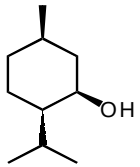
Metabolic Engineering

Metabolic engineering of *Escherichia coli* for terpene production

Jay D. Keasling
University of California
Berkeley, CA 94708

> 50,000 terpenoids identified to date

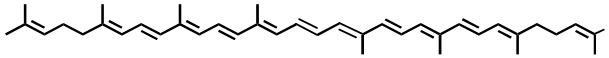
Essential oils



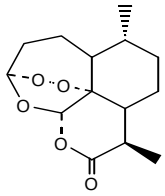
Menthol
C-10 Monoterpene

Carotenoids

Lycopene
C-40 Tetraterpene

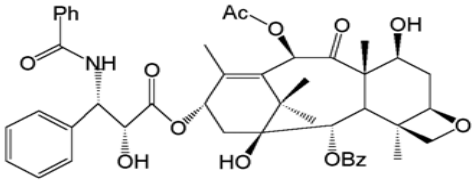


Anti-malarial agents



Artemisinin
C-15 Sesquiterpene

Chemotherapeutics

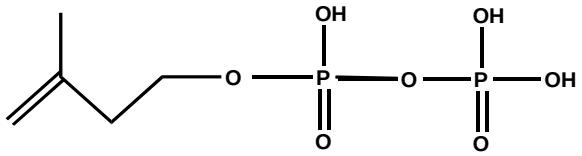


Taxol
C-20 diterpene

What are terpenoids?

Compounds synthesized from the basic 5-C building block

Isopentenyl pyrophosphate (IPP)



Function

For the plant: *Interaction with other organisms*

Signal molecules (pheromones, pigments), defense (chemical, physical), development (hormone analogues interfere with insect development).

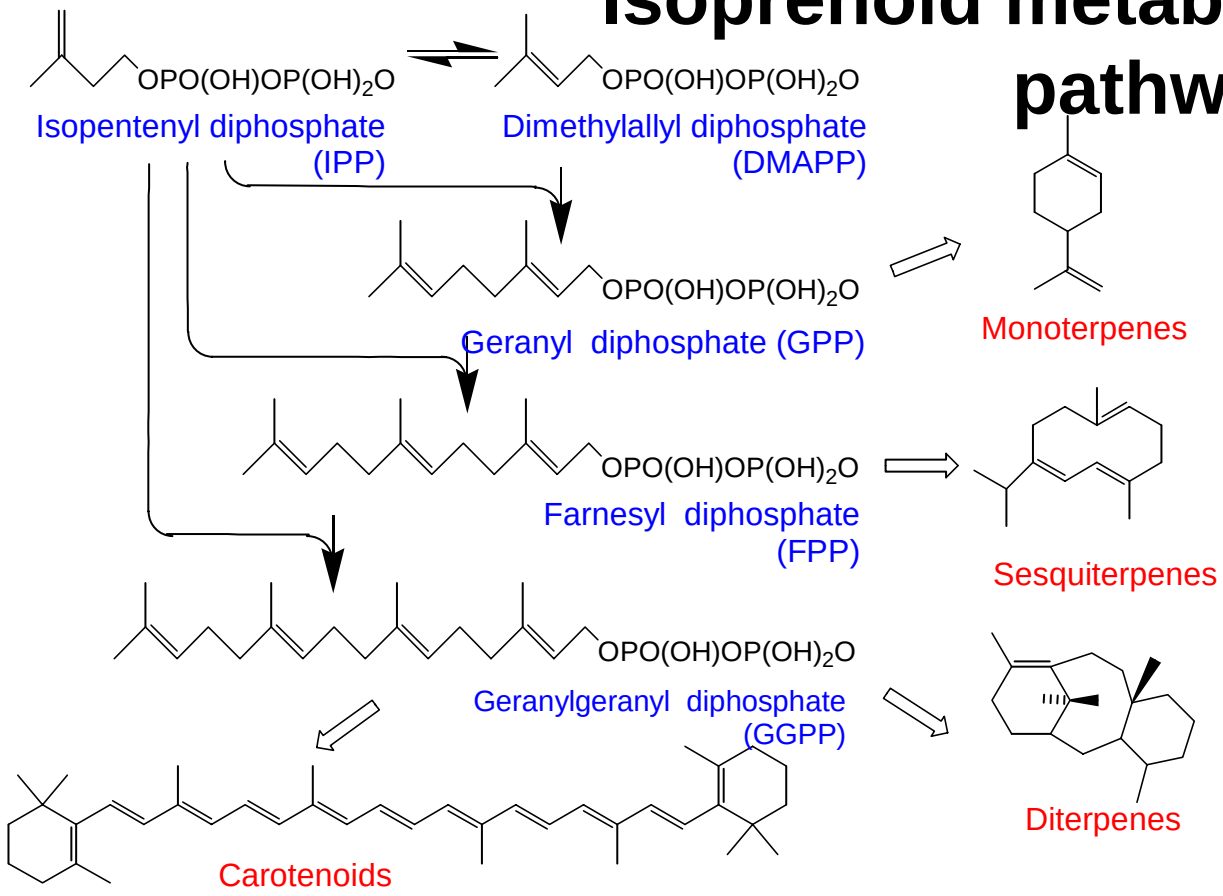
For us: Function as anti-malarial and anti-tumor agents, hormones, flavor, fragrance and pigments

Commercial potential is vast but may be limited by:

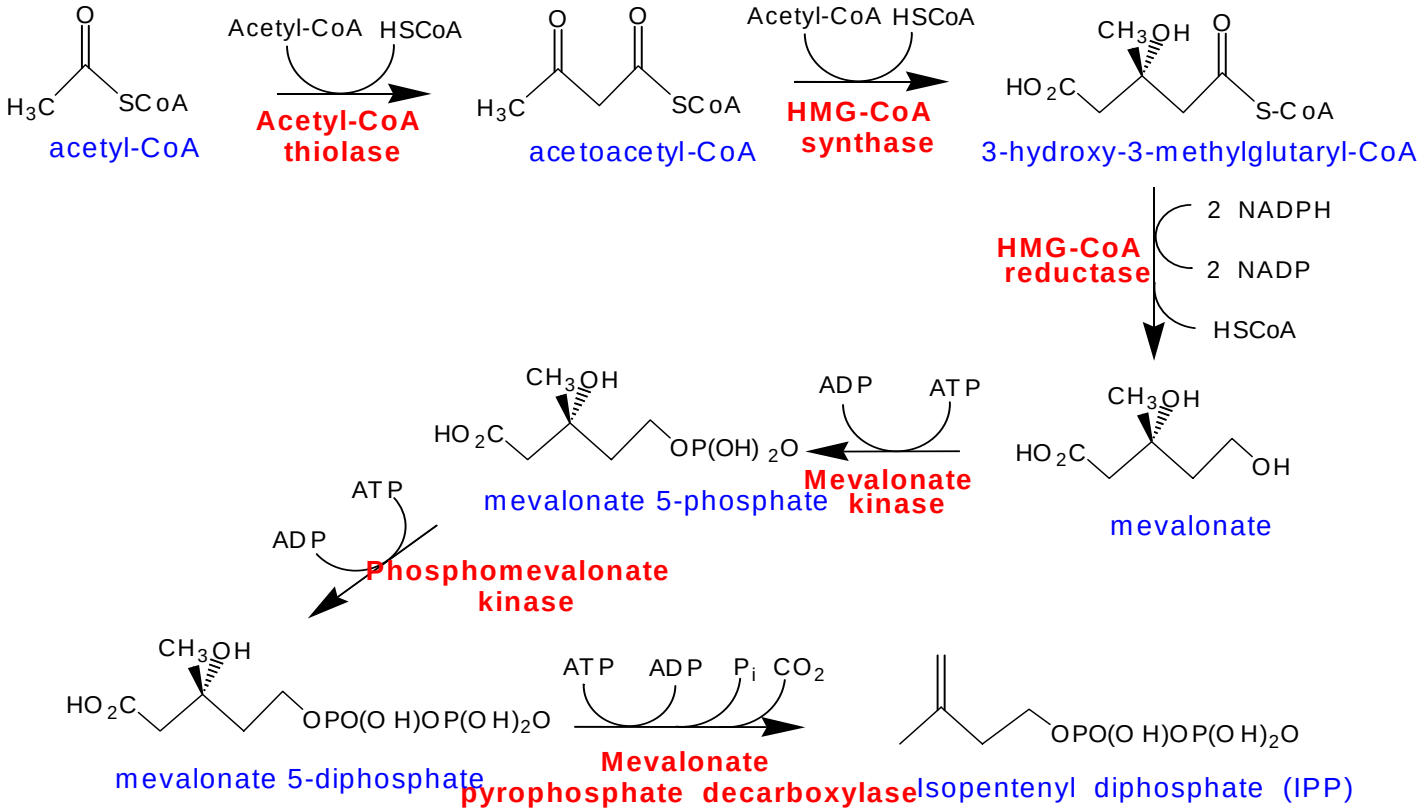
Yields from natural sources

Difficult chemical synthesis (many structural and stereoisomers)

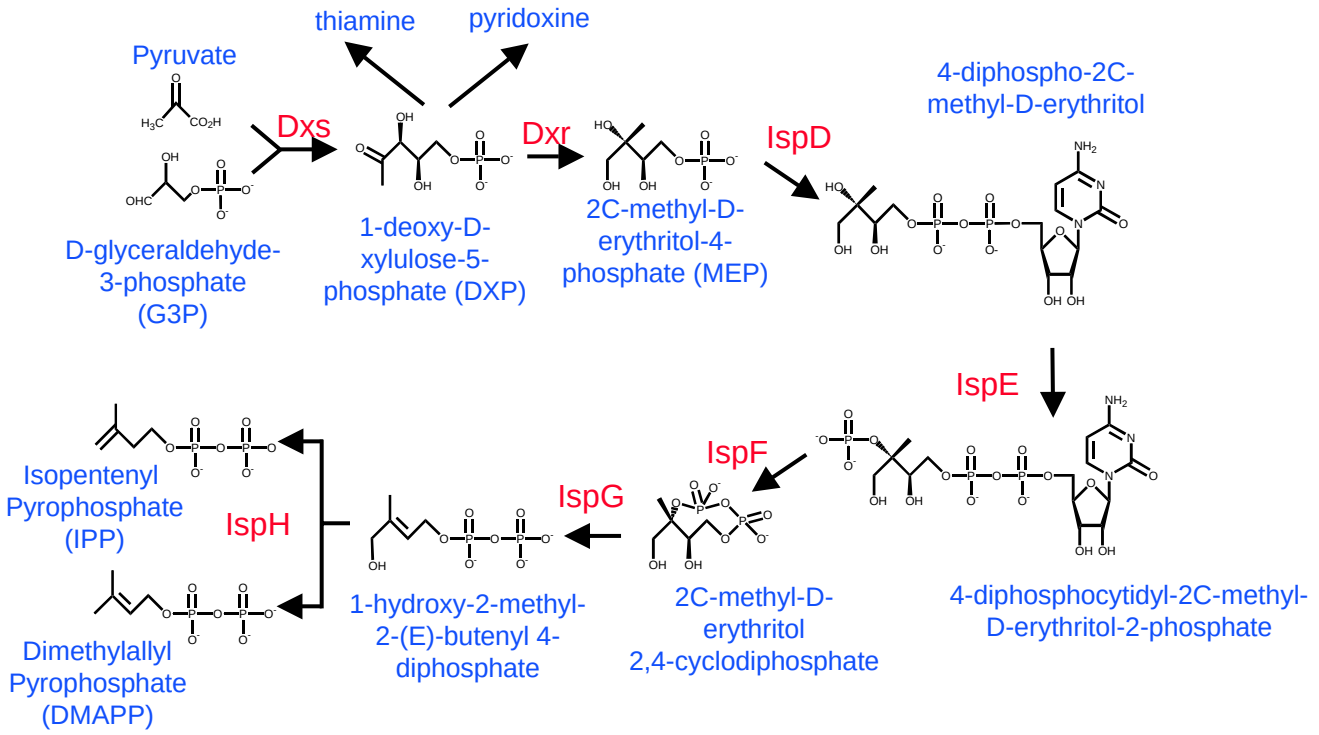
Isoprenoid metabolic pathways



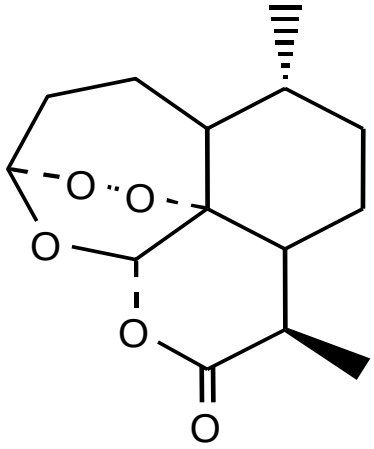
Mevalonate pathway



DXP pathway

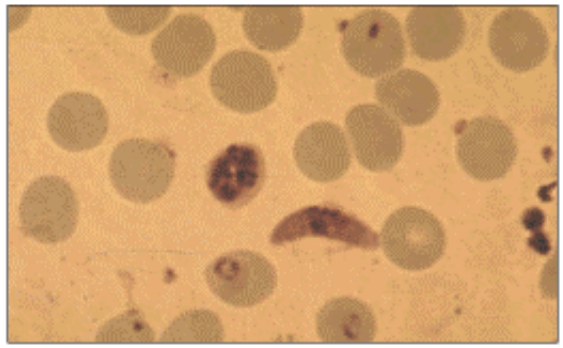

thiamine
pyridoxine
Pyruvate
Dxs
Dxr
IspD
4-diphospho-2C-methyl-D-erythritol
D-glyceraldehyde-3-phosphate (G3P)
1-deoxy-D-xylulose-5-phosphate (DXP)
2C-methyl-D-erythritol-4-phosphate (MEP)
IspE
IspF
IspG
IspH
Isopentenyl Pyrophosphate (IPP)
Dimethylallyl Pyrophosphate (DMAPP)
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate
2C-methyl-D-erythritol 2,4-cyclodiphosphate
4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate

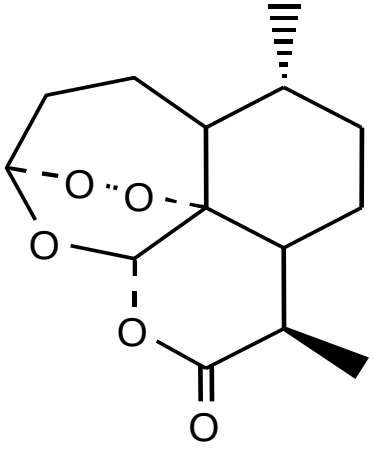
Artemisinin



Malaria

- Caused by *Plasmodium*, a single-cell protozoan
  - –Transmitted by Anopheles mosquito
  - –Destroys red blood cells
- 1.5-2.7 million people die of malaria every year
  - –90% of the victims are children
  - –40% of the world's population is at risk

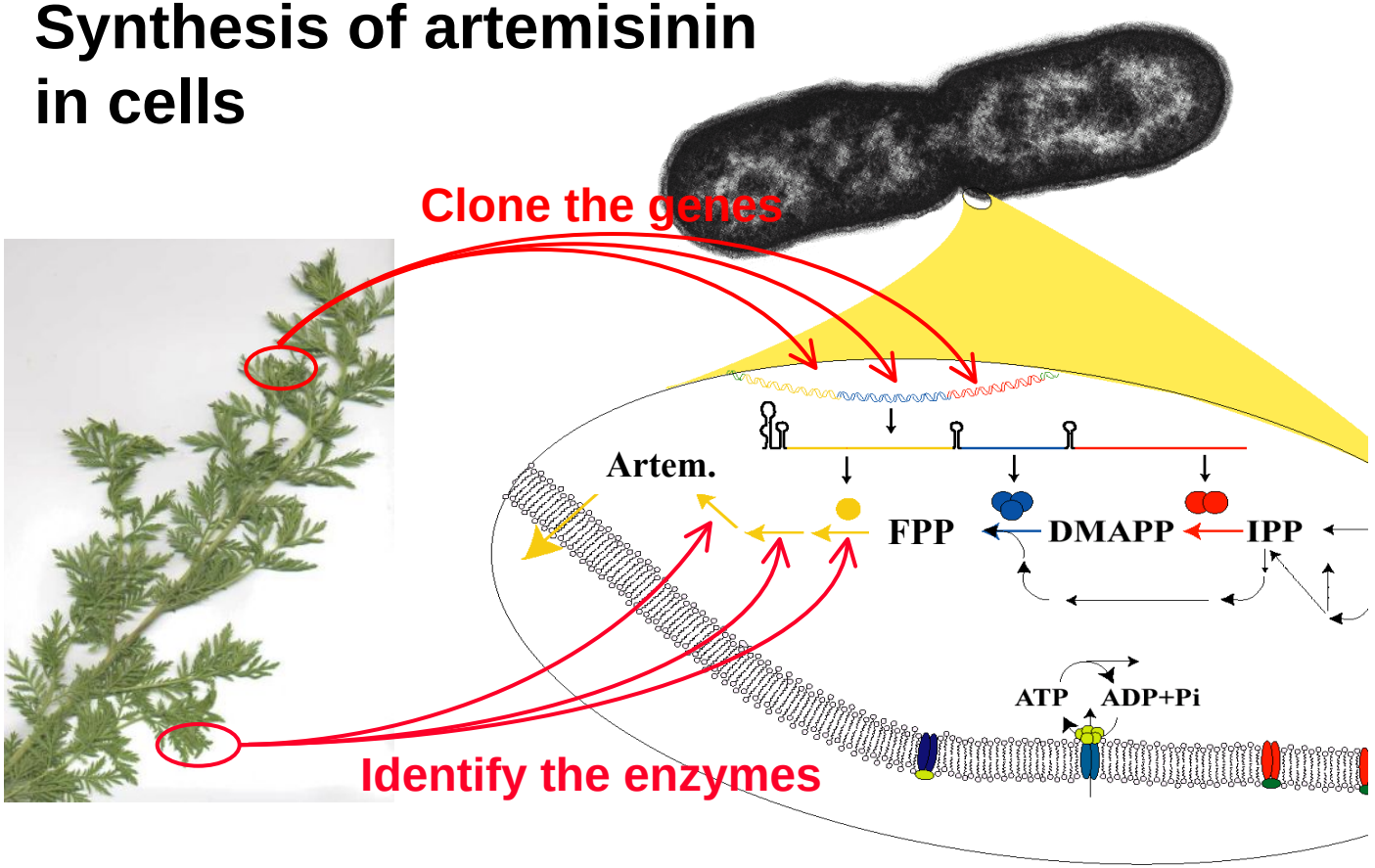




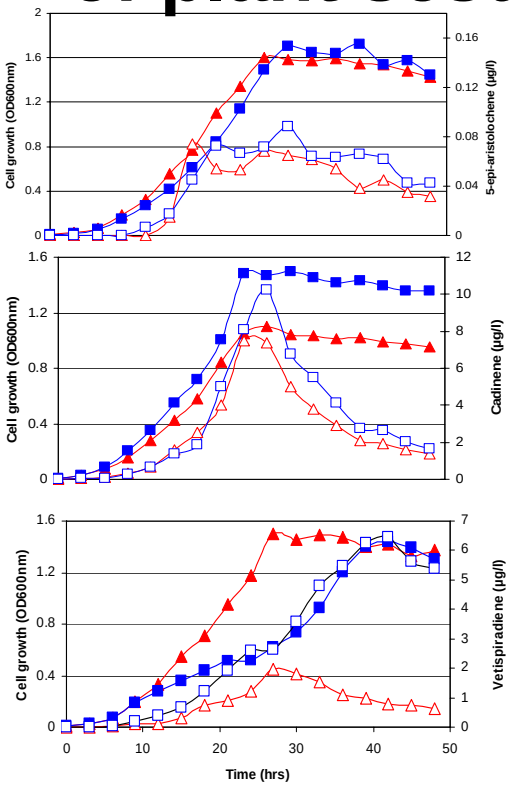
Artemisinin-based drugs

- The current cost for an artemisinin-based drug is approximately $2.25.
  - 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

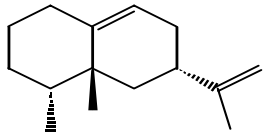
Synthesis of artemisinin in cells



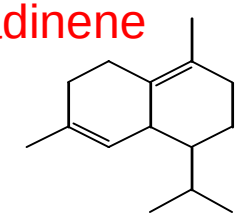
Poor performance of plant sesquiterpene cyclases



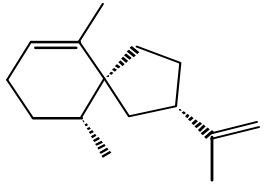
5-epi-aristolochene



Cadinene



Vetispiradiene



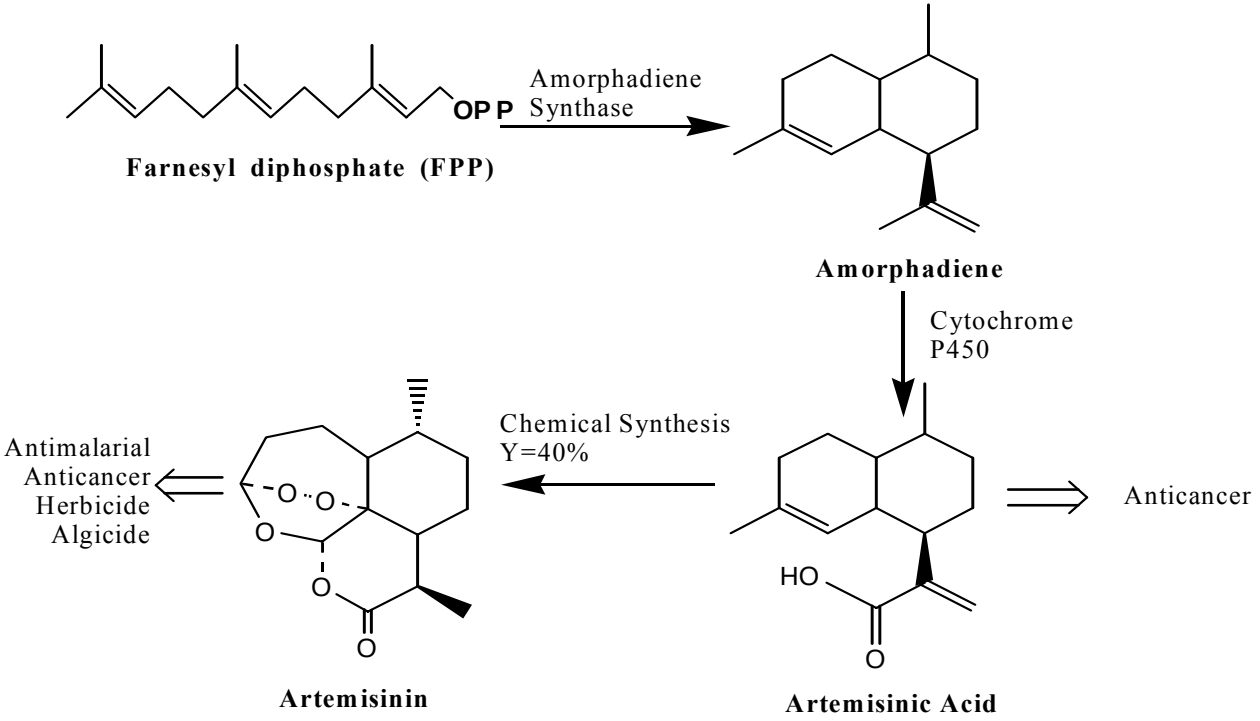
Low yields:

0.05 to 0.7 ng/mL/OD

Expression of rare *E. coli* codon tRNA did not much help

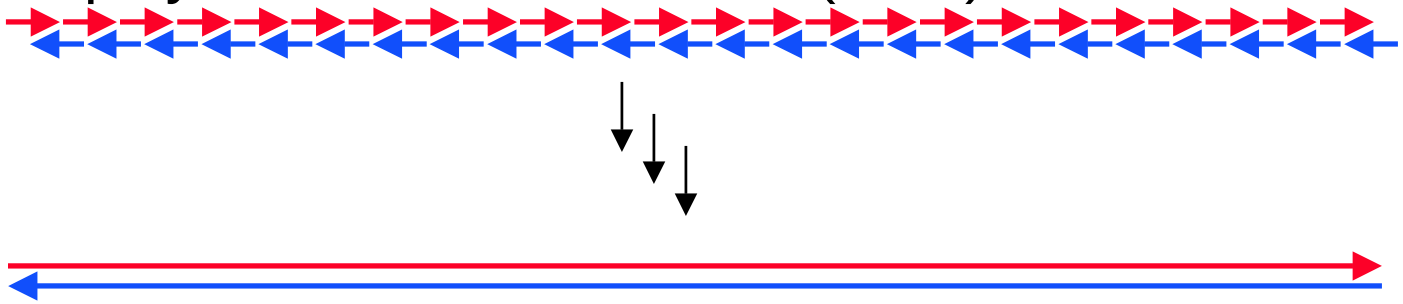
Martin et al., Biotech. Bioeng. 2001

Amorphadiene and artemisinin biosynthetic pathway

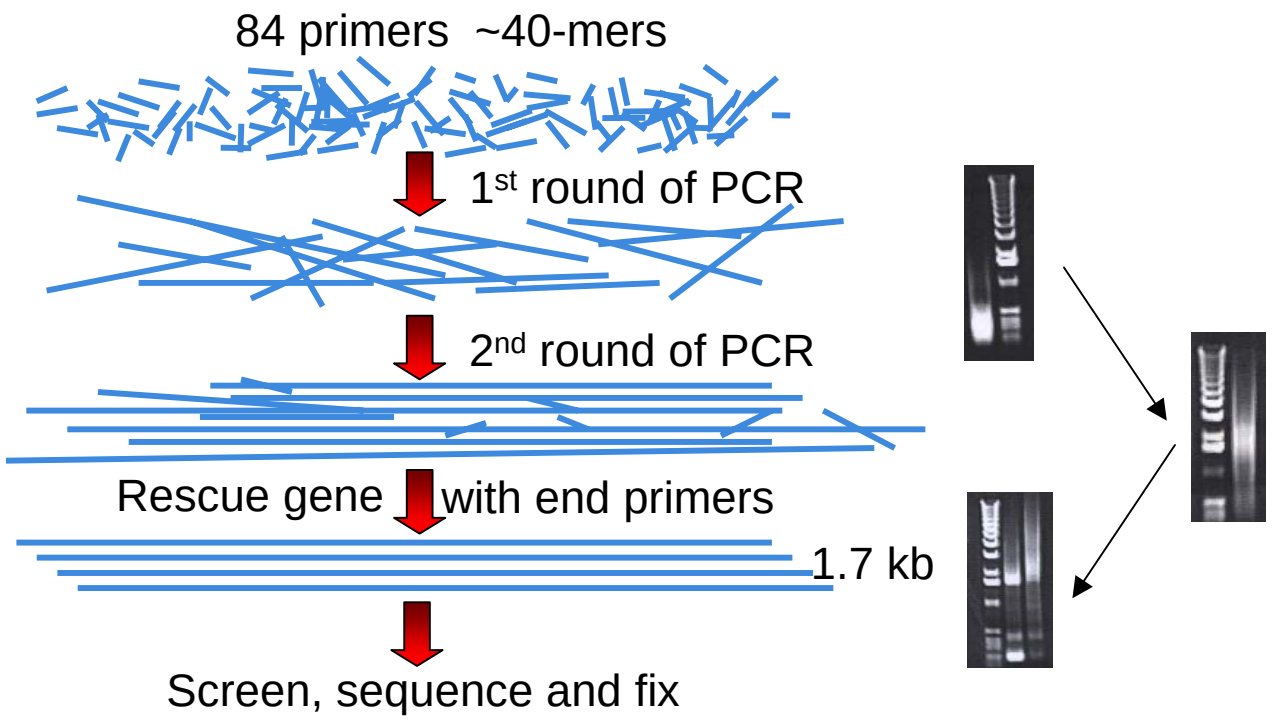


Assembly of rcAmorphadiene Cyclase

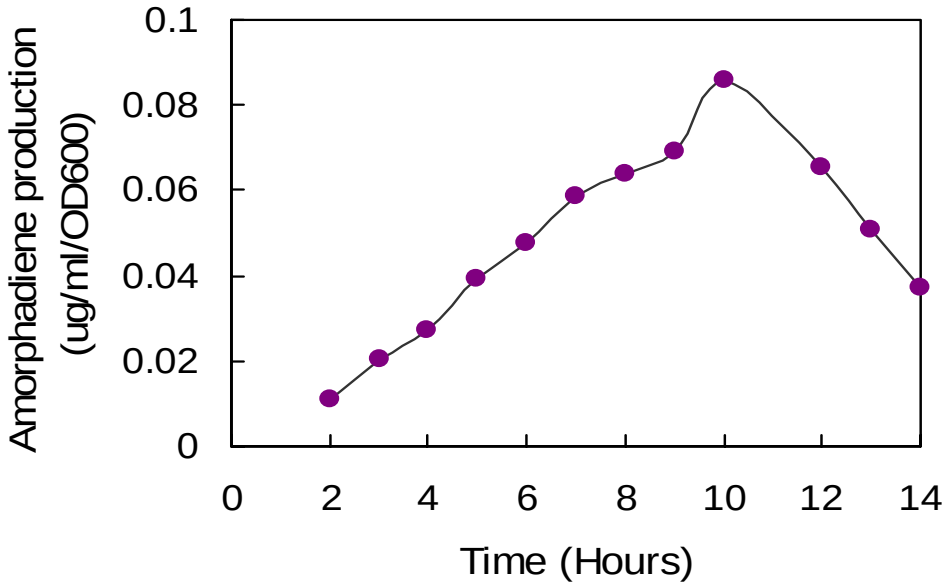
- Take gene sequence from patent
- Optimize sequence for expression in desired host
- Synthesize 84 oligonucleotides of ~40 basepairs each
- Assemble into complete gene using the polymerase chain reaction (PCR)



Terpene cyclase gene assembly

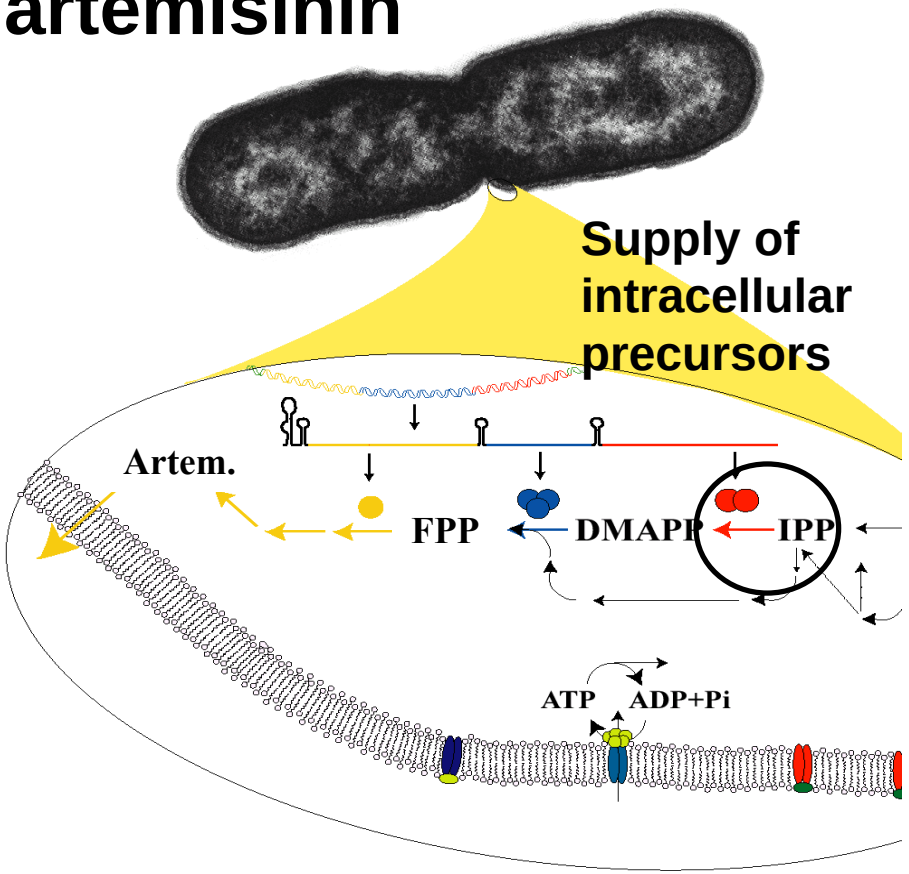


Amorphadiene production by the synthetic amorphadiene cyclase

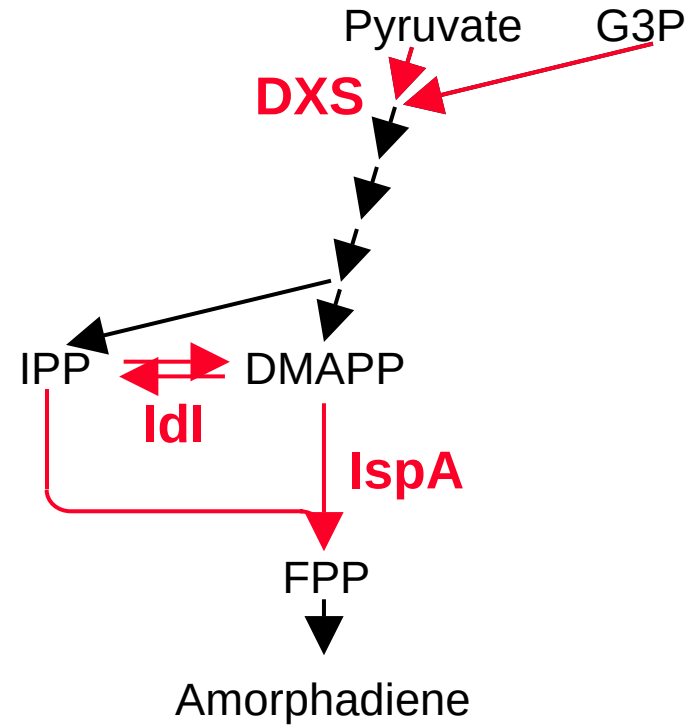


142-fold improvement over other native cyclases (100 ng/mL/OD)

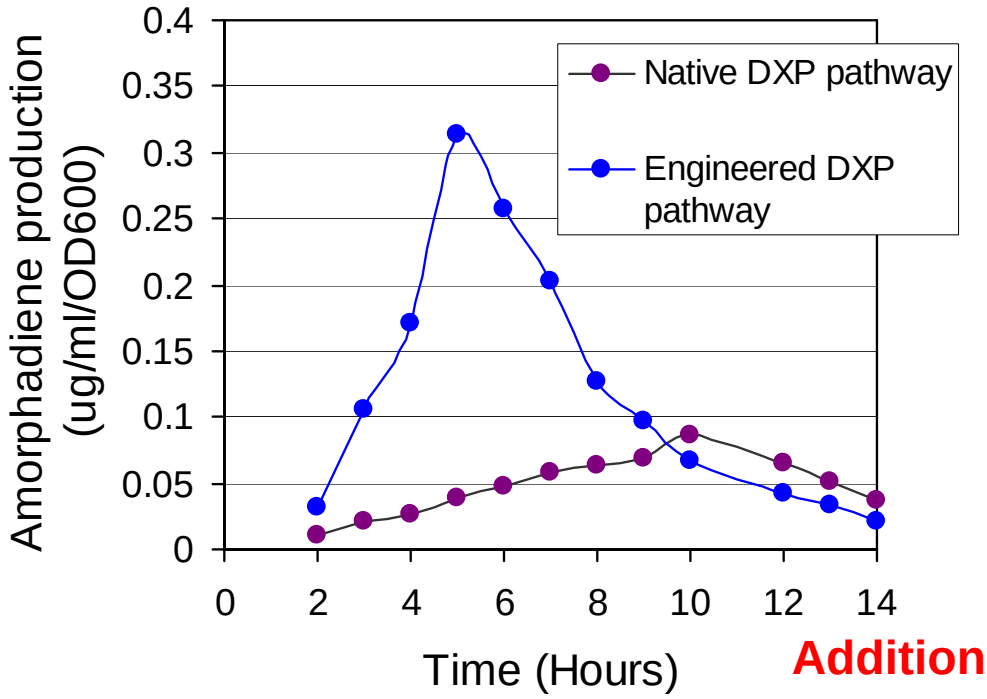
Synthesis of artemisinin in cells



Expression of genes known to limit production



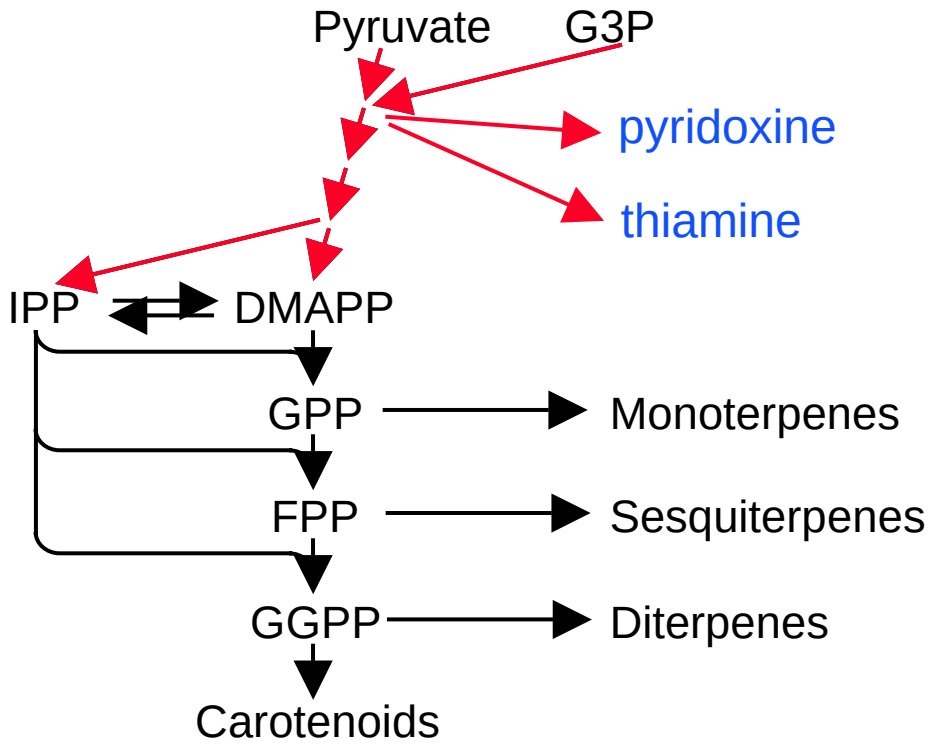
Amorphadiene production by the synthetic amorphadiene cyclase



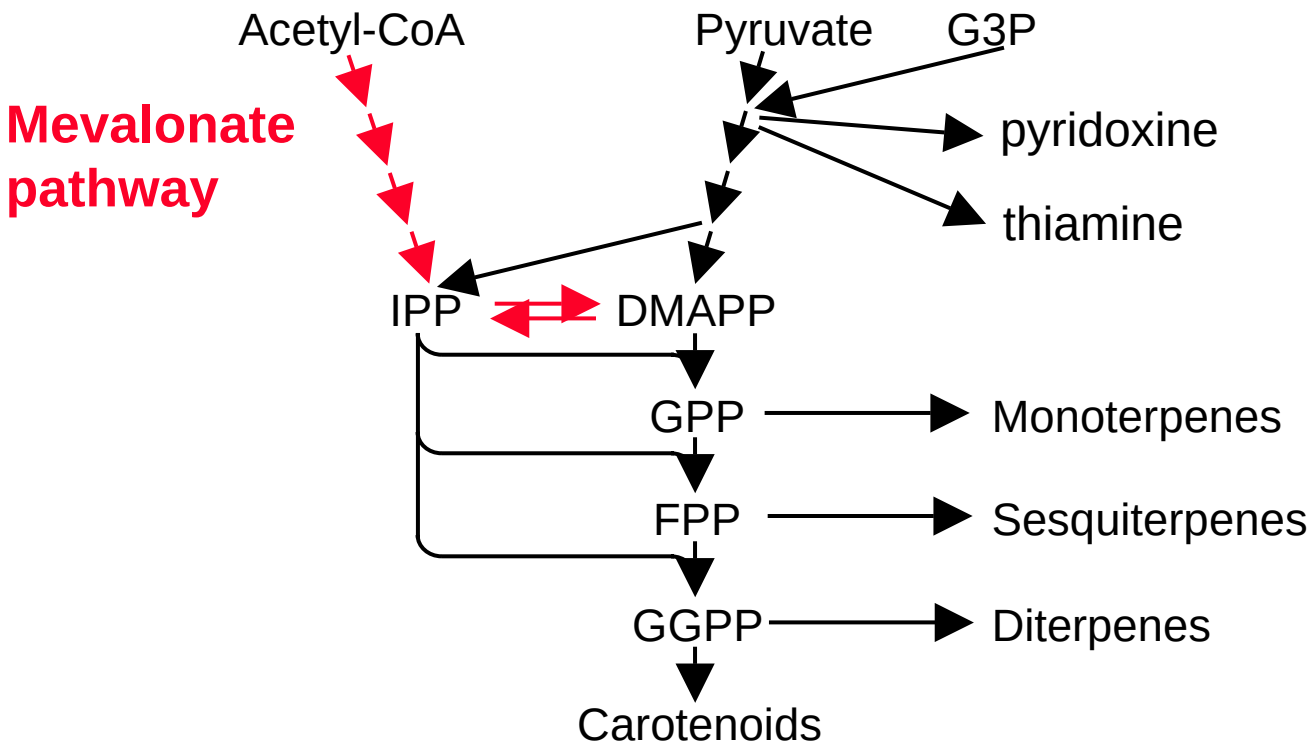
Additional 3-fold (300 ng/mL/OD)

Intermediates in the DXP pathway are necessary for growth

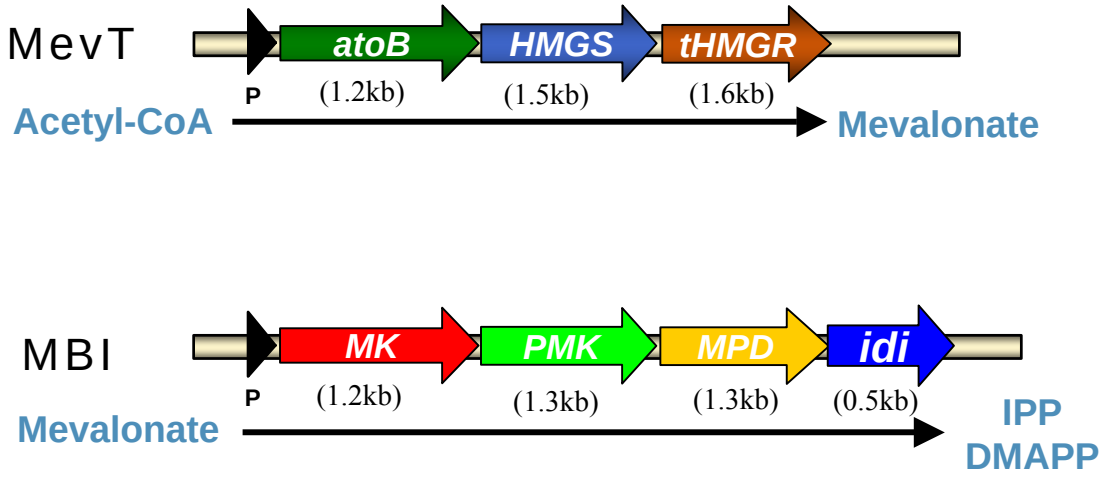
DXP Pathway



Engineering precursor production

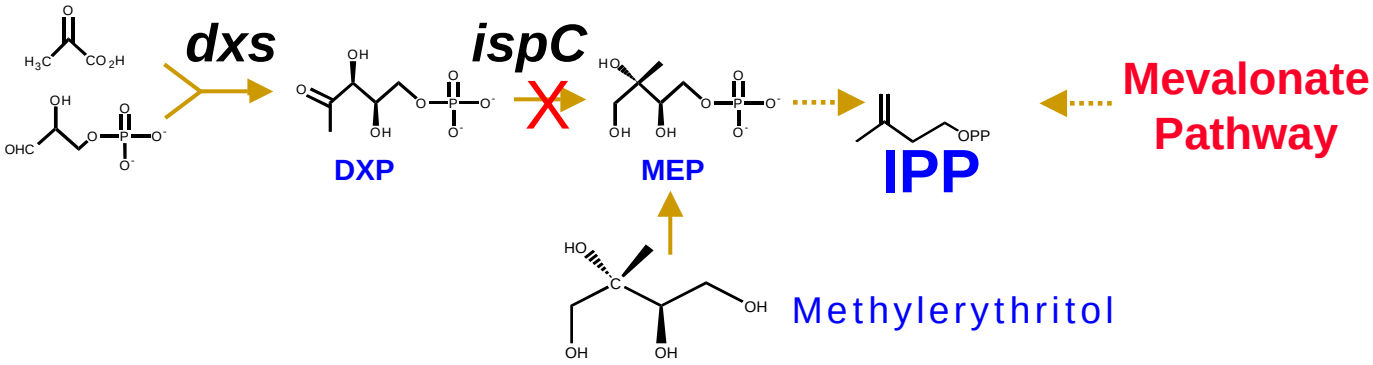


Construction of synthetic mevalonate pathway operons



Selection for mevalonate pathway

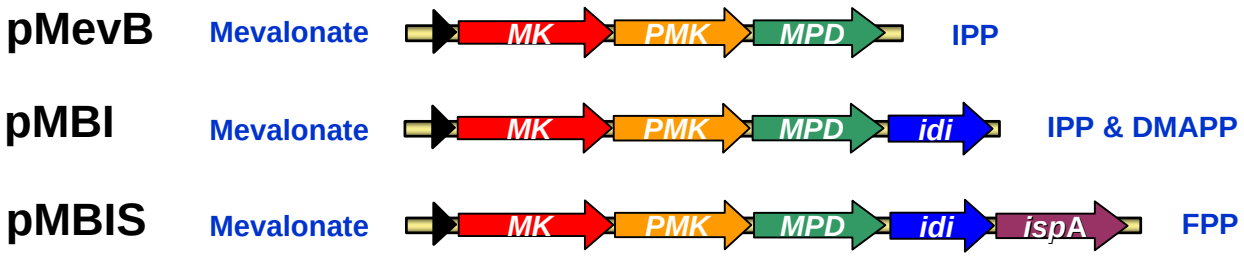
Inactivation of *E. coli's* DXP pathway

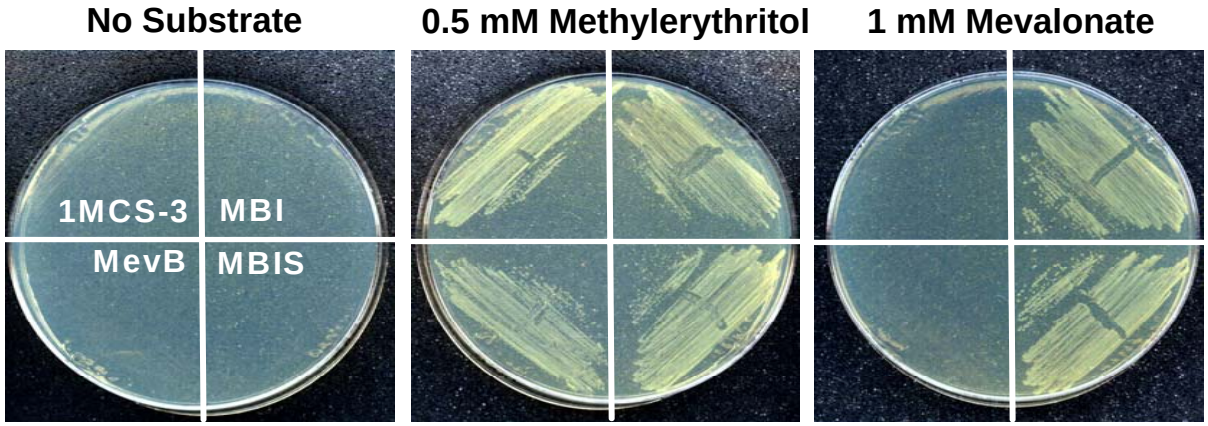


ispC knock out strain

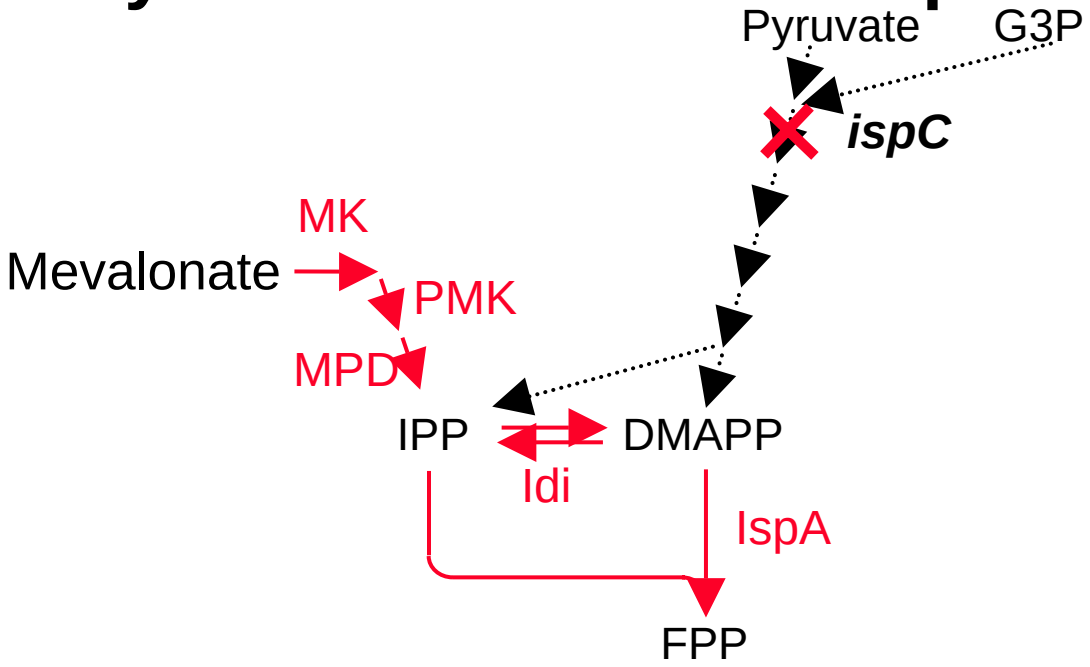
- Lethal, but *E. coli* will grow in the presence of methylerythritol (ME)
- Can be complemented by a functional mevalonate pathway

Bottom operon complements an *ispC* mutation in *E. coli*

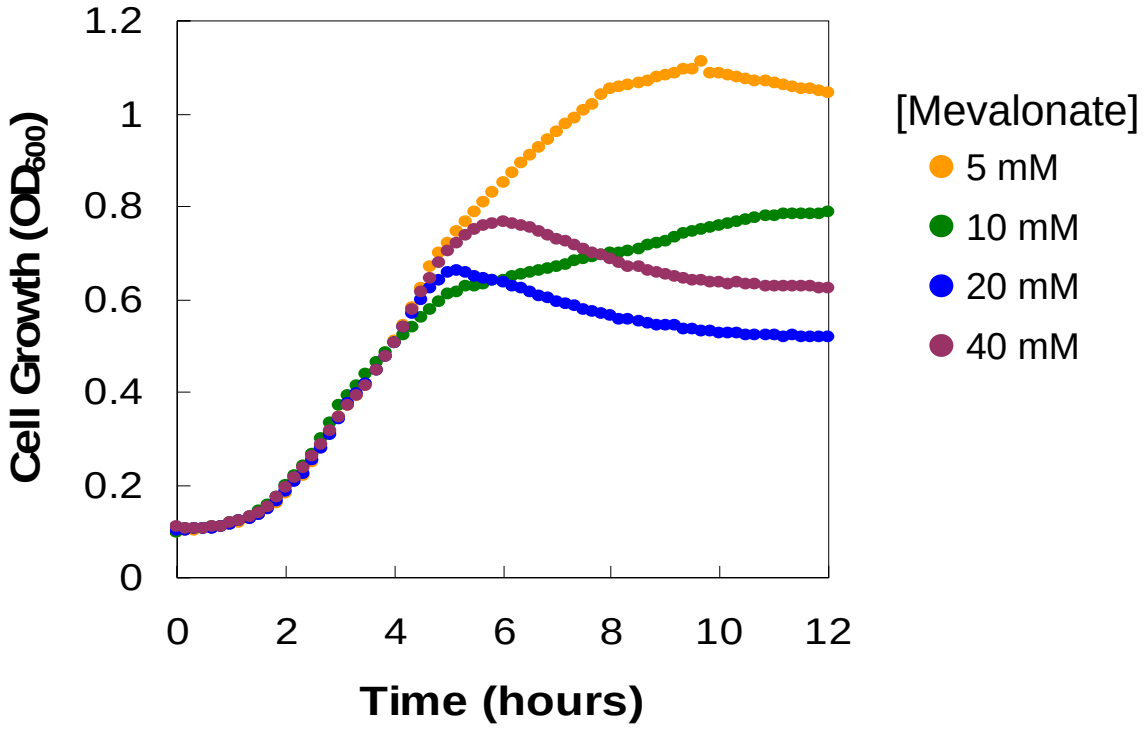




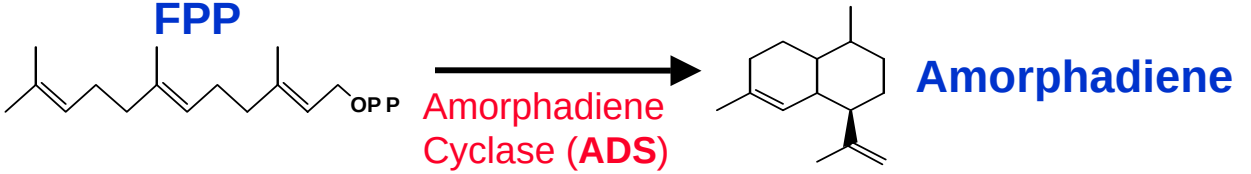
Idi is essential to replace the DXP pathway with the mevalonate pathway

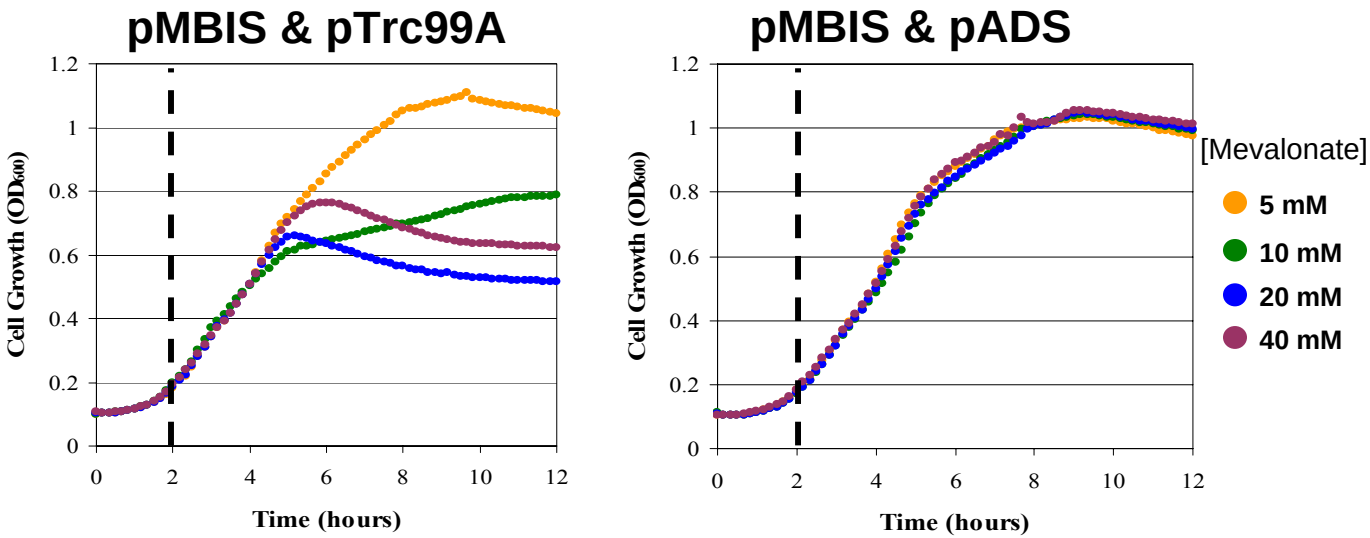


… but increasing concentrations of mevalonate inhibit growth

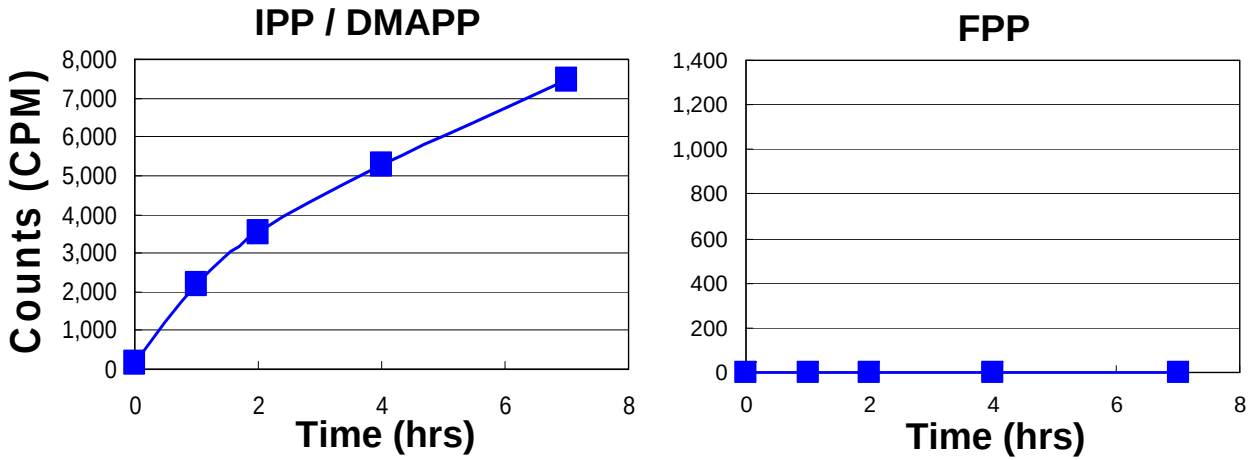


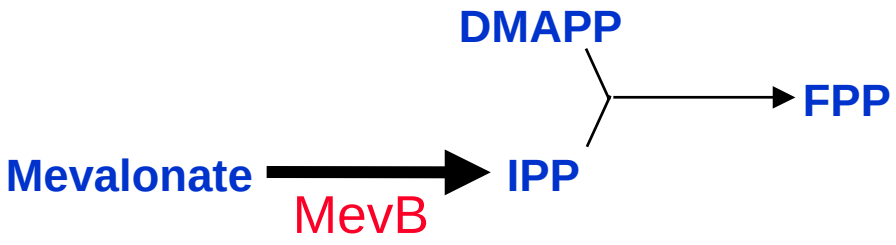
Co-expression of sesquiterpene cyclase alleviates growth inhibition



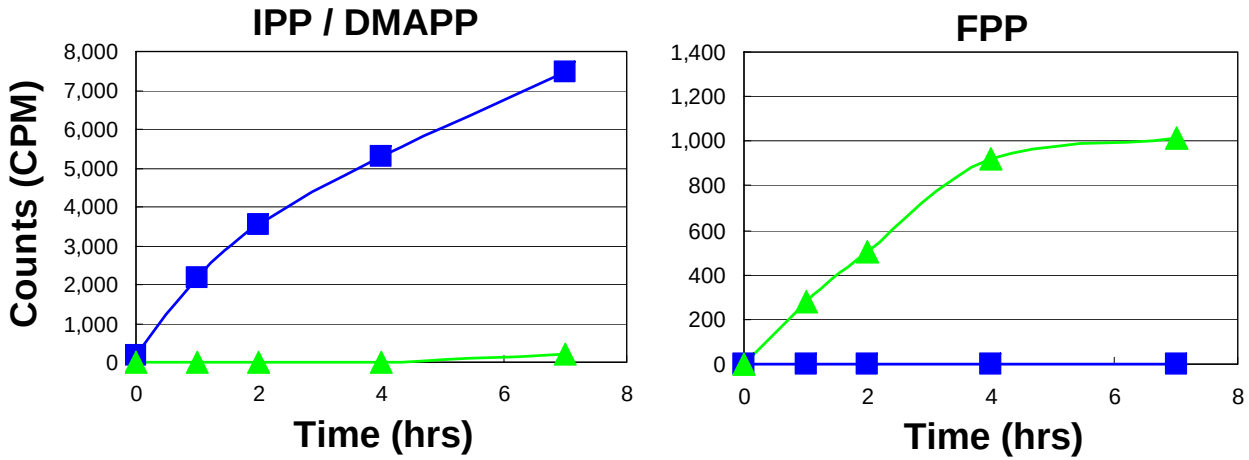


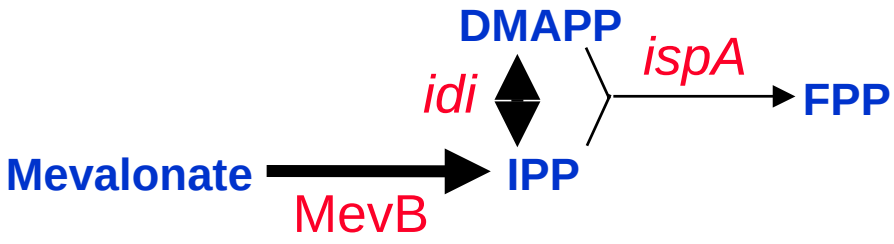
Intracellular prenyl pyrophosphates in MevB-supplemented strains



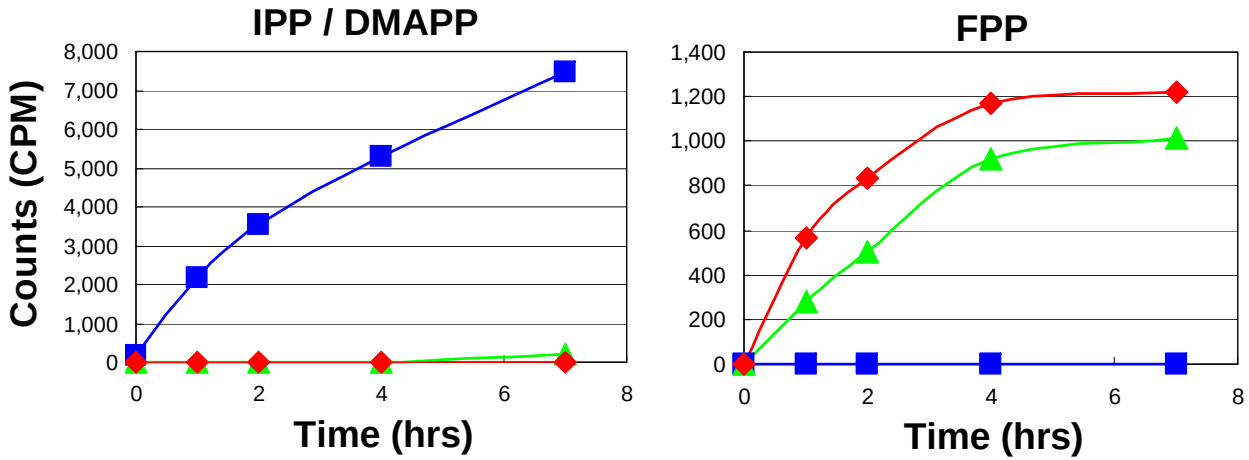


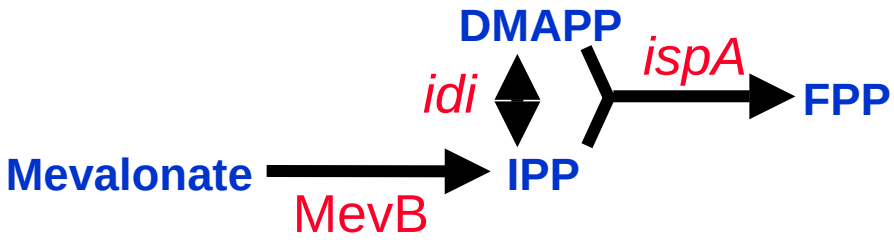
Intracellular prenyl pyrophosphates in MevB-supplemented strains



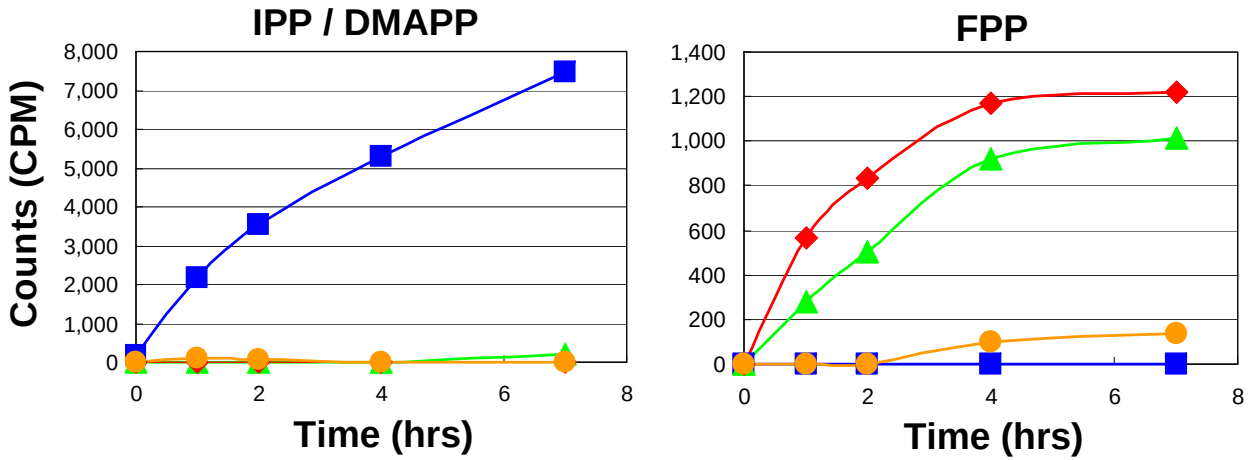


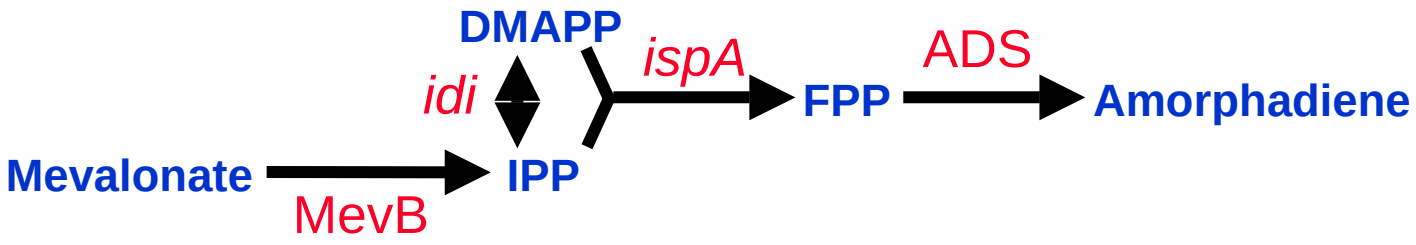
Intracellular prenyl pyrophosphates in MevB-supplemented strains



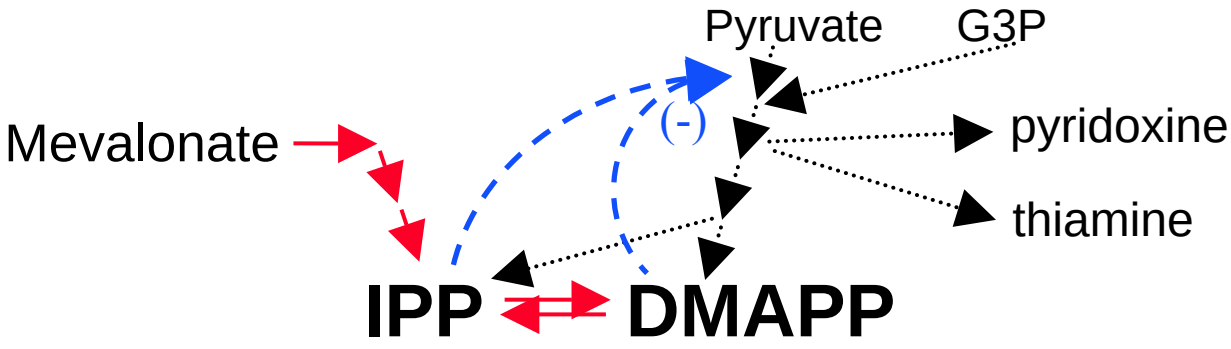


Intracellular prenyl pyrophosphates in MevB-supplemented strains

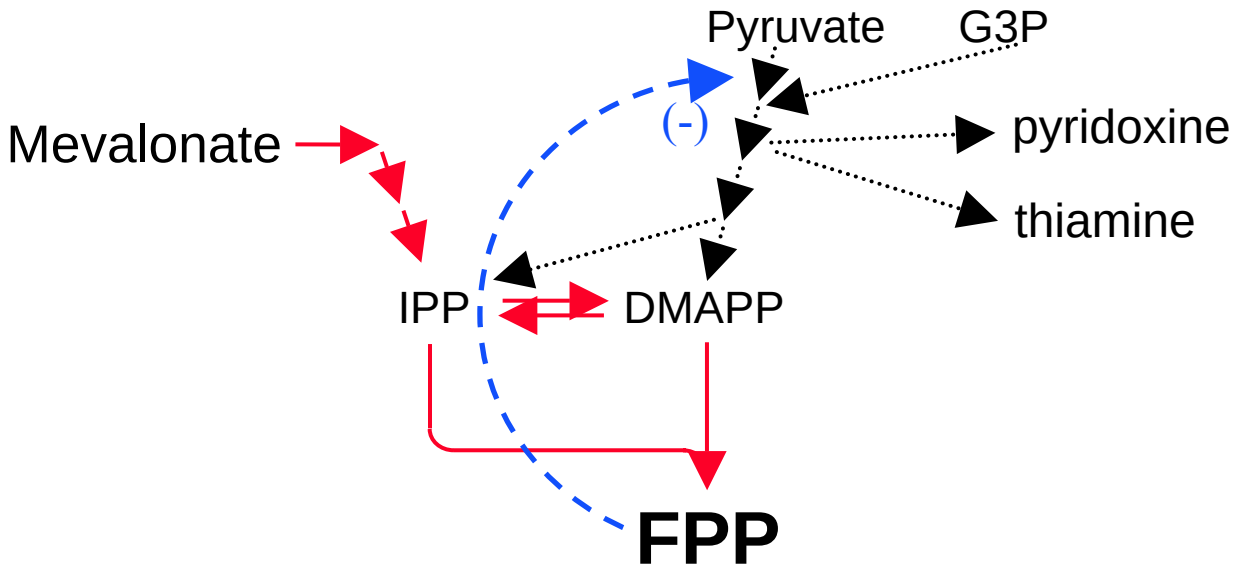




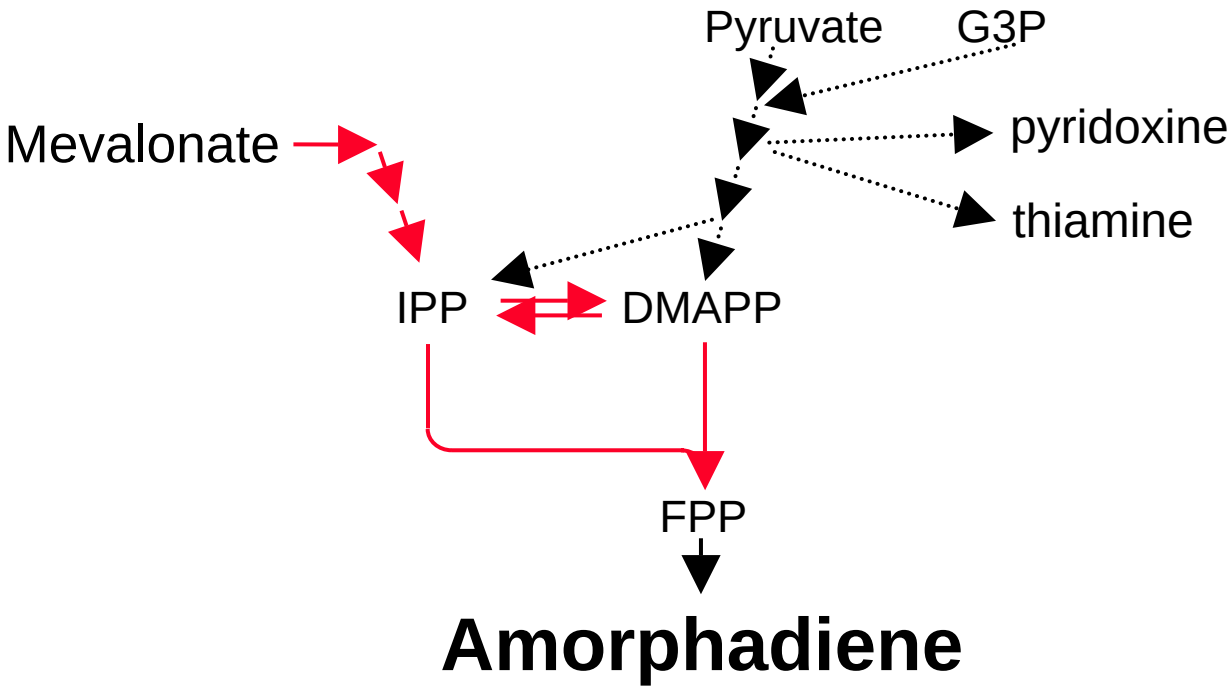
Accumulation of IPP/DMAPP inhibits growth



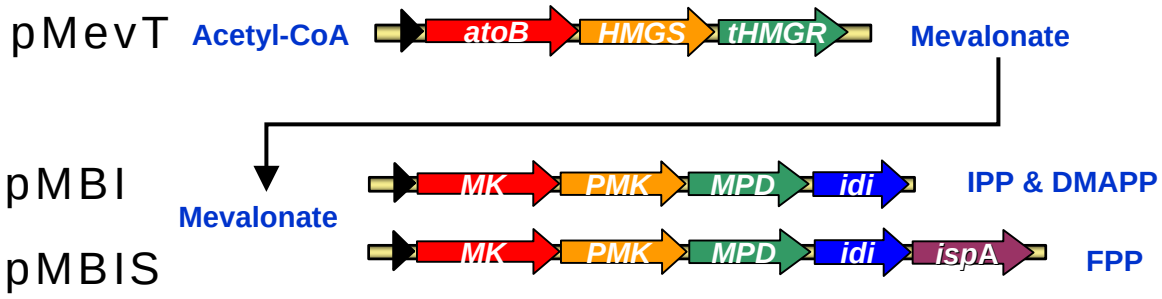
Accumulation of FPP also inhibits growth

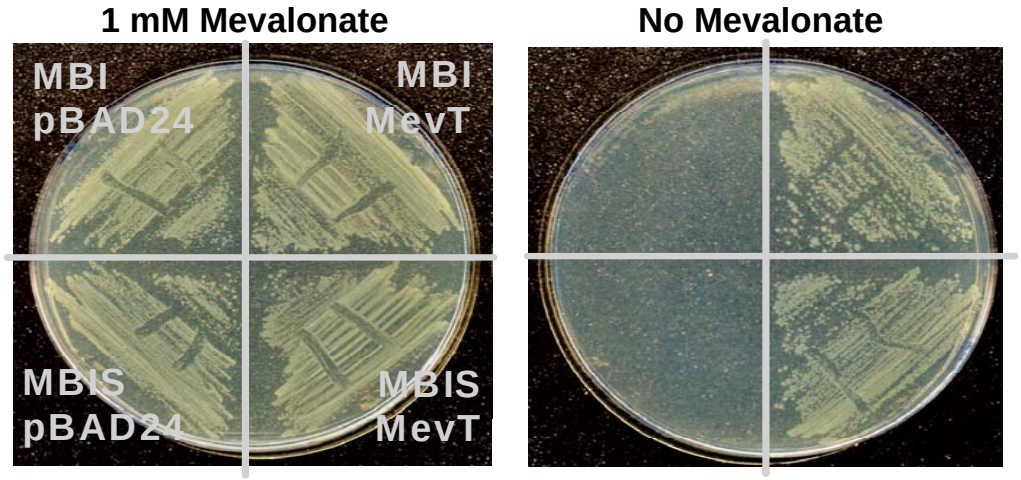


Pulling on precursors improves growth

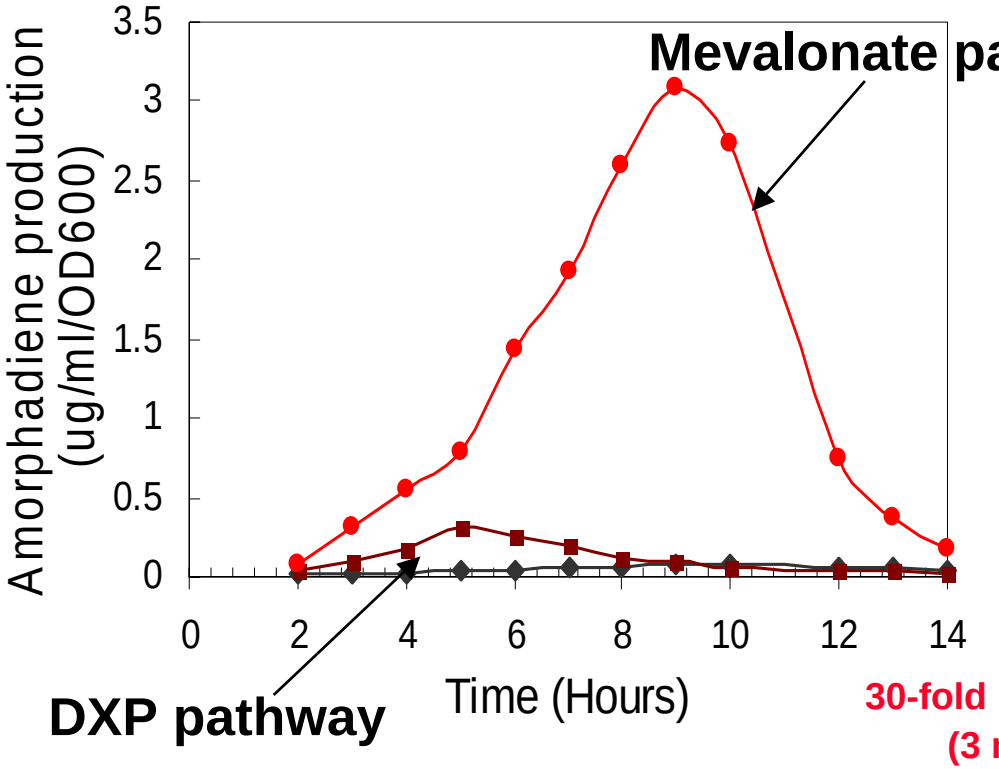


Function of the full pathway



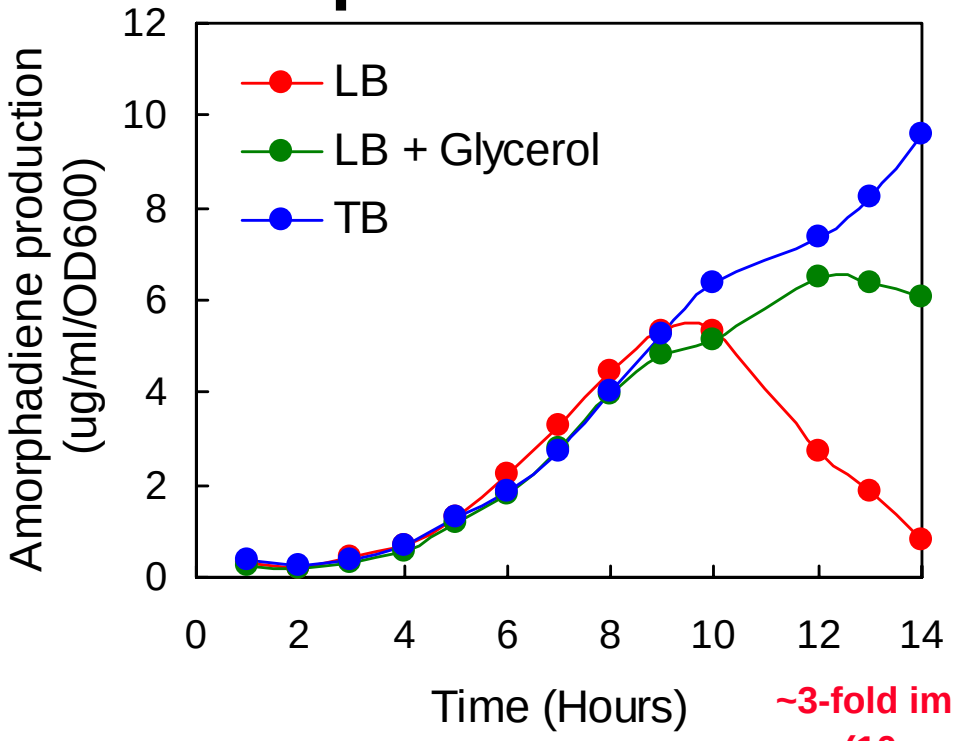


Amorphadiene from the full mevalonate pathway



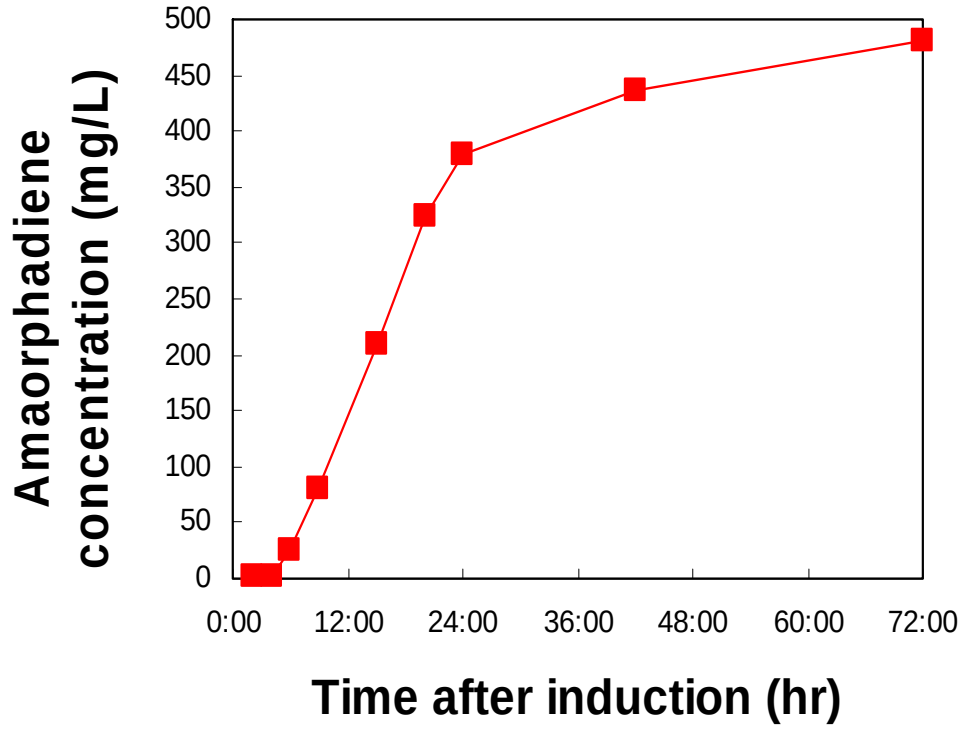
30-fold improvement (3 mg/L/OD)

Preliminary shake flask optimization

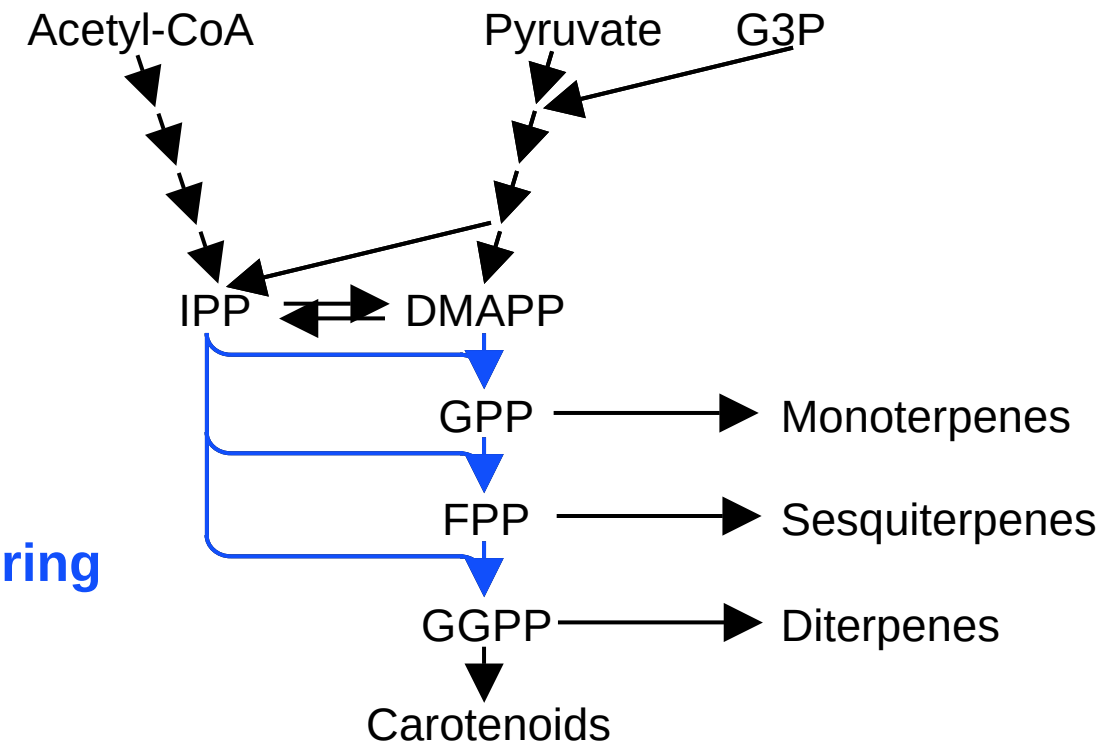


~3-fold improvement (10 mg/L/OD)

Amorphadiene production in a two-phase fermentation

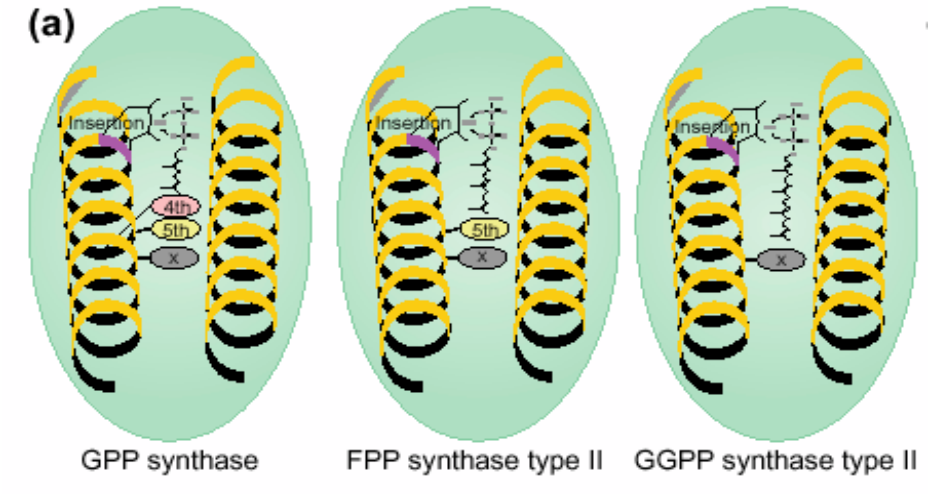


Production of mono- and diterpenes



Enzyme engineering

Design of GPP and GGPP synthases



WT *ips*A 75-ECIHA**YS**LIH**DD**LPAM**D**DDDLRRGLP-100

Y80D 75-ECIHA**DS**LIH**DD**LPAM**D**DDDLRRGLP-100

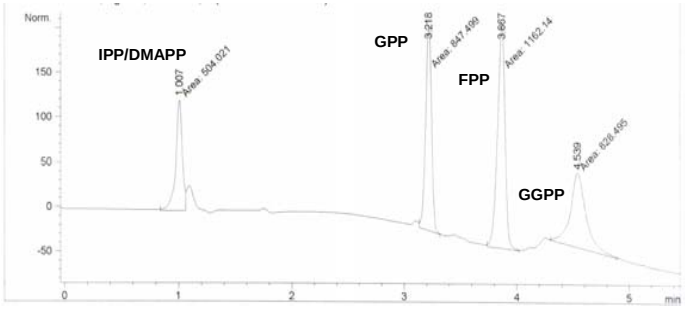
S81F 75-ECIHA**YF**LIH**DD**LPAM**D**DDDLRRGLP-100

-- XXxxx**DDxxxxD -- – Type 2**

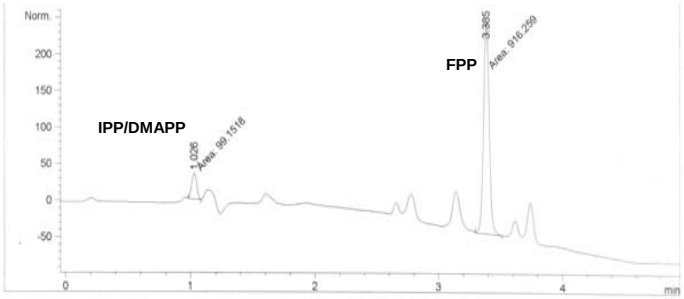
5^{th} 4^{th}

Product specificities of GPP/FPP/GGPP synthases

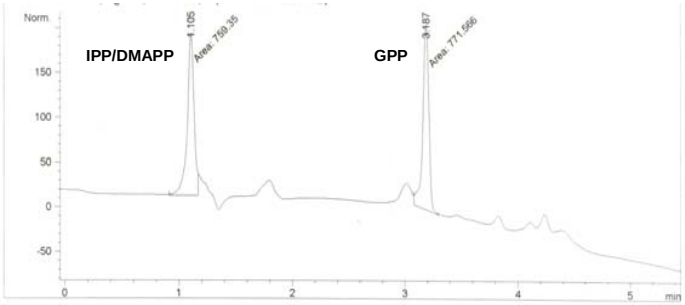
Standard



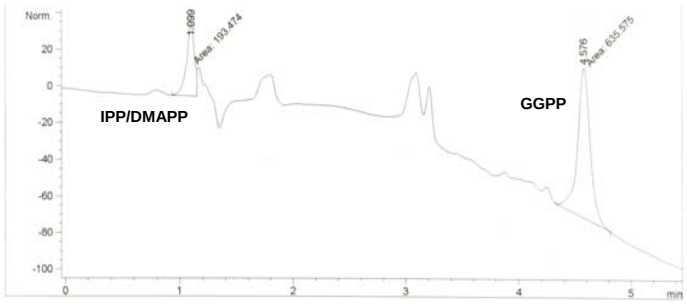
WT (FPP synthase)



S81F (GPP synthase)



Y80D (GGPP synthase)

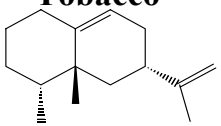


Expression of plant mono- sesqui- and diterpenes cyclases in *E. coli*

FPP ➡ Sesquiterpenes

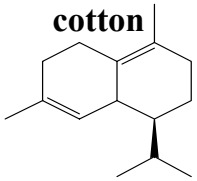
5-*epi*-aristolochene

Tobacco



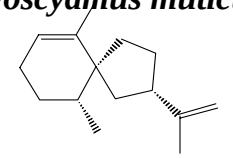
δ-cadinene

cotton



Vetispiradiene

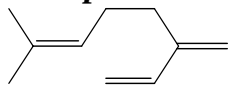
Hyoscyamus muticus



GPP ➡ Monoterpene

Myrcene synthase

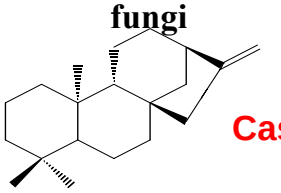
Arabidopsis thaliana



GGPP ➡ Diterpene

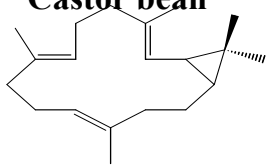
***ent*-Kaurene cyclase**

fungi

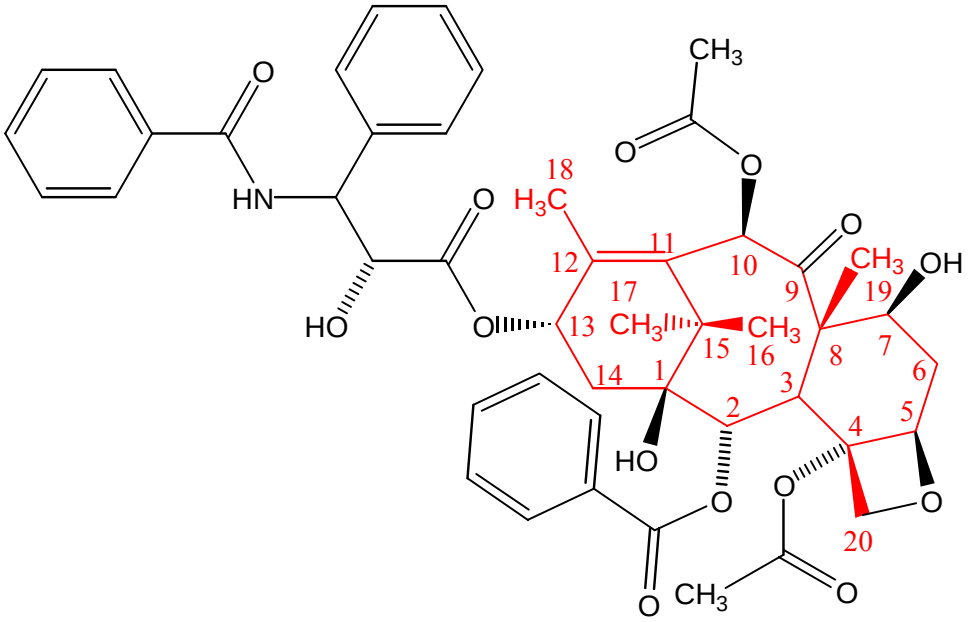


Casbene cyclase

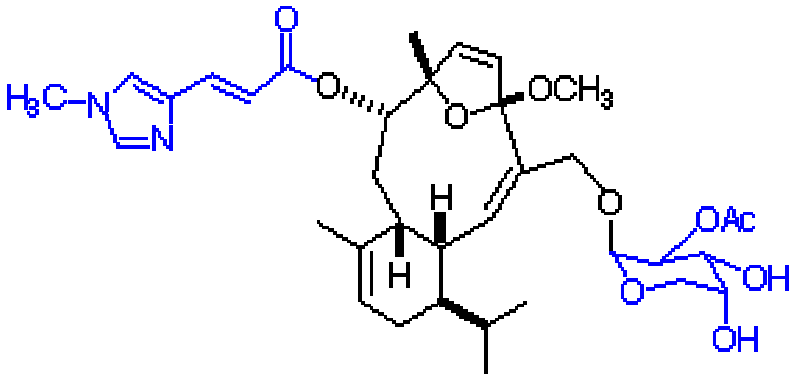
Castor bean



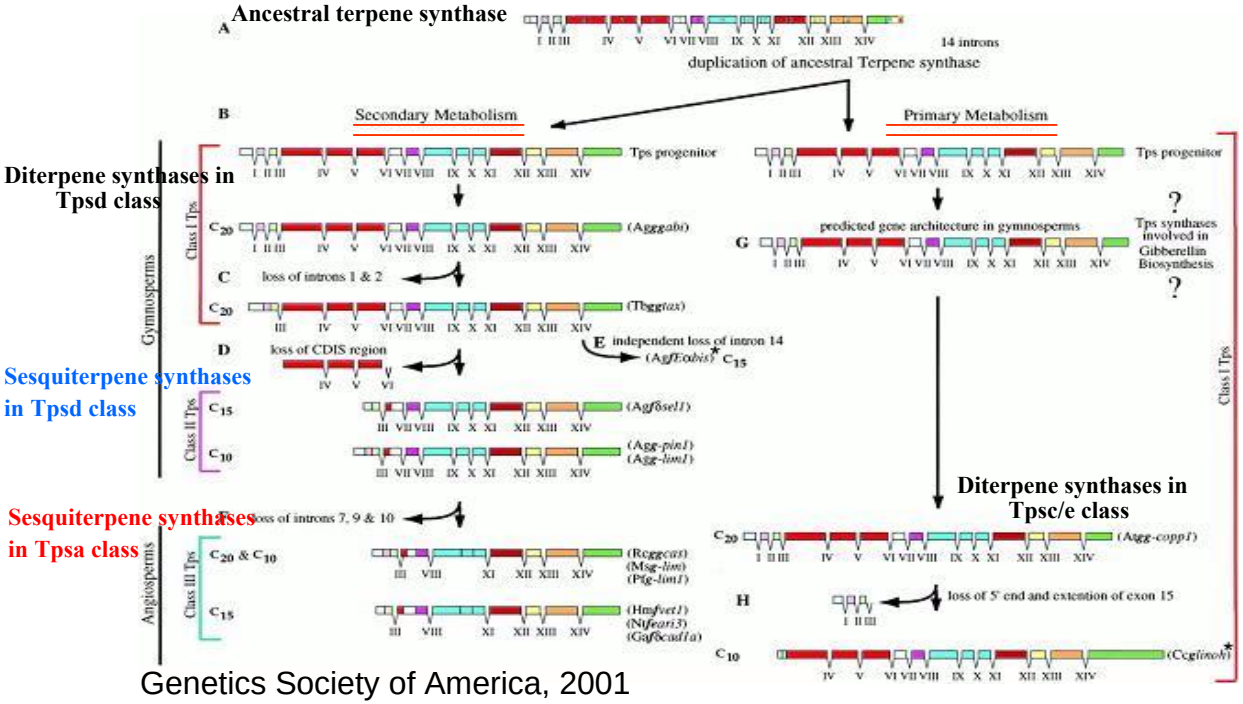
Taxol from the Pacific Yew



Eleutherobin from marine coral

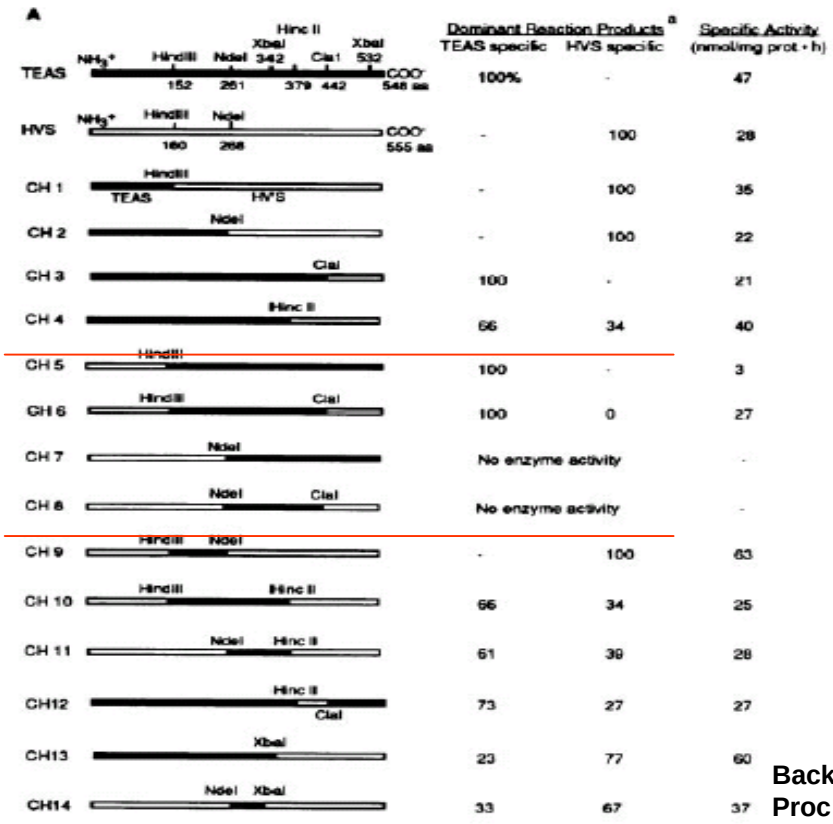


Exon organization of terpene synthases



Genetics Society of America, 2001
Trapp SC and Croteau R

Exon shuffling for new synthases



	Dominant Reaction Products[a]		Specific Activity
	TEAS specific	HVS specific	(nmol/mg prot • h)
TEAS	100%	-	47
HVS	-	100	28
CH 1	-	100	35
CH 2	-	100	22
CH 3	100	-	21
CH 4	66	34	40
CH 5	100	-	3
CH 6	100	0	27
CH 7	No enzyme activity		-
CH 8	No enzyme activity		-
CH 9	-	100	63
CH 10	66	34	25
CH 11	61	39	28
CH12	73	27	27
CH13	23	77	60
CH14	33	67	37

Back K, Chappell J.
Proc Natl Acad Sci USA. 1996 93(13):6841-5.

Products of δ-selinene and γ-humulene synthases

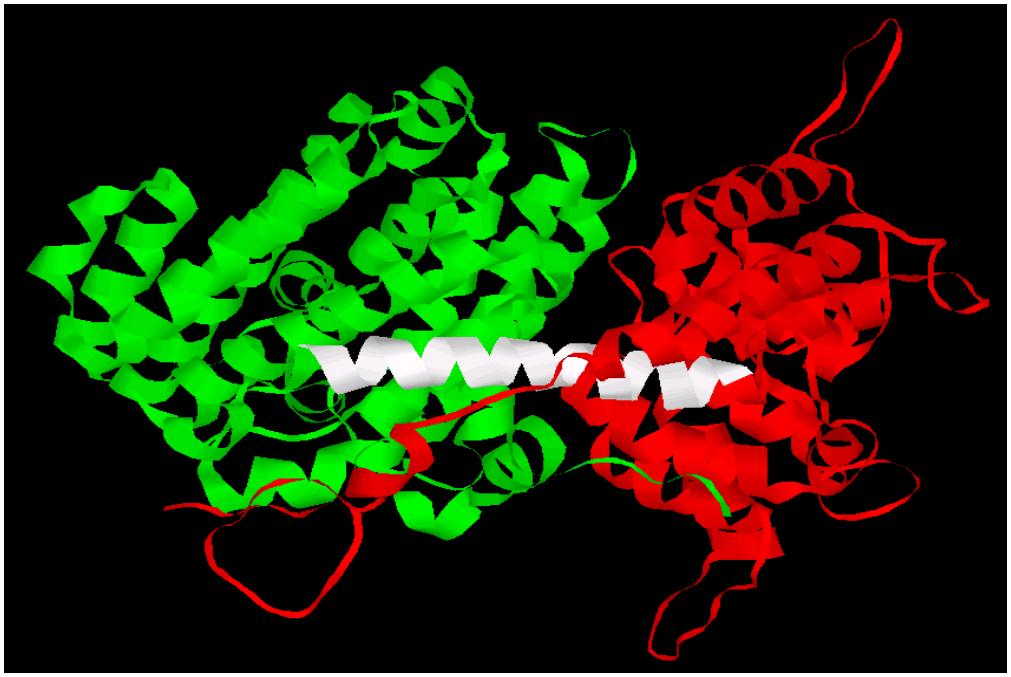
Compounds labeled as (tent.) were tentatively identified based on the mass spectrum alone.

δ-Selinene synthase (*ag4*)		γ-Humulene synthase (*ag5*)	
Sesquiterpene	%	Sesquiterpene	%
δ-Selinene	25.3	γ-Humulene	28.6
(*E,E*)-Germacrene B	17.4	Sibirene	15.1
Guaia-6,9-diene	9.7	Longifolene	11.8
Germacrene A	6.7	β-Himachalene	7.2
δ-Amorphene	6.4	γ-Himachalene	5.8
Unknown	4.7	α-Himachalene	4.8
Unknown	4.4	β-Bisabolene	3.9
Germacrene C	3.4	α-Longipinene	3.4
α-Amorphene	2.7	Sativene	3.1
Unknown	2.6	α-Ylangene	2.7
α-Selinene	1.7	β-Gurjunene	2.0
β-Caryophyllene	1.5	γ-Bisabolene	1.9
δ-Cadinene	1.4	β-Longipinene	1.5
Unknown	1.4	(*E*)-β-Farnesene	1.3
Unknown	1.3	(*E*)-α-Bisabolene	0.9
(*Z,E*)-Germacrene B (tent.)	1.3	δ-Amorphene	0.5
Seli-3,7(11)-diene	1.2	α-Amorphene	0.5
Germacrene D	0.8	β-Ylangene	0.3
α-Humulene	0.7	1,5,9-Trimethylcyclododeca-1,5,9-triene (tent. 2 isomers)	0.2
β-Bisabolene	0.7	Longicyclene	0.2
Sibirene	0.7	β-Cubebene	0.1
α-Guiaene	0.2	α-Copaene	0.1
α-Cadinene	0.2	α-Cubebene	0.1
α-Ylangene	0.1	(*Z,E*)-Germacrene B (tent.)	0.1
α-Copaene	0.1	Cyclosativene	<0.1
9 unknowns, each <1%	3.4	26 unknowns, each <1%	4.1

JBC, Vol273, No 4, 2078-2089,1998

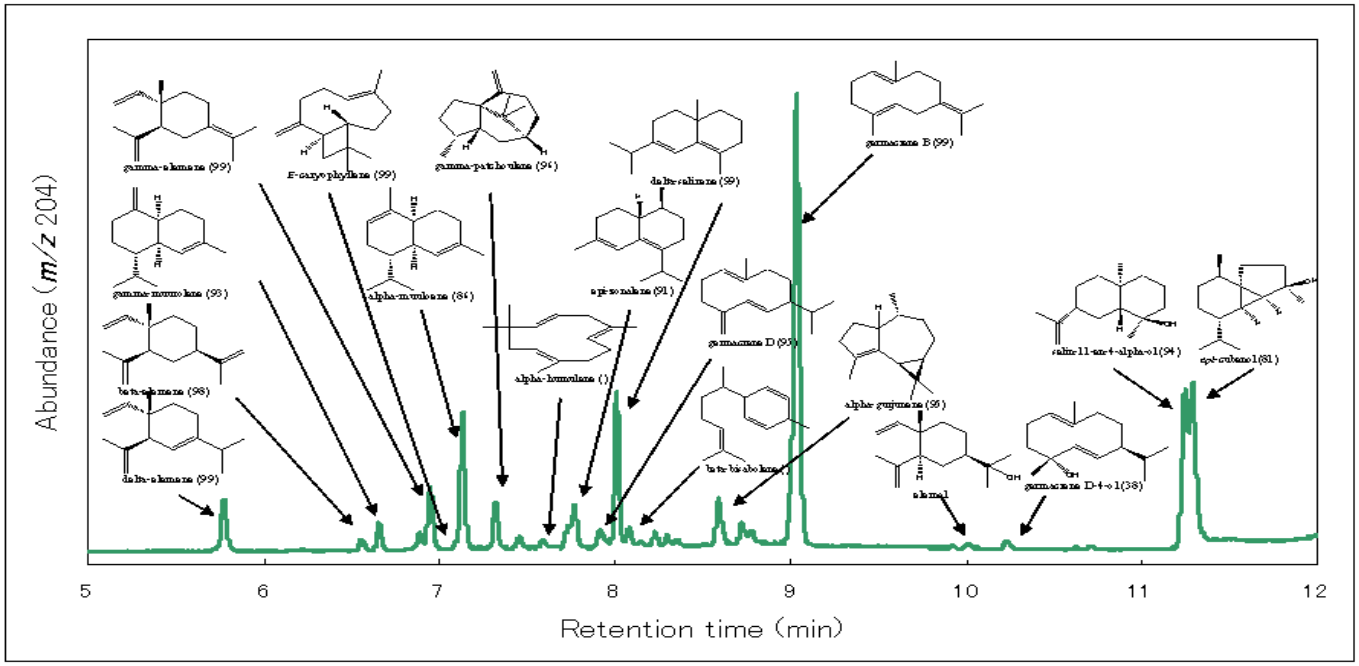
Steele C. L., Crock J, Bohlmann J, Croteau R

Exon shuffling for new synthases

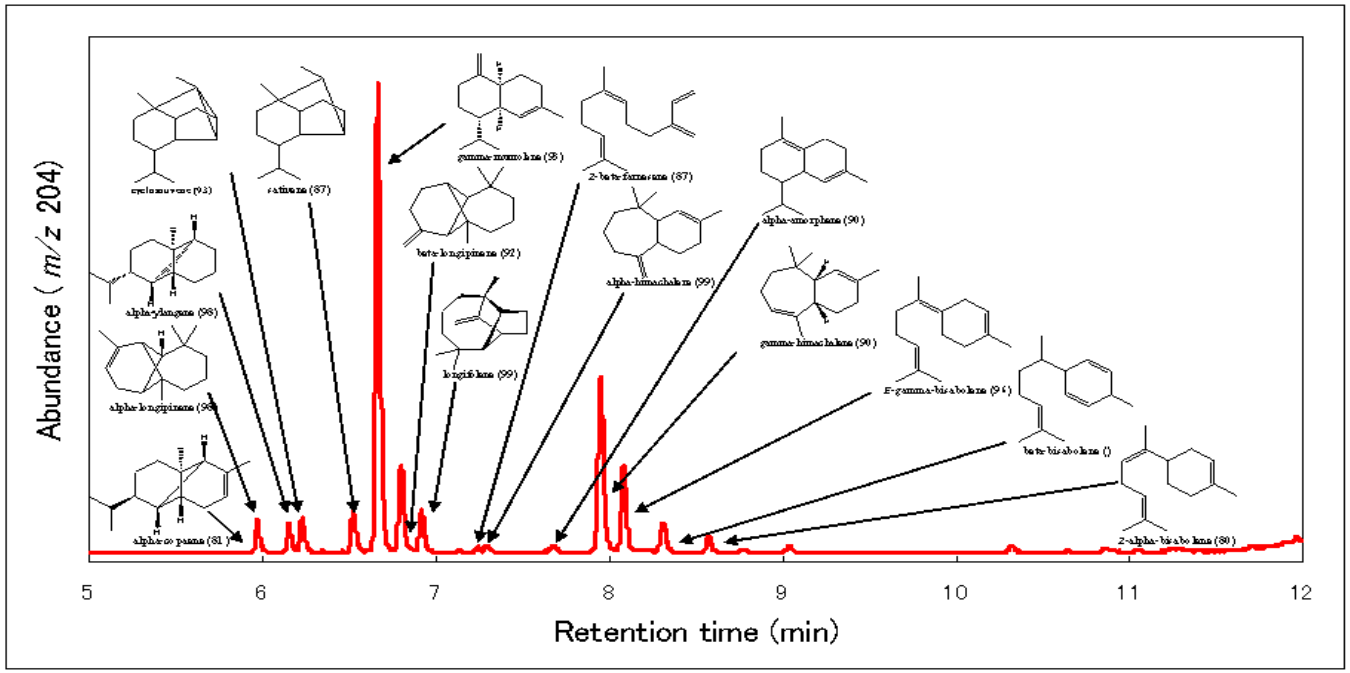


Predicted δ-selinene synthase

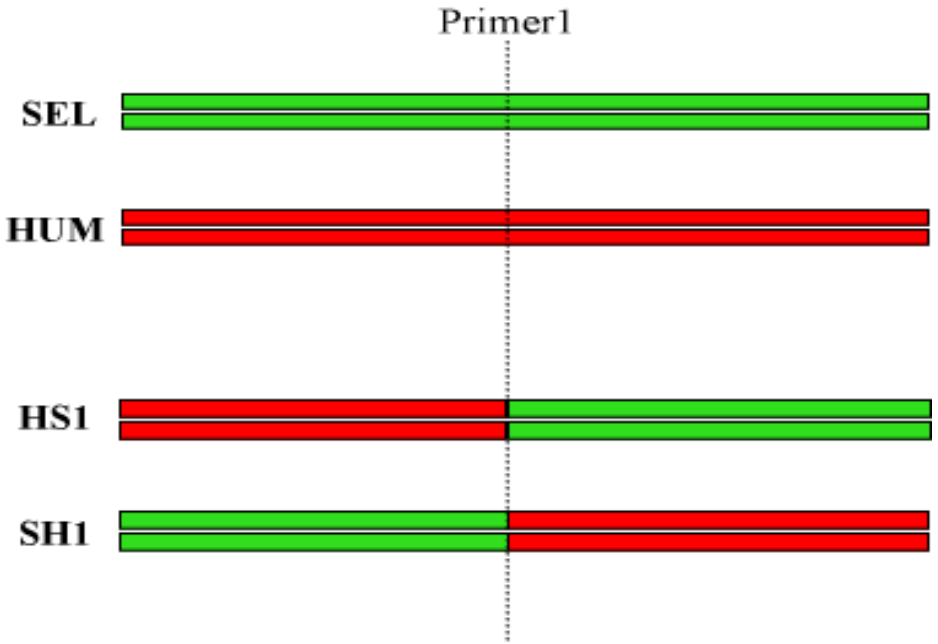
δ-Selinene synthase (SEL)



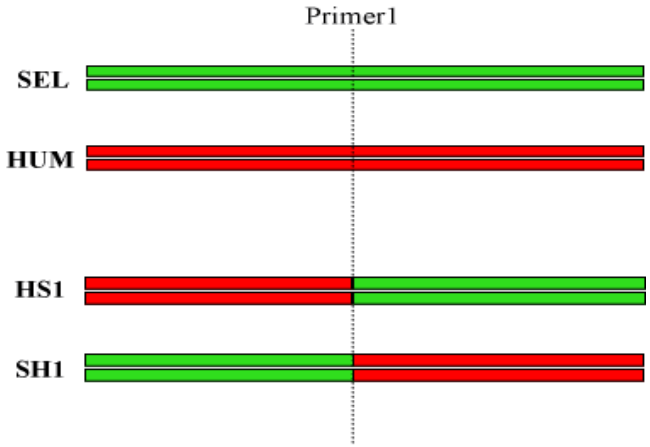
γ-Humulene Synthase (HUM)

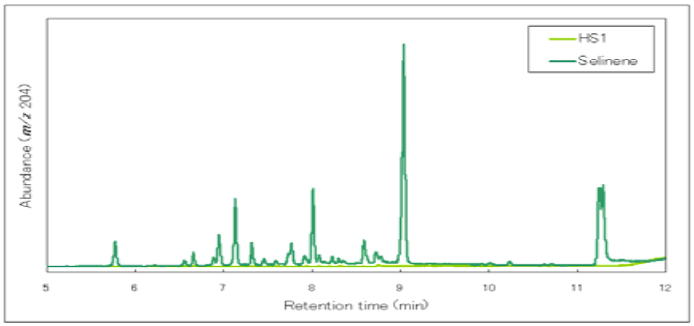


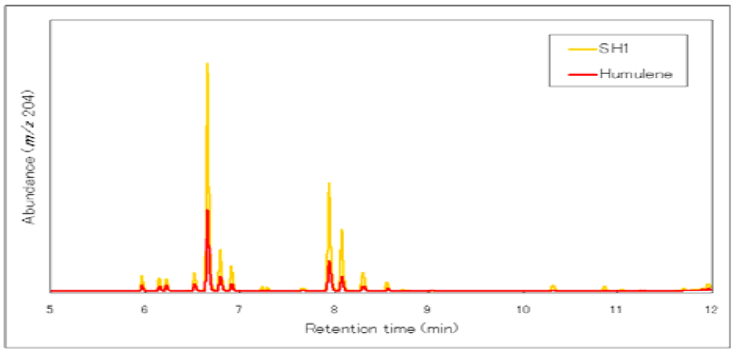
Domain swapping



Domain swapping



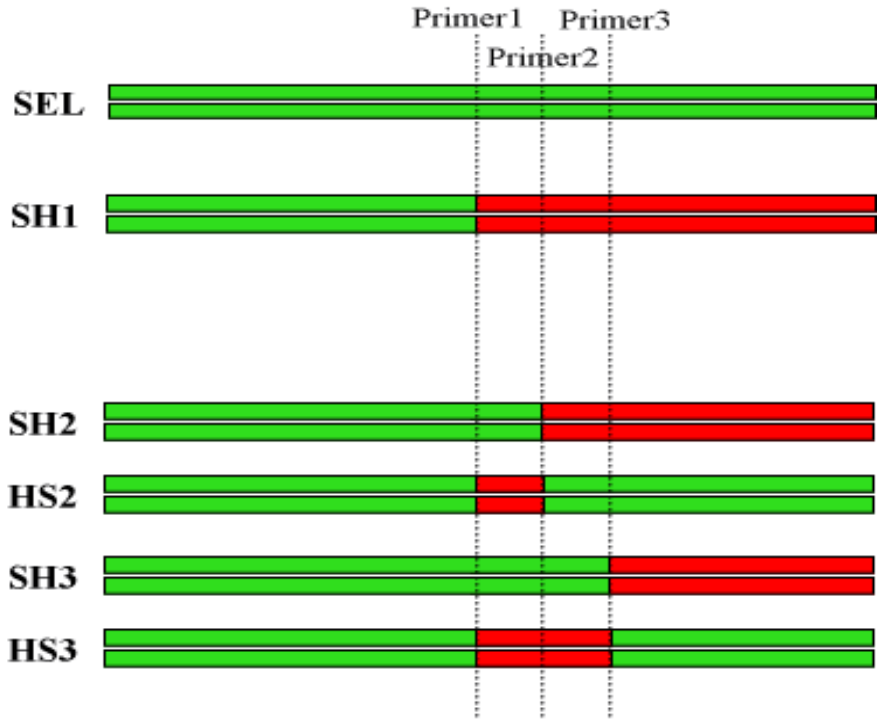




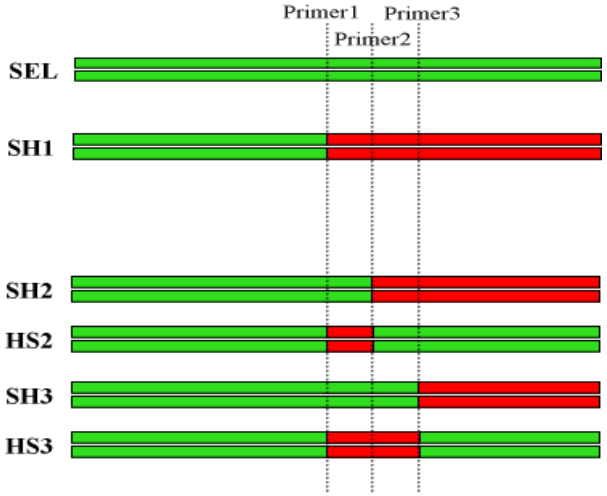
HS1 synthase is dead

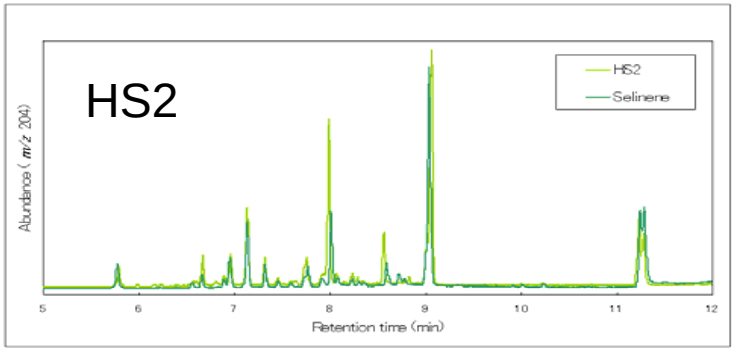
SH1 synthase is similar to HUM synthase

More Domain Swapping

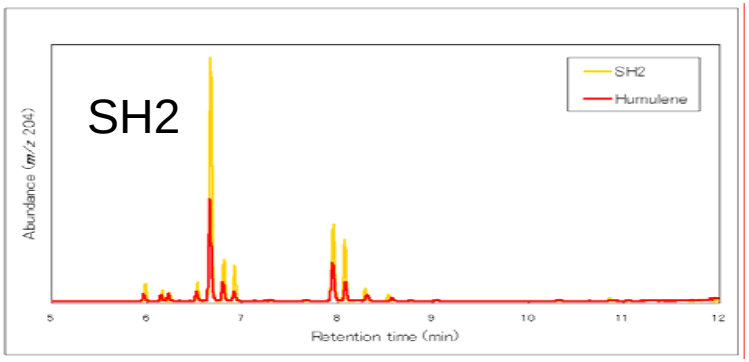


More Domain Swapping



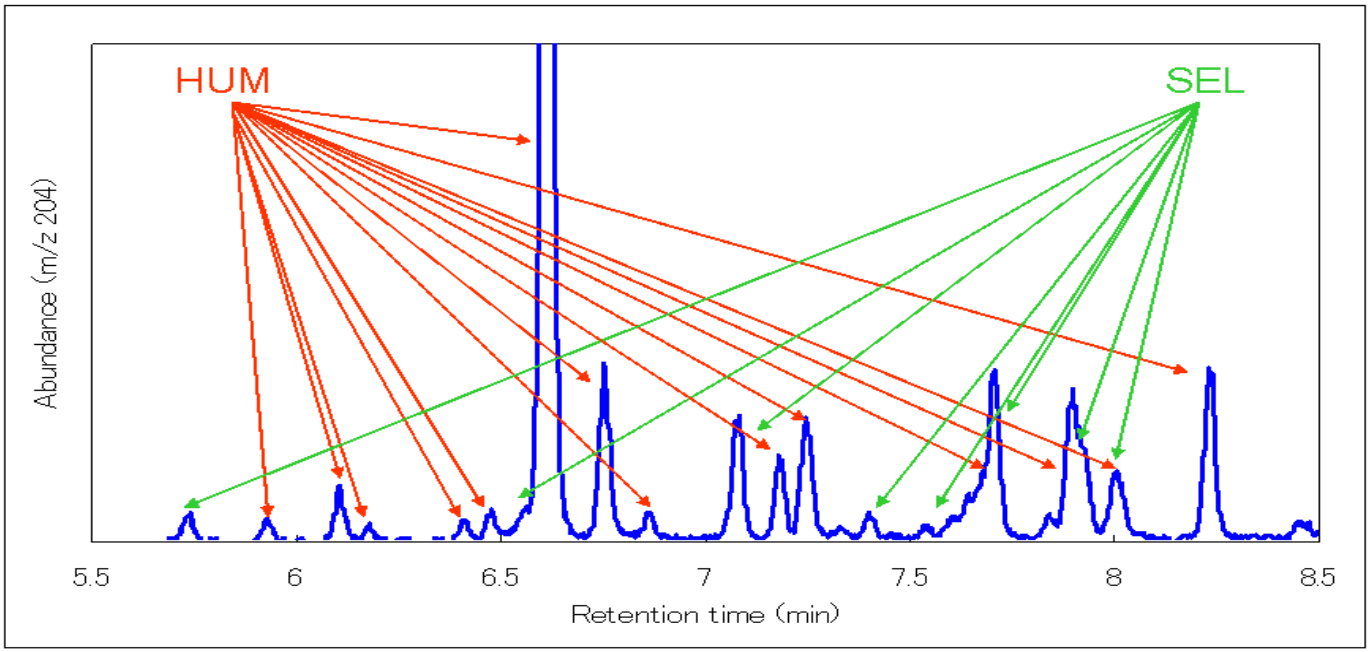


HS2 synthase is similar to SEL synthase

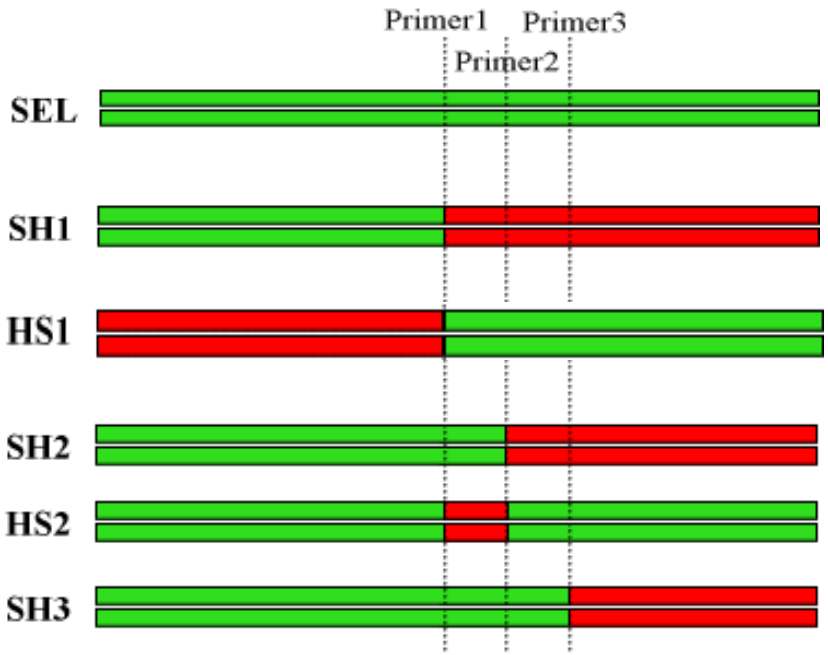


SH2 synthase is similar to HUM synthase

SH3 has properties of both synthases

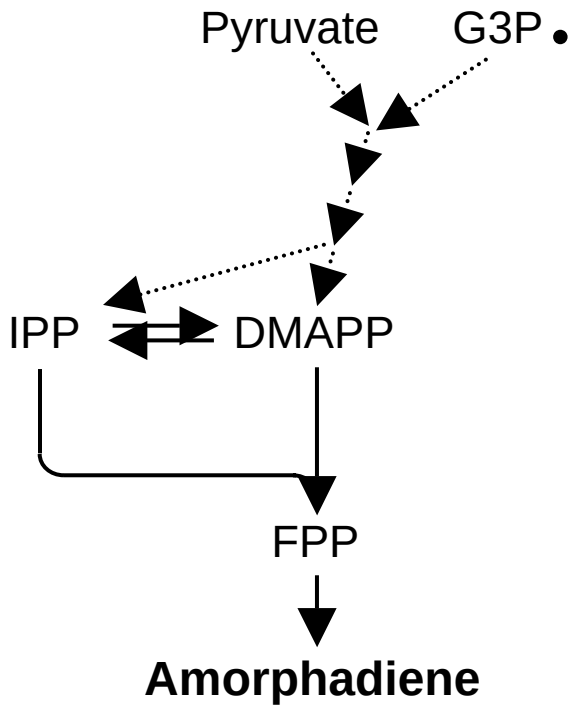


Domain Swapping Results



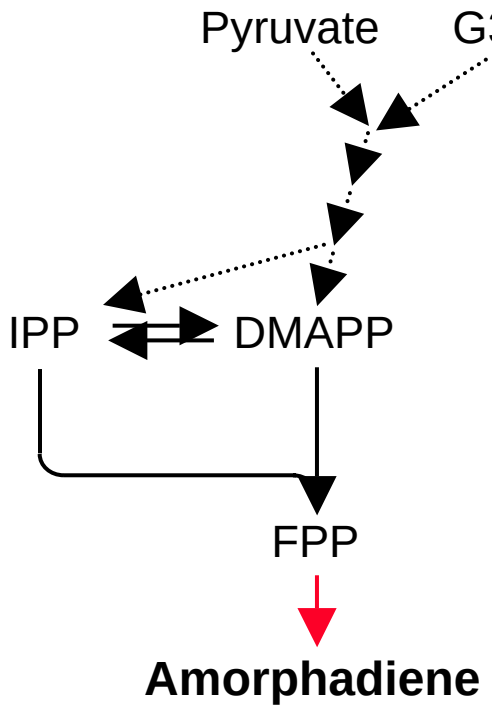
	SEL	HUM
SEL	X	
SH1		X
HS1		
SH2		X
HS2	X	
SH3	X	X

Summary



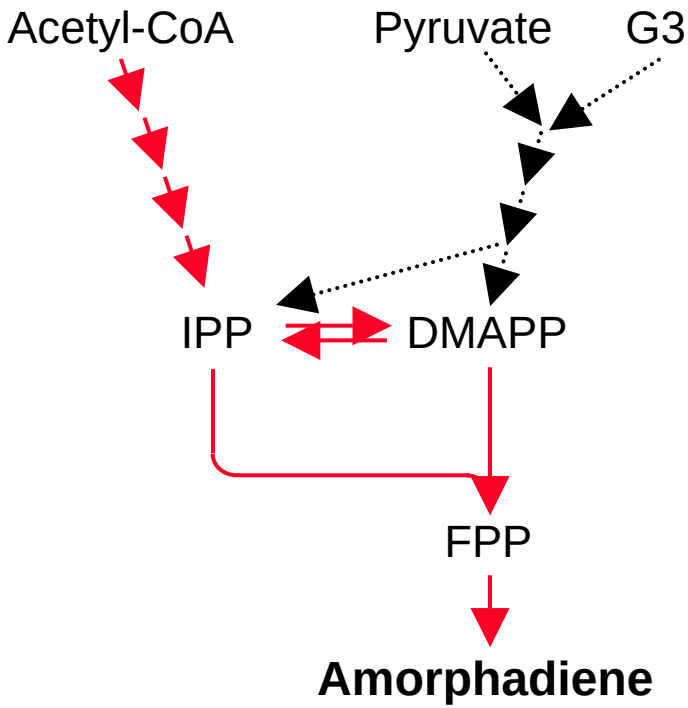
- Plant terpene cyclases perform poorly in *E. coli* (0.05-0.7 ng/mL)

Summary



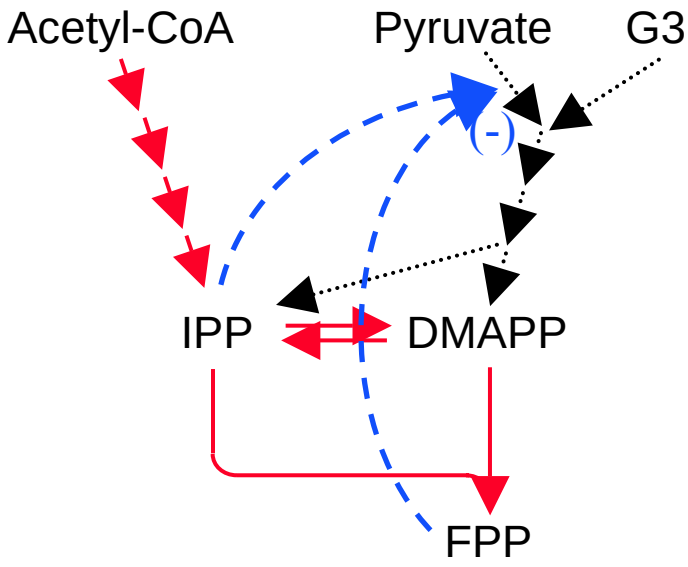
- Plant terpene cyclases perform poorly in *E. coli*
- Expression of codon-optimized synthetic cyclase gene leads to 142-fold improvement

Summary



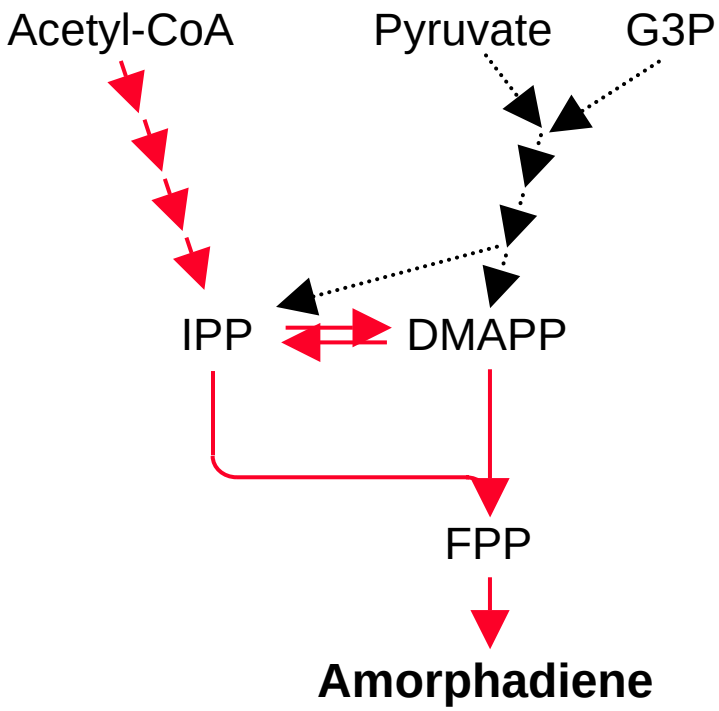
- Plant terpene cyclases perform poorly in *E. coli*
- Expression of codon-optimized synthetic cyclase gene leads to 142-fold improvement
- An engineered mevalonate pathway improves yields by another ~30 to 90-fold

Summary

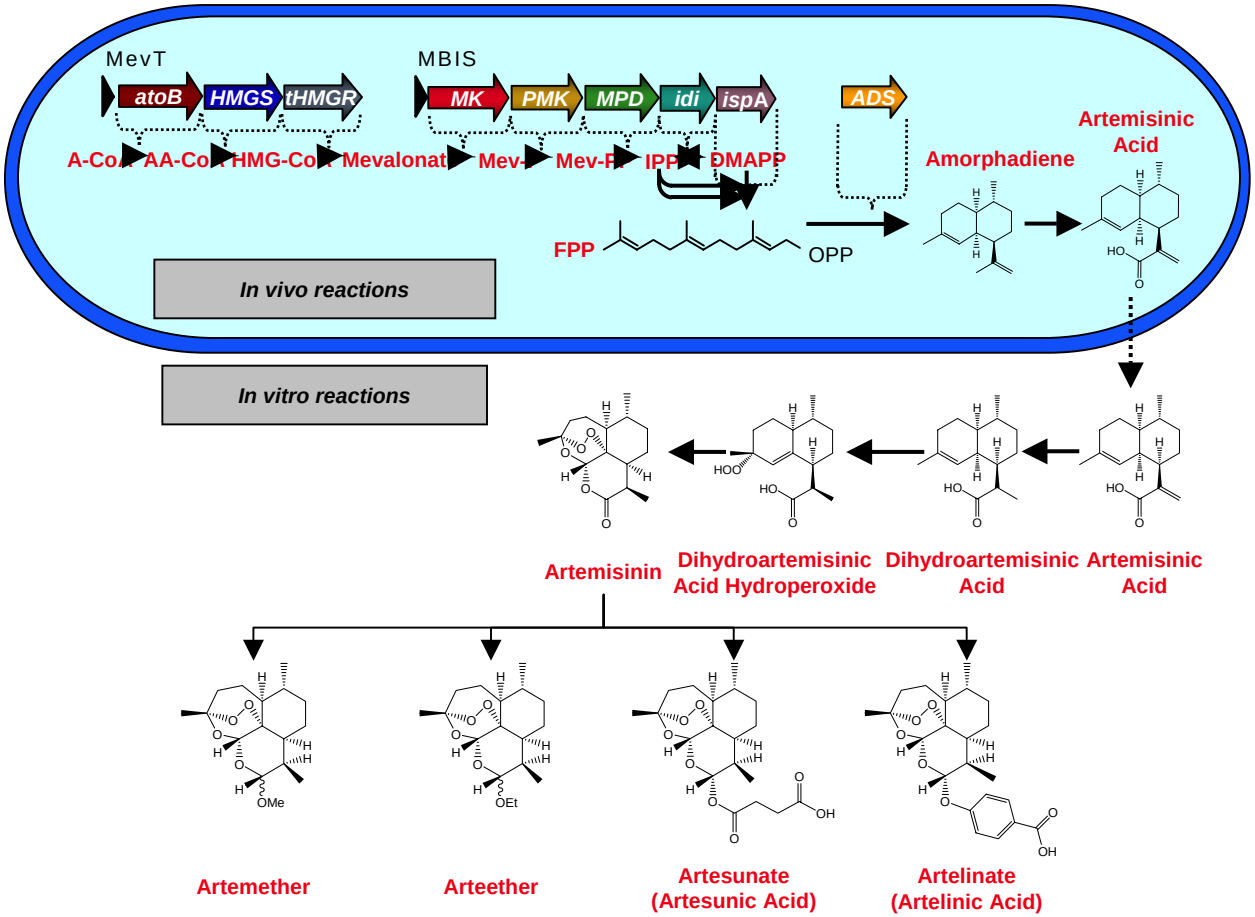


- Plant terpene cyclases perform poorly in *E. coli*
- Expression of codon-optimized synthetic cyclase gene leads to 142-fold improvement
- An engineered mevalonate pathway improves yields by another ~30 to 90-fold
- Excess intracellular prenyl-PP inhibits the growth of *E. coli* and induces mutations

Summary



- Plant terpene cyclases perform poorly in *E. coli*
- Expression of codon-optimized synthetic cyclase gene leads to 142-fold improvement
- An engineered mevalonate pathway improves yields by another ~30 to 90-fold
- Excess intracellular prenyl-PP inhibits the growth of *E. coli* and induces mutations
- Currently at ~0.5 g/L, a 10^5-10^7-fold increase


MevT
atoB
HMGS
tHMGR
MBIS
MK
PMK
MPD
idi
ispA
ADS
A-CoA
AA-CoA
HMG-CoA
Mevalonate
Mev-P
Mev-PP
IPP
DMAPP
FPP
OPP
Amorphadiene
Artemisinic Acid
In vivo reactions
In vitro reactions
Artemisinin
Dihydroartemisinic Acid Hydroperoxide
Dihydroartemisinic Acid
Artemisinic Acid
OMe
OEt
OH
Artemether
Arteether
Artesunate (Artesunic Acid)
Artelinate (Artelinic Acid)

Acknowledgements

Vincent Martin

Doug Pitera

Syd Withers

Yasuo Yoshikuni

Jack Newman

Kinkead Reiling

Funding

ONR, NSF (MEWG Program)