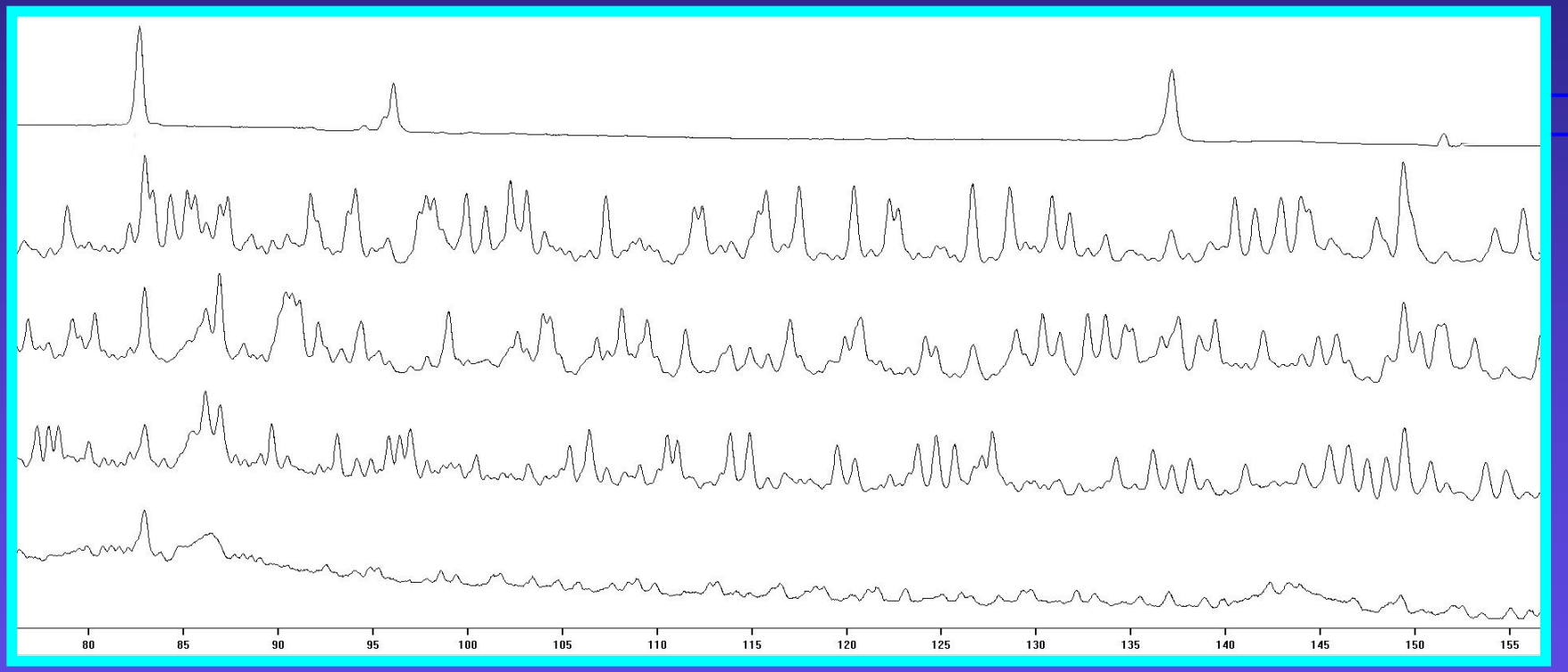
CIRCE-1 CIRCE-1 CIRCE-2 CIRCE-2 +1

TTT**TTAGCACCC**TCAACAGTT**GAGTGCTGGCACTC**TCGGGGGTA**GAGTGCCAA**A**T**AGGTTGTT

-35 -10

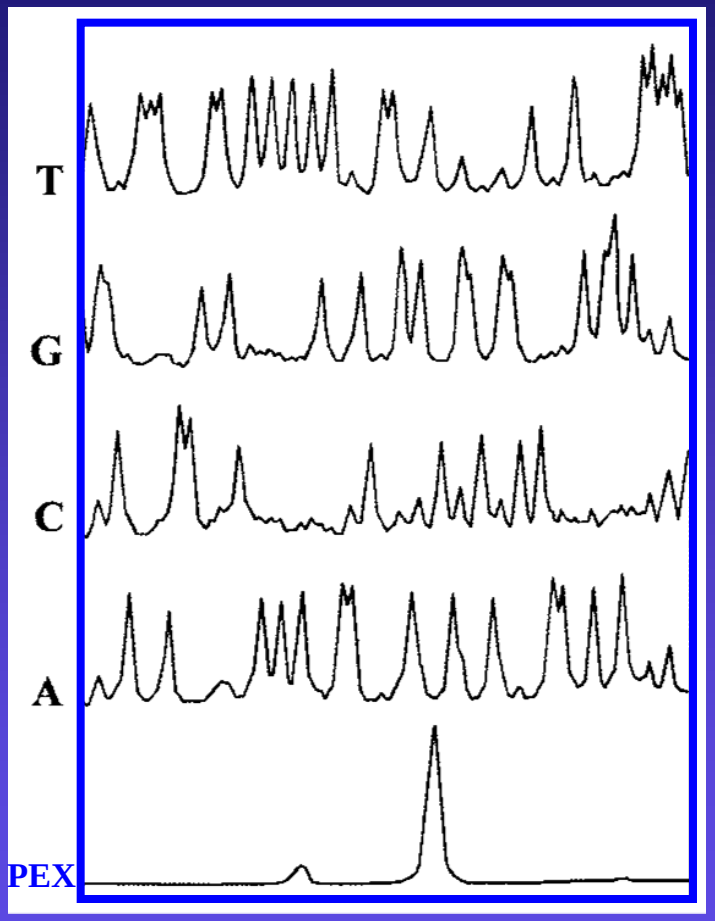
Primer extension

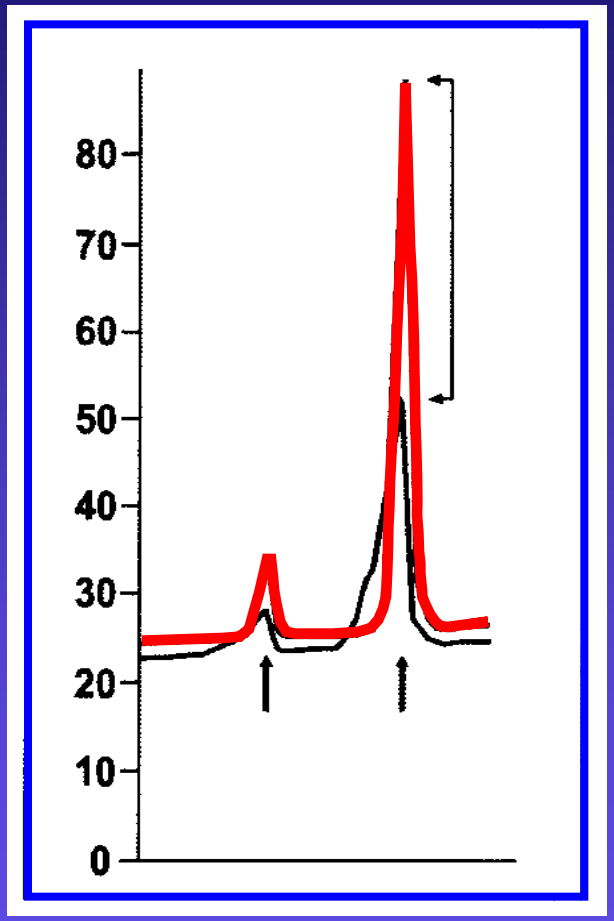
1) mRNA from *C. glutamicum*, cultivation + stress, PEX
2) ***in vitro*** **transcription + specific sigma, PEX**



Promoter *P-dnaK*: transcription start point (TSP)

- Vegetative promoter regulated negatively by HspR
- SigH-dependent promoter regulated positively by SigH



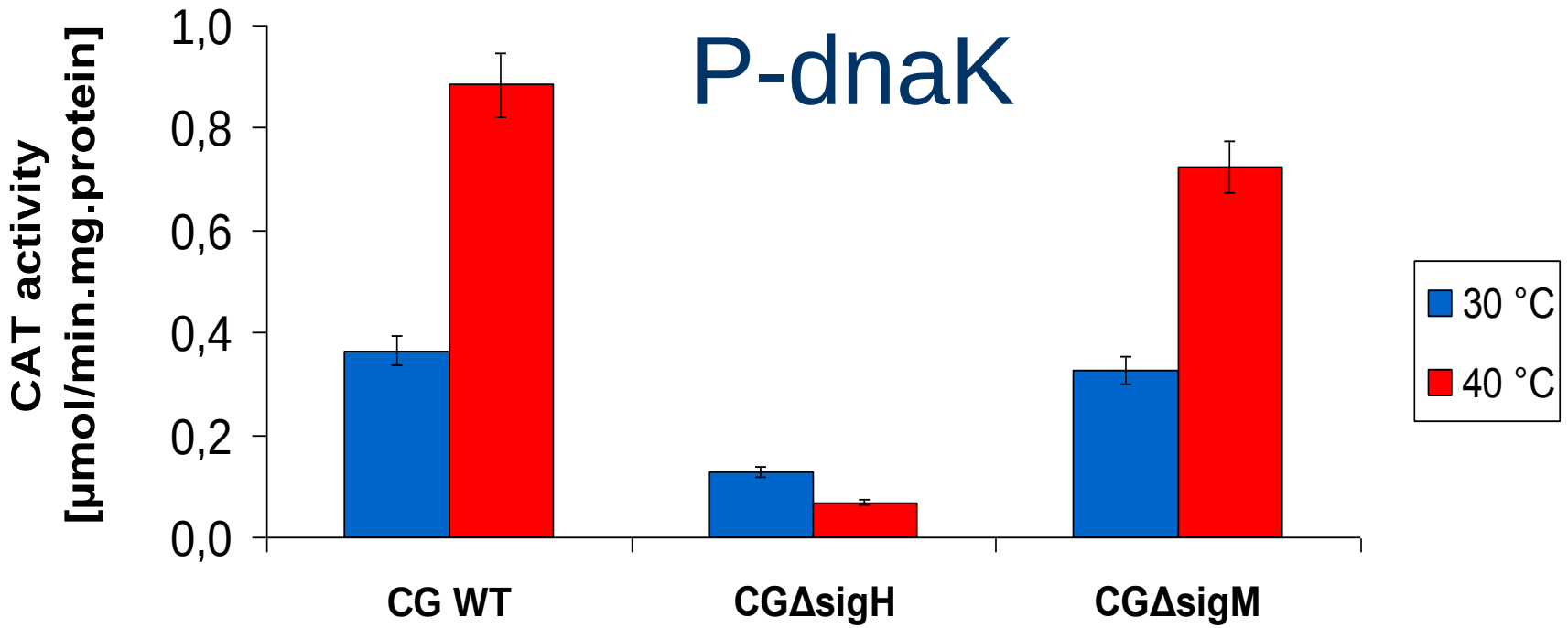


TCTAGTTGGAACAACTTTGTGGCATTACCGTTGCTATATATGTAAGCTTGAGTCAGGCAGGCTCAATGAG

-35 -35 -10 -10

TSP2 TSP1 HAIR HAIR

Promoter P-dnaK (*dnaK-grpE-dnaJ-hspR* operon)
(DnaK, GrpE, DnaJ = heat shock proteins, chaperones)

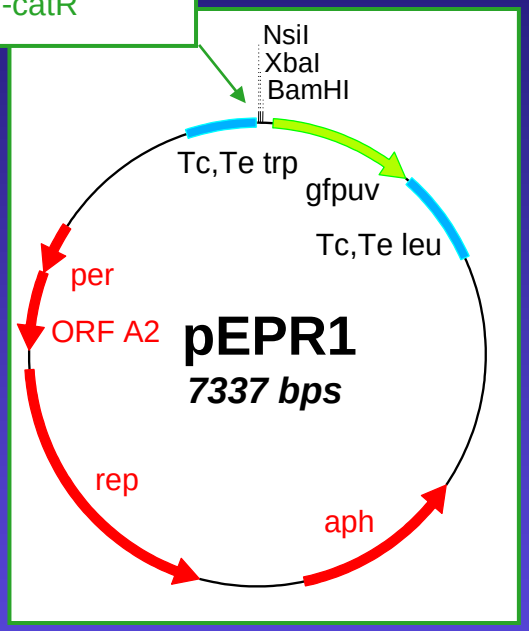


Transcription of the *dnaK-grpE-dnaJ-hspR* operon genes depends on SigH, especially after heat shock

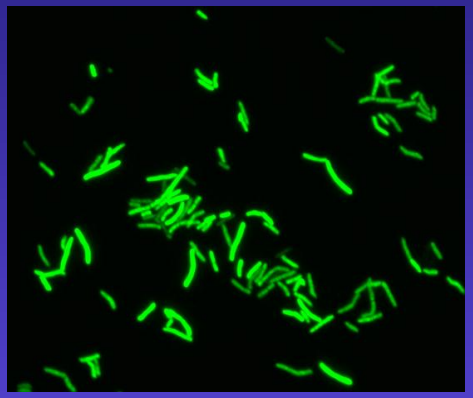
Promoter-probe vector pEPR1
(*E. coli – Corynebacterium+Rhodococcus*)

P-pheA, P-pheR,

P-catA, P-catR

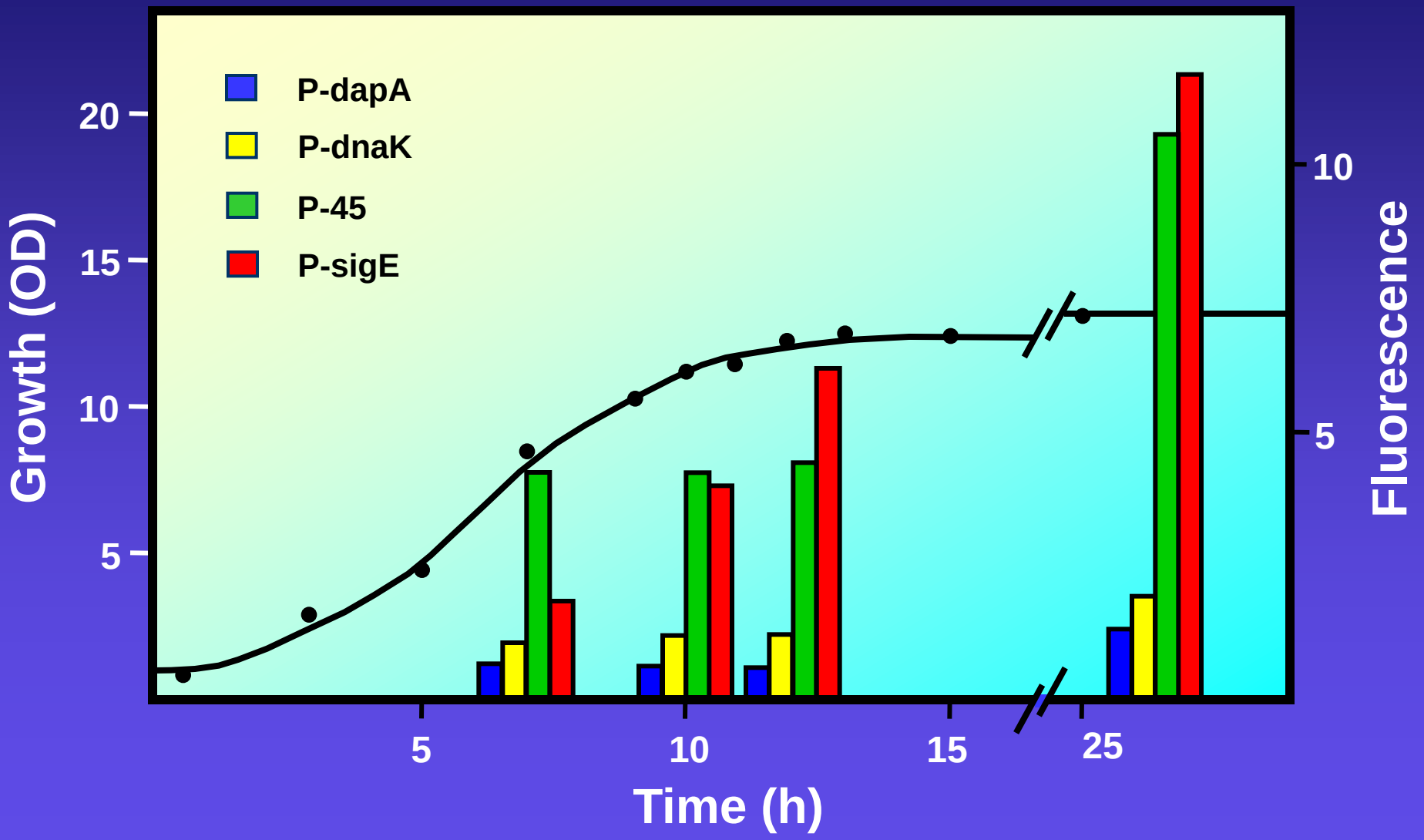


promoter activity
was measured as
intensity of fluorescence

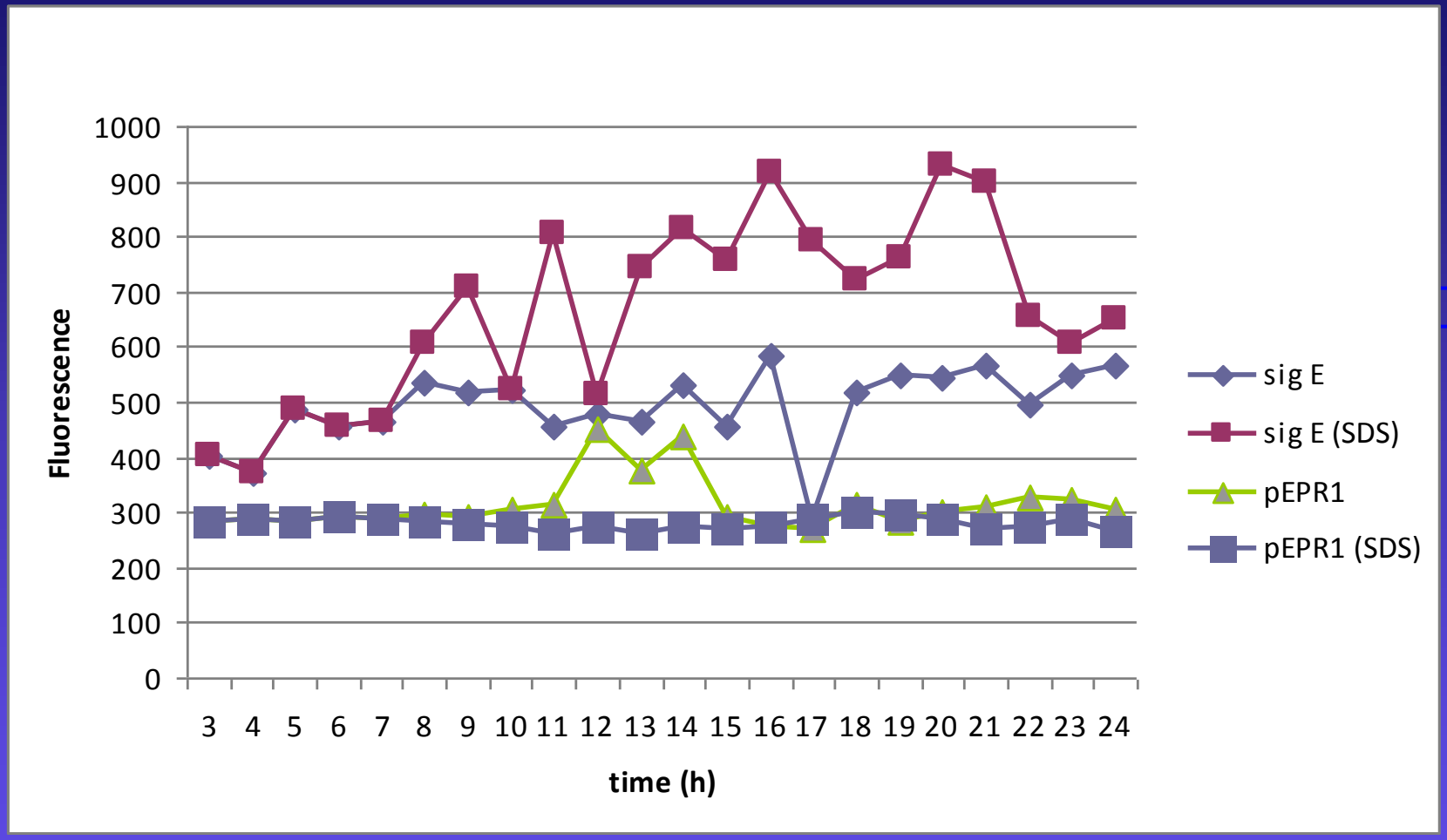


- Transcriptional fusion of the promoters with gene encoding green fluorescent protein
- *gfp*uv used as a reporter gene

Activity of *C. glutamicum* promoters in various growth phases



Activity of P*sigE* during batch cultivation



- P*sigE*: 4 SigA-dependent promoters, induced by surface stress

Reporter gene in a single-copy system: insertion into the chromosome (integation)

- Promoter with the reporter gene *cat* are inserted into the *C. glutamicum* chromosome
- Alternative approach: insertion of the *gfp* gene in transcription fusion with the studied promoter.

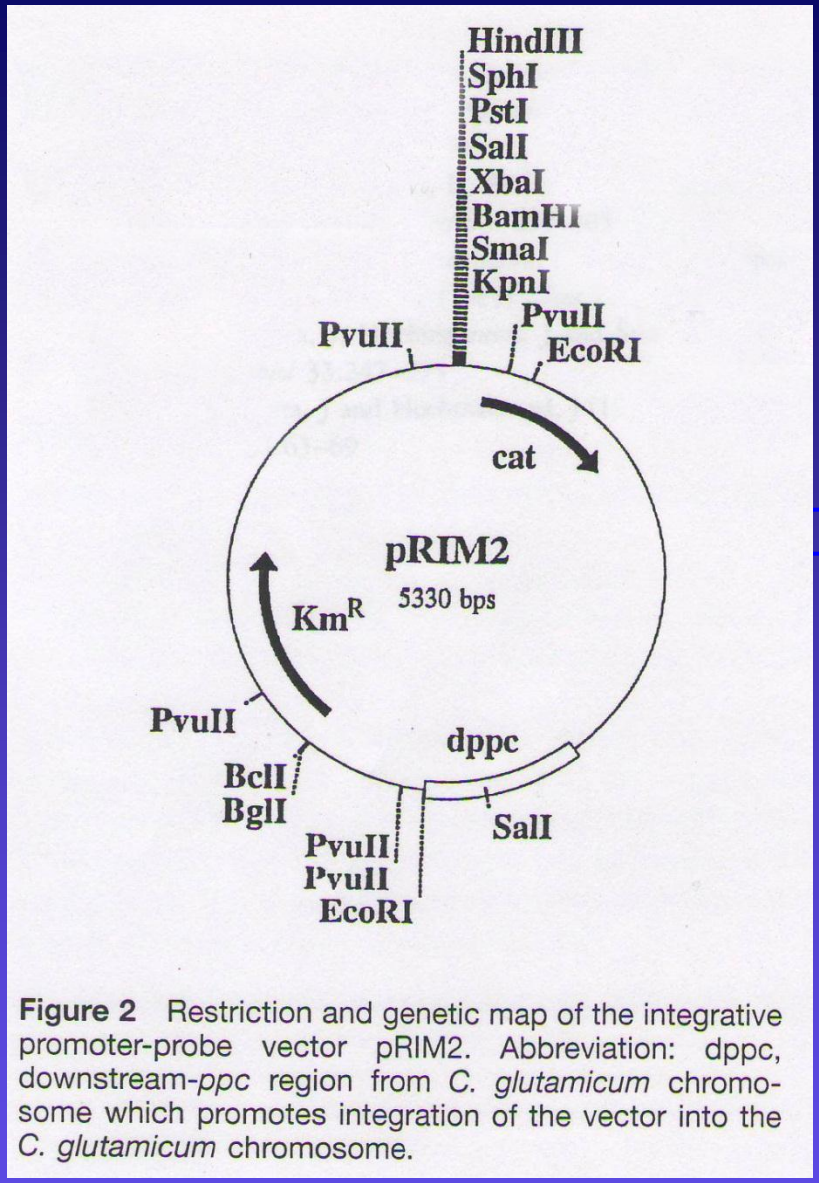


Figure 2 Restriction and genetic map of the integrative promoter-probe vector pRIM2. Abbreviation: dppc, downstream-*ppc* region from *C. glutamicum* chromosome which promotes integration of the vector into the *C. glutamicum* chromosome.

Integrative vector replicating in *E. coli*

Transfer: transformation

Genom *C. glutamicum* a metody globální analýzy (genome-wide analysis)

Table 1
General features of the *C. glutamicum* ATCC 13032 chromosome

Features of the chromosome	Property
Total size	3 282 708 bp
G+C content	53.8%
Coding sequences (CDS)-total	3002 (100%)
CDS encoding annotated proteins	2489 (83%)
CDS encoding putative cytosolic proteins	1518 (51%)
CDS encoding putative membrane proteins	660 (22%)
CDS encoding putative secreted proteins	311 (10%)
CDS encoding conserved hypothetical proteins	250
CDS encoding hypothetical proteins	263
Coding density	87%
Average gene length	952 bp
Ribosomal RNAs	6 operons (16S-23S-5S)
Transfer RNAs	42 different/60 genes
Other stable RNAs	2

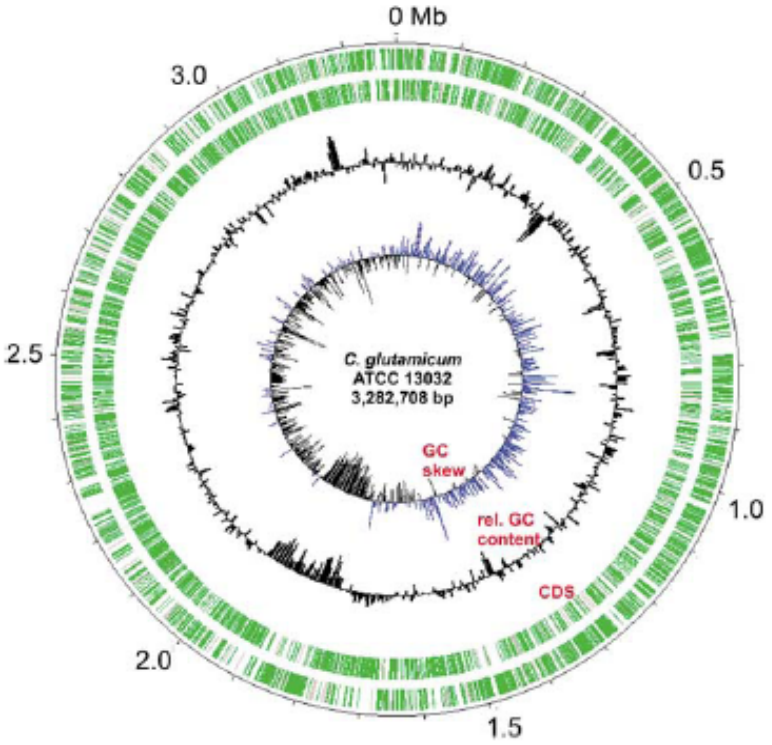


Fig. 1. Circular representation of the *C. glutamicum* ATCC 13032 chromosome. The concentric circles denote (from outward to inward): coding sequences (CDS) transcribed clockwise and counter-clockwise, relative G+C content, and GC skew. A positive deviation in G+C content from the average is shown by bars pointing outward and a negative deviation by bars pointing inward. The same holds for the GC skew plot where positive skew values are shown in blue color and negative values in black. The *C. glutamicum* ATCC 13032 genome sequence was deposited in the EMBL database.

Genes for biosynthesis and transport of amino acids in *C. glutamicum*

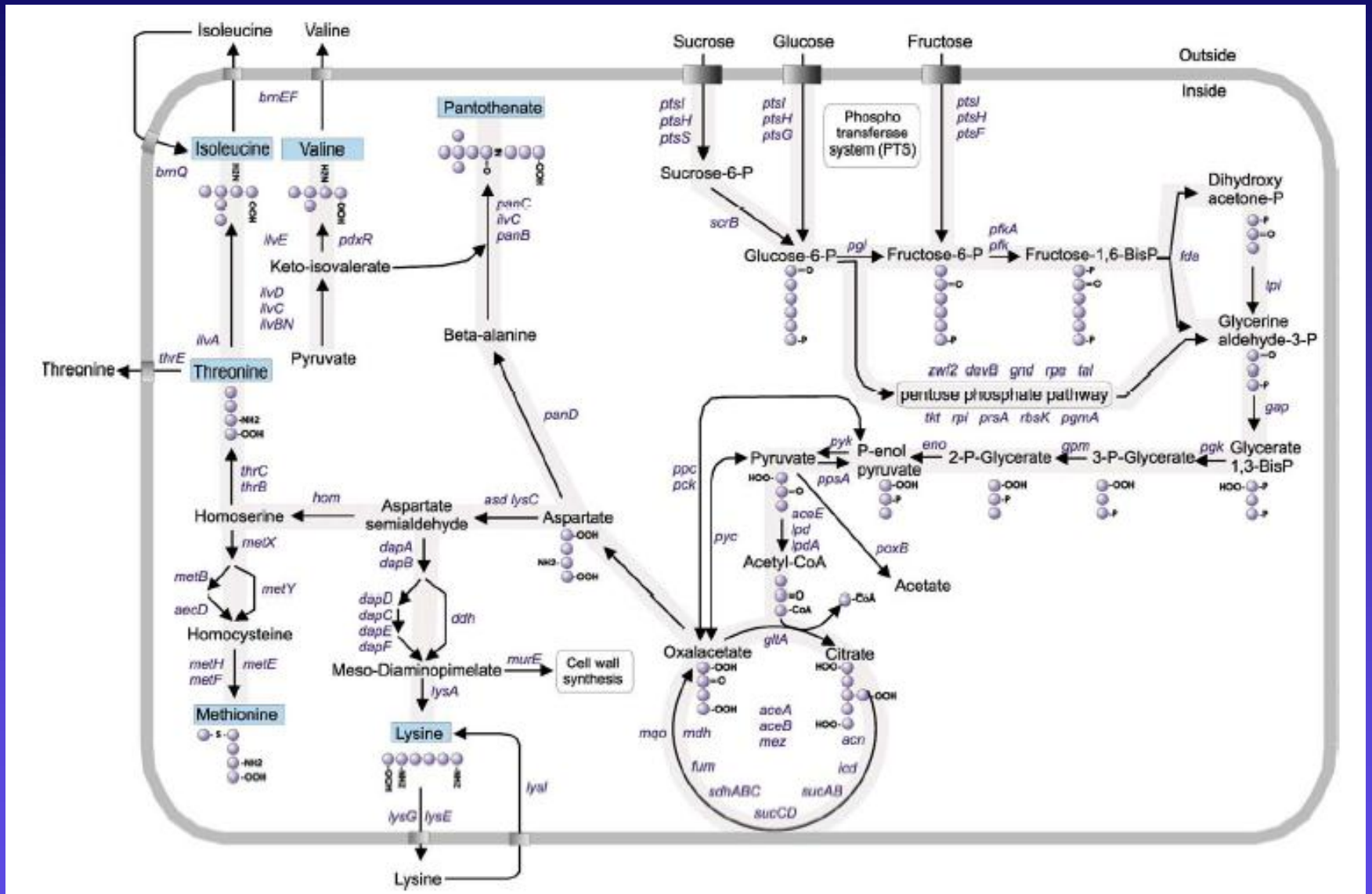


Fig. 3. Metabolic reconstruction of biosynthetic and transport pathways important for the overproduction of L-amino acids and vitamins derived from aspartate or pyruvate. A number of key metabolites are shown in structural formulas with bullets representing methyl groups. Details on the genes and their gene products are given in the text and in Table 3.

-Omics

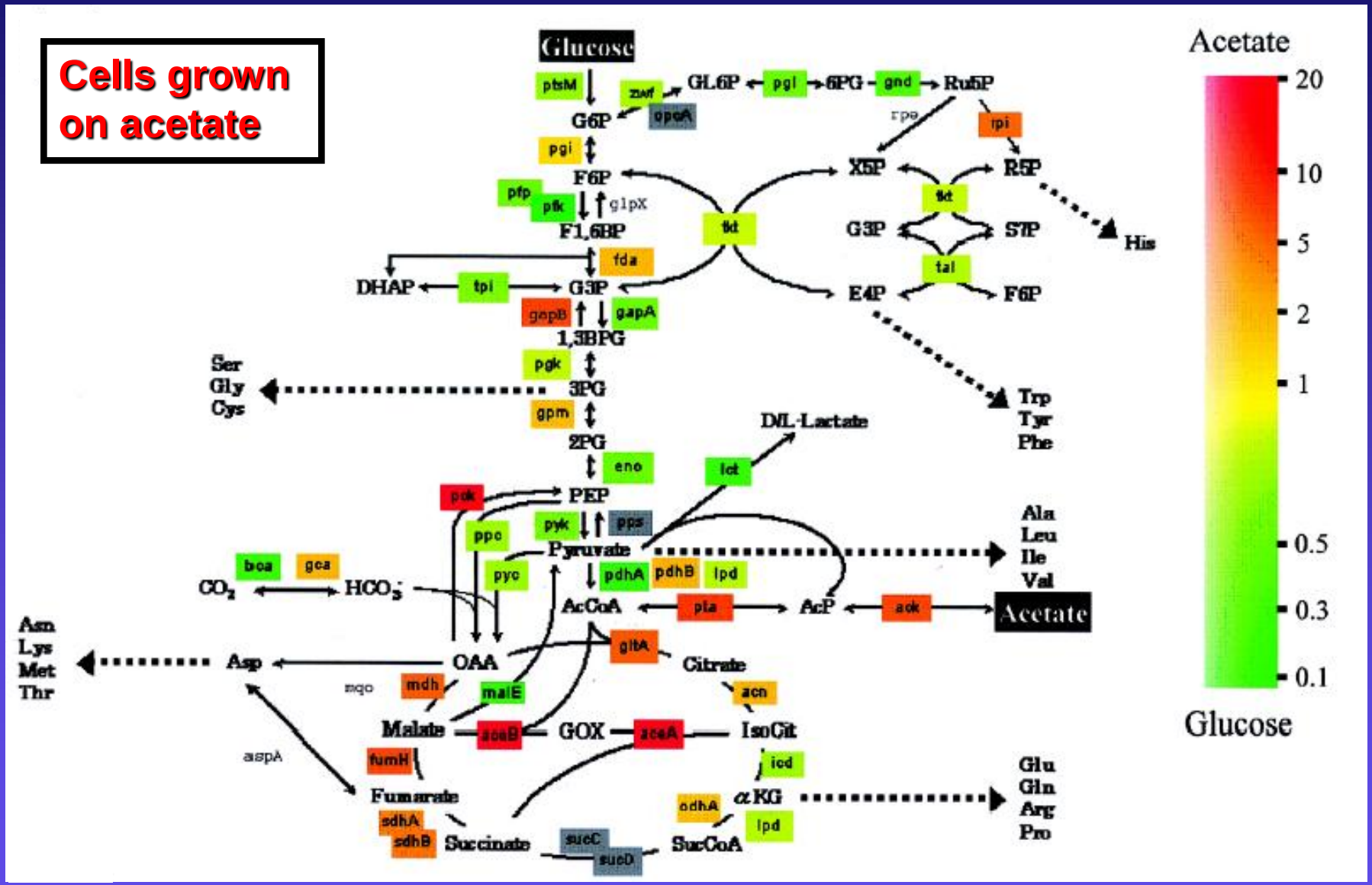
(A Journal of Integrative Biology)

- Genome - genomics
- Transcriptome - transkriptomics
- Proteome - proteomics
- Metabolom - metabolomics
- Fluxome - fluxomics

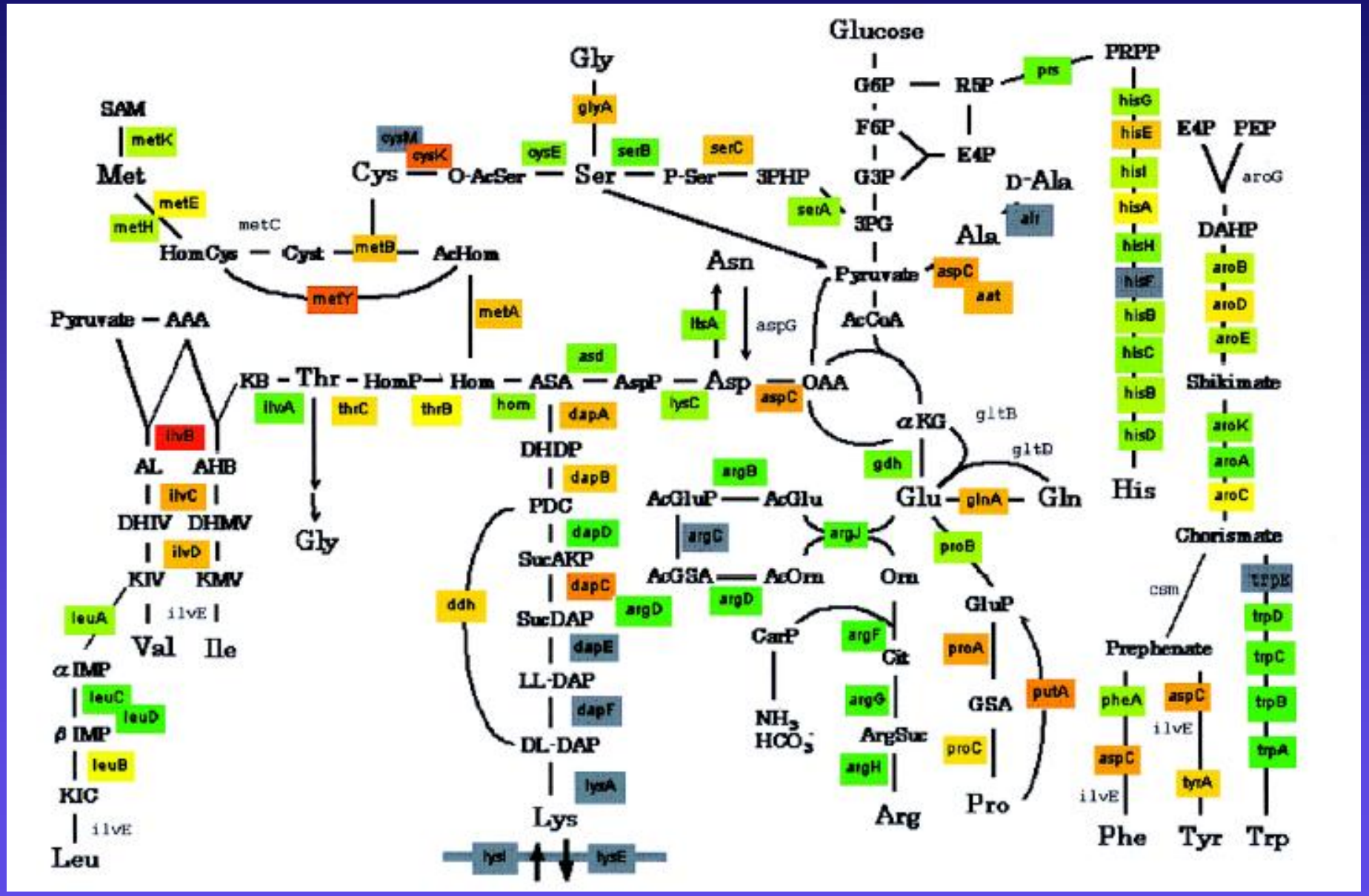
- Degradomics, foldomics, phosphatomics, interactomics, regulomics

- Informatics-driven research x Hypothesis-driven research

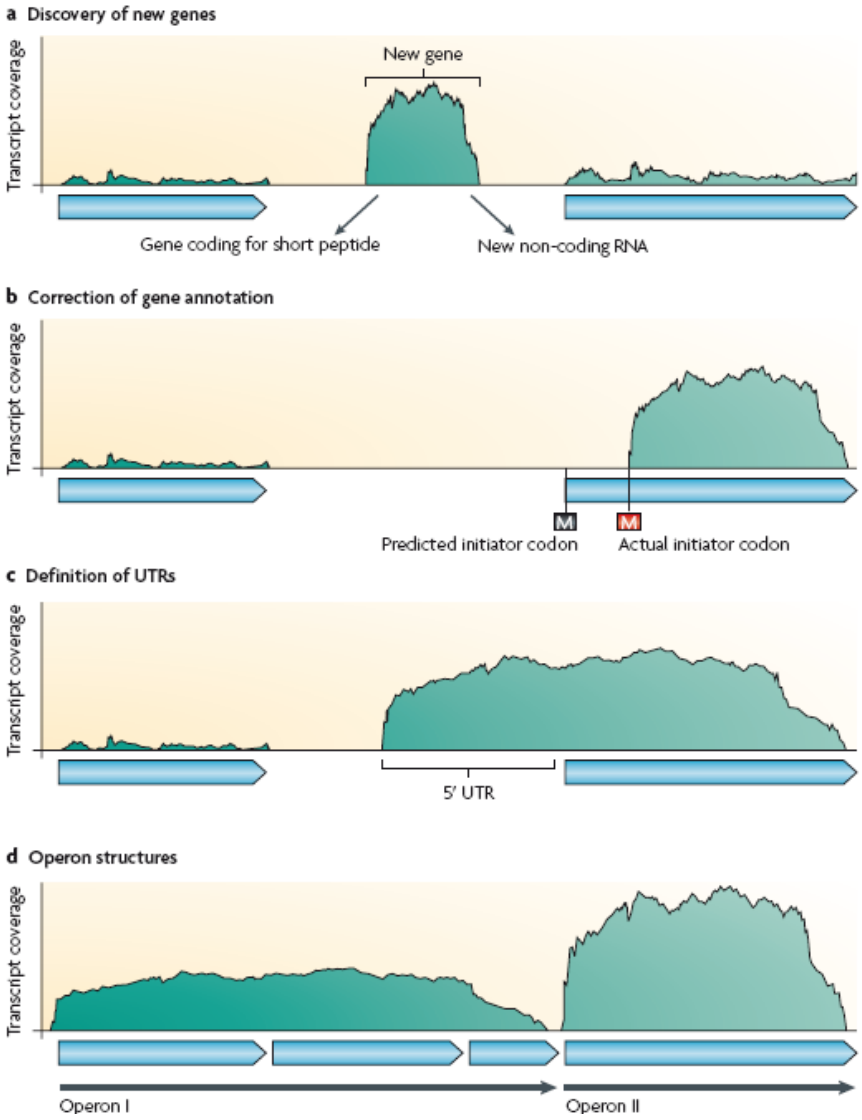
Transcriptional profiling of *C. glutamicum*



Transcriptional profiling of *C. glutamicum*



RNA-sequencing
(high throughput sequencing of cDNA libraries)



Genome-wide RNA-sequencing
- improve genome annotation
- new unexpected features of prokaryotic genome
-regulatory mechanism which involve RNA
- **Transcriptional landscape**

Figure 1 | **Contribution of transcriptomics to annotation of functional elements.** In all panels the x axis represents a schematic genomic region and the y axis represents transcript coverage as derived from RNA–seq or tiling array experiments. The light blue arrows depict annotated genes. Transcriptomic information can be used to improve genome annotation by enabling: the discovery of new genes (**a**); the correction of gene structure annotations (**b**; the black M depicts the predicted first methionine and the red M is the first methionine in the corrected annotation); the detection of UTRs and transcription start sites (**c**); and the determination of operon relationships (**d**).

Proteome of *C. glutamicum*

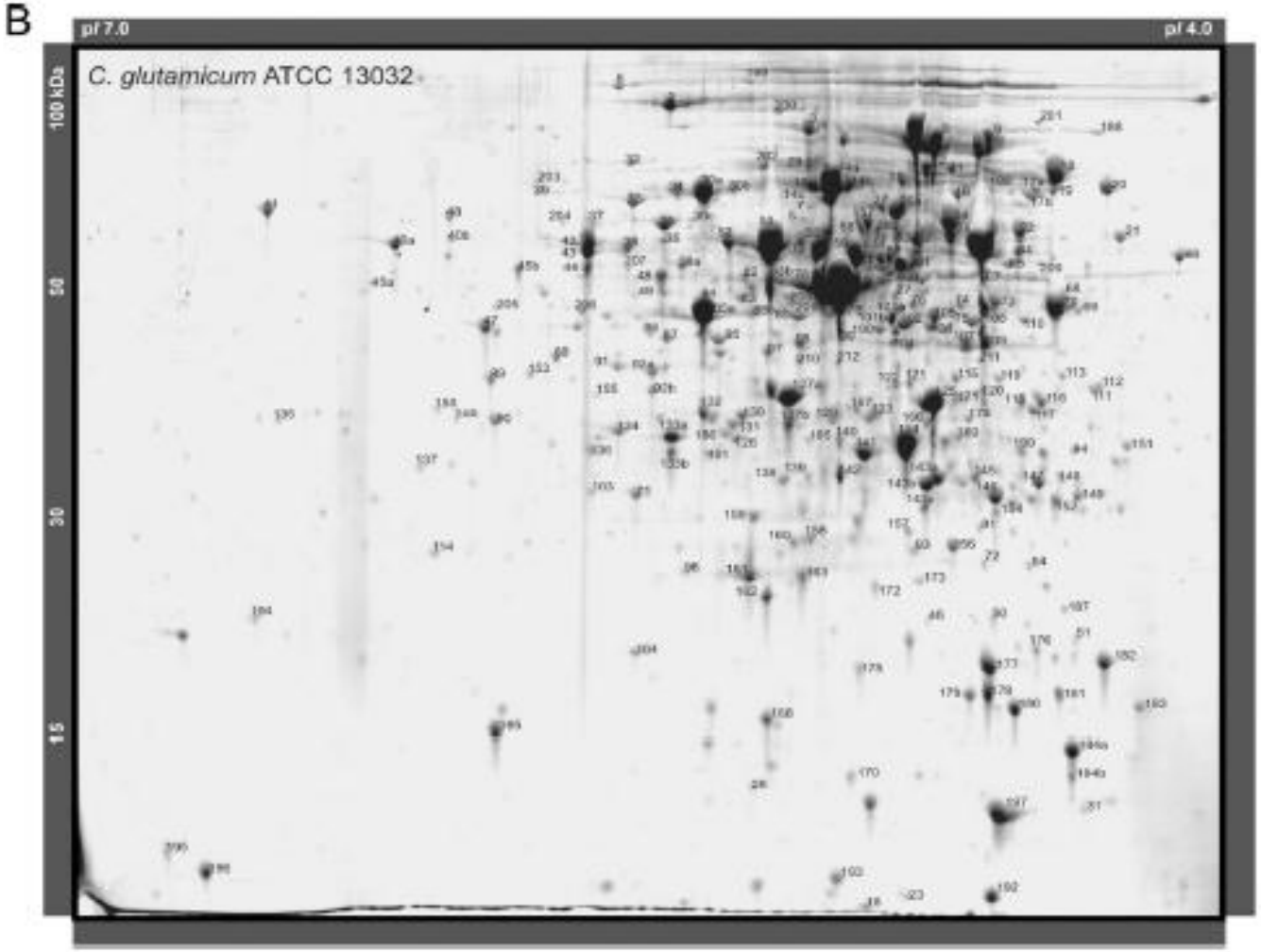
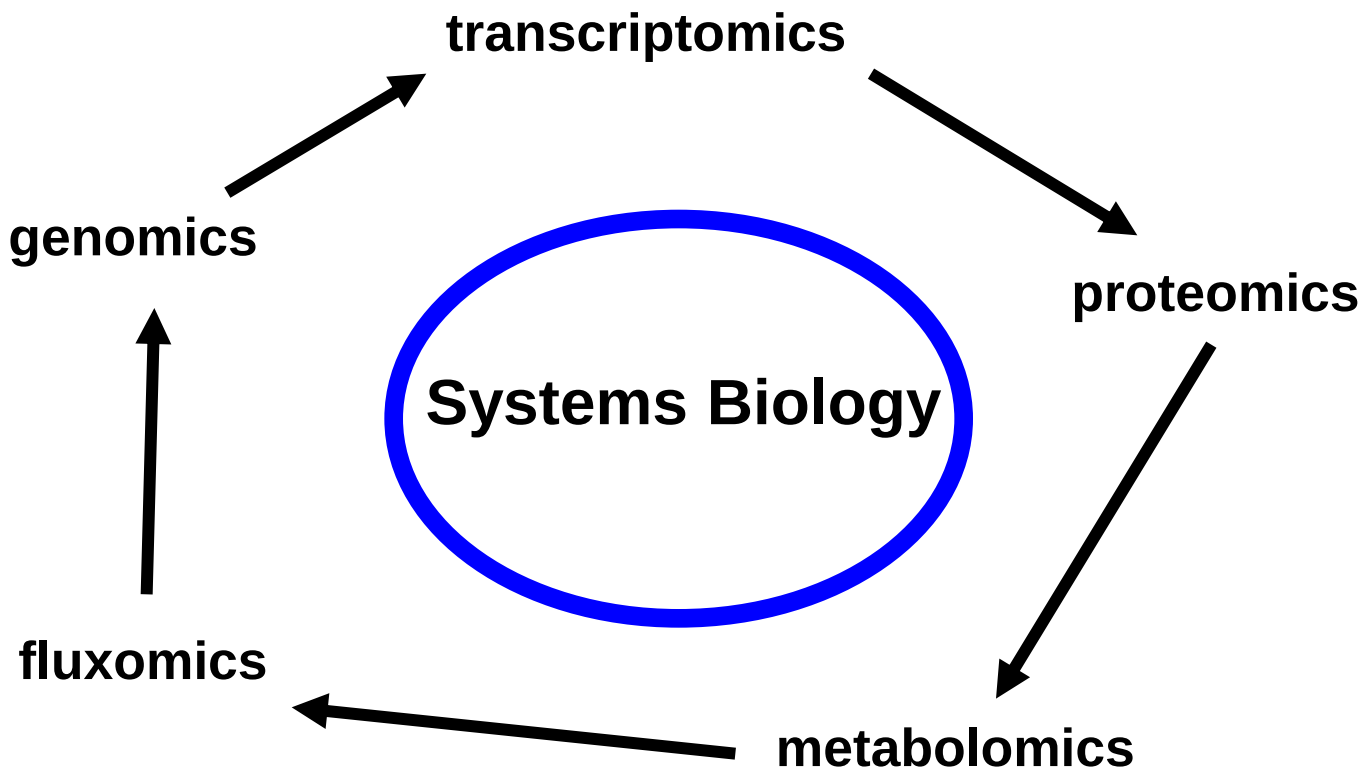


Figure 1. Reference 2-D maps of cytosolic proteins of *C. efficiens* YS-314 (A) and *C. glutamicum* ATCC 13032 (B). Cells were cultured in complex medium and collected at the transition point from exponential growth towards stationary phase. Proteins were separated with a 24-cm IPG stripe (pI range 4 to 7) and 12.5% SDS-PAGE finally stained with Coomassie. Protein spots are numbered. Numbers refer to detailed information presented in Table 1 for *C. efficiens* and in Supplementary Table S1 for *C. glutamicum*. Theoretical M_r and pI values are indicated. Letters indicate multiple spots of the same protein.

Systems biology



Systems Biology

- Systems-level understanding of biological systems that takes into account complex interactions of gene, protein, and cell element
- An academic field that seeks to integrate high-throughput biological studies to understand how biological systems function. By studying the relationships and interactions between various parts of a biological system (e.g. metabolic pathways, organelles, cells, physiological systems, organisms etc.) it is hoped that eventually an understandable model of the whole system can be developed.
- The study of the dynamic networks of interacting biological elements.
- The search for the syntax of biological information
- (syntaxe= skladba, vztahy skladebných prvků jazyka)
- Application of many disciplines to bring together information from the smallest units of the biological system (genes) to understand the function of the whole organism

Transcriptional regulatory network of *C. glutamicum*

- Corynebacterium glutamicum: prediction of 3002 protein-coding genes within the genome.
- Bioinformatics: 158 genes for proteins that act as transcriptional regulators of gene expression.
- DNA-binding transcriptional regulators
- General functions: sigma factors

- Basic components crucial to reconstruct the regulatory interactions in a bacterial cell:
- 1. DNA-binding transcriptional regulators
- 2. DNA-binding sites (operators) of the regulatory proteins in the genome sequence
- 3. Regulated target genes

Transcriptional regulators in the sequenced *Corynebacterium* genomes

Feature	*C. glutamicum*	*C. efficiens*	*C. diphtheriae*	*C. jeikeium*
	Industrial amino acid producer	Potential amino acid producer	Human pathogen (diphtheria)	Multiresistant nosocomial pathogen
Genome size	3.3 Mb	3.1 Mb	2.5 Mb	2.5 Mb
Number of coding sequences	3,002	2,950	2,320	2,104
Number of potential regulators	158	131	91	82
Percentageof potential regulators	5.3 %	4.4 %	3.9 %	3.9 %

Brune, et al., BMC Genomics 6, 86-95, (2005)

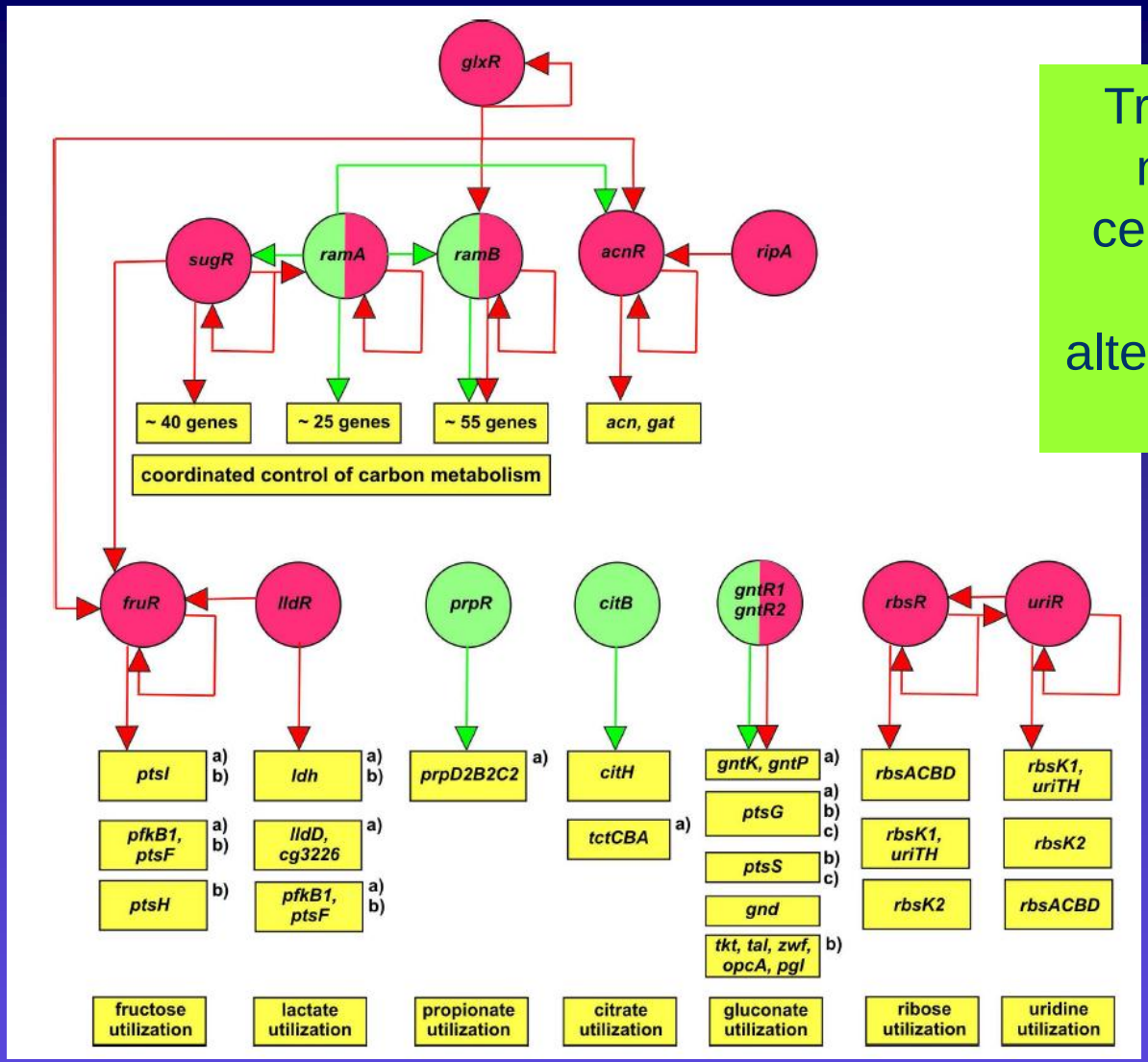
The transcriptional regulatory network model of *C. glutamicum*

The expression of many *C. glutamicum* genes is apparently controlled by coregulation:

158 genes are regulated by 2 TRs
46 genes by 3 TRs
15 genes by 4 or 5 TRs

Coregulation of gene expression is a prominent design principle in the transcriptional regulatory network of C. glutamicum

3 architectural types of TRs:
1) Local regulators – control small number of genes
2) Master regulators – a number of functionally related genes that belong to a corresponding functional module
3) Global regulators – control directly or indirectly large number of genes by hierachical cross-regulations (>20)



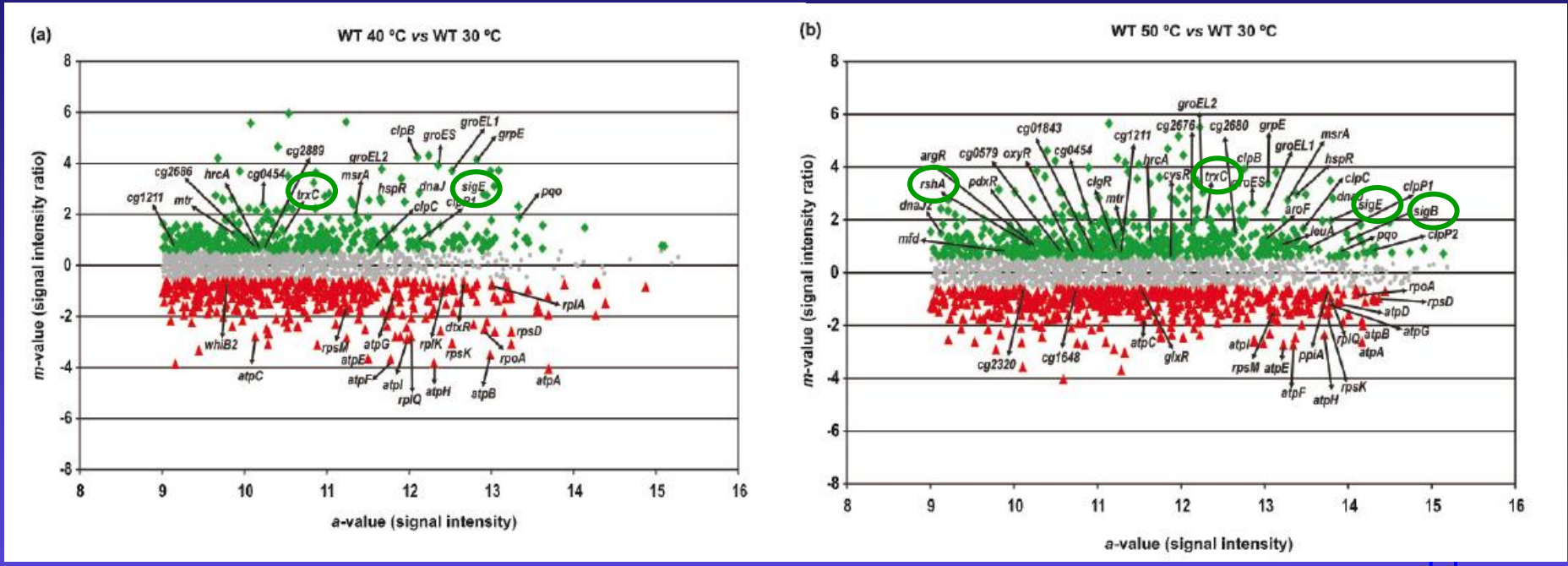
Transcriptional regulatory network controlling the central carbon metabolism and the utilization of alternative carbon sources in C. glutamicum.

GlxR protein: the only known global regulatory TR in *C. glutamicum*.
GlxR might be involved in direct transcriptional control of about 14% of the predicted genes of *C. glutamicum,* including 25 genes for TRs

Red node=repressor; green node=activator; green-red node=dual regulator; red line=repression; green line= activation. Several genes or operons controlled by a local regulator are moreover coregulated by the global regulator GlxR (a) and the master regulators SugR (b) and RamB (c).

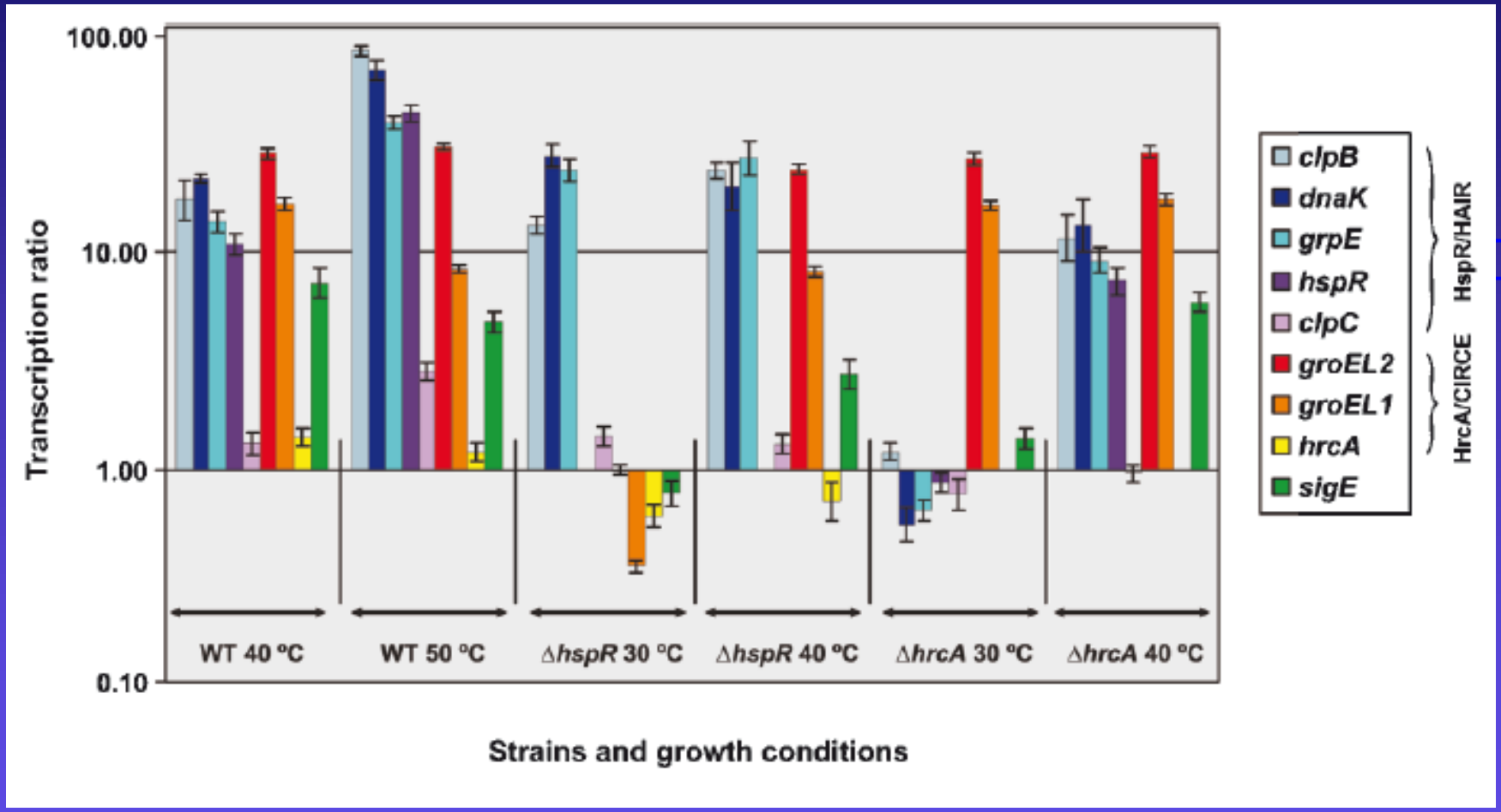
Microarray transcriptome profiling 1

- Ratio - intensity plot. Heatshock: a) 40 °C 60 min, b) 50 °C 10 min



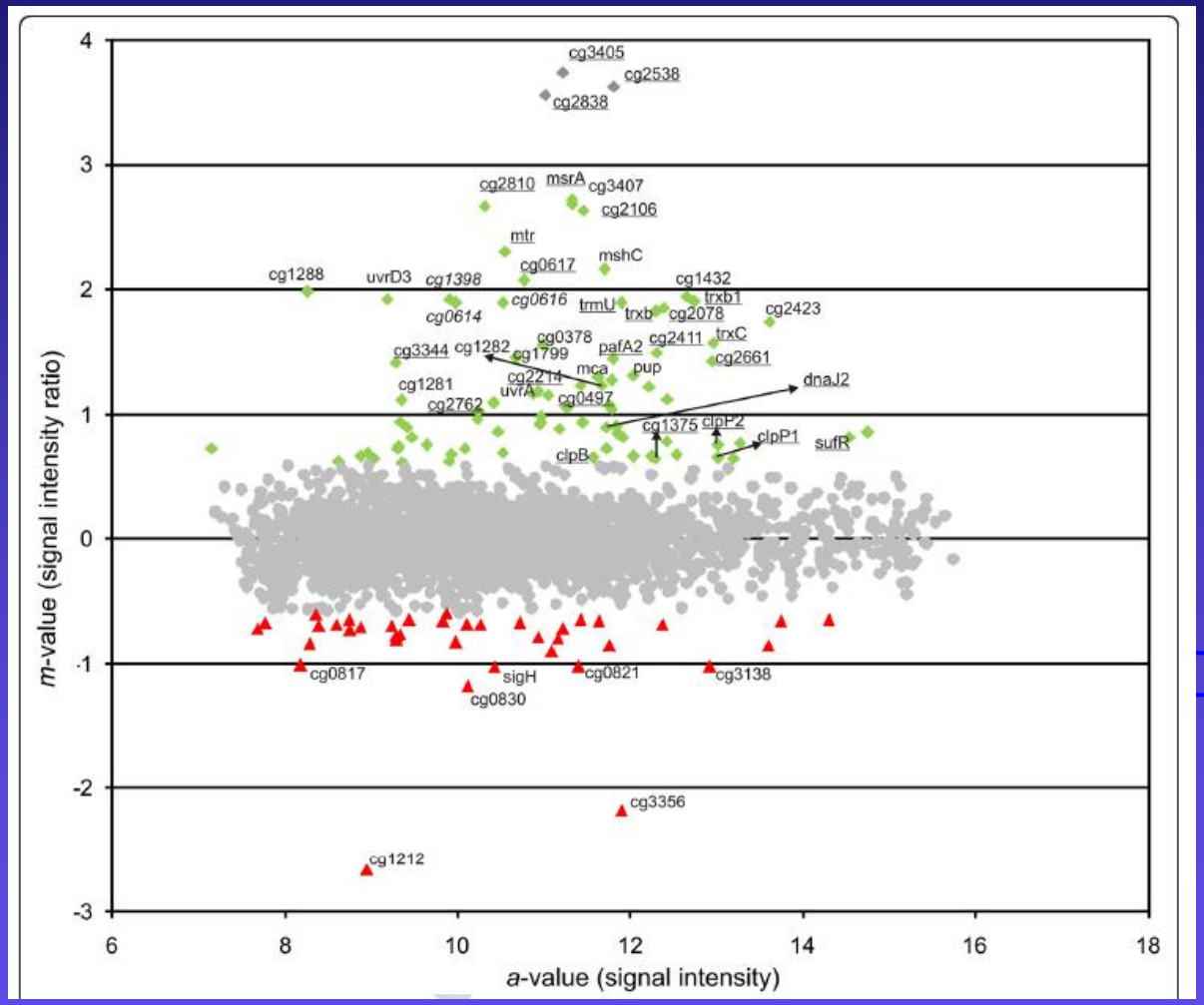
- Barreiro et al. 2009

Real time RT PCR (qPCR)



Microarray transcriptome profiling 2

- **Sequence Ratio: WT / delta *rshA* (anti-SigH) (Busche et al. 2012)**



- **SigH - first alternative sigma with defined promoter consensus SQ**

Real time RT PCR (qPCR) Δ*rshA* strain

- **SigH-dependent promoters: increased activity in the** Δ*rshA* strain

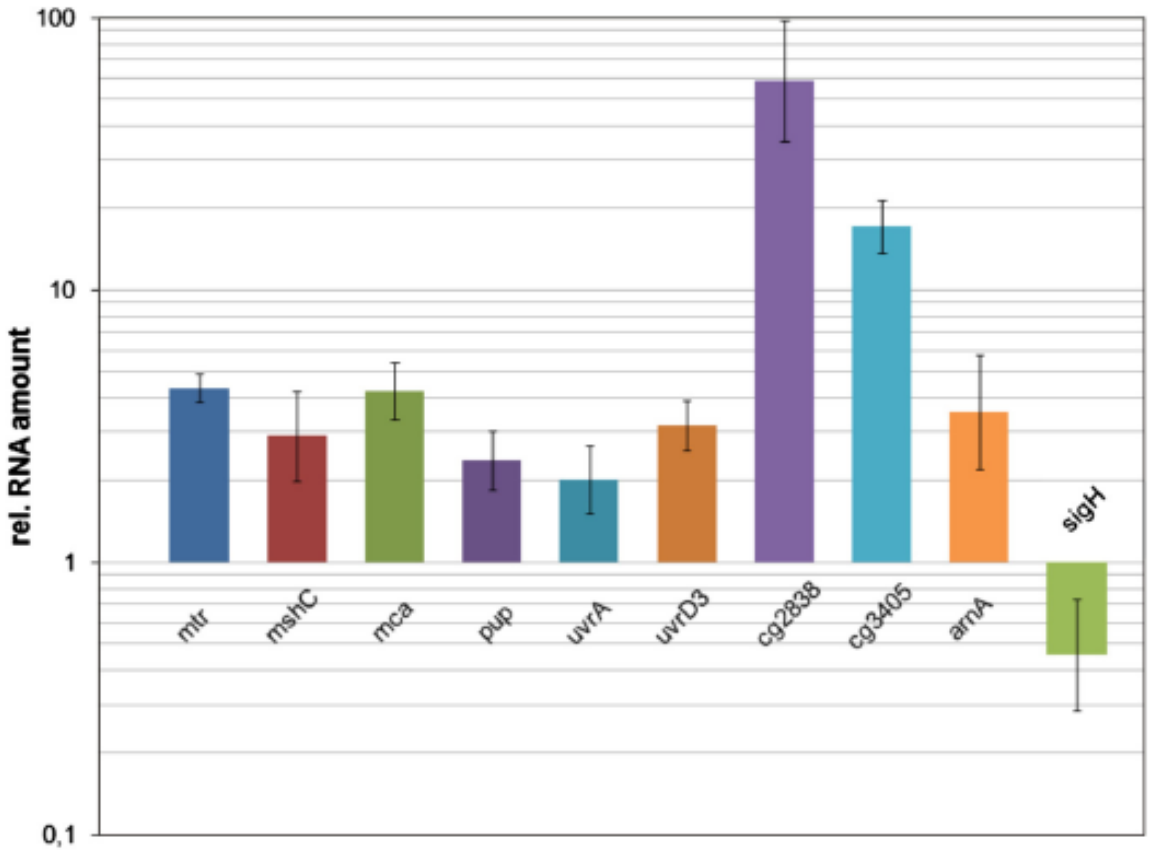
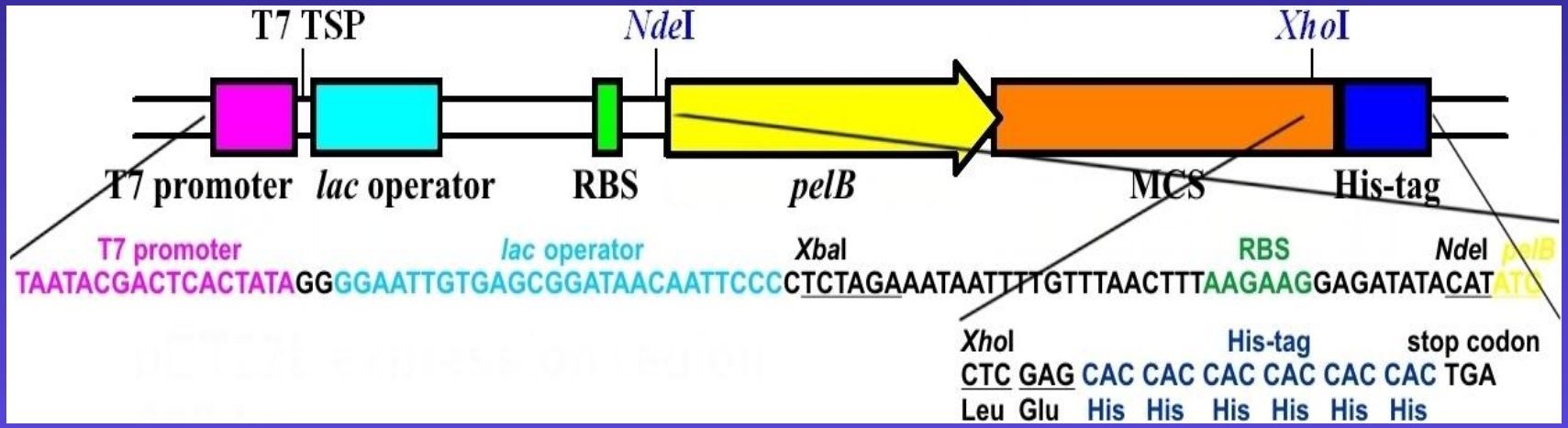


Figure 4 Relative transcript levels of selected potential SigH-dependent genes in *C. glutamicum* Δ*rshA*/*C. glutamicum* RES167 measured by q-RT-PCR. The data obtained for the RES167 strain served as a reference and the respective values were set to 1.0 on the logarithmic scale. Three biological replicates for the Δ*rshA* strain and four replicates for the RES167 strain were analysed in duplicate. SD values are shown as error bars.

- **Further studied promoters: P*mshC*, P*mca*, P*uvrA*, P*uvrD3* (P*arnA*?)**

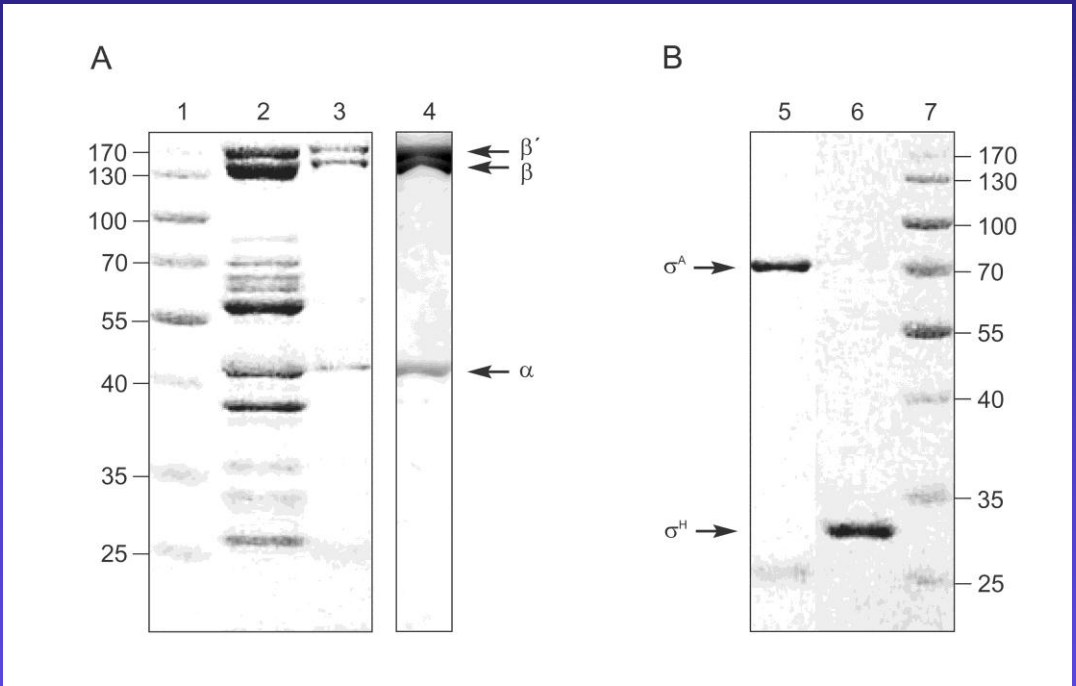
Construction of in-vitro transcription system of *C. glutamicum*

- **1. Cloning of the sigA and sigH genes in the pET22b vector and isolation of the sigma factors from *Escherichia coli* DE3 (IPTG-inducible overexpression) by affinity chromatography on Ni-beads**

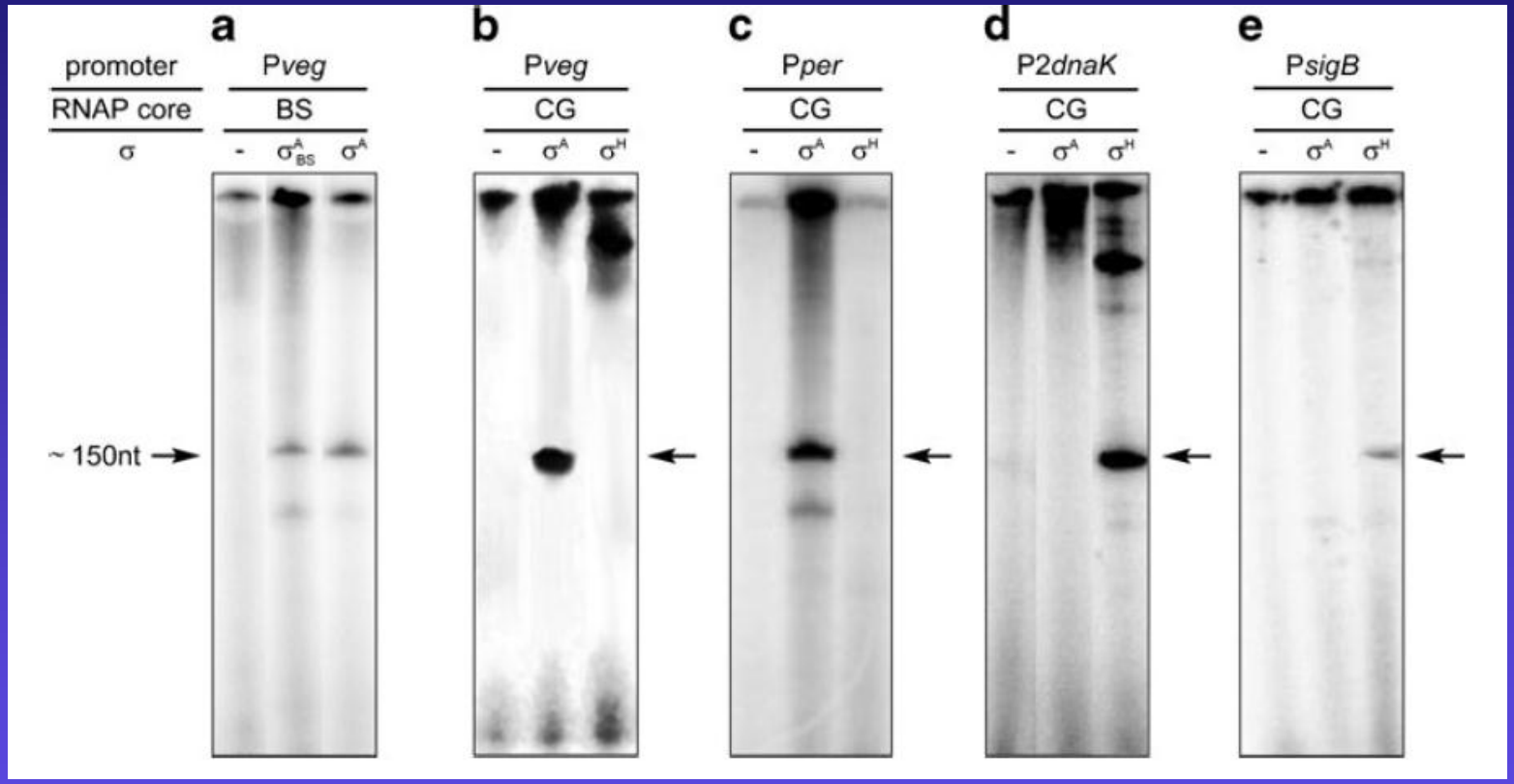


2. Isolation of core RNA polymerase (RNAP): construction of *C. glutamicum* strain expressing beta‘ subunit (RpoC) of RNAP carrying C-terminal His-tag and purification by affinity chromatography on Ni-beads

Isolation and purification of RNA polymerase from *C. glutamicum*

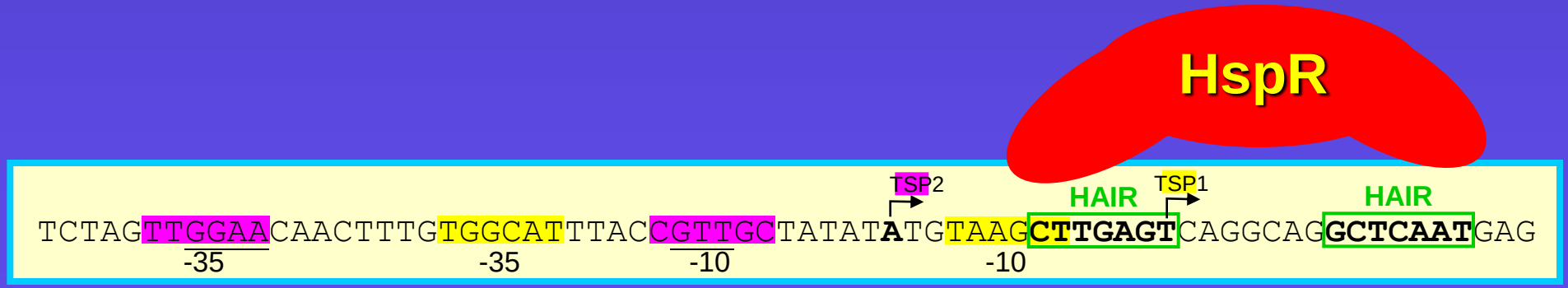


Use of in-vitro transcription system for *C. glutamicum*

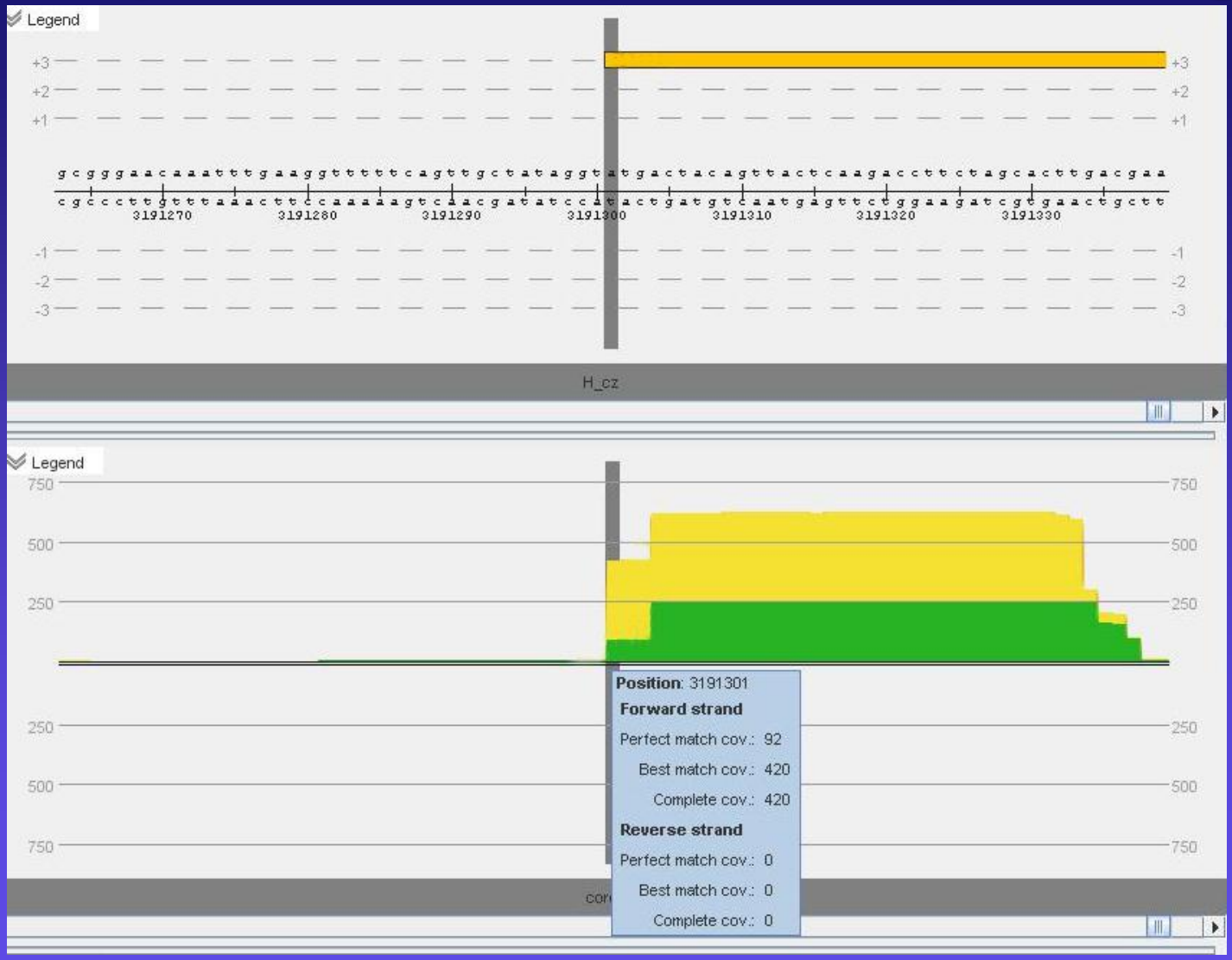


Promoter P*dnaK*

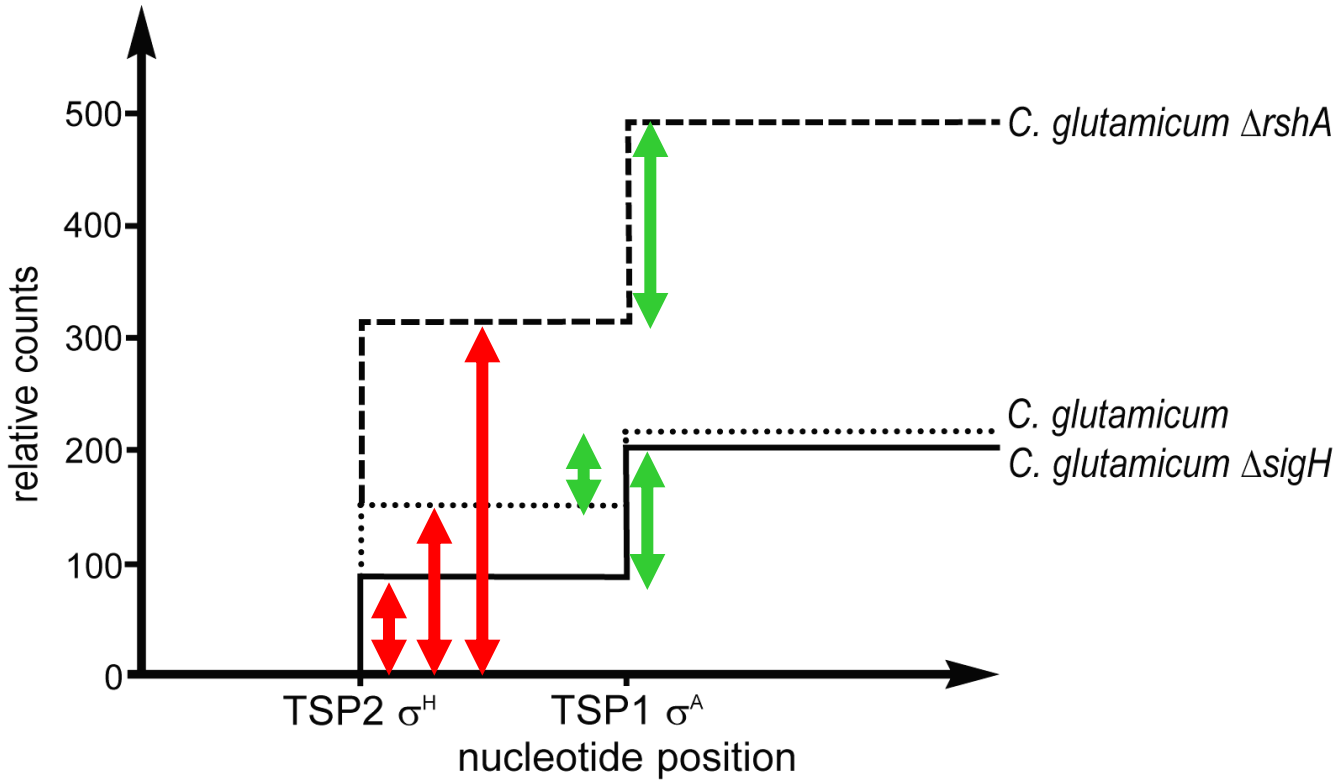
DnaK = chaperone
Heat shock induced promoter
2 promoters:P1=sigma A, P2=sigma H/E, dependent
Repressed by HspR
In vitro transcription P2: RNAP+σ^H, RNAP+σ^E
RNA sequencing



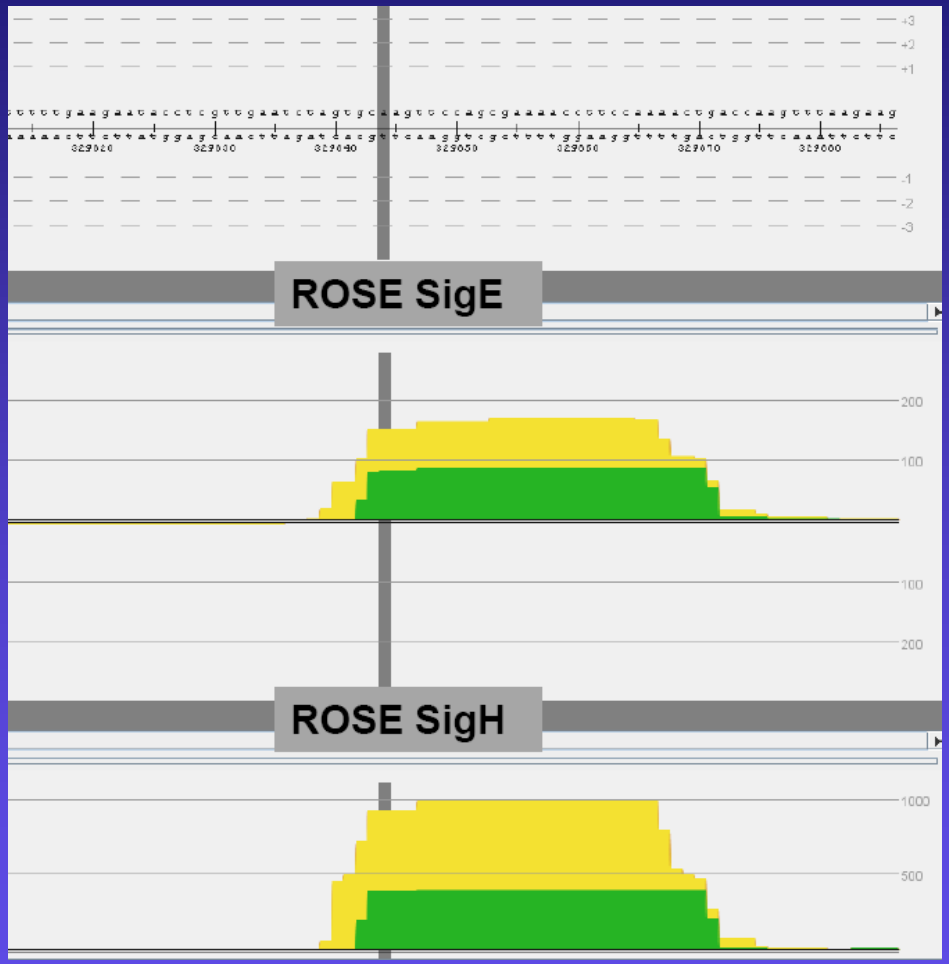
P1*dnaK*, P2*dnaK*: TSPs based on complete transcriptome sequencing 1



P1*dnaK*, P2*dnaK* TSPs based on complete transcriptome sequencing 2



In vitro transcription + specific sigma + RNA sequencing



Confirmed:

P*sigB* **GGAAC - 19 nt - GTT**
P*dnaK* **GGAAC - 19 nt - GTT**
P*sigA* **GGAAC - 18 nt - GTT**
P*cg0378* **GGAAC - 19 nt - GTT**
P*cg3344* **GGAAC - 19 nt - GTT**

Confirmed by *in vitro* transcription:

P*sigB*
P*dnaK*

SigH+SigE–dependent promoters

Pcg0211
Pcg1671
P2dnaJ2
PuvrA
PclgR

SigE–dependent promoters:

Pcg1595
Pcg0029
Pcg1121
Pcg1675

Pcg1671
Pcg2152
PcseA

SigH–dependent promoters

mca, uvrD, mshC, pup, rshA, clpB, arnA

Difference between consensus sequence for RNAP+σ^H and RNAP+σ^E

1) Comparisons of promoter sequences

σ^H-specific
σ^E-specific
σ^H + σ^E-specific

2) Mutagenesis:
GGAAC - 19 nt – GTT ⟶ GGAAT - 19 nt – GTT

σ^H-specific: GGAAT - 19 nt – GTT
σ^E-specific: GGAACnnAnTT- 13 nt – GTT