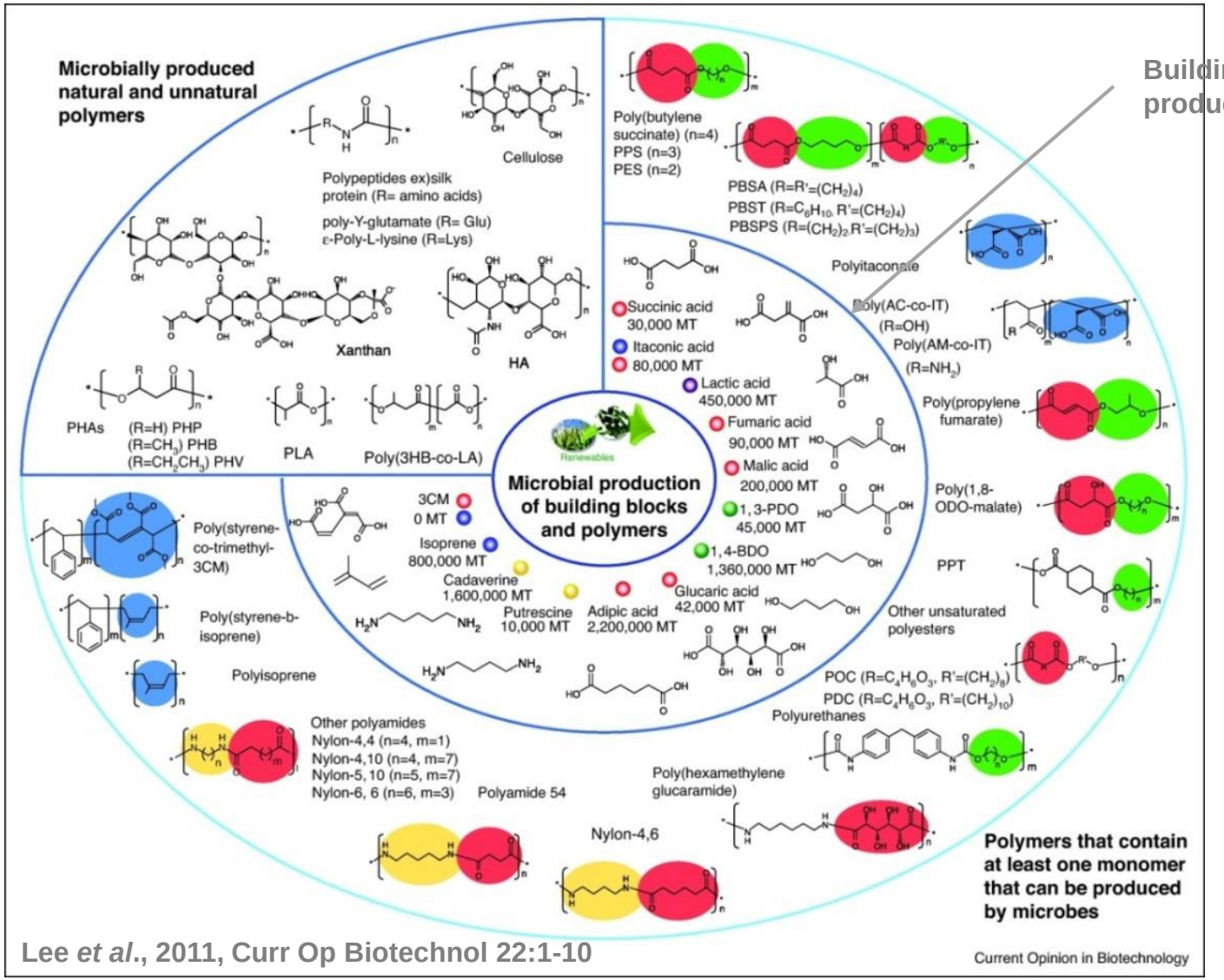
Metabolic engineering
Engineering of cell factories for industrial biotechnology II

Lecture 10

Biopolymers

- Microbially produced natural and unnatural polymers
  - Microbial cellulose
  - PHA (polyhydroxyalkanoates)
  - Silk (protein)
- Production of building blocks monomers by microbes
  - Lactic acid
  - Succinic acid
  - Isoprene
  - Glucaric acid
  - Monomers can be natural metabolites or biochemically tailored products

Microbially produced natural and non-natural chemical building blocks



Lee *et al*., 2011, Curr Op Biotechnol 22:1-10

Building blocks (Monomers) produced biotechnologically

Colored balls across layers indicate specific functional group(s) within chemical structures:
Red: dicarboxylic acids
Yellow: diamines
blue for alkenes or dienes
Purple: for carboxylic acids
Green: diols

Production of natural building block chemicals

- Most preferable way is to explore microorganisms in the nature that can efficiently produce them
  - Fumaric acid (*Rhizopus* sp)
  - itaconic acid (*Aspergilli*)
  - malic acid (*Aspergilli*)
  - lactic acid (*Lactobacilli*)
  - succinic acid (rumen bacteria)
- In order to reach commercially competitive titers
  - strain optimization by mutagenesis and screening
  - limited targeted strain engineering
  - bioprocess development

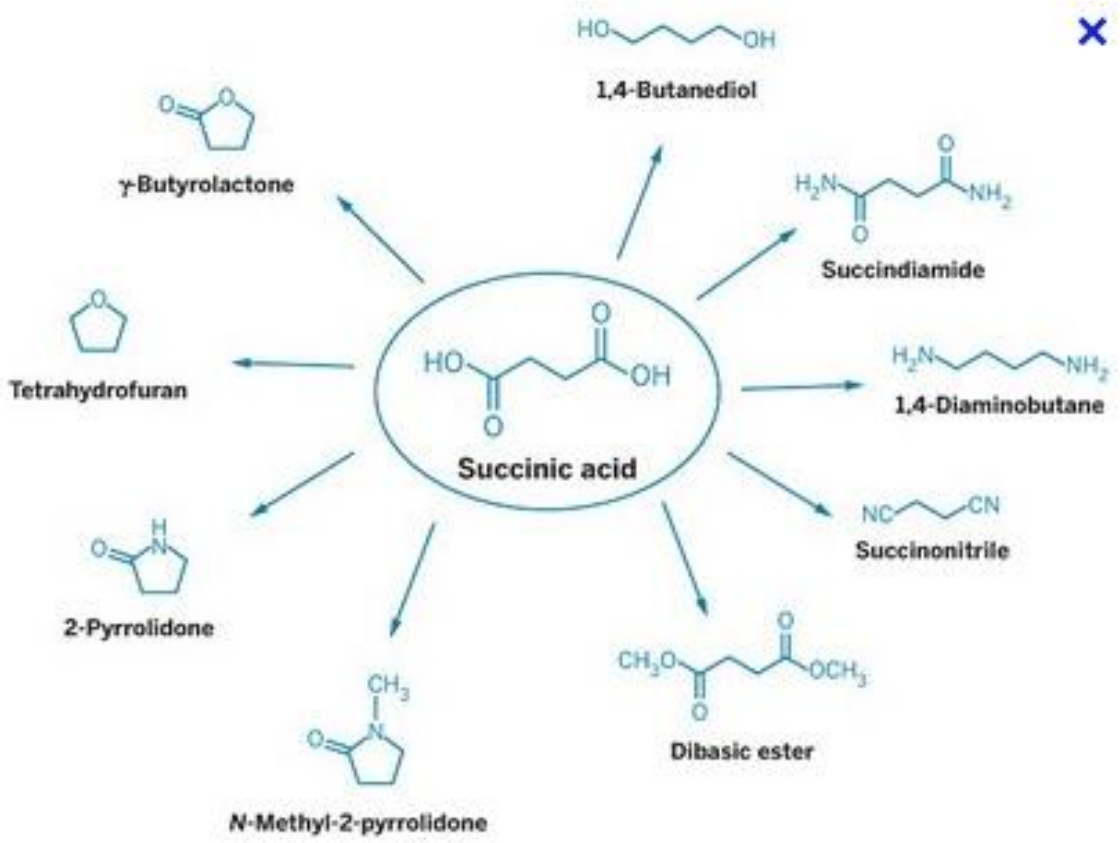
Succinic acid production in *E. coli*

Evolutionary metabolic engineering strategy

Microbial succinate production

- Fermentative production of succinate from renewable feedstock
- Succinate as substrate for transformation into plastics, solvents and other chemicals
- Natural producers such as *Actinobacillus succinogenes* can produce succinate at high rates but require complex media components
- Many recombinant *E. coli* strain produce succinate
  - Deletion of endogenous genes
  - Overexpression of endogenous and heterologous genes
  - But require often complex media components

Some applications for succinic acids…



https://www.bio-amber.com/bioamber/en/products

http://www.myriant.com/products/bio-succinic-acid.cfm

Combining Metabolic Engineering and Metabolic Evolution to Develop Nonrecombinant Strains of *Escherichia coli* C That Produce Succinate and Malate

Kaemwich Jantama,[1] M.J. Haupt,[1] Spyros A. Svoronos,[1] Xueli Zhang,[2] J.C. Moore,[2] K.T. Shanmugam,[2] L.O. Ingram[2]

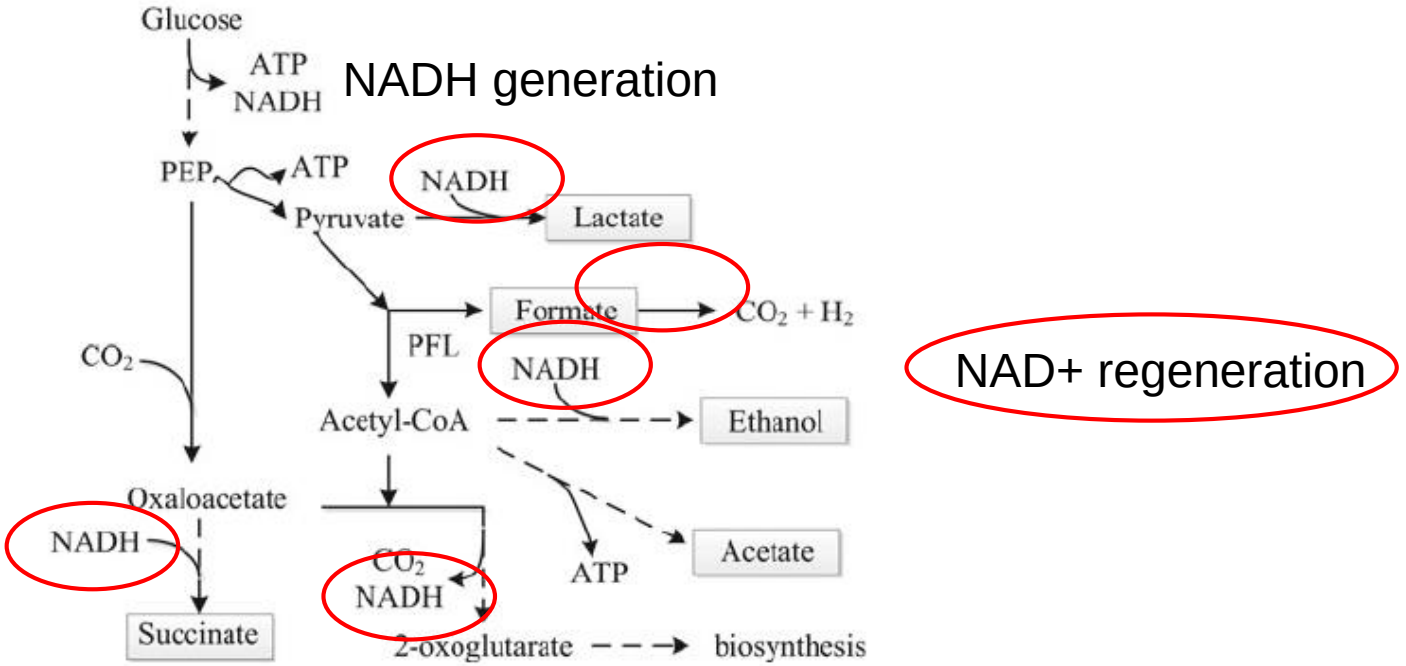
Biotechnology and Bioengineering, Vol. 99, No. 5, April 1, 2008

Goals:

- **Coupling of ATP production and growth to succinate and malate production**
- **No introduction of heterologous genes**

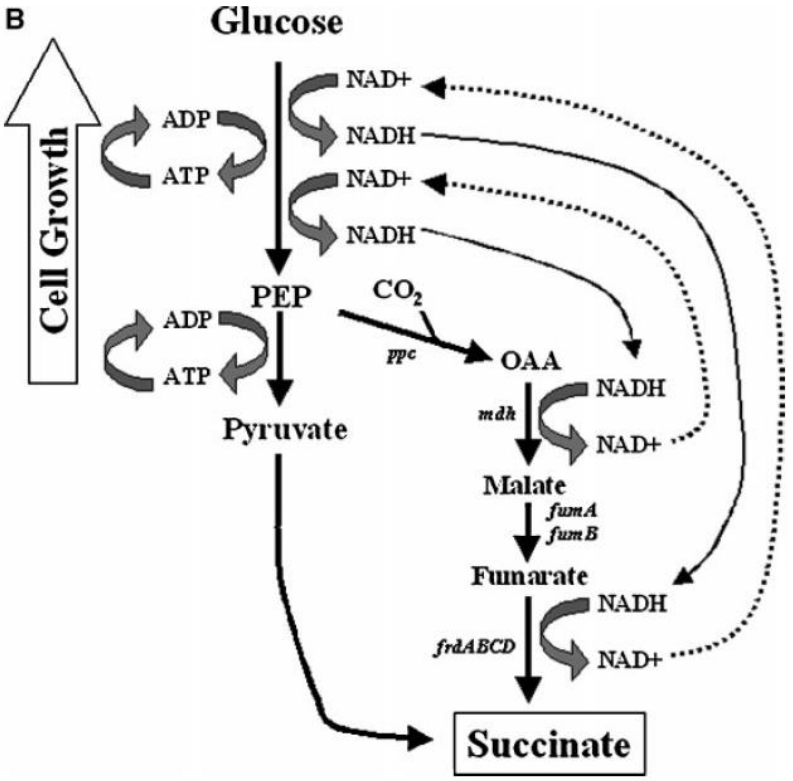
Fermentation of glucose to succinate

- During anaerobic growth in the absence of electron acceptors, glucose is fermented to form a mixture of products (boxed) including ethanol, acetate, lactate, succinate and formate by the model organism *E. coli* in order to regenerated NAD^+



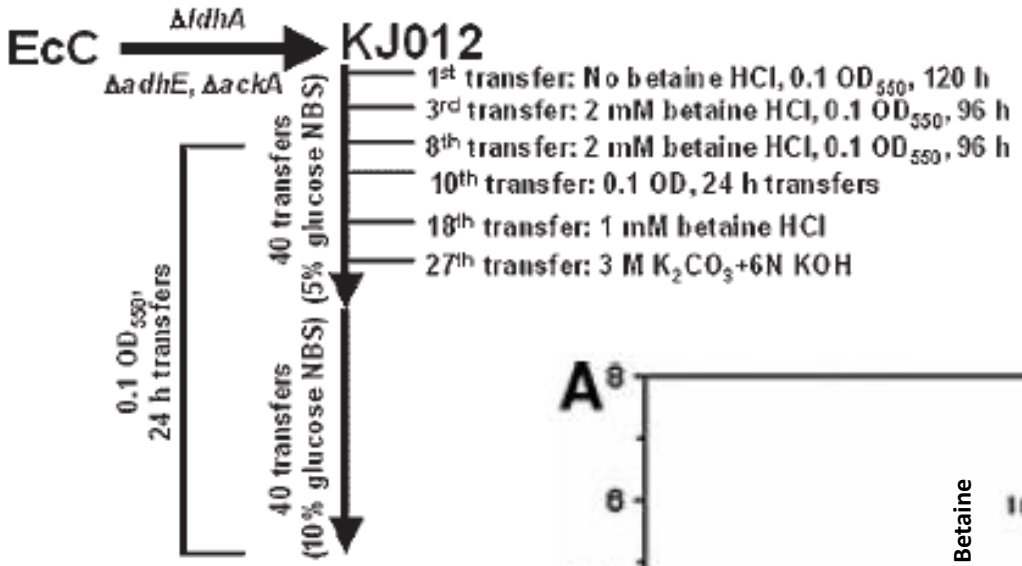
Coupling of ATP production and growth to succinate and malate production in engineered strains of *E. coli*

- In the deletion strain, no NAD^+ can be regenerated by fermentative pathways
- ATP production for growth is coupled to malate and succinate production during oxidation of NADH

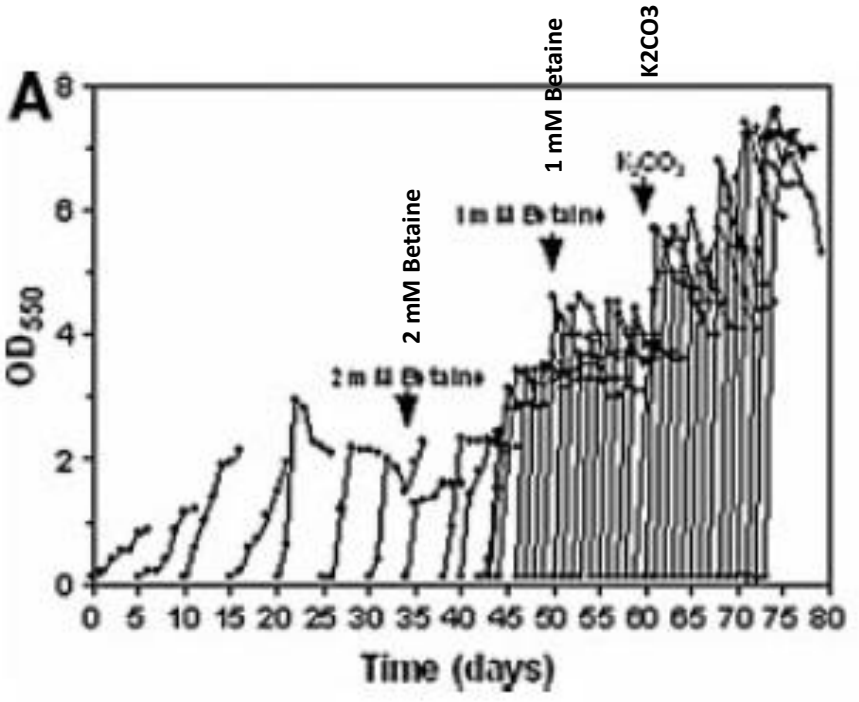


Selection for improved growth selects for improved production of succinate -> evolutionary metabolic engineering

Evolutionary metabolic engineering



CO2 used for succinate production via PEP carboxylase



Strain lacks:
Lactate dehydrogenase
Alcohol dehydrogenase
Acetate kinase

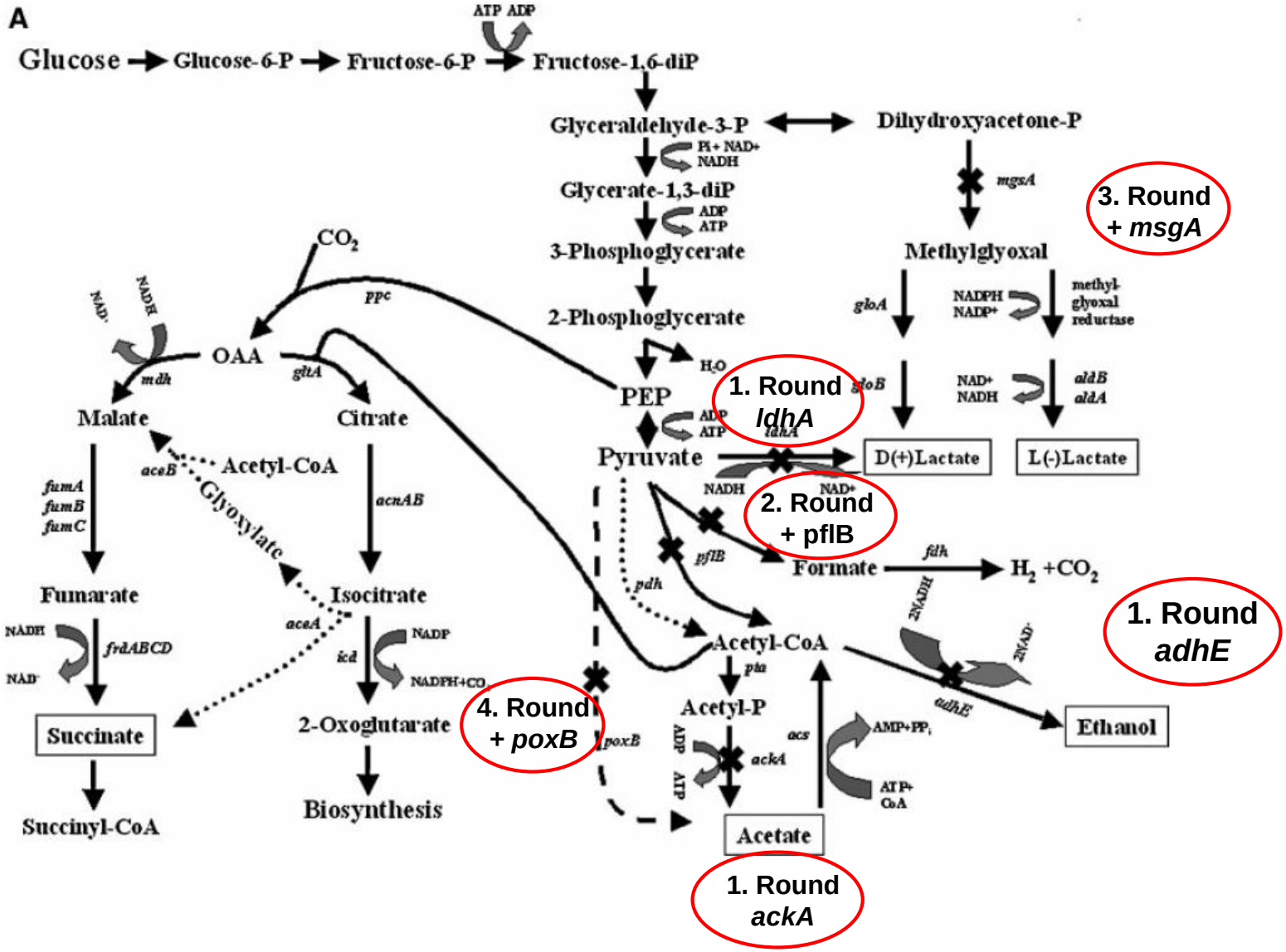
Evolutionary metabolic engineering results after 1. round

Strain[a]	Culture conditions	Media, gluc (w/v)	Cell yield[b] (g/L)	Succinate yield[c] mol/mol	Succinate yield[c] g/g	Average volumetric productivity[d] (g/L/h)	Fermentation products (mM)[e] Suc	Mal	Pyr	Ace	Lac	For
E. coli C wild type[f]	OD_{550}, 0.1 mM betaine	5%, NBS	2.0 ± 0.2	0.19 ± 0.02	0.12	0.12 ± 0.01	49 ± 3	—[g]	33 ± 10	152 ± 30	98 ± 24	262 ± 19
KJ012[f]	0.1 OD_{550}, 0.1 mM betaine	5% NBS	0.3 ± 0.1	0.20 ± 0.01	0.13	0.04 ± 0.01	6 ± 0.4	—	—	26 ± 1	—	—
KJ012	0.1 OD_{550}, 0.1 mM betaine shaken flask[h]	5% NBS + MOPS	1.5	0.10	0.06	0.02	10	—	—	226	—	16
KJ012	0.1 OD_{550} Luria Broth	5% LB	1.5	0.70	0.50	0.09	108	—	—	61	<2	14
KJ012 (*ldhA*, *ackA*, *adhE*) (KJ017)	1st TF: No betaine, 0.1 OD_{550}, 120 h transfers	5%, NBS	0.3	0.13	0.09	0.072	6	—	—	26	<2	—
	3rd TF: 2 mM betaine, 0.1 OD_{550}, 96 h transfers	5%, NBS	0.7	0.28	0.18	0.128	26	—	—	71	<2	—
	40th TF: 1 mM betaine, 0.1 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	5%, NBS	2.3	0.73	0.48	0.251	204	—	—	179	<2	151
	40th TF: 1 mM betaine, 0.1 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	10%, NBS	1.7	0.74	0.49	0.354	288	—	—	181	38	199

- Improved growth correlates with increased yield and productivity of succinate
- At 10% glucose accumulation of by-products, acetate, lactate and formate -> deletion of pflB

- Other deletions were introduced to reduce the carbon loss to undesired fermentation by-products

Fermentation of glucose to succinate



Fermentation of glucose to succinate

- These strains produced succinate titers of 668–733 mM, with a molar yield of 1.2–1.6 based on glucose metabolized
- Acetate, malate, and pyruvate accumulated as undesirable co-products and detracted carbon from the potential yield of succinate
- The maximum theoretical yield of succinate from glucose and CO_2 (excess) is 1.71 mol per mole glucose based on the following equation:

$$7\ C_6H_{12}O_6 + 6\ CO_2 \rightarrow 12\ C_4H_6O_4 + 6\ H_2O$$

- Deletion of additional five genes resulted in a strain producing nearly the maximal amount of succinate

Isoprene production

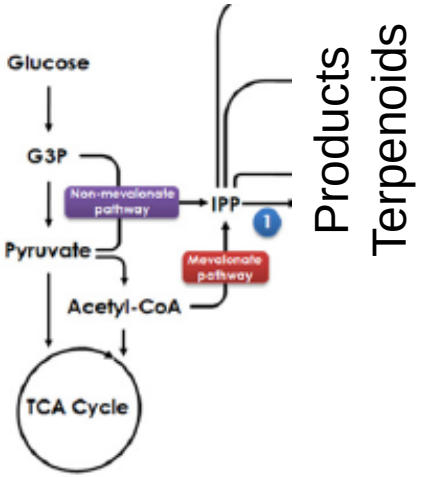
Effect of precursor molecule availability

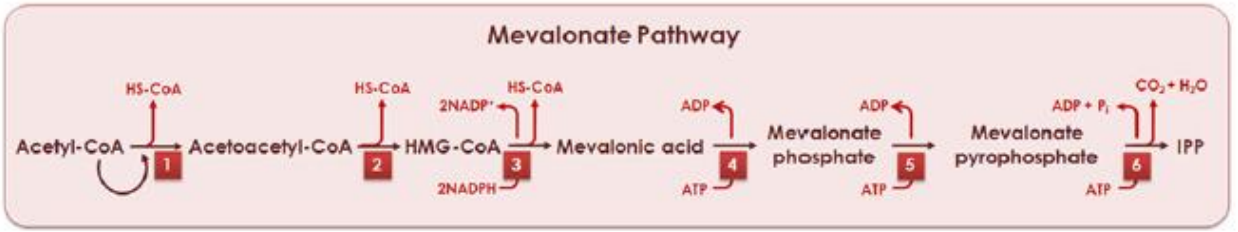
Isoprene production

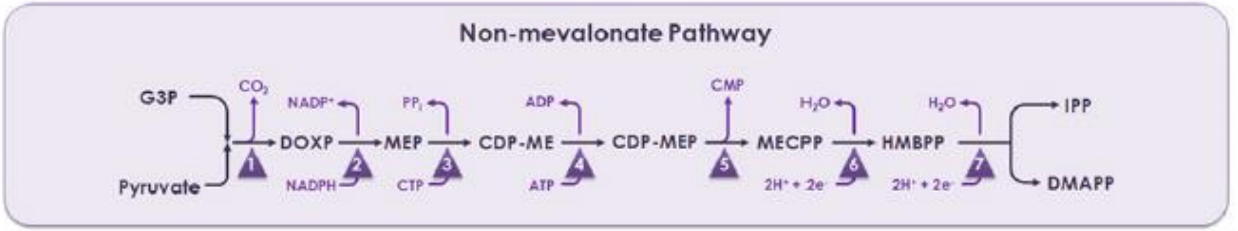
- In the biosphere isoprene is produced by plants
- Isoprene is one of the simplest members of isoprenoids, a valuable starting material for the synthesis of rubber, elastomers and isoprenoid medicines
- Isoprene is product of thermal cracking of oil
- Isoprene is volatile above 34°C

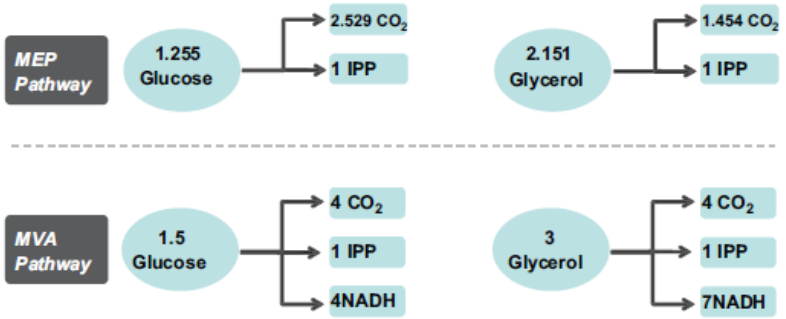
Pathways for isoprene production in *E. coli*

- The **mevalonate pathway** (MVA) and **2-C-methyl-D-erythritol 4-phosphate pathway** (MEP (=non-**mevalonate pathway**)) have been intensively studied and applied in the microbial production of isoprene.









Synthetic protein scaffolds provide modular control over metabolic flux

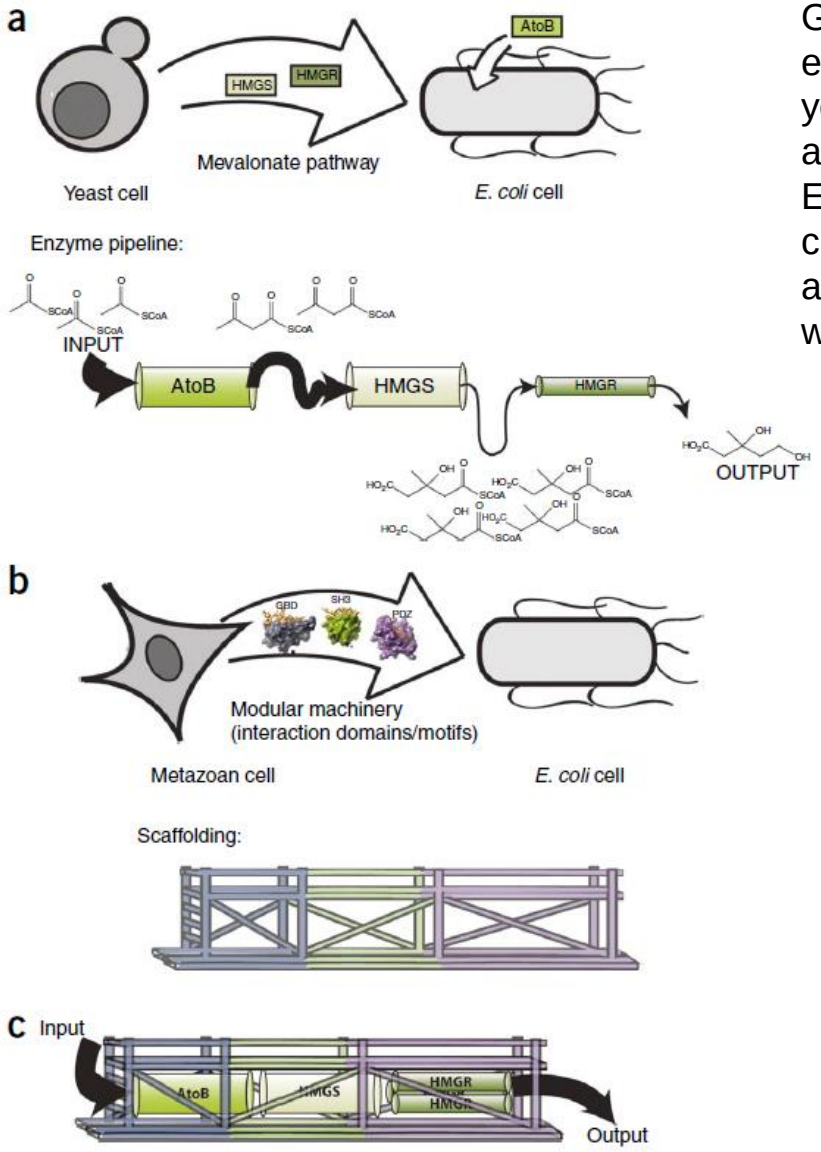
John E Dueber[1,2], Gabriel C Wu[1,2], G Reza Malmirchegini[1,9], Tae Seok Moon[3,4], Christopher J Petzold[5,6], Adeeti V Ullal[7], Kristala L J Prather[3,4] & Jay D Keasling[1,2,5–8]

Engineered metabolic pathways constructed from enzymes heterologous to the production host often suffer from flux imbalances, as they typically lack the regulatory mechanisms characteristic of natural metabolism. In an attempt to increase the effective concentration of each component of a pathway of interest, we built synthetic protein scaffolds that spatially recruit metabolic enzymes in a designable manner. Scaffolds bearing interaction domains from metazoan signaling proteins specifically accrue pathway enzymes tagged with their cognate peptide ligands. The natural modularity of these domains enabled us to optimize the stoichiometry of three mevalonate biosynthetic enzymes recruited to a synthetic complex and thereby achieve 77-fold improvement in product titer with low enzyme expression and reduced metabolic load. One of the same scaffolds was used to triple the yield of glucaric acid, despite high titers (0.5 g/l) without the synthetic complex. These strategies should prove generalizeable to other metabolic pathways and programmable for fine-tuning pathway flux.

NATURE BIOTECHNOLOGY VOLUME 27 NUMBER 8 AUGUST 2009

Generate scaffold to arrange the enzymes in close proximity and correct stoichiometry

Target the first 3 steps of the mevalonate pathway (MVA) using genes from *E. coli* and *S. cerevisiae*

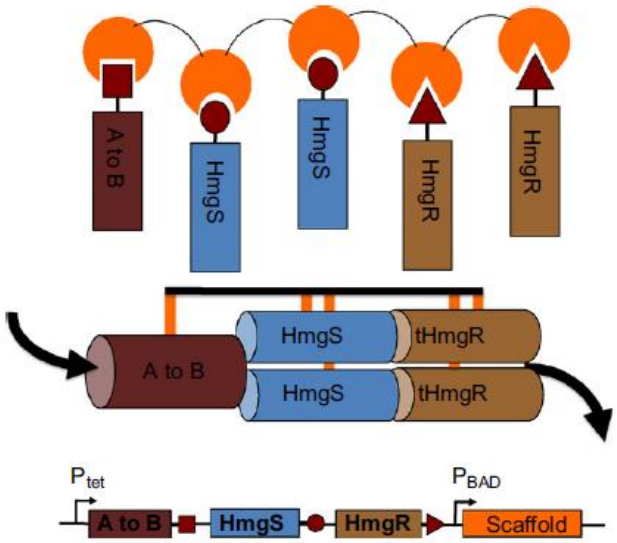


Genes encoding the mevalonate pathway enzymes (HMGS and HMGR) were taken from yeast (*S. cerevisiae*) and inserted into *E. coli* along with the *E. coli* gene encoding AtoB. Enzymes have different levels of activity, creating a bottleneck that results in accumulation of the intermediate HMG-CoA, which is toxic to *E. coli* at high concentrations

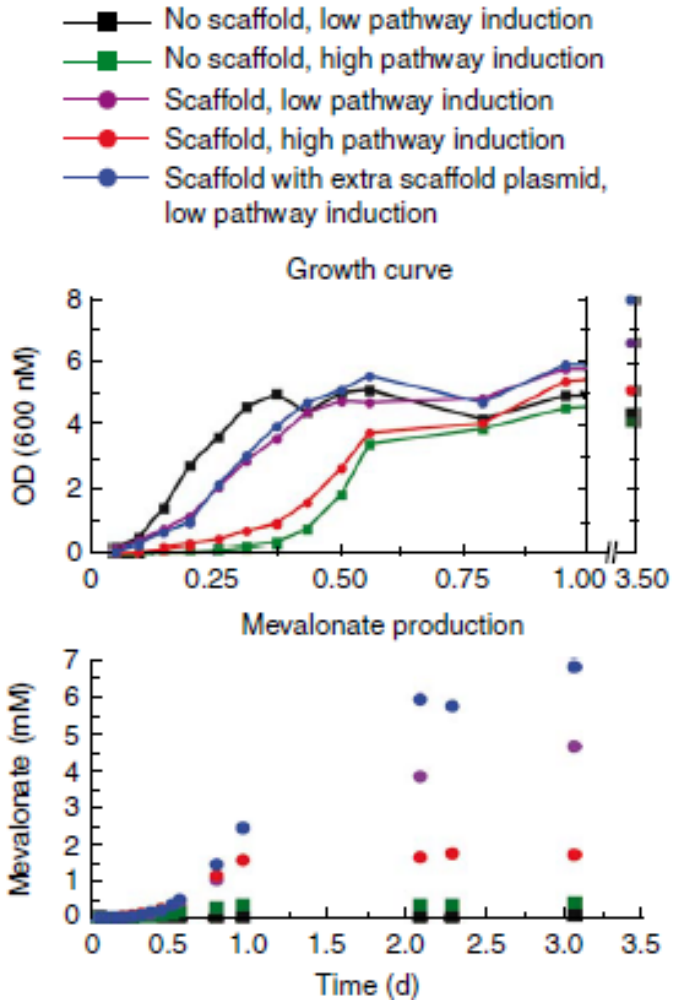
Protein-protein interaction domains and ligands from metazoan cells (mouse SH3 and PDZ domains and rat GBD)

The scaffolded pathway is more efficient as a result of co-localizing the mevalonate enzymes to the same complex as well as optimizing the enzyme stoichiometry to balance the units of activity at the complex

Scaffold structure and genetic construct used to arrange the three first enzymes of the mevalonate-based IPP biosynthetic pathway



- Reduce spatial distribution of enzymes
- Increases local concentration of substrate
- Modulate enzyme stoichiometry



Improved efficiency from pathway scaffolding allows higher titers to be achieved with faster growth of the production host.

High induction of the mevalonate biosynthetic pathway results in HMG-CoA accumulation which is inhibitory.

OPEN ACCESS Freely available online

PLOS ONE

Combination of Entner-Doudoroff Pathway with MEP Increases Isoprene Production in Engineered *Escherichia coli*

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1 Energy and Environment Fusion Technology Center, Department of Energy and Biotechnology, Myongji University, Cheoin-gu, Yongin-si, Gyeonggi-do, Republic of Korea, **2** Division of Bioscience and Bioinformatics, Myongji University, Cheoin-gu, Yongin-si, Gyeonggi-do, Republic of Korea

Liu *et al*., 2013, PLOS 8:e83290

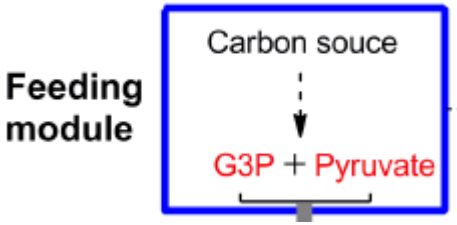
Goal:

Four glycolytic pathways,

- Embden-Meyerhof pathway (EMP),
- Entner-Doudoroff Pathway (EDP),
- Pentose Phosphate Pathway (PPP) and
- Dahms pathway

were tested as MEP feeding modules for isoprene production.

These pathways differ in terms of their modes of G3P and pyruvate generation.

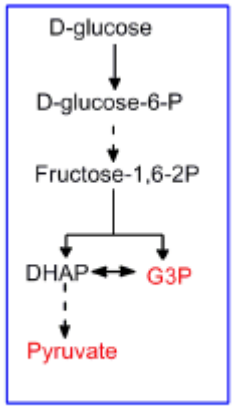


Improving isoprene production by optimal feeding of precursor molecules

- MEP starts with the condensation of pyruvate and glyceraldehyde-3-phosphate (G3P) to form 1-deoxy-D-xylulose-5-phosphate (DXP) and is typically found in most bacteria.
- study particularly aims to find a more efficient feeding module for MEP that would improve the isoprene production
- Four glycolytic pathways in *E*. *coli* were investigated
  - (1) Embden–Meyerhof–Parnas pathway (EMP)
  - (2) Entner-Doudoroff pathway (EDP)
  - (3) Pentose Phosphate Pathway (PPP)
  - (4) Dahms pathway
- These pathways differ in terms of their modes of G3P and pyruvate generation.

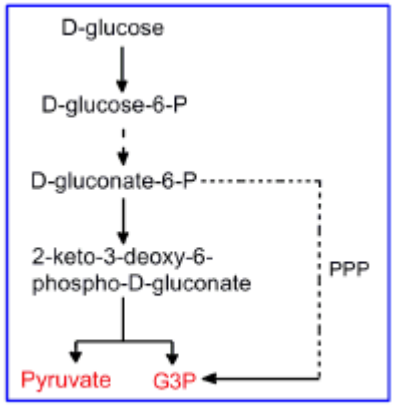
Liu *et al*., 2013, PLOS 8:e83290

Feeding module producing pyruvate and G3P



module 1 (EMP)

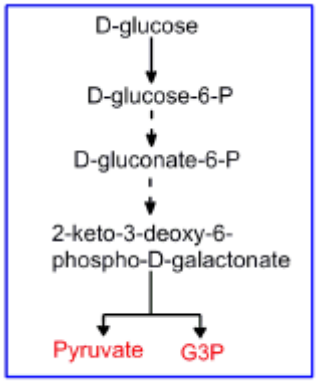
- For feeding module 1, EMP is native in *E. coli* and was hardly affected by other pathways when glucose is used as the sole carbon source.



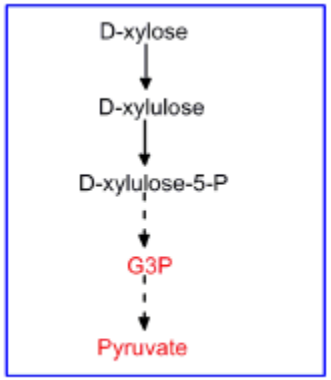
module 2 (EDP + PPP)

- Feeding module 2, EMP was blocked by disrupting the ***pgi*** gene to activate EDP. Previous studies indicated that glucose was mainly metabolized through PPP and partially through EDP after *pgi* was disrupted.

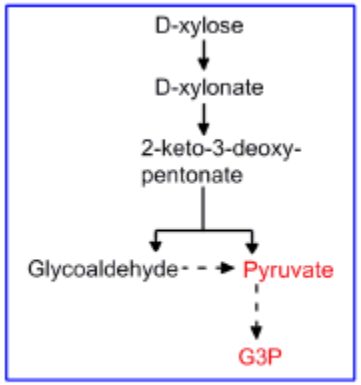
Liu *et al*., 2013, PLOS 8:e83290



module 3 (EDP)



module 4 (PPP)

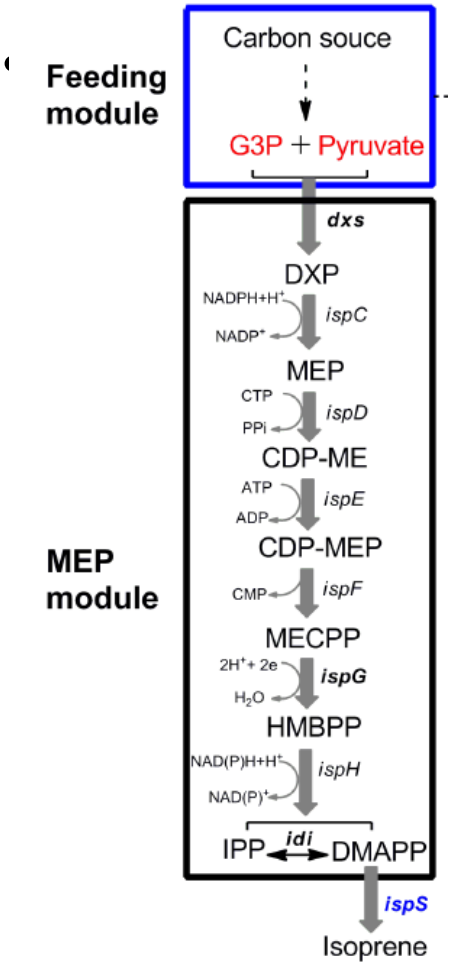


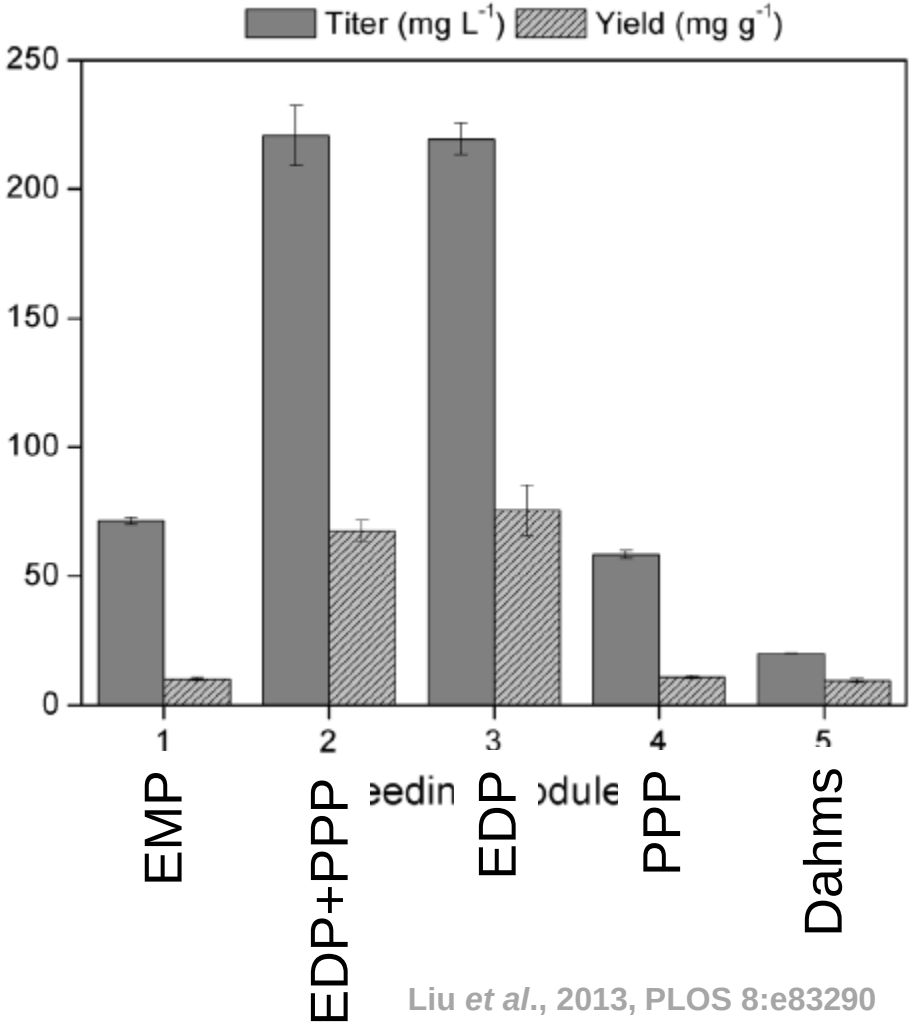
module 5 (Dahms pathway)

- Feeding module 3, both ***pgi*** and ***gnd*** genes were disrupted to block EMP and PPP, respectively, leaving out EDP as the only available pathway for glucose glycolysis.
- Feeding module 4, PPP is the only active glycolytic pathway in native *E*. *coli* when D-xylose is used as the sole carbon source.
- Feeding module 5, the ***xylA*** was disrupted to block the PPP for D-xylose metabolism, followed by the introduction of a D-xylose dehydrogenase (**Xdh**) encoding gene from *Caulobacter crescentus* into xylA-disrupted strain for the conversion of D-xylose to D-xylonate, an intact Dahms pathway was constructed by combining Xdh encoding gene with *E*. *coli* native D-xylonate catabolic pathway.

Liu *et al*., 2013, PLOS 8:e83290

Isoprene production





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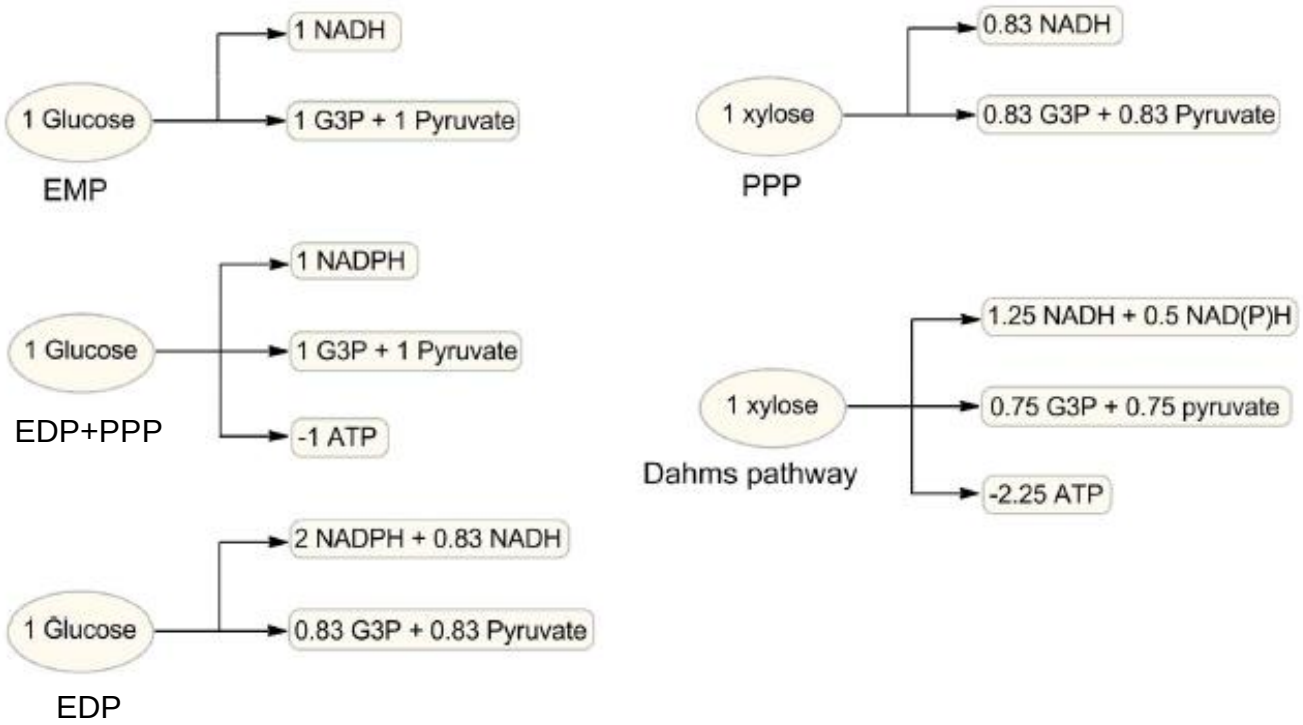
Theoretical yields of pyruvate and G3P



- Precursor synthesis has impact on isoprene production
- Difficult to account improvements solely on the introduced modifications
  - In modules 2 and 3, EMP blockage can also affect the carbon flux in TCA cycle and other secondary pathways, which could result in the redistribution between pyruvate and G3P pools in the cell.

Liu *et al*., 2013, PLOS 8:e83290

Secondary metabolites - antibiotics

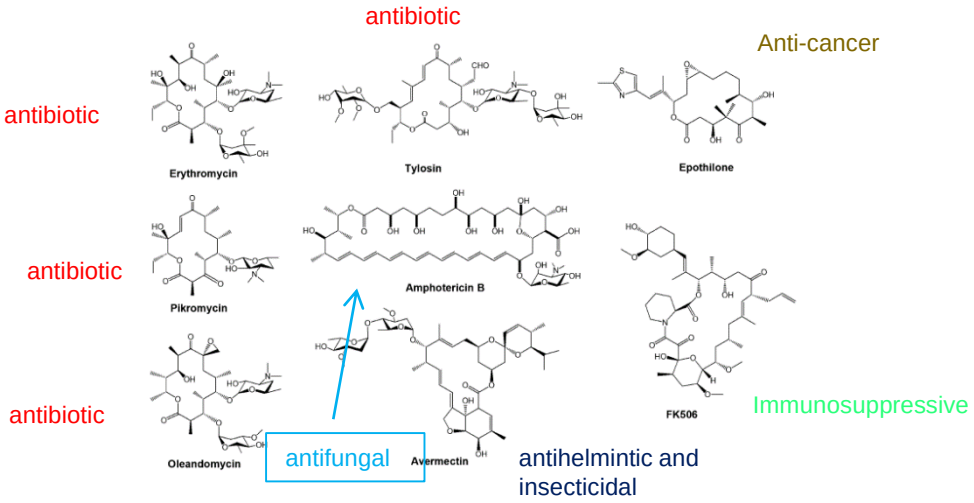
Shuffling of DNA sequences

Secondary Metabolites

- Secondary metabolites
  - Not essential for growth
  - Formation depends on growth conditions, produced as growth slows/stops
  - Produced as a group of related compounds
  - Often significantly overproduced
  - Often produced by spore-forming microbes during sporulation
  - Precursor / building blocks derived from the central metabolic pathways
- Antibiotics are of major industrial importance
- Producers: Actinomycetes (eg Streptomyces), Fungi (eg Penicillium)

Generation of novel antibiotics - Polyketide synthesis and engineering

- Polyketide are a remarkable class of natural products exhibiting a staggering range of functional and structural diversity,
- antibiotic, anticancer, antifungal, antiparasitic, immunosuppressive properties



Staunton and Weissman 2001 Nat. Prod. Rep., 18, 380-416

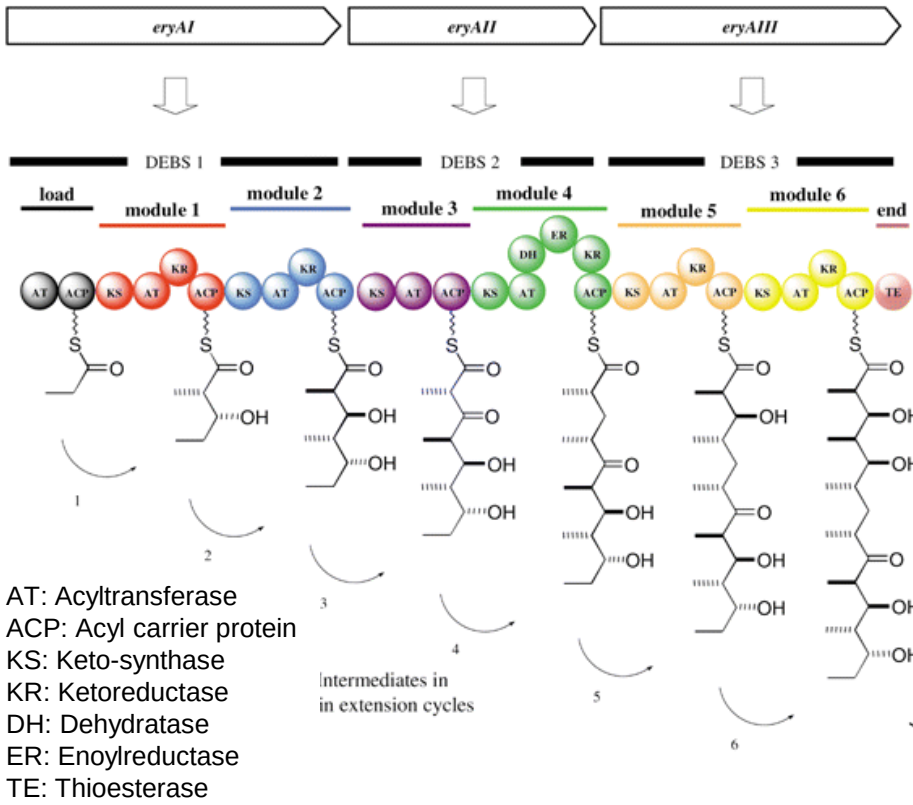
Domain organisation of the erythromycin polyketide synthase

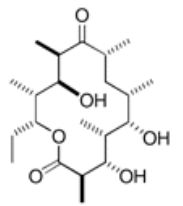
All polyketides are assembled by successive rounds of decarboxylative condensation between an acyl thioester and an a-carboxythioester in a fashion similar to fatty acid biosynthesis

Starting or loading module: AT-ACP-

Elongation or extending modules: -KS-AT-[DH-ER-KR]-ACP-

Termination or releasing module: -TE

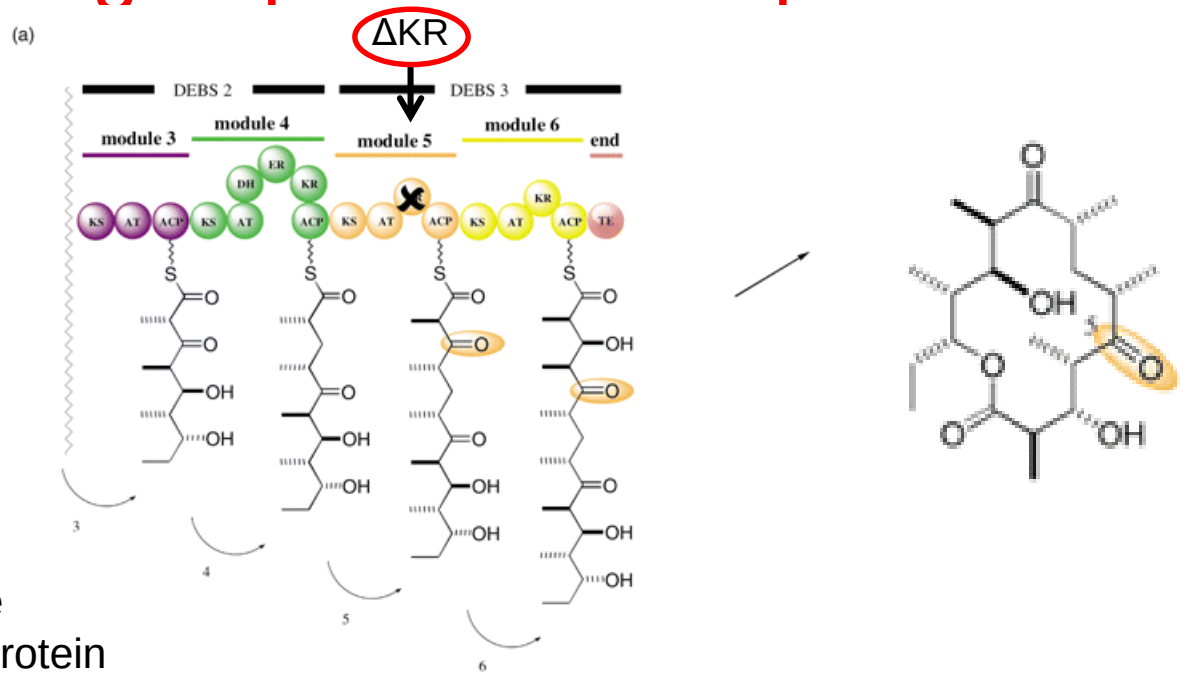




6-Deoxyerythronolide B
16

Staunton and Weissman 2001 Nat. Prod. Rep., 18, 380-416

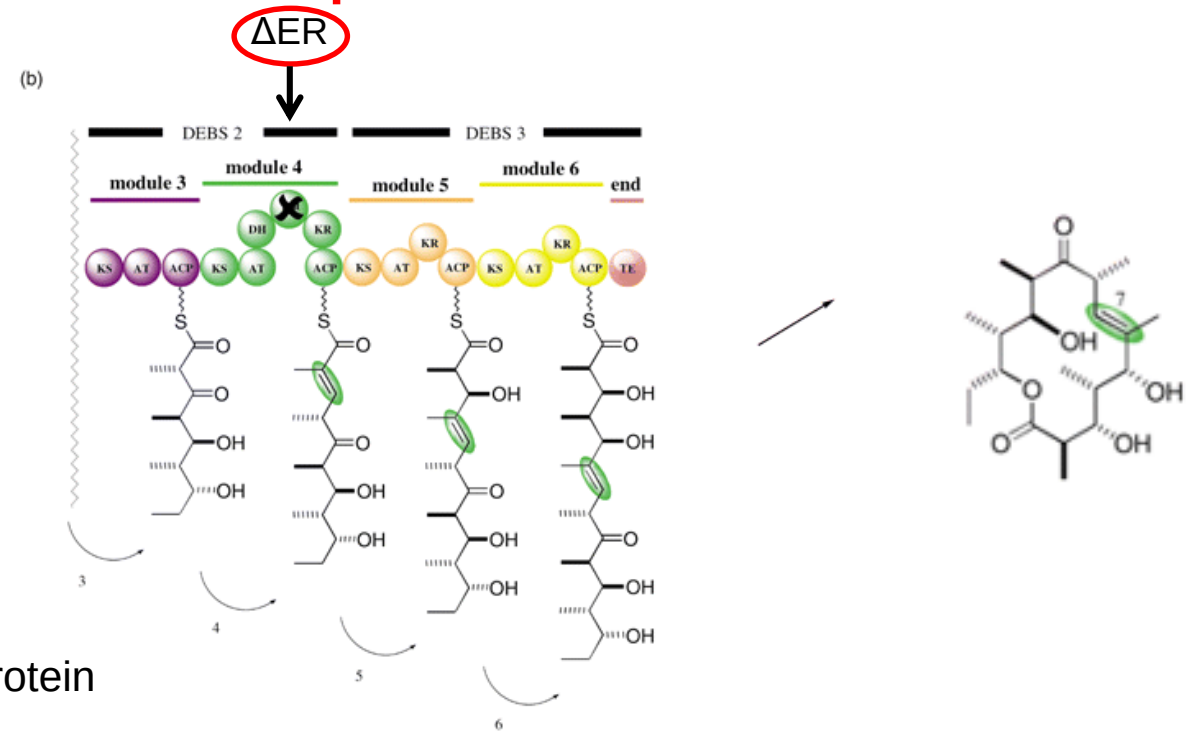
Inactivation of KR5 of DEBS resulted in the production of erythromycin analogues with keto groups at the C-5 position.



AT: Acyltransferase
ACP: Acyl carrier protein
KS: Keto-synthase
KR: Ketoreductase
DH: Dehydratase
ER: Enoylreductase
TE: Thioesterase

Staunton and Weissman 2001 Nat. Prod. Rep., 18, 380-416

Inactivation of ER4 resulted in an analogue of erythromycin with a double bond at the expected site



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Nat. Prod. Rep., 2001, **18**, 380-416

Staunton and Weissman 2001 Nat. Prod. Rep., 18, 380-416

Chromosomal repositioning of the thioesterase domain to locations within the erythromycin PKS

AT: Acyltransferase
ACP: Acyl carrier protein
KS: Keto-synthase
KR: Ketoreductase
DH: Dehydratase
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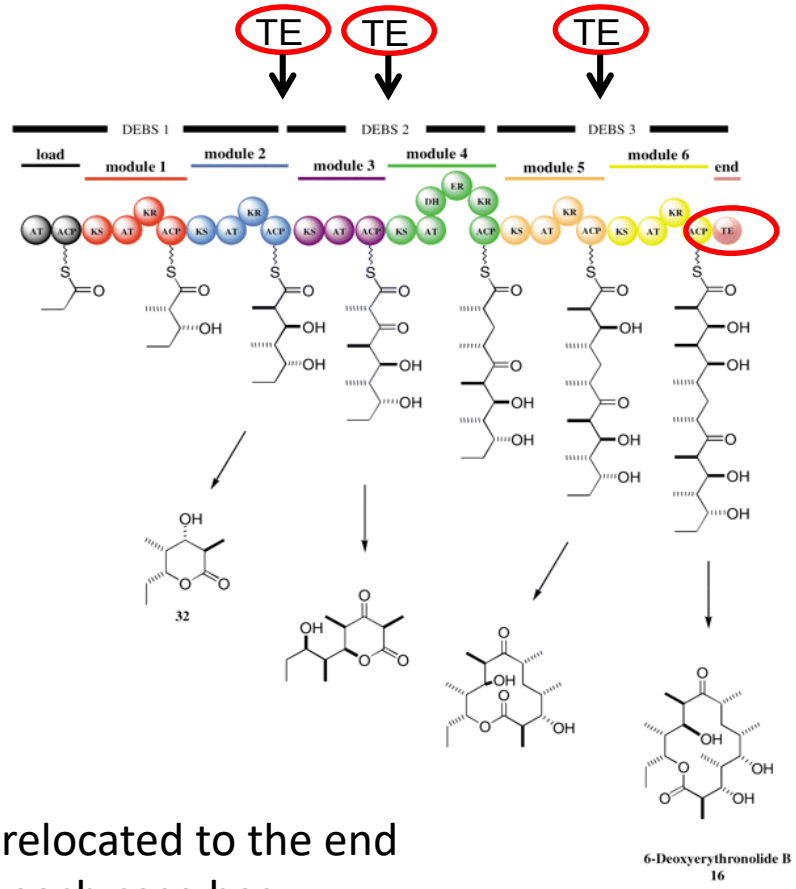
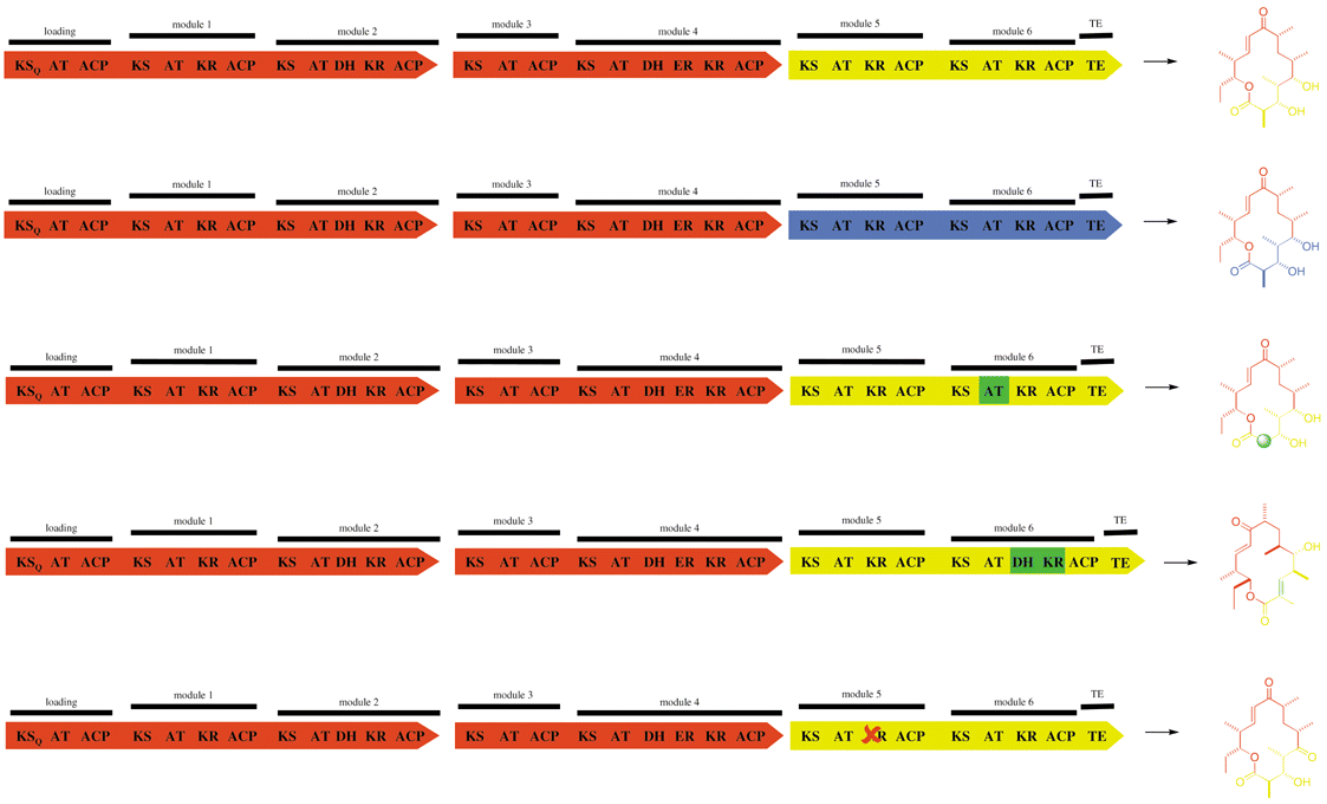


The TE has been successfully relocated to the end of modules 2, 3 and 5, and in each case has catalysed the formation of the anticipated lactone.

Staunton and Weissman 2001 Nat. Prod. Rep., 18, 380-416

Hybrid PKSs produced by recombining pks operons



whole protein subunits from the pikromycin (red), DEBS (yellow) and oleandomycin (blue) synthases.
PKSs were also generated using altered versions of DEBS 3 containing domains from RAPS (shown in green) or a domain deletion

- Combinatorial approach (=shuffling of DNA sequences) enables production of novel compounds!
- Applicable to any set of homologous gene clusters

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