Metabolic Engineering of Microorganisms

Degradation of Organophosphate Contaminants

Synthesis of Isoprenoids

Metabolic Engineering Working Group

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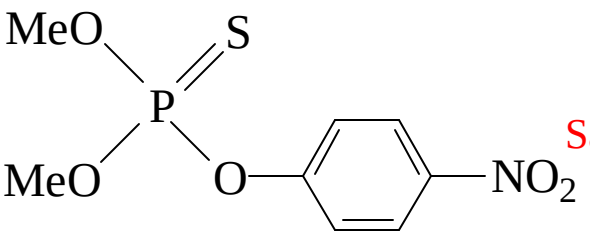
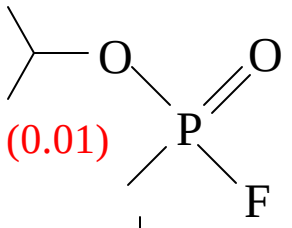
Degradation of organophosphates

Goal -

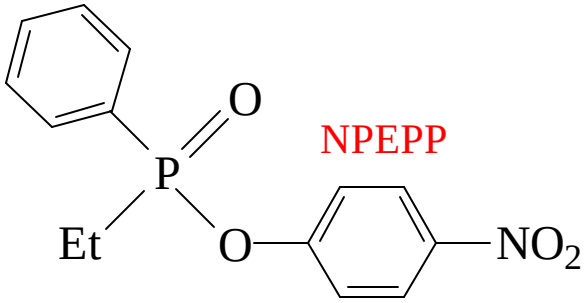
- to develop the experimental and theoretical methods to introduce multiple, heterologous, biodegradation pathways into a single organism
- to optimize the flux through those pathways for the remediation of toxic or recalcitrant organic contaminants.



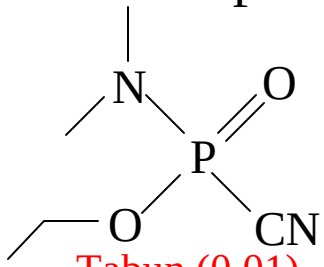
Methyl Parathion (14)



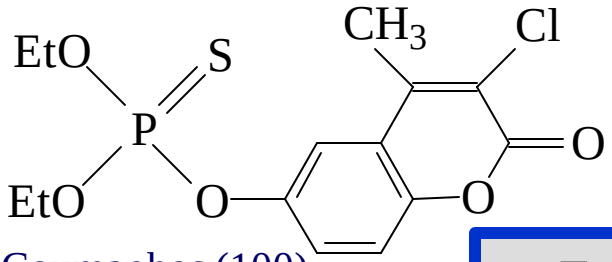
Sarin (0.01)



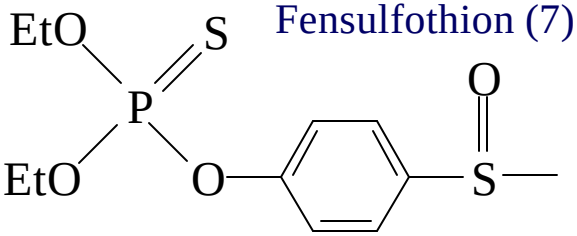
NPEPP



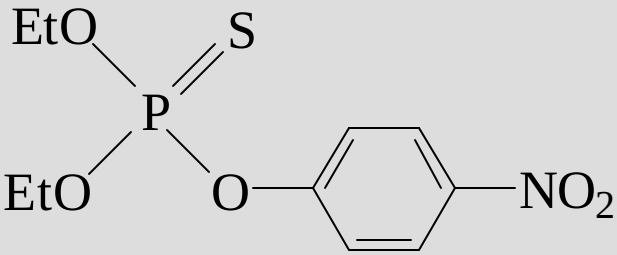
Tabun (0.01)



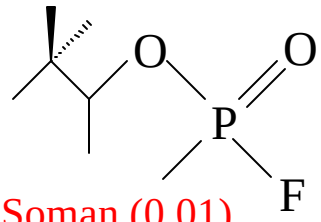
Coumaphos (100)



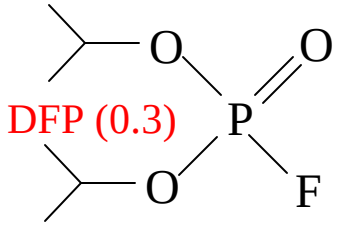
Fensulfothion (7)

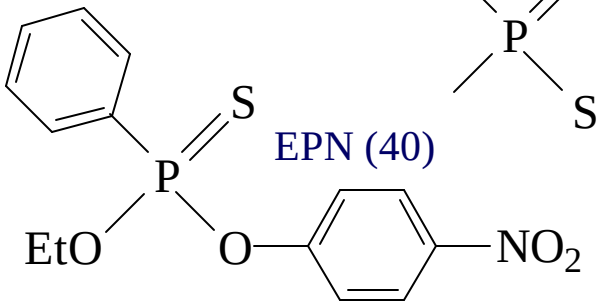


Parathion (LD_{50} 13 mg/kg)

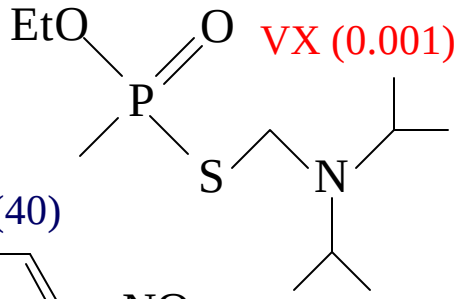


Soman (0.01)

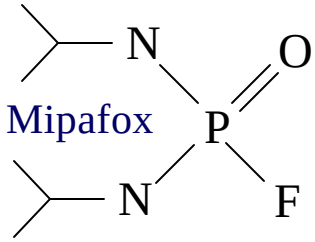


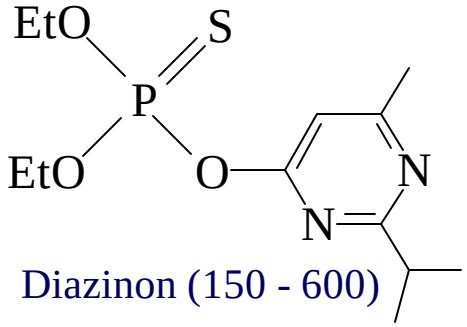


EPN (40)



VX (0.001)





Diazinon (150 - 600)

Justification

Pesticides

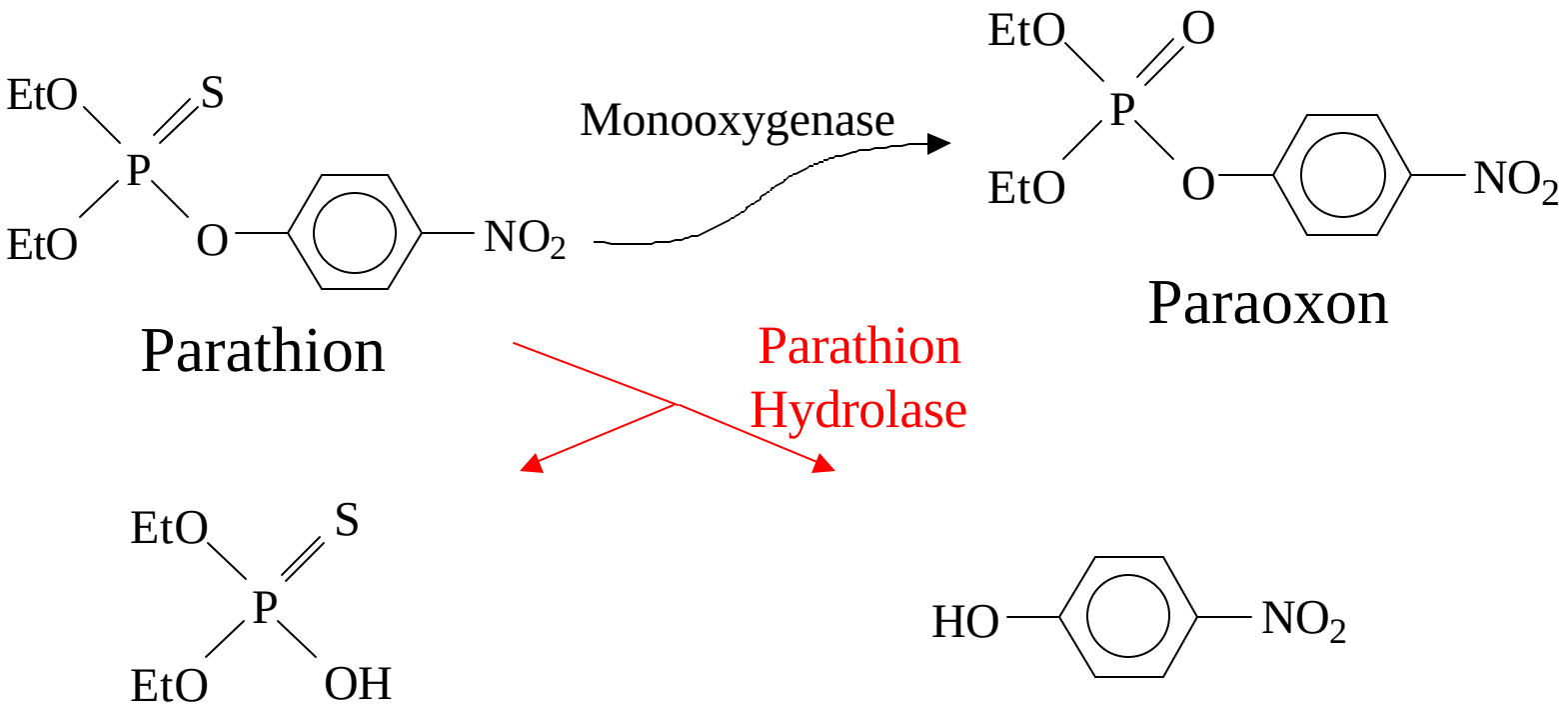
- ~ 60,000 tons of organophosphate pesticides are produced annually in the US
- U.S. Geological Survey reported 54.4% of groundwater sites sampled were contaminated with pesticides (1998)

Chemical Warfare Agents

- Chemical Weapons Convention calls for destruction of all chemical warfare stockpiles (1993)
- 30,000 metric tons of chemical agents to be destroyed in US

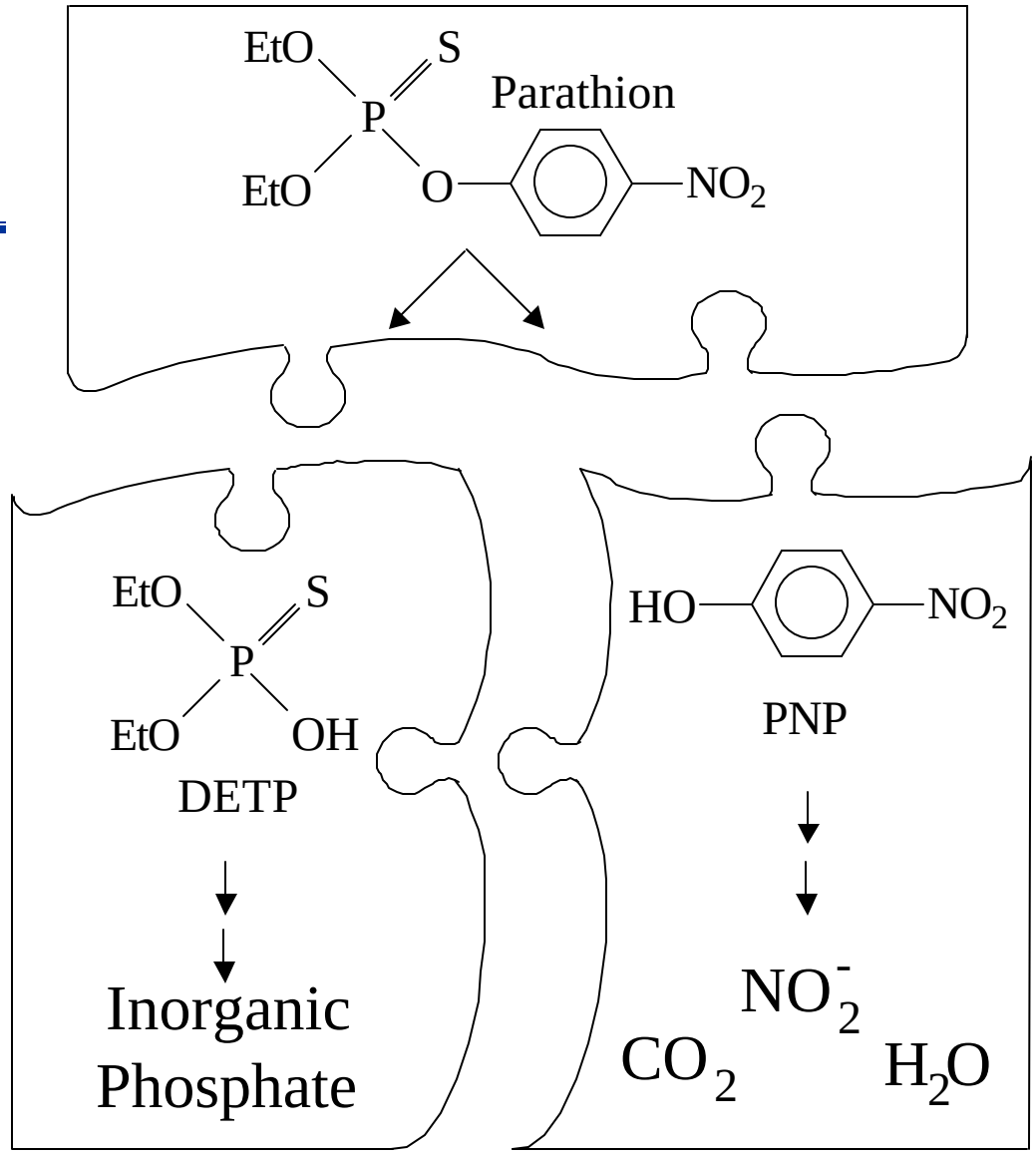
Parathion Degradation Background

- One of the most highly toxic compounds certified by EPA
- 4-7 million pounds are produced annually in the U.S.

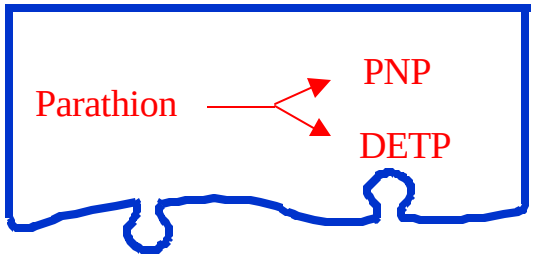


Parathion Degradation

A 3 piece puzzle:



Parathion Hydrolysis

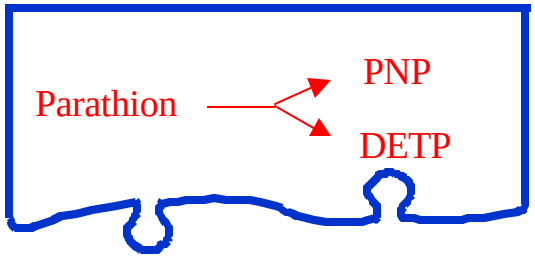


- Past work on parathion degradation has focused on initial hydrolysis
- Gene coding for parathion hydrolase (*opd*) has been cloned & sequenced from both *Pseudomonas* and *Flavobacterium*
- Two forms of *opd*:

Native – contains coding region for N-terminal leader sequence

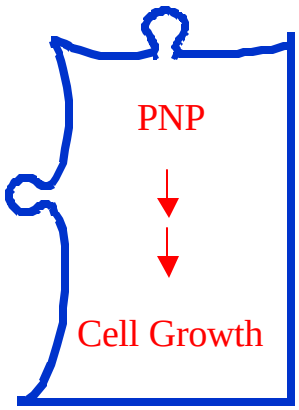
"Modified" – coding region for leader sequenced removed

Parathion Hydrolysis



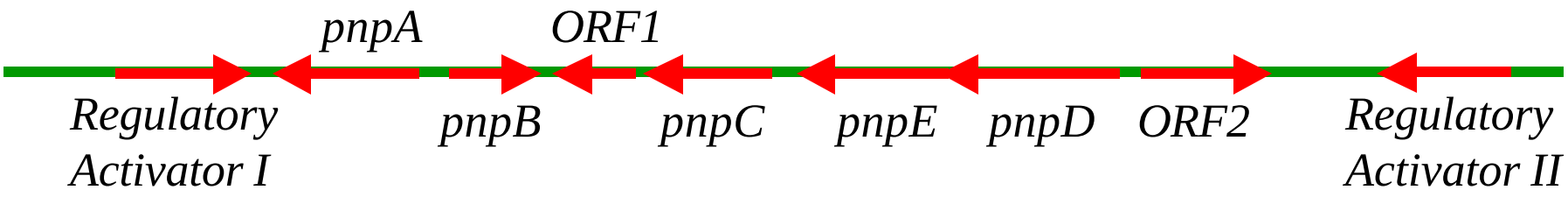
Plasmid:	pAWW01	pAWW02	pAWW04
Promoter:	P_{taclac}	P_{taclac}	P_{tac}
opd gene type:	"modified"	native	native

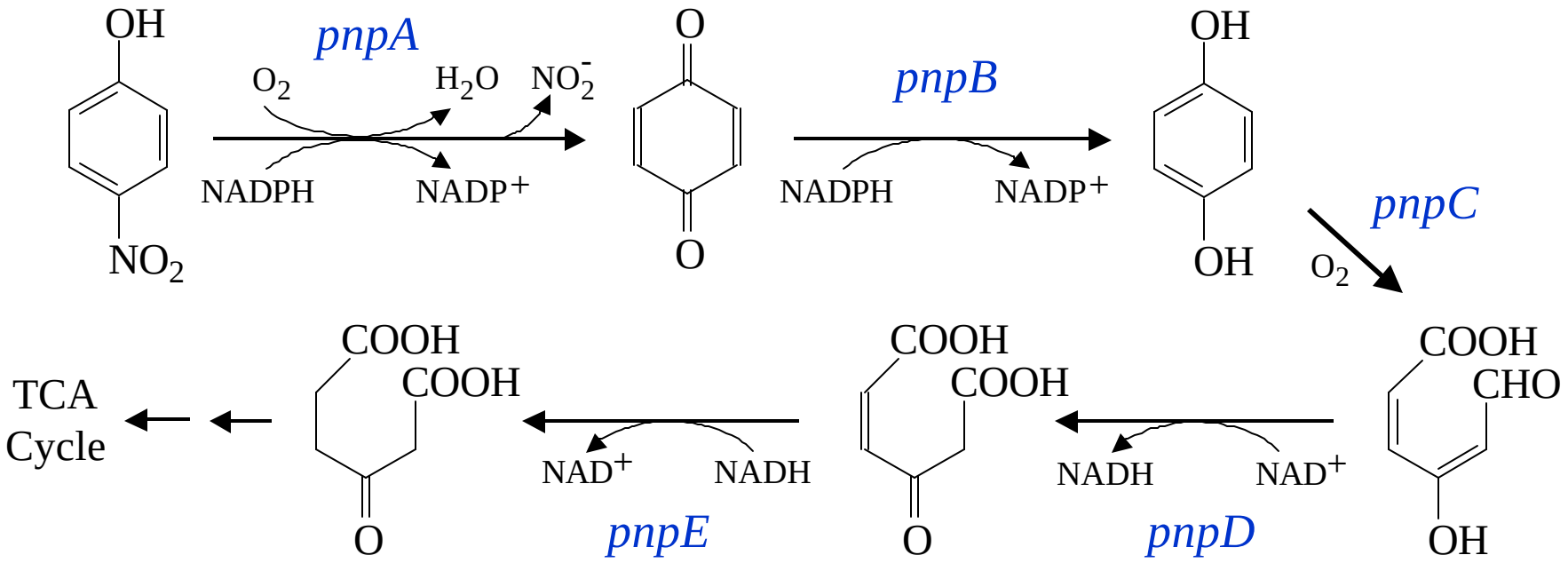
	Spec. Activity (μM/hour-OD)	Spec. Activity (μM/hour-OD)	Spec. Activity (μM/hour-OD)
E. coli DH5α:			
No induction	36.8	3.8	6.3
Full induction	88.5	10.2	13.9
P. putida KT2442:			
No induction	1.7	--	6.9
Full induction	1.8	--	7.3

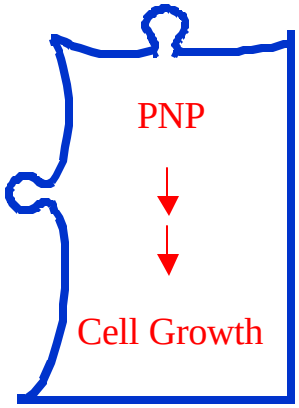


PNP Degradation

Bang & Zylstra isolated 18kb sequence from a Pseudomonad containing PNP degrading genes

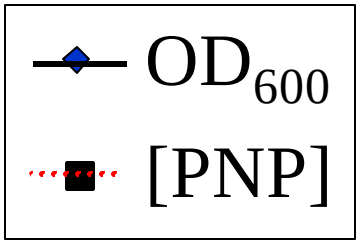


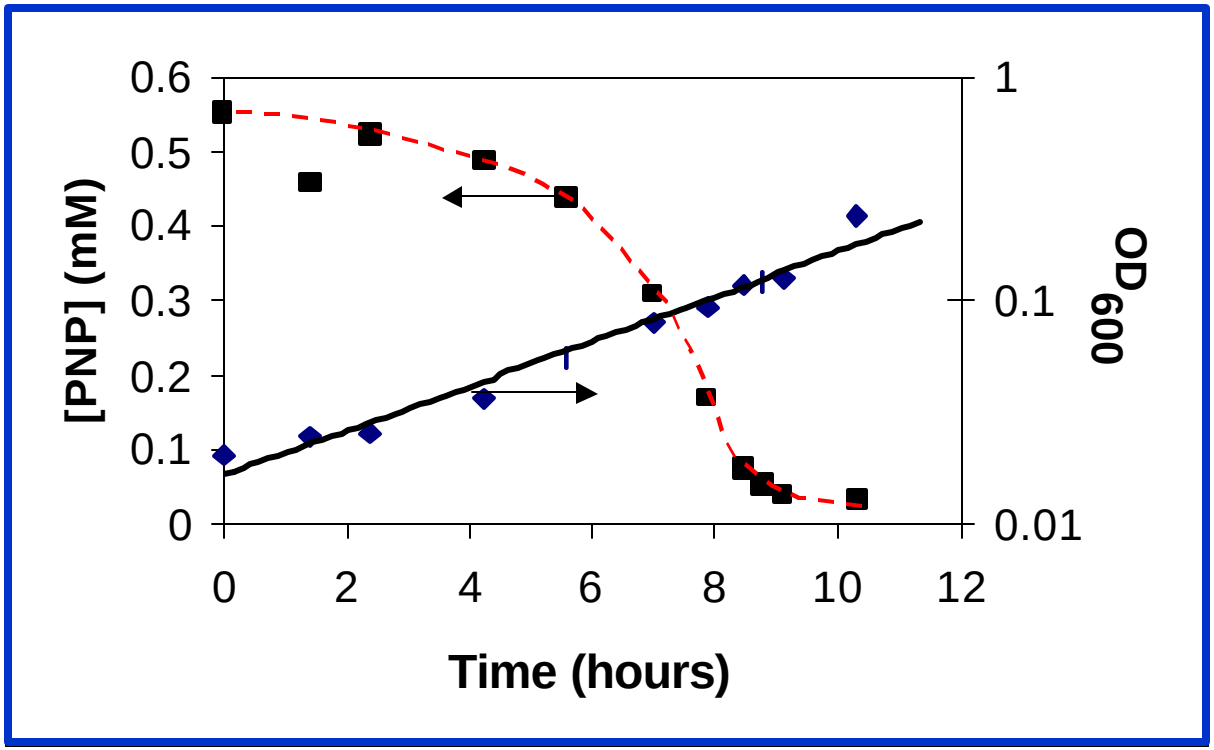




PNP Degradation

PNP degradation by *P. putida* KT2440 with pPNP

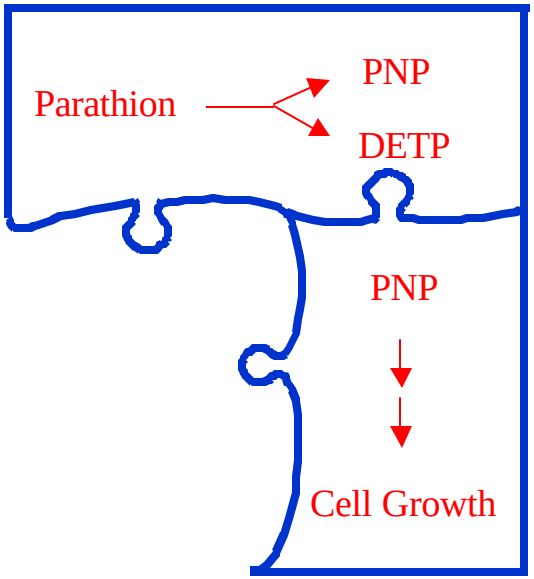




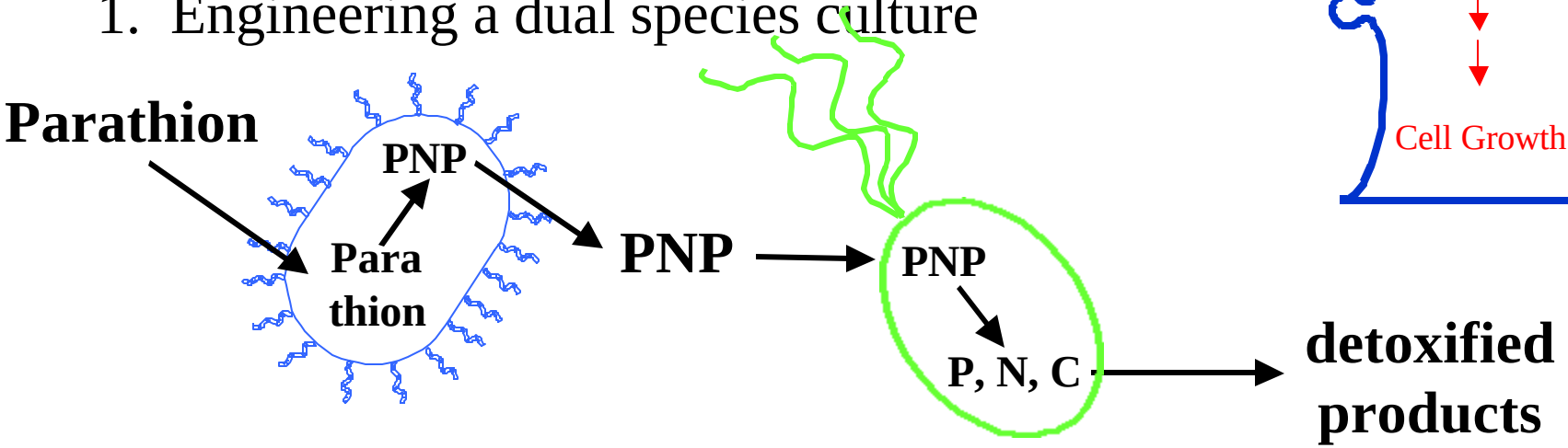
Specific Degradation Rate: 66 µmole/min-gDCW

Specific Growth Rate: 0.23 hour $^{-1}$

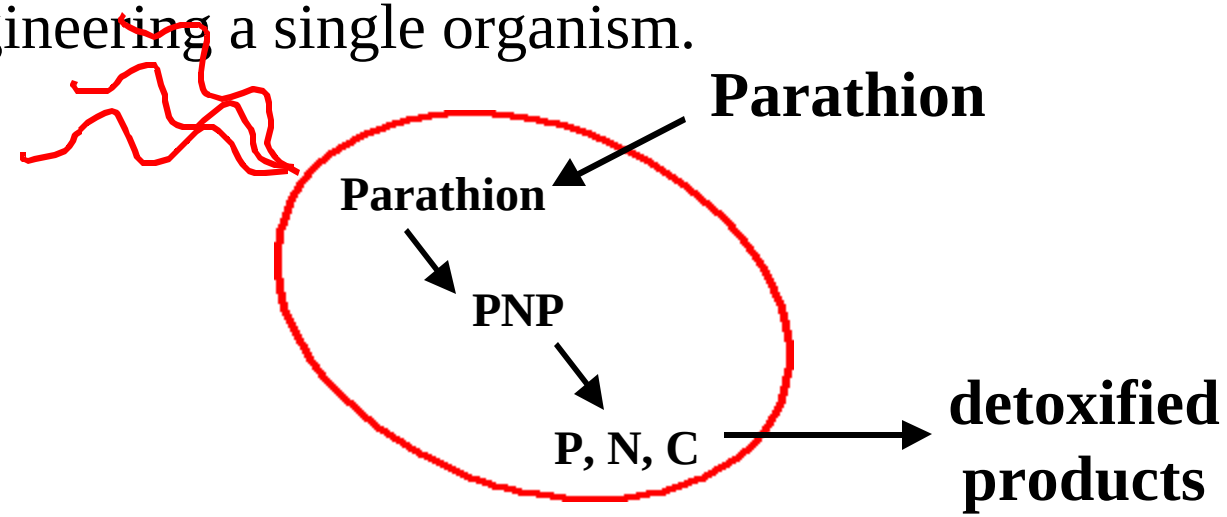
Two ME strategies



1. Engineering a dual species culture

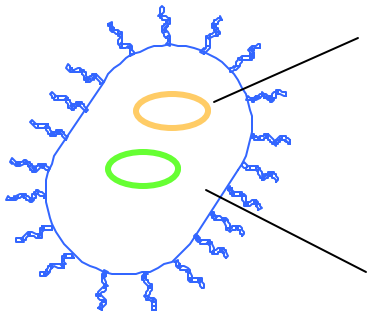


2. Engineering a single organism.



A dual species culture

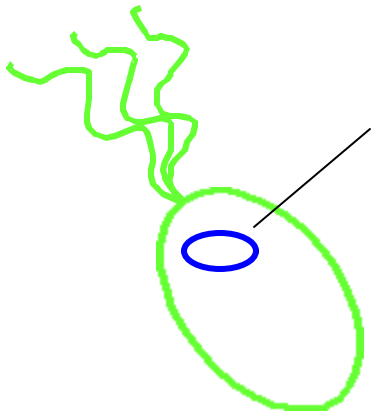
***E. coli* SD2**
hydrolyzes parathion



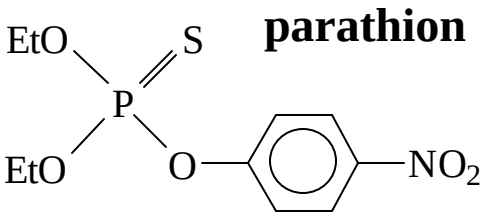
pWM513:
- parathion hydrolysis (*opd* genes)
- ampicillin resistance

pMAG1:
- *gfp* gene
- tetracycline resistance

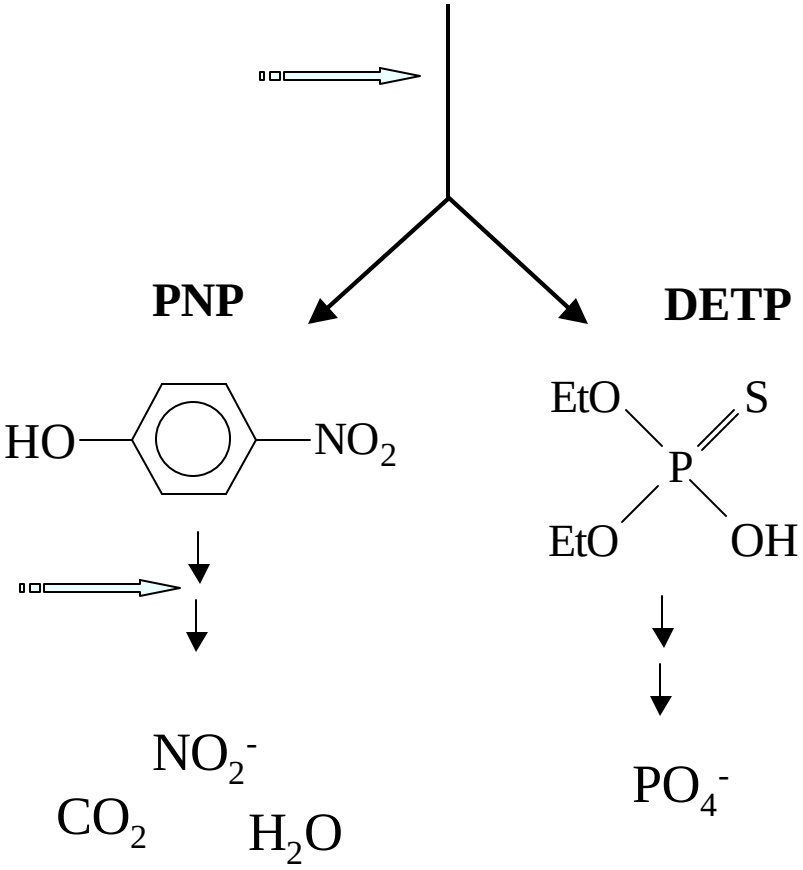
***Pseudomonas* KT2440**
mineralizes *p*-nitrophenol (PNP)



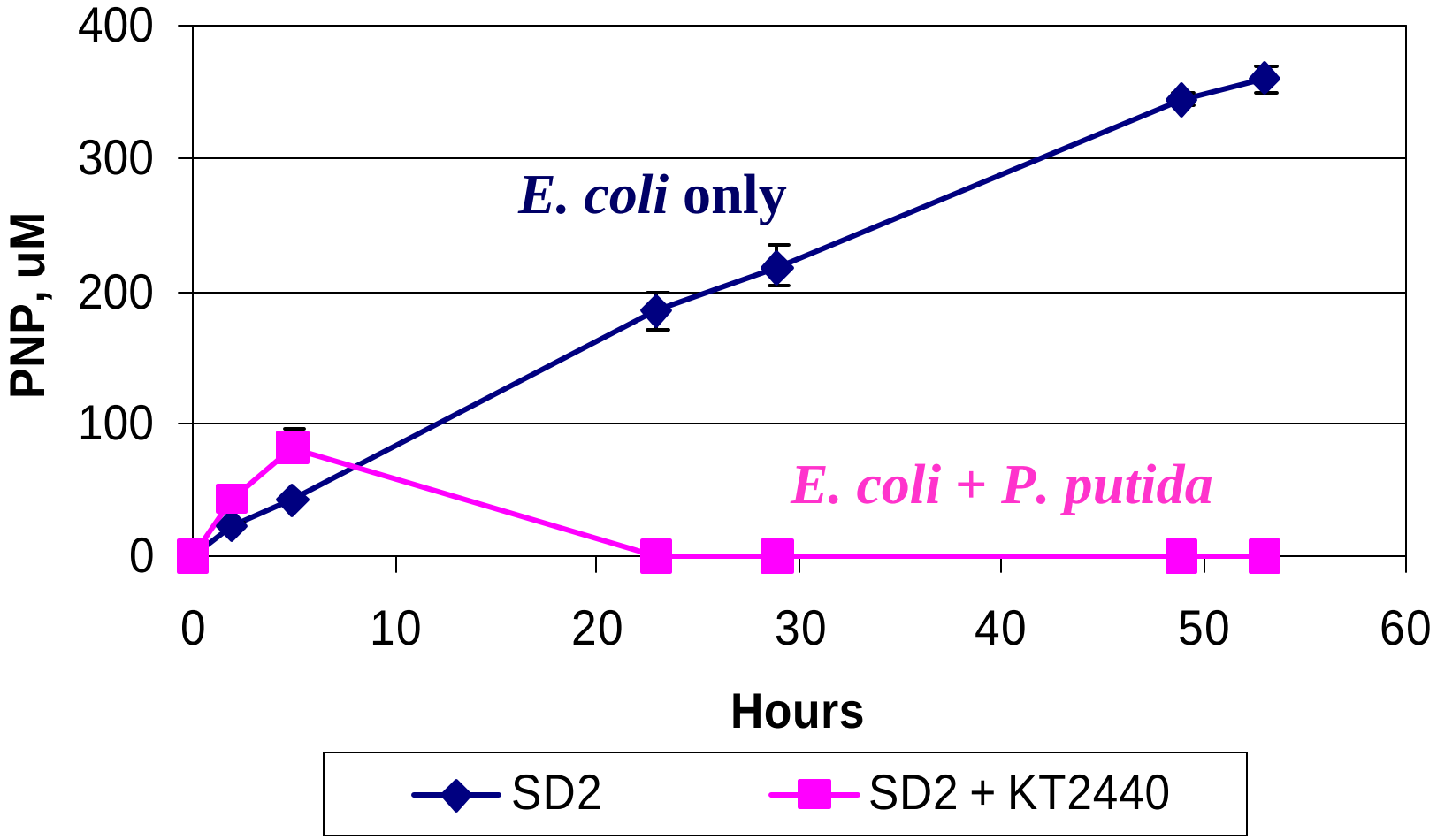
pPNP:
- PNP degradation
- tetracycline resistance
- natural ampicillin resistance



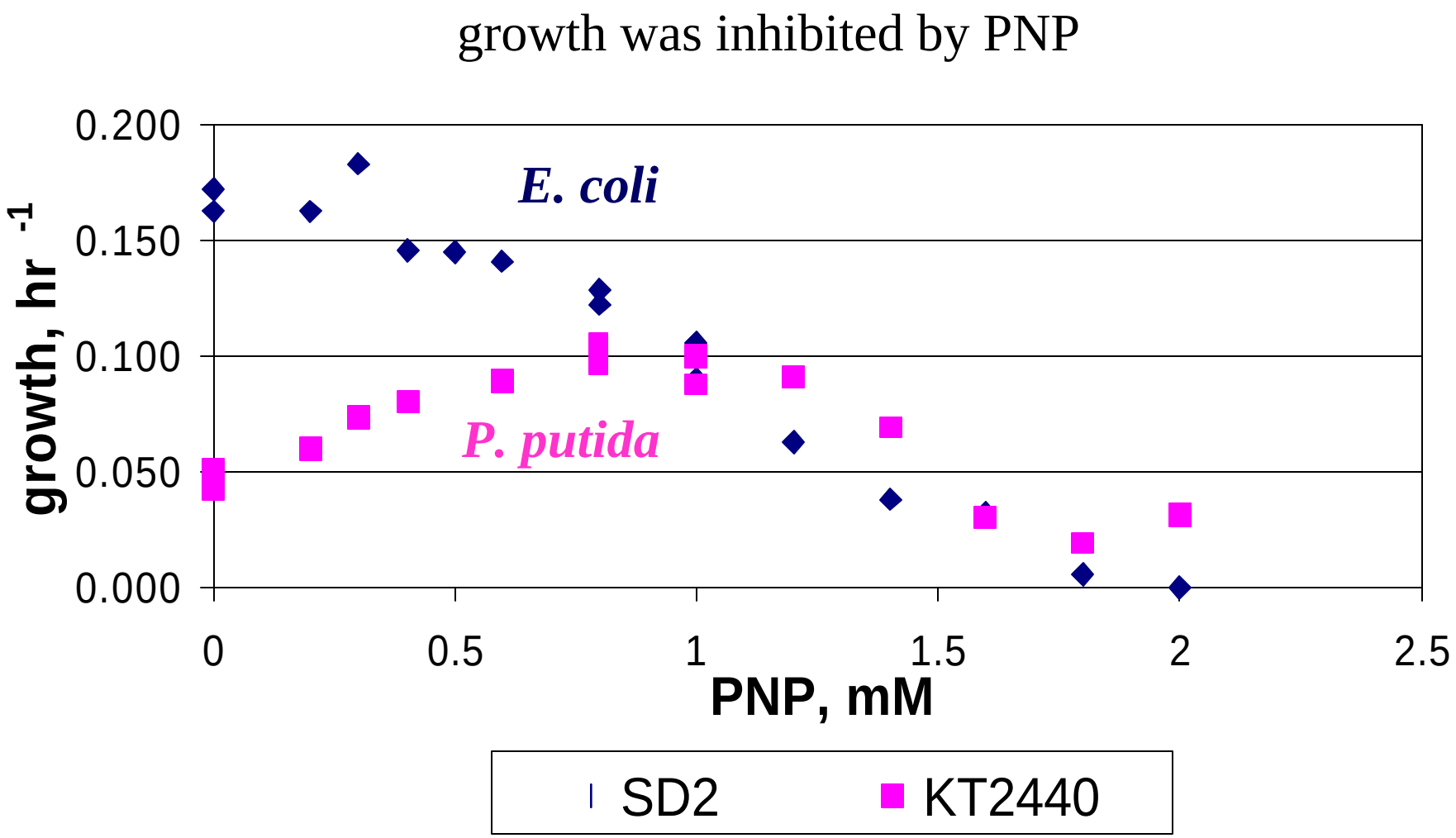
parathion



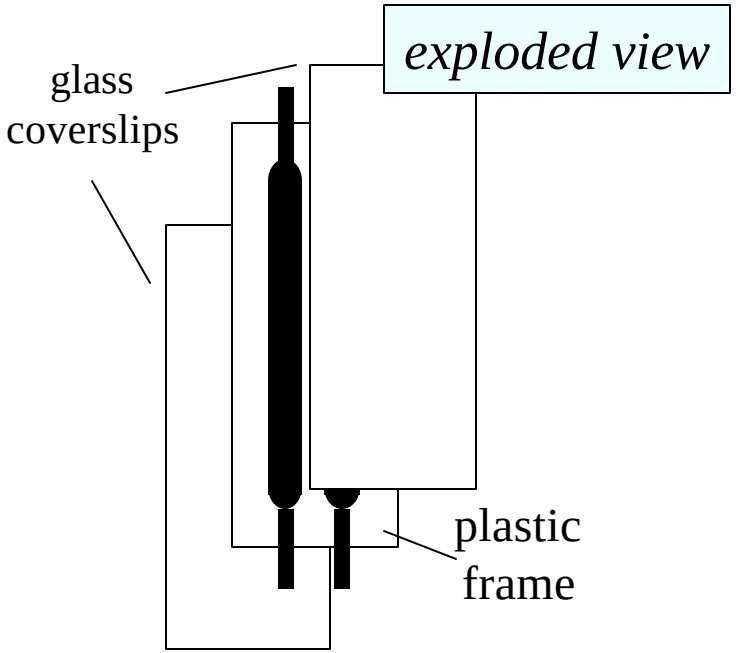
Biodegradation of parathion in suspended culture



Effect of PNP on cell growth

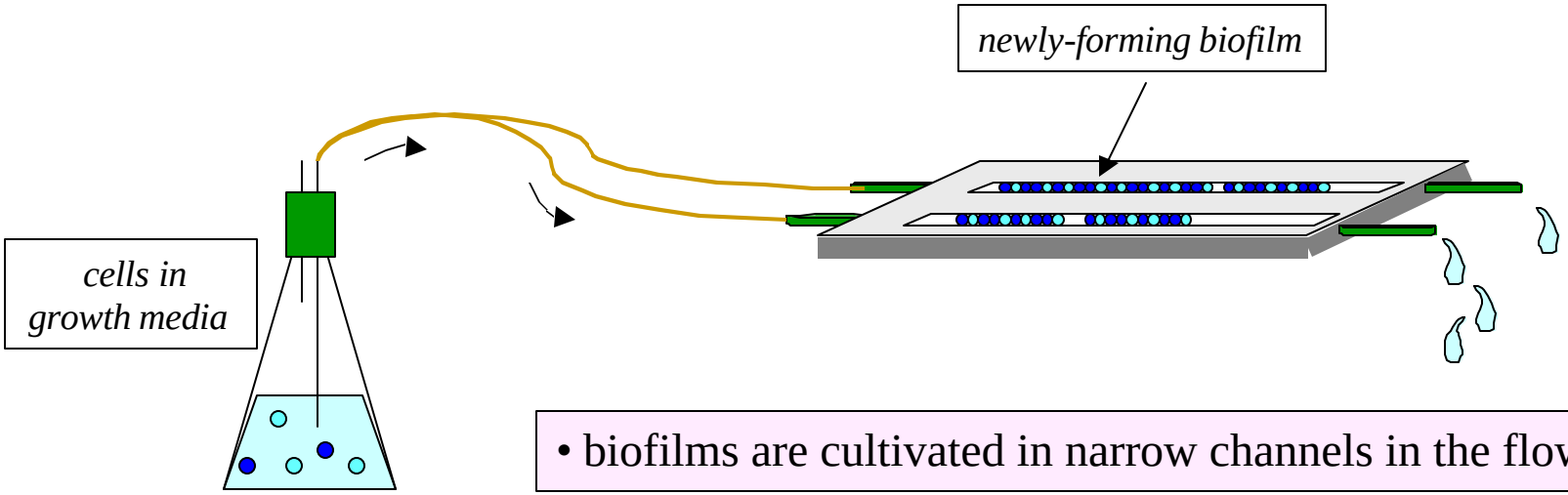


Flow cell for culturing biofilms



measurements

chamber size:
46 mm × 4 mm × 2 mm
chamber volume: 0.37 mL
flow: 0.86 mL min^{-1}
velocity: 41 mm min^{-1}



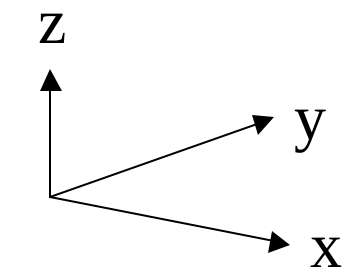
• biofilms are cultivated in narrow channels in the flow cell

Development of a coculture biofilm for parathion biodegradation

red: *P. putida* KT2440

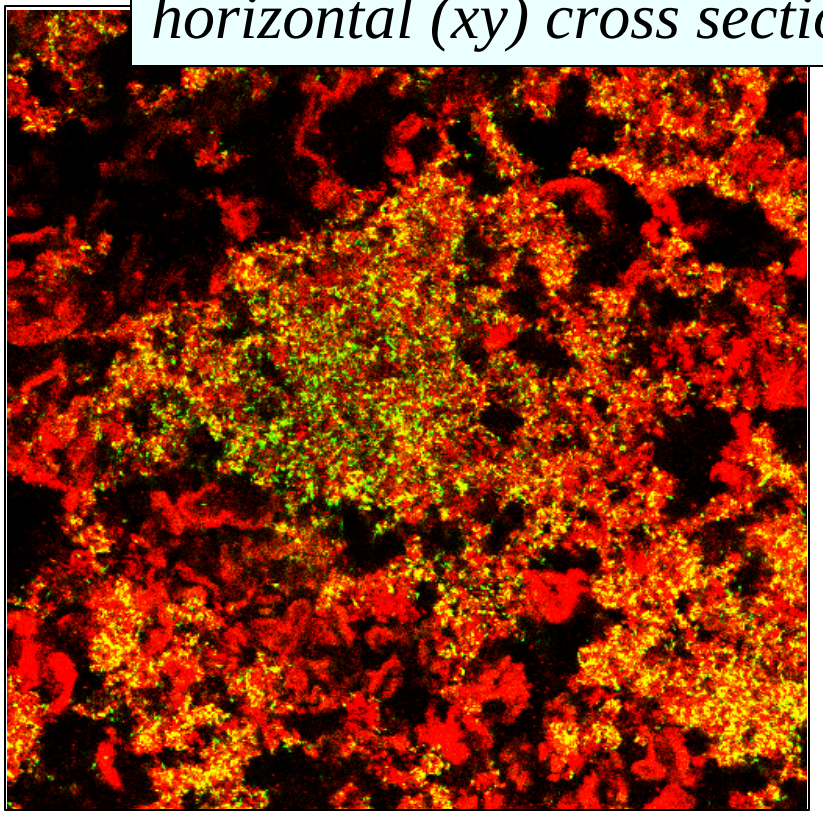
yellow/green: *E. coli* SD2

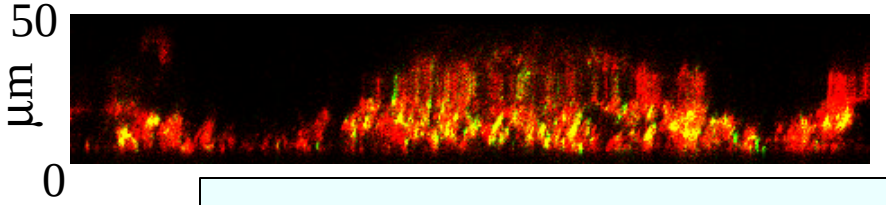
black: voids within the biofilm



orientation

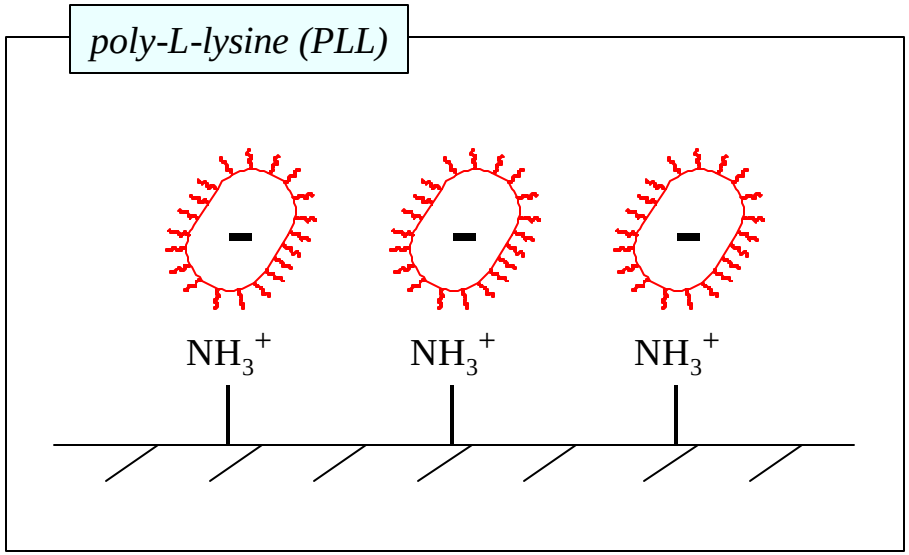
horizontal (xy) cross section





vertical (xz) cross section

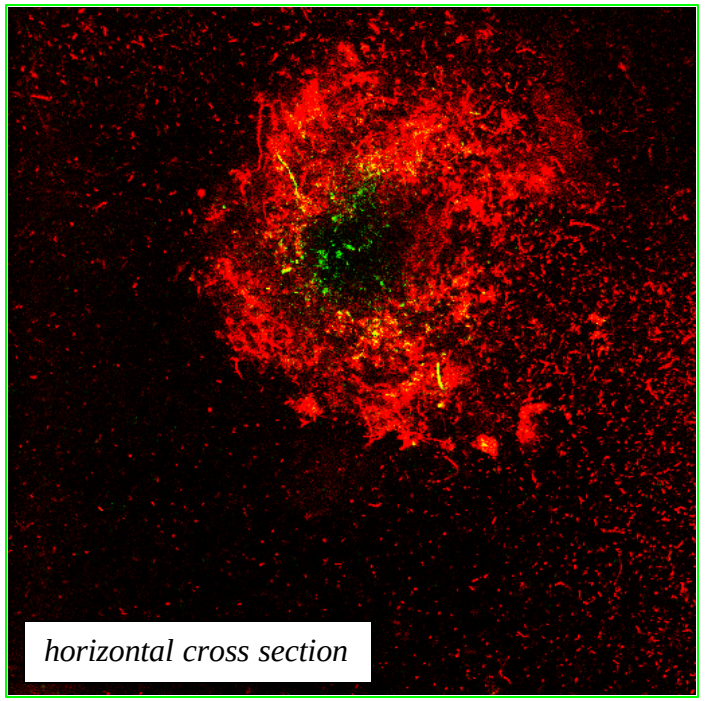
Biofilm engineering

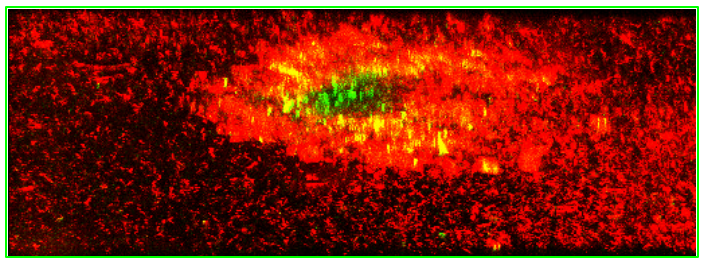


yellow/green: *E. coli* SD2 attached to glass sphere with PLL

red: *P. putida* KT2440

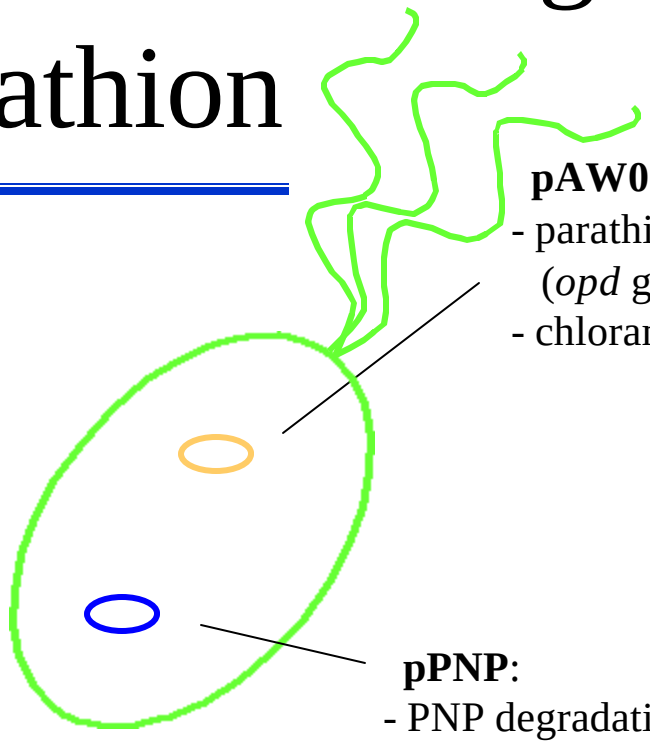
- strains were sequentially applied





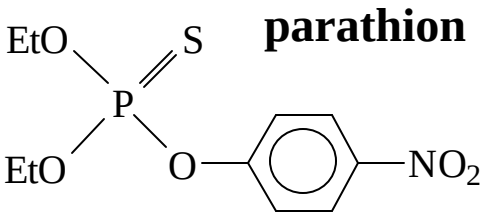
70 degree tilted projection

Engineering a single organism to degrade parathion



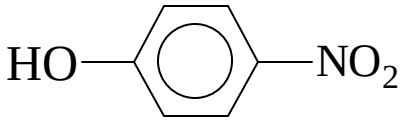
pAW02:
- parathion hydrolysis (*opd* genes)
- chloramphenicol resistance

pPNP:
- PNP degradation
- tetracycline resistance

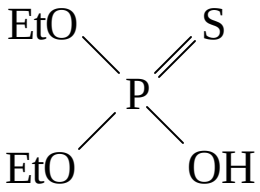


parathion

PNP



DETP



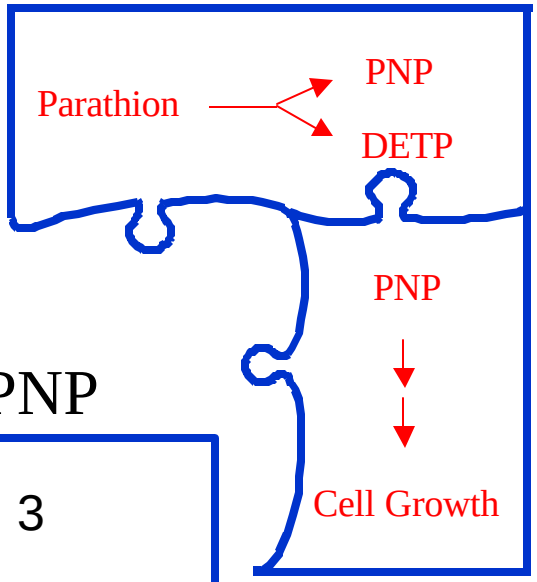
NO_2^-

CO_2 H_2O

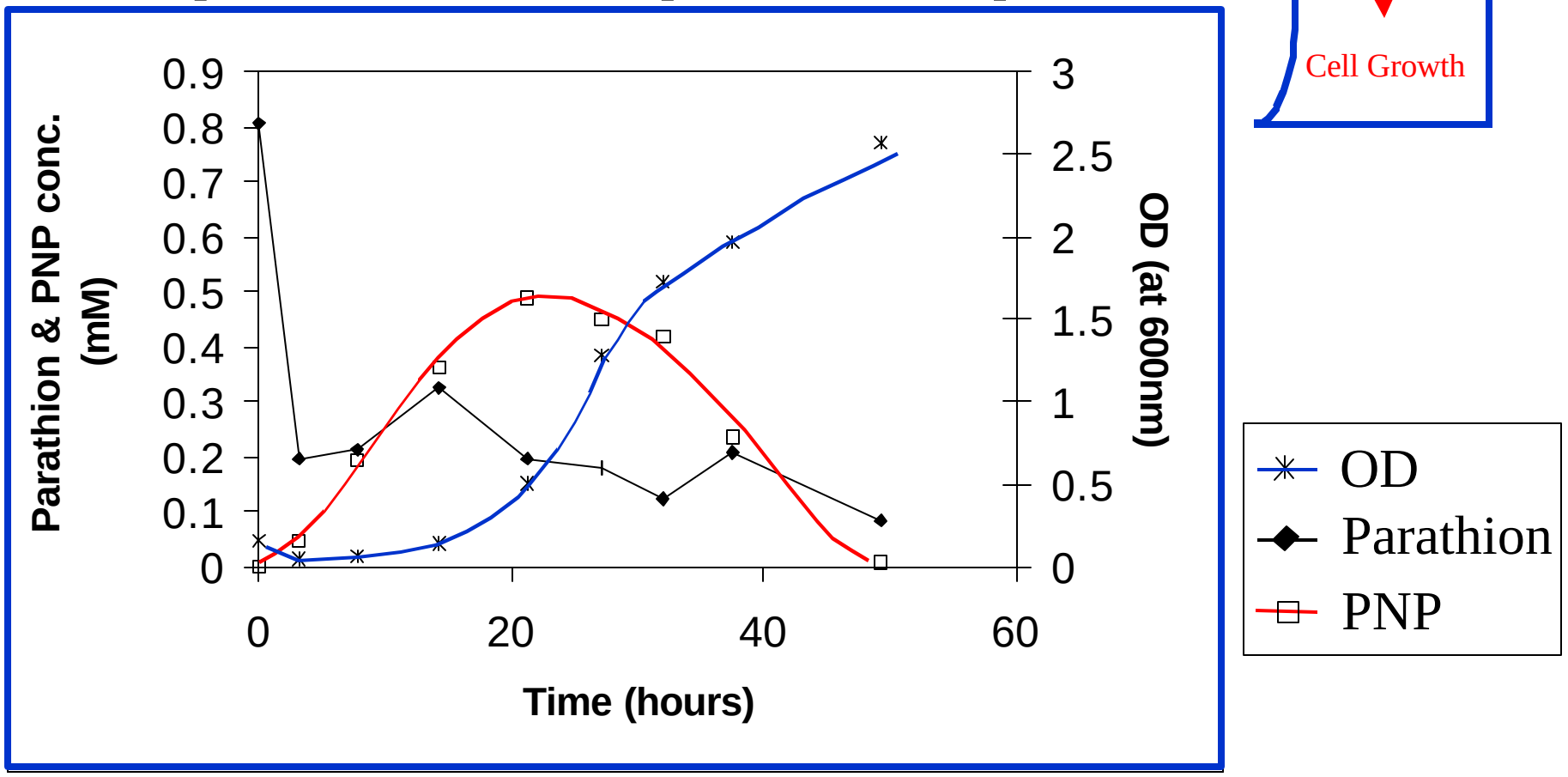
PO_4^-

***Pseudomonas* KT2440**
mineralizes *p*-nitrophenol (PNP)

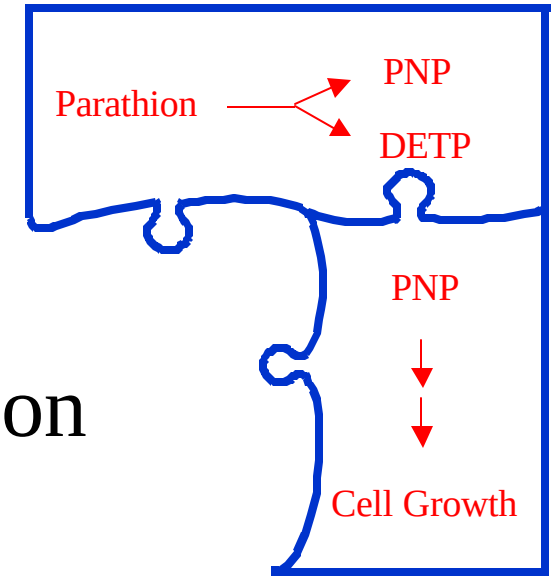
Parathion Utilization as a Carbon Source



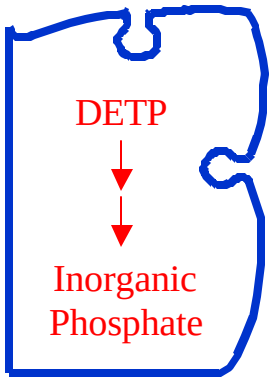
Parathion Degradation by
P. putida KT2442 with pAWW04 and pPNP



Parathion Utilization as a Carbon Source

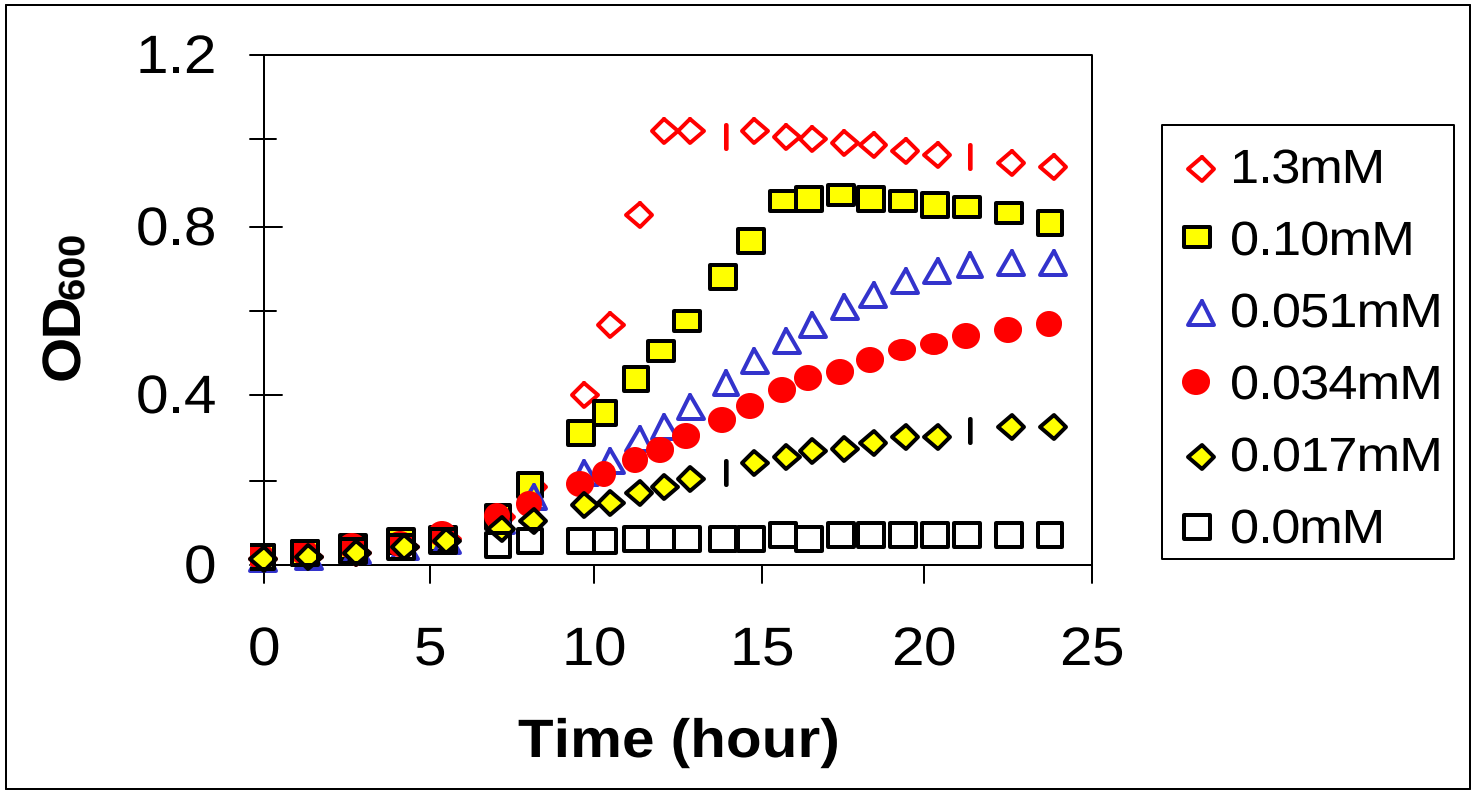


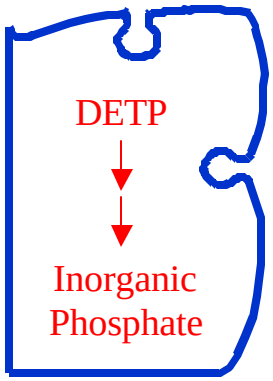
- Parathion is utilized as a carbon and energy source
- Parathion forms DNAPL, but is still bioavailable
- Measurement of aqueous phase parathion concentration is not a good indicator as to whether parathion degradation is occurring



DETP Degradation

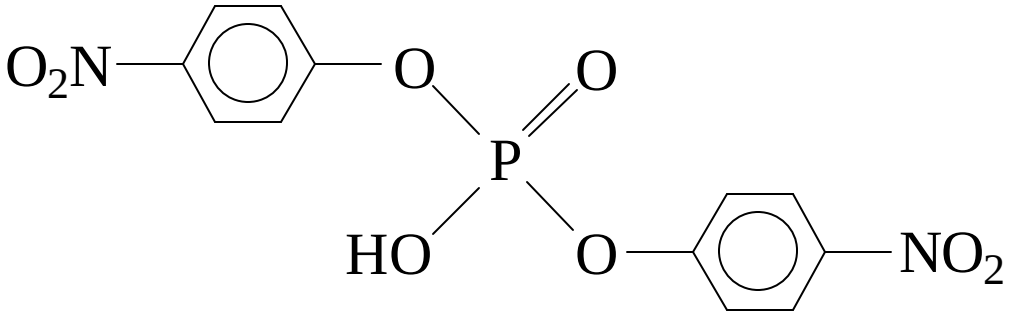
- *Comamonas acidovorans* is capable of utilizing DETP as a P-source:



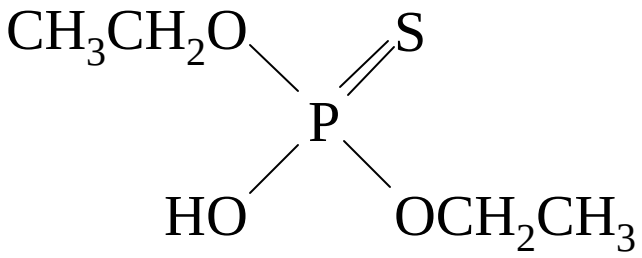


DETP Degradation

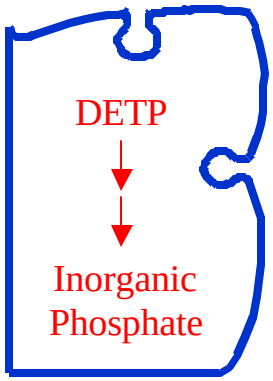
DETP degradation rates were estimated using a DETP analog, bis-(*p*-nitrophenol) phosphate (BNP).



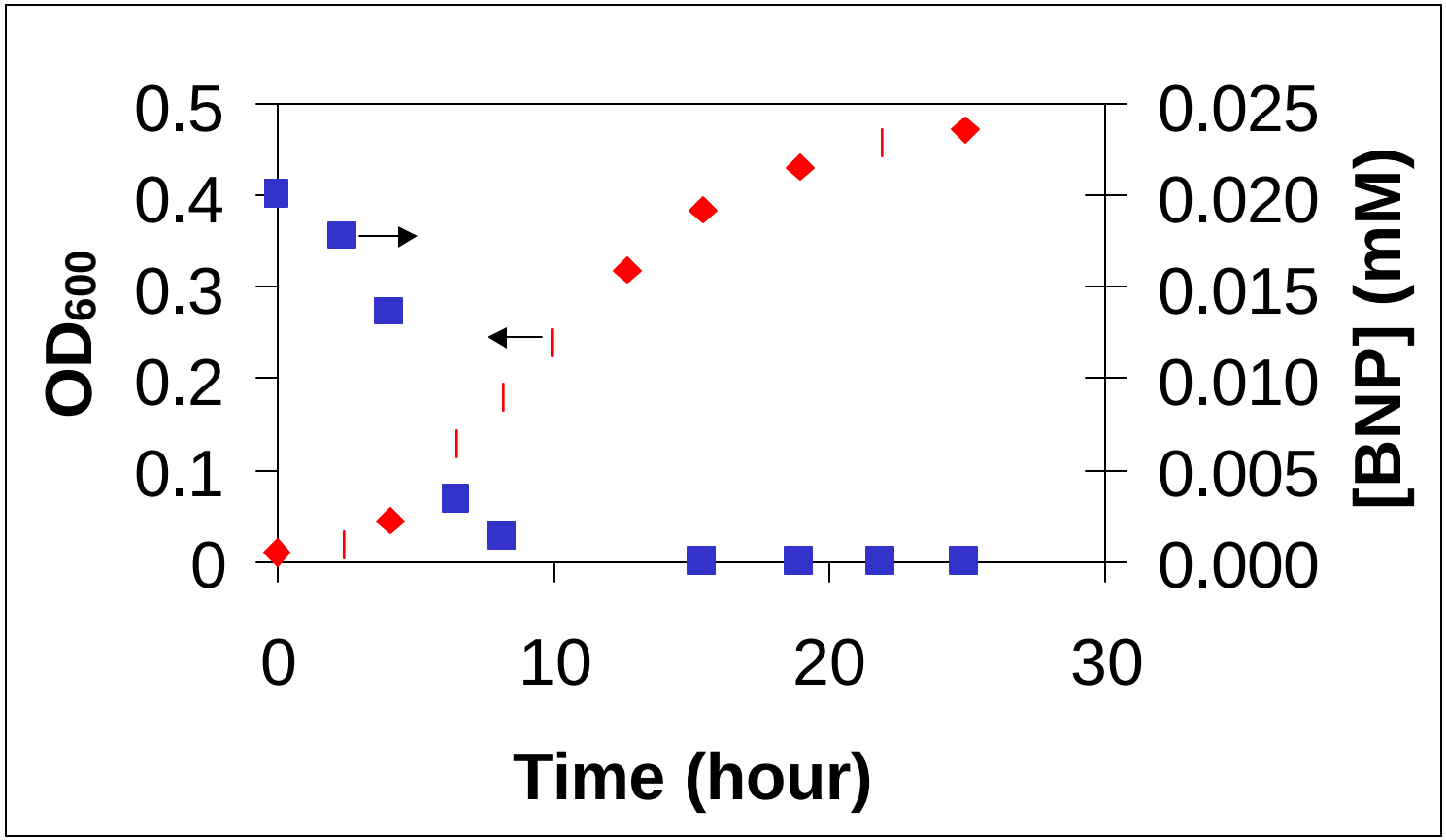
BNP



DETP



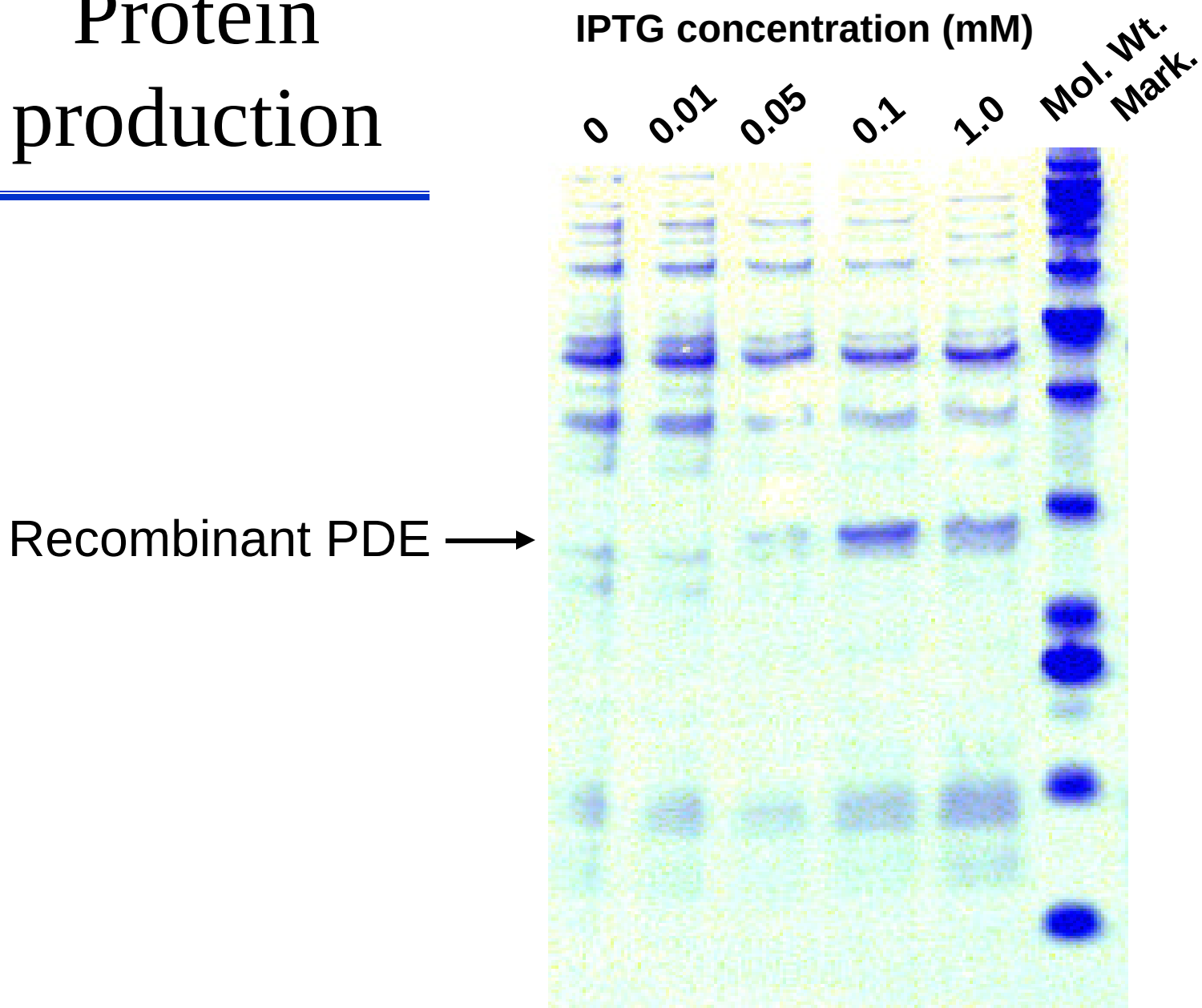
C. acidovorans growth and BNP disappearance



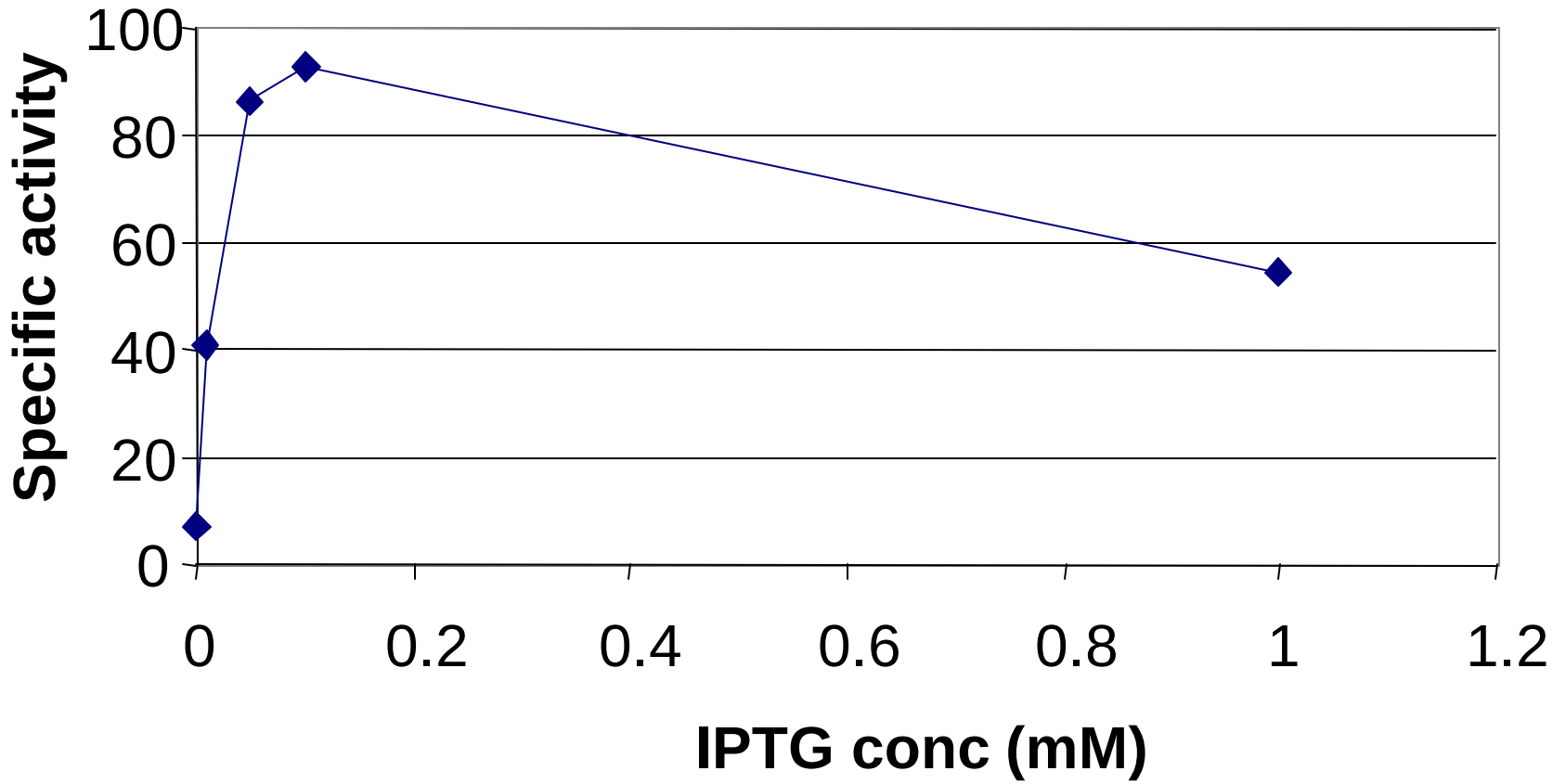
Purification and characterization of phosphodiesterase

- The phosphodiesterase was purified to homogeneity
  - Monomer of 65 KDa
  - Most active toward phosphodiesters, less activity on phosphomonoesters and phosphotriesters
- N-terminal sequenced
- Degenerate primers synthesized
- Gene cloned
  - Low homology to nucleotide phosphodiesterases
- Overexpression in *E. coli* results in high phosphodiesterase activity and growth on diethyl phosphate as a sole phosphate source

Protein production

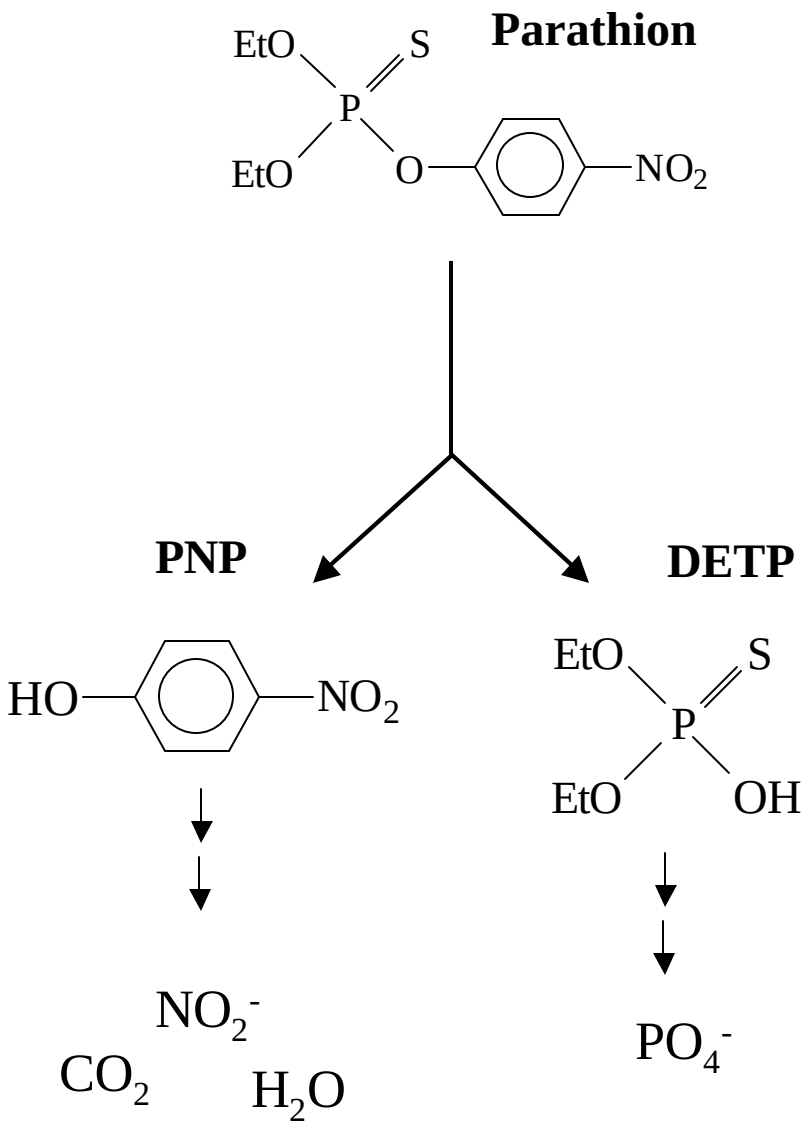


Induction studies



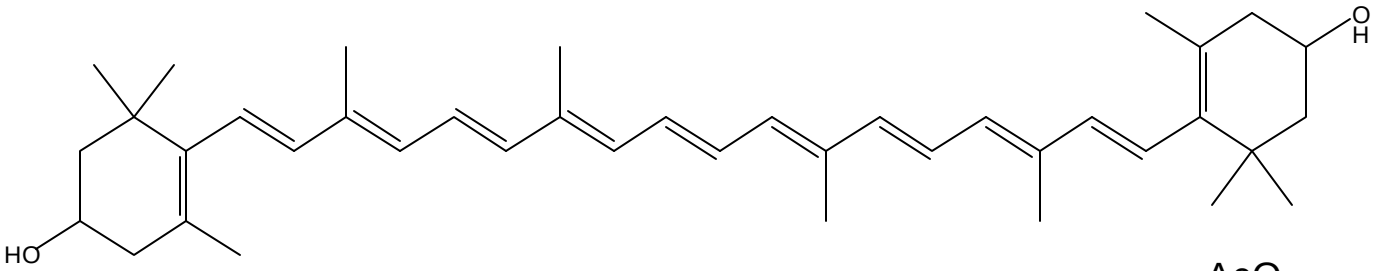
What's left?

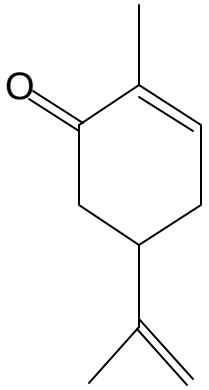
- Combine all genes for complete mineralization of paraoxon.
- Identify, purify, and characterize the gene encoding the enzyme that catalyzes P=S to P=O.
- Combine all genes into a single organism for parathion degradation.

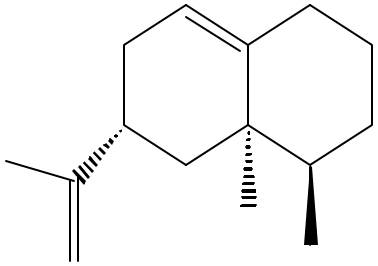


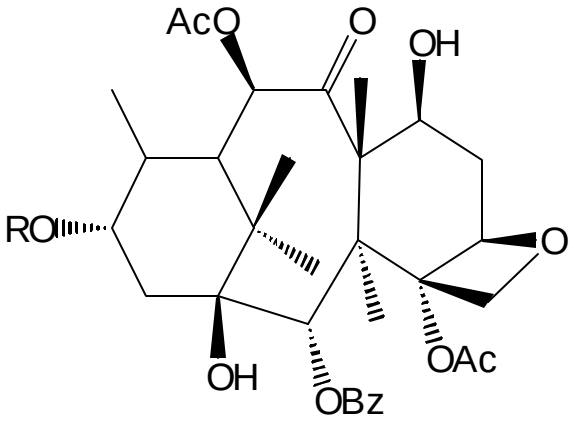
Isoprenoids

- Extremely diverse family of compounds
- Includes carotenoids and terpenoids









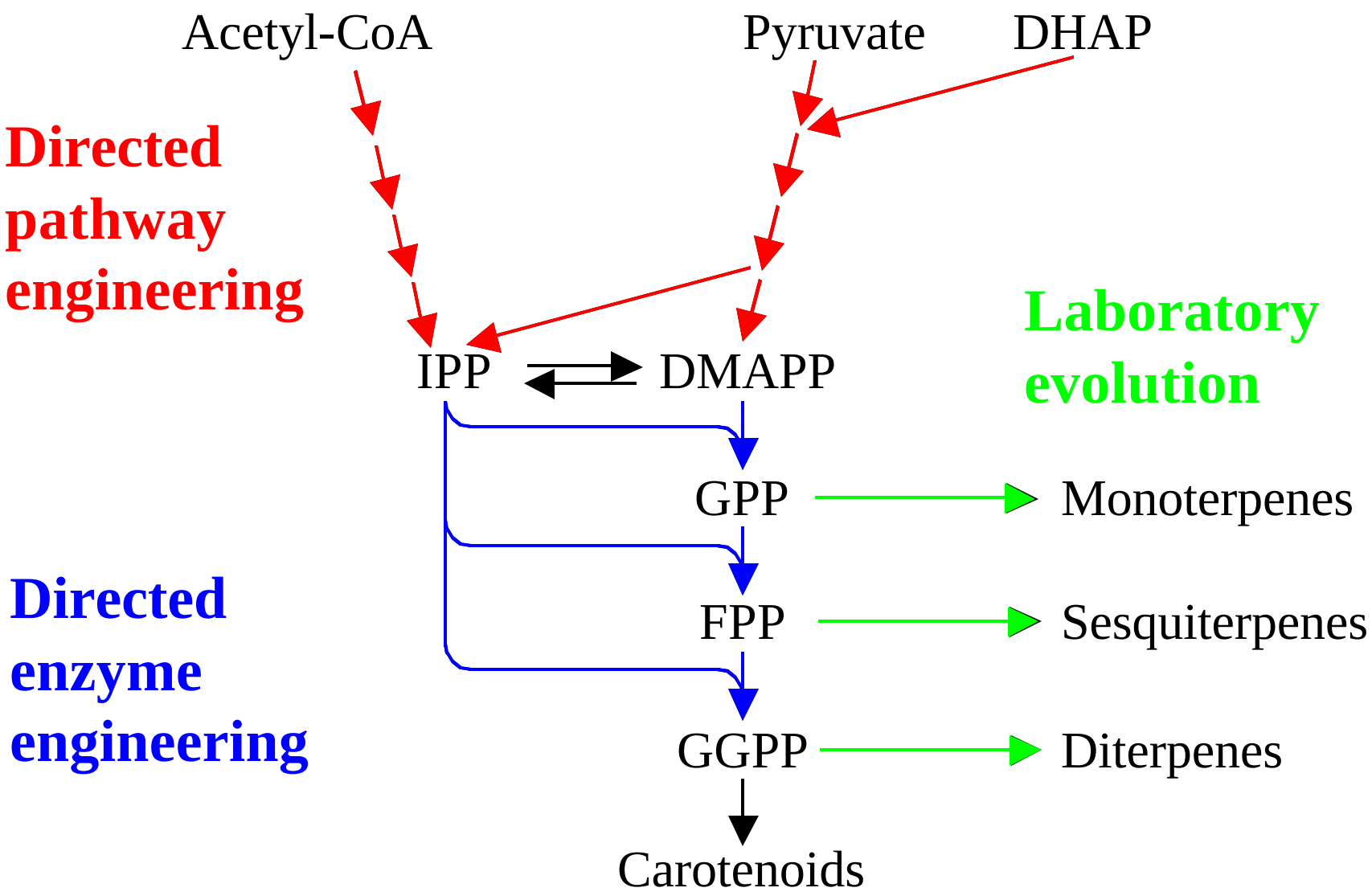
R = N-benzoyl-3-phenylisoserine

Synthesis of Isoprenoids

Goal -

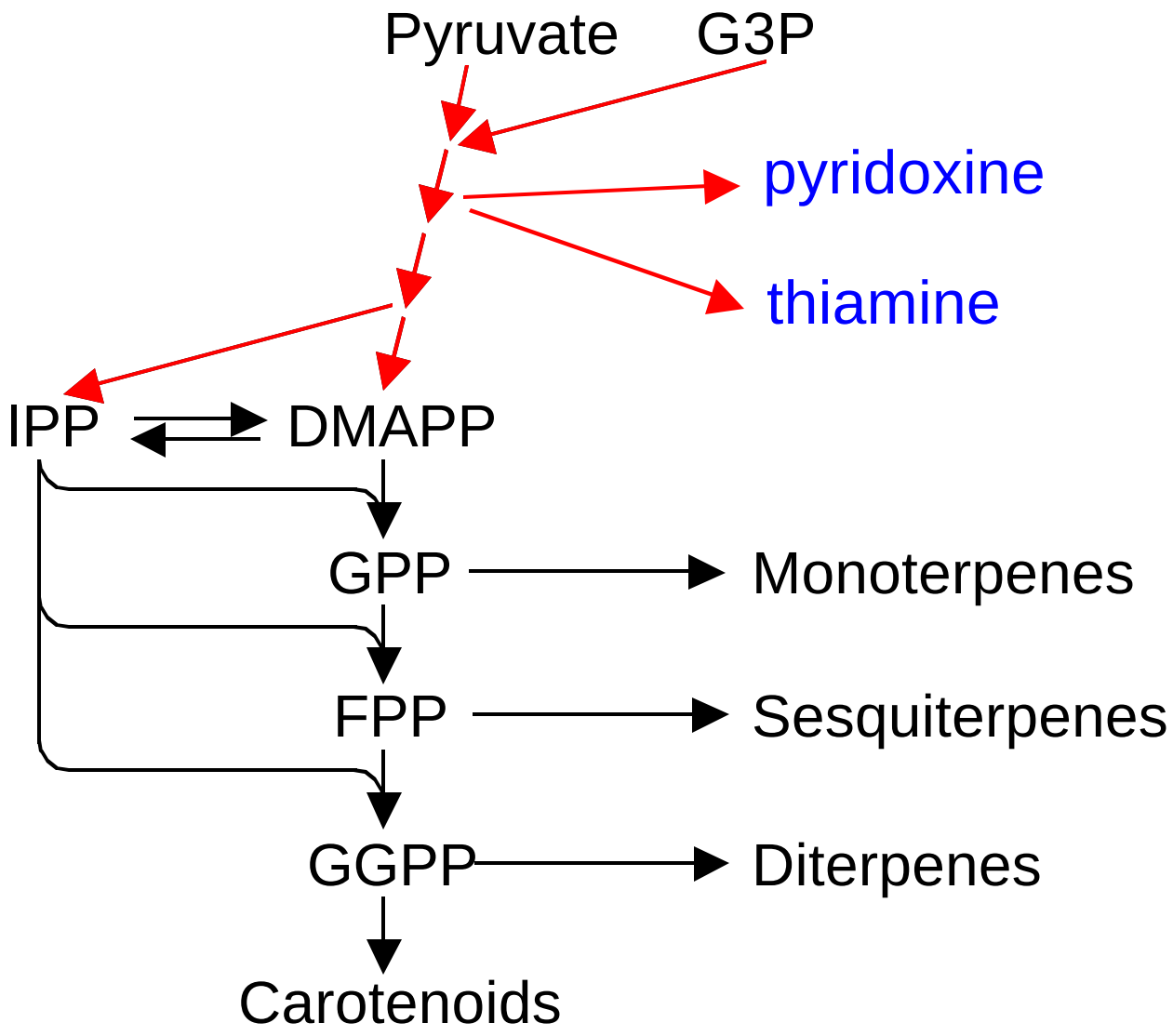
- to engineer the isoprenoid precursor pathways for enhanced production
- to introduce into *E. coli* the genes for carotenoid and terpenoid synthesis
- to evolve terpene cyclase genes

A multi-faceted approach

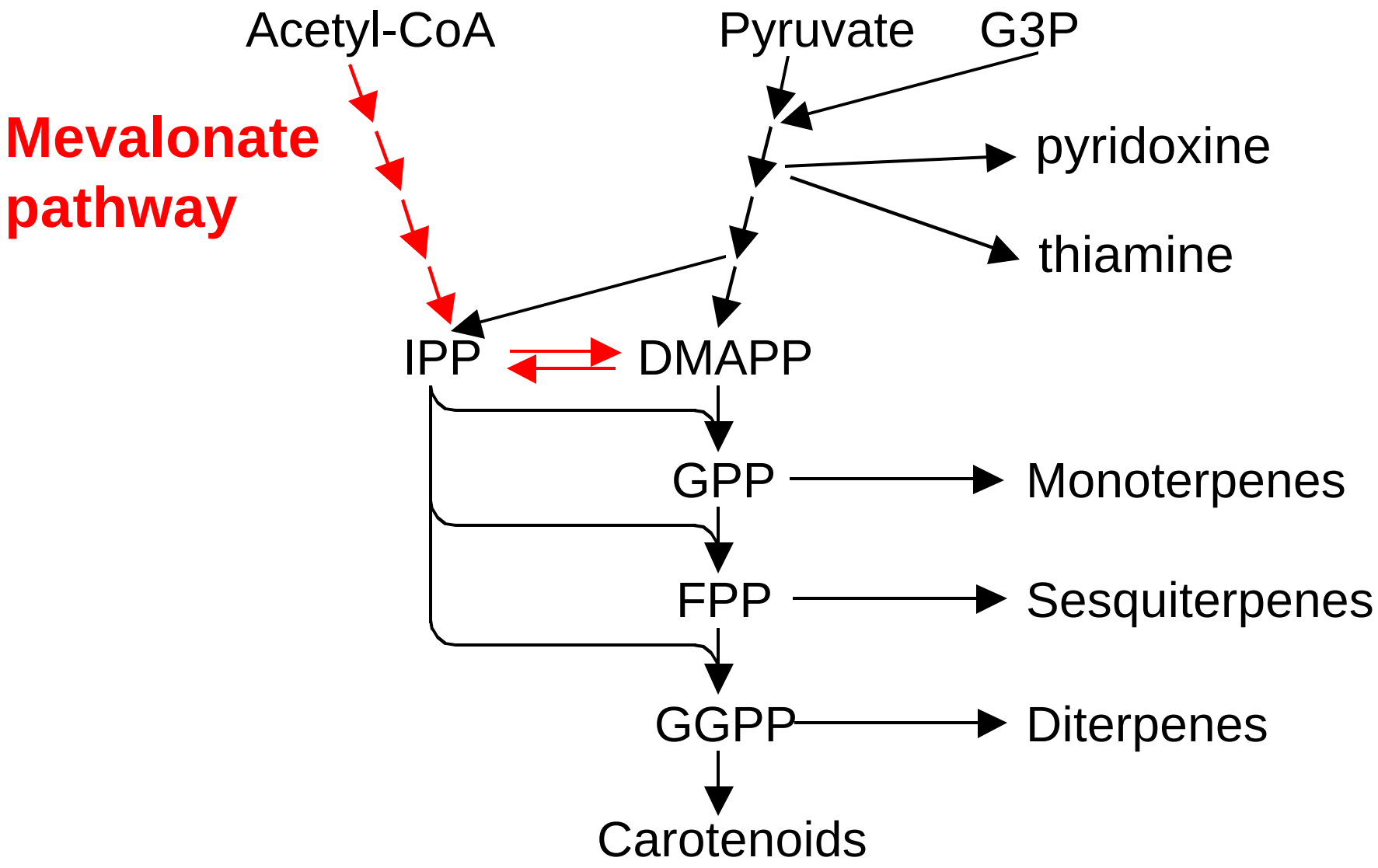


Engineering precursor production

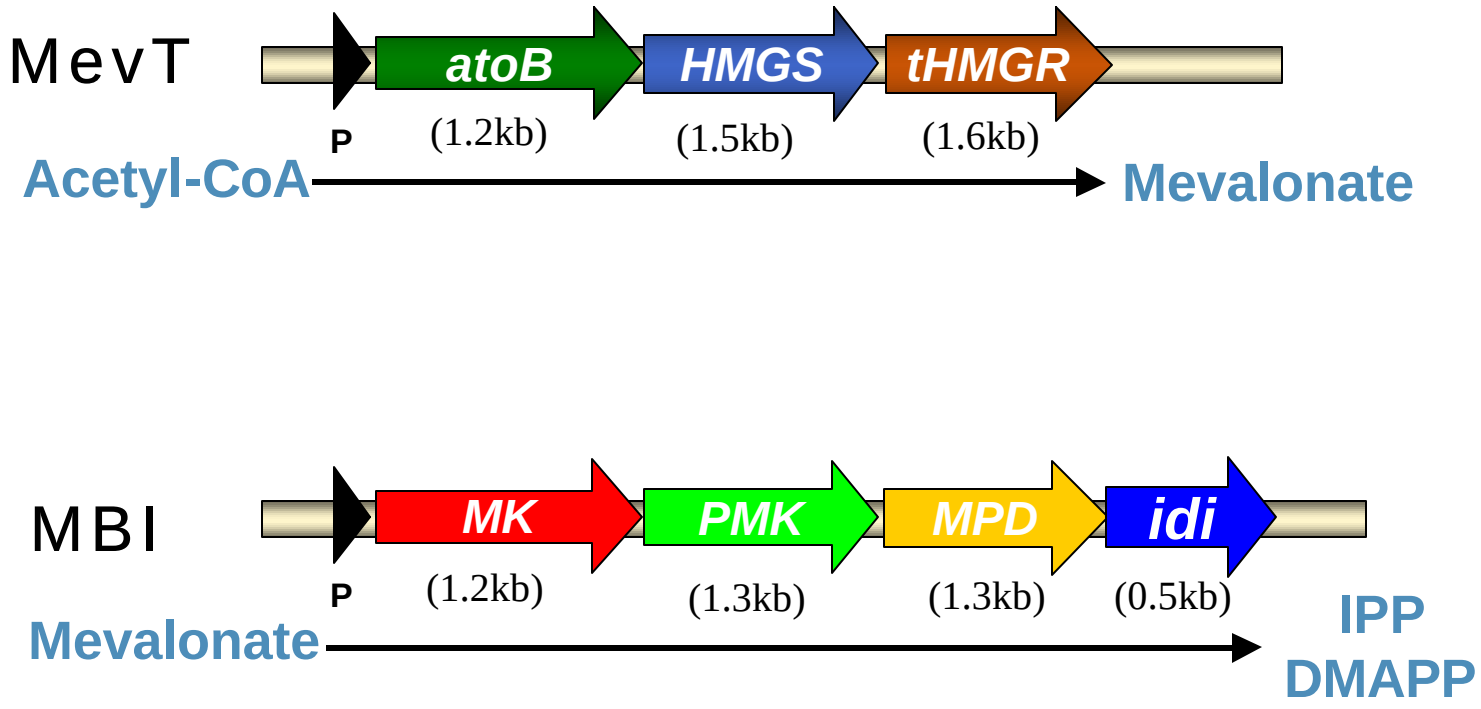
DXP Pathway



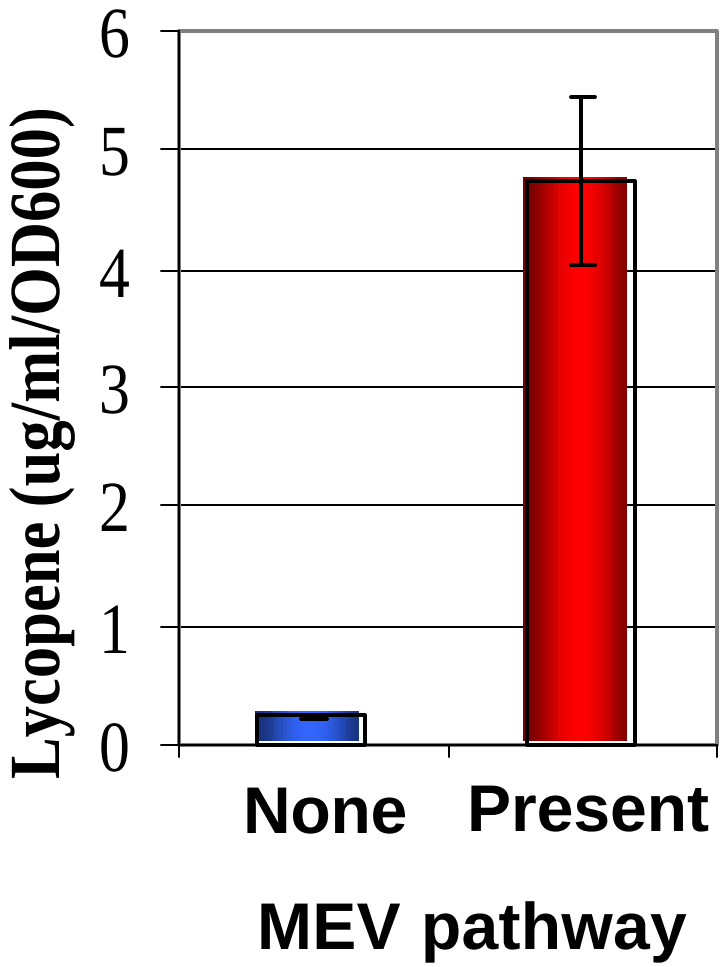
Engineering precursor production

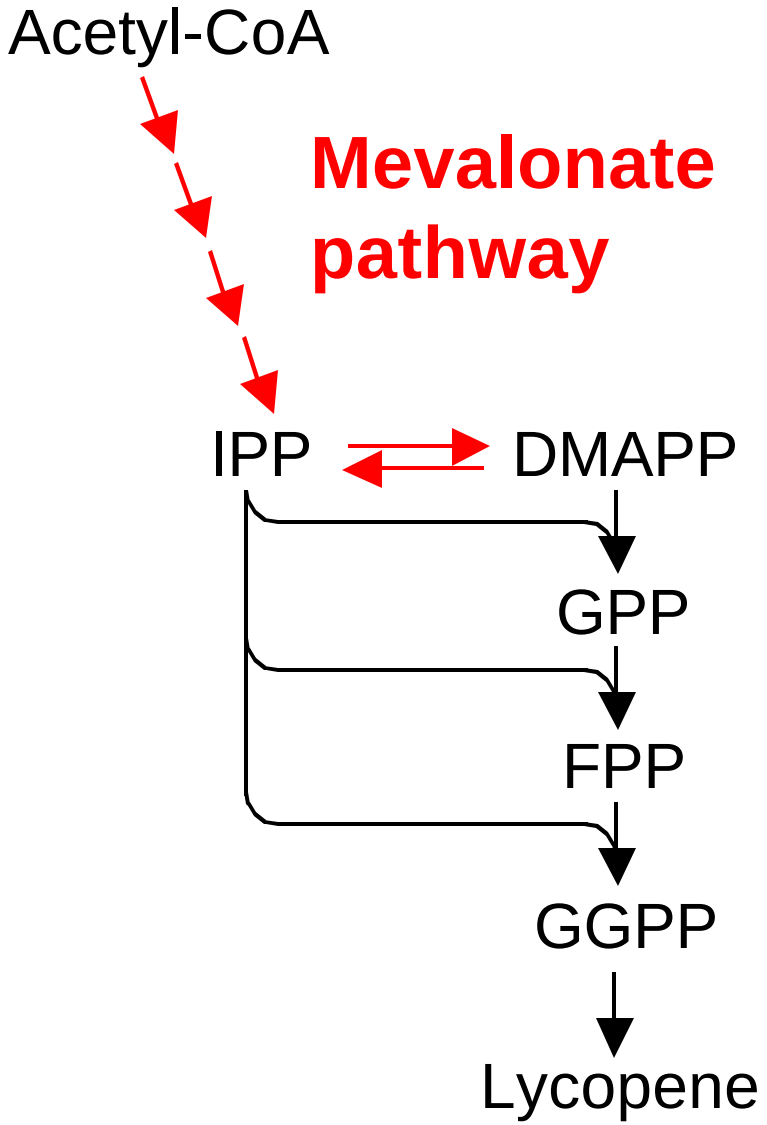


Construction of synthetic mevalonate pathway operons

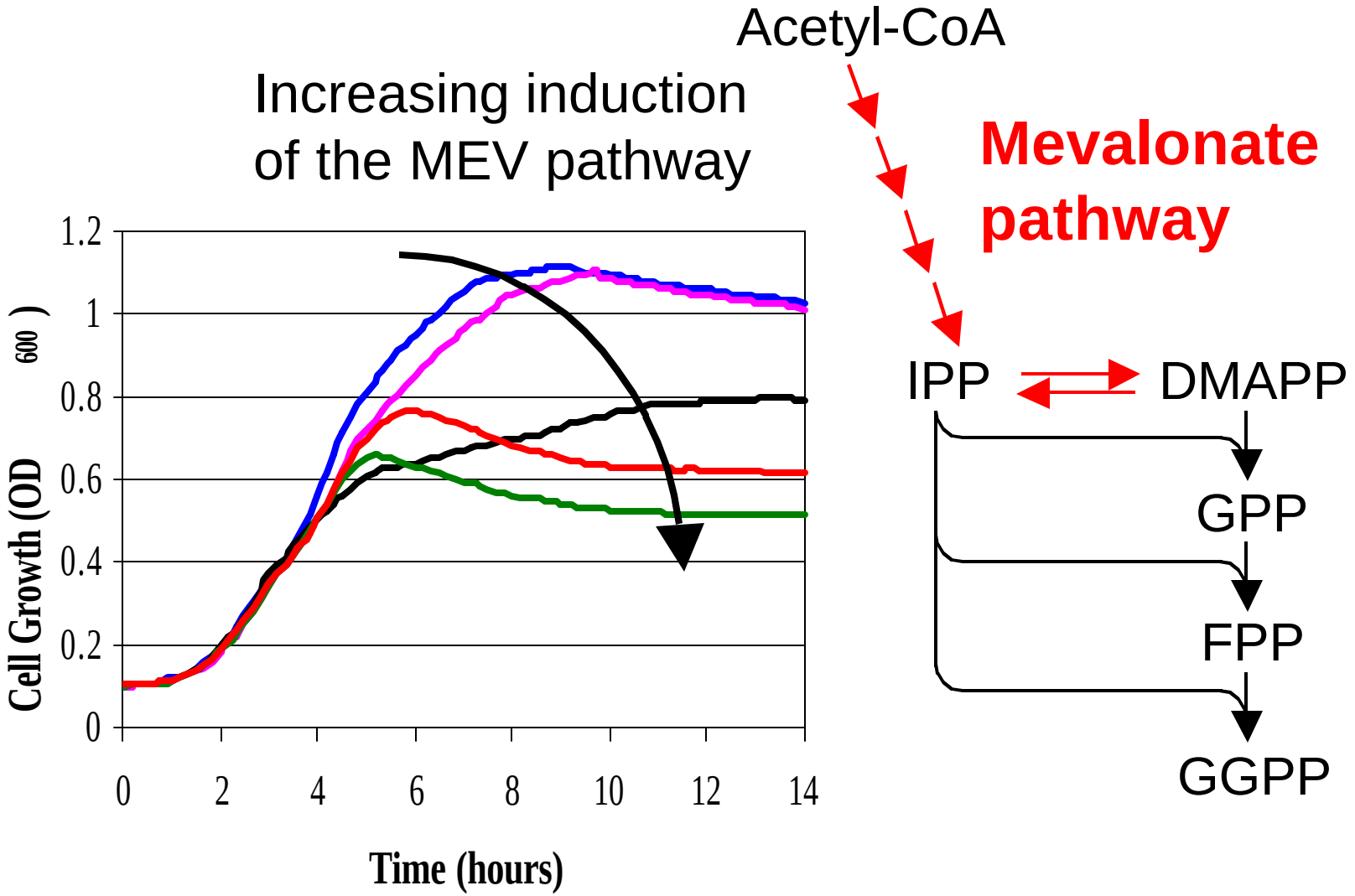


Engineering precursor production

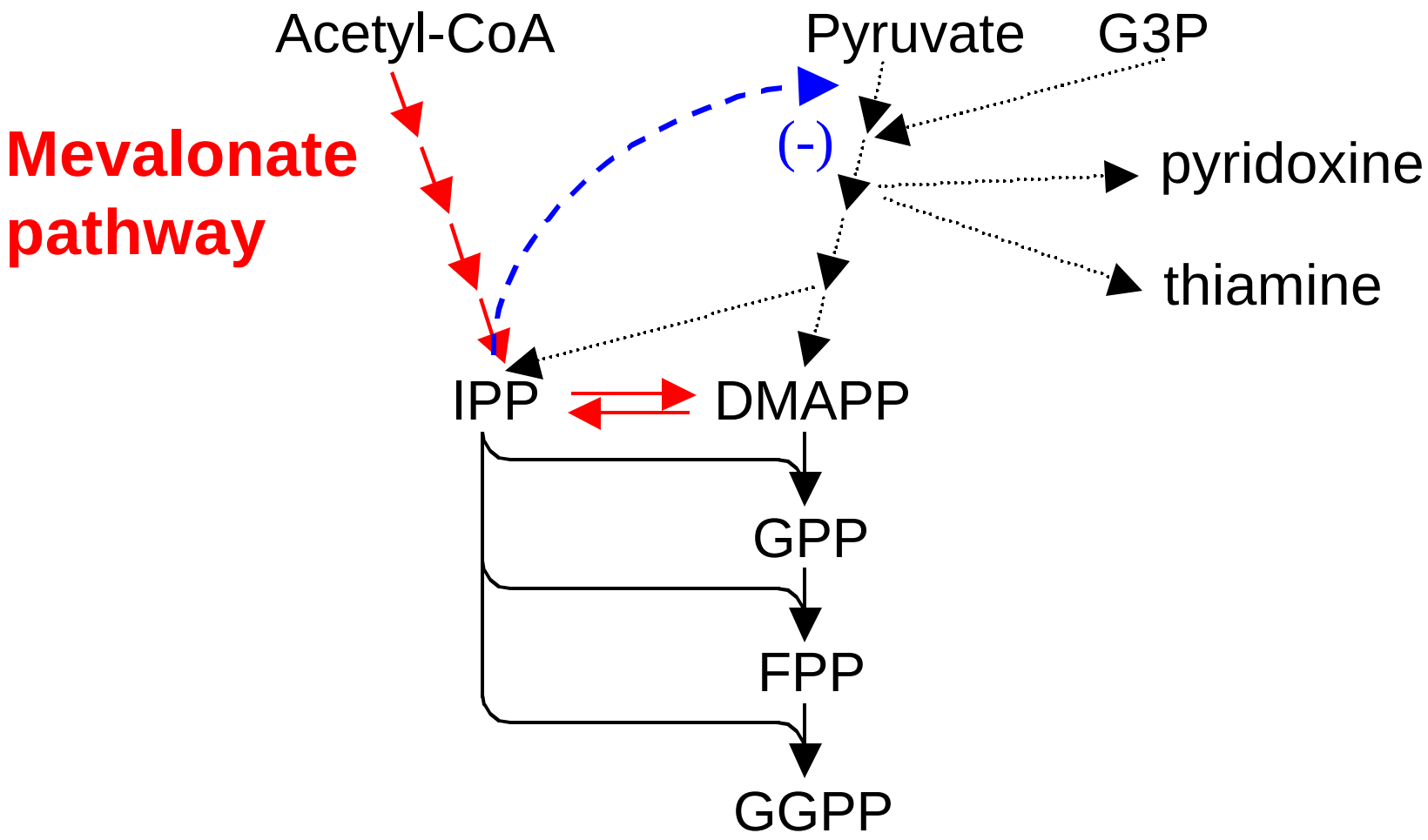




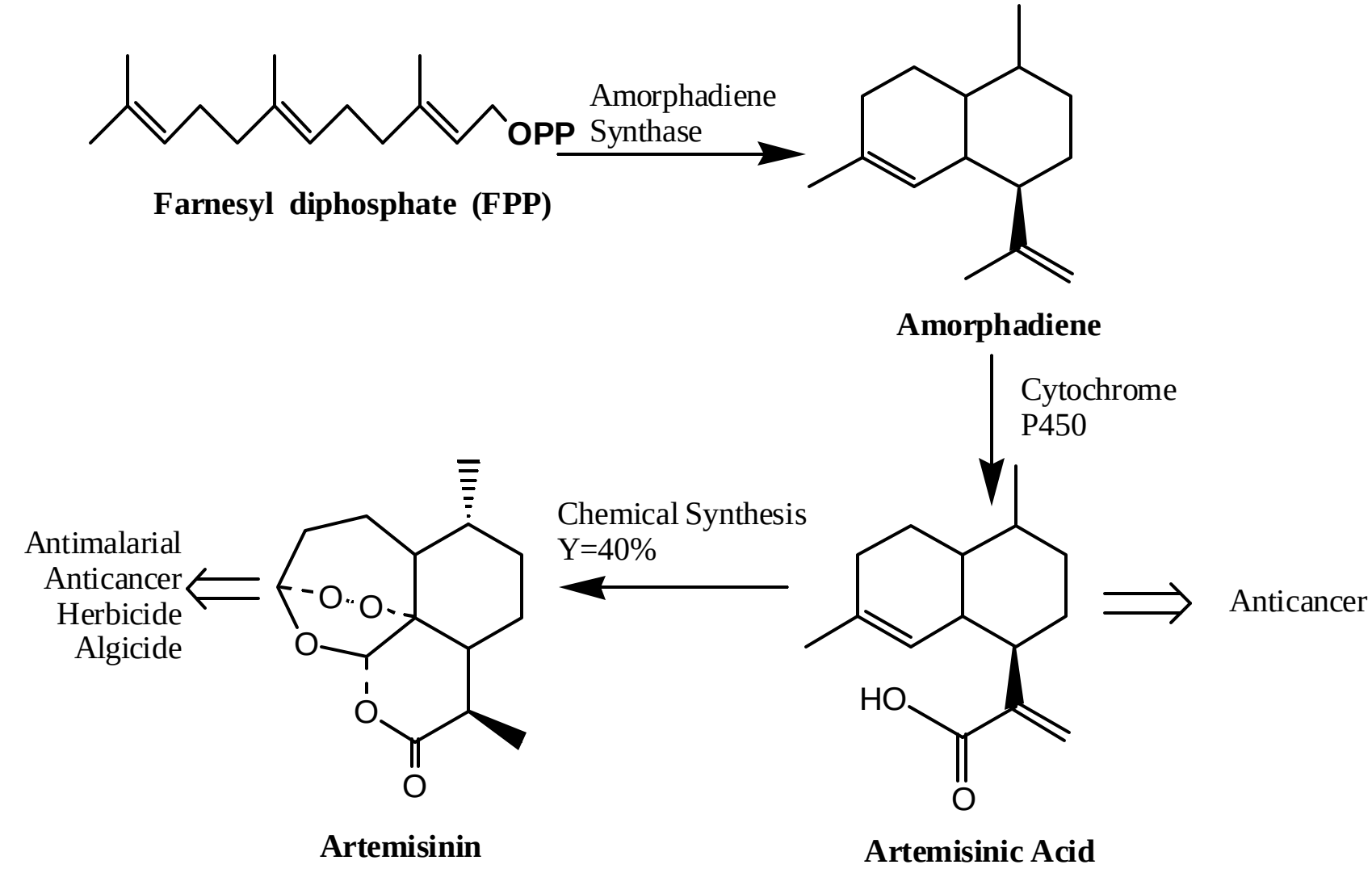
Engineering precursor production



Engineering precursor production

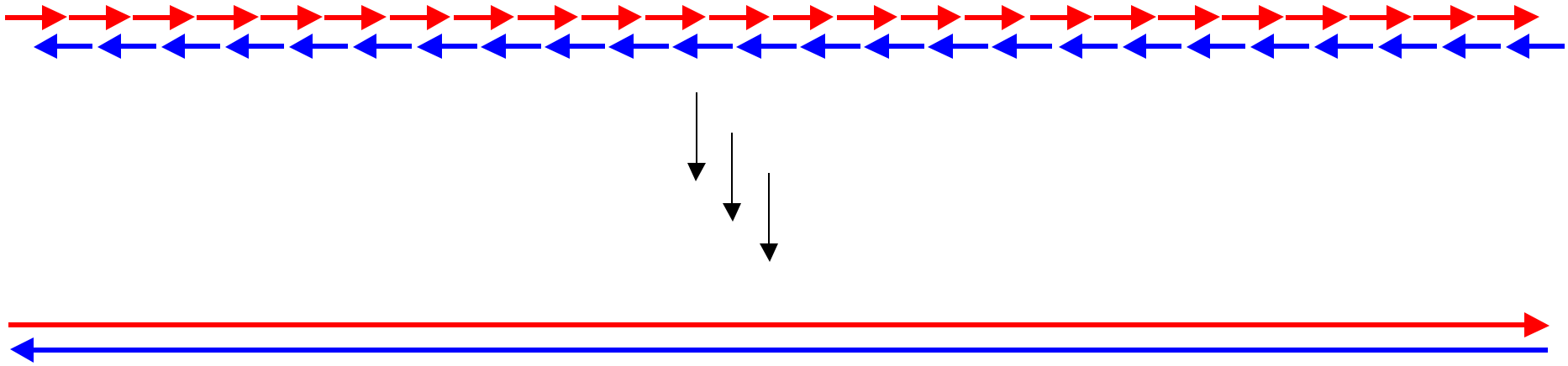


Amorphadiene and artemisinin biosynthetic pathway

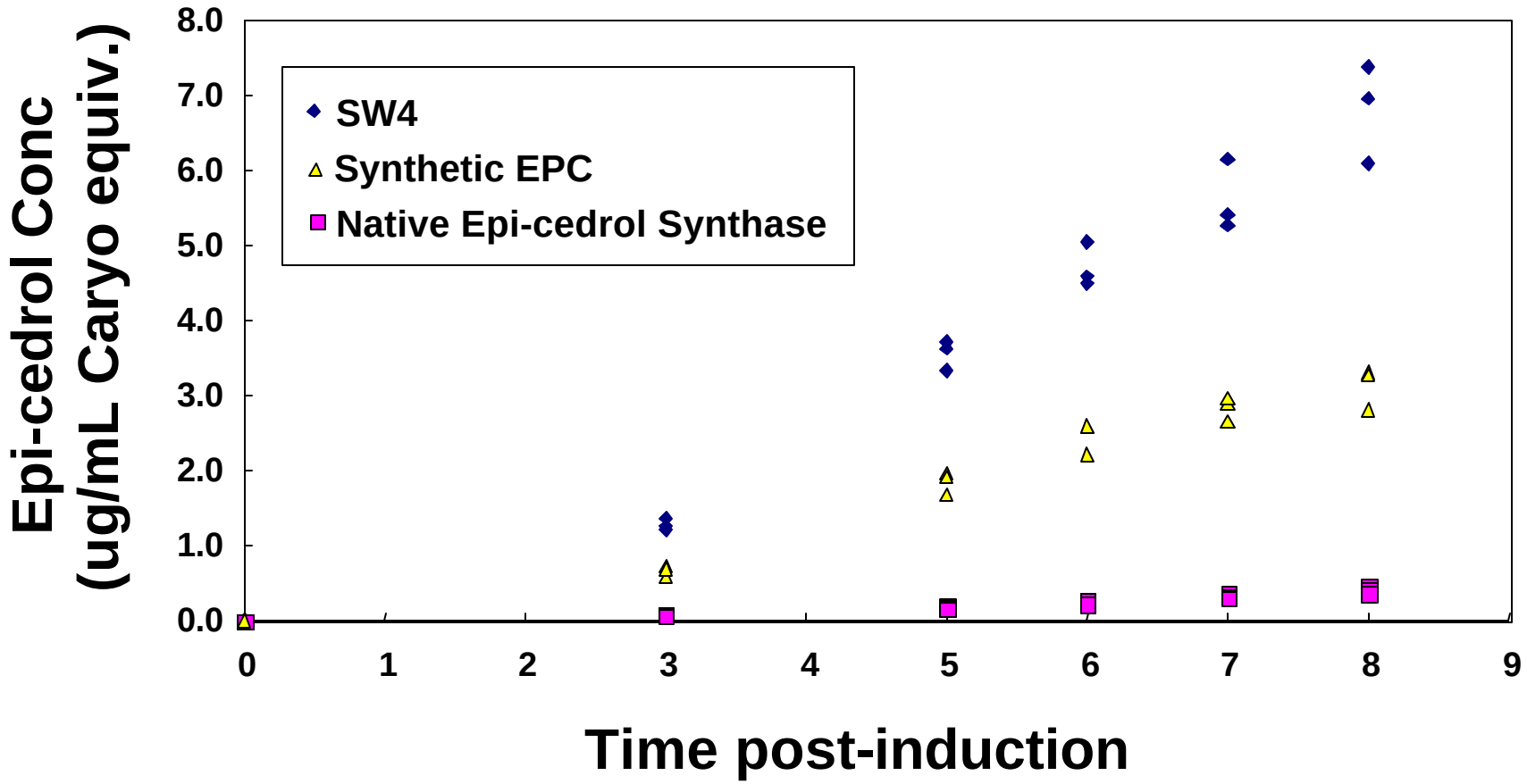


Assembly of rcAmorphadiene Cyclase

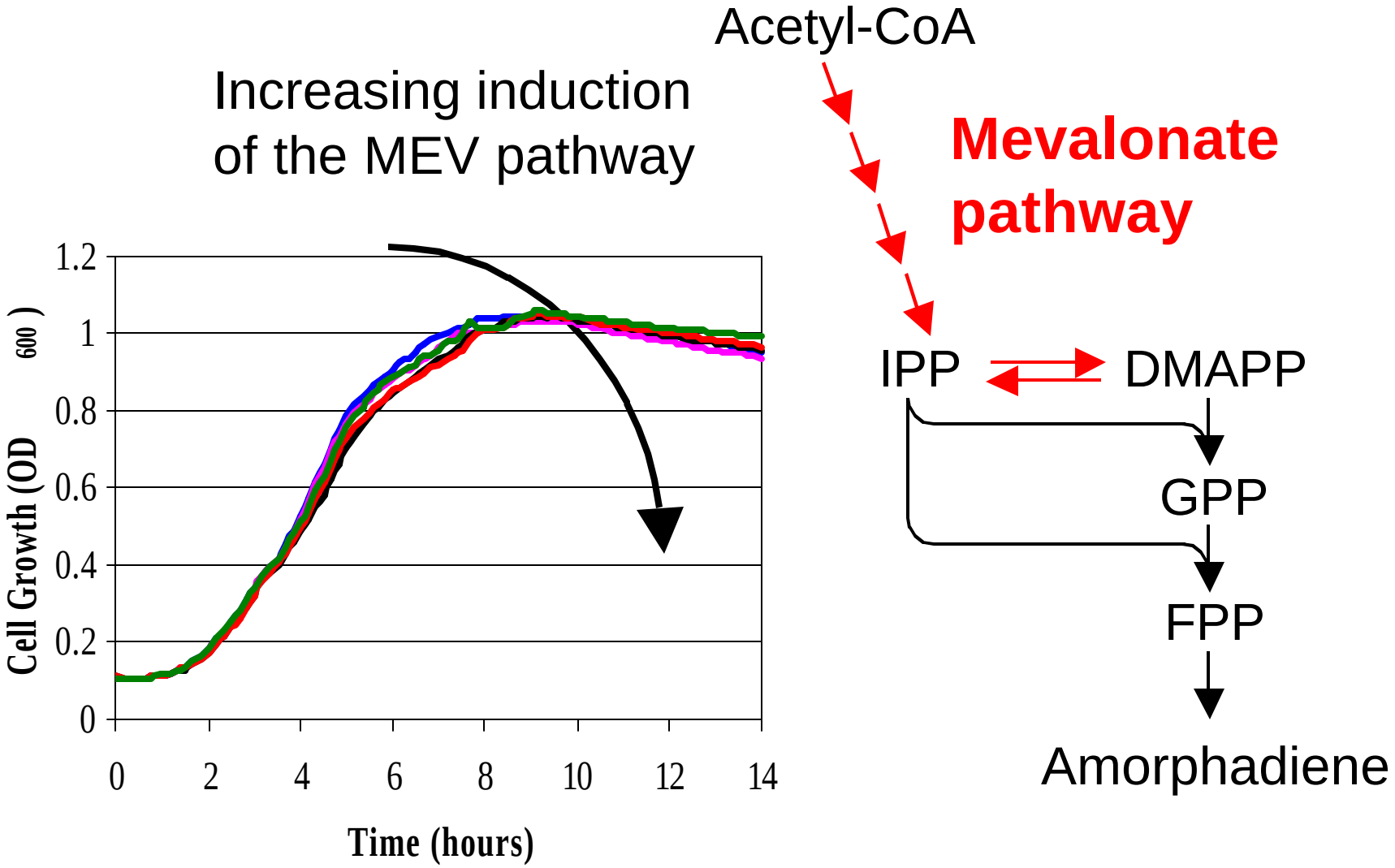
- Take gene sequence from patent
- Optimize sequence for expression in desired host
- Synthesize 84 oligonucleotides of ~40 basepairs each
- Assemble into complete gene using the polymerase chain reaction (PCR)



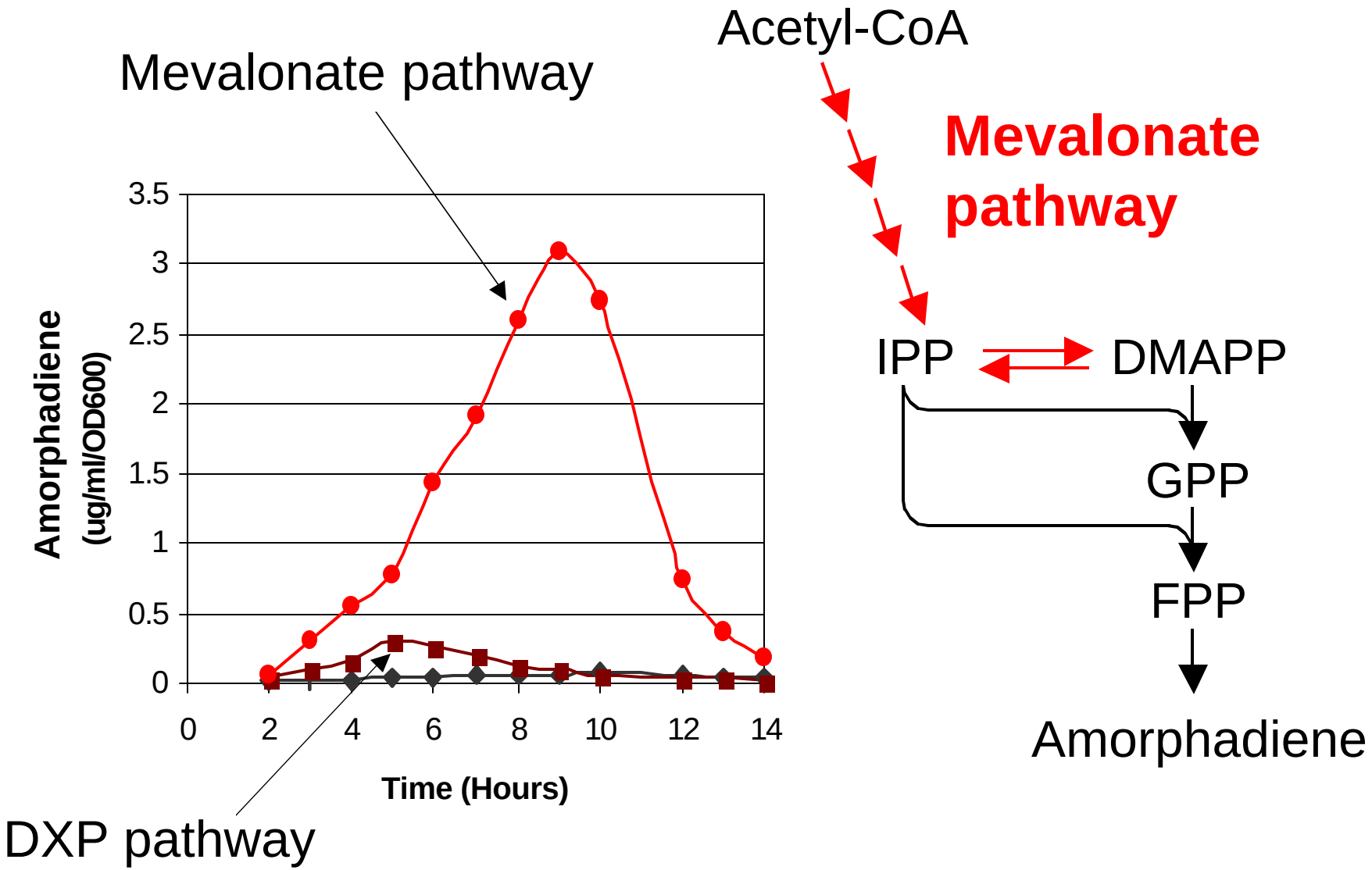
Screen clones by GC-MS



Engineering precursor production



Engineering precursor production

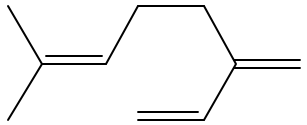


Expression of plant mono- sesqui- and diterpenes cyclases in *E. coli*

GPP ➡Monoterpene

Myrcene synthase

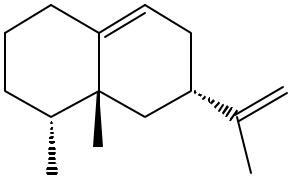
Arabidopsis thaliana



FPP ➡ Sesquiterpenes

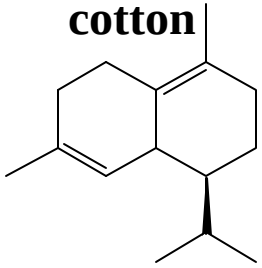
5-*epi*-aristolochene

Tobacco



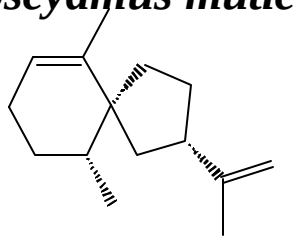
d-cadinene

cotton



Vetispiradiene

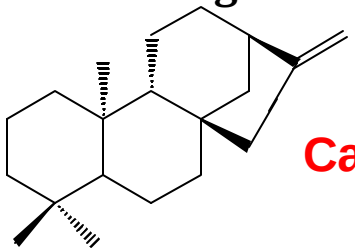
Hyoscyamus muticus



GGPP ➡ Diterpene

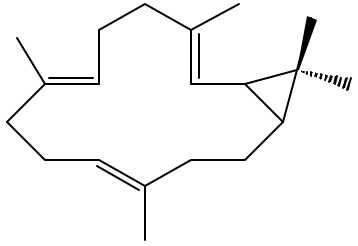
***ent*-Kaurene cyclase**

fungi



Casbene cyclase

Castor bean



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