Accademia Nazionale dei Lincei
Symposium on the
International Year of Chemistry

Milano, October 3, 2011

Metabolic Engineering: synthetic chemistry for the 21st century

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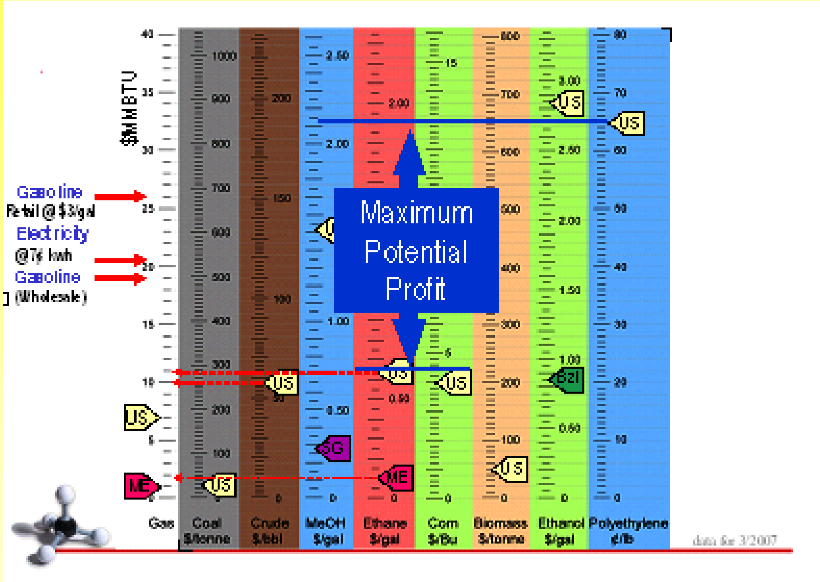
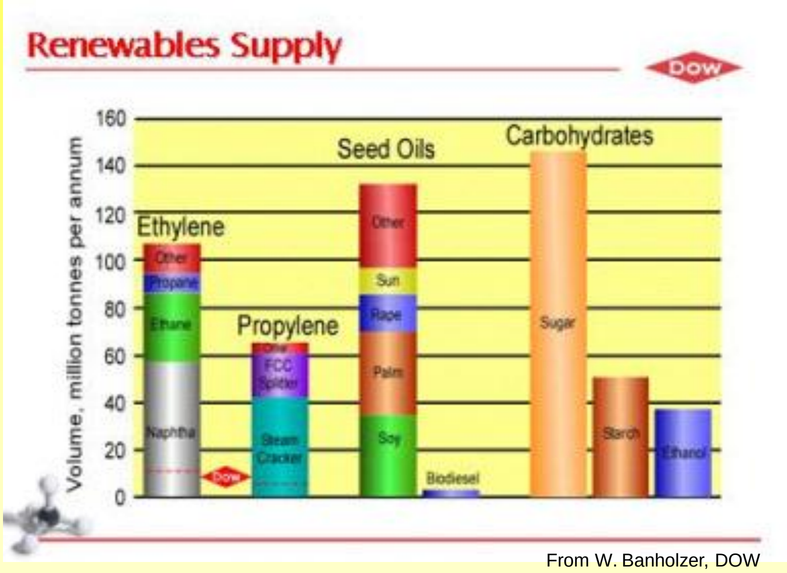
MIT

Acknowledgements

0. A primer on feedstocks and chemical products



From W. Banholzer, DOW



From W. Banholzer, DOW

A new biobased economy: *Principally, a feedstock, market and technology story*

- Sugar-biomass based:
  - Great availability
  - Alternative sources. No need to compete with food
  - Dramatically alters decision making with regards to resource utilization
- Biotechnology is natural technology for sugar modification and upgrade to bio-products
- Growing market for products with low carbon footprint

Metabolic Engineering, the biotech revolution, and the chemical-fuels industry (White Biotech)

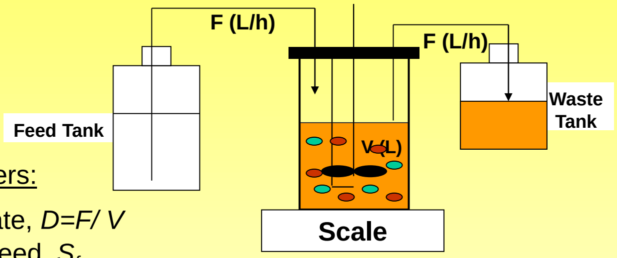
- Fuels and chemicals were the initial biotech target
  - Cetus (Chiron), Genex, Biogen
- More *challenging* technical problem than insulin
  - Switch of emphasis to medical applications
- Changing boundary conditions
  - Emphasis on renewable resources
  - Robust US federal funding ⇒ Applied mol. biology
  - Genomics
  - Systems Biology: a new mindframe in biological research
  - Metabolic Engineering
- Exploit applications of biology beyond medicine

Comparing Chemistry vs. Biotechnology

- Biotechnology: Higher selectivity
  - Much better in converting sugars to products
  - Generally, smaller plants, lower capital cost
- Biotechnology: Exquisite specificity in carrying out difficult reactions
- Biotechnology: Better at *new synthesis*
- Chemistry: Faster
- Chemistry: Better in converting petroleum-fossil feedstocks
- Chemistry: Better in operating in adverse conditions

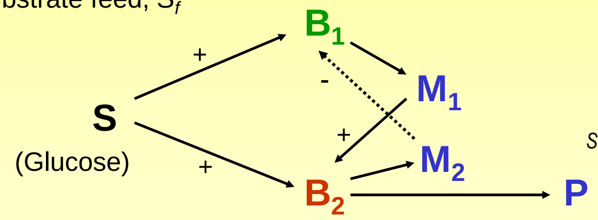
I. Origins of Metabolic Engineering

Mixed cultures: Multi-step synthesis within a single tank



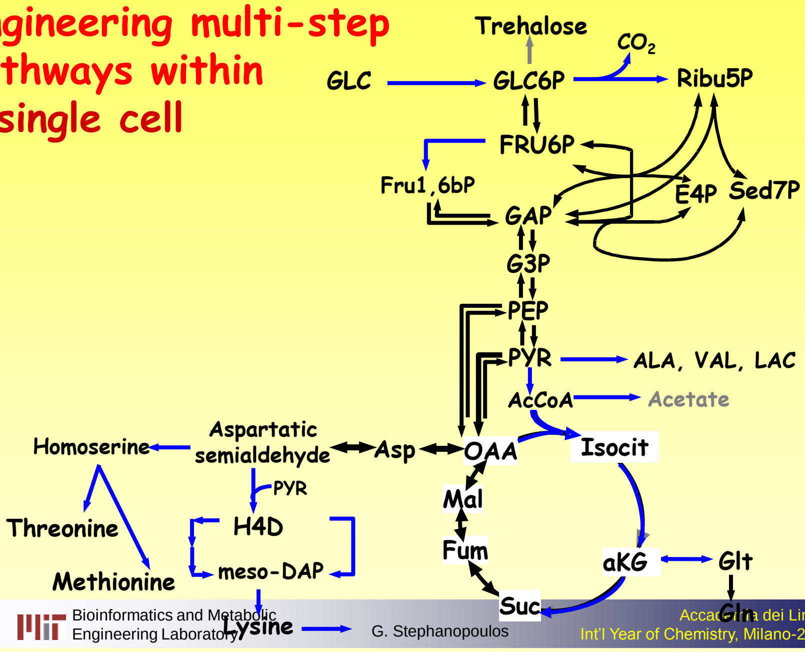
Parameters:

- Dilution Rate, $D=F/V$
- Substrate feed, S_f



Science, **213:** 972-979 (1981)

Metabolic Engineering: Engineering multi-step pathways within a single cell



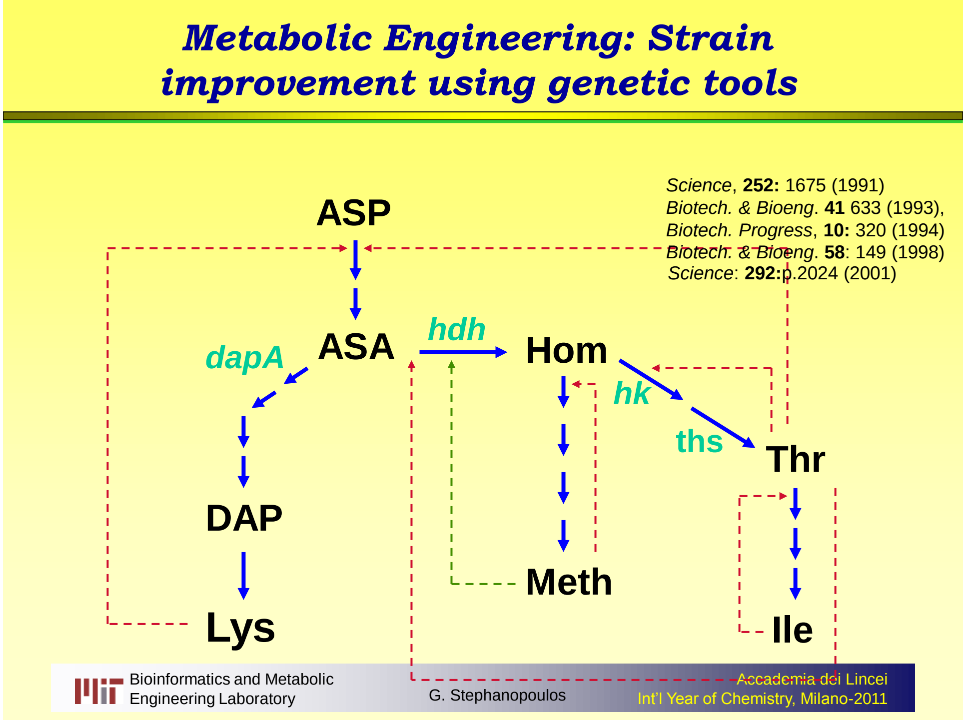
Leaders

- Jay Bailey
- Tony Sinskey
- Terry Papoutsakis
- Lonnie Ingram
- Jim Liao
- Ka-Yiu San
- European group (Nielsen, Heijnen)
- Korean-Japanese researchers (Sang Yup Lee)

Metabolic Engineering as a new Organic Chemistry

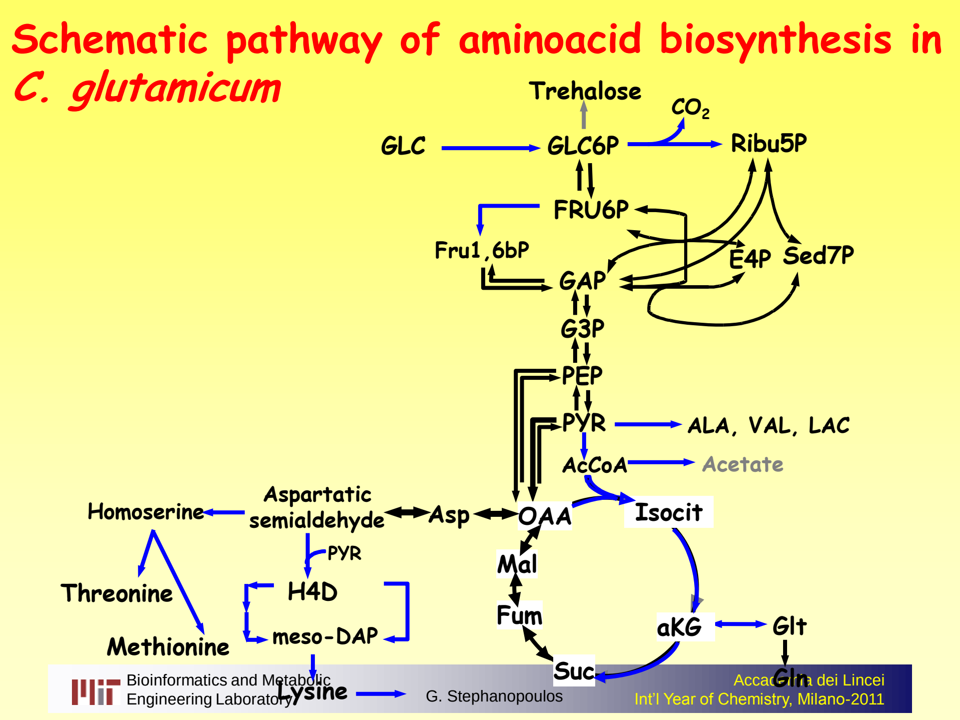
Metabolic Engineering: Making improved biocatalysts capable of:

- **Enhanced production of a *native* product to a microorganism**
- **Formation of a product that is *new* to the microorganism**
- **Synthesizing *novel products***



Metabolic Engineering: Strain improvement using genetic tools

Science, **252:** 1675 (1991)
Biotech. & Bioeng. **41** 633 (1993),
Biotech. Progress, **10:** 320 (1994)
Biotech. & Bioeng. **58**: 149 (1998)
Science: **292:**p.2024 (2001)



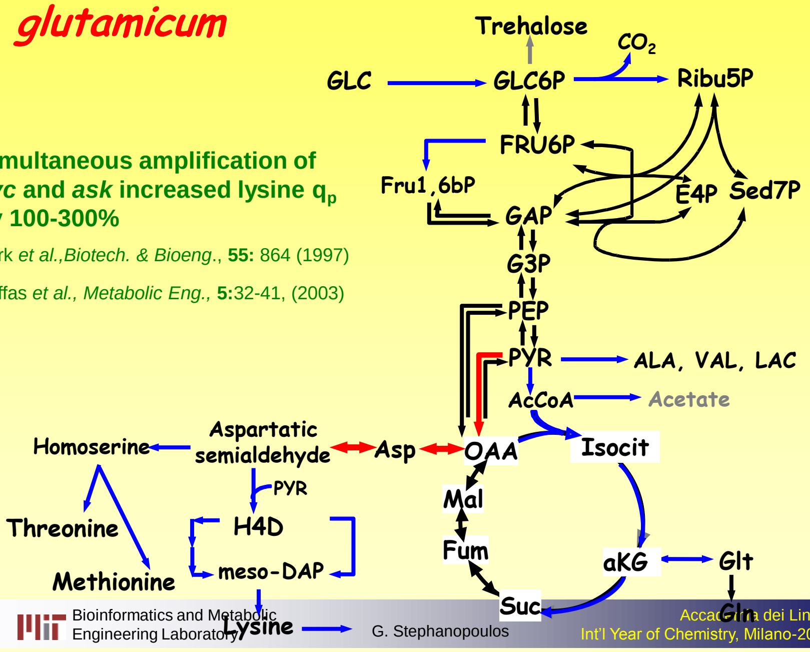
Schematic pathway of aminoacid biosynthesis in *C. glutamicum*

Probing metabolic pathways using isotopic tracers

Capable of:

- Reconstructing metabolic networks
- Calculating pathway fluxes
- Identifying rate-controlling steps

Schematic pathway of aminoacid biosynthesis in *C. glutamicum*



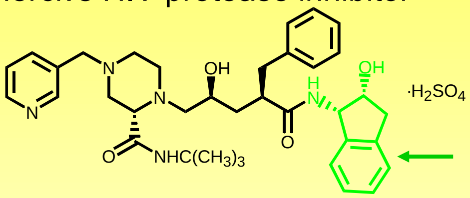
Simultaneous amplification of *pyc* and *ask* increased lysine q_p by 100-300%

Park *et al.,Biotech. & Bioeng*., **55:** 864 (1997)

Koffas *et al., Metabolic Eng.,* **5:**32-41, (2003)

Example: Indene biocatalysis for the synthesis of Crixivan precursor

- Crixivan™: Merck's HIV protease inhibitor

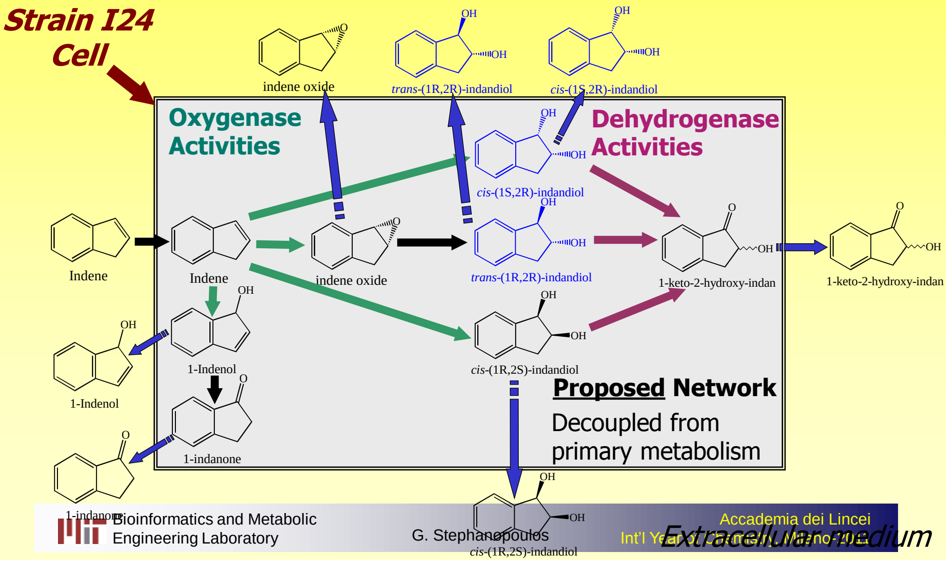


Aminoindanol

- Chemical synthesis of aminoindanol requires the use of expensive catalyst and gives low yields.
- Increased production is desired to meet patient needs (1 kg/patient/year).
- Bioconversion can potentially result in 100% yield.
- Project focus on production of 2R-indandiol.

Indene Bioconversion by Rhodococcus

Stafford *et al.*, *PNAS*, **99:**1801-1806, (2002);
European J. of Biochemistry, **278:** 1450-1460 (2001)

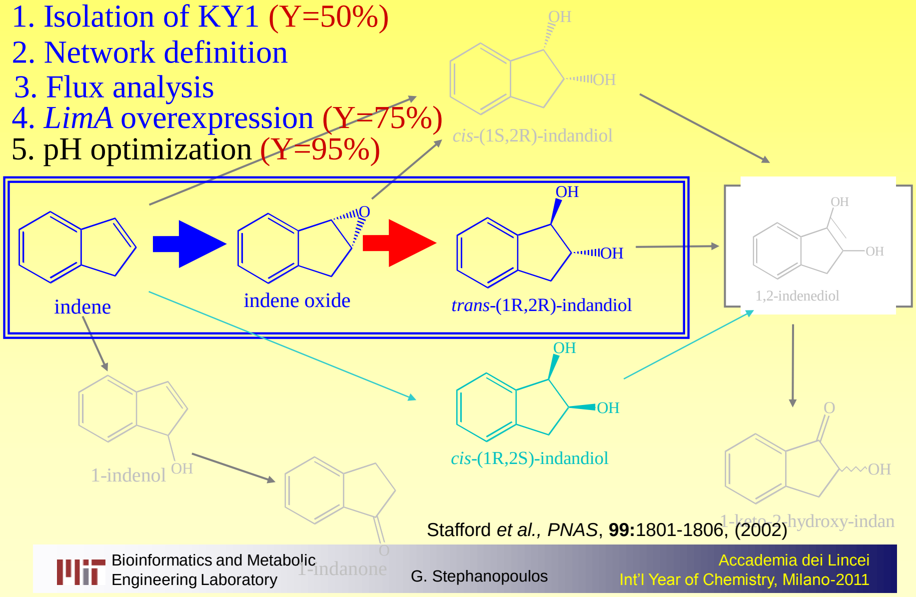


Proposed Network

Decoupled from primary metabolism

ME of Indene Bioconversion: Summary

1. Isolation of KY1 (Y=50%)
2. Network definition
3. Flux analysis
4. *LimA* overexpression (Y=75%)
5. pH optimization (Y=95%)



Stafford *et al.*, *PNAS*, **99:**1801-1806, (2002)

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20 Years of Metabolic Engineering

- **20 *very* productive years**
- **Recognized for high quality. Evidence:**
  - ME conference
  - Journal publications
  - *Metabolic Engineering* journal
  - Good record of real accomplishments
- **Recent successes have emboldened new research in higher risk areas**
- **Established identity of ME with distinct goals and intellectual content**

II. Differentiating characteristics of Metabolic Engineering

How does Metabolic Engineering differ from Genetic Engineering?

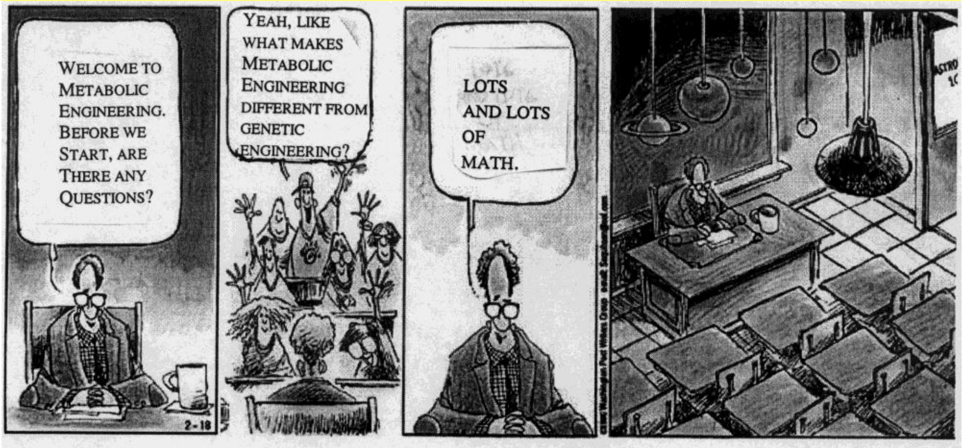
… Metabolic engineering differs from Genetic Engineering and related molecular biological sciences in that it concerns itself with the properties of the *entire metabolic network* as opposed to individual genes and enzymes.

"Metabolic Engineering: Issues and Methodologies," *Trends in Biotechnology,* Vol. 11, pp. 392-396 (1993)

Differentiation of Metabolic Engineering

- Integration. Concern about the function of the entire pathway (Systems biology)
- Pathway optimization using concepts from Chemical Reaction Engineering (not stitching genes together)
- Established identity of ME with distinct goals and intellectual content

What is Metabolic Engineering?



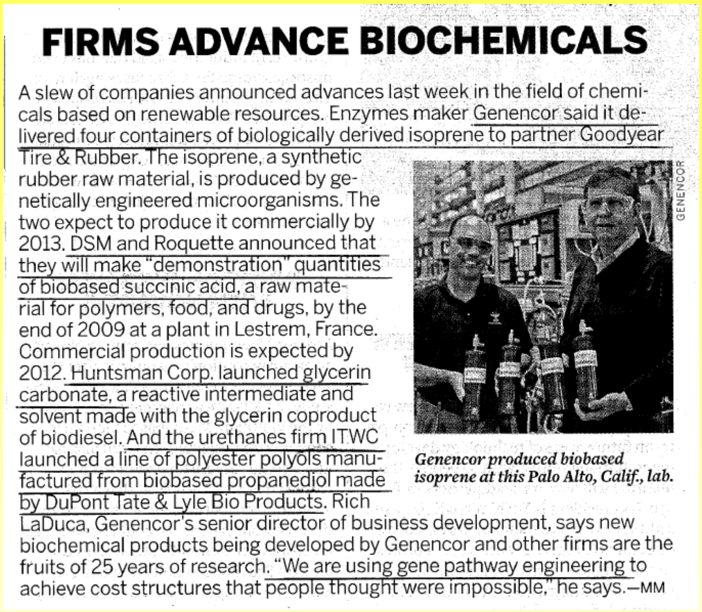
Technologies of Metabolic Engineering

1. Flux determination in metabolic networks. Identification of bottlenecks in pathways
2. Precise control of gene expression and metabolic fluxes
3. Constructing new metabolic pathways
   - Cloning or synthesizing genes (codon optimized) from various sources and transferring them to the host cell
   - Identifying gene targets
4. Eliciting tolerance to various stresses
5. Inverse Metabolic Engineering

III. Accomplishments of Metabolic Engineering

Past record of Metabolic Engineering

- Aminoacids: Increases in lysine spec. productivity by 120-300%
- Ethanologenic *E. coli* (also: *butanologenic E. coli)*
- Biopolymers *(Metabolix-ADM)*
- 1,3 propane diol *(DuPont-Tate and Lyle)*
- Indan-diol production (precursor of Crixivan-HIV protease inhibitor): Yield increased from 25% to >95%
- Artemisinin (amorphadiene) production by yeast and *E. coli*
- Lycopene production in *E. coli:* Increase from 4,500 to ~25,000 ppm of CDW, fermentations > 250 mg/L
- Many other applications:
  - Succinate
  - 3-HPA
  - Threonine
  - Tamiflu precursor



FIRMS ADVANCE BIOCHEMICALS

A slew of companies announced advances last week in the field of chemicals based on renewable resources. Enzymes maker Genencor said it delivered four containers of biologically derived isoprene to partner Goodyear Tire & Rubber. The isoprene, a synthetic rubber raw material, is produced by genetically engineered microorganisms. The two expect to produce it commercially by 2013. DSM and Roquette announced that they will make "demonstration" quantities of biobased succinic acid, a raw material for polymers, food, and drugs, by the end of 2009 at a plant in Lestrem, France. Commercial production is expected by 2012. Huntsman Corp. launched glycerin carbonate, a reactive intermediate and solvent made with the glycerin coproduct of biodiesel. And the urethanes firm ITWC launched a line of polyester polyols manufactured from biobased propanediol made by DuPont Tate & Lyle Bio Products. Rich LaDuca, Genencor's senior director of business development, says new biochemical products being developed by Genencor and other firms are the fruits of 25 years of research. "We are using gene pathway engineering to achieve cost structures that people thought were impossible," he says.—MM

GENENCOR

Genencor produced biobased isoprene at this Palo Alto, Calif., lab.

C&E News, Business Concentrates, March 16, 2009

III. Accomplishments of Metabolic Engineering:

Expressing whole pathways, natural or unnatural, in microorganisms

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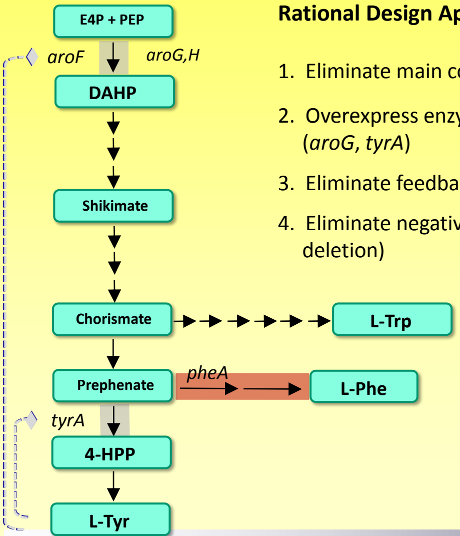
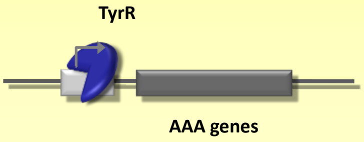
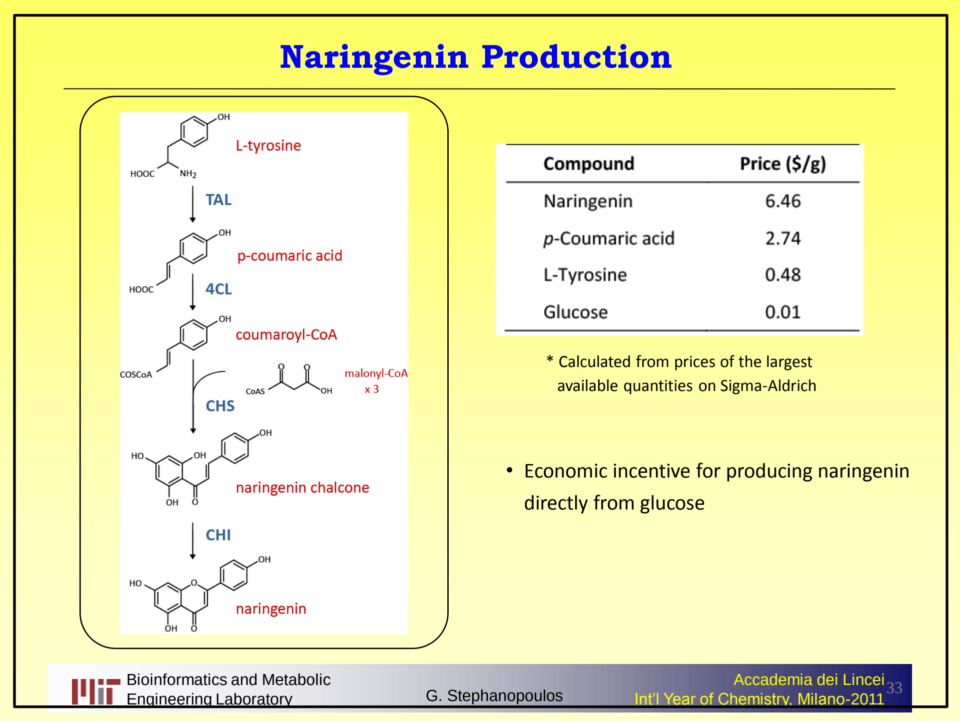
Aromatic amino acid biosynthetic pathway



Rational Design Approaches

1. Eliminate main competing reactions (*pheA* deletion)
2. Overexpress enzymes constituting major bottlenecks (*aroG*, *tyrA*)
3. Eliminate feedback repression of enzymes (*aroG*^{fbr}, *tyrA*^{fbr})
4. Eliminate negative transcriptional regulator (TyrR deletion)



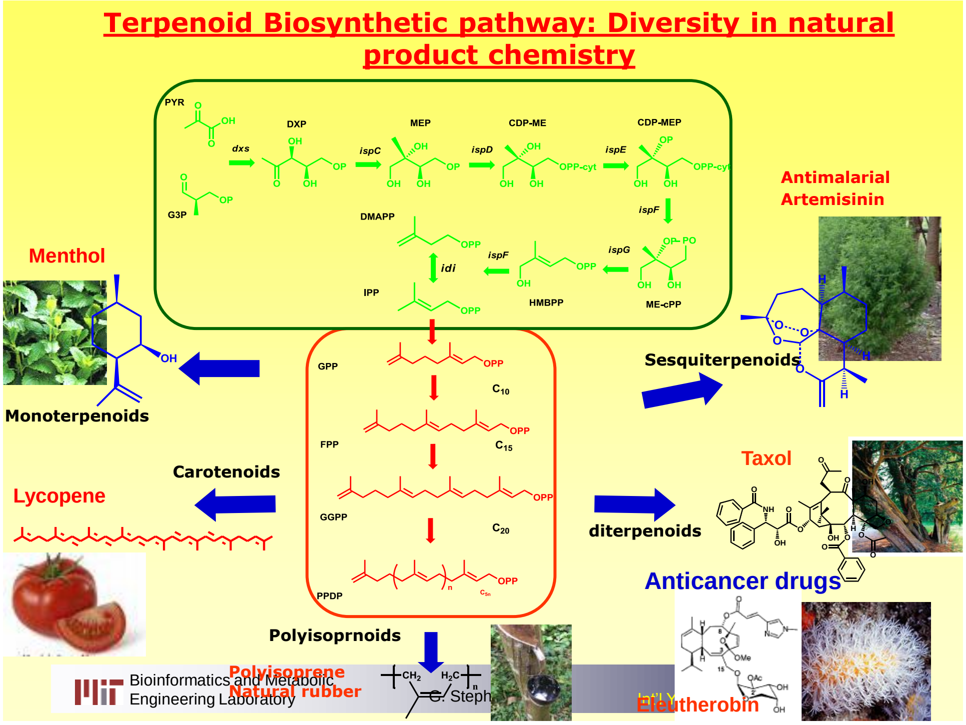


Naringenin Production

Compound	Price ($/g)
Naringenin	6.46
p-Coumaric acid	2.74
L-Tyrosine	0.48
Glucose	0.01

* Calculated from prices of the largest available quantities on Sigma-Aldrich

- Economic incentive for producing naringenin directly from glucose



Terpenoid Biosynthetic pathway: Diversity in natural product chemistry

III. Accomplishments of Metabolic Engineering:

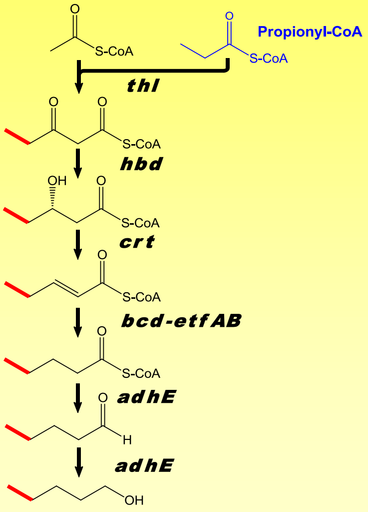
Conducting difficult and new chemistry

Pentanol Synthesis

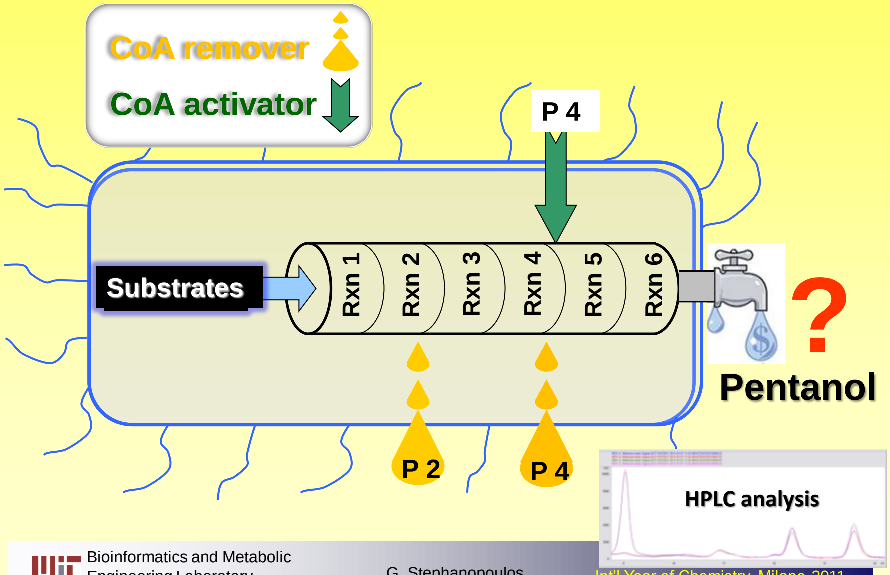
Challenges:

1. Supply of building block (Propionyl-CoA)
2. Condensation reaction of C2 + C3
3. Acceptance of 5-carbon substrates for the rest of pathway enzymes

Proposed Pentanol Pathway

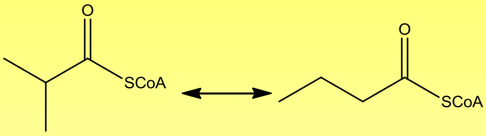


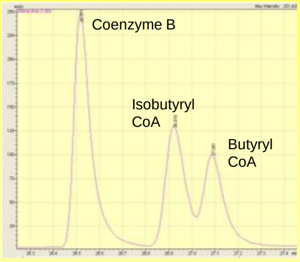
Redirecting Carbon Flux



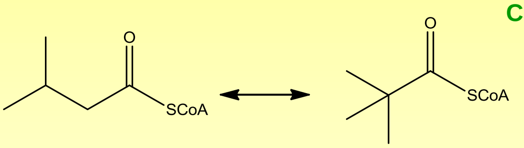
Mutases: The branching enzymes

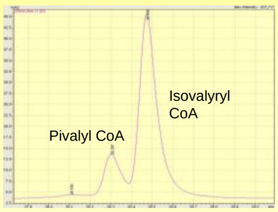
C4



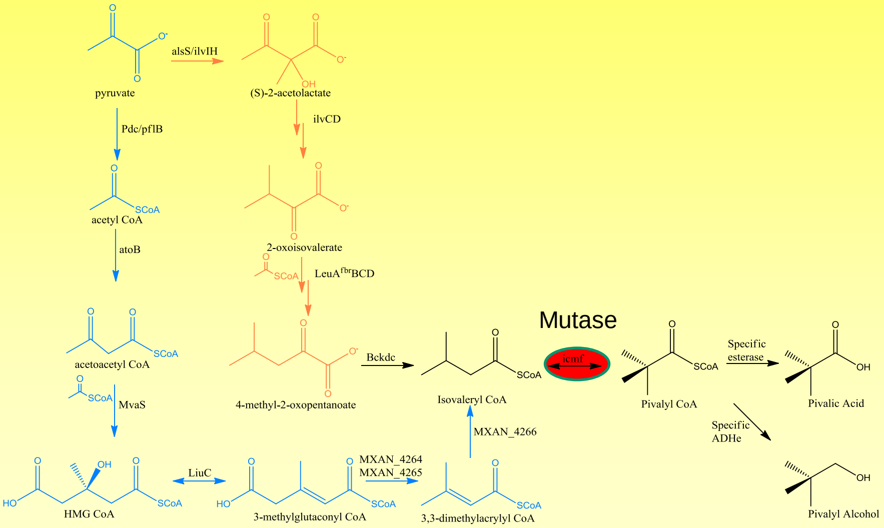


C5

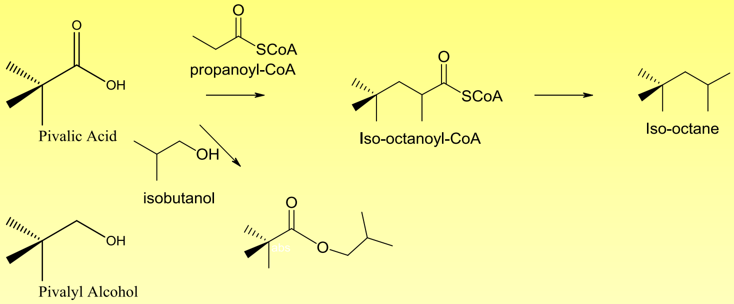




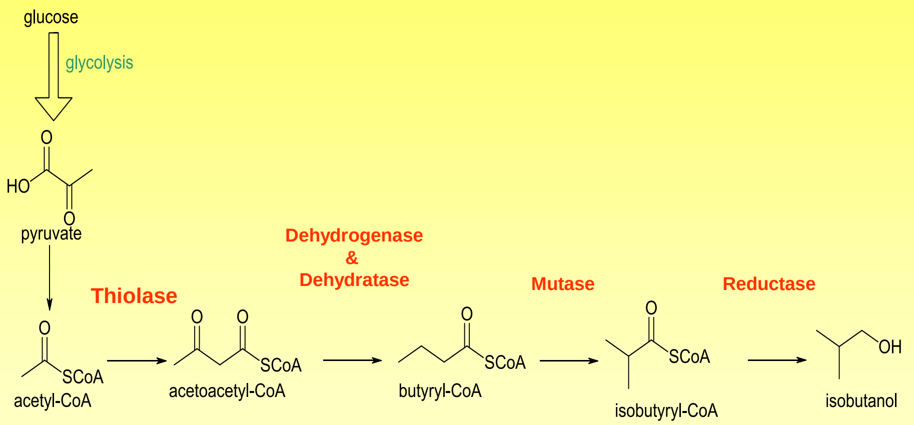
Pivalic acid pathway



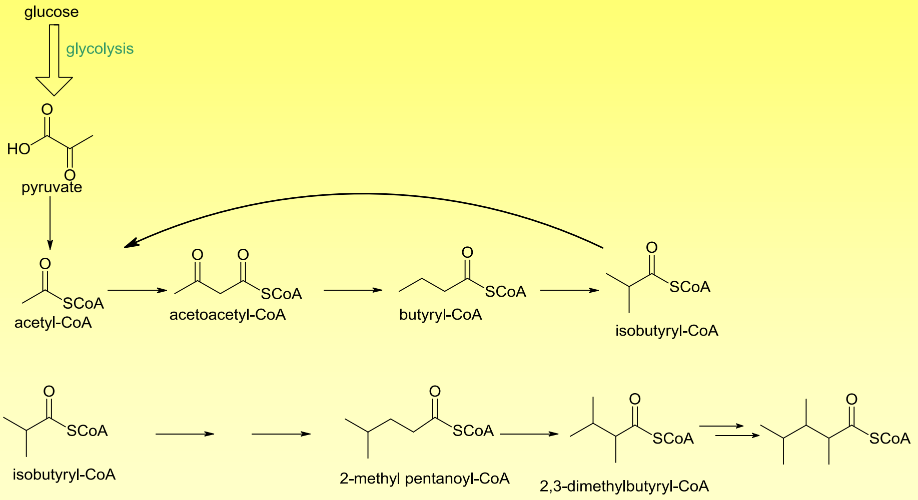
Beyond C5



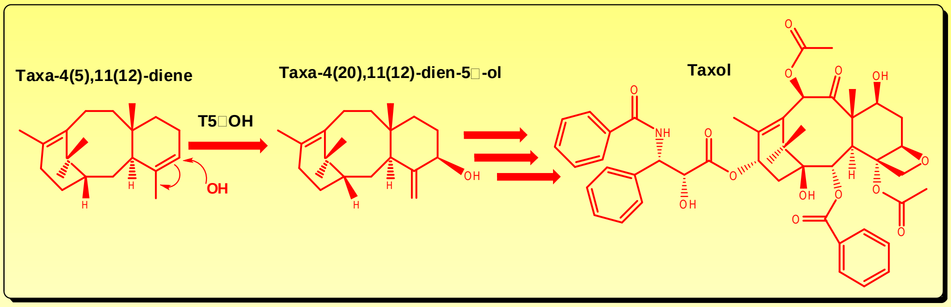
Biofuels toolkit



Biofuels toolkit

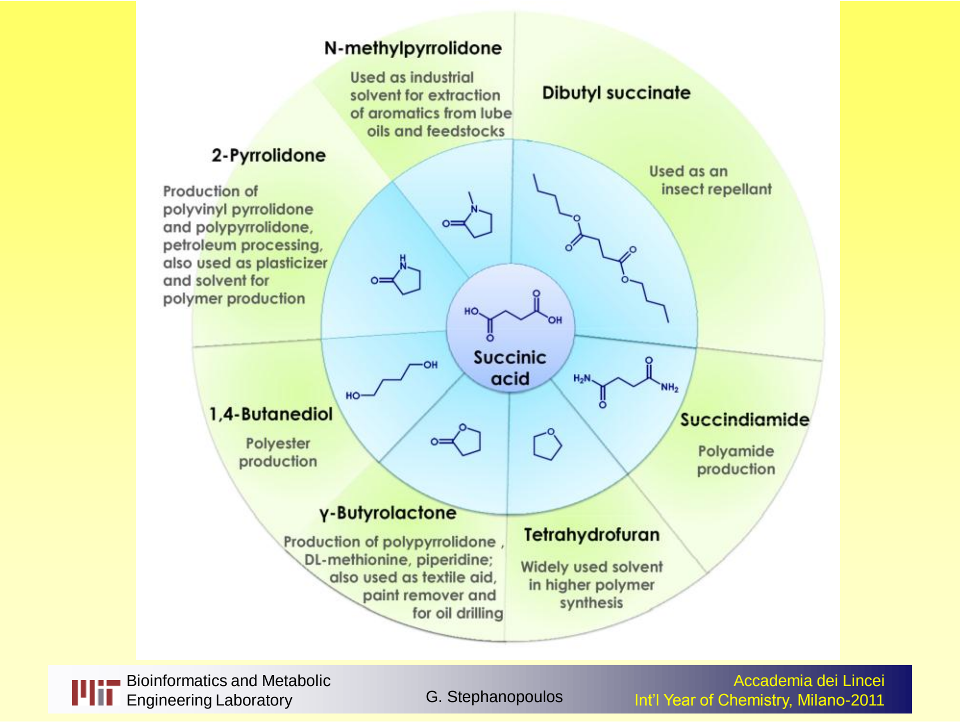


P450 oxidation: First step in the pathway to taxol



Metabolic Engineering:

Platform for a Biobased economy



Succinic acid

N-methylpyrrolidone
Used as industrial solvent for extraction of aromatics from lube oils and feedstocks

Dibutyl succinate
Used as an insect repellant

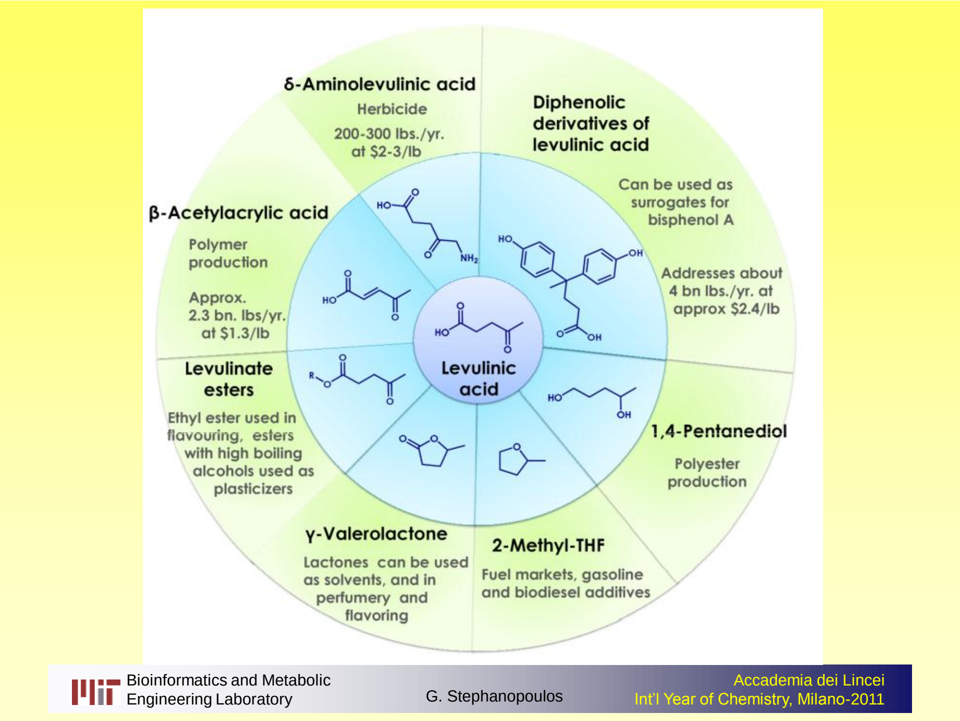
Succindiamide
Polyamide production

Tetrahydrofuran
Widely used solvent in higher polymer synthesis

γ-Butyrolactone
Production of polypyrrolidone , DL-methionine, piperidine; also used as textile aid, paint remover and for oil drilling

1,4-Butanediol
Polyester production

2-Pyrrolidone
Production of polyvinyl pyrrolidone and polypyrrolidone, petroleum processing, also used as plasticizer and solvent for polymer production



Levulinic acid

δ-Aminolevulinic acid
Herbicide
200-300 lbs./yr. at $2-3/lb

Diphenolic derivatives of levulinic acid
Can be used as surrogates for bisphenol A
Addresses about 4 bn lbs./yr. at approx $2.4/lb

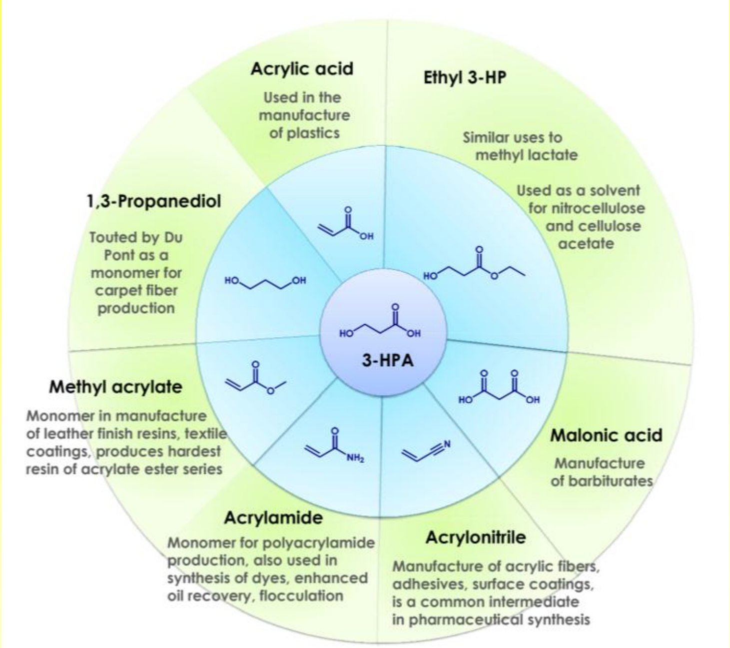
1,4-Pentanediol
Polyester production

2-Methyl-THF
Fuel markets, gasoline and biodiesel additives

γ-Valerolactone
Lactones can be used as solvents, and in perfumery and flavoring

Levulinate esters
Ethyl ester used in flavouring, esters with high boiling alcohols used as plasticizers

β-Acetylacrylic acid
Polymer production
Approx. 2.3 bn. lbs/yr. at $1.3/lb



3-HPA

Acrylic acid
Used in the manufacture of plastics

Ethyl 3-HP
Similar uses to methyl lactate

Used as a solvent for nitrocellulose and cellulose acetate

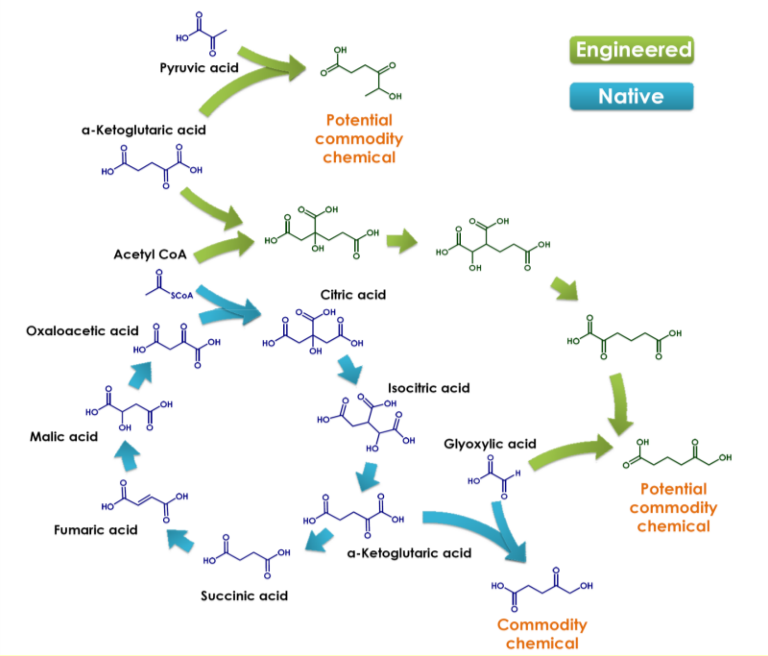
1,3-Propanediol
Touted by Du Pont as a monomer for carpet fiber production

Methyl acrylate
Monomer in manufacture of leather finish resins, textile coatings, produces hardest resin of acrylate ester series

Malonic acid
Manufacture of barbiturates

Acrylamide
Monomer for polyacrylamide production, also used in synthesis of dyes, enhanced oil recovery, flocculation

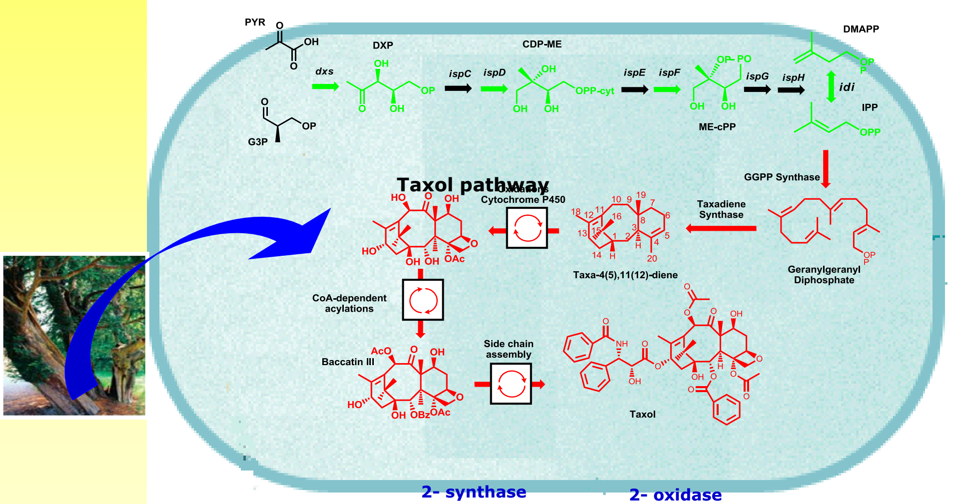
Acrylonitrile
Manufacture of acrylic fibers, adhesives, surface coatings, is a common intermediate in pharmaceutical synthesis



Metabolic Engineering:

Platform for discovery *and* production of new therapeutics

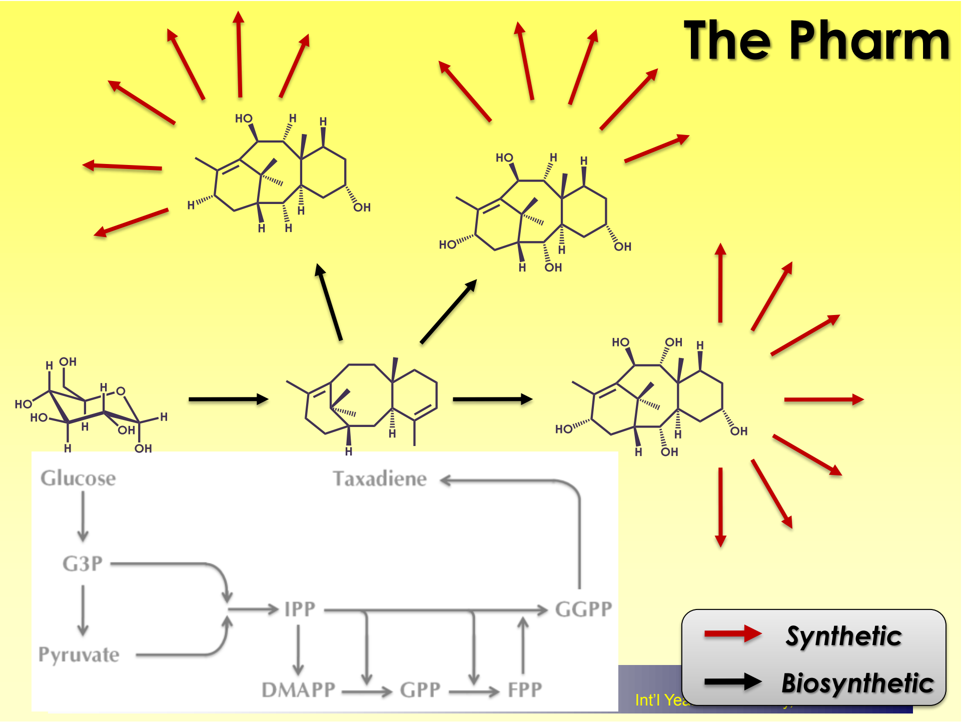
Future directions of ME-2



19- enzymes from IPP and DMAPP

2- synthase
8 – hydroxylase
5 – transferase

2- oxidase
1 - Aminomutase
1 - Co-ligase

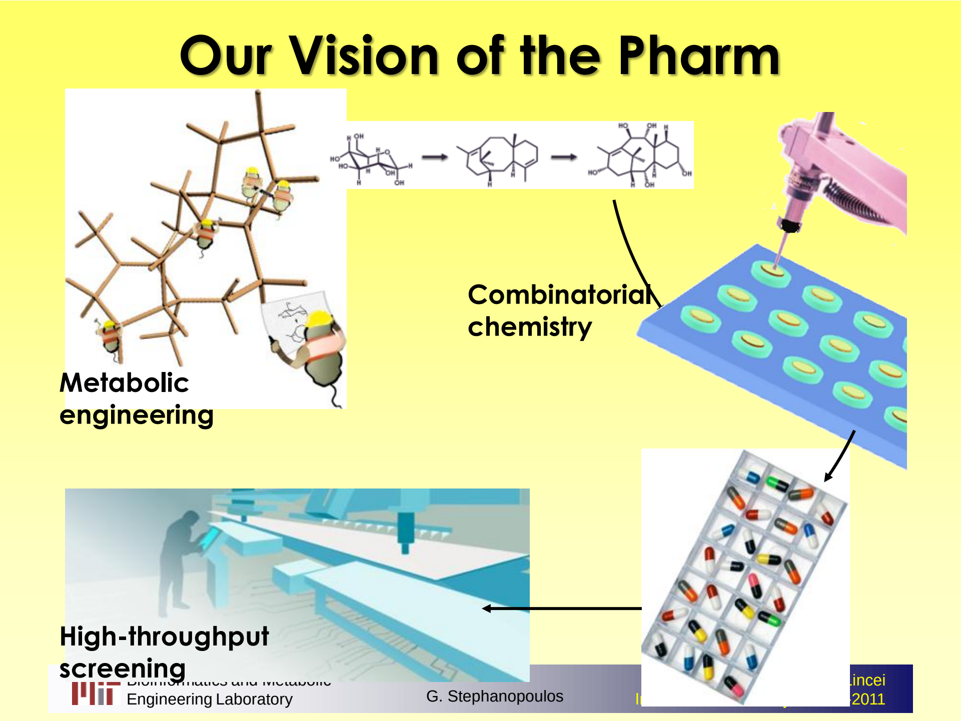


The Pharm

Glucose
G3P
Pyruvate
IPP
DMAPP
GPP
FPP
GGPP
Taxadiene

Synthetic
Biosynthetic

Int'l Yea



Our Vision of the Pharm

Metabolic engineering

Combinatorial chemistry

High-throughput screening

Engineering Laboratory

G. Stephanopoulos

incei
2011

A few illustrative examples

Example 1

Engineering an obese microbe for
oil overproduction

Converting sugars to oil for *Biodiesel* production

- Key points:
  - It is a bad idea to use vegetable oils for biodiesel
  - Sustainable biodiesel production MUST be based on carbohydrates

	Gallons GE/acre/year
Soybeans	48
Sesame	74
Jatropha	202
Cellulosic ethanol	533
Sugarcane ethanol	566
Algae	~6,000

  - Need organisms capable of converting sugars to fats and lipids (or Free Fatty Acids, FFA)

Conversion Yield is critical

1 Gallon of biodiesel = 3.8 Liters = 3.4 Kgs

Assuming a Theoretical Yield of 31%, 3.4 Kgs can be produced from 3.4 / 0.31 = 11 Kgs of Glucose

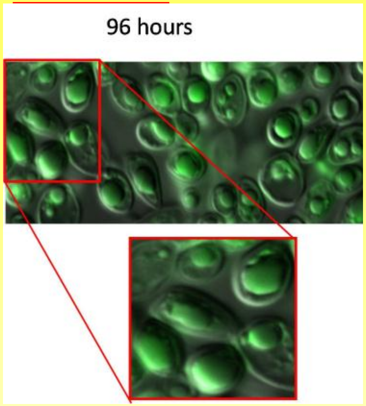
that costs ~ $1.20-1.40

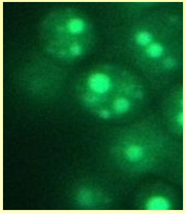
- Hence, biodiesel can be produced from sugars at an estimated total cost of $1.80-2.00

- **Key: Achieving yields as close to maximum as possible**

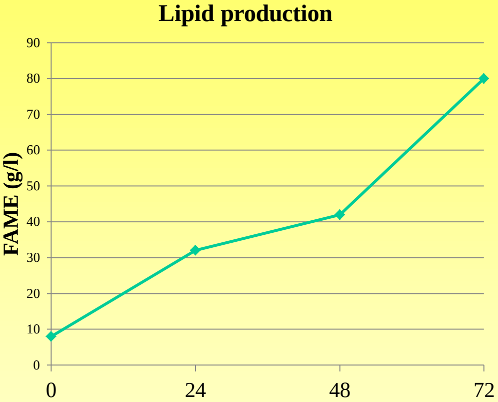
Some results on recombinant oil producing microbe

Recombinant





Wild type



Total sugar consumed: 312 g/L
Total oil produced: 80g/L in 72 hours
Yield: 29.4%
Theoretical Maximum Yield: 31%

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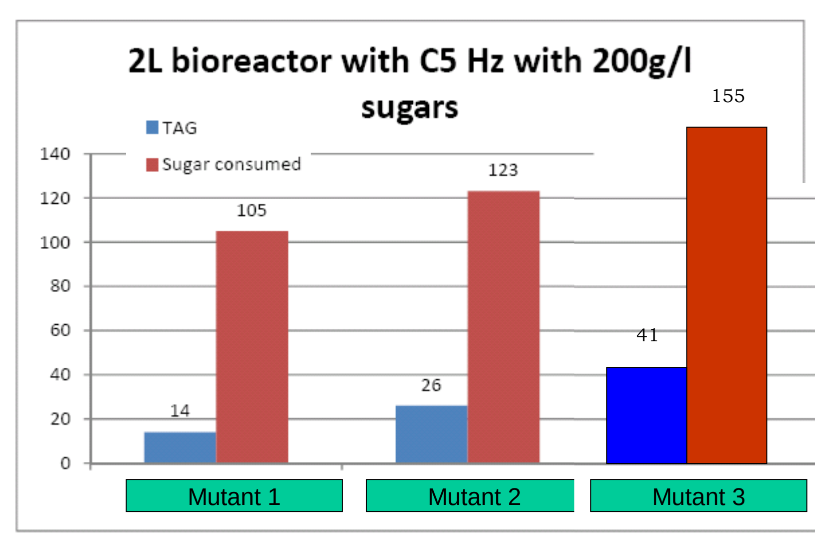
72 hour fermentation. C5 Xz supplemented with 200g/L of glucose.
Yield for mutant 3 is 41/155 = 26.5%



Example 2

Engineering the isoprenoid pathway for natural product overproduction

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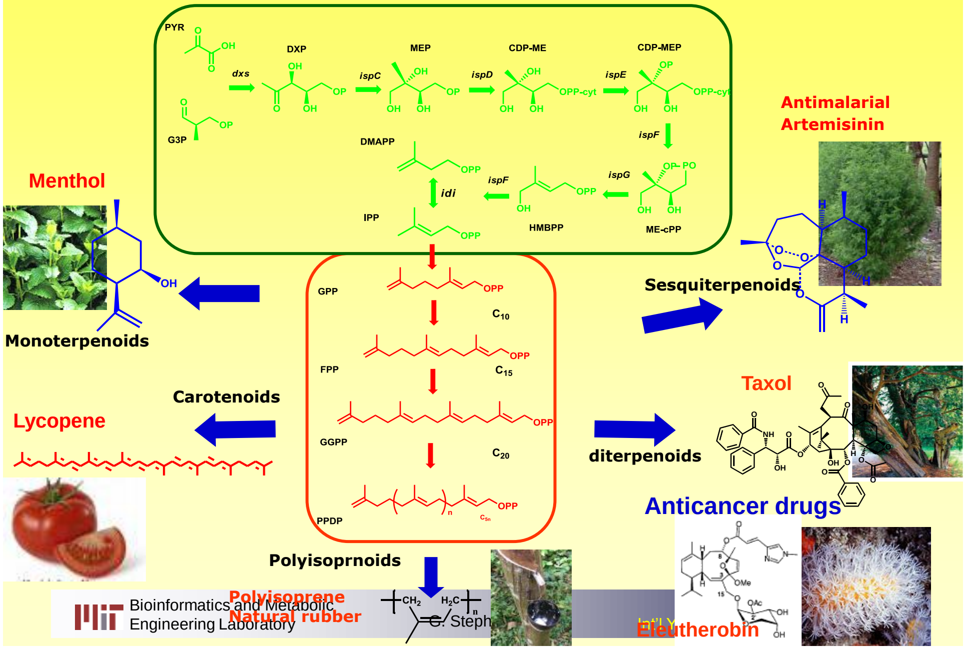
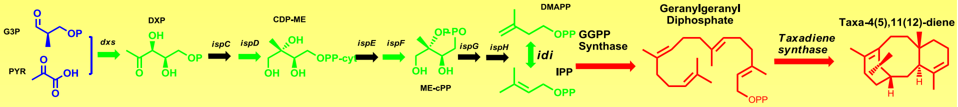
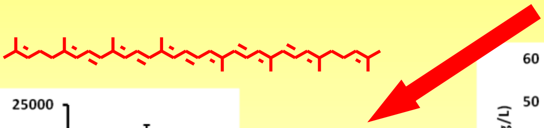
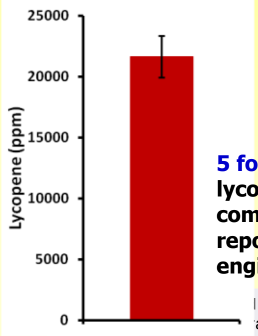
Terpenoid Biosynthetic pathway: Diversity in natural product chemistry



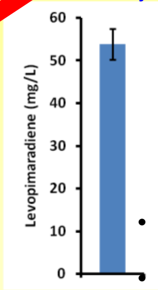
Engineering other terpenoid pathways in isoprenoid precursor pathway engineered *E. coli*



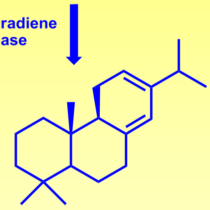




5 fold increase in lycopene production compared previously reported pathway engineered strain



Levopimaradiene Synthase



Leopimaradiene

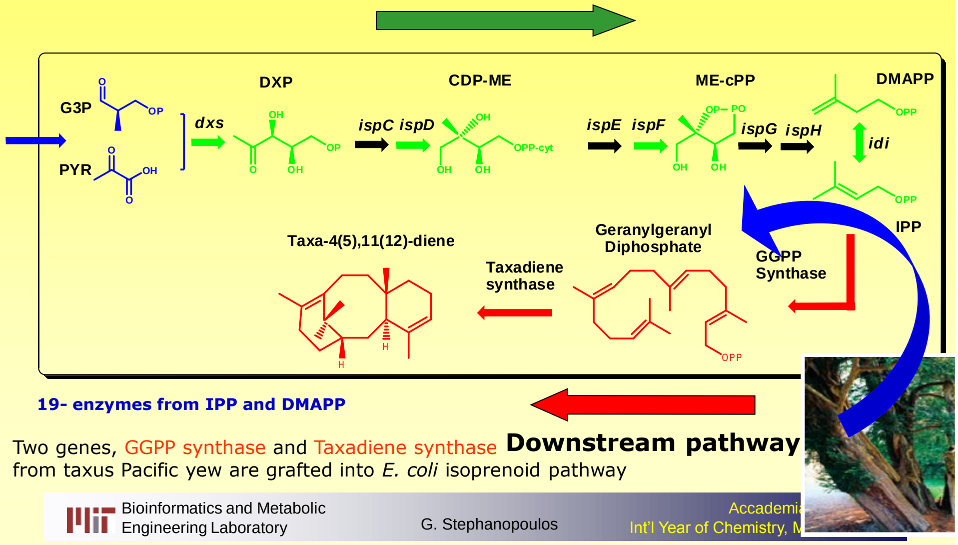
- **Levopimaradiene – precursor for Ginkolide**
- **Ginkolide – pharmacological ly active against Alzheimer's and dementia**

How efficient is the MEP Pathway?

- Prior studies yielded modest results (a few mg/L of taxadiene)
- Is the MEP pathway somehow deficient in isoprenoid production?

Engineering Taxol biosynthetic pathway in *E. coli*
– most challenging and complex chemistry in natural products

Upstream pathway

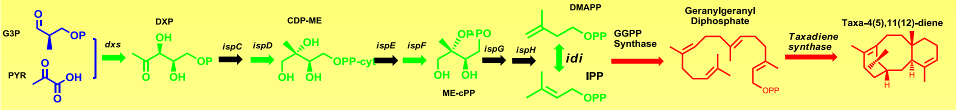


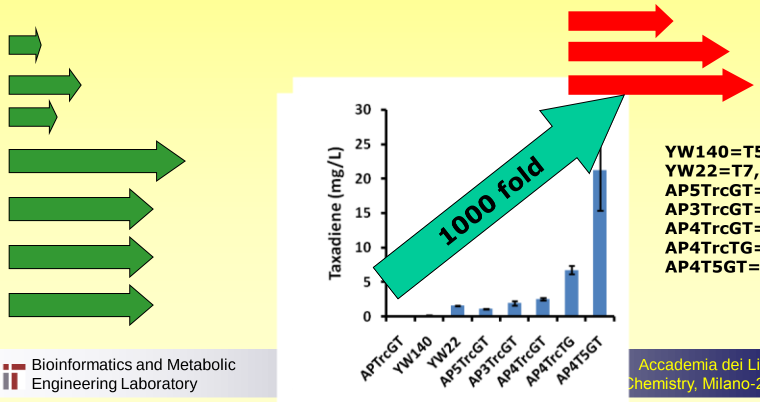
19- enzymes from IPP and DMAPP

Two genes, GGPP synthase and Taxadiene synthase from taxus Pacific yew are grafted into *E. coli* isoprenoid pathway

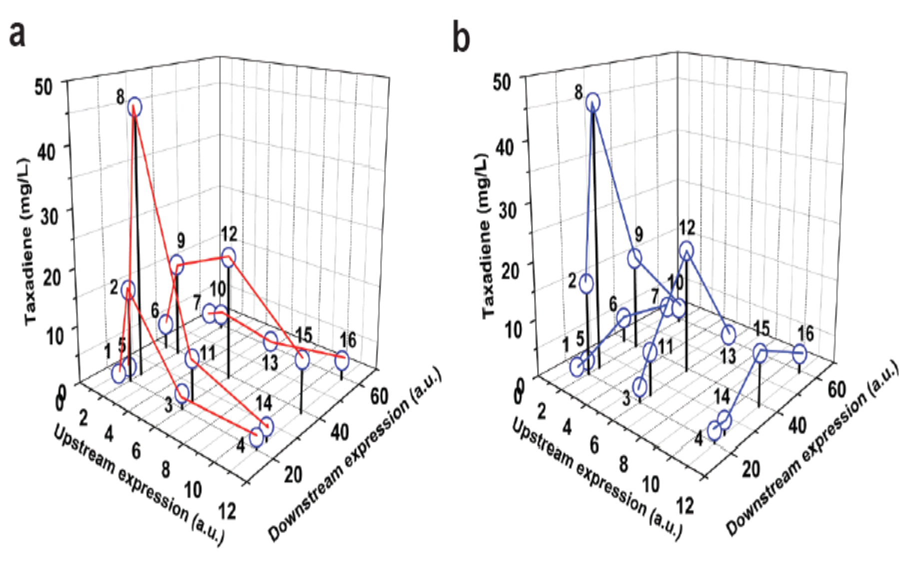
Downstream pathway

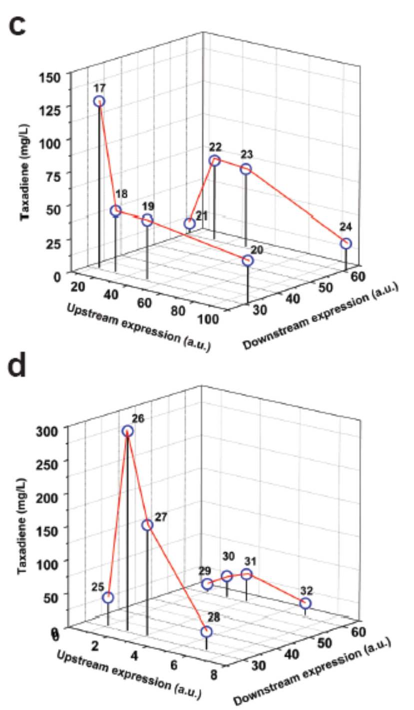
Modulating the upstream and downstream pathway for amplifying taxadiene production





YW140=T5 single
YW22=T7, single
AP5TrcGT=single
AP3TrcGT=10 copies
AP4TrcGT=5 copies
AP4TrcTG=5 copies
AP4T5GT=5 copies



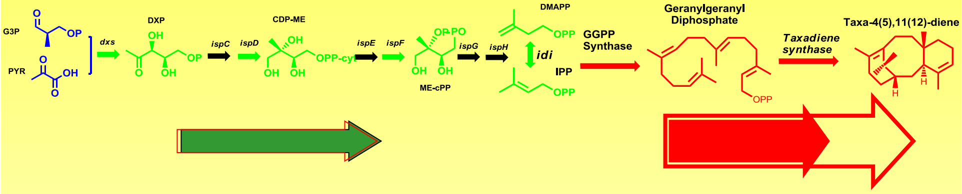


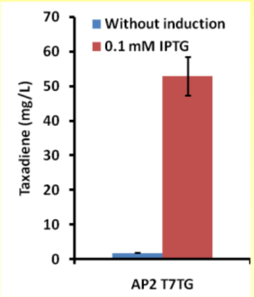
e

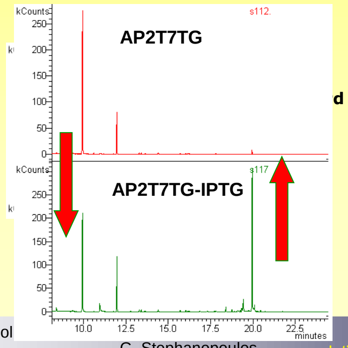
1. Ep20TrcGT
2. ECh1TrcMEPp20GT
3. Ep5TrcMEPp20TrcGT
4. Ep10TrcMEPp20TrcGT
5. Ep20TrcTG
6. Ep20T5GT
7. Ep20T5GTTrcT
8. ECh1TrcMEPp20TrcTG
9. ECh1TrcMEPp20T5GT
10. ECh1TrcMEPp20T5GTTrcT
11. Ep5TrcMEPp20TrcTG
12. Ep5TrcMEPp20T5GT
13. Ep5TrcMEPp20T5GTTrcT
14. Ep10TrcMEPp20TrcTG
15. Ep10TrcMEPp20T5GT
16. Ep10TrcMEPp20T5GTTrcT
17. EDE3p10TrcMEPp5T7TG
18. EDE3p20TrcMEPp5T7TG
19. EDE3p20T5MEPp5T7TG
20. EDE3p20T7MEPp5T7TG
21. EDE3p5TrcMEPp10T7TG
22. EDE3p20TrcMEPp10T7TG
23. EDE3p20T5MEPp10T7TG
24. EDE3p20T7MEPp10T7TG
25. EDE3p5T7TG
26. EDE3Ch1TrcMEPp5T7TG
27. EDE3Ch1T5MEPp5T7TG
28. EDE3Ch1T7MEPp5T7TG
29. EDE3p10T7TG
30. EDE3Ch1TrcMEPp10T7TG
31. EDE3Ch1T5MEPp10T7TG
32. EDE3Ch1T7MEPp10T7TG

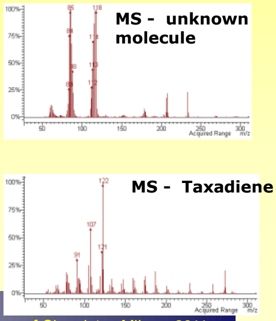
Why the optimal expression for high taxadiene production?

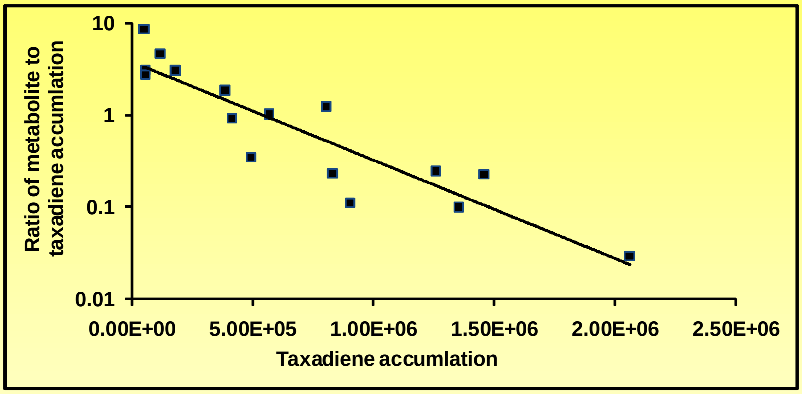
Presence of unknown inhibitory molecule in the pathway





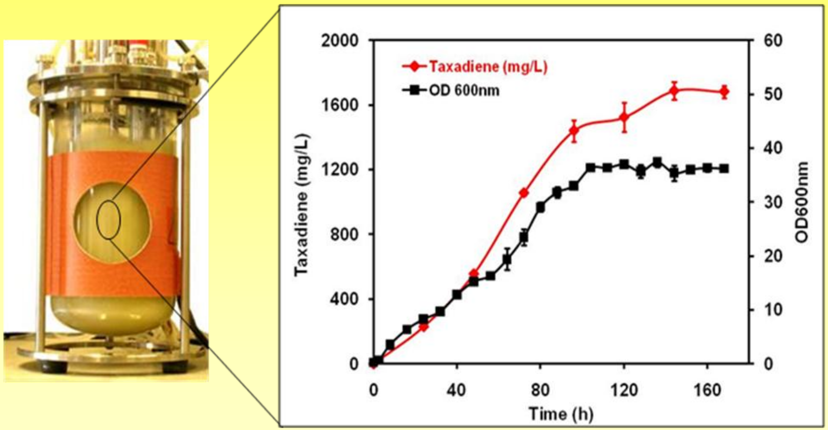






Science, **330:** 70-74 (2010).

Fermentation of taxadiene producing strain AP2T7TG

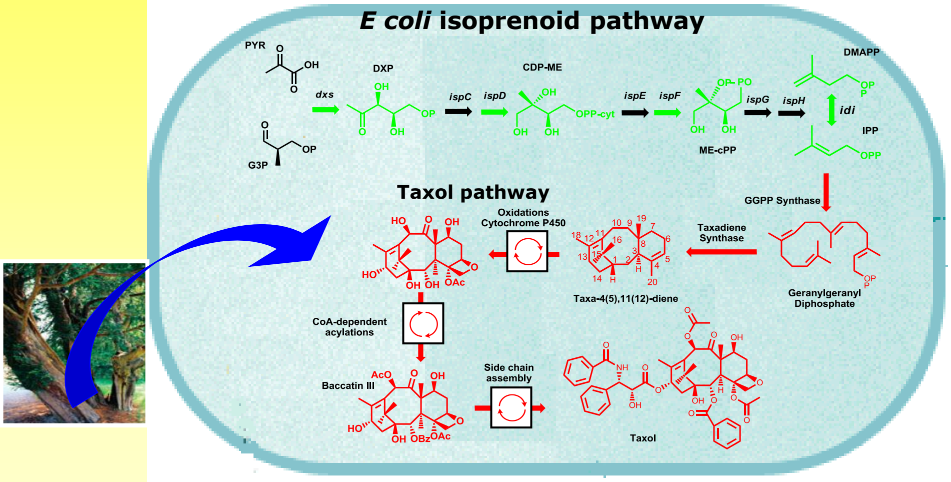


Science, **330:** 70-74 (2010)

- **Taxadiene production: ~1,700 mg/L**

Engineering Taxol biosynthetic pathway in *E. coli*

– most challenging and complex chemistry in natural products



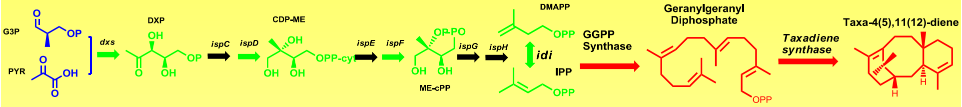
19- enzymes from IPP and DMAPP

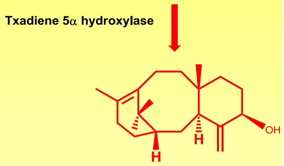
2- synthase
8 – hydroxylase
5 – transferase

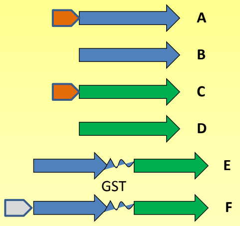
2- oxidase
1 - Aminomutase
1 - Co-ligase

Next Step: Taxadiene to Taxa-4(20),11,12-dien-5a-ol

Engineering P450 hydroxylases towards the biosynthesis of taxol

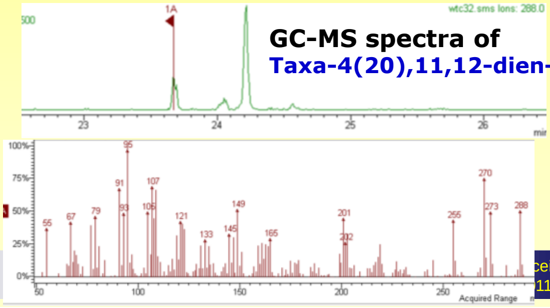




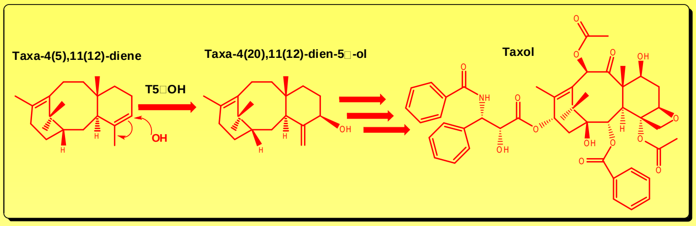


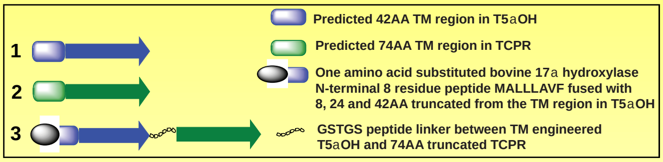
The N-terminal of the P450 and P450 reductases truncated and constructed fusion protein with linker for optimal expression in *E coli*

- ⬠ mammalian-derived leader sequence, MALLLLAVF, the eight-residue mammalian peptide derived from the N-terminal region of the bovine 17a hydroxylase
- ⬠ N-terminal region identified for 45 Amino acid and 74 Amino acid truncations in Taxoid 5α-hydroxylase and Taxoid NADPH p450 reductase

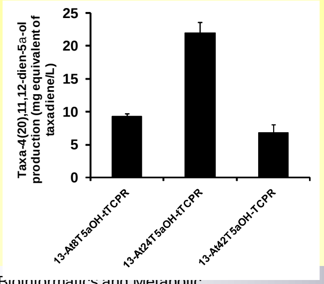


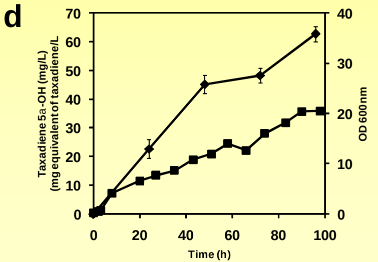
GC-MS spectra of
Taxa-4(20),11,12-dien-5a-ol





- Predicted 42AA TM region in T5aOH
- Predicted 74AA TM region in TCPR
- One amino acid substituted bovine 17a hydroxylase N-terminal 8 residue peptide MALLLAVF fused with 8, 24 and 42AA truncated from the TM region in T5aOH
- GSTGS peptide linker between TM engineered T5aOH and 74AA truncated TCPR





Example 3

Engineering *Escherichia coli* to overproduce tyrosine directly from glucose

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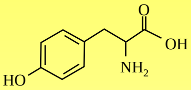
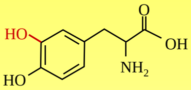
L-tyrosine is a valuable compound

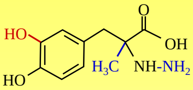
Dietary supplement



L-tyrosine

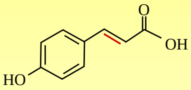


L-DOPA (levodopa)



Carbidopa

Parkinson's disease treatment



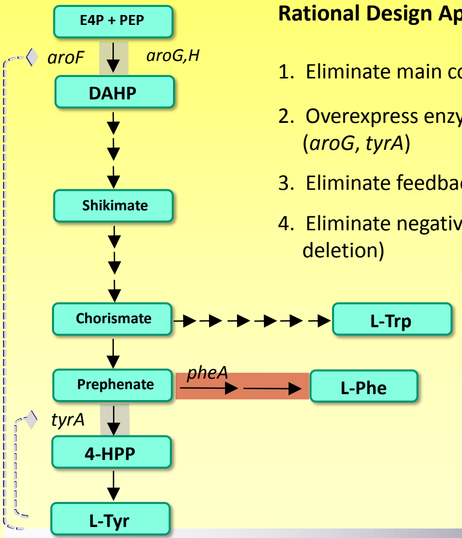
p-coumaric acid

Specialty chemicals and polymers

Flavonoids

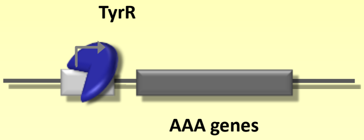
anti-allergic
anti-inflammatory
antimicrobial
anti-cancer
antioxidant

Aromatic amino acid biosynthetic pathway



Rational Design Approaches

1. Eliminate main competing reactions (*pheA* deletion)
2. Overexpress enzymes constituting major bottlenecks (*aroG*, *tyrA*)
3. Eliminate feedback repression of enzymes (*aroG*^{fbr}, *tyrA*^{fbr})
4. Eliminate negative transcriptional regulator (TyrR deletion)



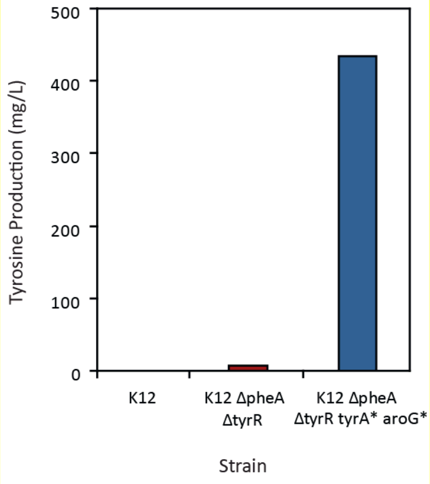
Rational design is a good start…

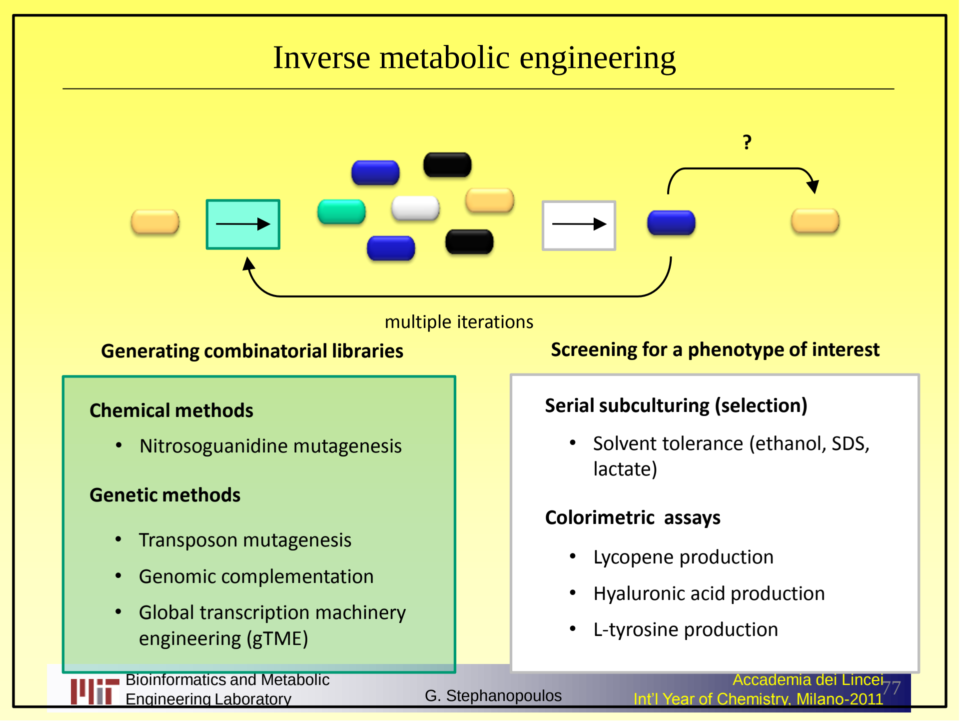
Rational Design Approaches

1. Eliminate main competing reactions (*pheA* deletion)
2. Eliminate negative transcriptional regulator (TyrR)
3. Overexpress enzymes constituting major bottlenecks (*aroG*, *tyrA*)
4. Eliminate feedback repression of enzymes (*aroG*^{fbr}, *tyrA*^{fbr}) (T. Lutke)

… but can Inverse Metabolic Engineering get us further?

Rationally-Designed Strains





Inverse metabolic engineering

multiple iterations

Generating combinatorial libraries

Screening for a phenotype of interest

Chemical methods

- Nitrosoguanidine mutagenesis

Genetic methods

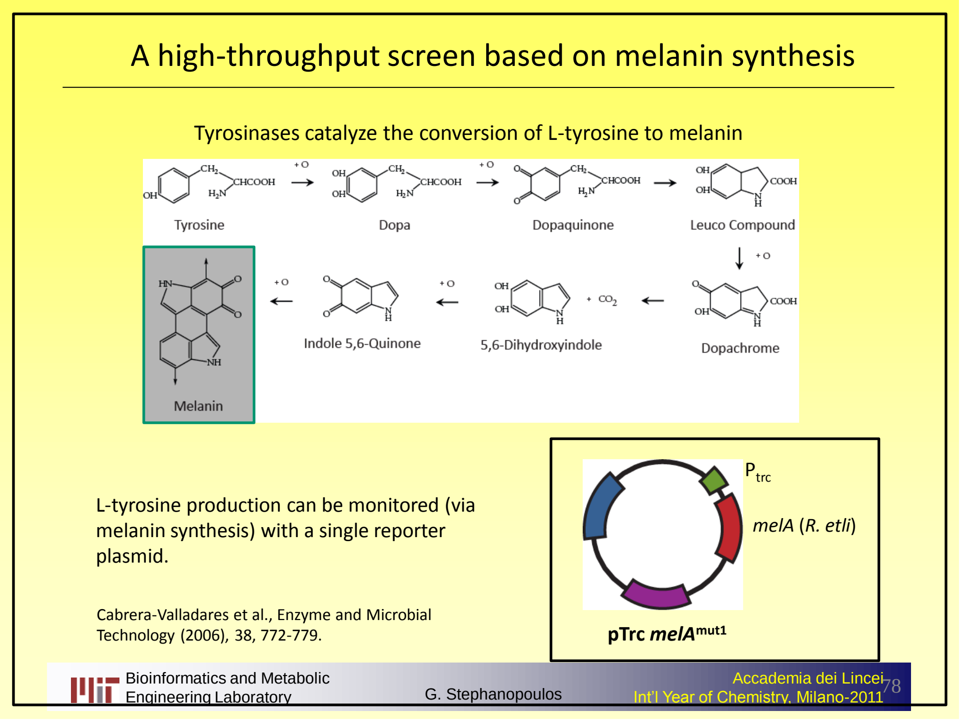
- Transposon mutagenesis
- Genomic complementation
- Global transcription machinery engineering (gTME)

Serial subculturing (selection)

- Solvent tolerance (ethanol, SDS, lactate)

Colorimetric assays

- Lycopene production
- Hyaluronic acid production
- L-tyrosine production



A high-throughput screen based on melanin synthesis

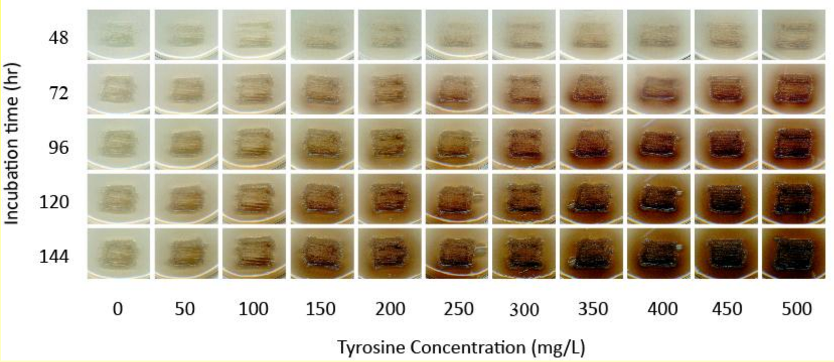
Tyrosinases catalyze the conversion of L-tyrosine to melanin

L-tyrosine production can be monitored (via melanin synthesis) with a single reporter plasmid.

Cabrera-Valladares et al., Enzyme and Microbial Technology (2006), 38, 772-779.

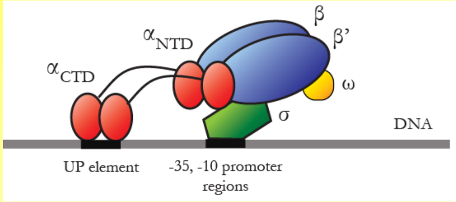
Melanin synthesis on agar plates

Plates with different L-tyrosine concentrations can be easily differentiated based on the amount of melanin produced.



Global Transcription Machinery Engineering (gTME)

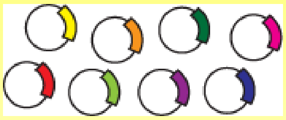
RNA polymerase



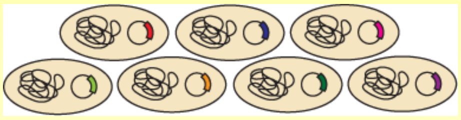
rpoA – α subunit *rpoD* – sigma factor 70

rpoA^{WT}/ *rpoD*^{WT}

Error-prone PCR



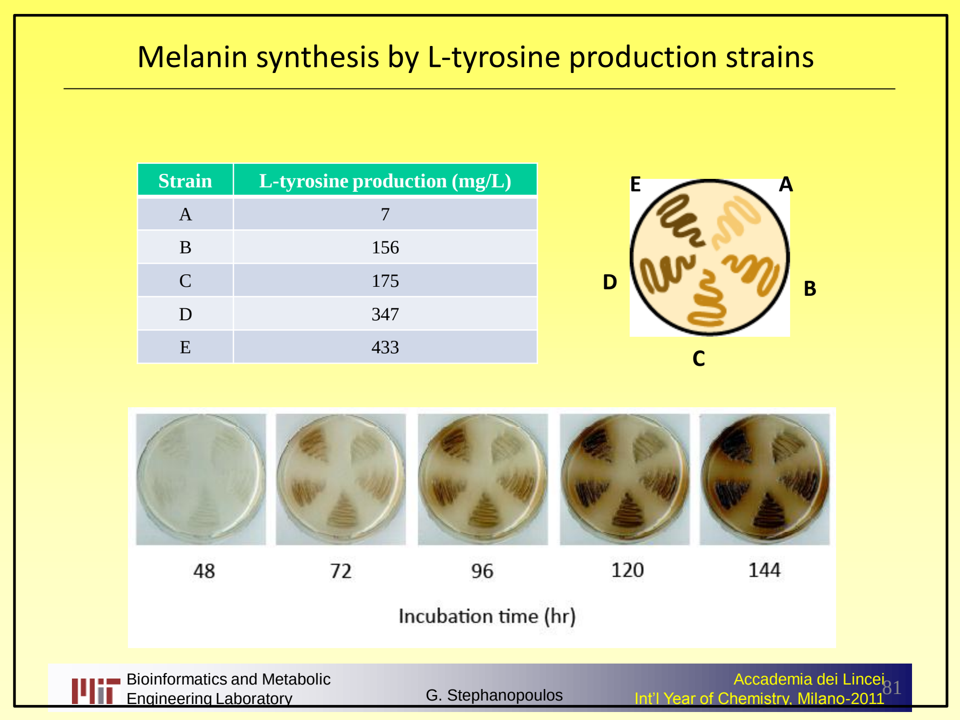
Transform into *E. coli*



rpoA library size: 7.5×10^5 viable colonies

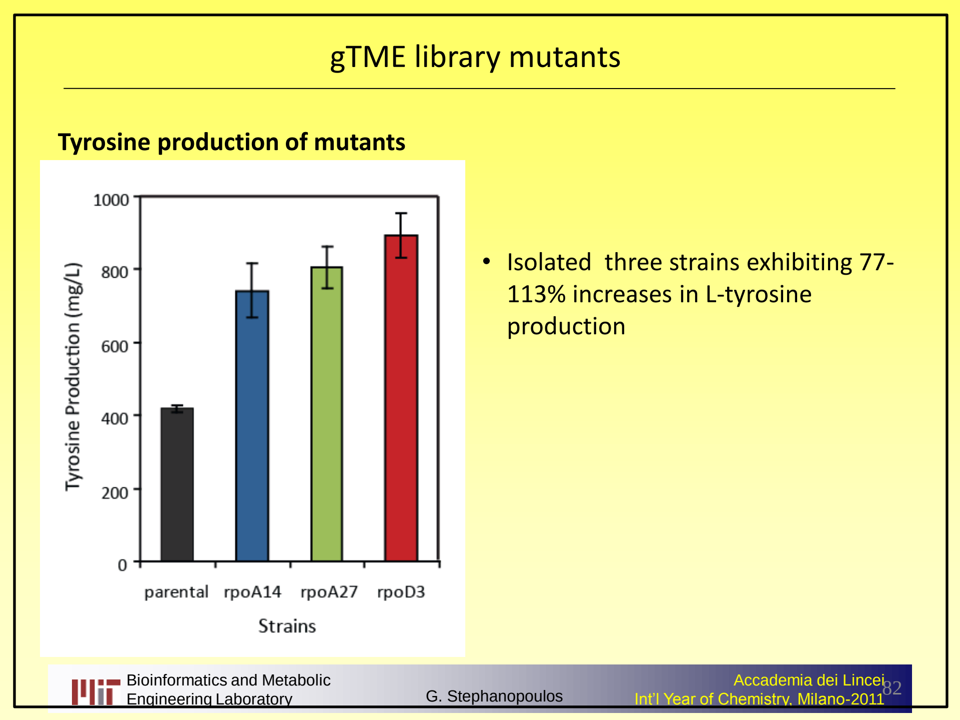
rpoD library size: 3.1×10^6 viable colonies

Parental strain: K12 Δ*pheA* *tyrR*::*tyrA*^{fbr}*aroG*^{fbr} *lacZ*::*tyrA*^{fbr}*aroG*^{fbr}



Melanin synthesis by L-tyrosine production strains

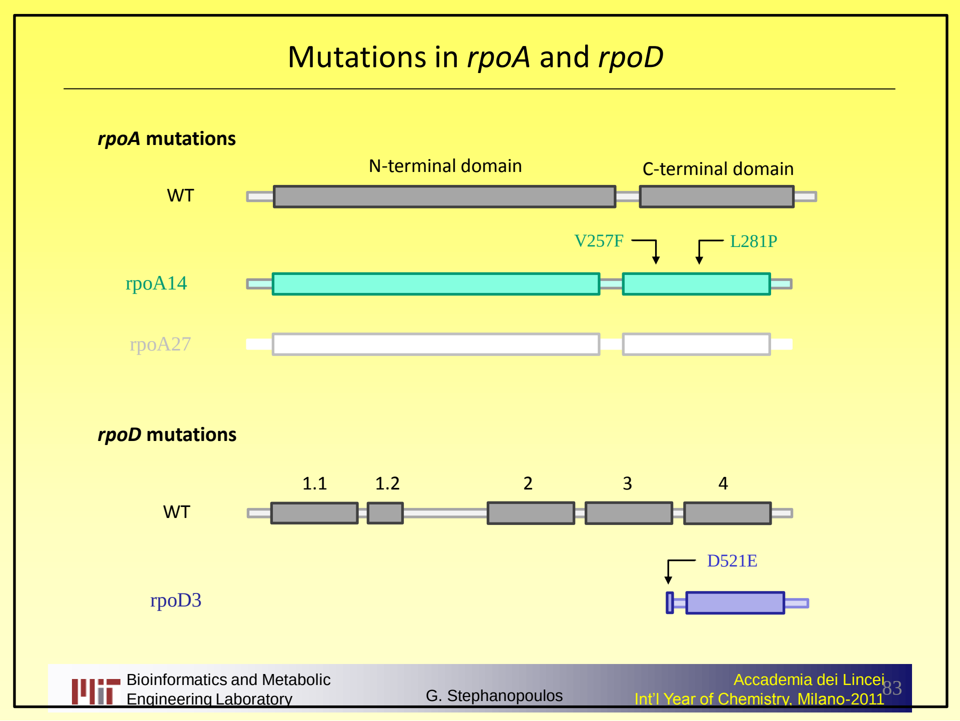
Strain	L-tyrosine production (mg/L)
A	7
B	156
C	175
D	347
E	433



gTME library mutants

Tyrosine production of mutants

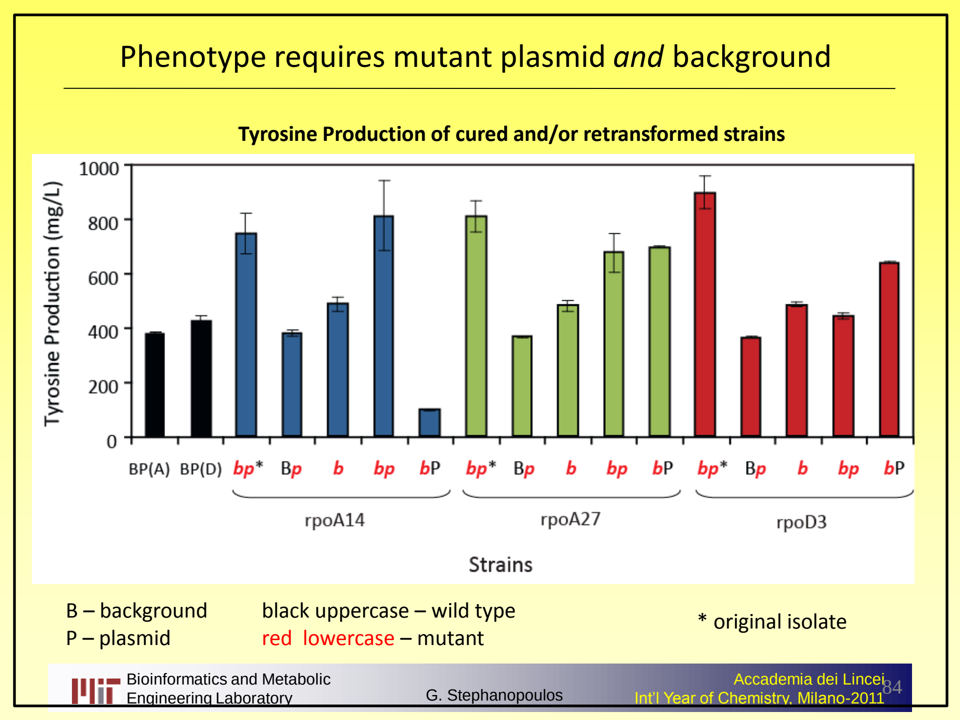
- Isolated three strains exhibiting 77-113% increases in L-tyrosine production



Mutations in *rpoA* and *rpoD*

***rpoA* mutations**

***rpoD* mutations**



Phenotype requires mutant plasmid *and* background

Tyrosine Production of cured and/or retransformed strains

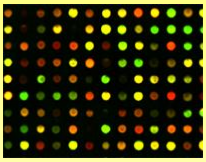
B – background
P – plasmid

black uppercase – wild type
red lowercase – mutant

* original isolate

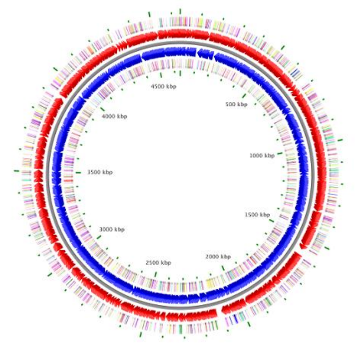
‘Omics approaches for strain characterization

Transcriptional analysis (microarrays)



Look for patterns of differential gene expression

Whole genome sequencing



Scan the entire genome for sequence variations

Summary: strain equivalences

Original isolates (~1,000 mg/L of tyrosine) =

Mutant background + *rpoA* or *rpoD* mutant plasmids =

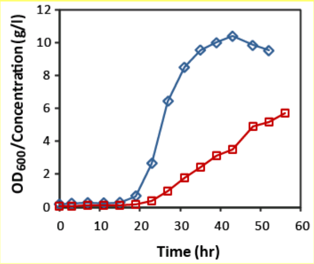
Mutant background + overexpression of *EvgA* or *RelA* (regulators of AR or stringent response via ppGpp) =

Wild type *E. coli* harboring *HisA* or *PurF* mutations + mutant plasmids or *EvgA/RelA* overexpression

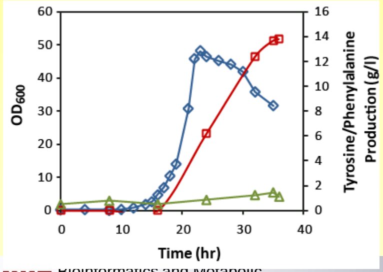
(a completely genetically defined strain overproducing tyrosine)

1.5-l Fermentations in MOPS and R media

MOPS minimal medium



R medium



Media Formulation	MOPS	MOPS	R
Fermentation scale	50 ml	1.5-l	1.5-l
L-tyrosine	902 mg/l	5.71 g/l	13.8 g/l
Glucose consumed (g)	5 g	28	115
Yield (g Tyr/g Glc)	0.180	0.204	0.120
Maximum Productivity (mg Tyr/g DCW/hr)	-	92.6	188
Maximum Productivity (g Tyr/L/hr)	-	0.280	2.06
Growth rate (hr^{-1})	0.296	0.275	0.405
Maximum OD_{600}	3.72	10.4	48.1

Trade-off between yields and maximum productivities/titers

◇ OD_{600} □ L-tyrosine △ L-phenylalanine

IV. What is in the future?

Future applications drivers

- **Sustained interest in utilization of renewable resources**
  - **Pressure on commodities will continue**
  - **Climate change concerns will persist**
  - **Biotechnology is better than chemistry in utilizing carbohydrates**

Future applications drivers

- **New technology push:**
  - **Chemical synthesis of heterologous genes**
  - **Increased appreciation of systemic approaches to pathway engineering (mind-frame of *Systems Biology*)**
  - **Increased experimentation with pathway construction harboring random DNA combinations (Synthetic DNA)**
  - **Inverse Metabolic Engineering**
  - **Development of High-Throughput screens for chemicals production**

Future directions of ME-1

- **Expand portfolio with numerous new applications:**
  - Invasion to the core of the chemical industry at oil prices greater than $100/bbl (xylenes, terpenes, isoprene, butadiene,…)
  - Best technology for specialty chemicals (specific oxidations, acylations, amidations, stereo-specific compounds, API's, …)
  - Tremendous *diversity* of new products (isoprenoid pathway, glycosylated compounds,

---

Future directions of ME-2

Providing platform
for discovery *and* production
of new therapeutics

The end

Questions?

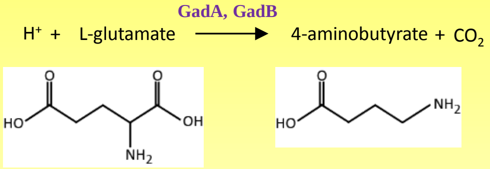
Upregulated genes identified by microarrays

			rpoD3	**rpoA27**
Gene Name	**Gene ID**	**Function**	**Log Fold Change**	**Log Fold Change**
b3517	*gadA*	glutamate decarboxylase	2.236	1.974
b1493	*gadB*	glutamate decarboxylase	2.699	2.515
b1492	*gadC*	glutamate:γ-aminobutyrate antiporter	2.027	-
b3512	*gadE*	transcriptional activator	2.137	2.160
b3510	*hdeA*	acid stress chaperone	1.950	-
b3509	*hdeB*	acid stress chaperone	2.115	-
b3511	*hdeD*	acid resistance membrane protein	1.707	-
b3506	*slp*	starvation lipoprotein	1.609	1.897

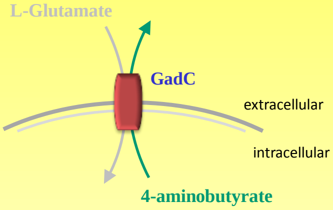
- 4 out of 7 upregulated genes in rpoA27 were related to acid resistance
- 8 out of 17 upregulated genes in rpoD3 were related to acid resistance

Acid resistance (AR) proteins and function

***gadABC* – glutamate-dependent AR system**



$$H^+ + \text{L-glutamate} \xrightarrow{\text{GadA, GadB}} \text{4-aminobutyrate} + CO_2$$



***hdeAB* – acid stress chaperone proteins**

- Prevent aggregation of perisplasmic proteins at acidic pH
- Form mixed aggregates with proteins that have failed to solubilize at acidic pH and allow their subsequent solubilization at neutral pH

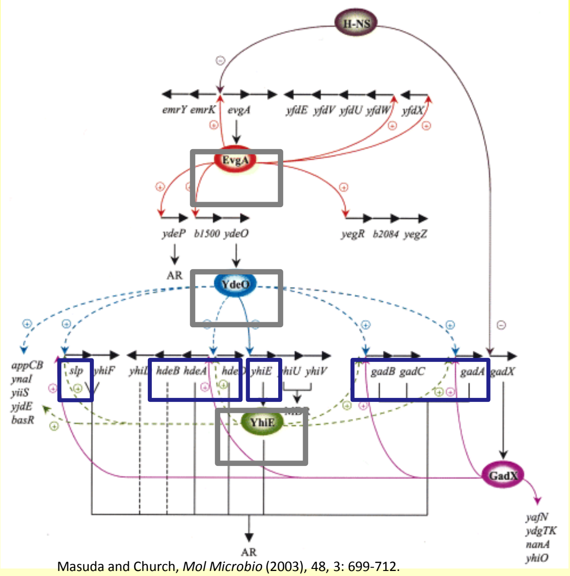
Transcriptional network for acid resistance system

Upregulated Genes
gadA
gadB
gadC
gadE (*yhiE*)
hdeA
hdeB
hdeD
slp

Transcriptional regulators

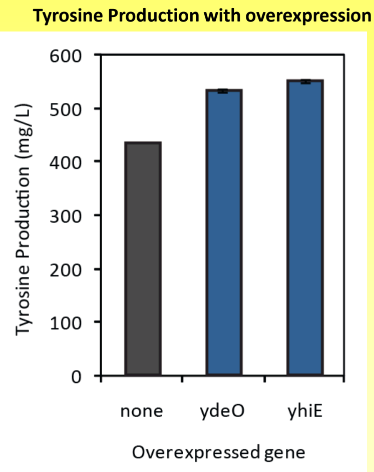
EvgA, YdeO

GadE (YhiE)



Masuda and Church, *Mol Microbio* (2003), 48, 3: 699-712.

Overexpression of acid resistance regulators

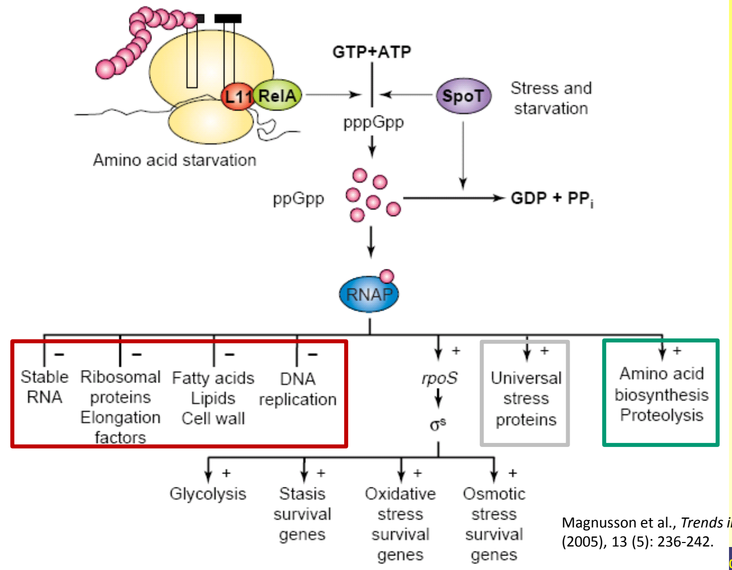

Tyrosine Production with overexpression

Only modest (22-26%) increases in L-tyrosine production

Downregulated pathways identified by microarrays

Pathway	rpoD3	rpoA14	rpoA27
Arginine synthesis	✓	✓	✓
Isoleucine synthesis	✓	✓	✓
Leucine/valine synthesis	✓	✓	✓
Histidine synthesis	✓		✓
Tryptophan synthesis	✓		✓
Lysine synthesis	✓	✓	
Glutamate synthesis	✓	✓	✓
De novo purine/pyrimidine biosynthesis	✓		
DNA replication	✓		✓
Ribosomal proteins and RNA	✓	✓	✓
Fatty acid elongation		✓	

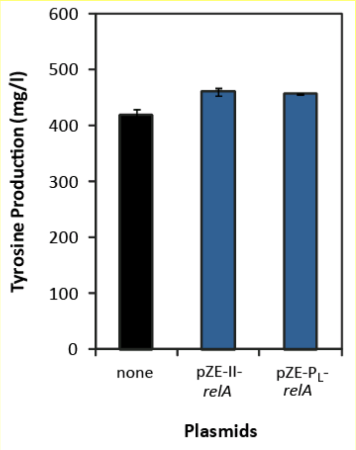
A role for (p)ppGpp and the stringent response



Magnusson et al., *Trends in Microbiology* (2005), 13 (5): 236-242.

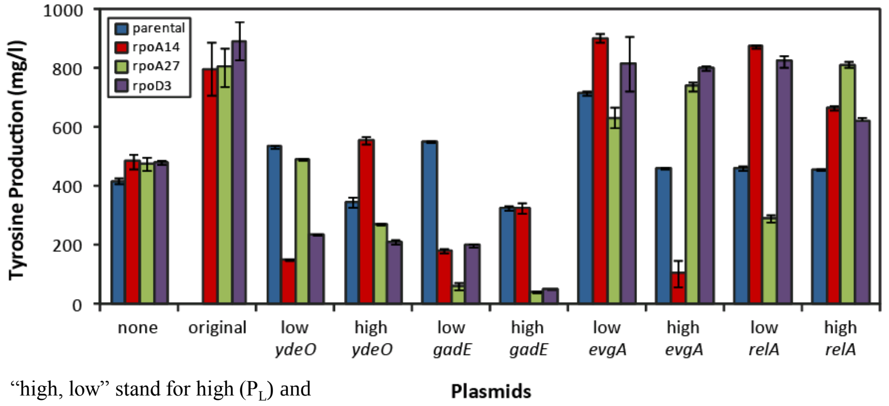
Overexpression of *relA*

Tyrosine production with *relA*



Only modest increases (~25%) in L-tyrosine production

Overexpression of targets in mutant backgrounds



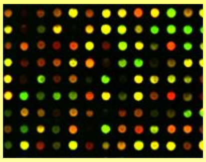
"high, low" stand for high (P_L) and low strength promoter

EvgA, YdeO, GadE – acid resistance transcriptional regulators

RelA – ppGpp synthetase

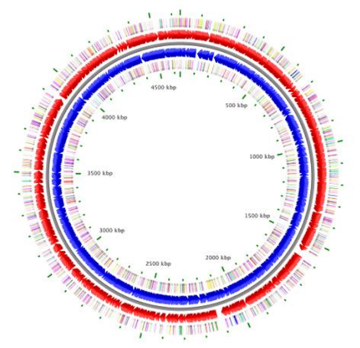
'Omics techniques for strain characterization

Transcriptional analysis (microarrays)



Look for patterns of differential gene expression

Whole genome sequencing

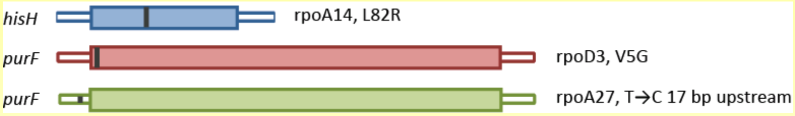


Scan the entire genome for sequence variations

Whole Genome Sequencing – SNP summary

Position	rpoD3		rpoA14		rpoA27		Annotation
	R	S	R	S	R	S	
2092803			T	G			*hisH*; imidazole glycerol phosphate synthase subunit, glutamine amidotransferase (histidine biosynthesis)
2428247	A	C					*purF*; amidophosphoribosyl transferase (*de novo* purine biosynthesis)
2428277					T	C	

R: Reference, S: Substituted base pair



- Histidine and purine biosynthetic pathways have two shared precursors/intermediates
- Both *hisH* and *purF* share similar enzyme functions and glutamate utilization

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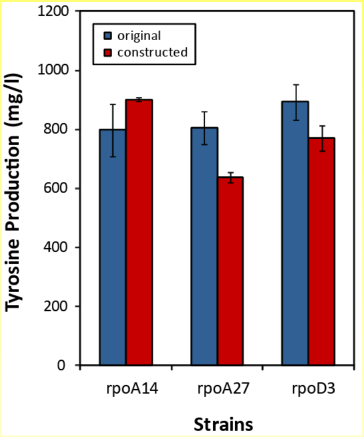
Verification of SNPs

Tyrosine Production with *purF* and *hisH* mutations



All mutations are capable of enhancing L-tyrosine production when combined with *rpoA*/*rpoD* expression