Engineering microbes for production of low-cost, effective, anti-malarial drugs

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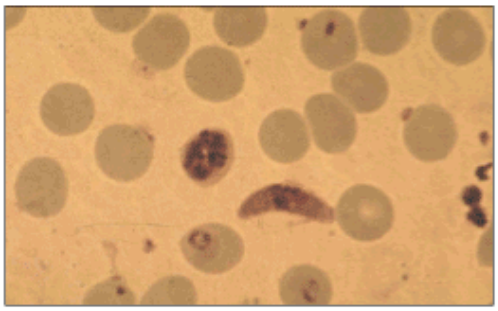
MCB 113

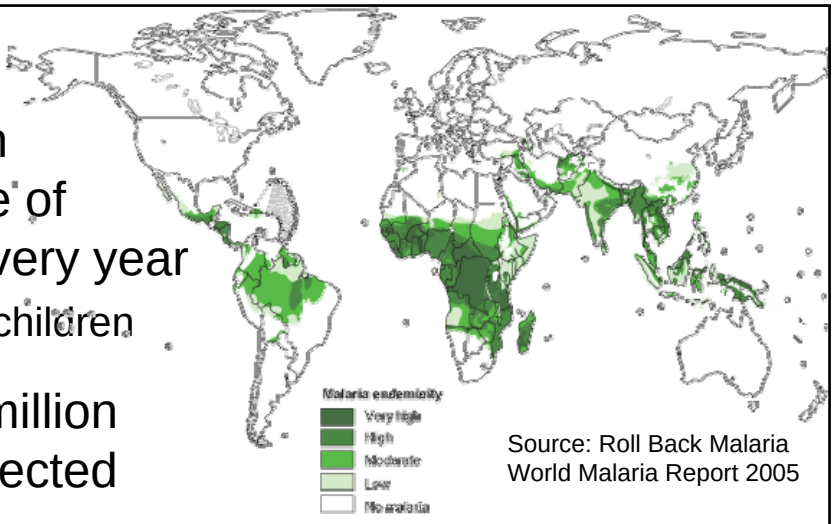
Malaria

- Caused by *Plasmodium*, a single-cell protozoan
  - –Transmitted by Anopheles mosquito
  - –Destroys red blood cells



Malaria

- 1-3 million people die of malaria every year
  - –90% are children
- 300-500 million people infected

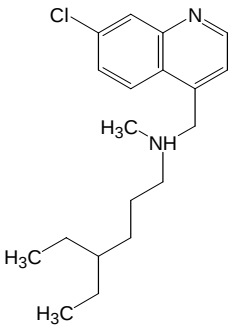


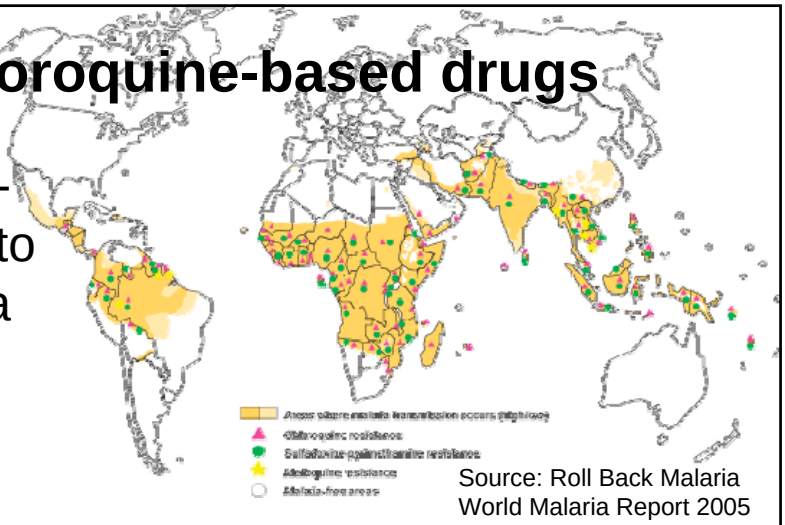
Source: Roll Back Malaria
World Malaria Report 2005

- Economists have proposed that malaria decreases the GDP of affected countries by as much as 50%.

Chloroquine-based drugs

- Most widely-used drugs to treat malaria



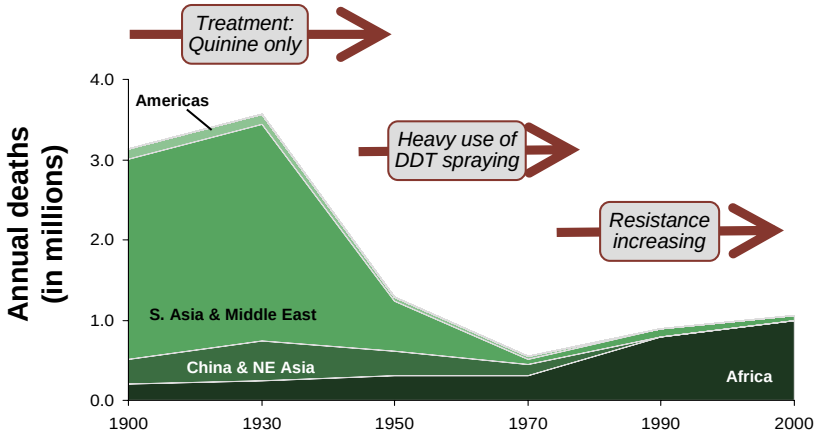


Source: Roll Back Malaria
World Malaria Report 2005

- *Plasmodium* in many parts of the world is largely resistant to chloroquine

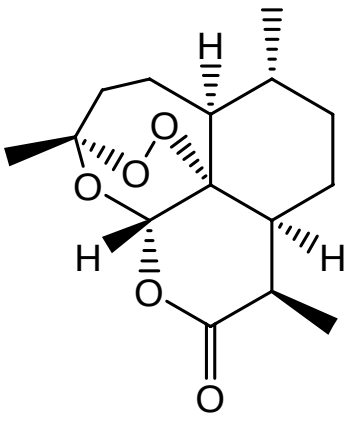
Disease burden on the rise as resistance grows

Trends in Africa Particularly Troubling

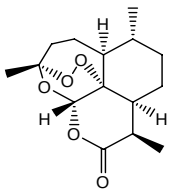


Source: Roll Back Malaria

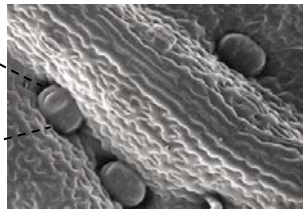
Artemisinin



Artemisinin is produced by trichomes in *Artemisia annua* leaves



Artemisinin...



...is produced by trichomes...

...found on *Artemisia annua* leaves...

A brief history of artemisinin

168 B.C. *Recipes For 52 Kinds Of Diseases* found in the Mawangdui Han Dynasty tomb

→**Hemorrhoids**

340 A.D. *Zhou Hou Bei Ji Fang (Handbook of Prescriptions for Emergency Treatments)*

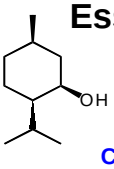
→**Fevers (malaria)**

1972 Active ingredient (artemisinin) isolated

Isoprenoids

> 50,000 known molecules

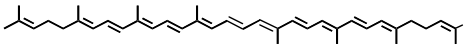
Essential oils



Menthol
C-10 Monoterpene

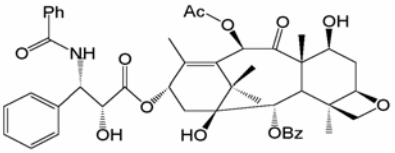
Carotenoids

Lycopene
C-40 Tetraterpene



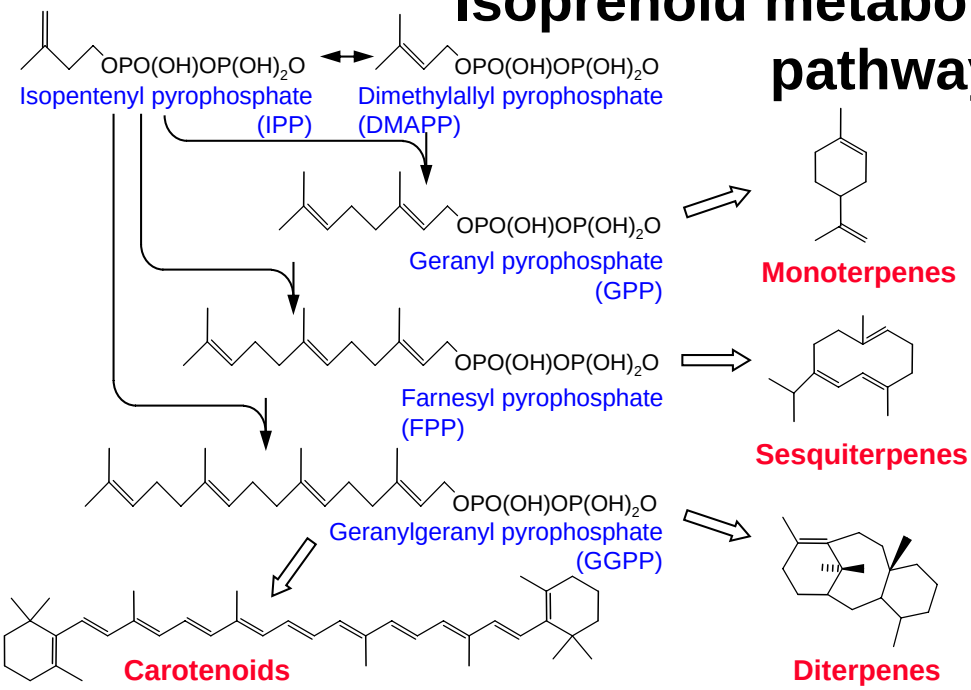
Chemotherapeutics

Eleutherobin
C-20 Diterpene

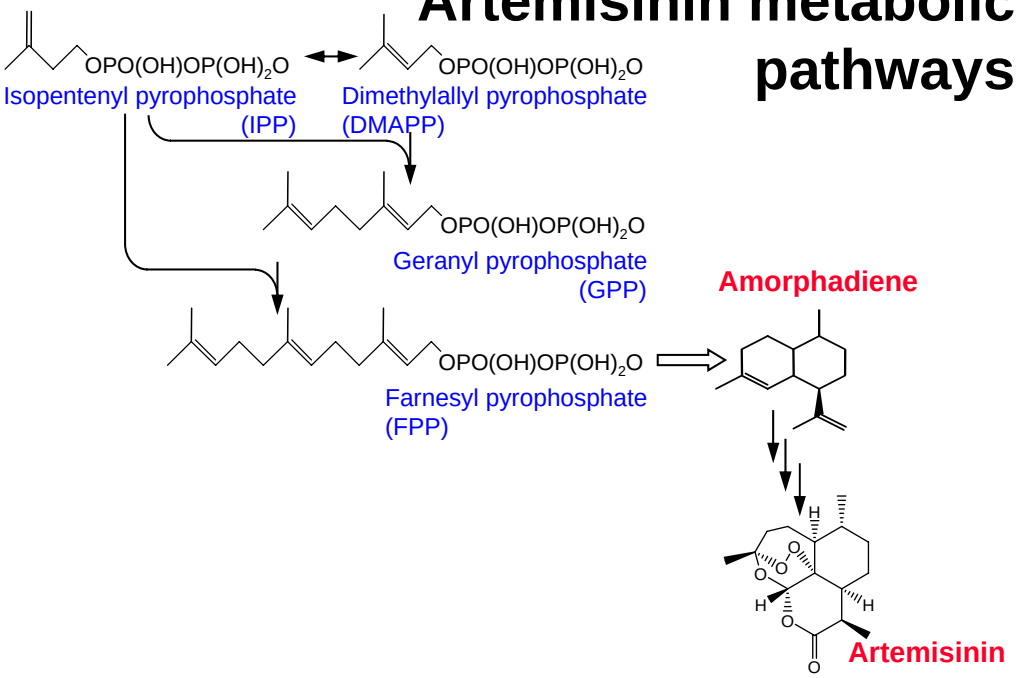


Taxol
C-20 Diterpene

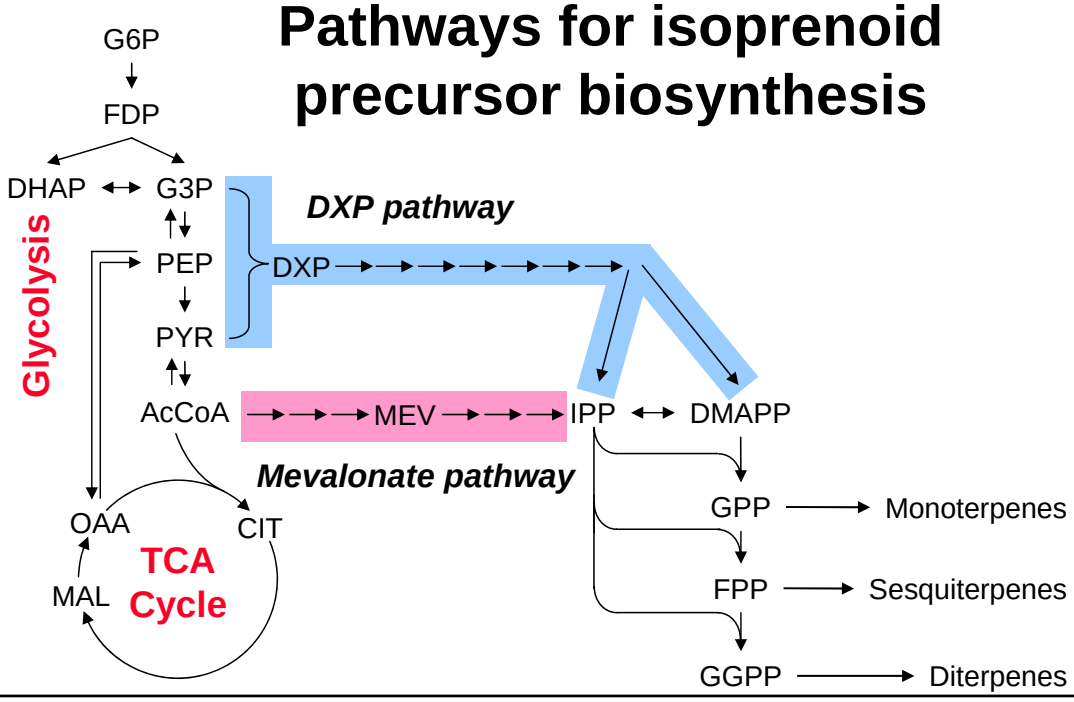
Isoprenoid metabolic pathways

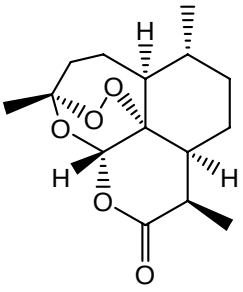


Artemisinin metabolic pathways



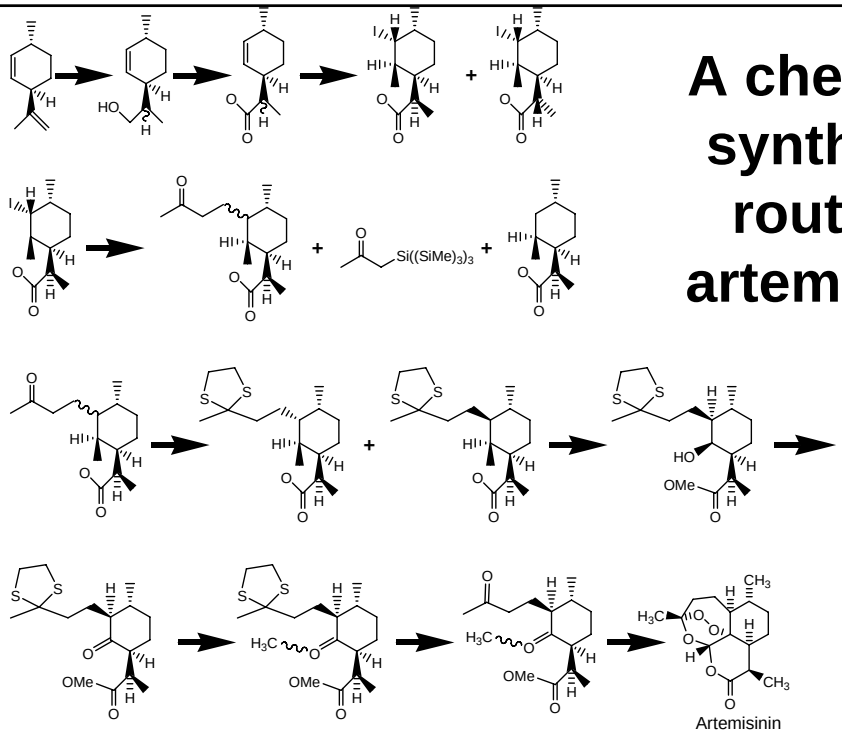
Pathways for isoprenoid precursor biosynthesis





Artemisinin-based drugs

- The current cost for an artemisinin-based drug is approximately $2.50.
  - –Artemisinin generally adds $1.00-1.50 to the cost for drugs
  - –Most developing countries spend less than $4/person/year on health care
- As many as 10-12 treatments are needed for each person annually
- World Health Organization estimates that 700 tons will be needed annually



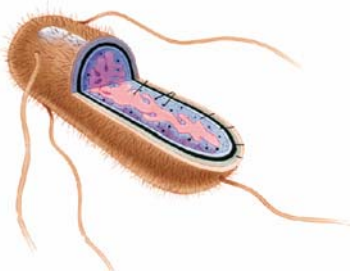
A chemical synthesis route to artemisinin

Goal

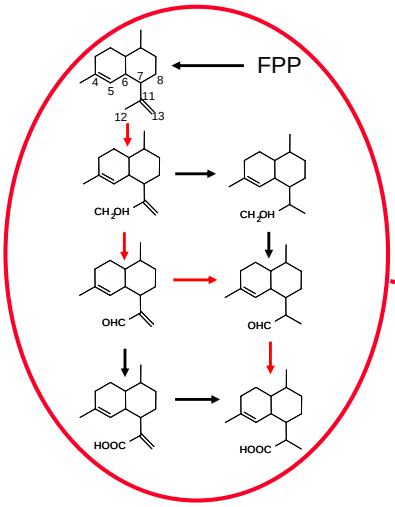
Reduce the cost of artemisinin-based anti-malarial drugs by an order of magnitude.

Approach

Engineer a microorganism to produce artemisinin from an inexpensive, renewable resource.

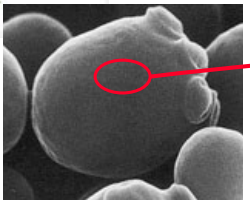


Designing a microbe to synthesize artemisinin

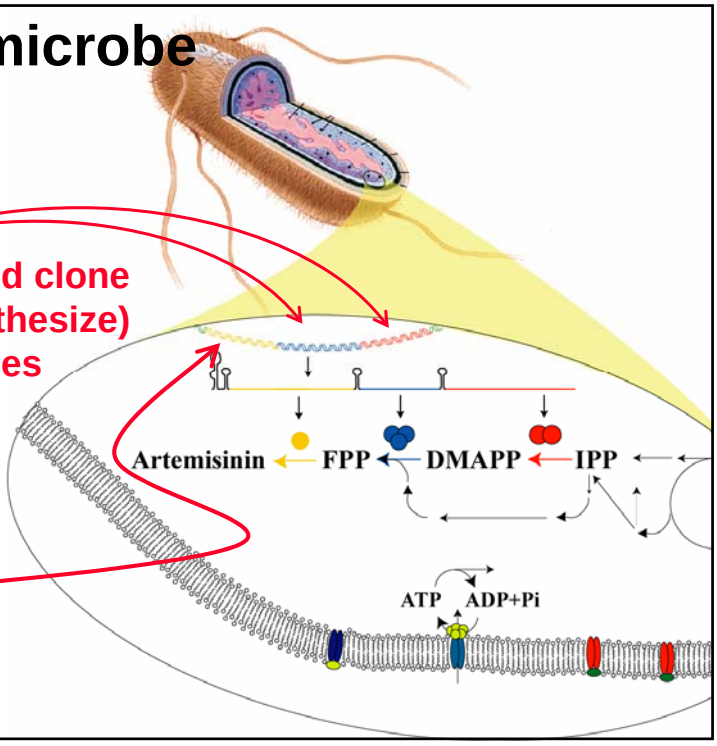


Identify the chemistry

Designing a microbe to synthesize artemisinin

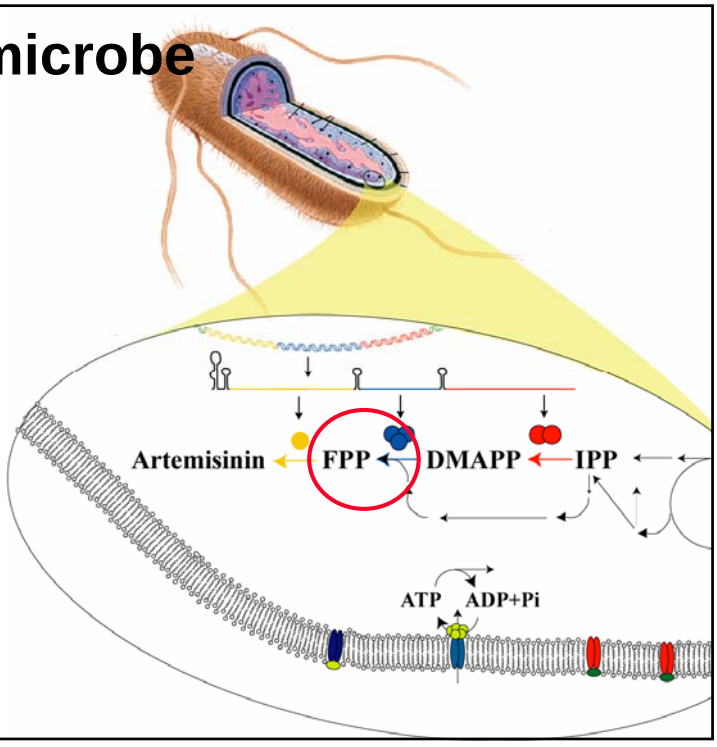


Find and clone (or synthesize) the genes



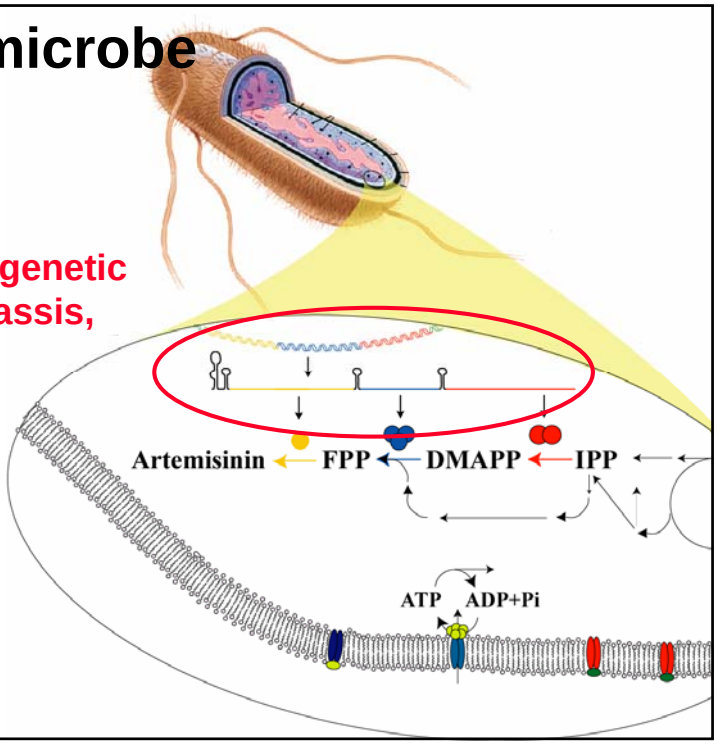
Designing a microbe to synthesize artemisinin

Supply of intracellular precursors



Designing a microbe to synthesize artemisinin

Well characterized genetic control system (chassis, parts, devices)



Microbial production of artemisinin

Advantages

- Production not affected by weather conditions
- Consistent supply
- Pure product can be made (free of other contaminating terpenes)
- Access to artemisinin can be controlled to minimize production of mono-therapies.

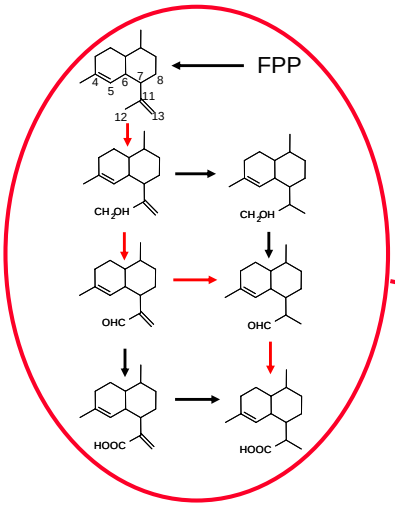
Microbial production of artemisinin

Challenges

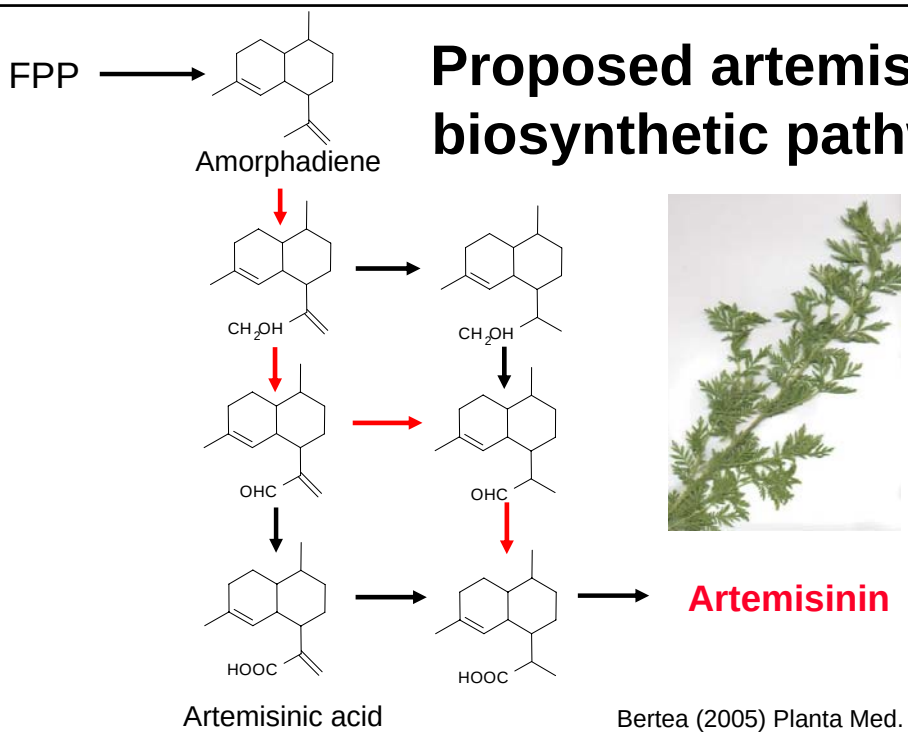
- Need the genes for all of the enzymes in the pathway
- Not always simple to express in microbes the genes from very different organisms
- Need to balance metabolic pathways to optimize production
- Need a platform host and a host of metabolic engineering tools

Designing a microbe to synthesize artemisinin

Identify the chemistry

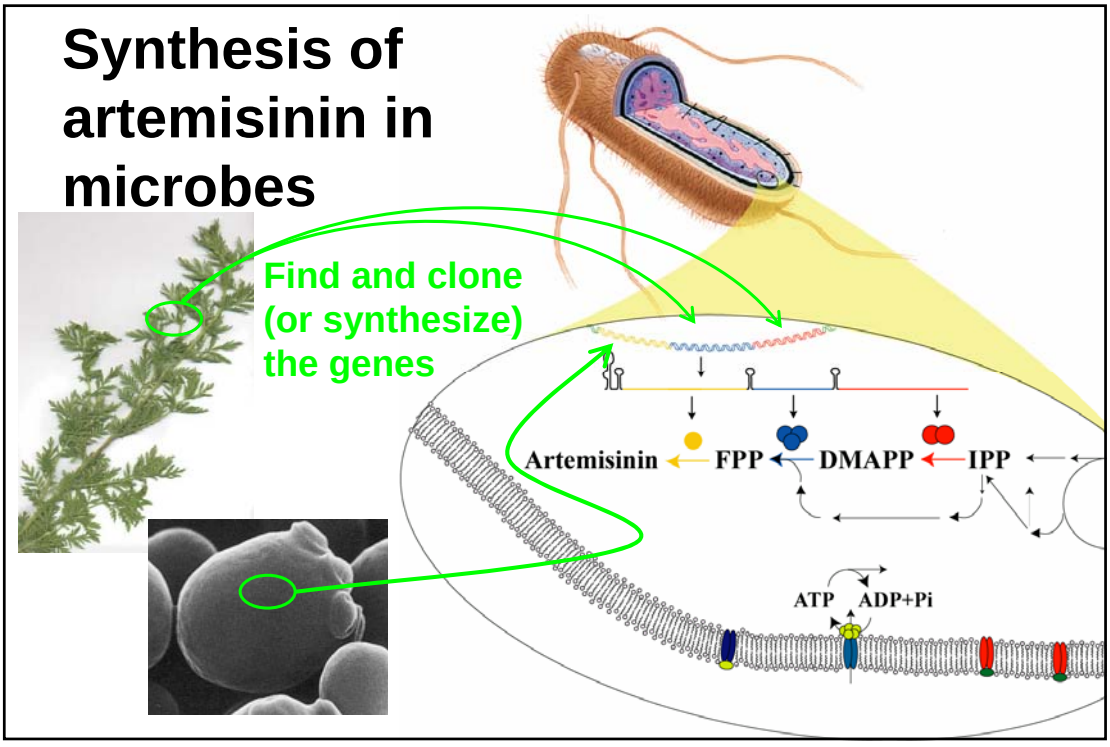


Proposed artemisinin biosynthetic pathway

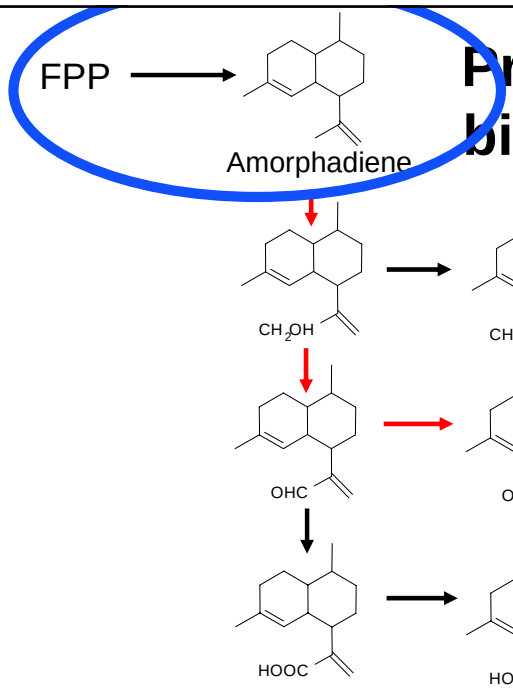


Bertea (2005) Planta Med. **71**:40-47

Synthesis of artemisinin in microbes



Proposed artemisinin biosynthetic pathway



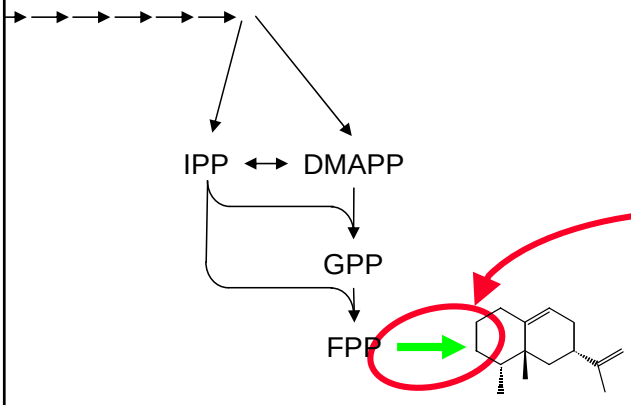
Artemisinin

Artemisinic acid

Bertea (2005) Planta Med. **71**:40-47

Production of a model isoprenoid in *E. coli*

DXP pathway

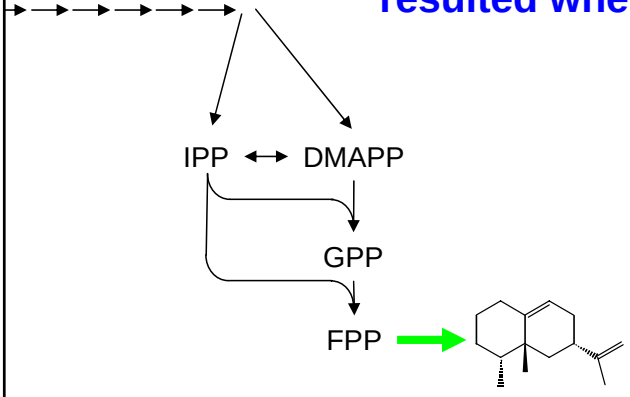


Tobacco as an early model

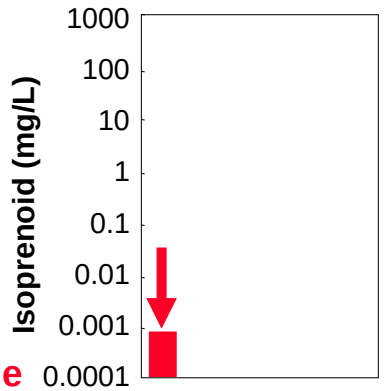
5-*epi*-aristolochene

Production of a model isoprenoid in *E. coli*

DXP pathway



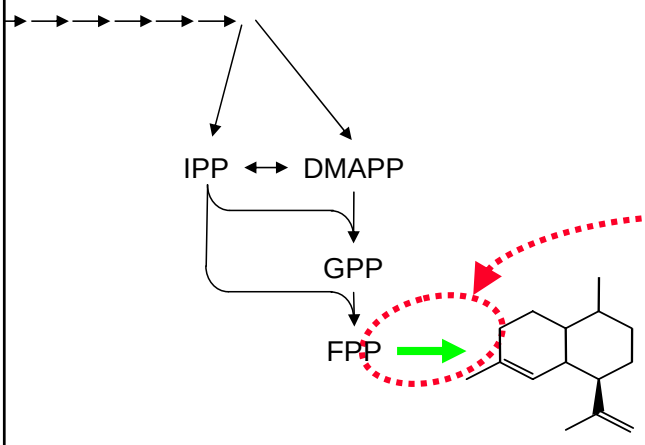
Very low production of isoprenoid resulted when using the native gene



5-*epi*-aristolochene

Production of the artemisinin precursor (amorphadiene) in *E. coli*

DXP pathway



amorphadiene

atcttgtgat catcccaaga caaaaccaga gaaaaagacc tgtctgtttt tttaagaagt
ctttatatta tttttttttgt cggagaatct tataagcatg gcttcaggag gatcaaagtc
ggcagctttc atgcttctga tgctgaatct tggtctctat ttcgtcatca ccatcatcgc
ttcttgggct gttaatcacg gcatcgagag aactcgcgag tctggtaact aacaaagata
acaactgatt aagtaacaat taatccaacg ttagaaaatg tcatcatcaa tcttcttttt
gtggtatttt gcagcgtcga cactgtcact tccggcgaag atattcccga tatacttccc
ggtggggaac atggcgaccg gttttttcgt aatattcacg ttaatcgccg gcgtcgtcgg

Gene synthesis

- Remove rare codons
- Insert restriction sites

atcttgtgat catcccaaga caaaaccaga gaaaaagacc tgtctgtttt tttaaga
ctttatatta tttttttttgt cggagaatct tataagcatg gcttcaggag gatcaaa
ggcagctttc atgcttctga tgctgaatct tggtctctat ttcgtcatca ccatcat
ttcttgggct gttaatcacg gcatcgagag aactcgcgag tctggtaact aacaaag
acaactgatt aagtaacaat taatccaacg ttagaaaatg tcatcatcaa tcttctt
gtggtatttt gcagcgtcga cactgtcact tccggcgaag atattcccga tatactt
ggtggggaac atggcgaccg gttttttcgt aatattcacg ttaatcgccg gcgtcgt

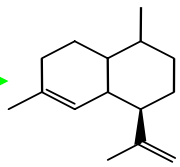
Gene resynthesis improves amorphadiene production

DXP pathway

IPP ↔ DMAPP

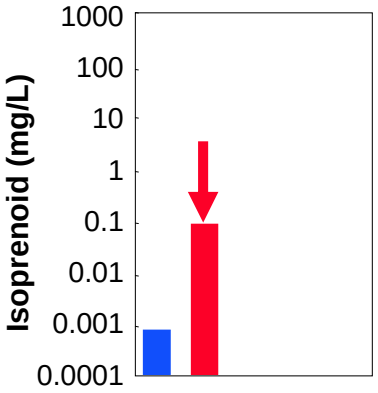
GPP

FPP

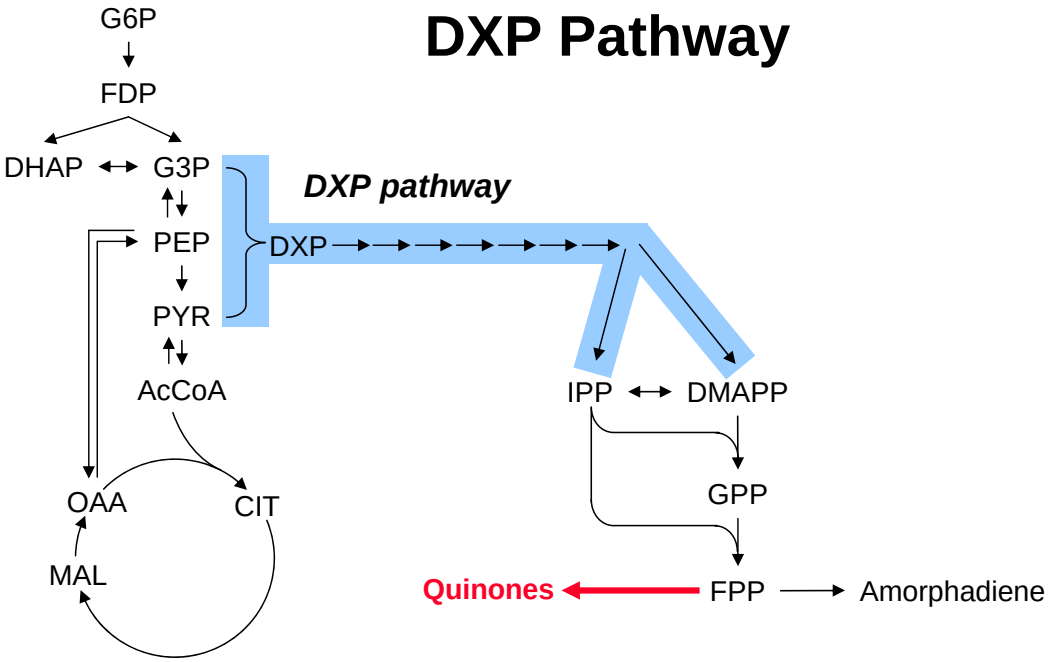


amorphadiene

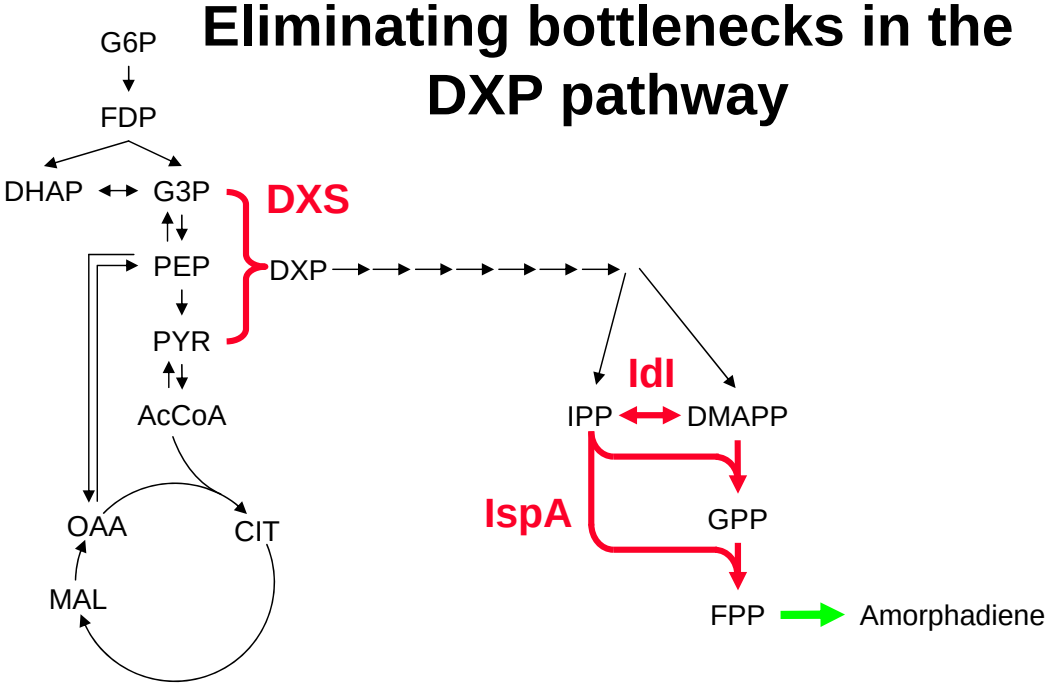
142-fold improved production!



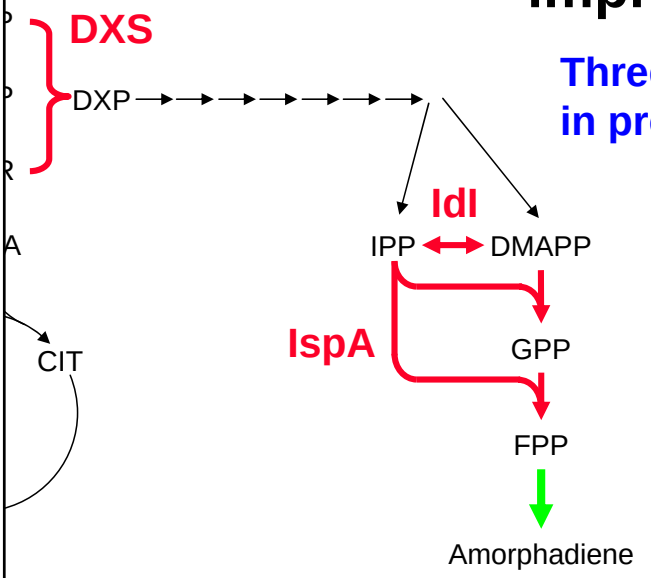
DXP Pathway



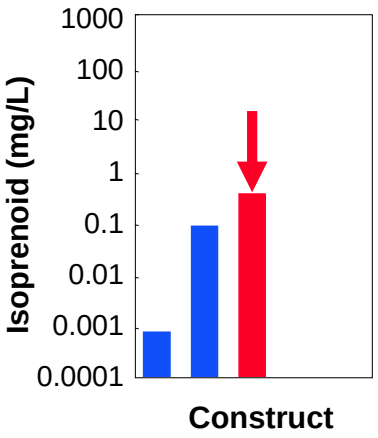
Eliminating bottlenecks in the DXP pathway



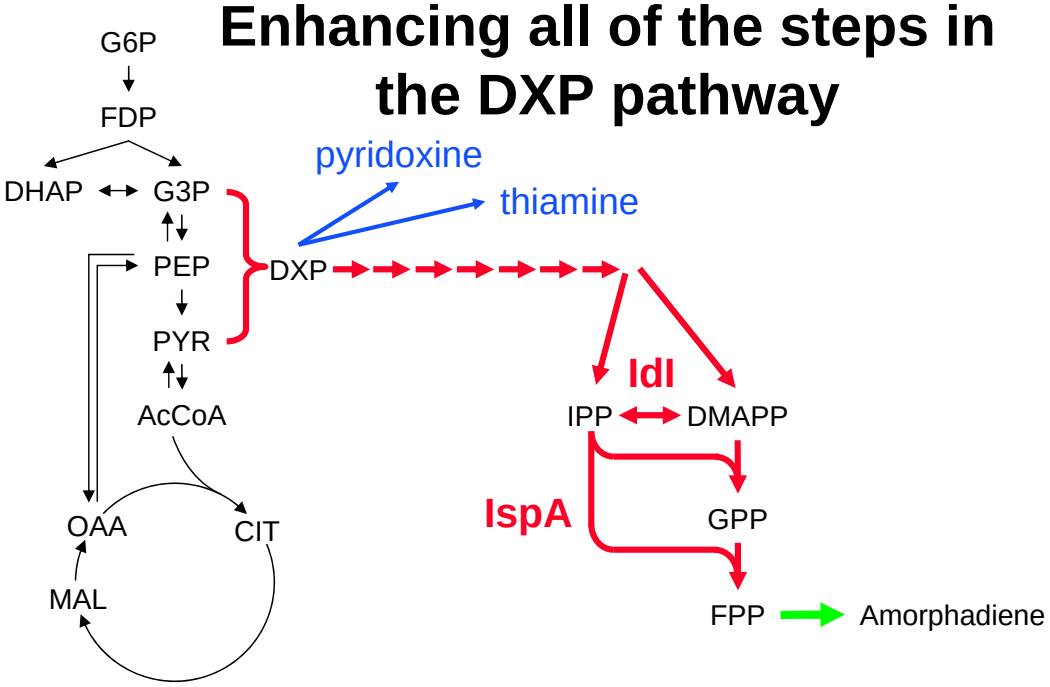
Engineering *E. coli's* native pathway improves production



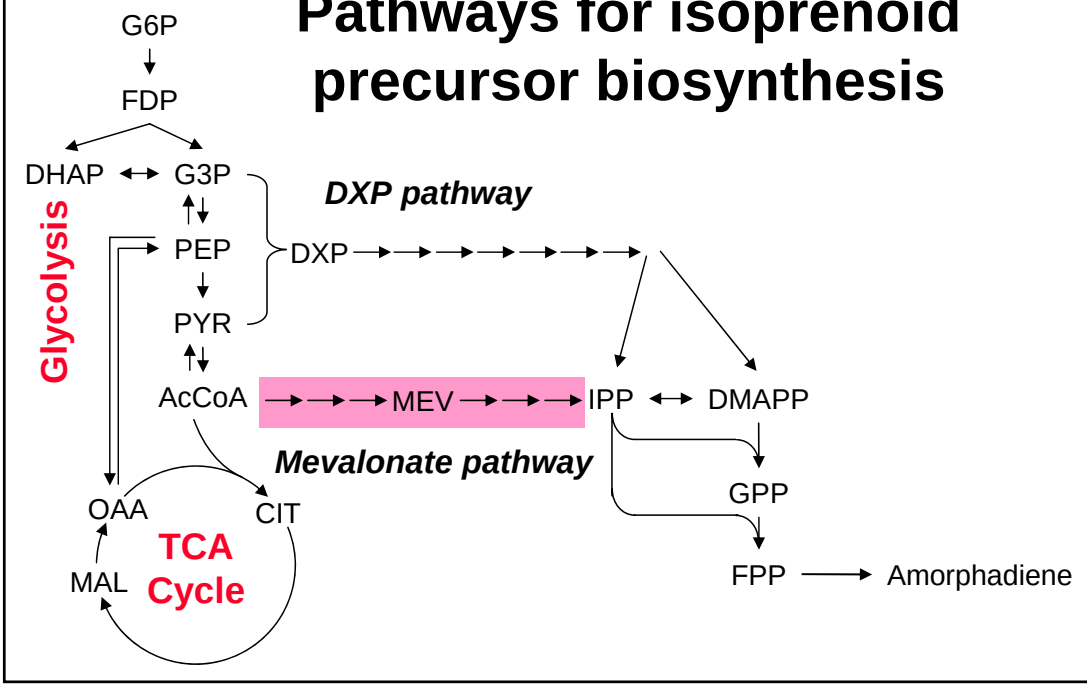
Three-fold improvement in production



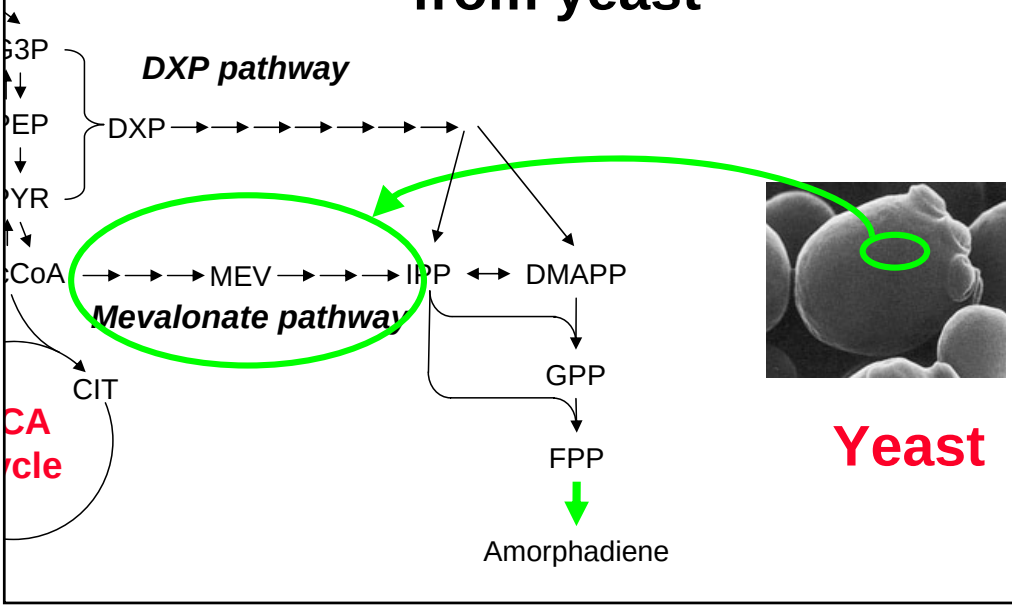
Enhancing all of the steps in the DXP pathway



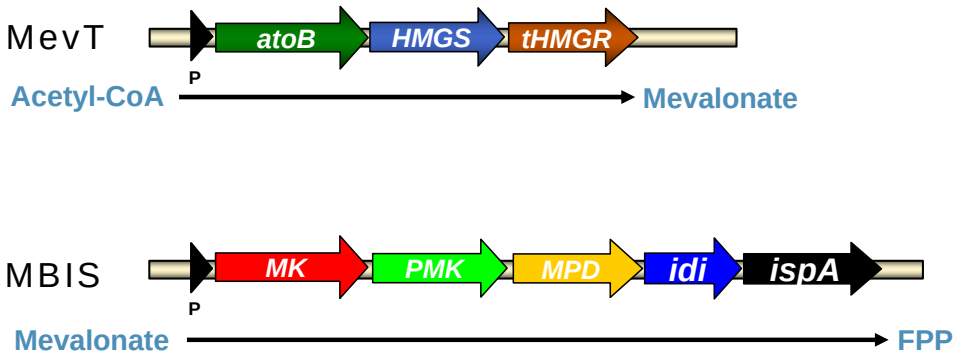
Pathways for isoprenoid precursor biosynthesis



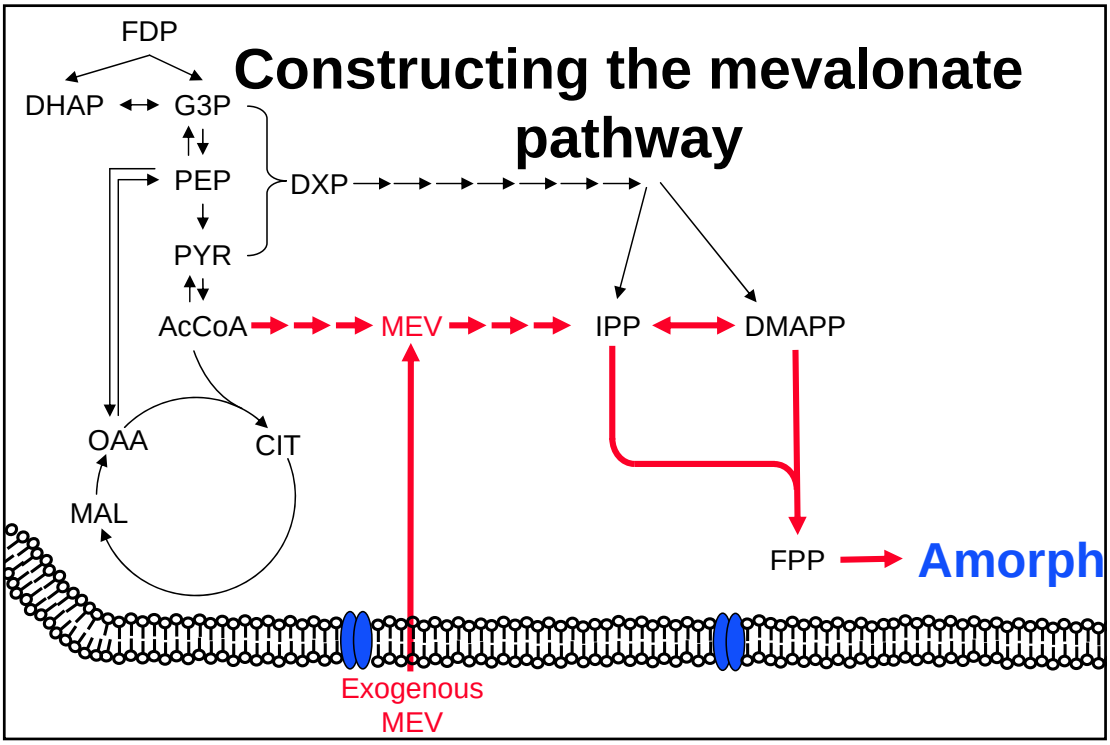
Recruiting the mevalonate pathway from yeast



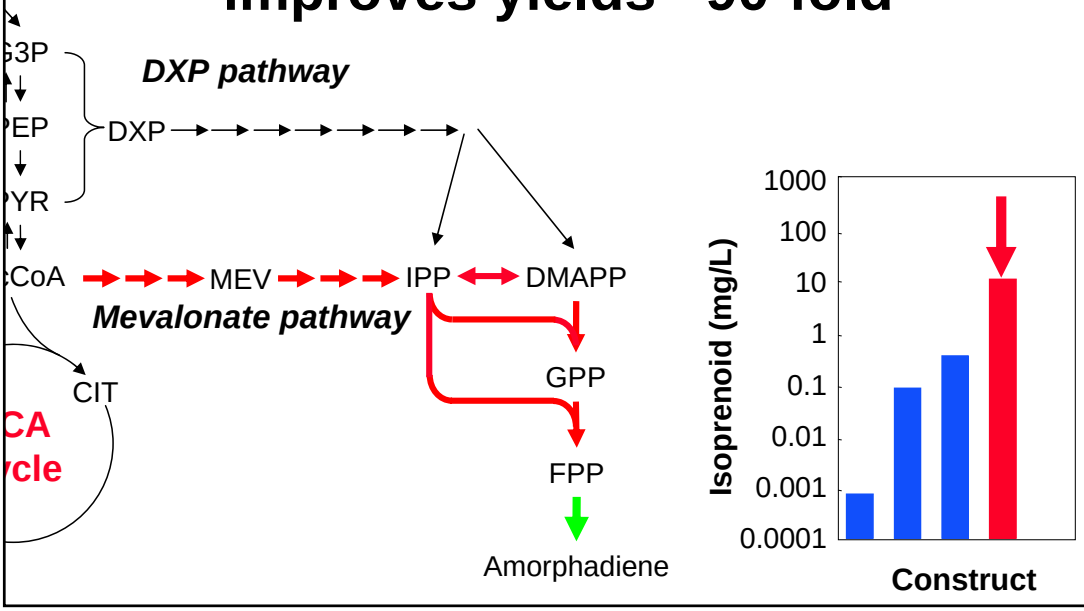
Construction of synthetic mevalonate pathway operons



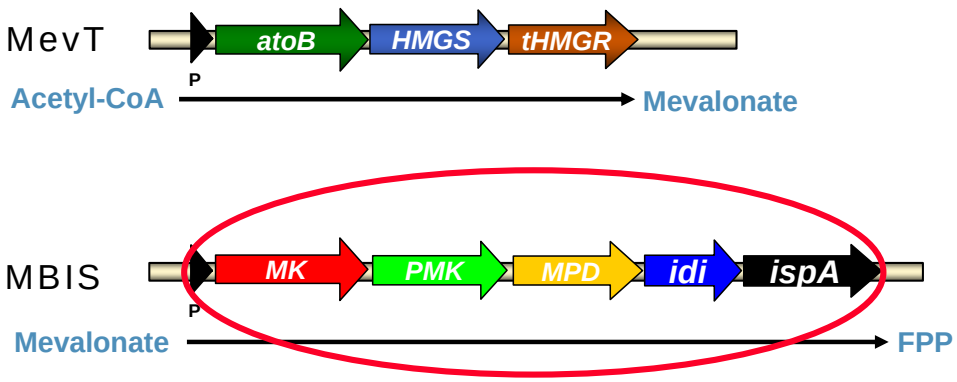
Constructing the mevalonate pathway

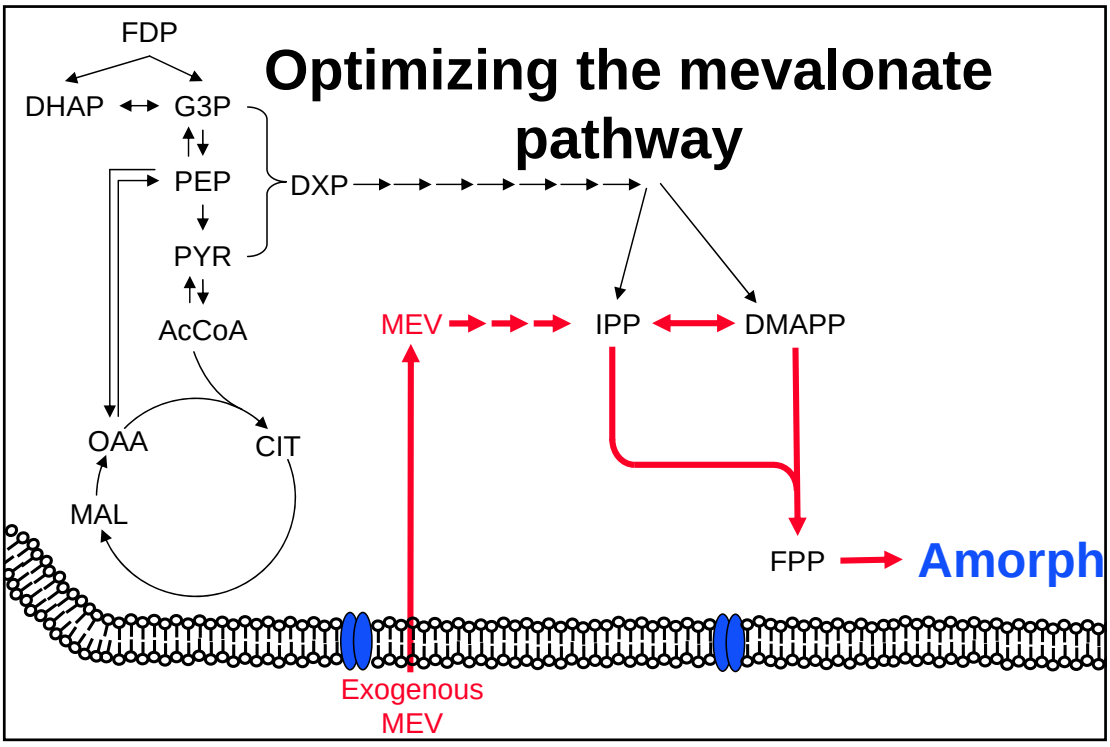


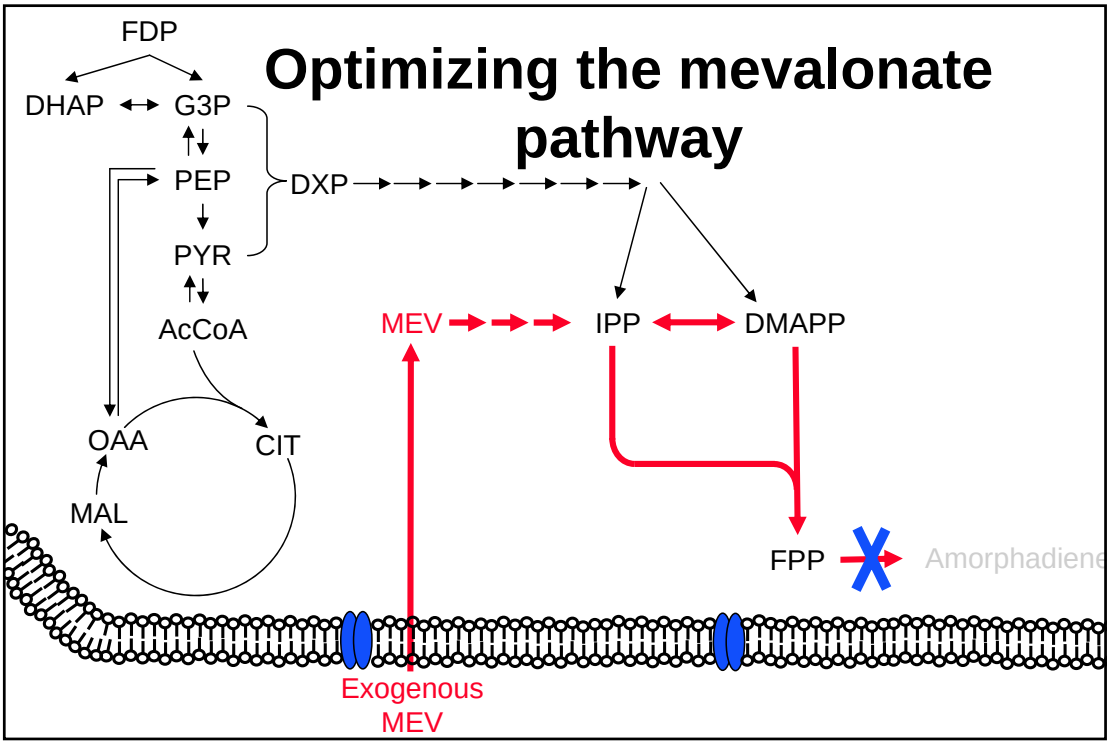
The yeast mevalonate pathway improves yields ~90-fold



Optimizing the bottom part of the mevalonate pathway




Optimizing the mevalonate pathway
FDP
DHAP
G3P
PEP
PYR
AcCoA
OAA
CIT
MAL
DXP
MEV
IPP
DMAPP
FPP
Amorph
Exogenous MEV


Optimizing the mevalonate pathway
FDP
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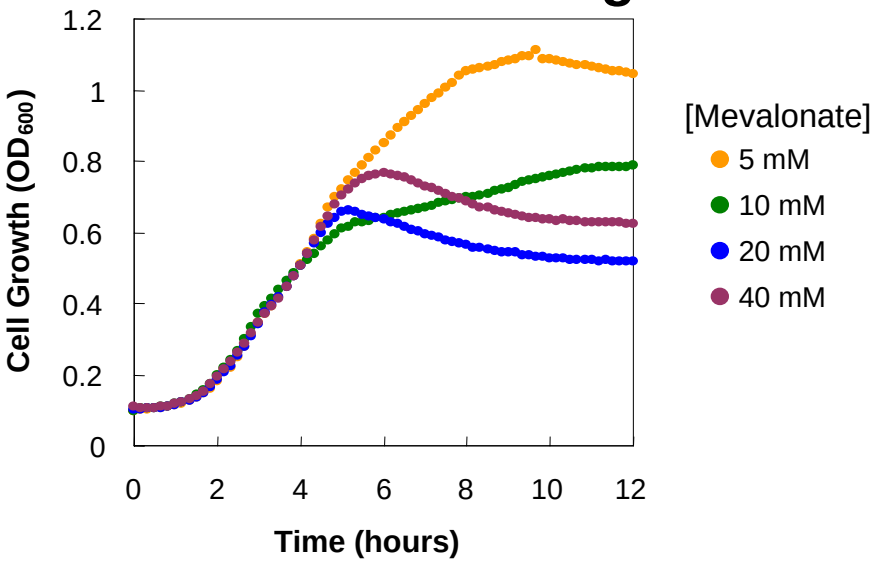
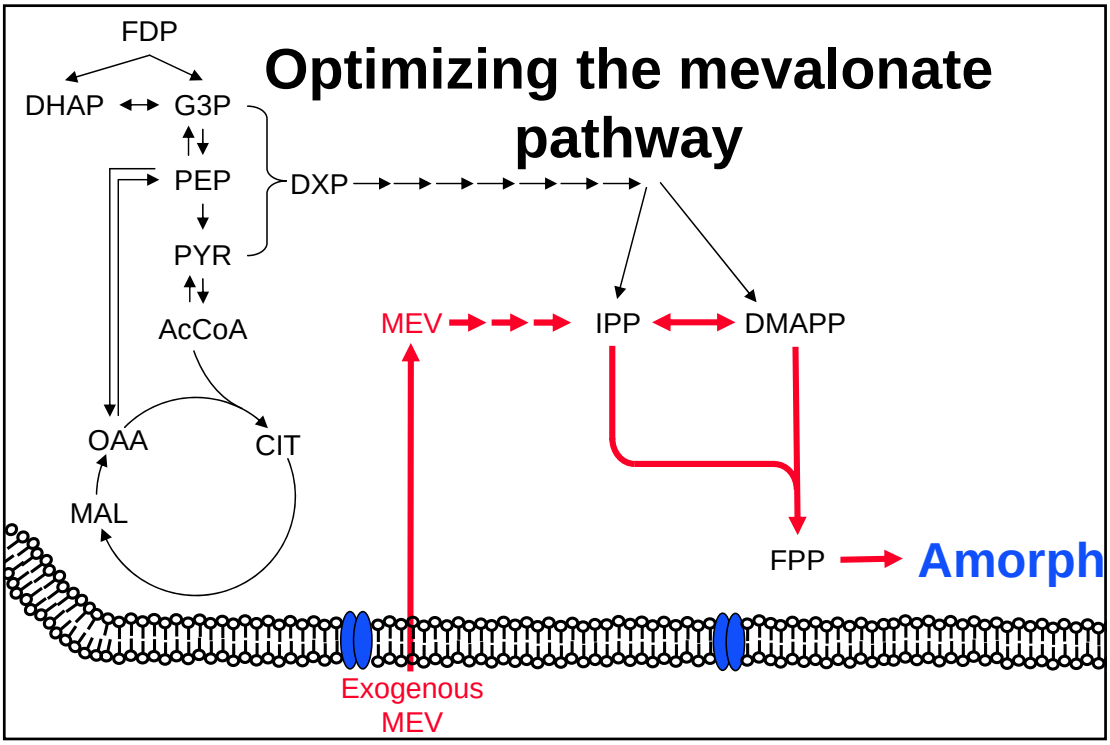
AcCoA
OAA
CIT
MAL
DXP
MEV
IPP
DMAPP
FPP
Amorphadiene
Exogenous MEV

Increasing concentrations of mevalonate inhibit growth

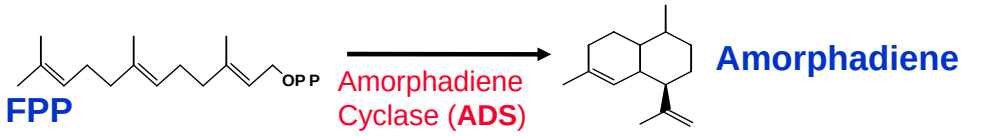


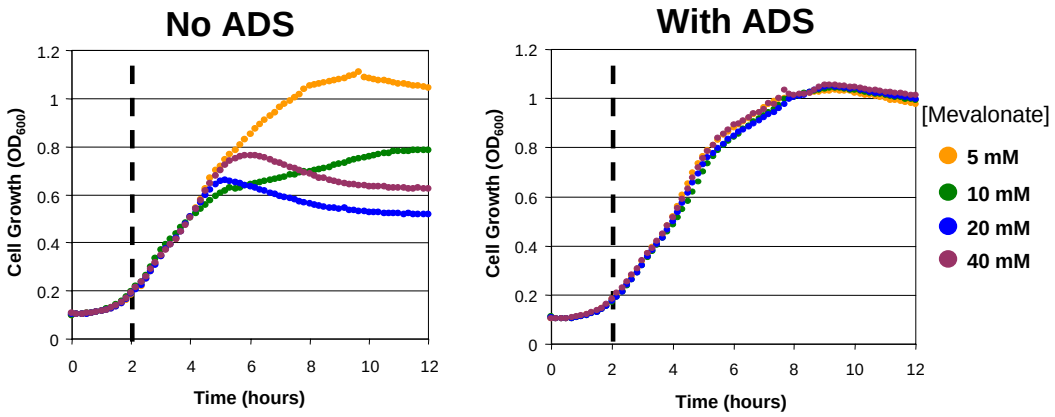
Martin et al. 2003. *Nat. Biotechnol.* 21:796-802.

Optimizing the mevalonate pathway

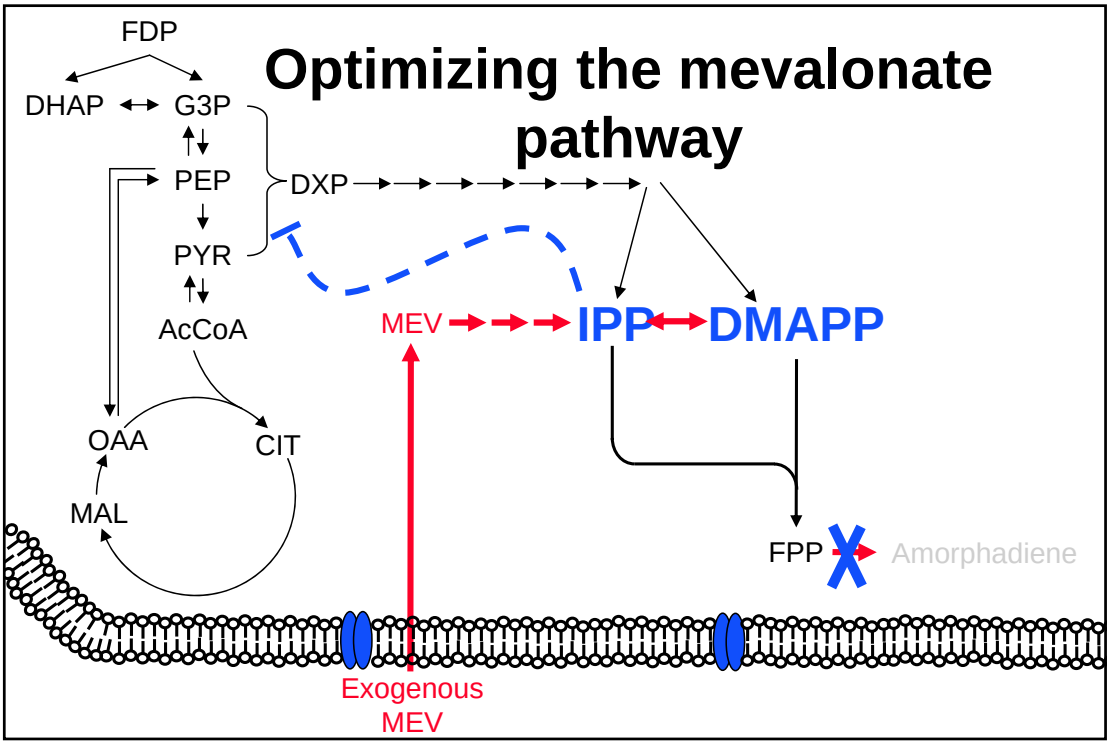


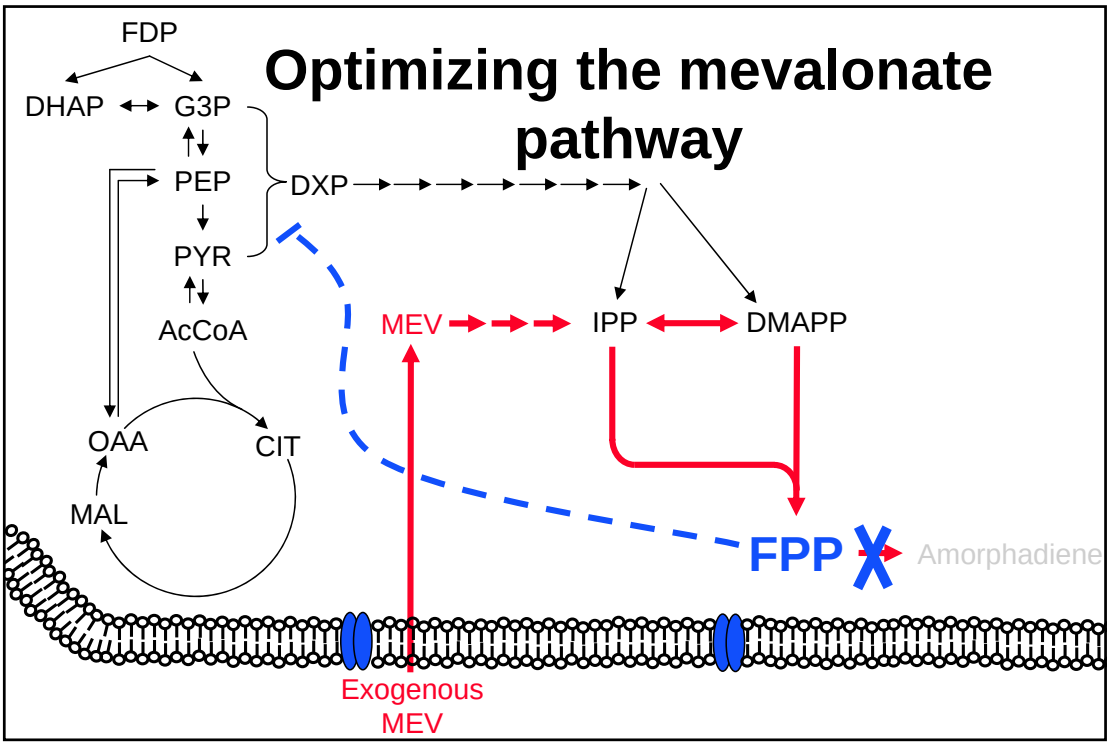
Co-expression of sesquiterpene cyclase alleviates growth inhibition

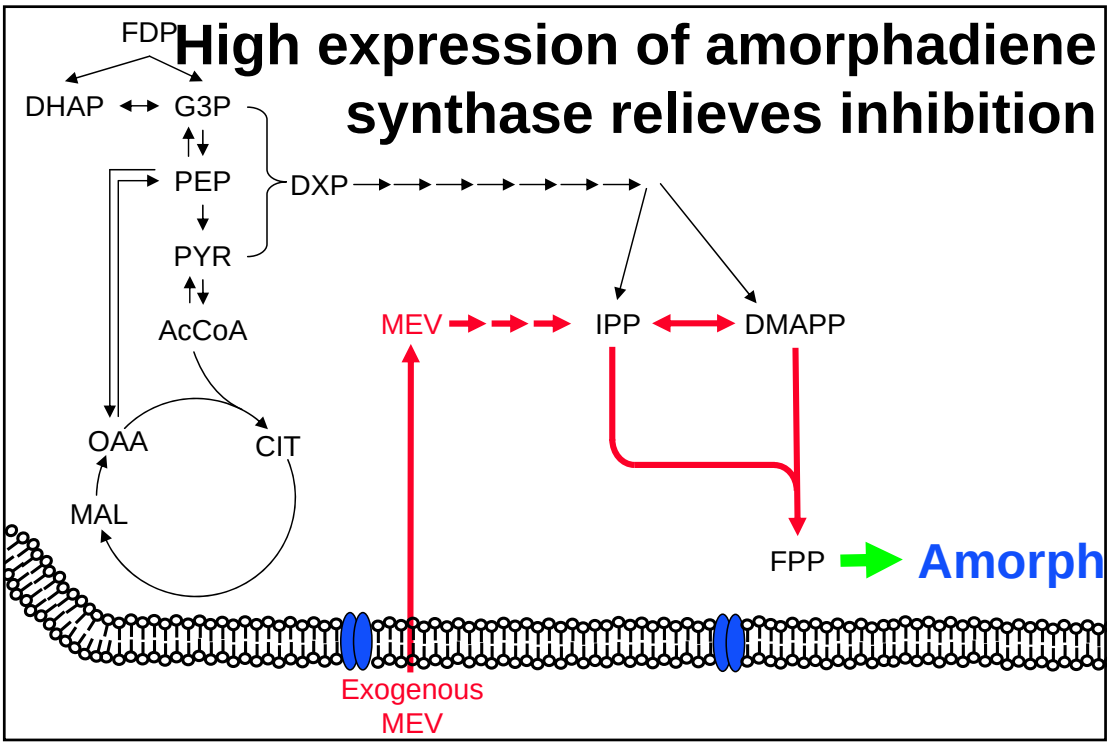




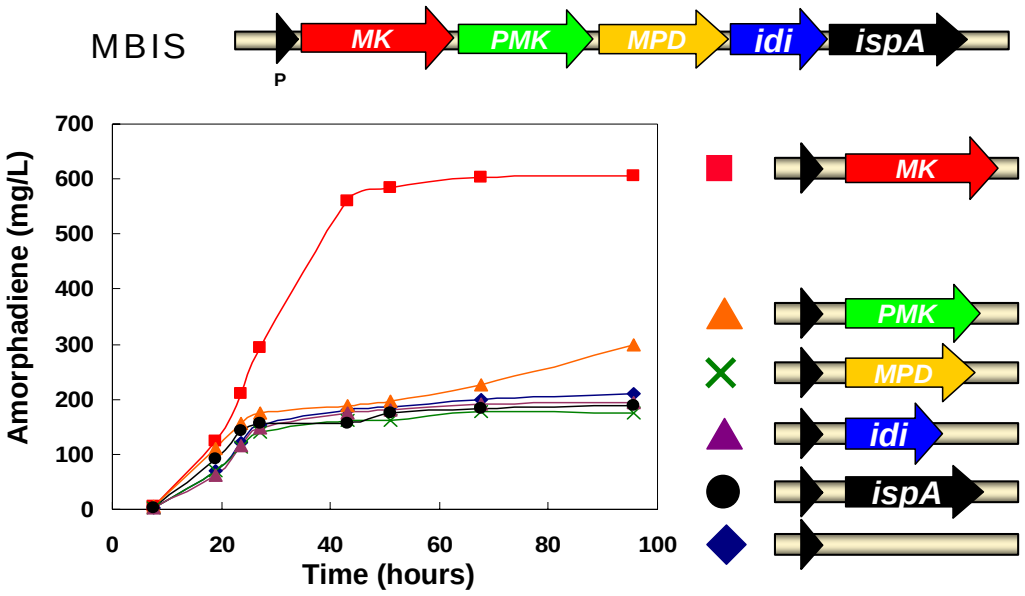
Optimizing the mevalonate pathway

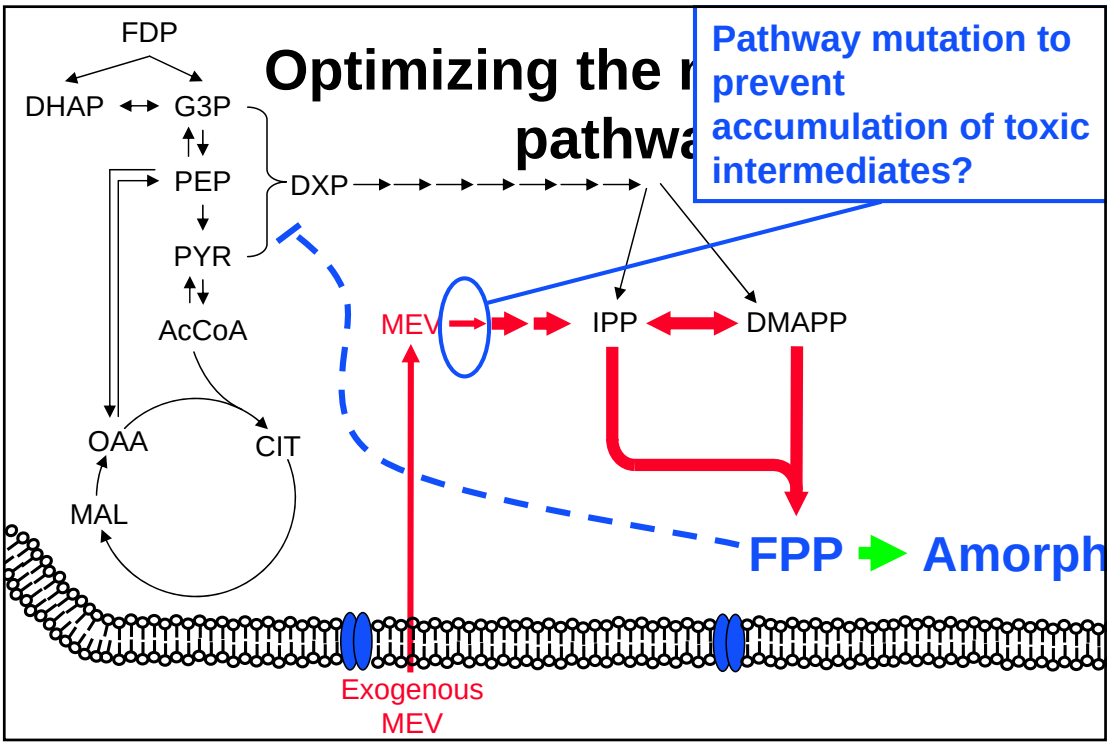



Optimizing the mevalonate pathway
FDP
DHAP
G3P
PEP
PYR
AcCoA
OAA
CIT
MAL
DXP
MEV
IPP
DMAPP
FPP
Amorphadiene
Exogenous MEV


High expression of amorphadiene synthase relieves inhibition
FDP
DHAP
G3P
PEP
PYR
AcCoA
OAA
CIT
MAL
DXP
MEV
IPP
DMAPP
FPP
Amorph
Exogenous MEV

Mevalonate kinase limits amorphadiene production

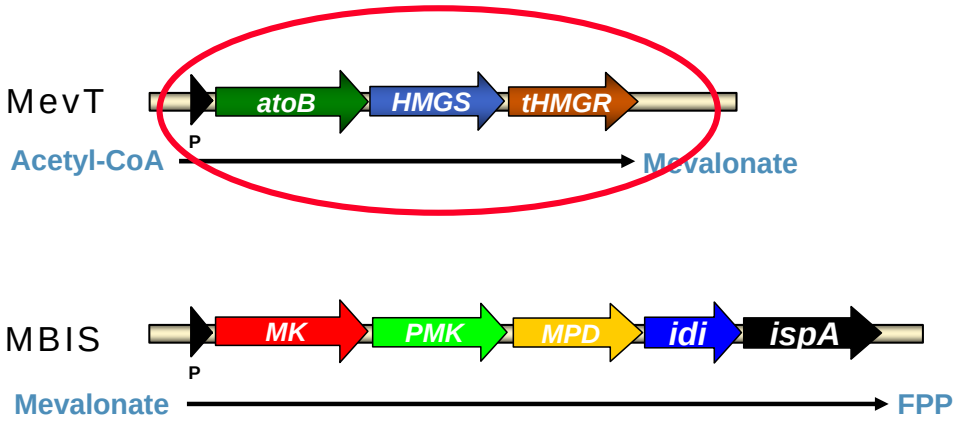




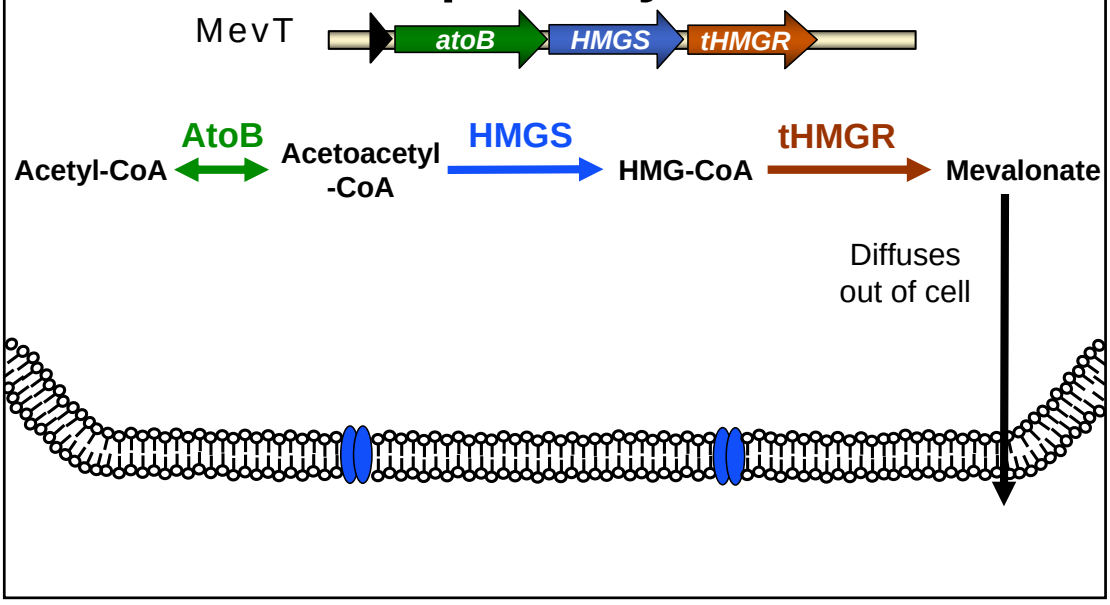
Mini-summary: Two lessons

- Even native metabolic intermediates can be toxic at high concentrations.
- “Pulling” on a pathway is just as important as “pushing”.

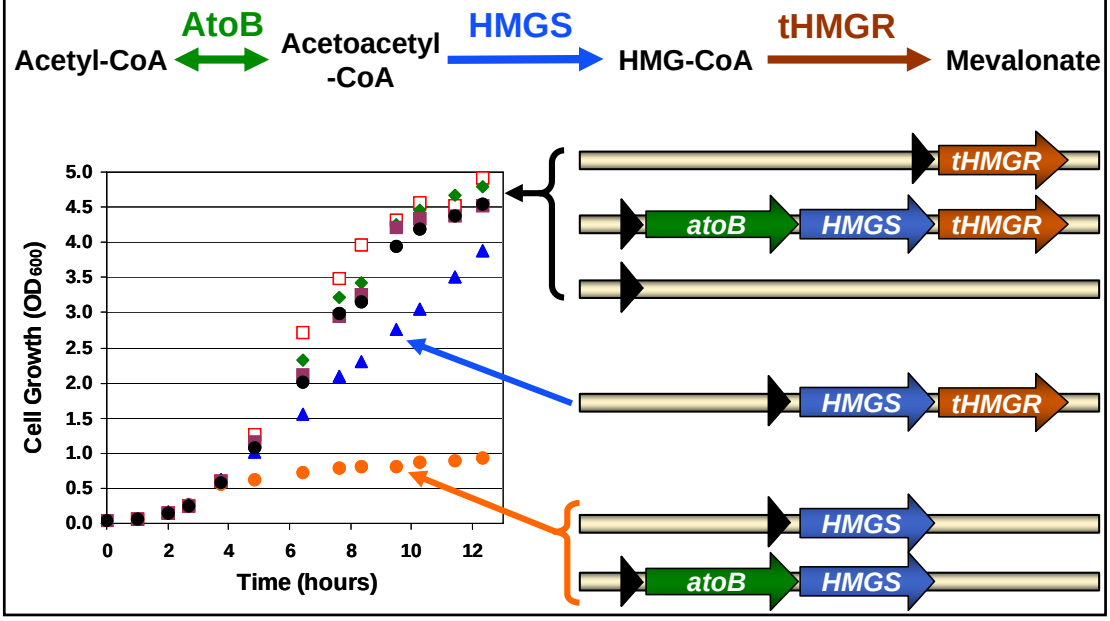
Optimizing the top part of the mevalonate pathway



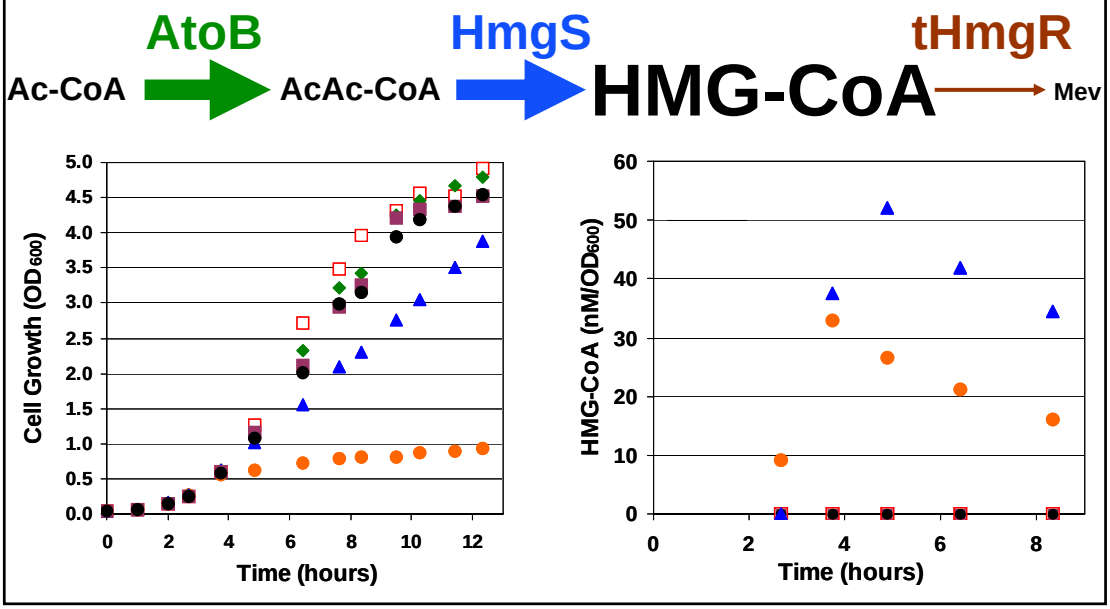
The top part of the mevalonate pathway



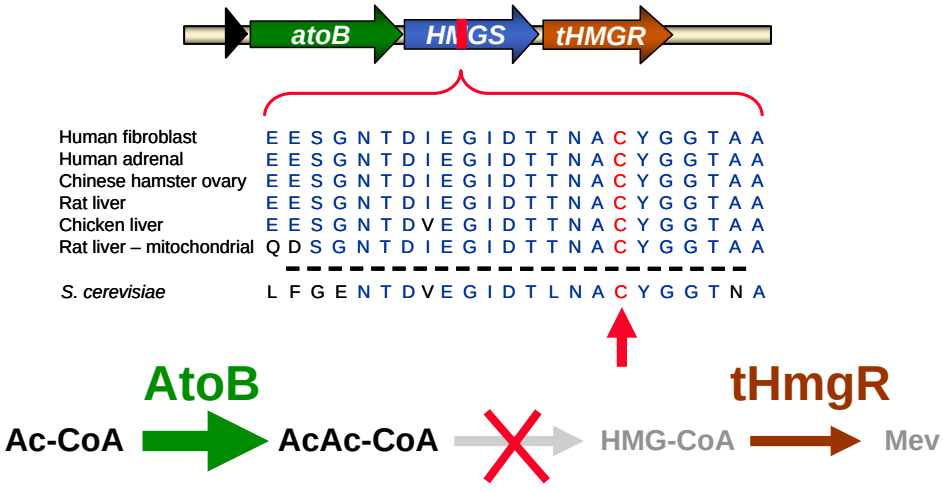
Growth inhibition by MevT pathway

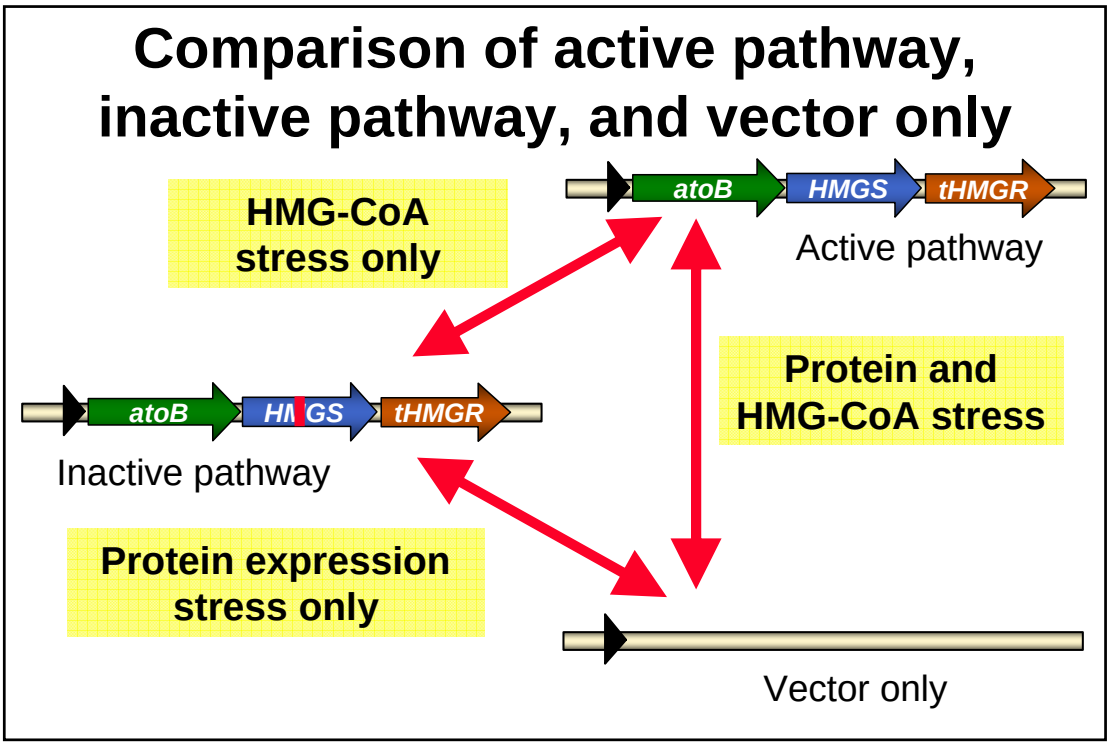


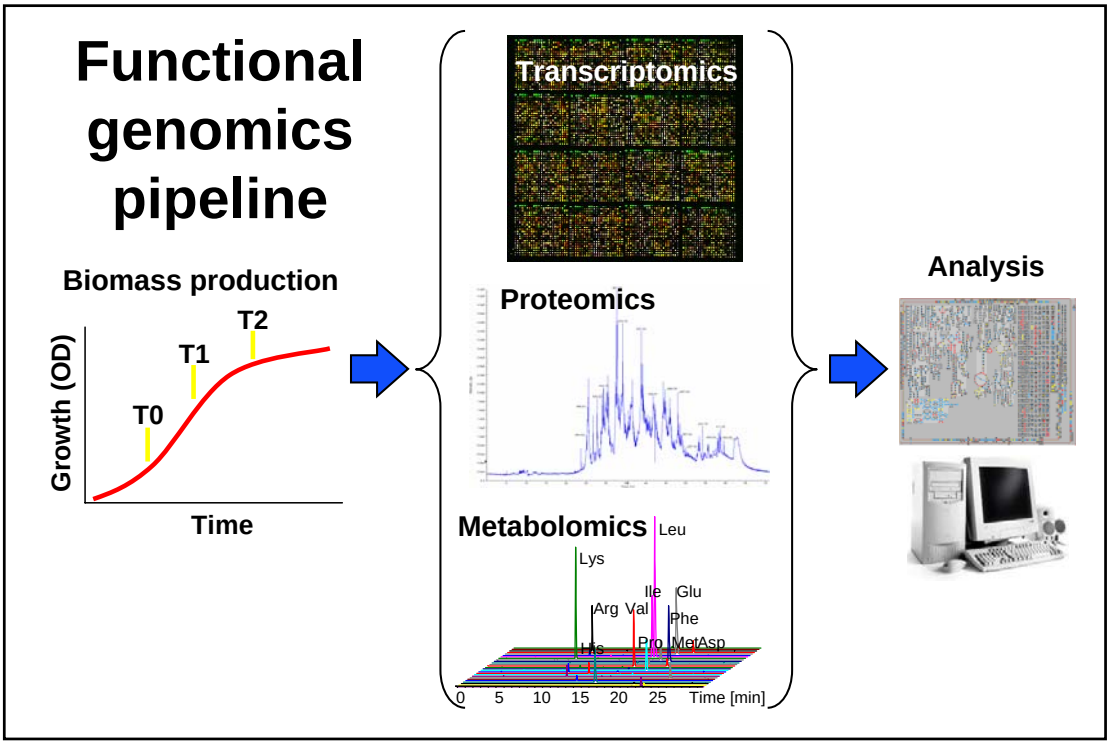
Accumulation of the toxic intermediate Hmg-CoA



C159A mutation in *HMGS* knocks out activity



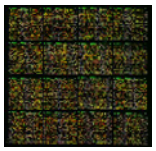

Comparison of active pathway, inactive pathway, and vector only
HMG-CoA stress only
atoB
HMGS
tHMGR
Active pathway
Protein and HMG-CoA stress
atoB
HMGS
tHMGR
Inactive pathway
Protein expression stress only
Vector only


Functional genomics pipeline
Biomass production
Growth (OD)
T0
T1
T2
Time
Transcriptomics
Proteomics
Metabolomics
Leu
Lys
Ile
Glu
Arg
Val
Phe
Pro
Met
Asp
0
5
10
15
20
25
Time [min]
Analysis

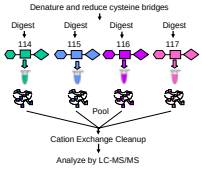
Functional genomics pipeline

Biomass production

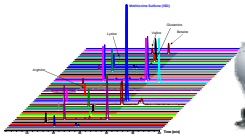
Transcripts



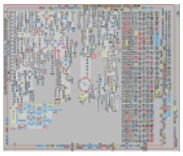
Proteins



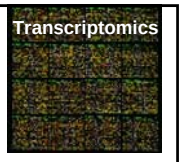
Metabolites

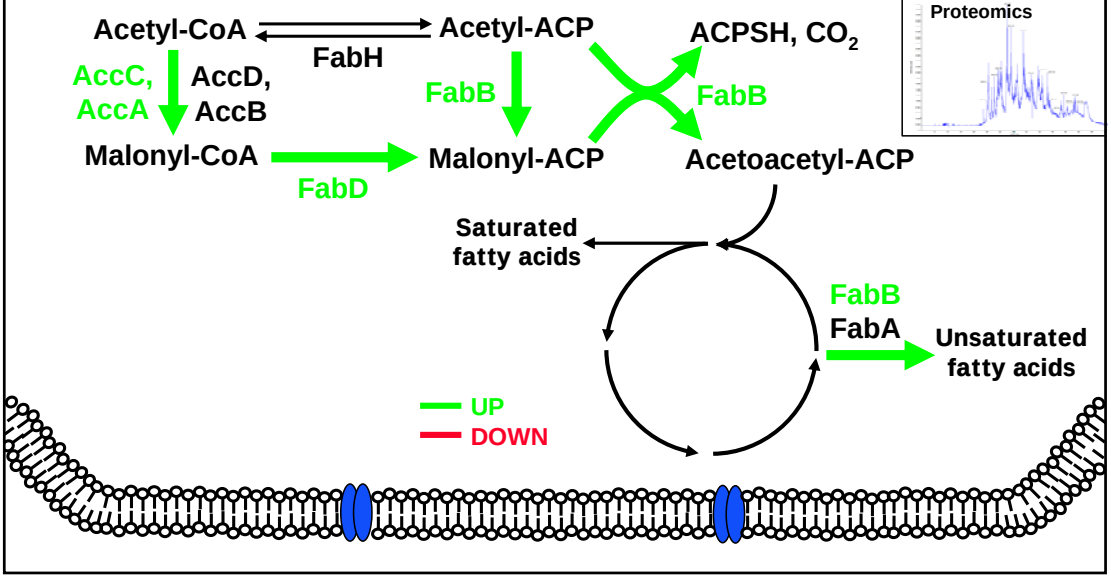


Analysis

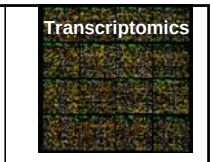


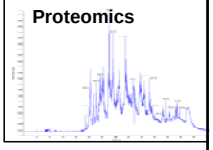
MevT up-regulates genes involved in fatty acid biosynthesis





Stress regulons up-regulated in MevT strain





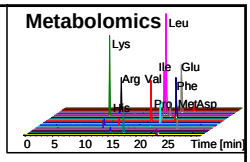
Sideophores
NADPH Generation
Fe-S Cluster Repair
Hydroperoxidases
Oxidative Stress

HMG-CoA

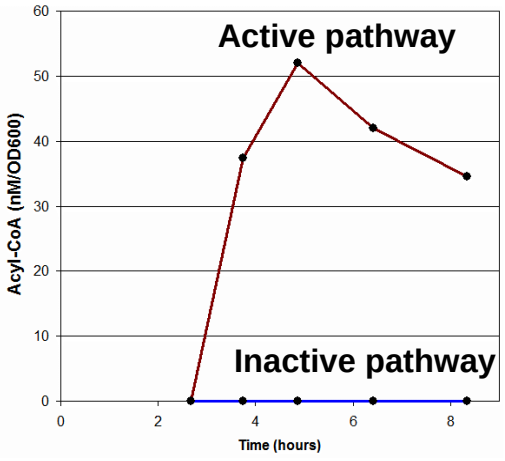
Proteases
Chaperones
Inclusion Body Proteins
Protein Misfolding

Membrane/Osmotic Stress
Protectants
PSP
Porins
Porins

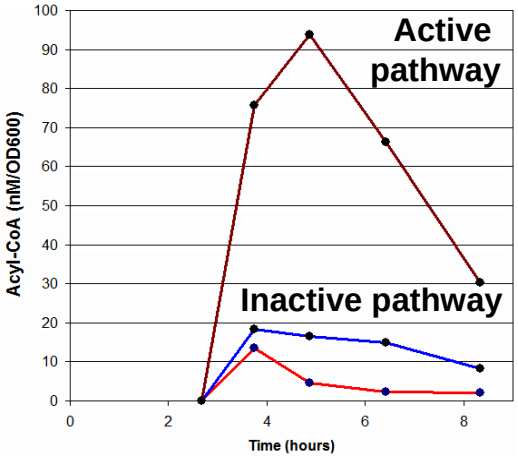
HMG-CoA and malonyl-CoA accumulation



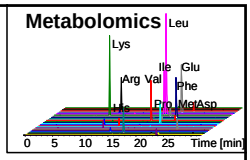
HMG-CoA

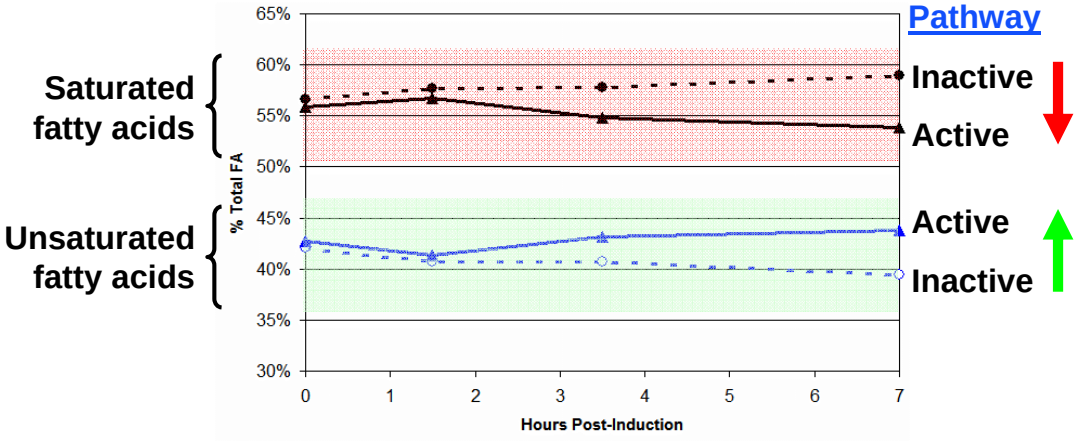


Malonyl-CoA

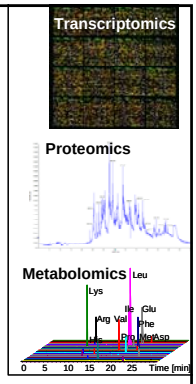


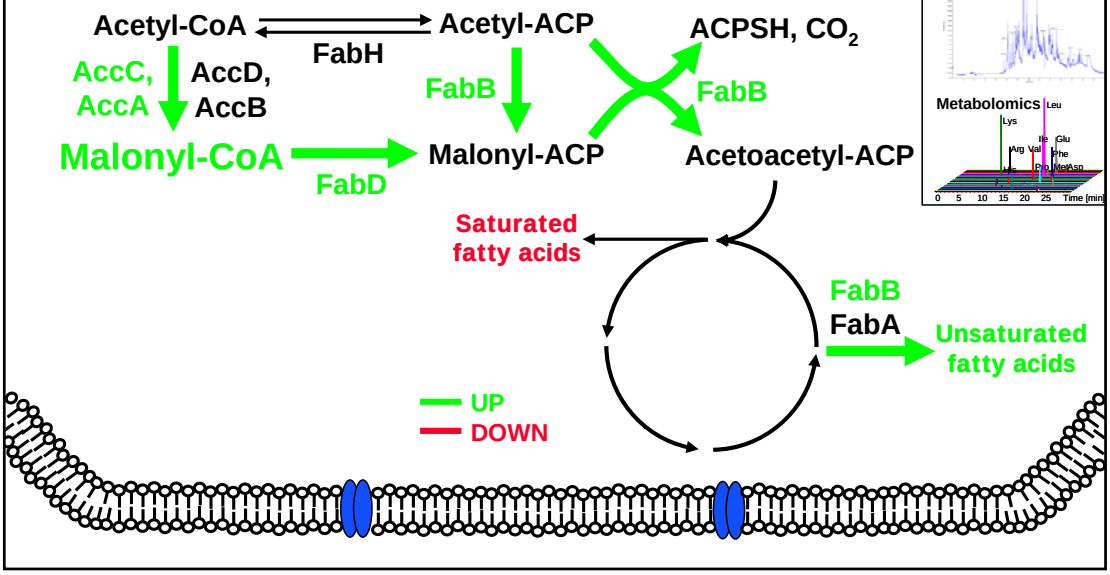
Presence of MevT enriches unsaturated fatty acids





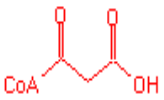
MevT up-regulates fatty acid biosynthesis



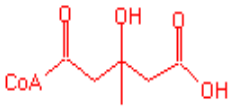


Three additional pieces of information

- Malonyl-CoA is very similar in structure to HMG-CoA



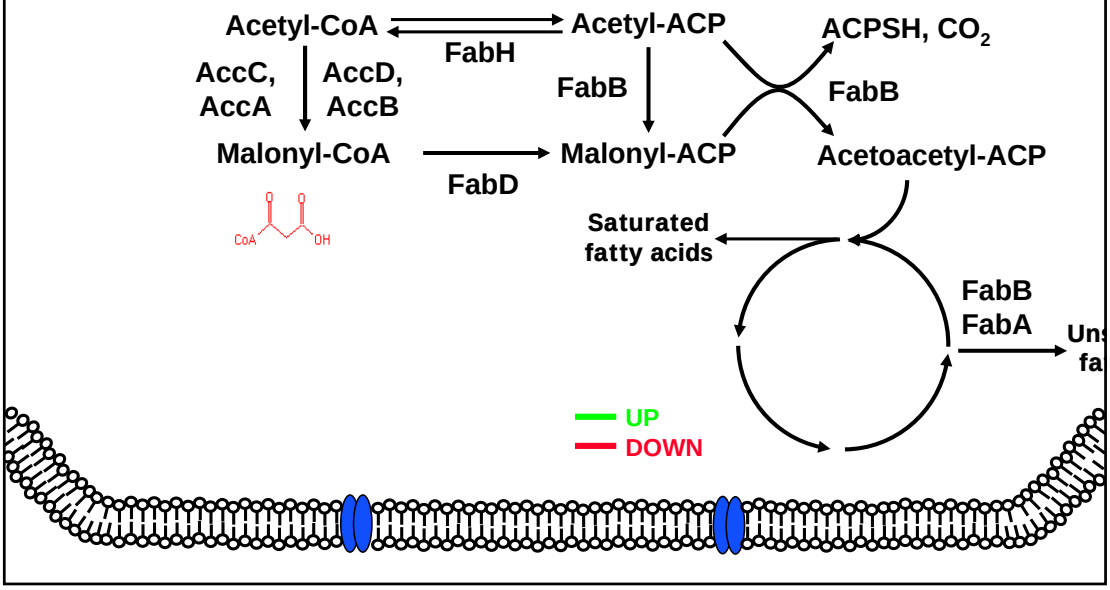
Malonyl-CoA

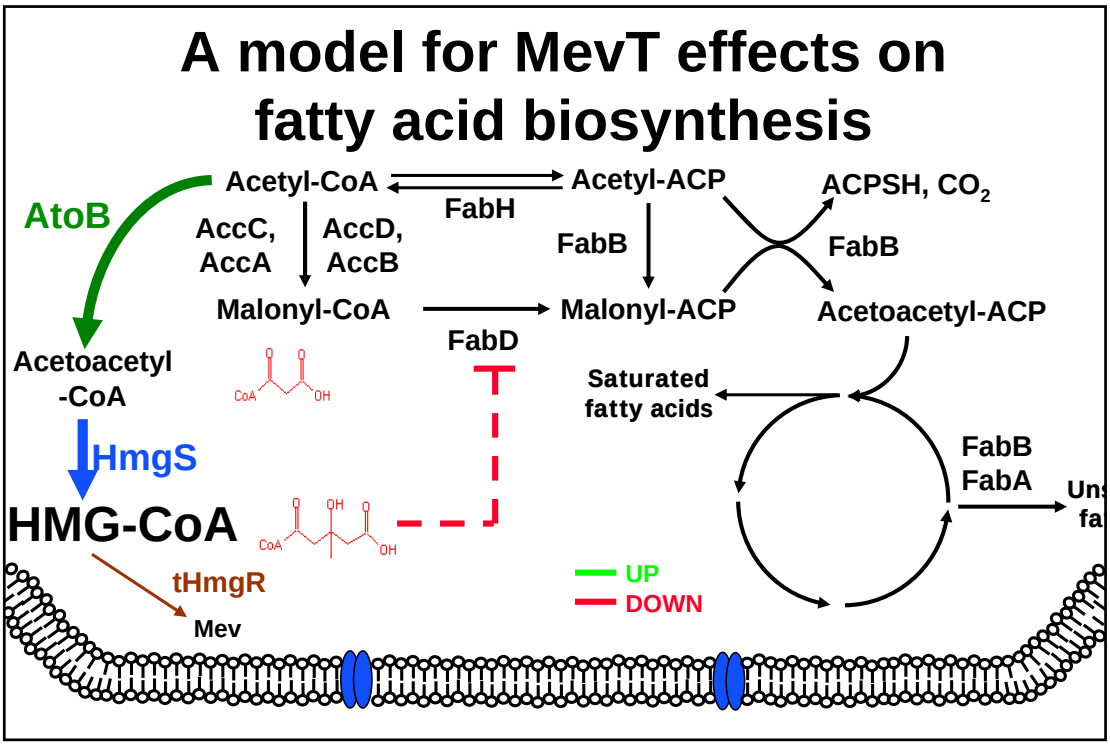


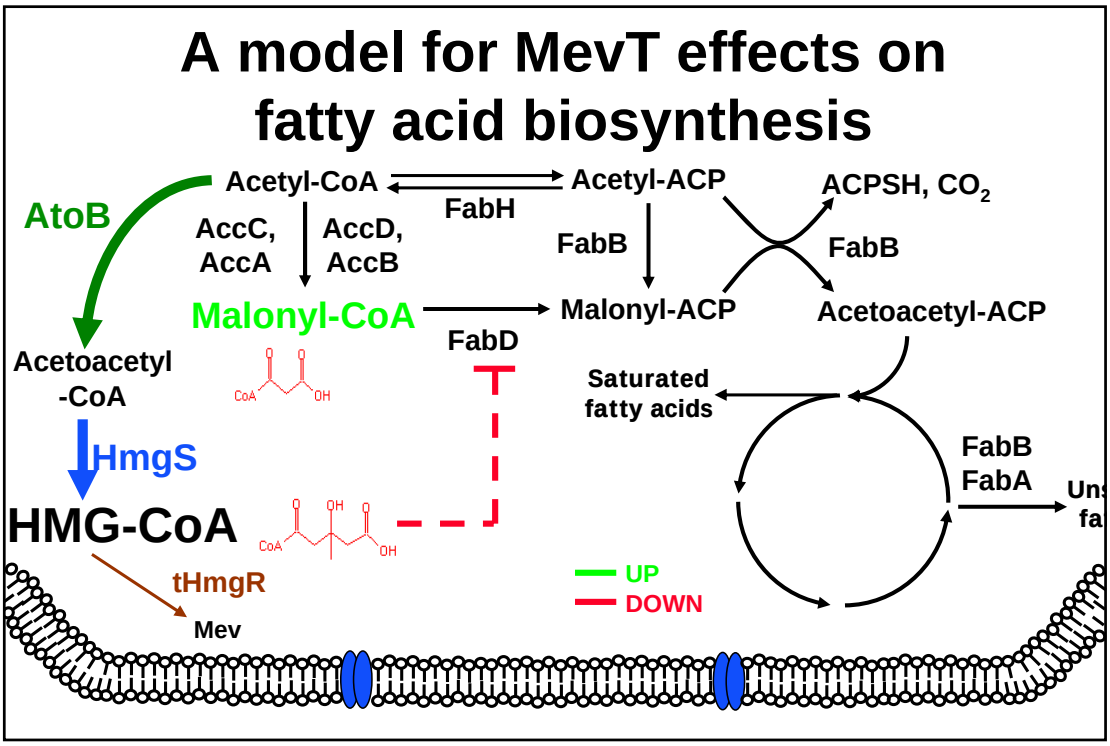
HMG-CoA

- HMG-CoA reductase (tHMGR) activity increases with induction, then decreases sharply.
- HMG-CoA accumulation in organisms that produce it natively is known to cause oxidative stress.

A model for MevT effects on fatty acid biosynthesis




A model for MevT effects on fatty acid biosynthesis
Acetyl-CoA
Acetyl-ACP
FabH
ACPSH, CO2
AtoB
AccC, AccA
AccD, AccB
FabB
FabB
Malonyl-CoA
Malonyl-ACP
Acetoacetyl-ACP
FabD
Acetoacetyl -CoA
Saturated fatty acids
HmgS
FabB FabA
HMG-CoA
tHmgR
UP
DOWN
Mev


A model for MevT effects on fatty acid biosynthesis
Acetyl-CoA
Acetyl-ACP
FabH
ACPSH, CO2
AtoB
AccC, AccA
AccD, AccB
FabB
FabB
Malonyl-CoA
Malonyl-ACP
Acetoacetyl-ACP
FabD
Acetoacetyl -CoA
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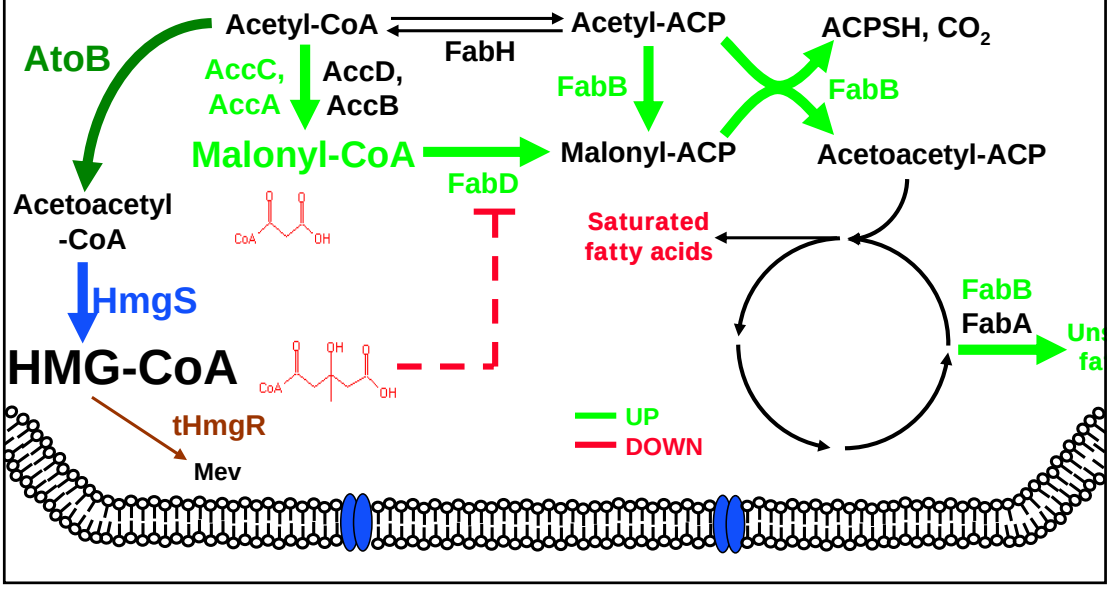
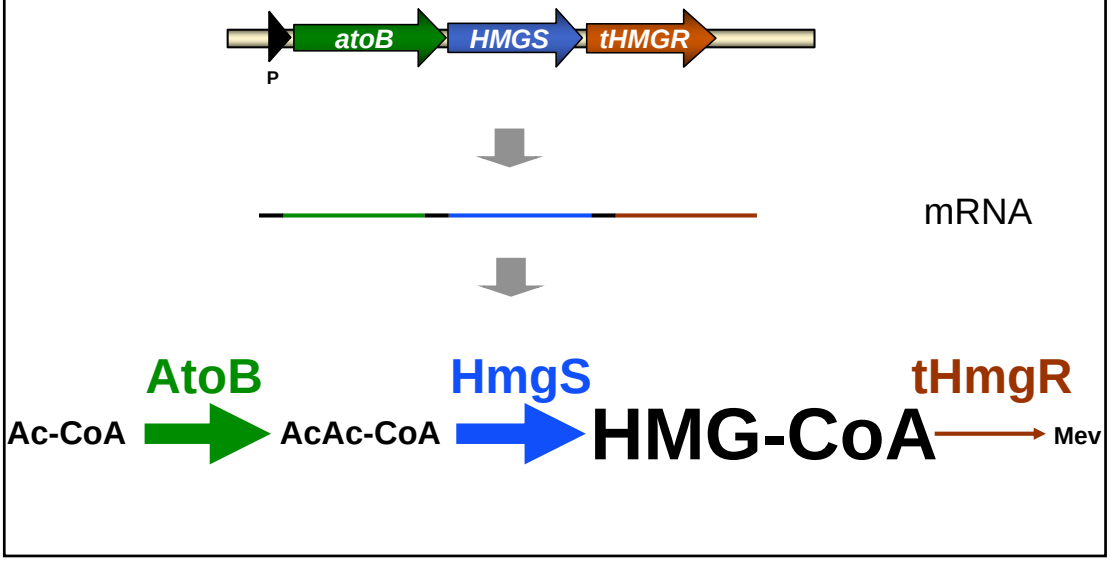
HmgS
FabB FabA
HMG-CoA
tHmgR
UP
DOWN
Mev

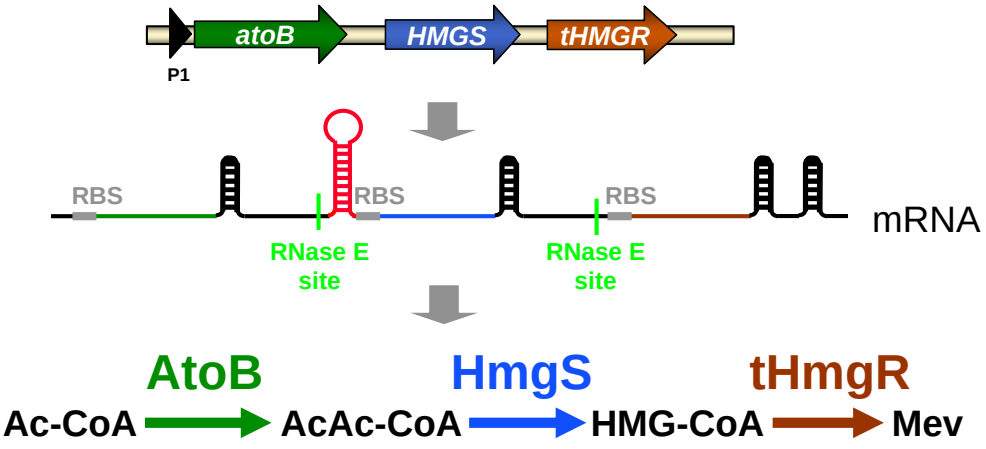
A model for MevT effects on fatty acid biosynthesis



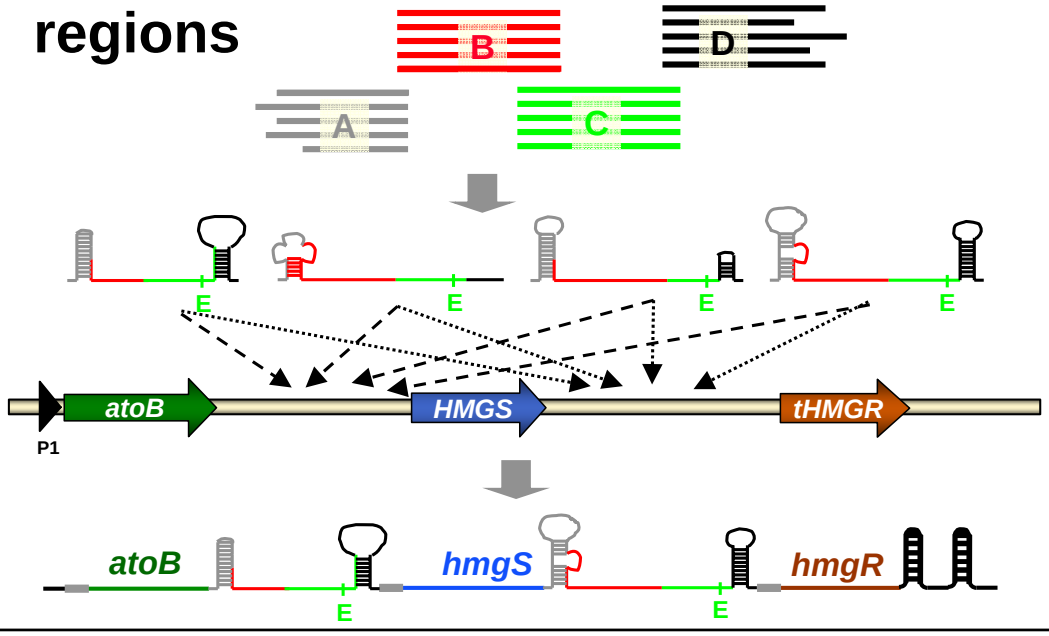
Accumulation of the toxic intermediate HMG-CoA



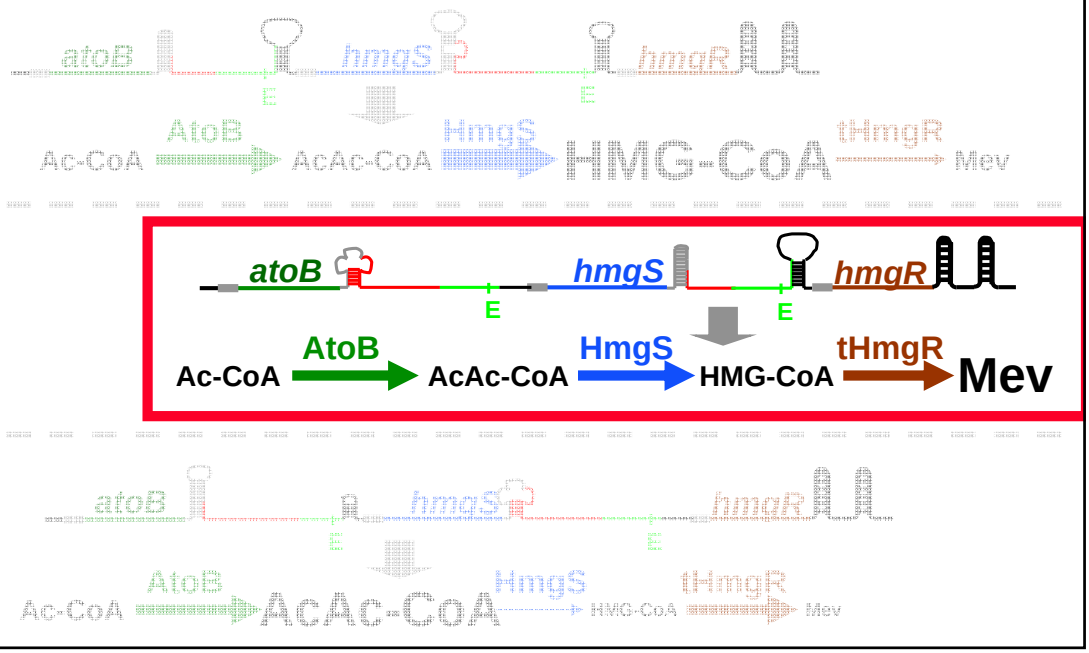
Balancing the mevalonate pathway by tuning mRNA stability and translation efficiency



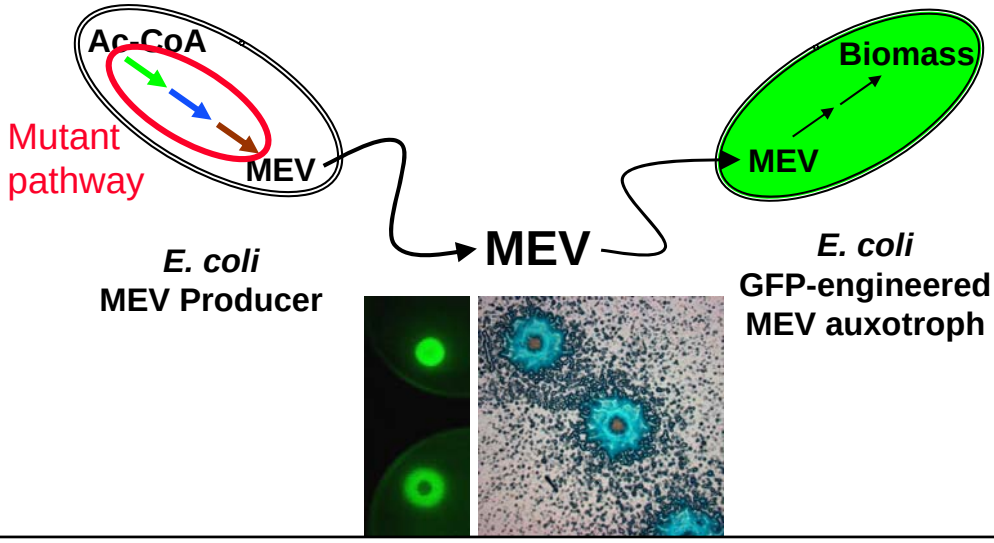
Combinatorial design of intergenic regions



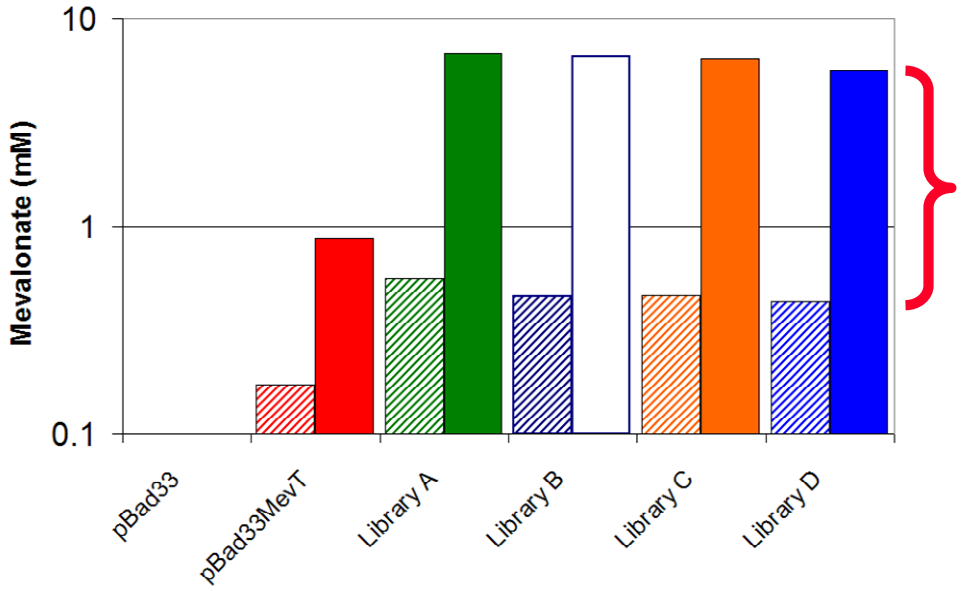
How do we choose?



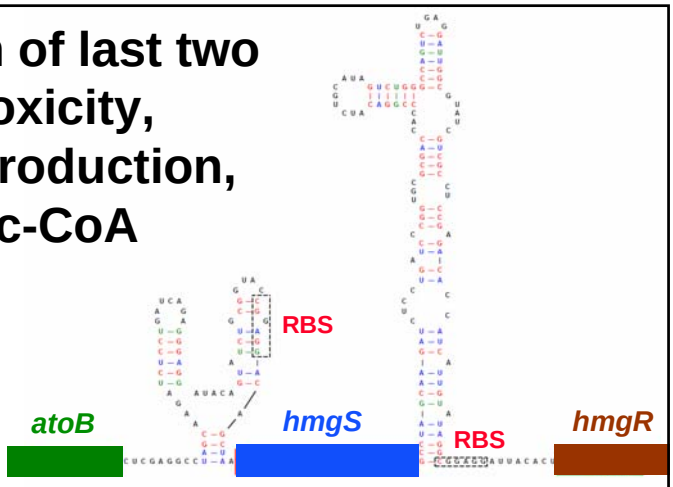
A fluorescent screen for improved mevalonate production



Best producers make about 7X more mevalonate



Down-regulation of last two genes relieves toxicity, increases Mev production, and increases Ac-CoA concentration

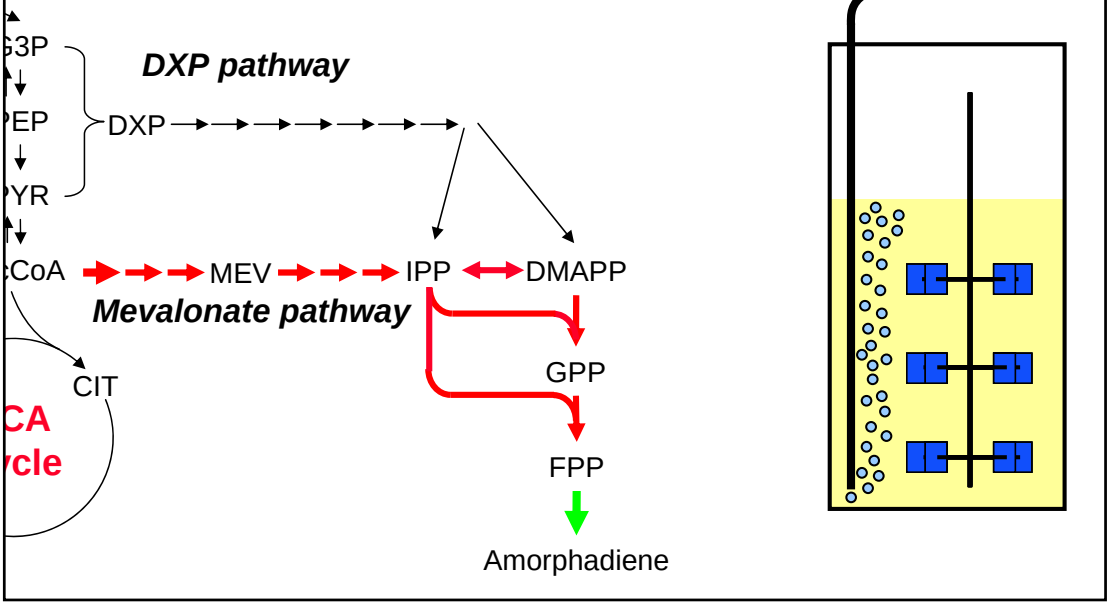


Ac-CoA —(AtoB)→ AcAc-CoA —(HmgS)→ HMG-CoA —(HmgR)→ Mev

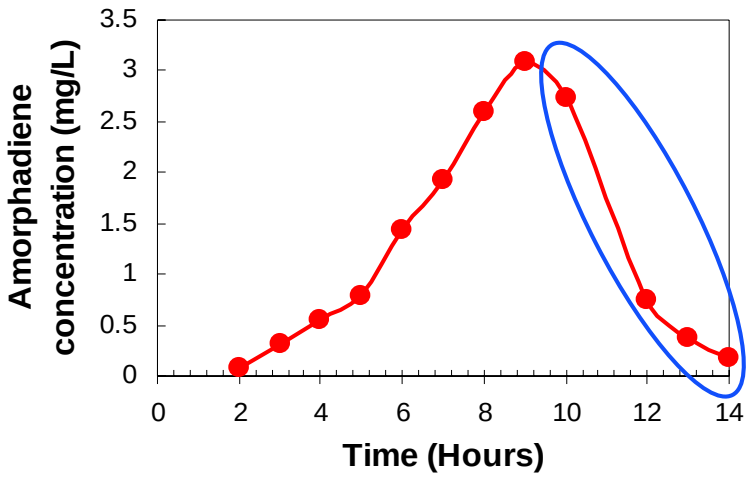
Mini-summary: Two more lessons

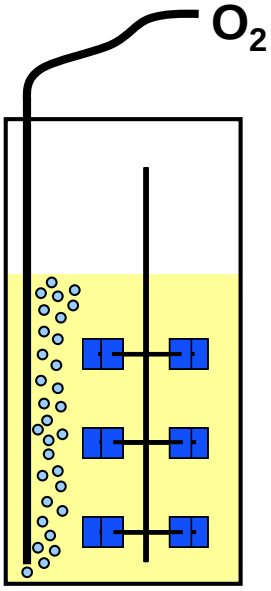
- Functional genomics is a useful tool for diagnosing problems in engineered cells.
- Pathway balancing is much harder than pathway construction!

Large-scale production of amorphadiene

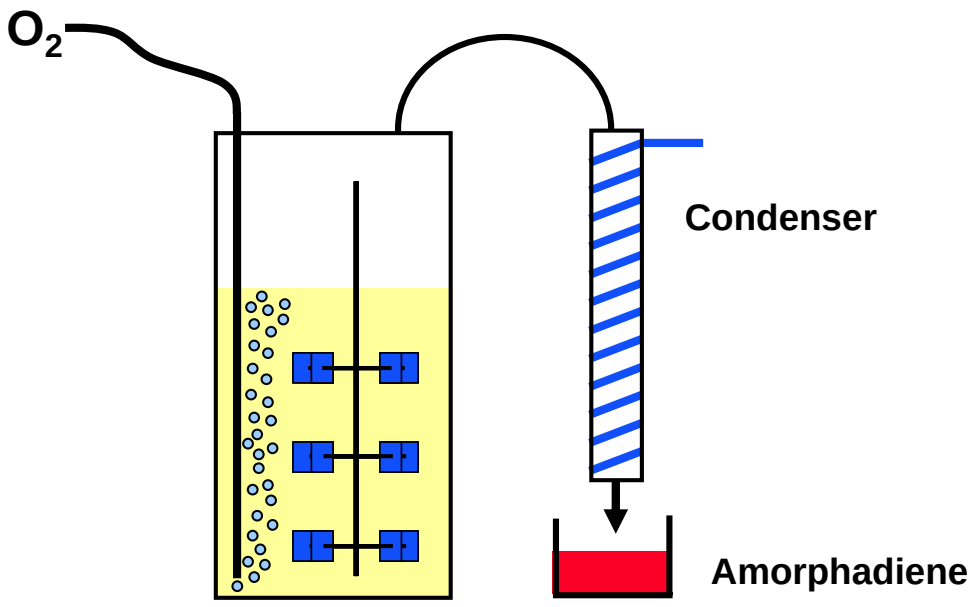


Amorphadiene is volatile and is lost from bioreactors

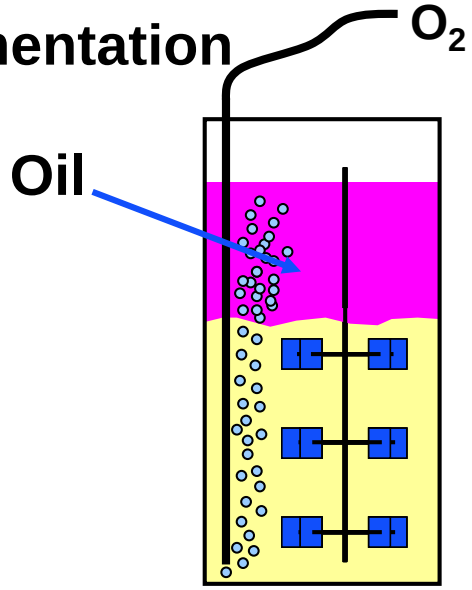




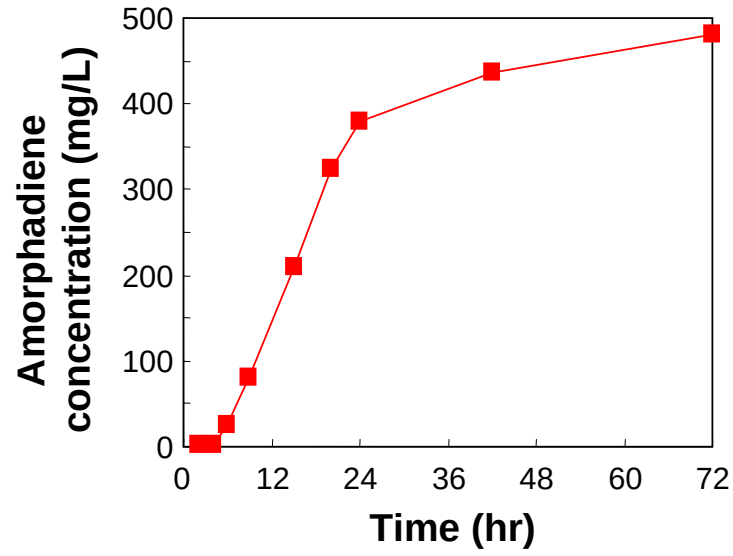
Amorphadiene is lost from bioreactors

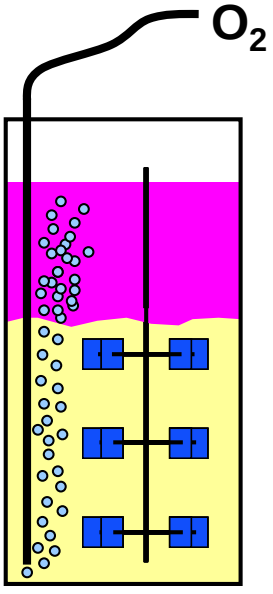


Amorphadiene production in a two-phase fermentation

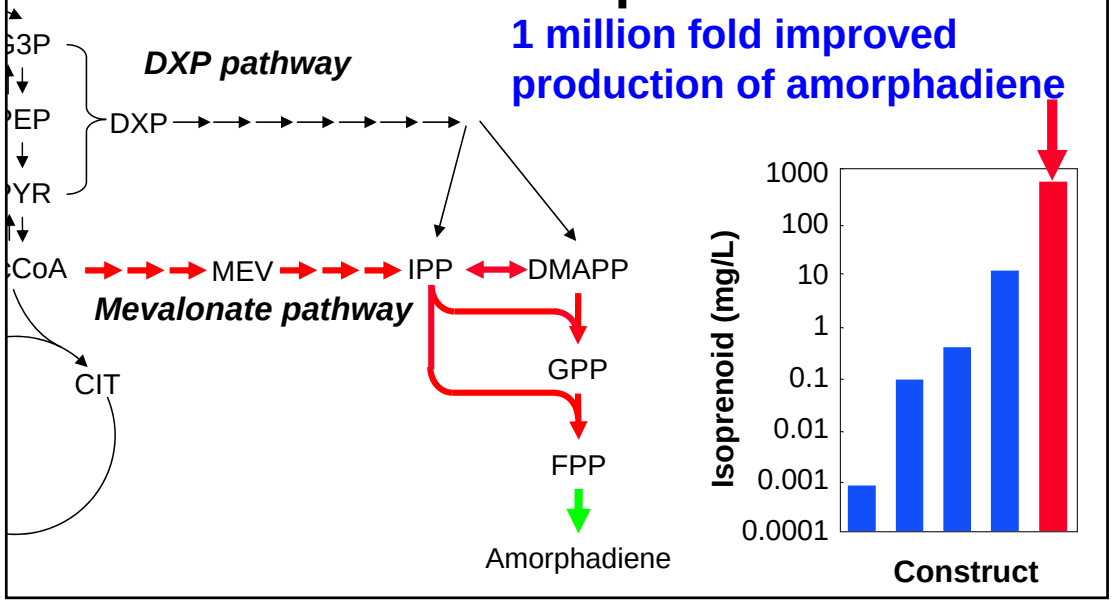


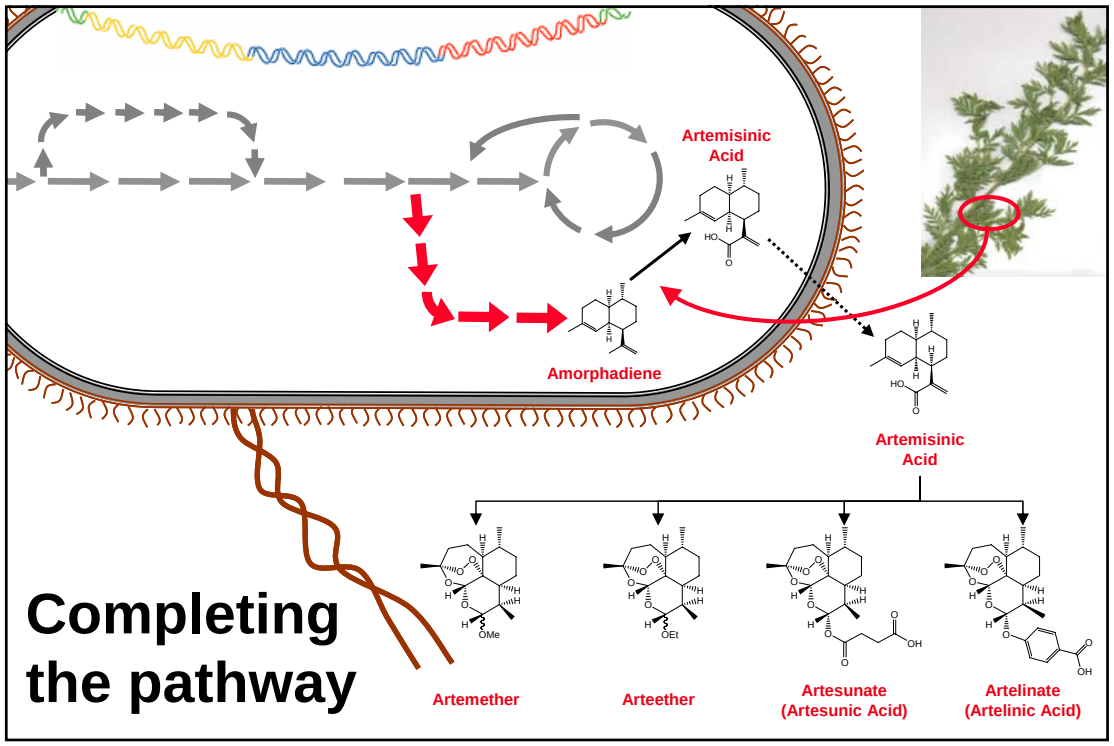
Amorphadiene production in a two-phase fermentation



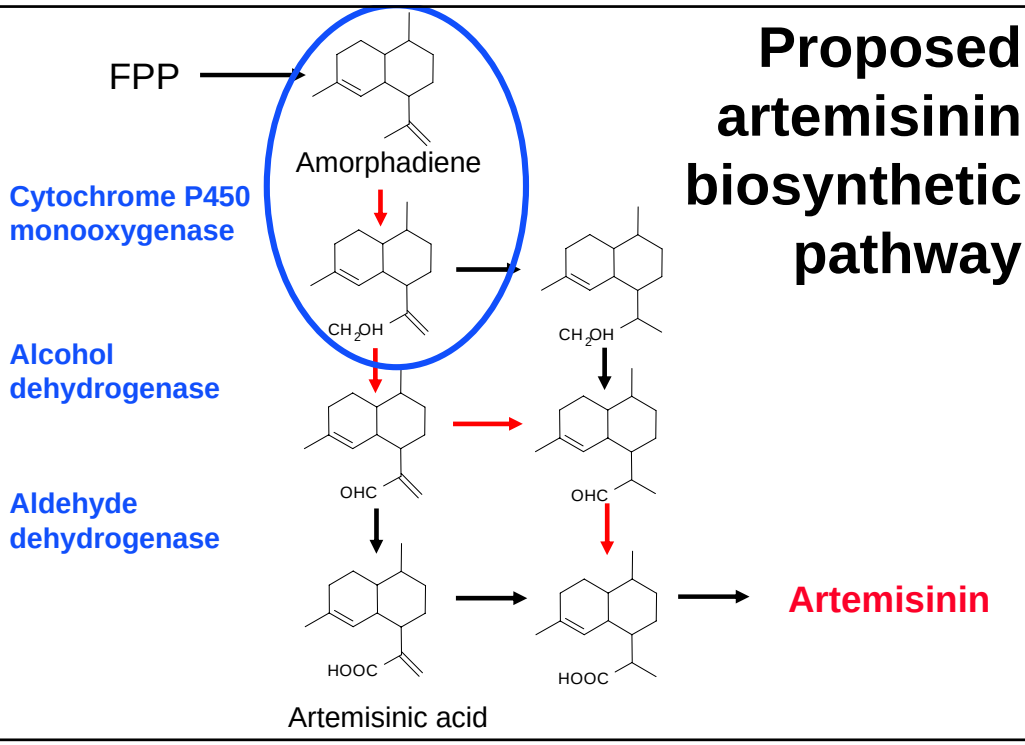


A two-phase fermentation collects all of the amorphadiene



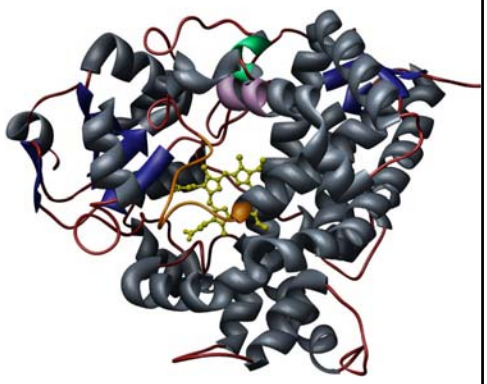


Proposed artemisinin biosynthetic pathway



Cytochrome P450's

- Heme-containing oxygenases
- Important for metabolizing toxic compounds in the human body
- Often required as one of the initial steps in natural products synthesis
- Plant- and animal-derived versions require additional proteins for function
  - Cytochrome P450 reductase partners
- Often bound to the membrane

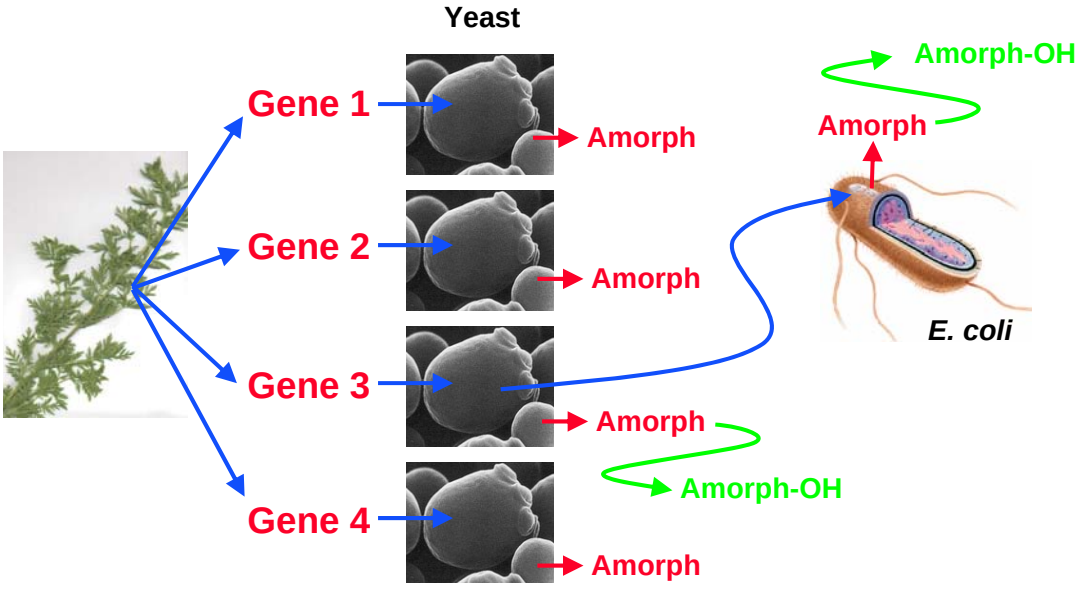


http://www.p450.kvl.dk/gallery/CYP3A4.jpg

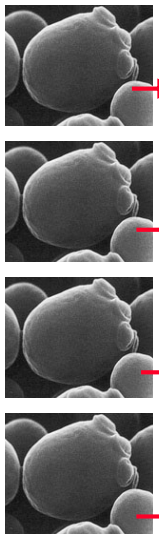
Cloning the remaining genes in the Artemisinin biosynthetic pathway

- Cytochrome P450's are traditionally difficult to express in *E. coli*
- A good host for cytochrome P450 expression and screening would aid in elucidating the remaining steps in the biosynthetic pathway
  - **A *Saccharomyces cerevisiae* (yeast) strain capable of producing amorphadiene would be very beneficial**

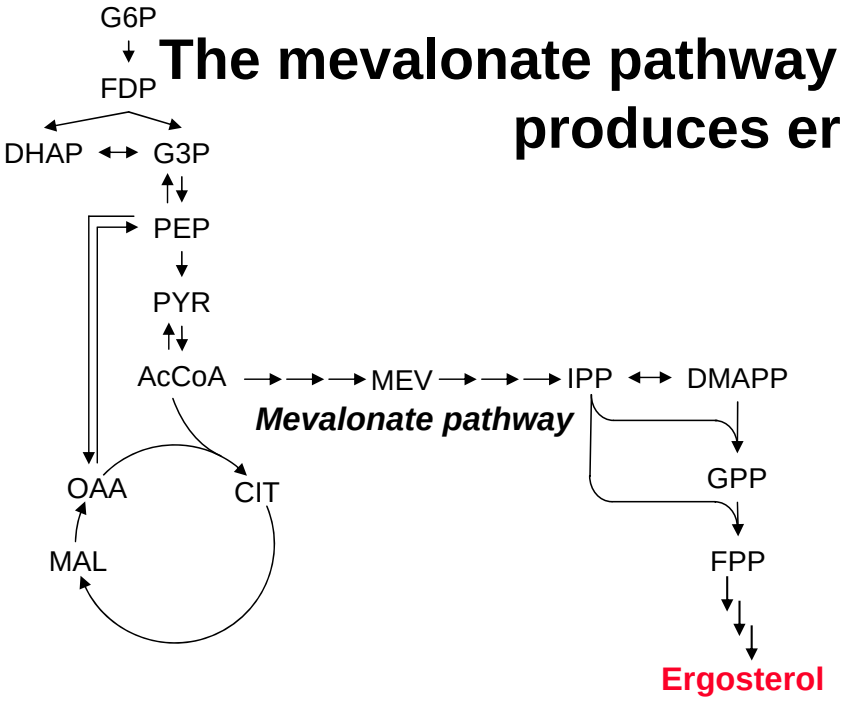
An experimental path for identifying the P450



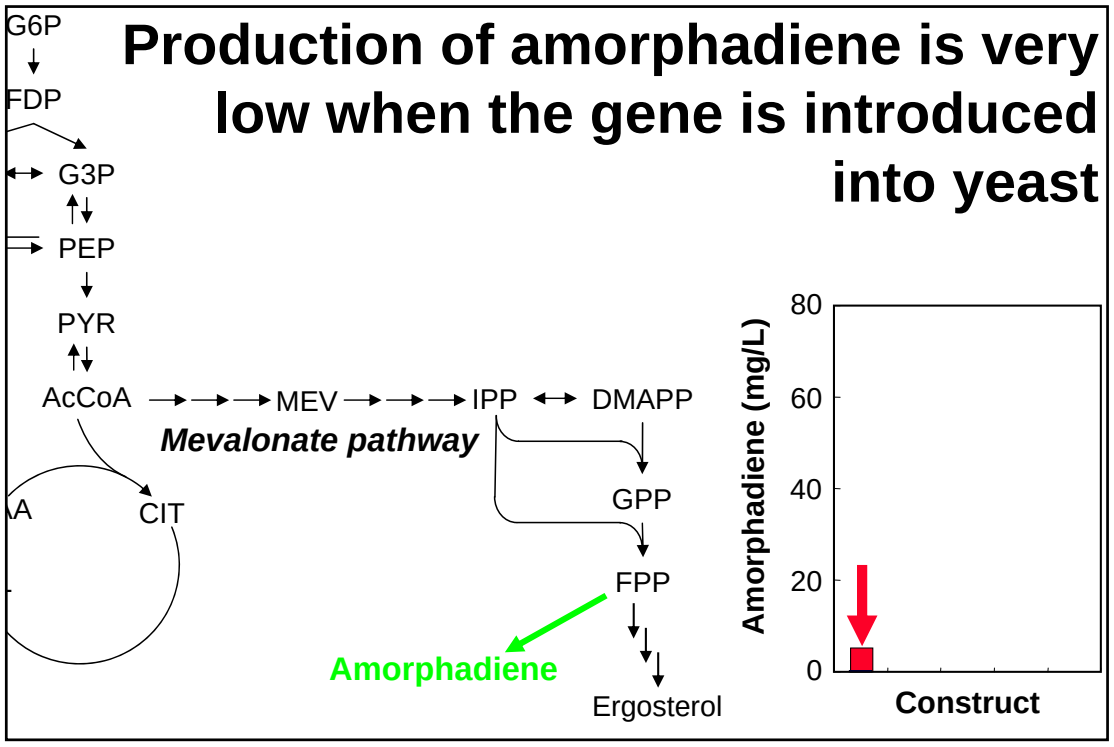
Construction of a yeast that produces amorphadiene



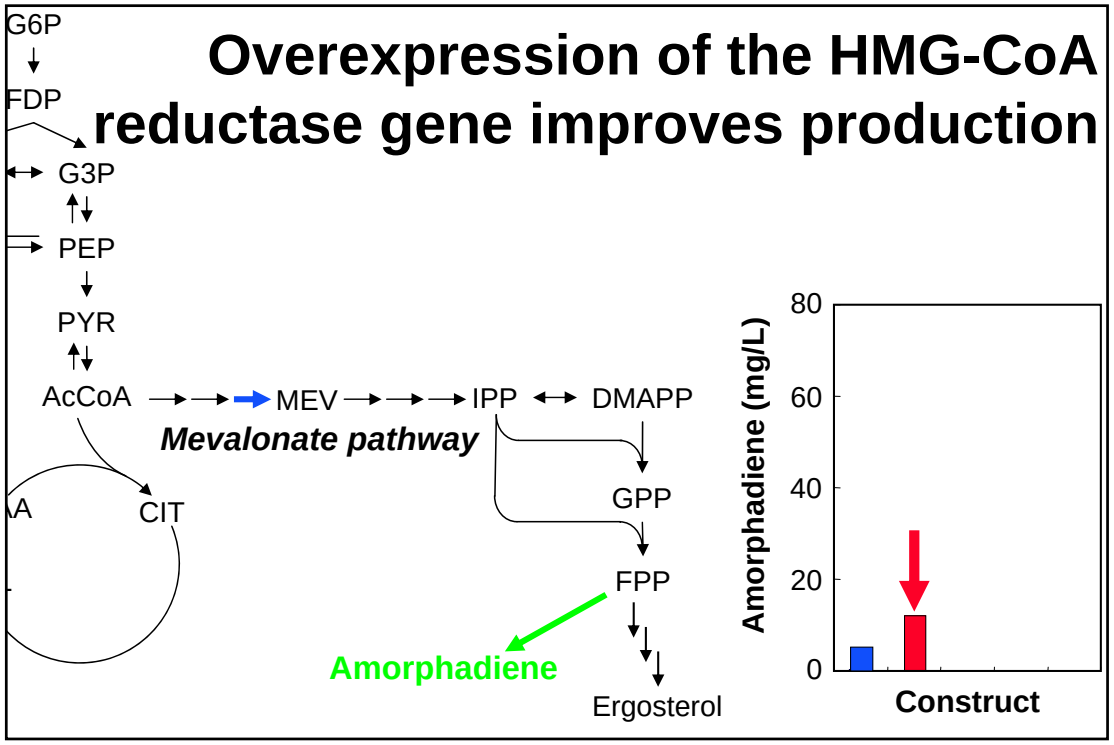
The mevalonate pathway in yeast produces ergosterol

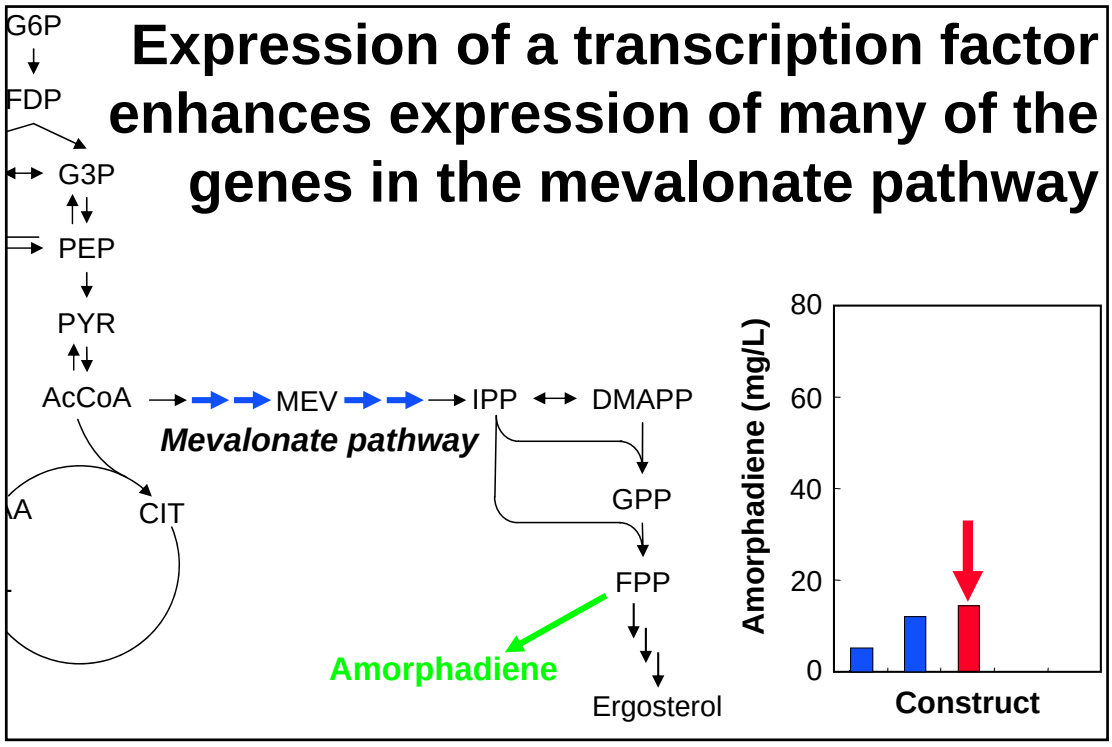


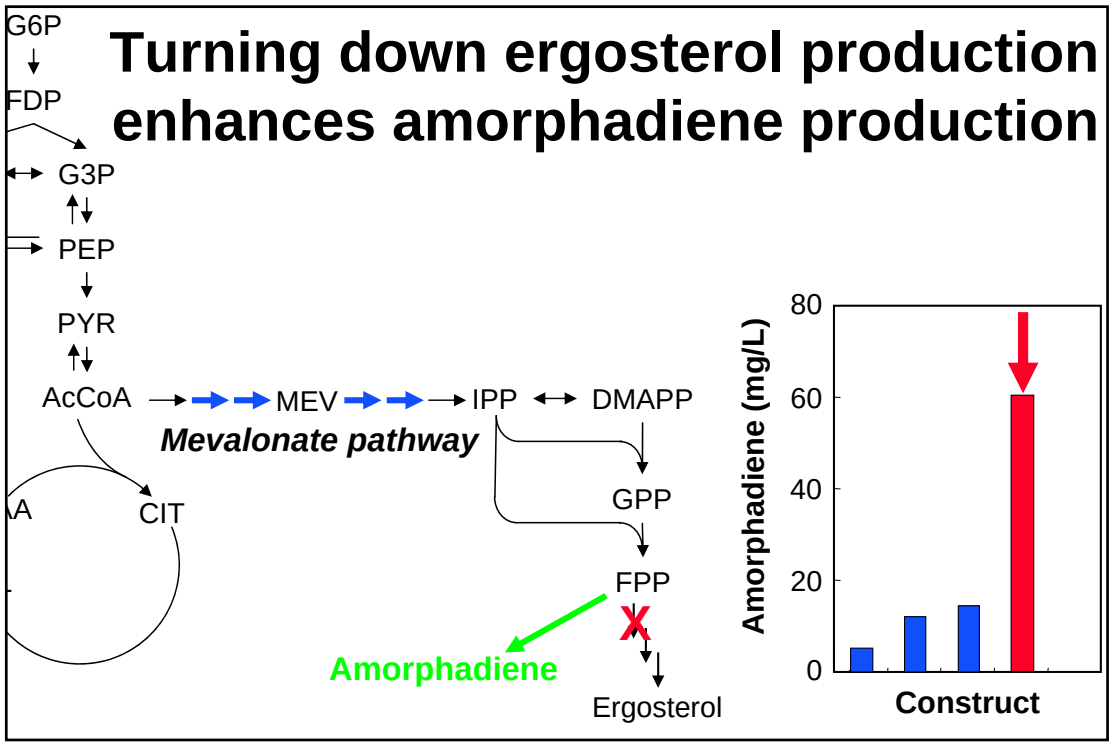
Production of amorphadiene is very low when the gene is introduced into yeast



Overexpression of the HMG-CoA reductase gene improves production

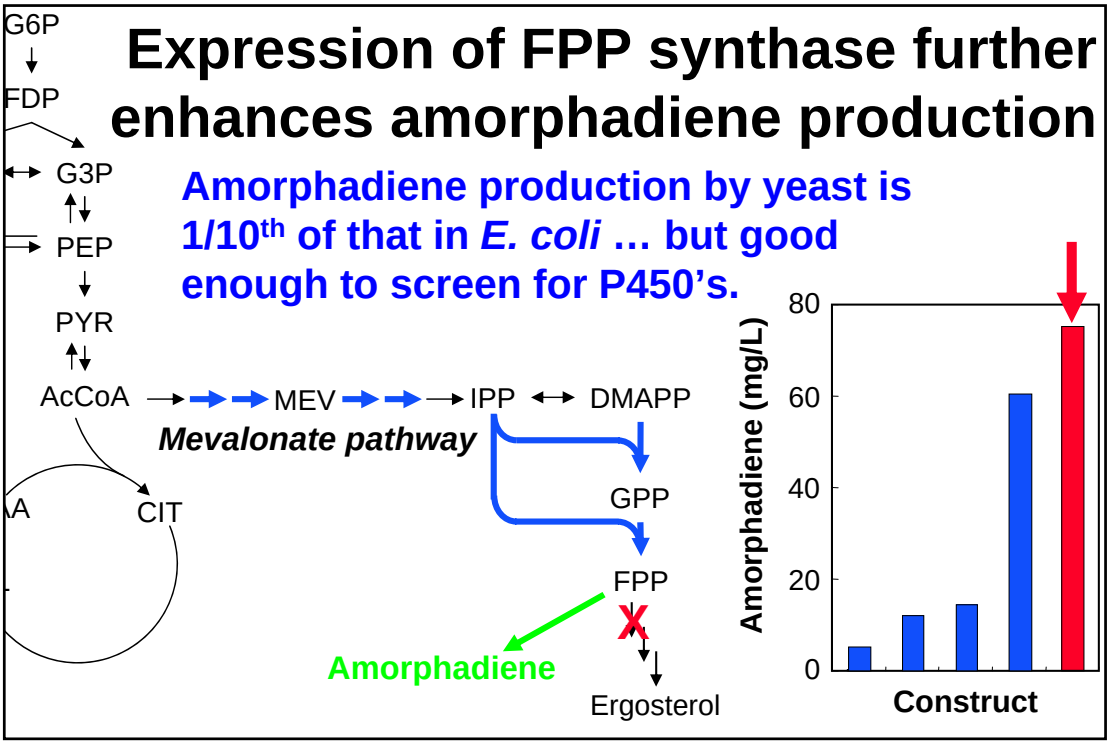



Expression of a transcription factor enhances expression of many of the genes in the mevalonate pathway
G6P
FDP
G3P
PEP
PYR
AcCoA
MEV
IPP
DMAPP
Mevalonate pathway
CIT
GPP
FPP
Amorphadiene
Ergosterol
Amorphadiene (mg/L)
80
60
40
20
0
Construct

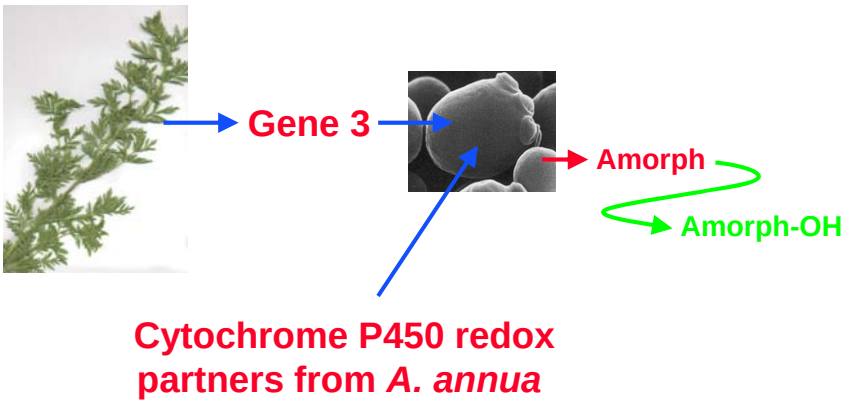

Turning down ergosterol production enhances amorphadiene production
G6P
FDP
G3P
PEP
PYR
AcCoA
MEV
IPP
DMAPP
Mevalonate pathway
CIT
GPP
FPP
Amorphadiene
Ergosterol
Amorphadiene (mg/L)
80
60
40
20
0
Construct

Expression of FPP synthase further enhances amorphadiene production

Amorphadiene production by yeast is 1/10th of that in *E. coli* … but good enough to screen for P450's.



The genes encoding the P450 and redox partners must be present in the same cell



Finding the remaining genes in the biosynthetic pathway

Gene 1

Gene 2

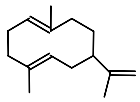
Gene 3

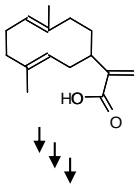
Gene 4

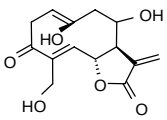
Lettuce, chicory, and sunflower produce isoprenoids like artemisinin

Germacrene A
(Chicory, sunflower and lettuce)

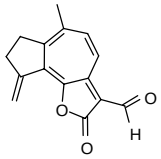
Amorphadiene
(*Artemisia annua*)





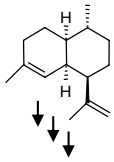


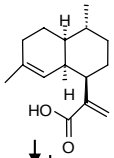
niveusin A

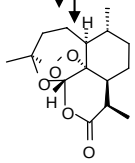


lettucenin A

P450's involved

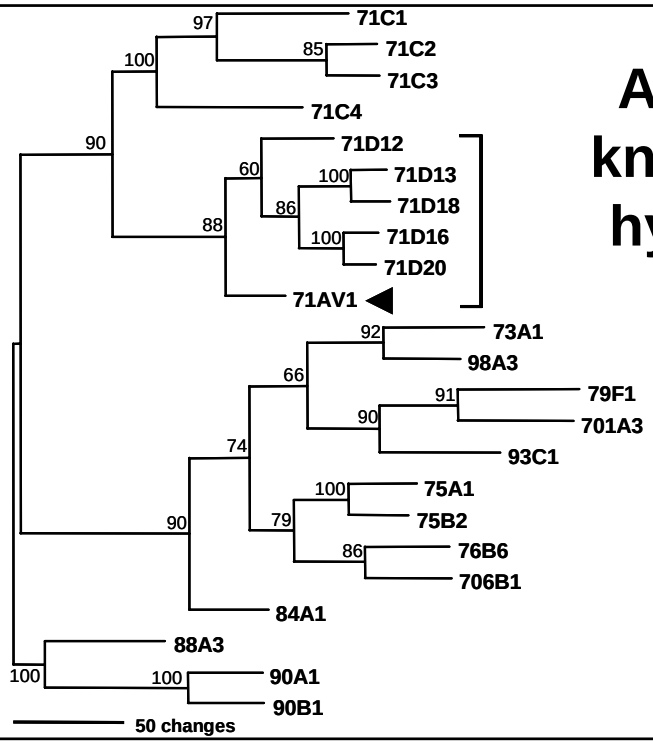




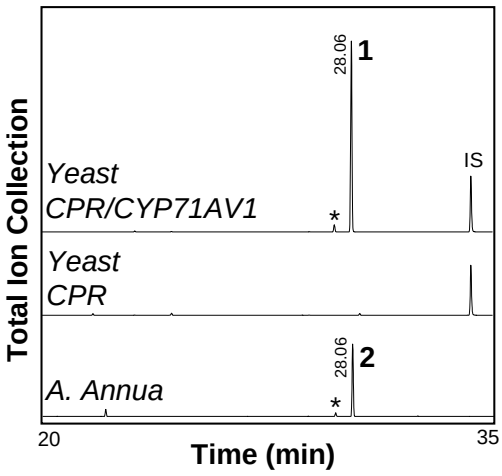


Artemisinin

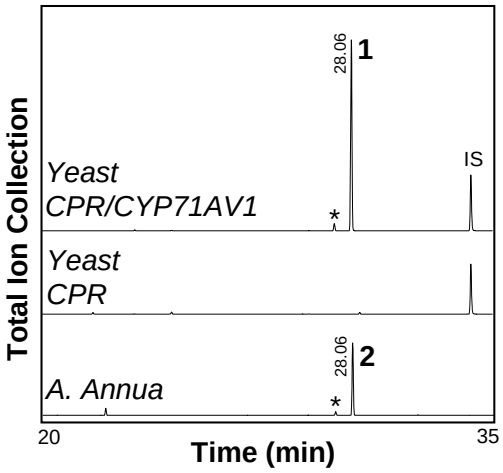
Alignment of known terpene hydroxylases

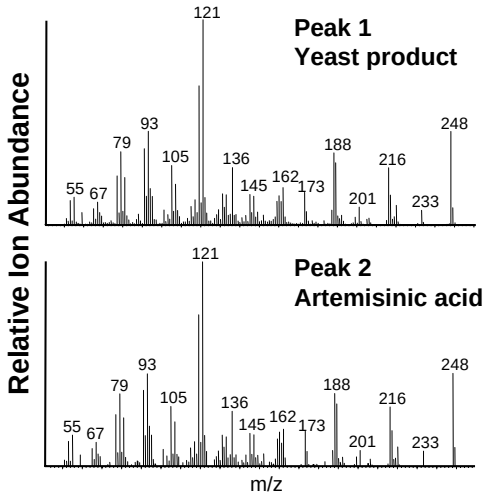


P450 candidate is functional in yeast!

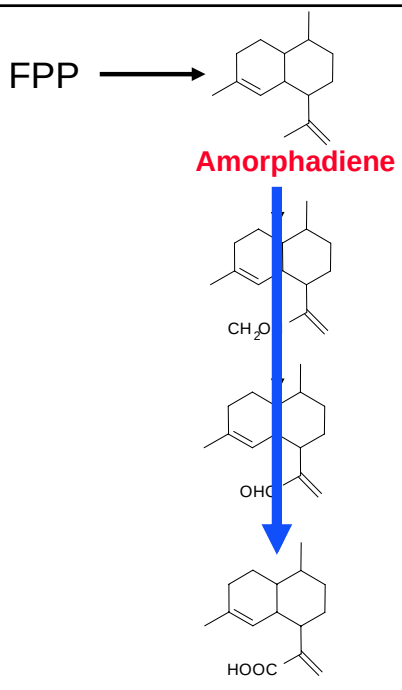


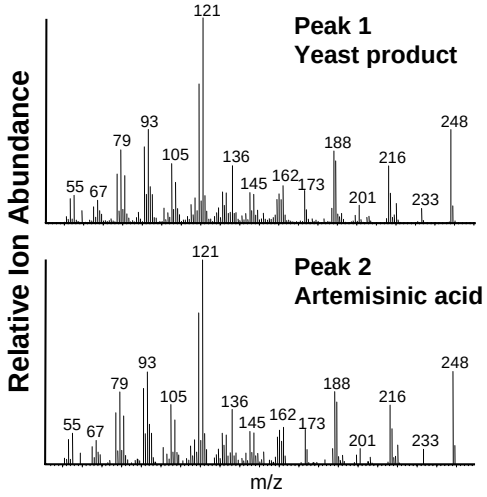
P450 candidate produces artemisinic acid!

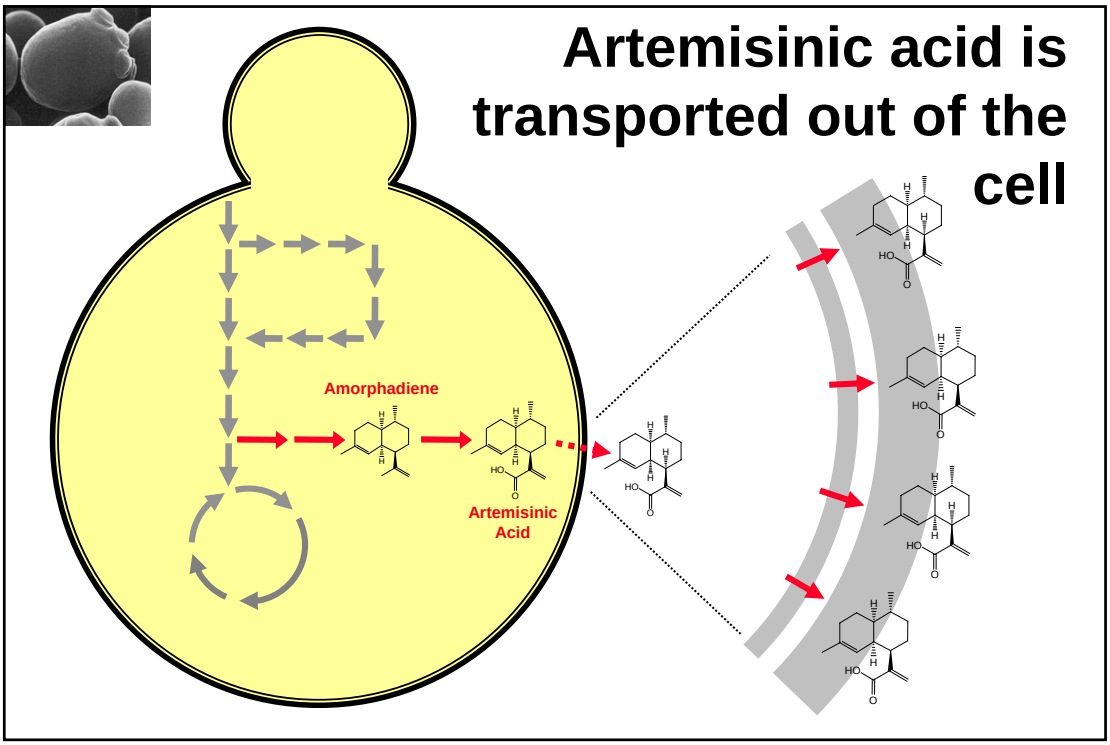




Proposed artemisinin biosynthetic pathway

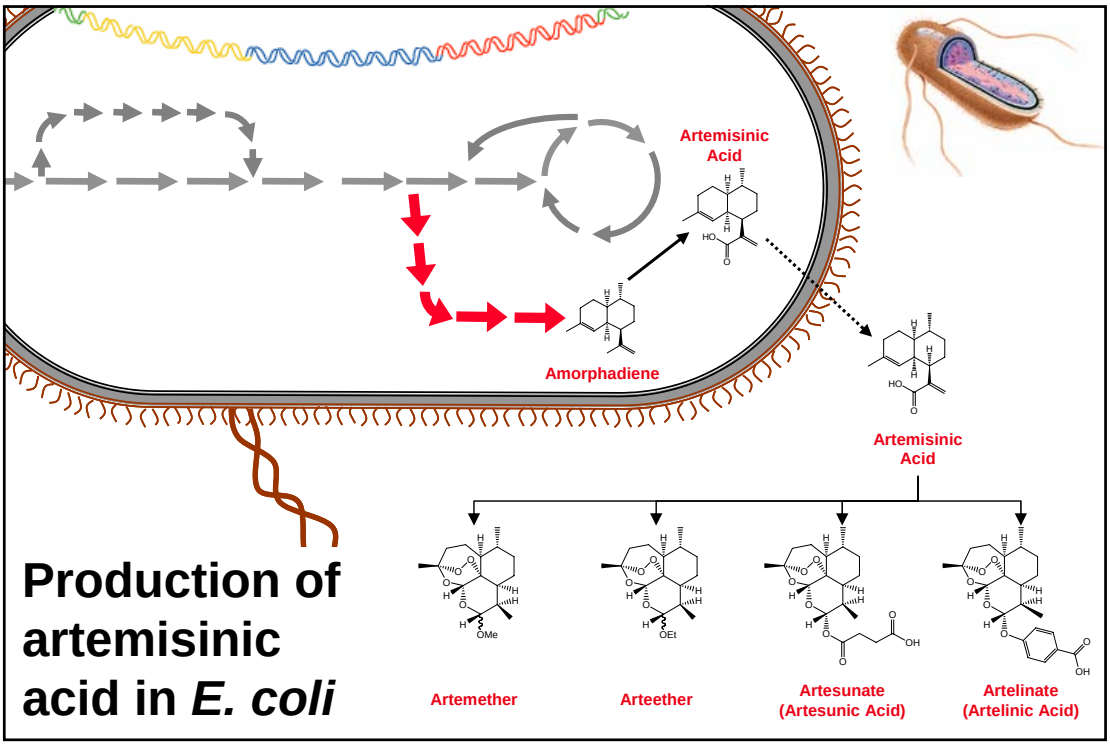






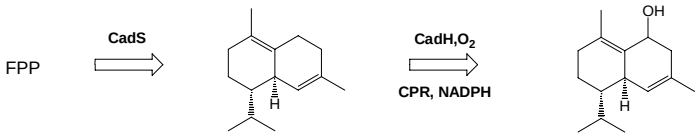
Expression of amorphadiene oxidase in yeast

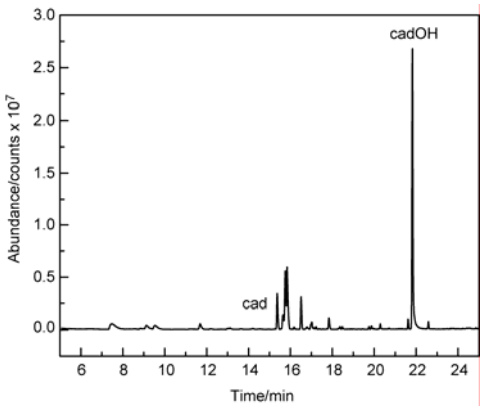
- Amorphadiene oxidase catalyzes all three steps
  - amorphadiene → alcohol → aldehyde → artemisinic acid
- The amorphadiene oxidase is functional in vivo
- Artemisinic acid is secreted from yeast and adsorbs to the outside of the cells
- Artemisinic acid can readily released from the cells by a pH change



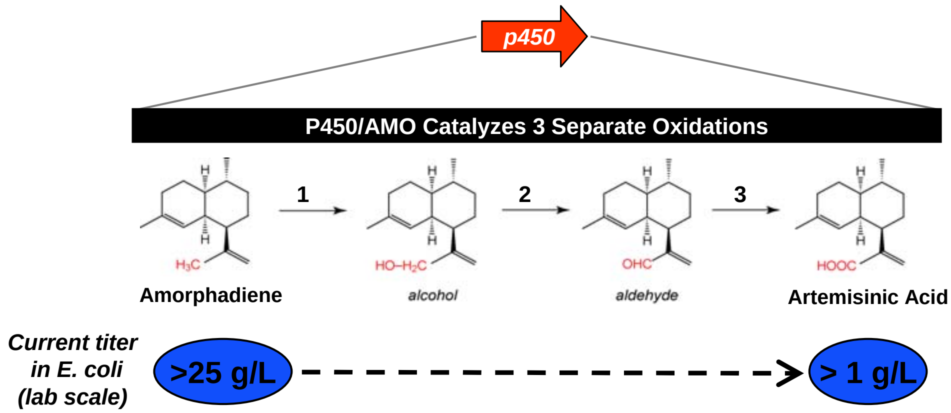
Production of artemisinic acid in *E. coli*

Production of hydroxylated terpenes in *E. coli*

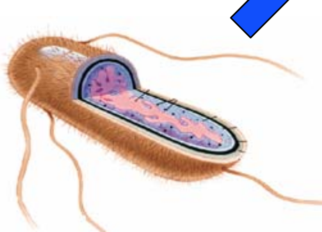




Completing the biosynthetic pathway in *E. coli*



Research, Development & Delivery



Keasling Laboratory

Amyris Biotechnologies

Institute for OneWorld Health

Artemisinin costs

Current cost of API	**$1.00/g**
Cost with new process	**$.10/g**

Conclusions

- *E. coli* has been engineered to produce amorphadiene at yields in excess of 0.5 g/L.
- *S. cerevisiae* has been engineered to produce amorphadiene at yields of approximately 0.1 g/L.
- A cytochrome P450 and its redox partners can oxidize amorphadiene to artemisinic acid.
- *S. cerevisiae* expressing amorphadiene oxidase produces artemisinic acid, which is secreted from the cells.
- *E. coli* can functionally express cytochrome P450's that oxidize terpenes.

Acknowledgements

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