Eat Well, Live Well.

AJINOMOTO

AGRI

Ajinomoto-Genetika Research Institute

Combination of novel technologies with traditional strategies for microbial production of amino acids and related desirable compounds

Ajinomoto-Genetika Research Institute, Moscow, Russia

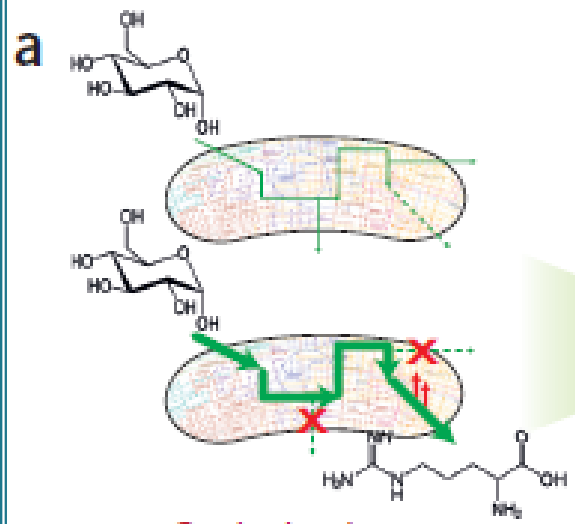
Nataliya V. Stoynova

October 04, 2018

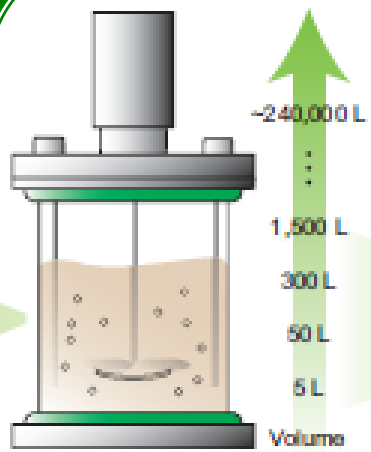
Amino acids production and usage

L-Glu	Fermentation	Food-use
DL-Met	Chemical synthesis	Food/Feed-use
L-Lys	Fermentation	Feed-use
Gly	Chemical synthesis	Pharmaceutical, food-use
L-Phe	Fermentation, enzymatic synthesis	Food-use, amino acid-based sweeteners
L-Asp	Enzymatic synthesis	Amino acid-based sweeteners
L-Thr	Fermentation	Feed-use
L-Cys	Extraction, chemical synthesis, fermentation	Pharmaceutical, food-use
DL-Ala	Chemical synthesis	Food-use, amino acid-based sweeteners
L-Arg	Fermentation	Pharmaceutical, food-use
L-Trp	Fermentation, enzymatic synthesis	Pharmaceutical, feed-use
L-Val	Fermentation	Pharmaceutical, food-use, feed-use
L-Leu	Fermentation	Pharmaceutical, food-use
L-Ile	Fermentation	Pharmaceutical, feed-use
L-His	Fermentation	Pharmaceutical, feed-use
L-Pro	Fermentation	Pharmaceutical
L-Ser	Fermentation	Pharmaceutical
L-Tyr	Extraction	Pharmaceutical

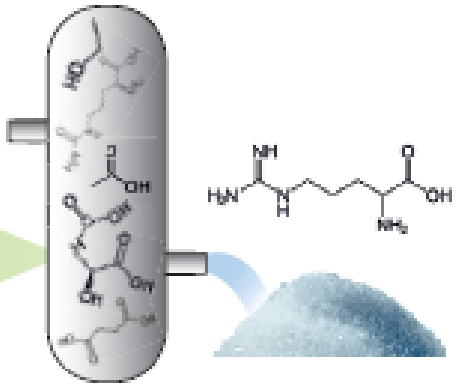
adapted from Nature Biotechnol 2015, 33:1061–1072



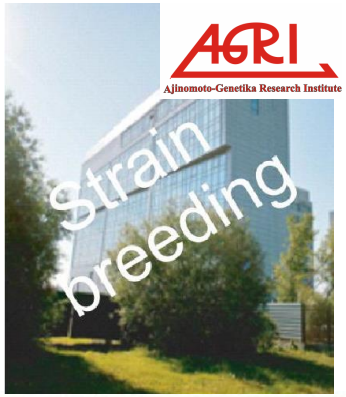
Strain development
(upstream process)



Fermentation
(midstream process)



Separation & purification
(downstream process)

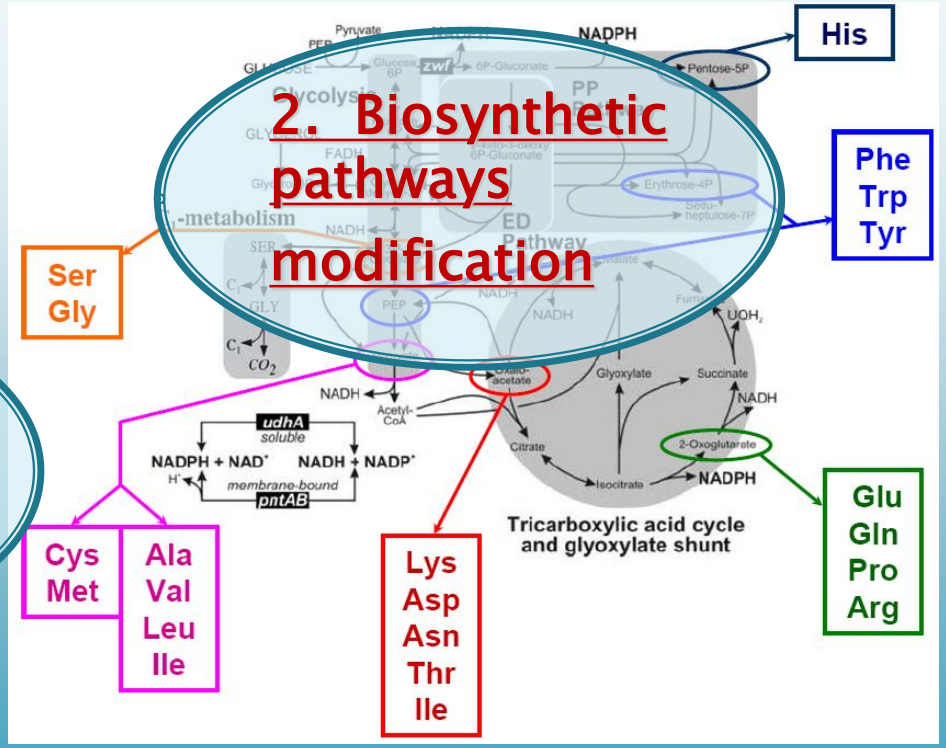


Ajinomoto-Genetika Research Institute (AGRI): R&D on fermentative microbes for amino acids, nucleotides and other products by means of traditional (pathway focused) metabolic engineering strategies and corresponding efficient tools along with omics-based and evolutionary approaches.

Rational design | Random editing | HTP screening | HTP analysis
Adaptive evolution | Genome analysis | Transcriptome analysis
Traditional selection | Molecular modeling | Genome editing tools | Cultivation optimization

Some aspects of AA-producing strains breeding

Substrates influx: C, N, O_2, P, S, etc



1. Host selection

2. Biosynthetic pathways modification

3. AA export

4. Substrate: transport, etc

5. Adaptation to CP scale

in

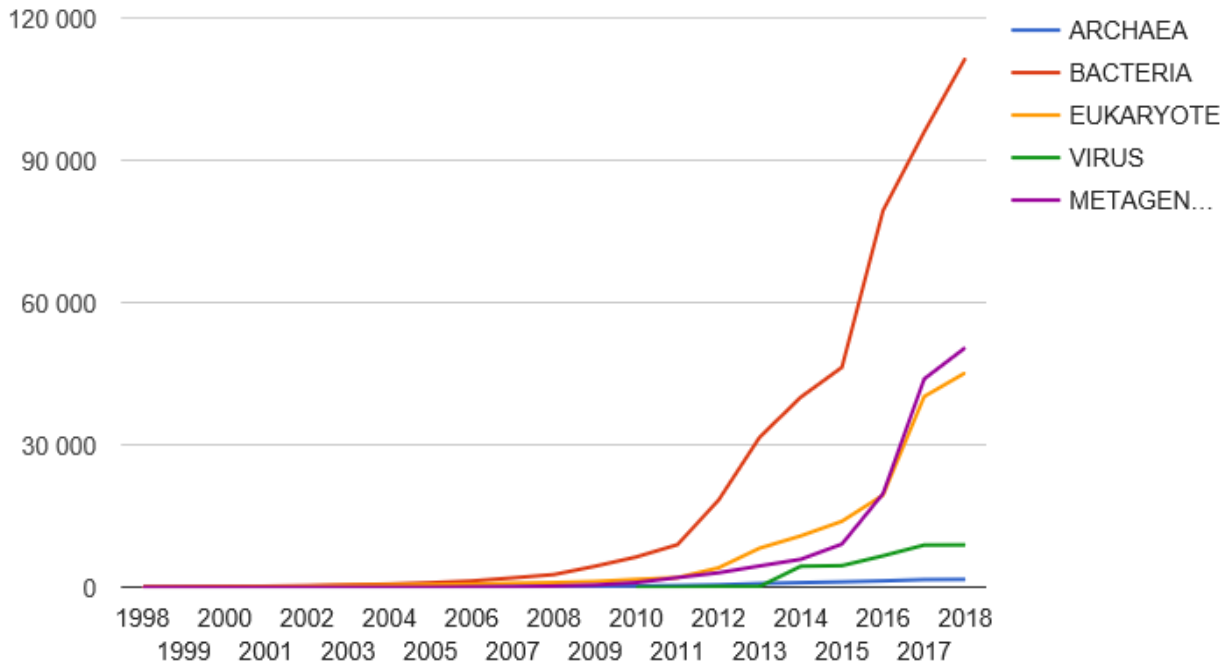
out

Efflux of target amino acid

1. Host selection

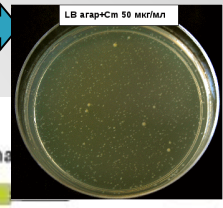
C. glutamicum, *E. coli* – traditional hosts for microbial synthesis of AA and their derivatives.
But now …..

Genome sequencing projects

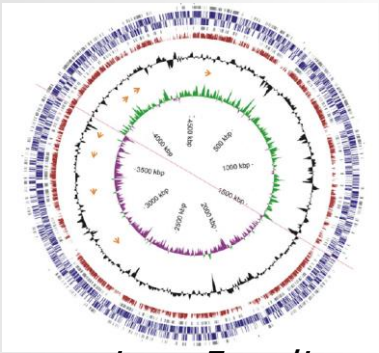


adapted from Genome OnLine Database

Host strain: an example of workable alternative



Pantoea ananatis: Gram-negative acidophilic bacterium belonging to the family *Enterobacteriaceae,* **capable of growing on a variety of sugars at acidic and neutral pH; resistant to L-Glu**



P. ananatis vs *E. coli*

Genome annotation

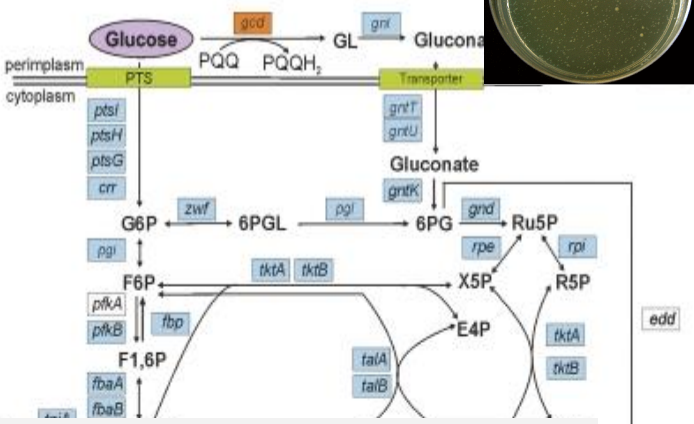
Hara Y, Kadotani N, Izui H, Katashkina JI, Kuvaeva TM, Andreeva IG, Golubeva LI, Malko DB, Makeev VJ, Mashko SV, Kozlov YI.

Appl Microbiol Biotechnol. 2012 Jan;93(1):331-41.

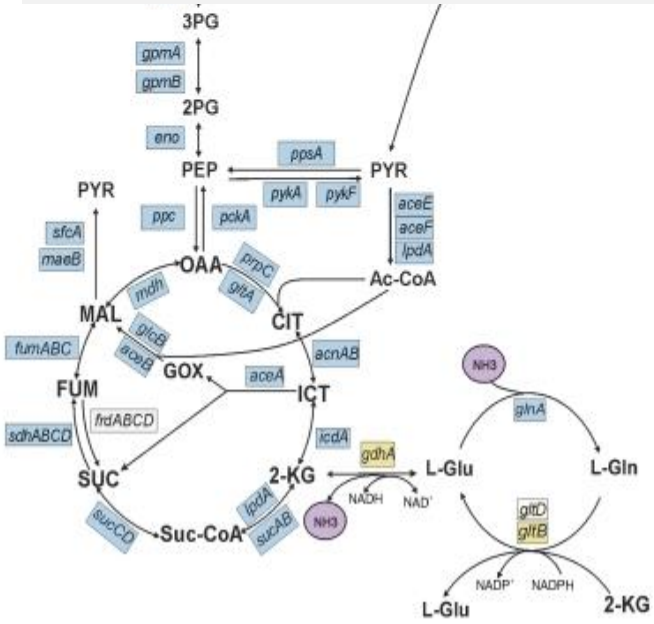
The complete genome sequence of Pantoea ananatis AJ13355, an organism with great biotechnological potential.

Genetic tools were developed

(Katashkina et al, 2009; Andreeva et al, 2011)

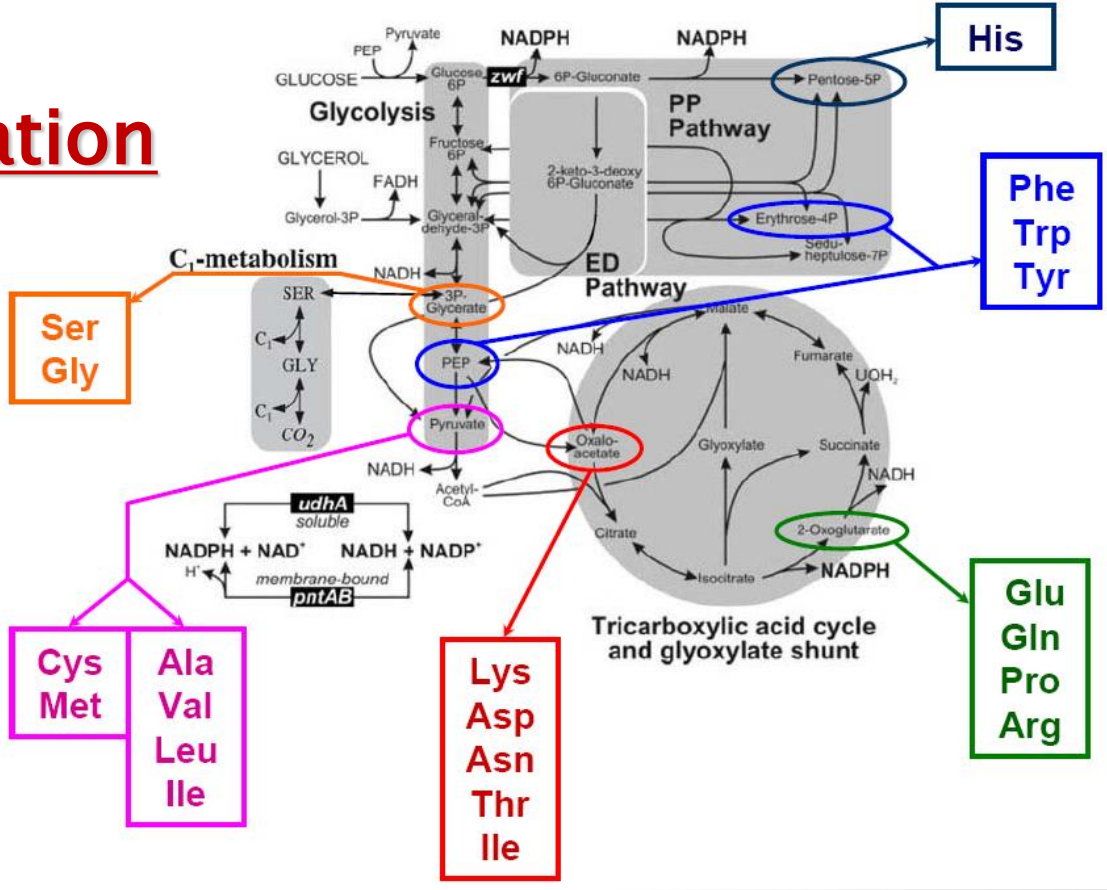


Genome-scale metabolic reconstruction



2. Biosynthetic pathways modification

- Feedback-resistant key enzymes
- Terminal pathway enhancement
- Blocking of degradation



Central metabolism perturbations to provide the target biosynthetic pathway with precursors, cofactors, etc

Examples

Desensitization to feedback inhibition: SAT example (I)

Protein Engineering, Design & Selection vol. 19 no. 4 pp. 163–167, 2006

Engineering of *Escherichia coli* L-serine O-acetyltransferase on the basis of crystal structure: desensitization to feedback inhibition by L-cysteine

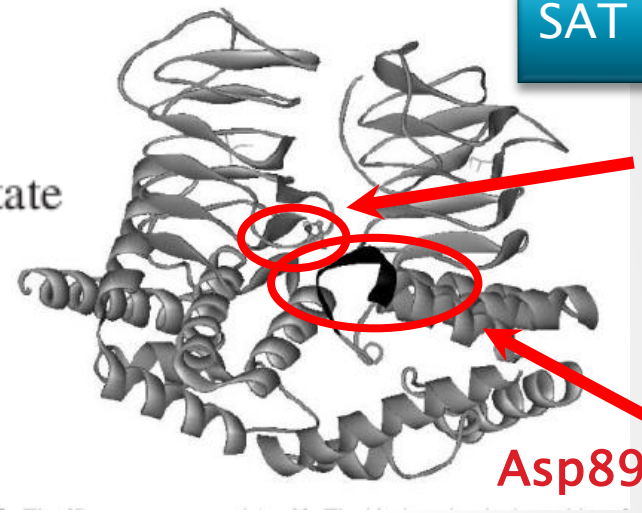
Y.Kai[1], T.Kashiwagi[1], K.Ishikawa[1], M.K.Ziyatdinov[2], E.I.Redkina[2], M.Y.Kiriukhin[2], M.M.Gusyatiner[2], S.Kobayashi[3], H.Takagi[3] and E.Suzuki[1,4]

SAT

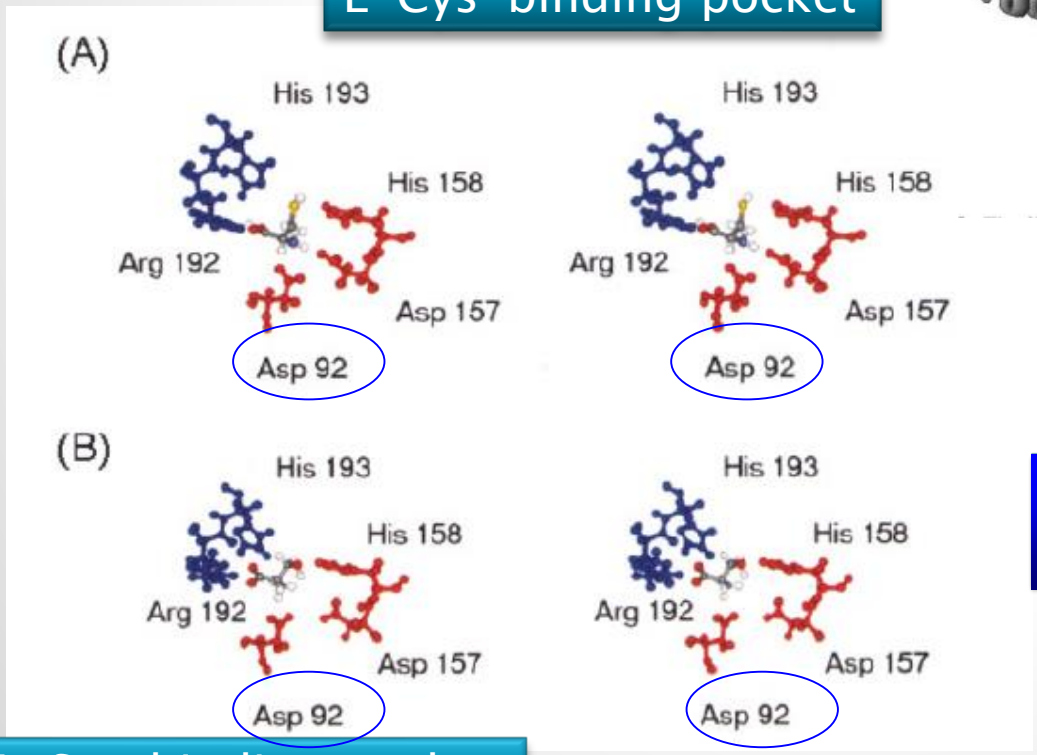
L-serine + acetyl-CoA → *O*-acetyl-L-serine + CoA

O-acetyl-L-serine + sulfide → L-cysteine + acetate

(−)



L-Cys-binding pocket



Docking study

Randomized fragment-directed mutagenesis

CysE mutants isolation

Desensitization to feedback inhibition: SAT example (II)

Table I. Comparison of the mutants activity and sensitivity to L-Cys inhibition

CysE gene on the plasmid	Specific activity, μM/min/mg	IC_{50}, μM	K_i, μM*
CysE wild-type	1680	0.8	0.6
CysE 256	1067	18.0	14.5
CysE 5	715	1100.0	950.0
CysE 12	1440	125.0	114.0
CysE 15	1470	550.0	510.0
CysE 1	1600	460.0	420.0
CysE 142	1220	20.0	15.0
CysE 10	2692	4.7	3.4
CysE 11-2	1900	410.0	395.0
CysE 15-2	1100	6.0	4.5

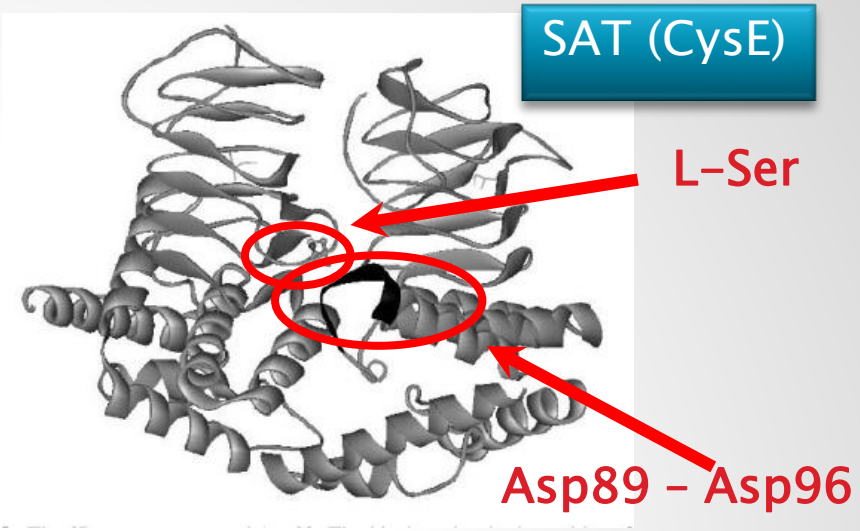


Table II. The mutants' sequence*

Allele of the *cysE* gene	Randomized sequence of the SAT protein (at positions 89–96)
CysE wild-type	Arg Thr Arg Asp Pro Ala Val Asp
CysE5	Arg Thr Arg Asp Pro Ala Arg Pro
CysE12	Arg Thr Arg Asp Pro Ala Gly Gly
CysE15	Arg Thr Arg Asp Pro Ala Leu Pro
CysE1	Pro Thr Arg Asp Pro Ala Val Asp
CysE142	Ser Leu Arg Asp Pro Ala Val Asp
CysE10	Arg Thr Arg Asp Pro Thr Val Asp
*CysE*11-2	His Val Arg Asp Ala Thr Val Asp
*CysE*15-2	Thr Arg Arg Asp Pro Ala Val Asp

Table III. Comparison of the catalytic properties with those by the previous studies

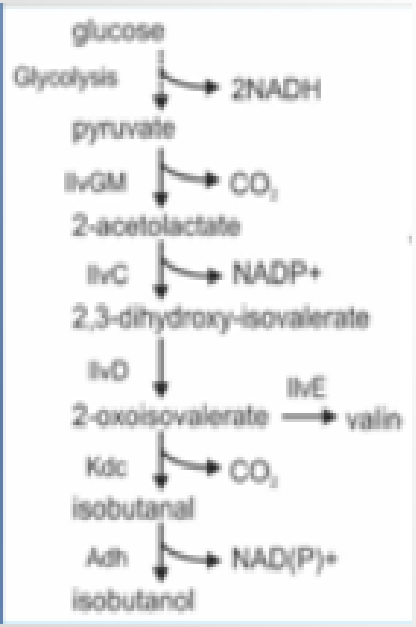
SAT	Activity versus *Escherichia coli* wild-type (%)	Relative activity for 100 μM L-cysteine added (%)
Arabidopsis thaliana SAT-m (Takagi *et al.*, 1999a)	1.2	100
pCEM256T (Nakamori *et al.*, 1998)	50.1	18.6
*CysE*5	42.5	100
*CysE*12	85.7	54.4
*CysE*15	87.5	89.3
*CysE*1	95.2	87.5
*CysE*142	72.6	31.6
*CysE*10	160.2	<10
*CysE*11-2	113.1	92.1
*CysE*15-2	65.5	<10

As a result: SATs with high enzymatic activity and extreme insensitivity to inhibition by L-Cys.

Gene engineering approaches for direct modification of metabolic pathways

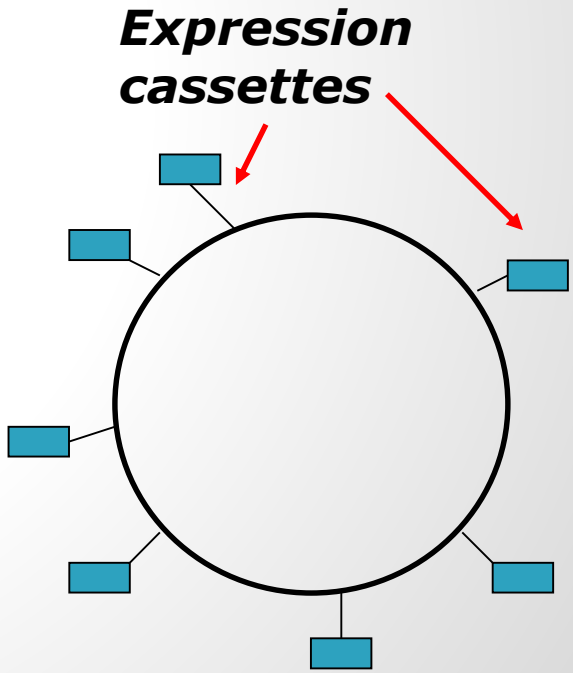
Requirements to strains exploited for large-scale fermentation processes nowadays: no plasmids, no antibiotic resistance genes, complete information about all genome modifications should be submitted.

Genetic tools should be developed and/or adapted, accordingly (*E. coli*, *M. methylotrophus*, *C. glutamicum*, *B. subtilis*, *B. amyloliquefaciens*, *P. ananatis*, etc).



Metabolic pathway

Tasks: controlled enhancement of the expression of genes encoding enzymes of target biosynthesis or central metabolism; attenuation of undesirable pathways.



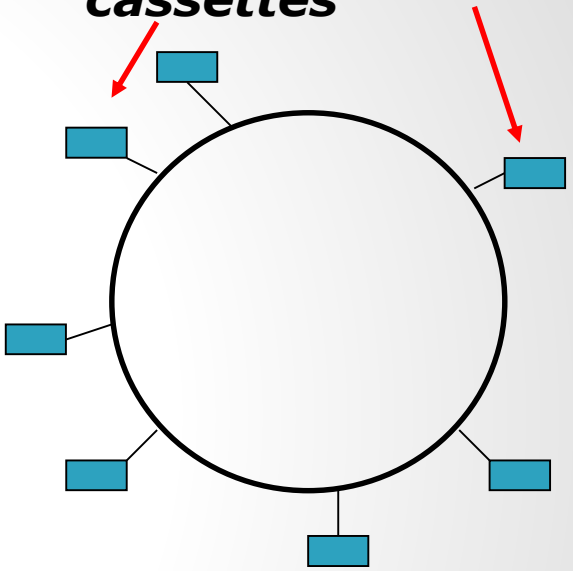
Chromosome of a producer strain

Mini-Mu-based random integration for construction of plasmid-less and marker-less AA-producing strains with completely known chromosomal structure

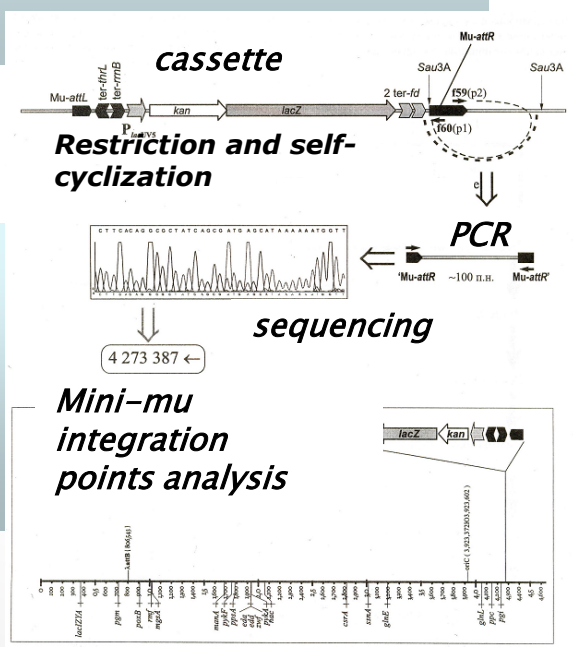
On the basis of phage-transposon Mu, an effective dual-component integration system for editing of Gram-negative bacteria genomes was developed.

Transposition and integrative modules are separated that allows effective random insertions of desired DNA fragments into a chromosome and their further amplification.

Mini-Mu expression cassettes



Chromosome of a producer strain

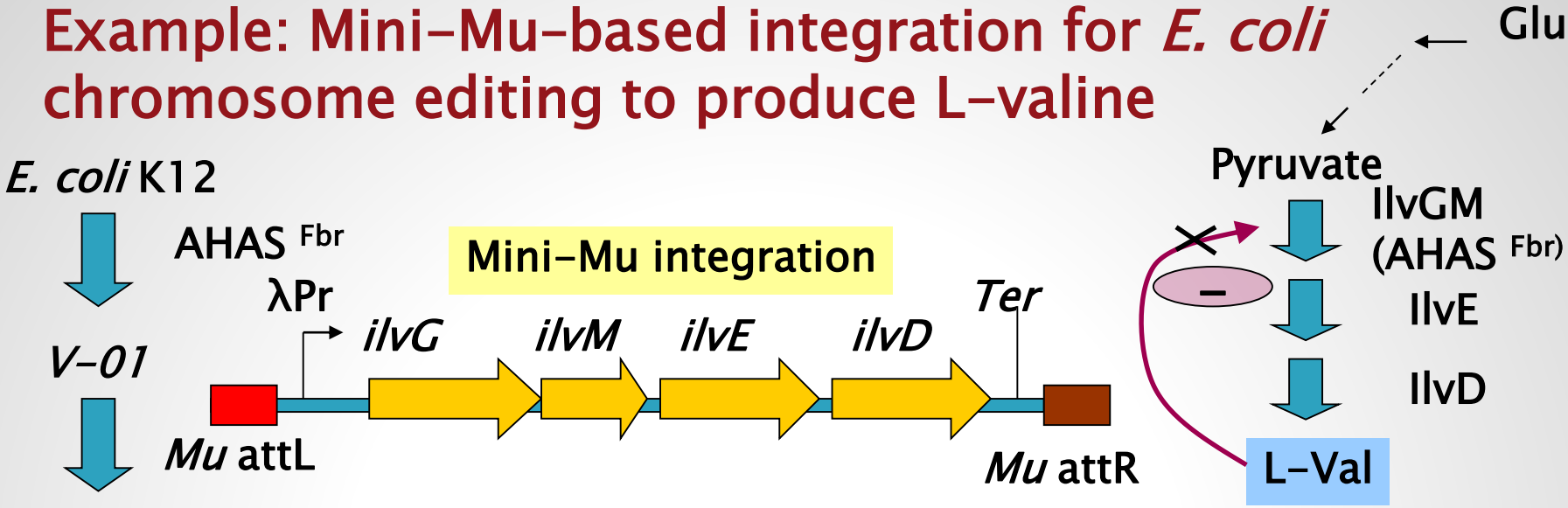


The procedure is followed by CGS or inversed-PCR analysis (Zimenkov et al, 2004) to find out the integration points.

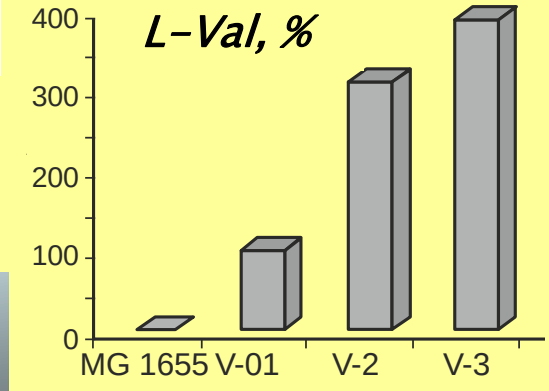
Genome of the final stable plasmid-less multi-integrant may contain transposition-mediated chromosomal rearrangements.

Reviewed in Akhverdyan et al, (2011) Appl Microbiol Biotechnol DOI 10.1007/s00253-011-3416y

Example: Mini-Mu-based integration for *E. coli* chromosome editing to produce L-valine



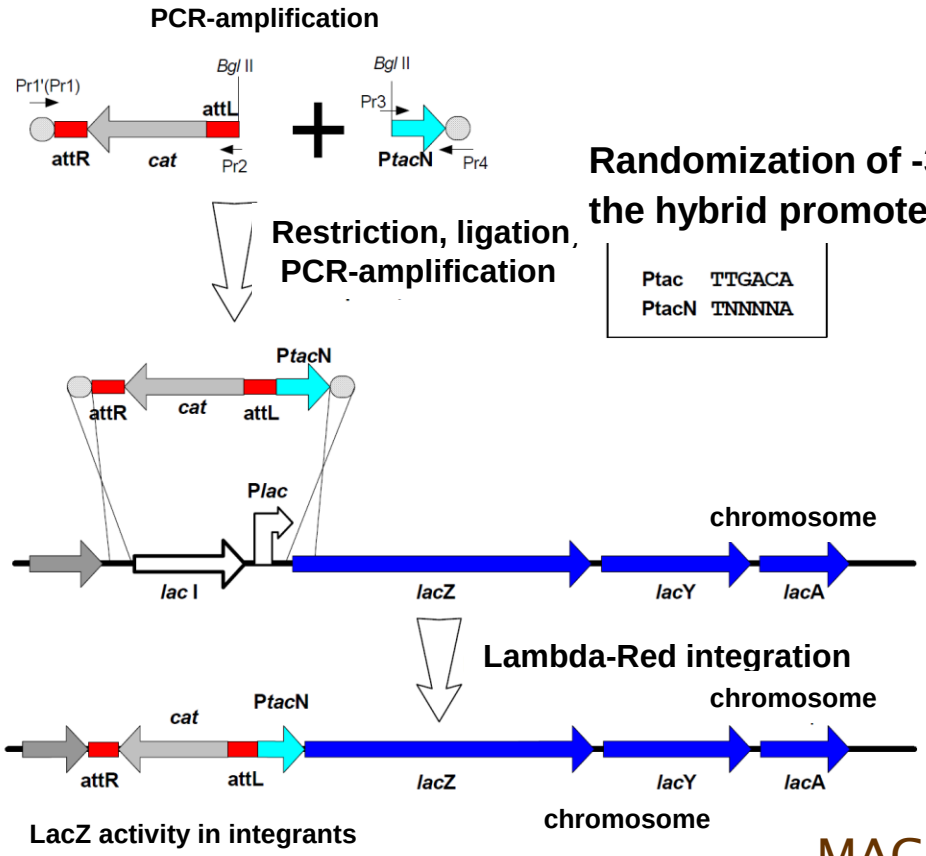
Step-by-step increasing of Val accumulation level



Dual-component integration system allows to amplify pre-existed mini-Mu cassettes.

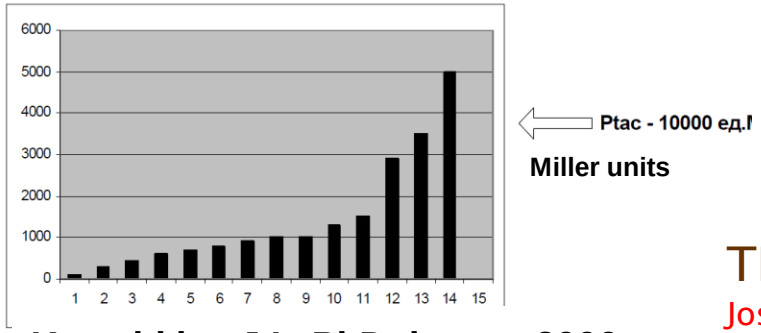
Genome of the final stable plasmid-less multi-integrant may contain transposition-mediated chromosomal rearrangements.

Optimization of genes expression in chromosome



Randomization of -35 of the hybrid promoter Ptac

Ptac	TTGACA
PtacN	TNNNNA



Katashkina J.I., PhD theses, 2006

"Recombineering"-based approaches (dsDNA, ssDNA) for chromosome editing:

- targeted (deletions, precise modifications of regulatory and/or structural parts of genes)

Based on pioneer works of D. Court's Lab, B. Wanner's Lab, etc.

- "multiplex" recombineering methods- MAGE, TRMR, CRMAGE (CRISPR/Cas9-optimized MAGE)

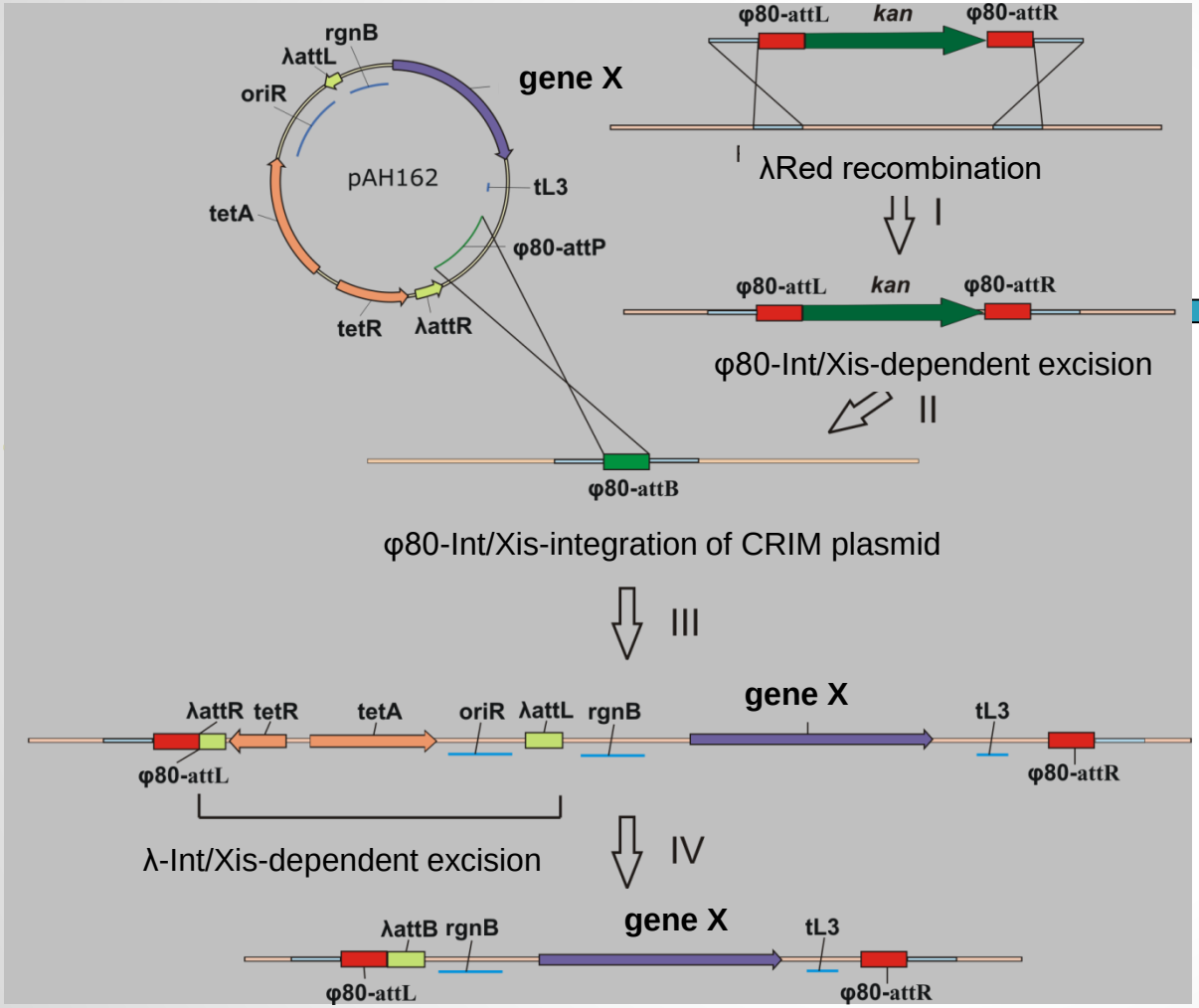
MAGE- (**m**ultiplex **a**utomated **g**enome **e**ngineering)

Harris H. Wang at al. "Programming cells by multiplex genome engineering and accelerated evolution", Nature 460, 894-898 (2009).

TRMR-(**tr**ackable **m**ultiplex **r**ecombineering)

Joseph R. Warner at al. "Rapid profiling of a microbial genome using mixtures of barcoded oligonucleotides", Nature BioteMAGEchnology vol 28, №8, 856-862 (2010).

Site-specific chromosomal integration mediated by bacteriophages recombination systems:

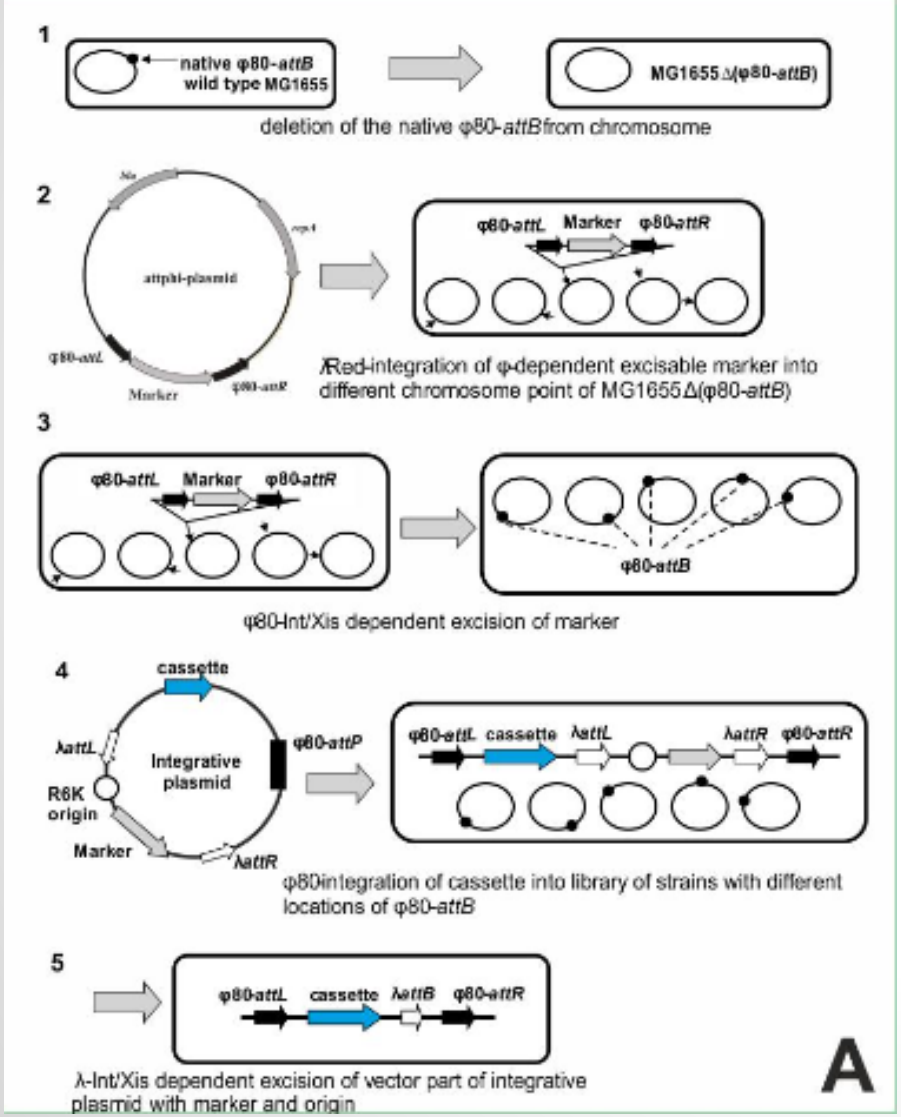


Dual-In/Out method

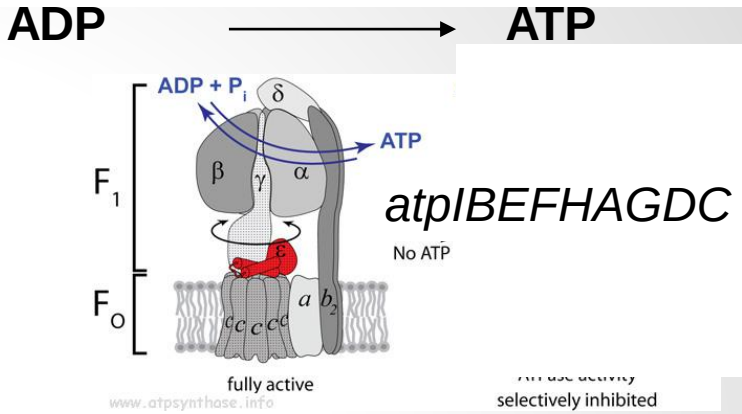
Library of φ80-attB "integration platforms" for locus-specific insertion of target genes was constructed (more than 20 platforms).

Minaeva et al, BMC Biotechnol, 2008

PCR-free cloning and targeted integration of any long DNA fragment



Minaeva et al, BMC Biotechnol, 2008



Ublinskaya et al, (2012) Journal of Microbiological Methods, 89, 167-173

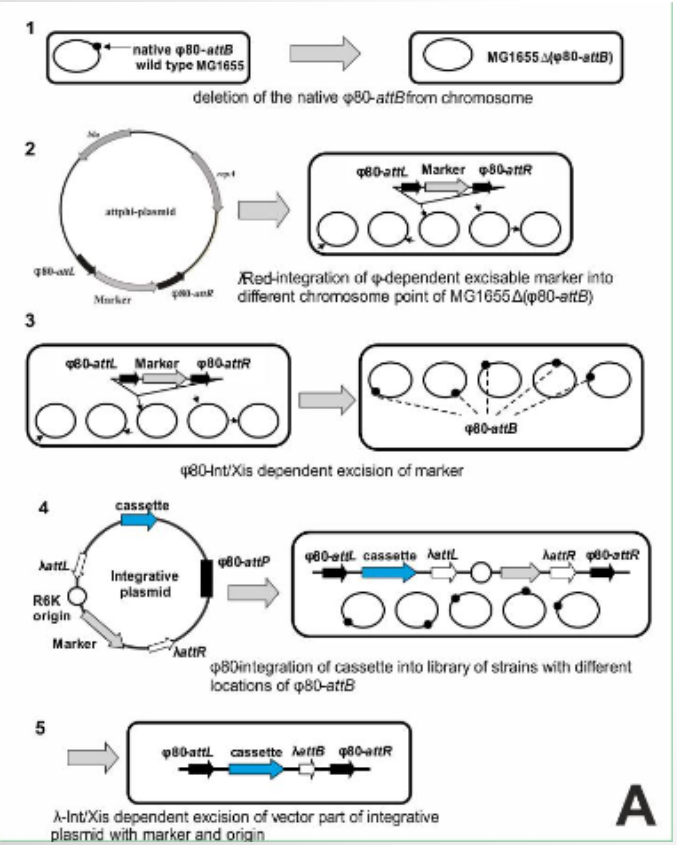
in vitro cloning, I-SceI-based

further developed in

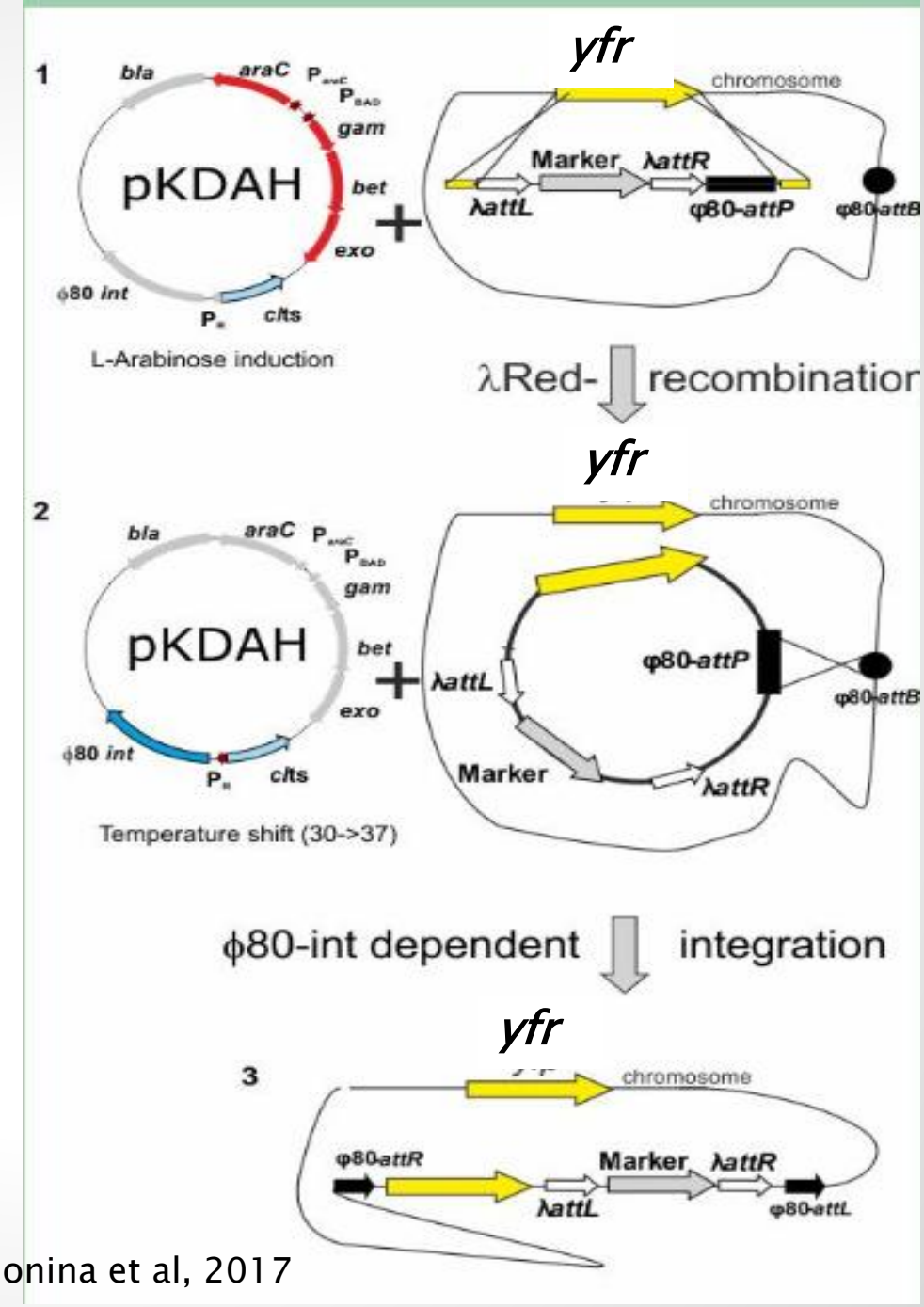
Hook et al, (2016) Journal of Microbiological Methods, 130, 83-91.

in vivo cloning

PCR-free cloning and targeted integration of any long DNA fragment

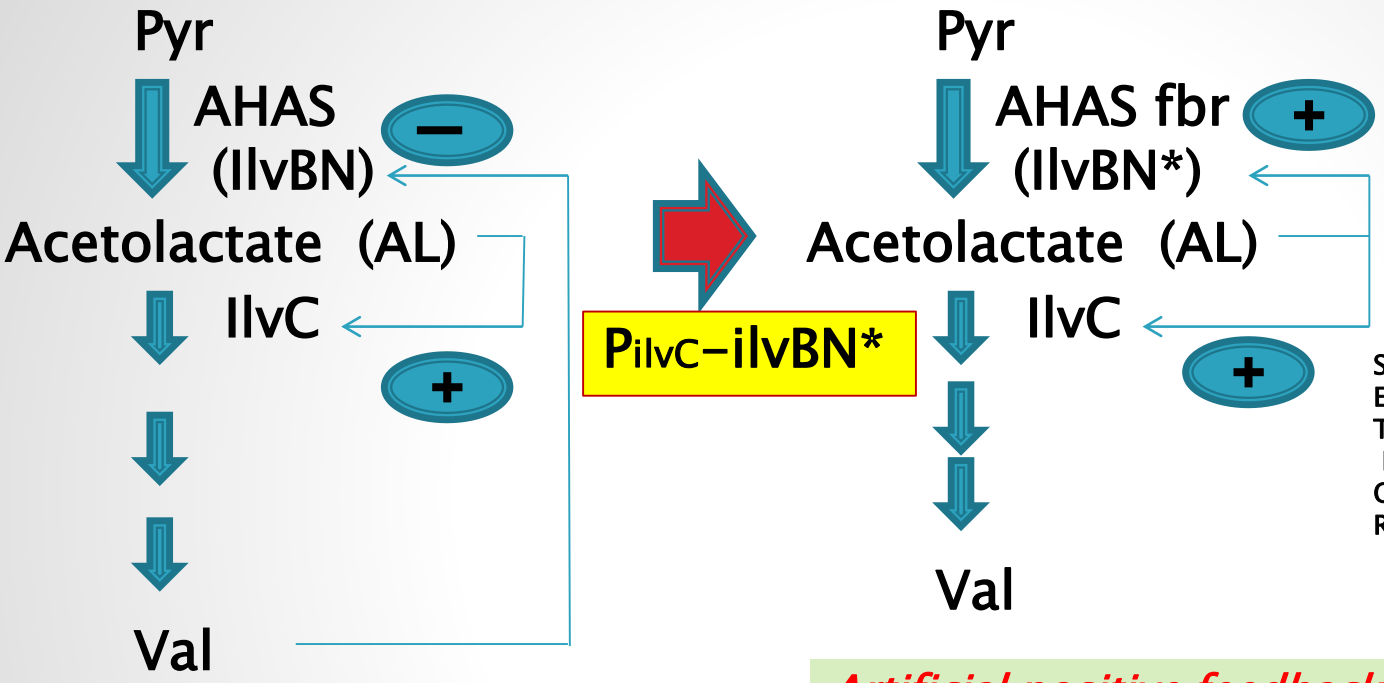


Minaeva et al, BMC Biotechnol, 2008



Igonina et al, 2017

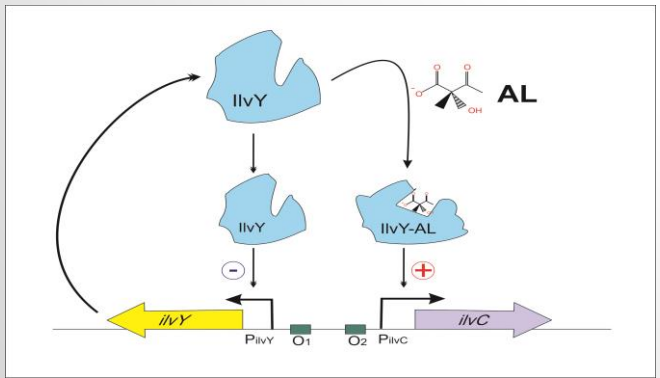
Metabolic regulation: artificial positive feedback loop in branched chain amino acids biosynthesis

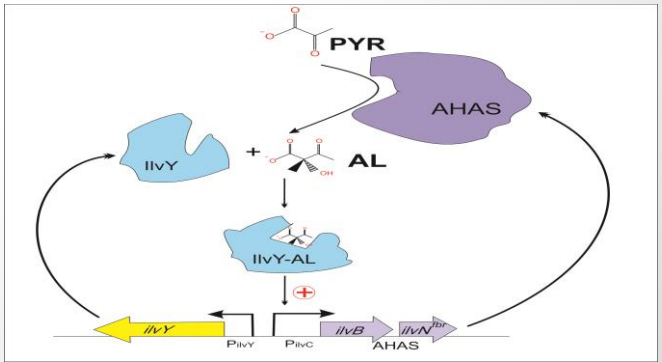


Sycheva et al (2015) AUTO-INDUCIBLE EXPRESSION SYSTEM, AND THE USE THEREOF FOR PRODUCING USEFUL METABOLITES USING A BACTERIUM OF THE FAMILY ENTEROBACTERIACEAE RU2549708, WO2013151174 (A1), etc

wild-type: negative regulation by final product

Artificial positive feedback module: metabolic intermediate (AL) triggers its own synthesis





Phosphoketolase reaction for Glu production – an example of metabolic grafting

JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 103, No. 3, 262–269. 2007
DOI: 10.1263/jbb.103.262

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Innovative Metabolic Pathway Design for Efficient L-Glutamate Production by Suppressing CO_2 Emission

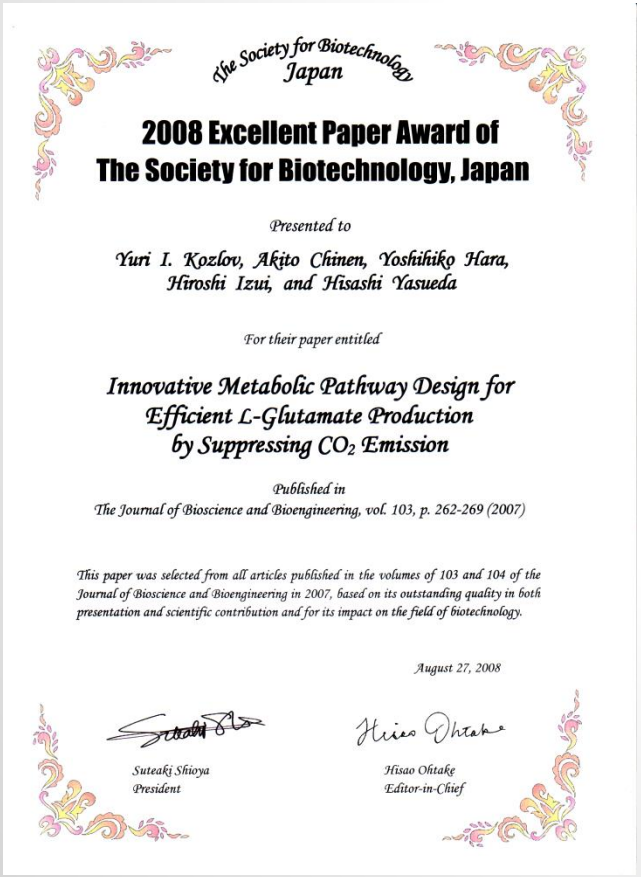
Akito Chinen,[1] Yuri I. Kozlov,[2] Yoshihiko Hara,[1]
Hiroshi Izui,[1] and Hisashi Yasueda[1]*

Fermentation and Biotechnology Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan[1] and Ajinomoto Genetika Research Institute, 1st Dorozhny proezd, 1, Moscow 117545, Russian Federation[2]

Received 13 October 2006/Accepted 23 December 2006

Prof. Yu.I.Kozlov
1944 - 2007

$$\text{Yield (Y)} = \frac{\text{amino acid}}{\text{glucose}}$$



The Society for Biotechnology Japan

2008 Excellent Paper Award of The Society for Biotechnology, Japan

Presented to

Yuri I. Kozlov, Akito Chinen, Yoshihiko Hara, Hiroshi Izui, and Hisashi Yasueda

For their paper entitled

Innovative Metabolic Pathway Design for Efficient L-Glutamate Production by Suppressing CO_2 Emission

Published in
The Journal of Bioscience and Bioengineering, vol. 103, p. 262-269 (2007)

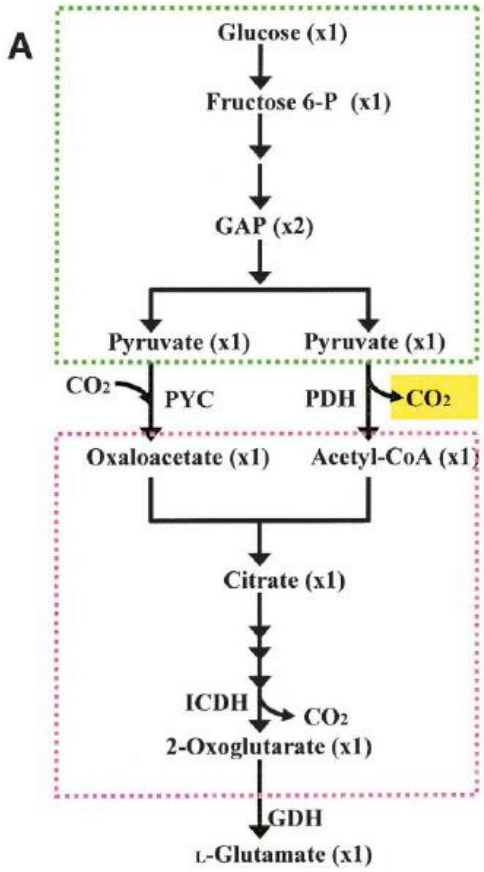
This paper was selected from all articles published in the volumes of 103 and 104 of the Journal of Bioscience and Bioengineering in 2007, based on its outstanding quality in both presentation and scientific contribution and for its impact on the field of biotechnology.

August 27, 2008

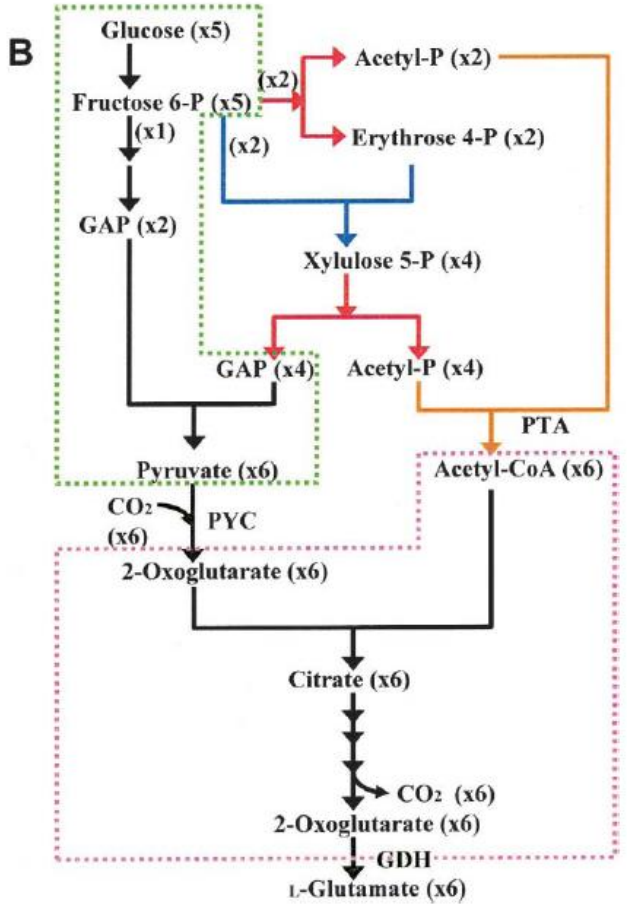
Suteaki Shioya
President

Hisao Ohtake
Editor-in-Chief

Y_{theor} = 81.7%



Y_{theor} = 98.0%



SsrA-like tagged alleles for controlled decreasing of undesirable enzyme activity

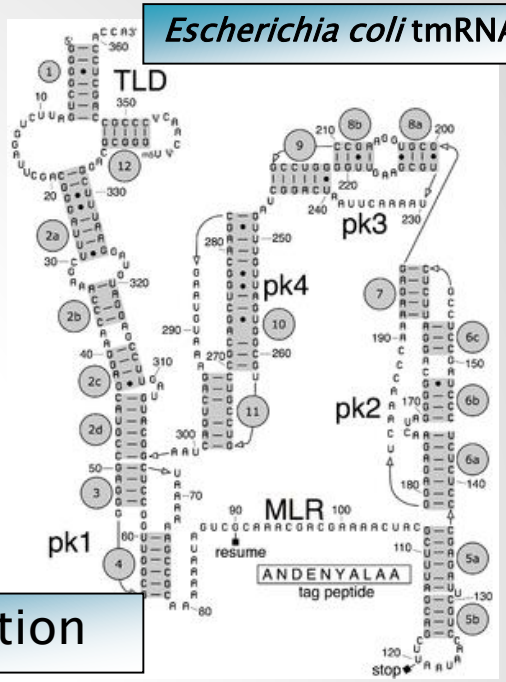
Construction of an L-phenylalanine-producing tyrosine-prototrophic *Escherichia coli* strain using *tyrA* ssrA-like tagged alleles

Vera G. Doroshenko · Rustem S. Shakulov · Svetlana M. Kazakova · Alexander D. Kivero · Tatyana A. Yampolskaya · Sergey V. Mashko

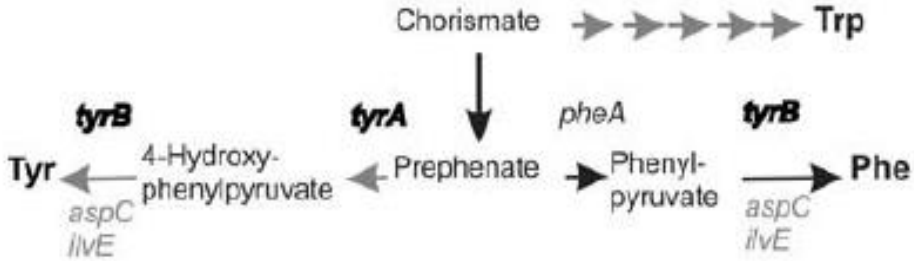
Biotechnol Lett (2010)
DOI 10.1007/s10529-010-0265-1

Escherichia coli tmRNA

ssrA product



Based on
Keiler et al, 1996;
Flinn et al, 2001



Trans-translation

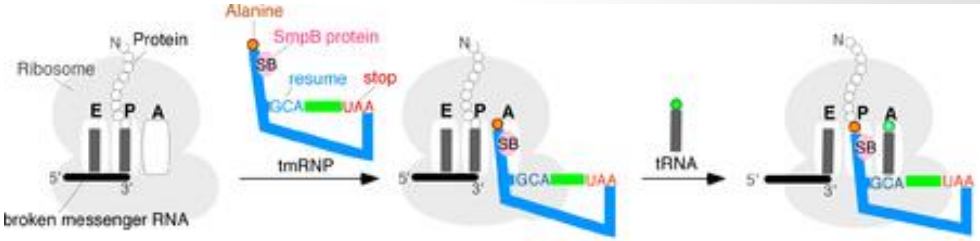
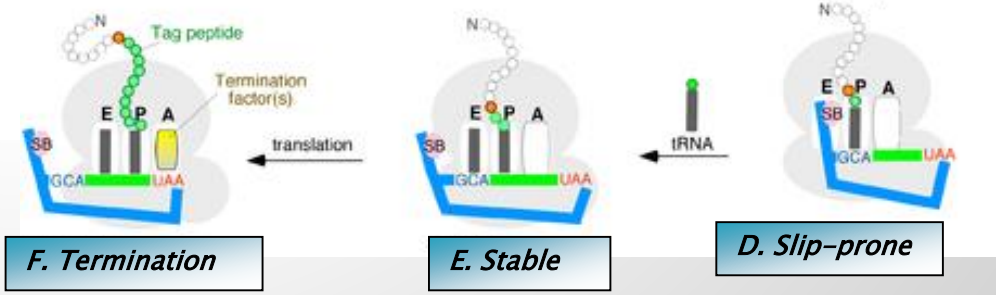


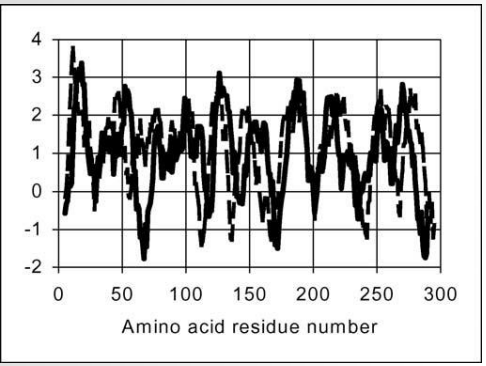
Table 2 Tyrosine (Tyr) production in cultures of DV269(*tyrA*-tag) *E. coli* strains

TyrA-tag	TyrA^{wt}	TyrA-LAA	TyrA-A-LAA	TyrA-LDD	TyrA-A-LDD
Tyr, mg/l[a]	50 ± 10	4 ± 2	<2	4 ± 2	4 ± 2
Tyr/Phe, %	1.3	0.06	<0.04	0.1	0.1

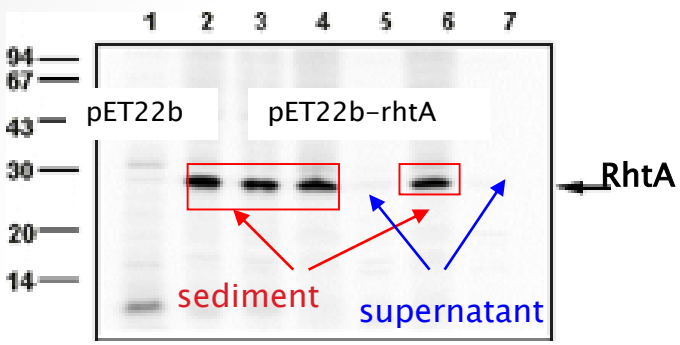


3. Search, identification and usage of genes that control amino acids efflux

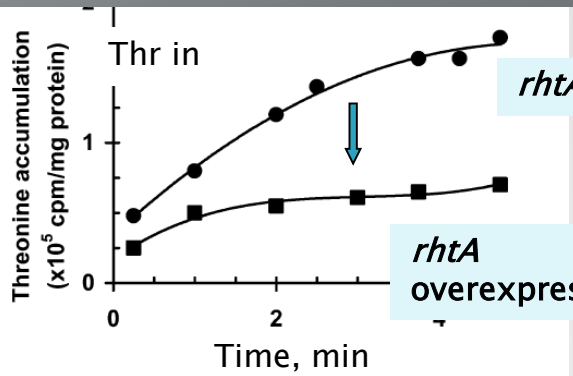
More than 20 years ago, in GosNIIGenetika, the gene *rhtA* from *Escherichia coli* was identified that encoded hydrophobic membrane protein responsible for new, previously unknown, function – efflux of amino acid and its precursor from bacterial cell.



Hydropathy plot of RhtA (solid line) and YdeD (dashed line)



Cellular localization of the RhtA protein (autoradiography)



Influence of overexpression of an exporter gene on intracellular amino acid concentration

From

Phenotype study of strains with altered expression of *rhtA*

To

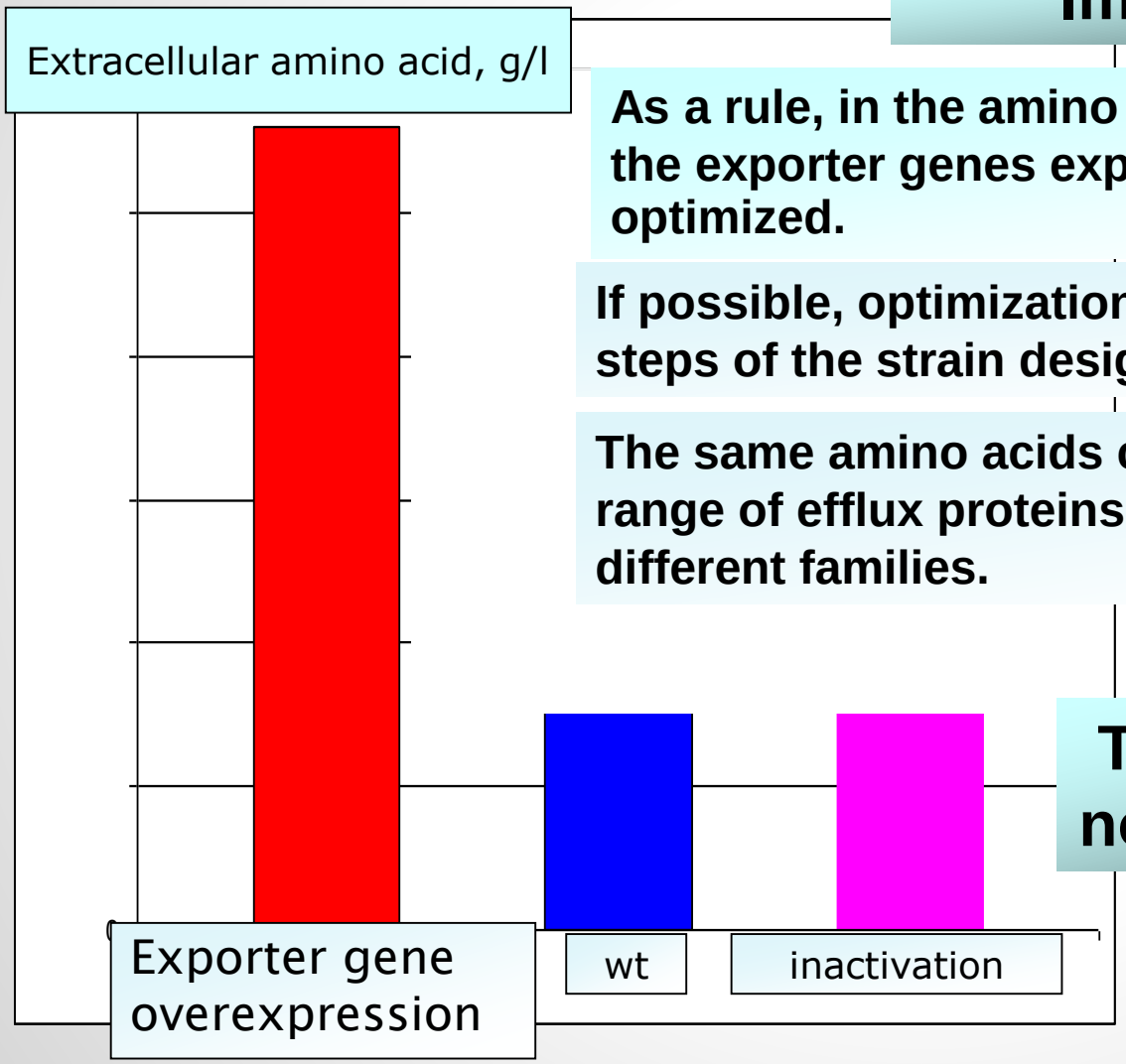
Methodology for search and identification of new genes that control the export of a wide range of intracellular metabolites

Amino acids exporters

Exporter/ gene	Family	Substrates	TMS	Regulation	Reference
Corynebacterium glutamicum					
LysE/*lysE*	LysE	lys, arg	6	Lys G	Vrljic *et al.*, 1996; Bellmann *et al.*, 2001
ThrE/*thrE*	ThrE	thr, ser	10		Simic *et al.*, 2001
BrnEF/*brnEF*	Liv-E	BCAA, met	4/7	Lrp	Kennerknecht *et al.*, 2002; Trötschel *et al.*, 2005
MscS/*NCgl1221*	MscS	glu, asp	4		Nakamura *et al.*, 2007
Escherichia coli					
RhtA/*rhtA*	DME	thr, hom, lys, pro etc.	10	Stress conditions	Zakataeva *et al.*, 1997; Livshits *et al.*, 2003
RhtB/*rhtB*	RhtB	thr, hom	6	Stress conditions	Aleshin *et al.*, 1999; Zakataeva *et al.*, 1999
RhtC/*rhtC*	RhtB	thr	6	Stress conditions	Aleshin *et al.*, 1999; Zakataeva *et al.*, 1999
LeuE/*yeaS*	RhtB	leu, met, his, ile, etc.	6	Lrp	Livshits et al., 2001; Kutukova *et al.*, 2005
EamA/*ydeD*	DME	OAS, cys, asn, gln	10		Daßler *et al.*, 2000
EamB/*yfiK*	RhtB	OAS, cys, AS, pro, thr	6		Livshits et al., 2001; Franke *et al.*, 2003
ArgO/*yggA*	LysE	arg, lys	6	ArgP	Livshits et al., 2001; Nandineni and Gowrishankar, 2004
YddG/*yddG*	DME	phe, tyr, trp	10		Livshits et al., 2003; Doroshenko *et al.*, 2007
YgaZH/*ygaZH*	Liv-E	BCAA, met	5/3	Lrp	Tabolina *et al.*, 2005; Park *et al.*, 2007
CydDC/*cydDC*	ABC	cys	6/6		Pittman et al., 2002; Cruz-Ramos et al., 2004

- identified and characterized in AGRI

Influence of the expression level of exporter gene on amino acid production by producer strain



Important lessons :

As a rule, in the amino acid producers, the level of the exporter genes expression should be specially optimized.

If possible, optimization is usually performed at early steps of the strain design.

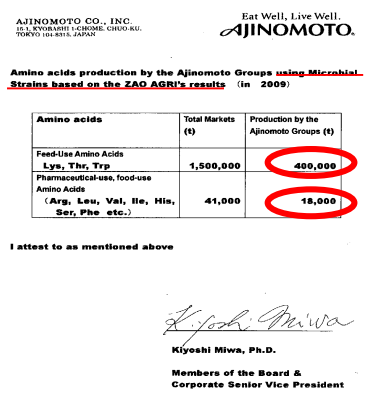
The same amino acids can be exported from a cell by a range of efflux proteins that sometimes belong to different families.

These conclusions are not limited by AA efflux

Some results:

The application of amino acids-producing strains obtained in this work, ensured in 2009 year the industrial production of feed-use amino acids (lysine, threonine, tryptophan) – 400 000 tons (27% of total world production and food-use and pharmaceutical-use amino acids (arginine, leucine, valine, isoleucine, histidine, serine, phenylalanine, etc) – 18 000 tons (44% of total world production).

(from Ajinomoto Co Ink Certificate)



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TOKYO 104-8315, JAPAN

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Amino acids production by the Ajinomoto Groups using Microbial Strains based on the ZAO AGRI's results (in 2009)

Amino acids	Total Markets (t)	Production by the Ajinomoto Groups (t)
Feed-Use Amino Acids Lys, Thr, Trp	1,500,000	400,000
Pharmaceutical-use, food-use Amino Acids (Arg, Leu, Val, Ile, His, Ser, Phe etc.)	41,000	18,000

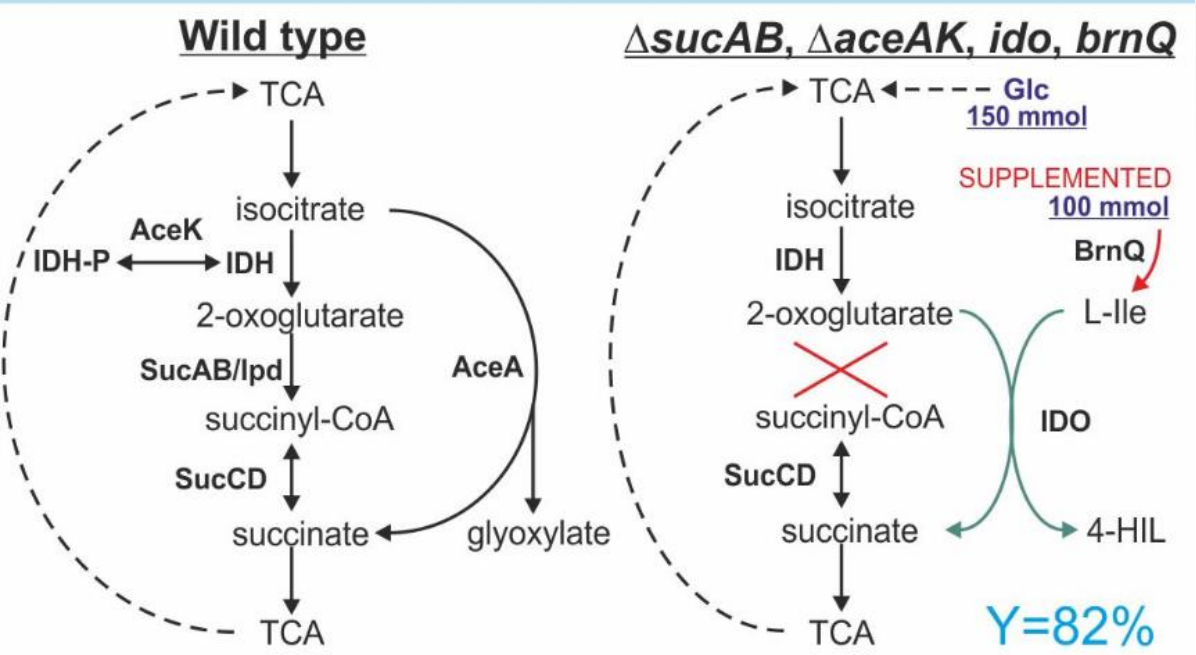
I attest to as mentioned above

Kiyoshi Miwa, Ph.D.

Members of the Board &
Corporate Senior Vice President

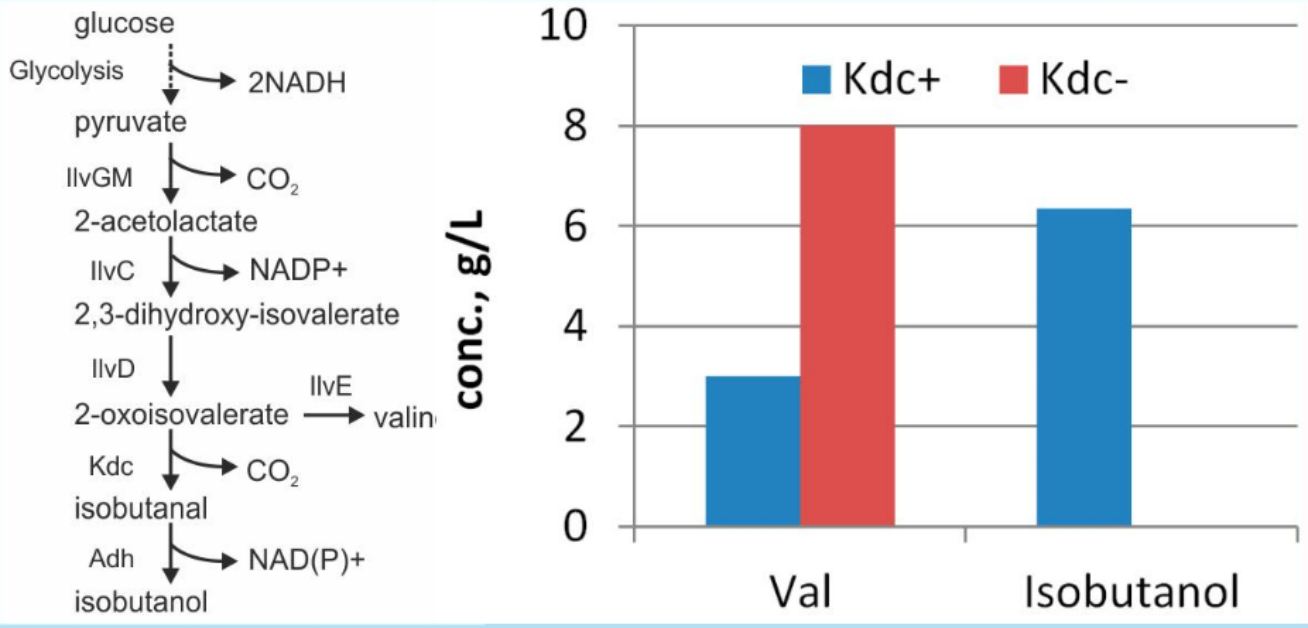
http://www.lysine.com/

Strains producing AA-related compounds



4-hydroxyisoleucine (4-HIL) as antidiabetic preparation

Kodera, Smirnov et al, 2009;
Smirnov, Kodera et al, 2010

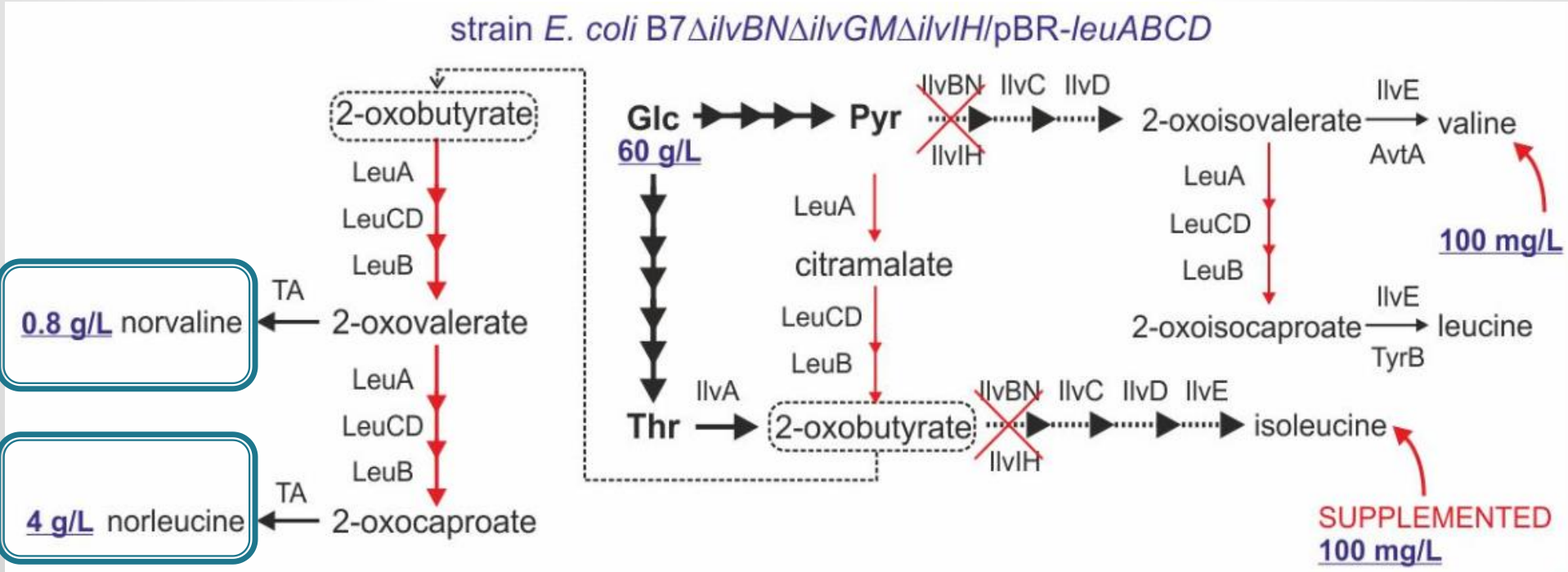


Isobutanol as biofuel

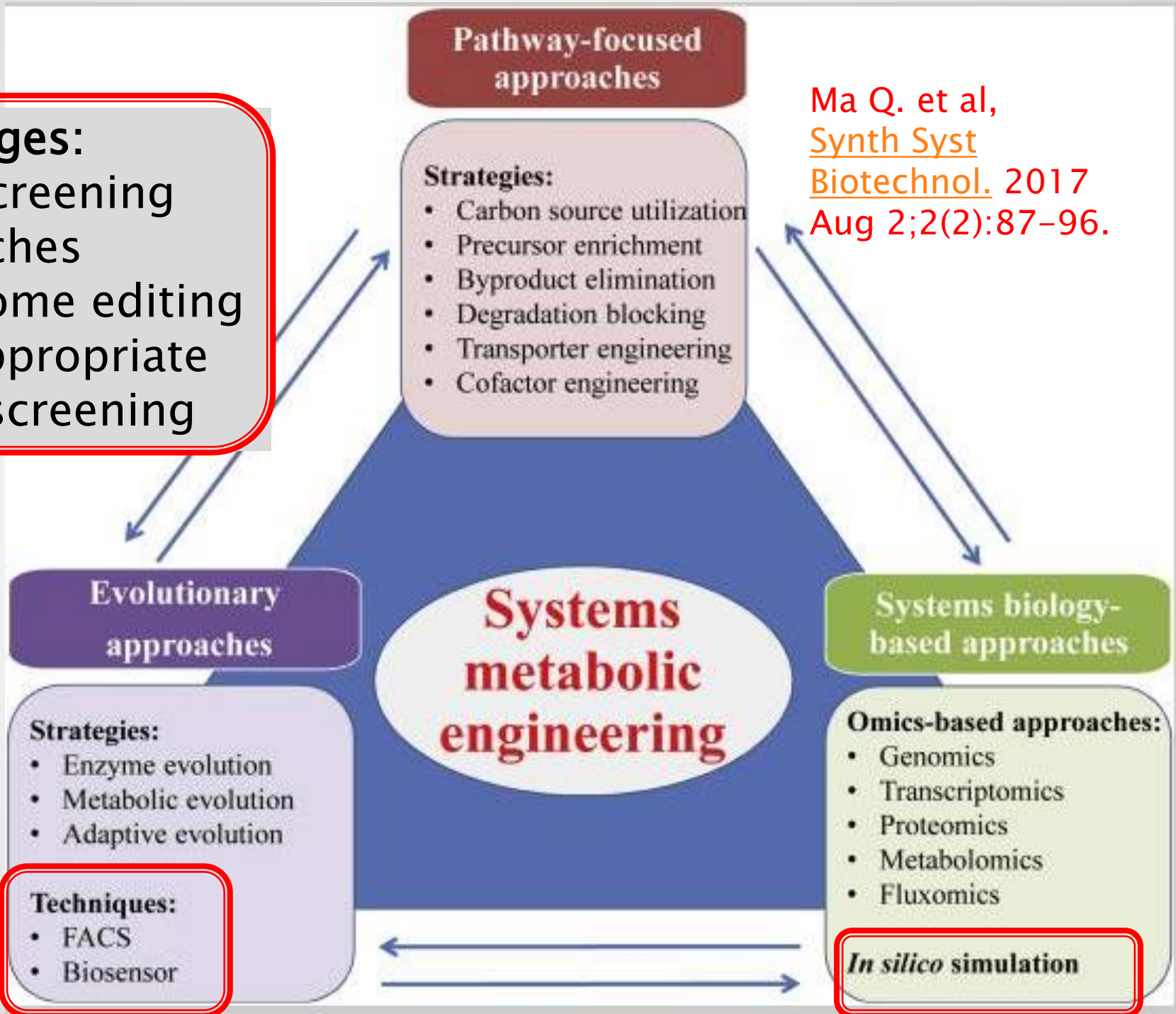
Savrasova et al, 2011

“Underground” metabolism

Over the past years, heighten interest in genetic code engineering allows the creation of different types of proteins with the novel biochemical properties or synthetic organisms that can incorporate noncanonical amino acids (NCAAs) instead of one of 20 proteinogenic canonical amino acids (CAAs) into a protein. A range of NCAAs which are structural analogs of the natural CAAs is known that can substitute CAAs in ribosomal translation process.



Challenges:
(i) HT screening approaches
(ii) genome editing tools appropriate for HT screening



Ma Q. et al, Synth Syst Biotechnol. 2017 Aug 2;2(2):87-96.

Acknowledgments:

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Thank you for attention