Metabolic Engineering of Microorganisms

Degradation of Organophosphate Contaminants

Synthesis of Isoprenoids

Coolfont

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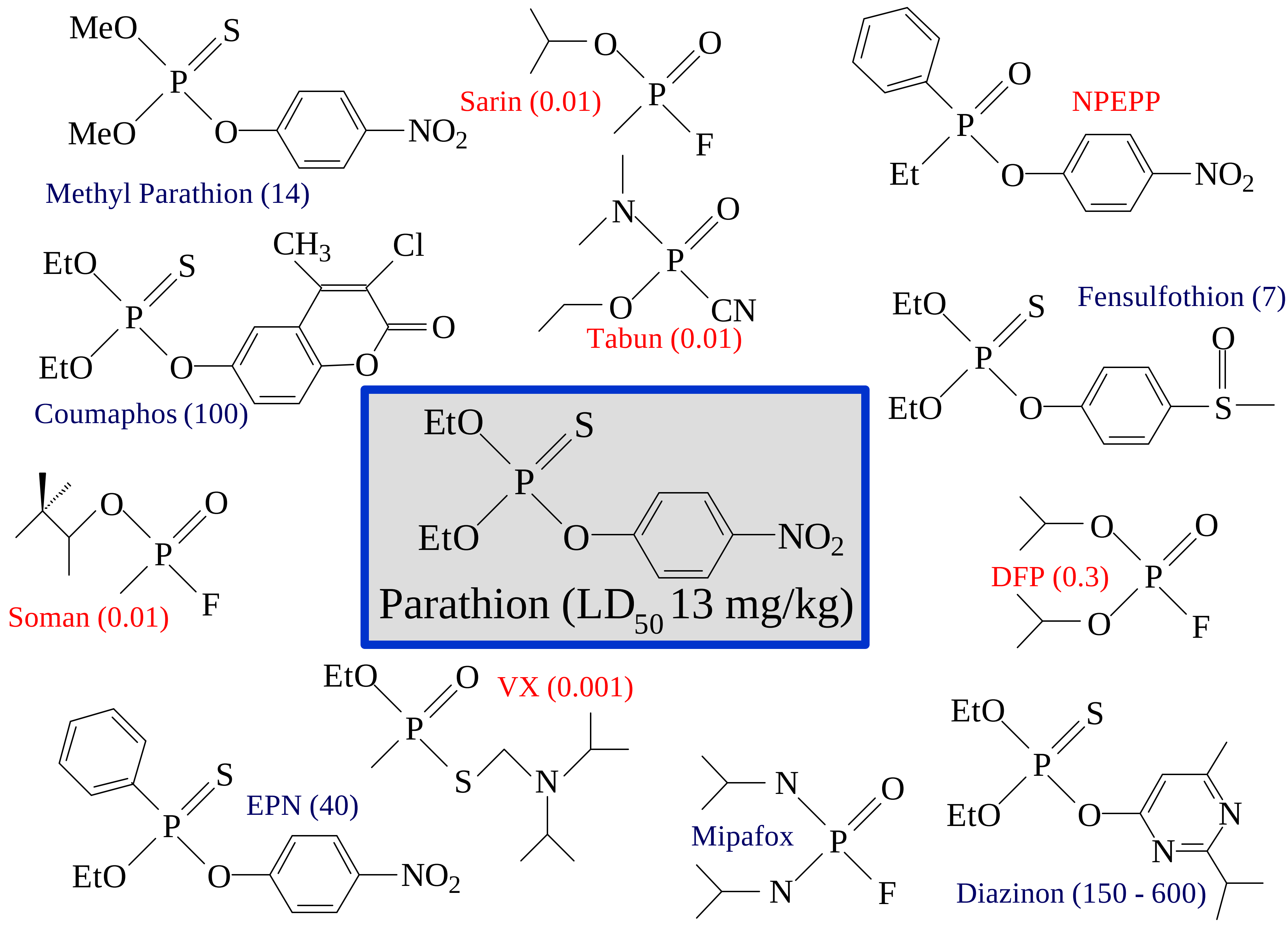
Degradation of organophosphates

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 (LD50 13 mg/kg)
Soman (0.01)
DFP (0.3)
VX (0.001)
EPN (40)
Mipafox
Diazinon (150 - 600)

Justification

Pesticides

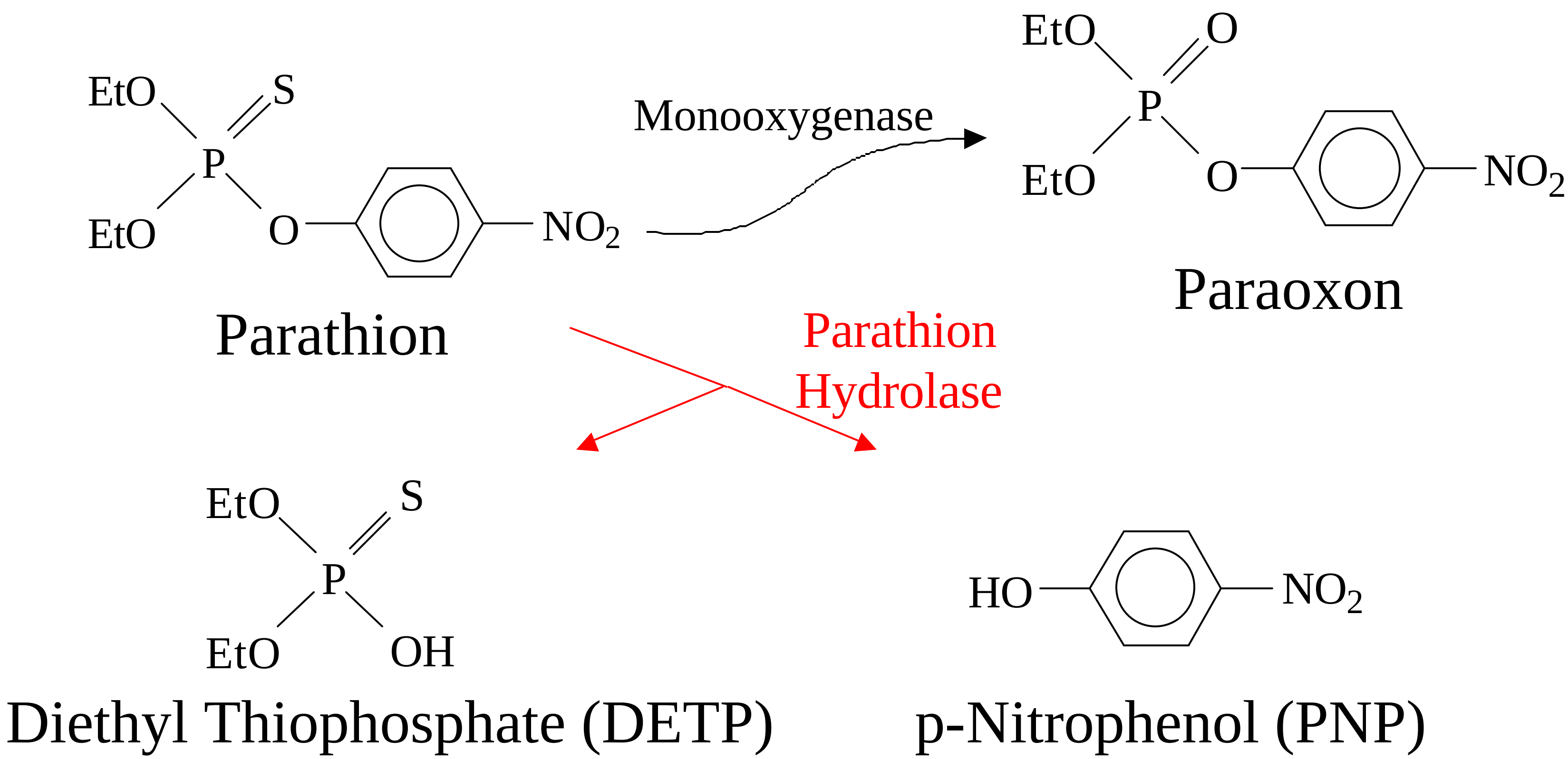
- ~ 60,000 tons of organophosphate pesticides are produced annually in the US
- U.S. Geological Survey reported 54.4% of groundwater sites sampled were contaminated with pesticides (1998)

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

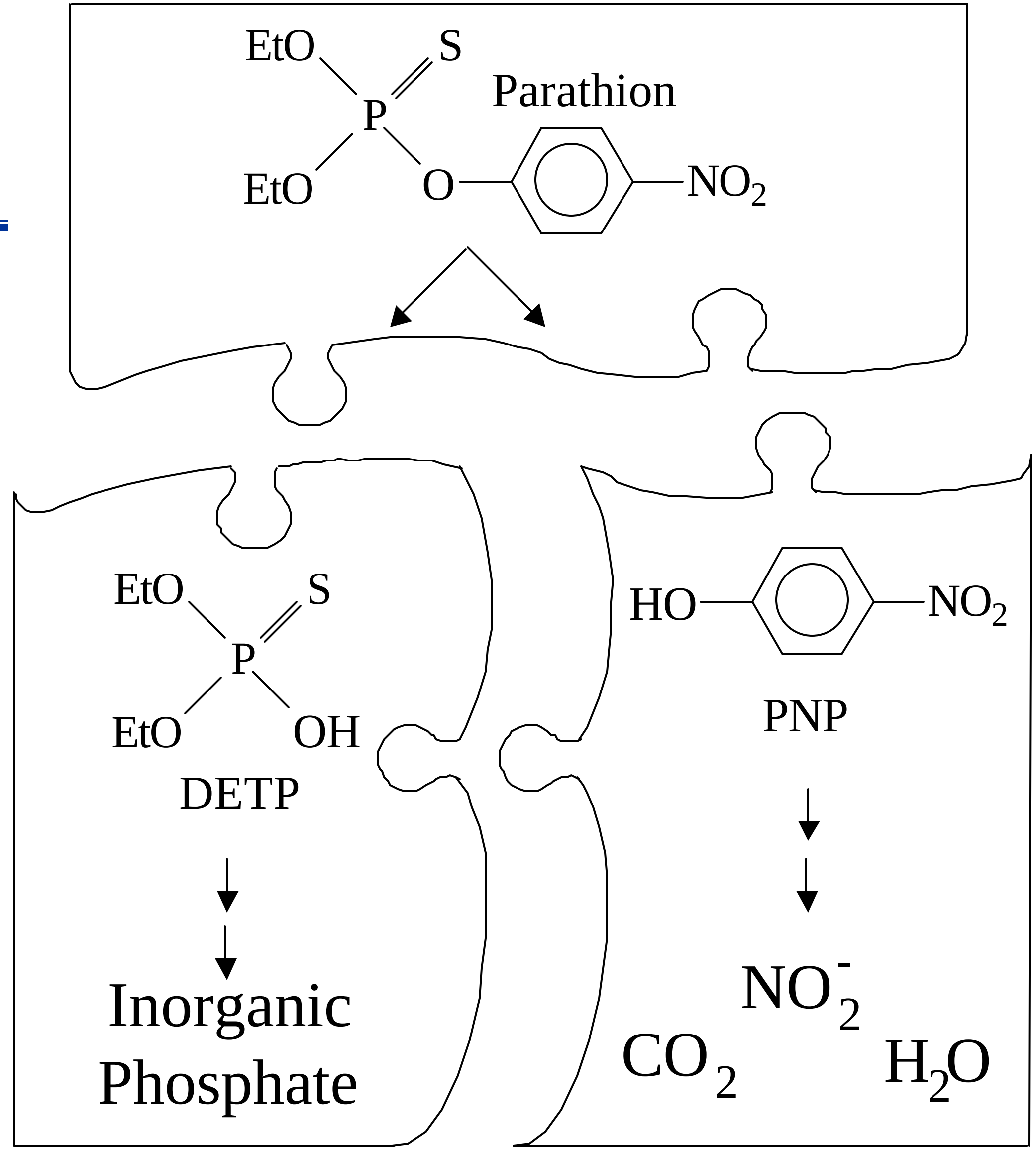
Parathion Degradation Background

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

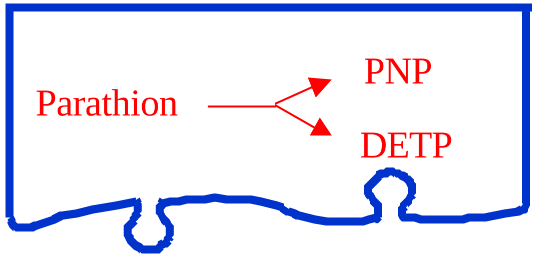


Parathion Degradation

A 3 piece puzzle:



Parathion Hydrolysis

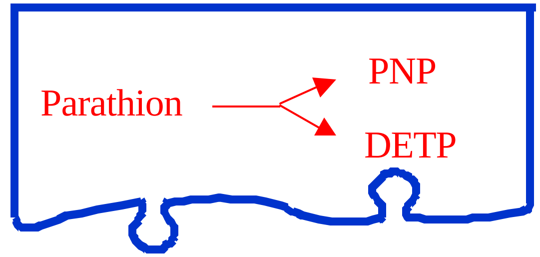


- Past work on parathion degradation has focused on initial hydrolysis
- Gene coding for parathion hydrolase (*opd*) has been cloned & sequenced from both *Pseudomonas* and *Flavobacterium*
- Two forms of *opd*:

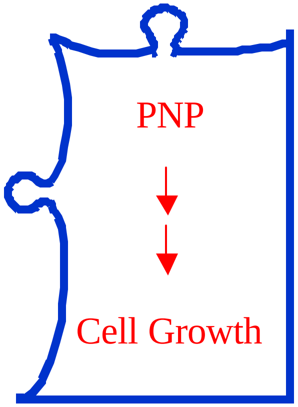
Native – contains coding region for N-terminal leader sequence

"Modified" – coding region for leader sequenced removed

Parathion Hydrolysis

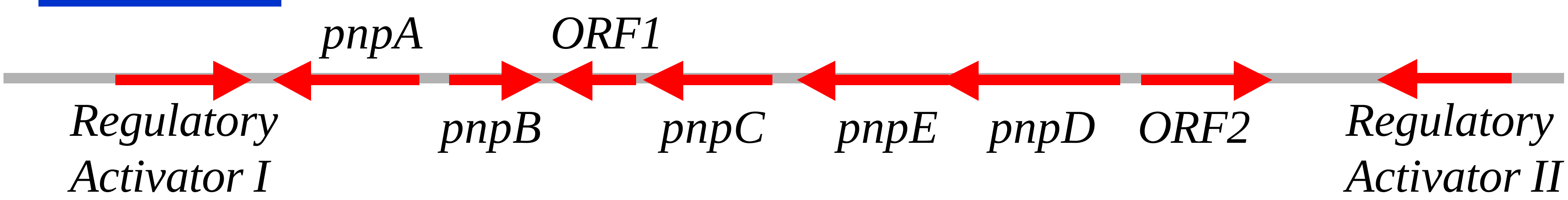


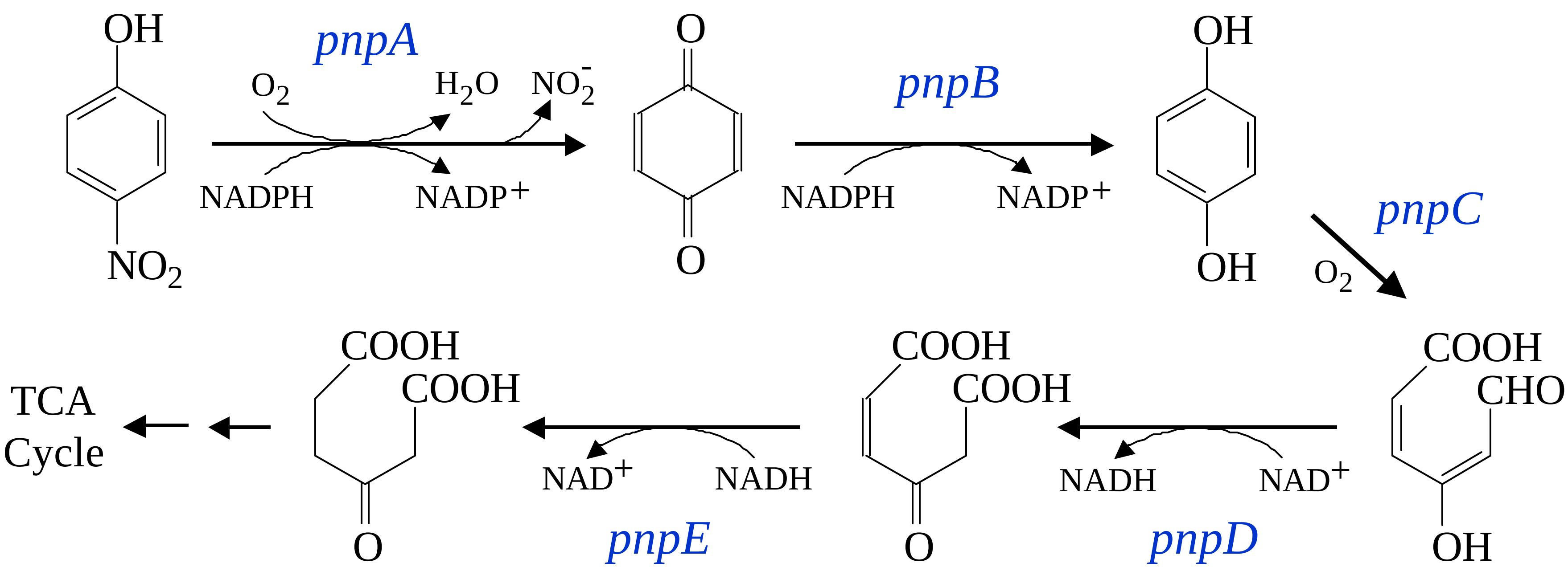
	pAWW01	pAWW02	pAWW04
Plasmid:	pAWW01	pAWW02	pAWW04
Promoter:	P_{taclac}	P_{taclac}	P_{tac}
opd gene type:	“modified”	native	native
	Spec. Activity (µM/hour-OD)	Spec. Activity (µM/hour-OD)	Spec. Activity (µM/hour-OD)
E. coli DH5α:			
No induction	36.8	3.8	6.3
Full induction	88.5	10.2	13.9
P. putida KT2442:			
No induction	1.7	--	6.9
Full induction	1.8	--	7.3

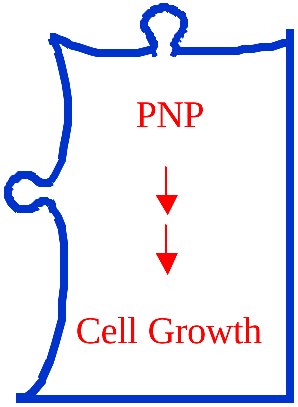


PNP Degradation

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

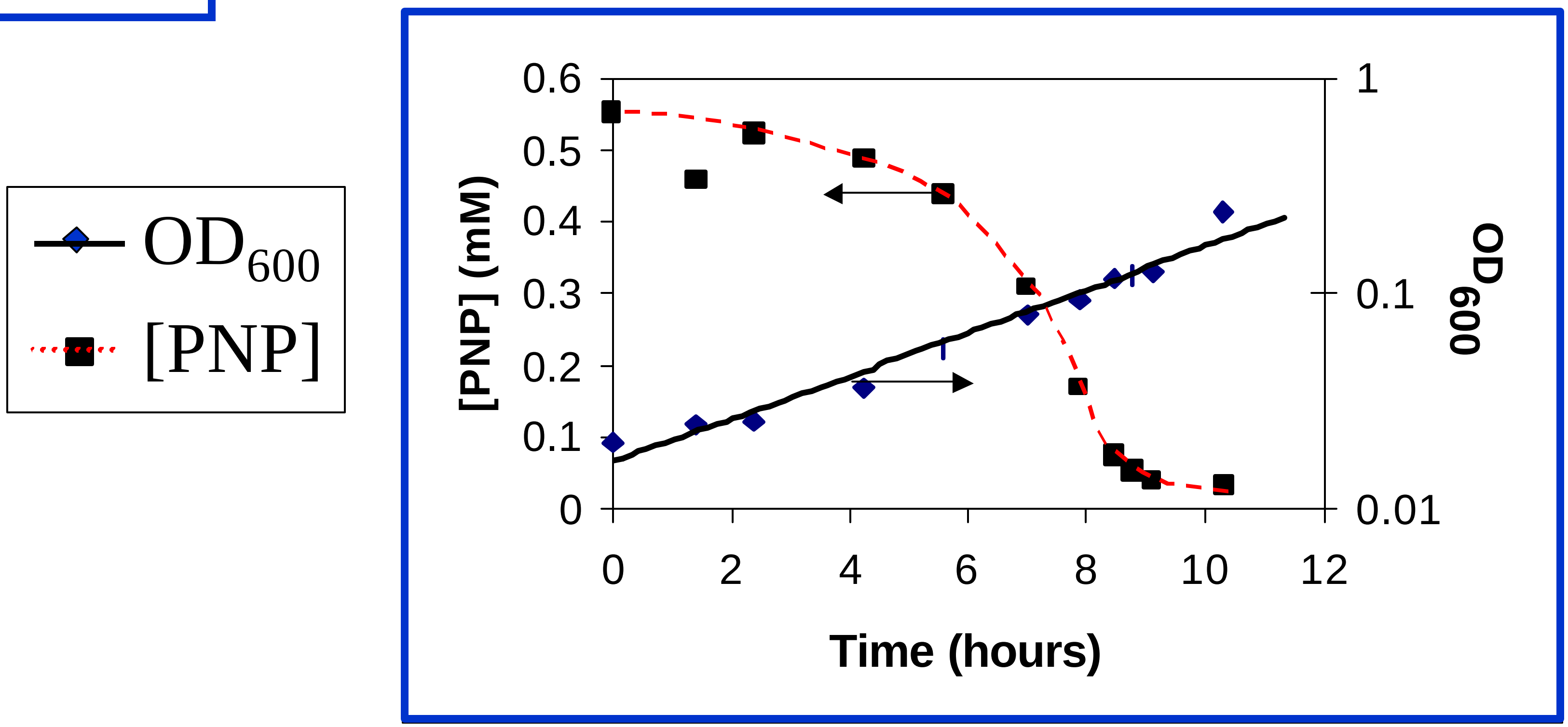






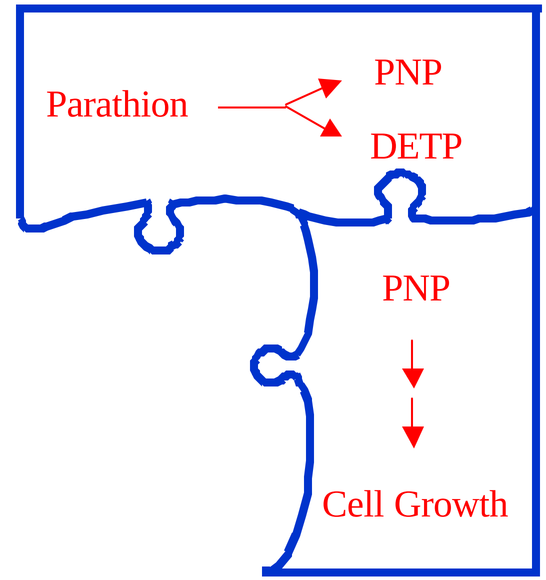
PNP Degradation

PNP degradation by *P. putida* KT2440 with pPNP

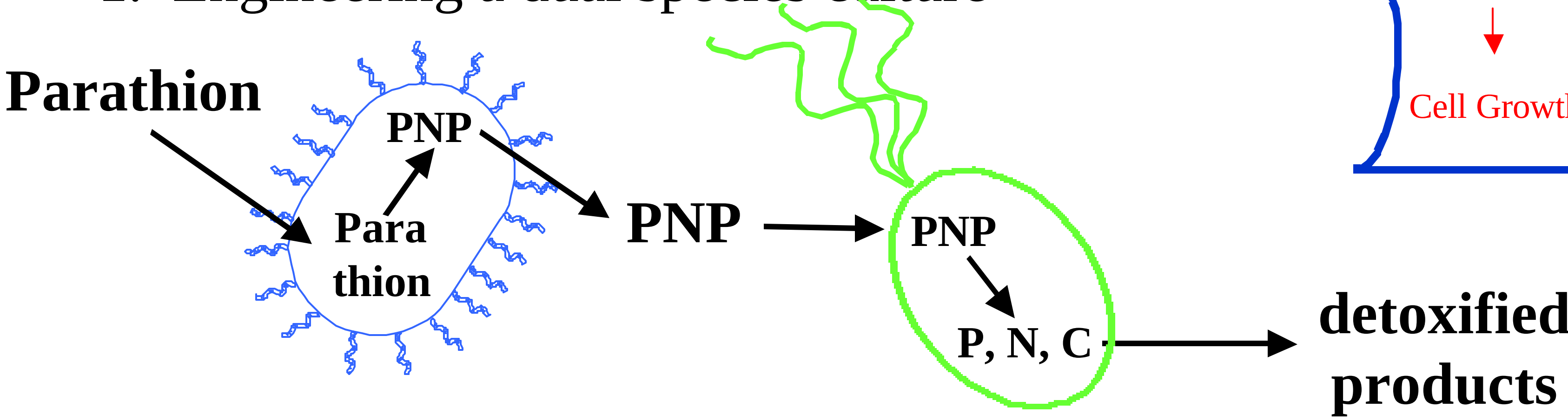


Specific Degradation Rate: 66 μmole/min-gDCW
Specific Growth Rate: 0.23 hour $^{-1}$

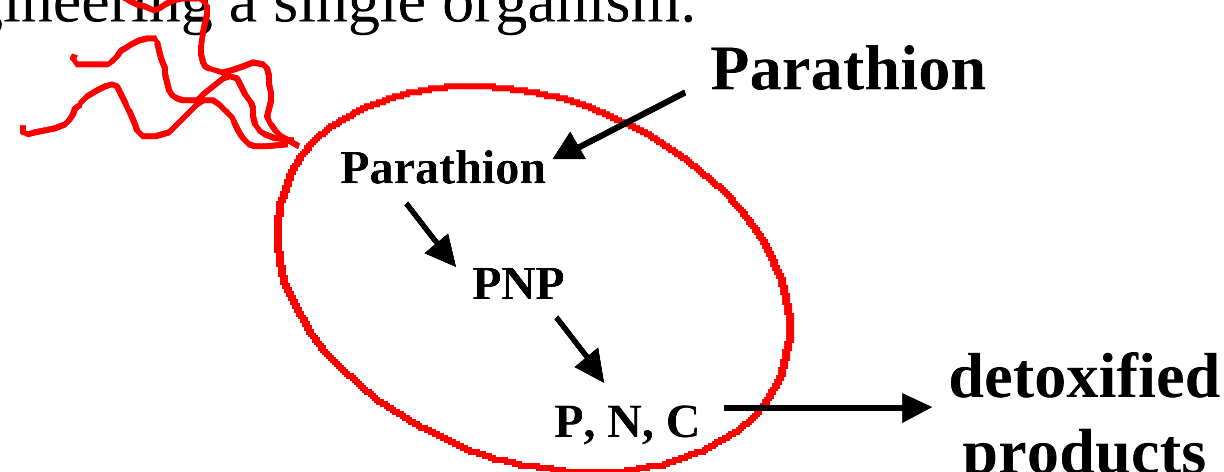
Two ME strategies



1. Engineering a dual species culture

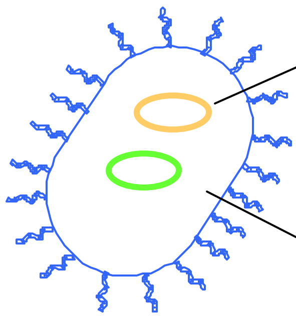


2. Engineering a single organism.



A dual species culture

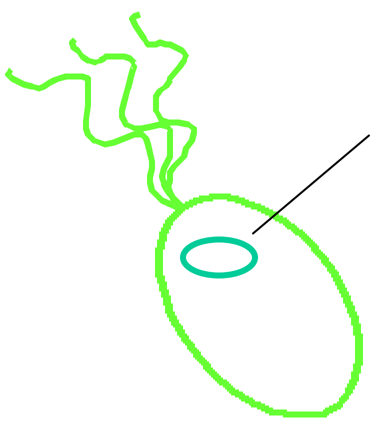
***E.coli* SD2**
hydrolyzes parathion



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

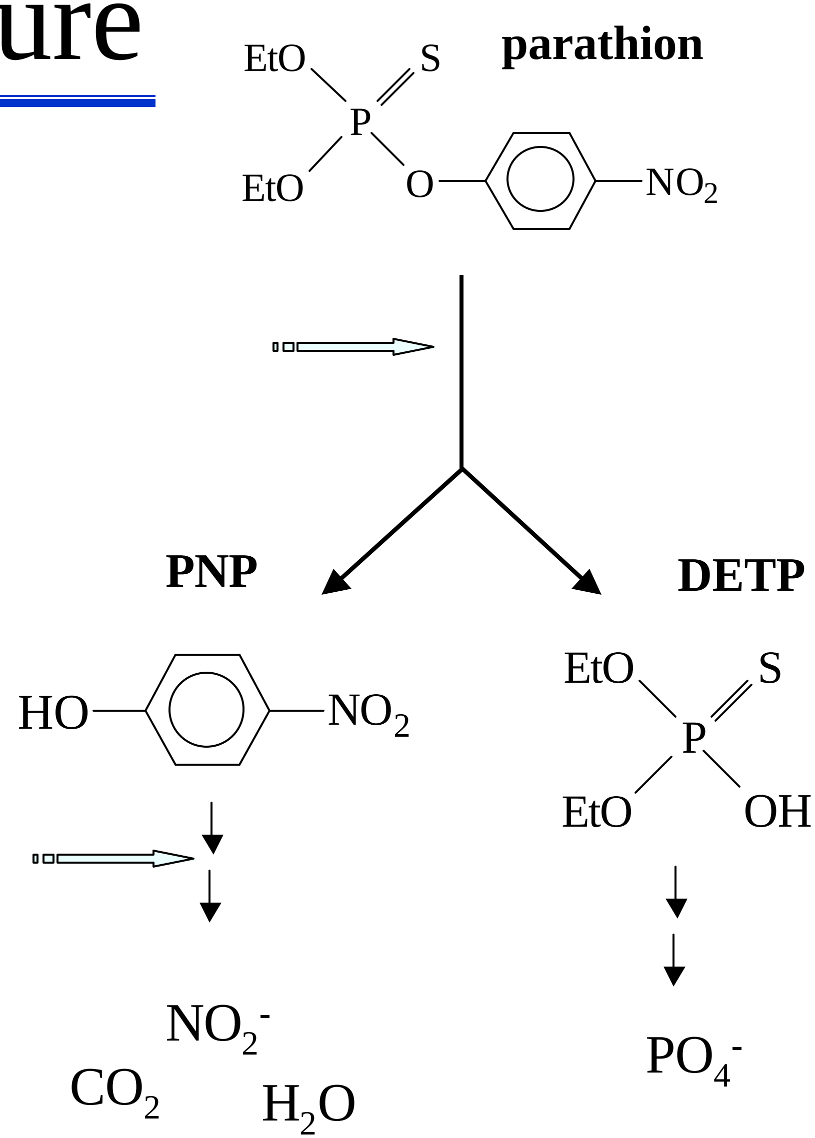
pMAG1:
- *gfp* gene
- tetracycline resistance

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

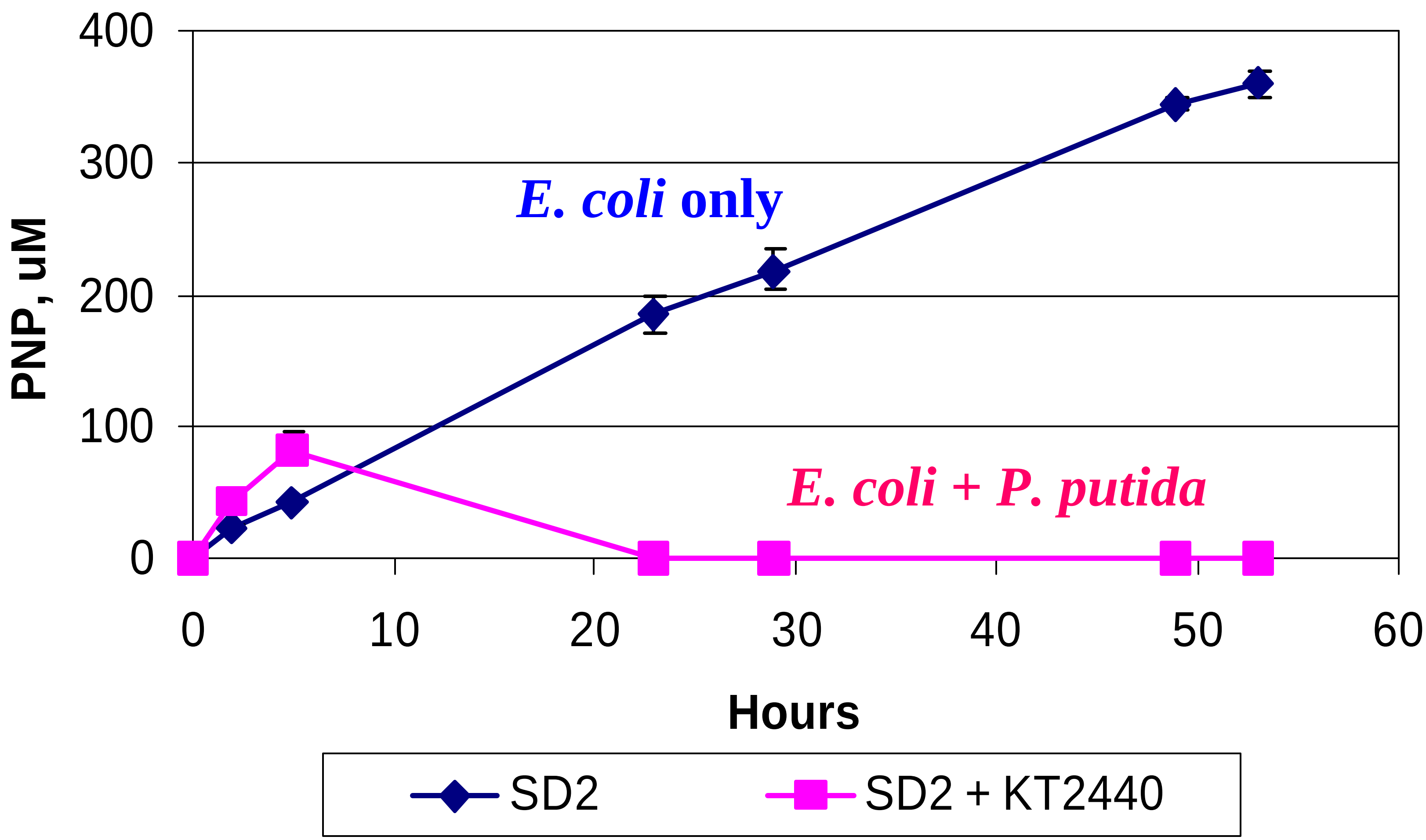


pPNP:
- PNP degradation
- tetracycline resistance

- natural ampicillin resistance

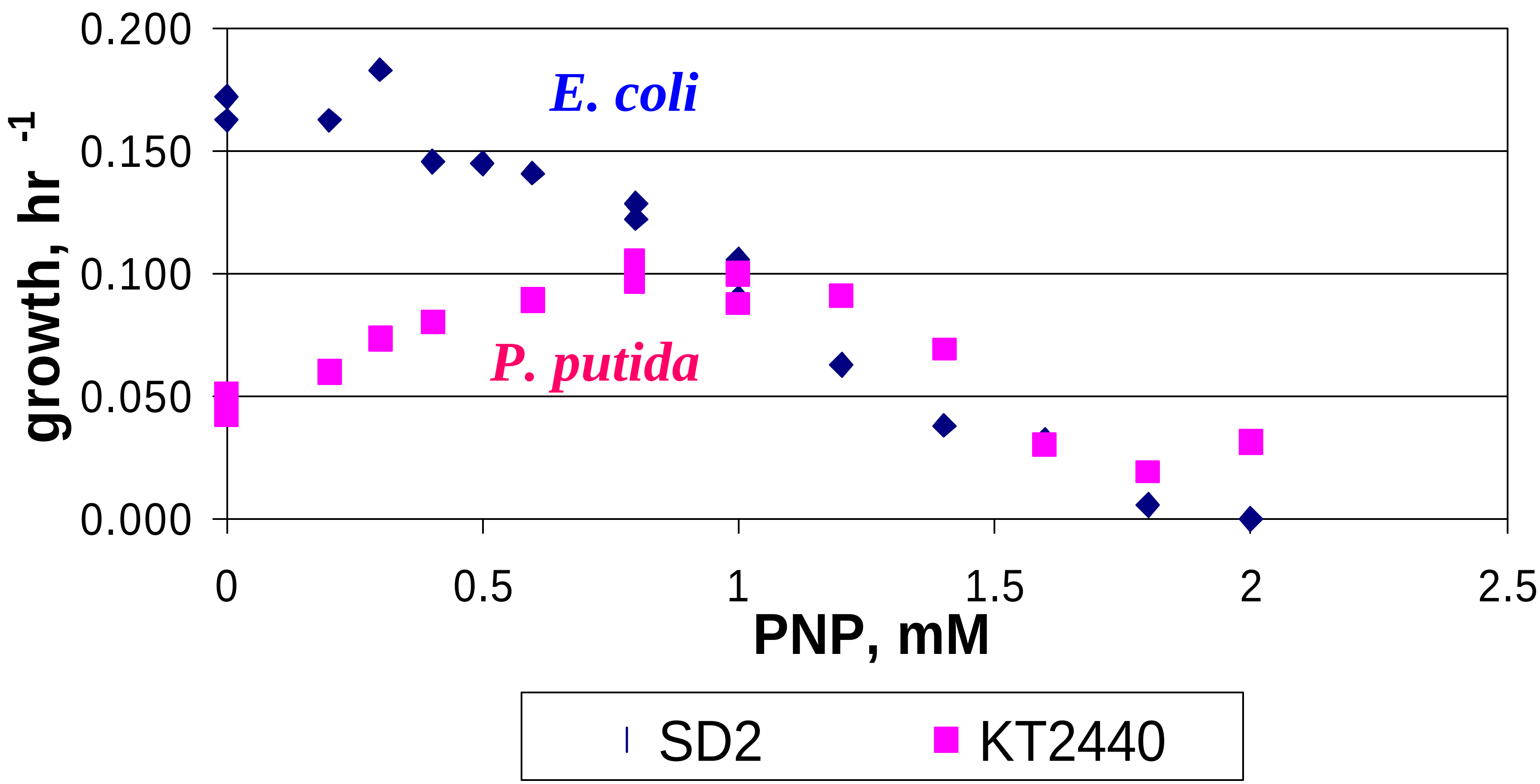


Biodegradation of parathion in suspended culture

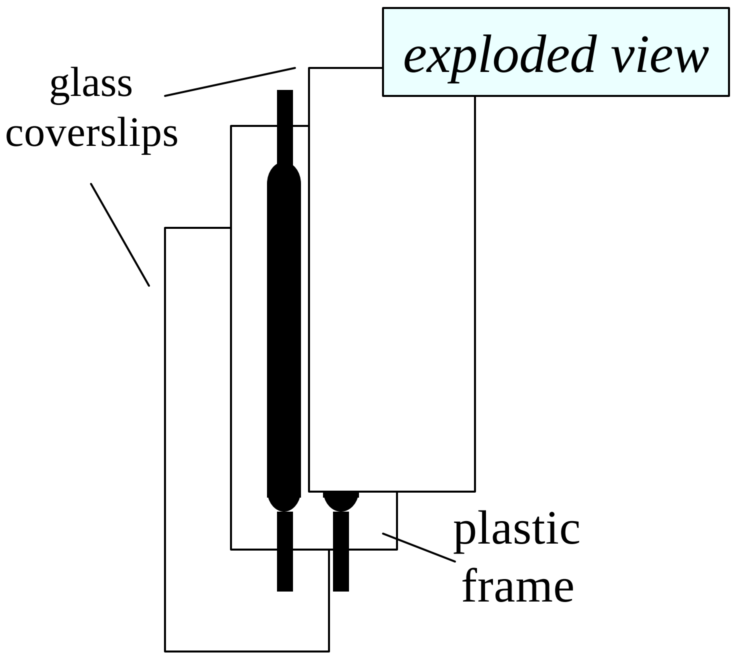


Effect of PNP on cell growth

growth was inhibited by PNP

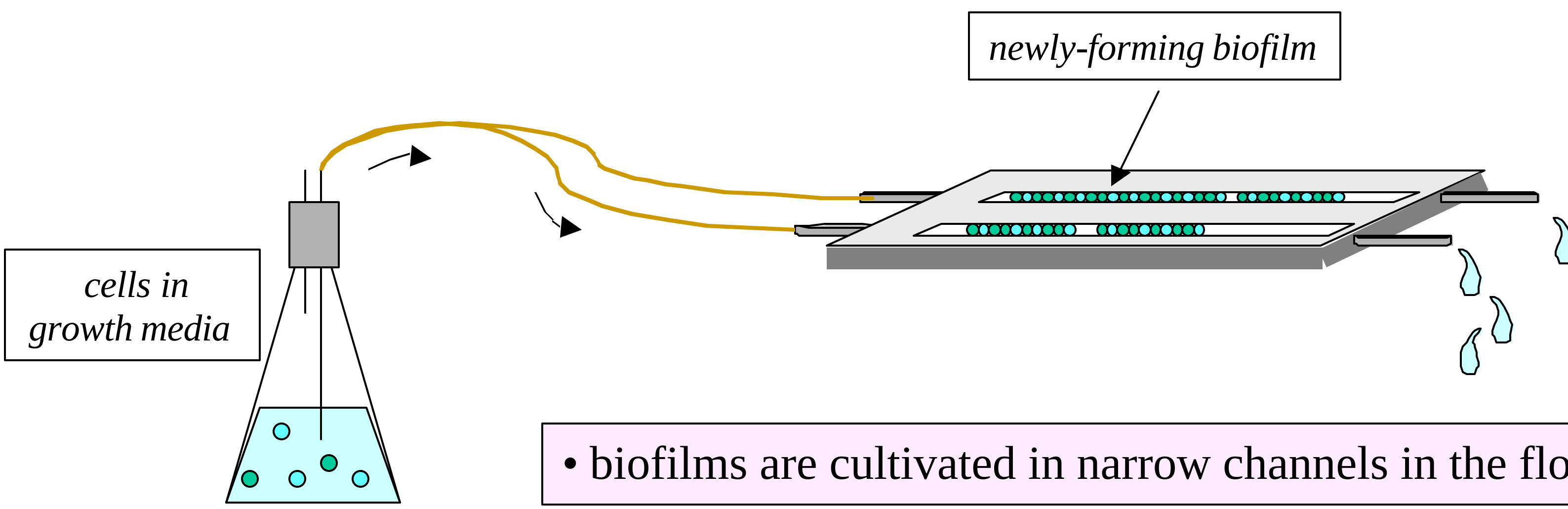


Flow cell for culturing biofilms



measurements

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



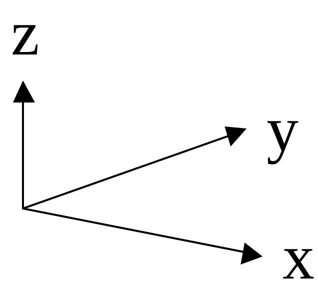
• biofilms are cultivated in narrow channels in the flow cell

Development of a coculture biofilm for parathion biodegradation

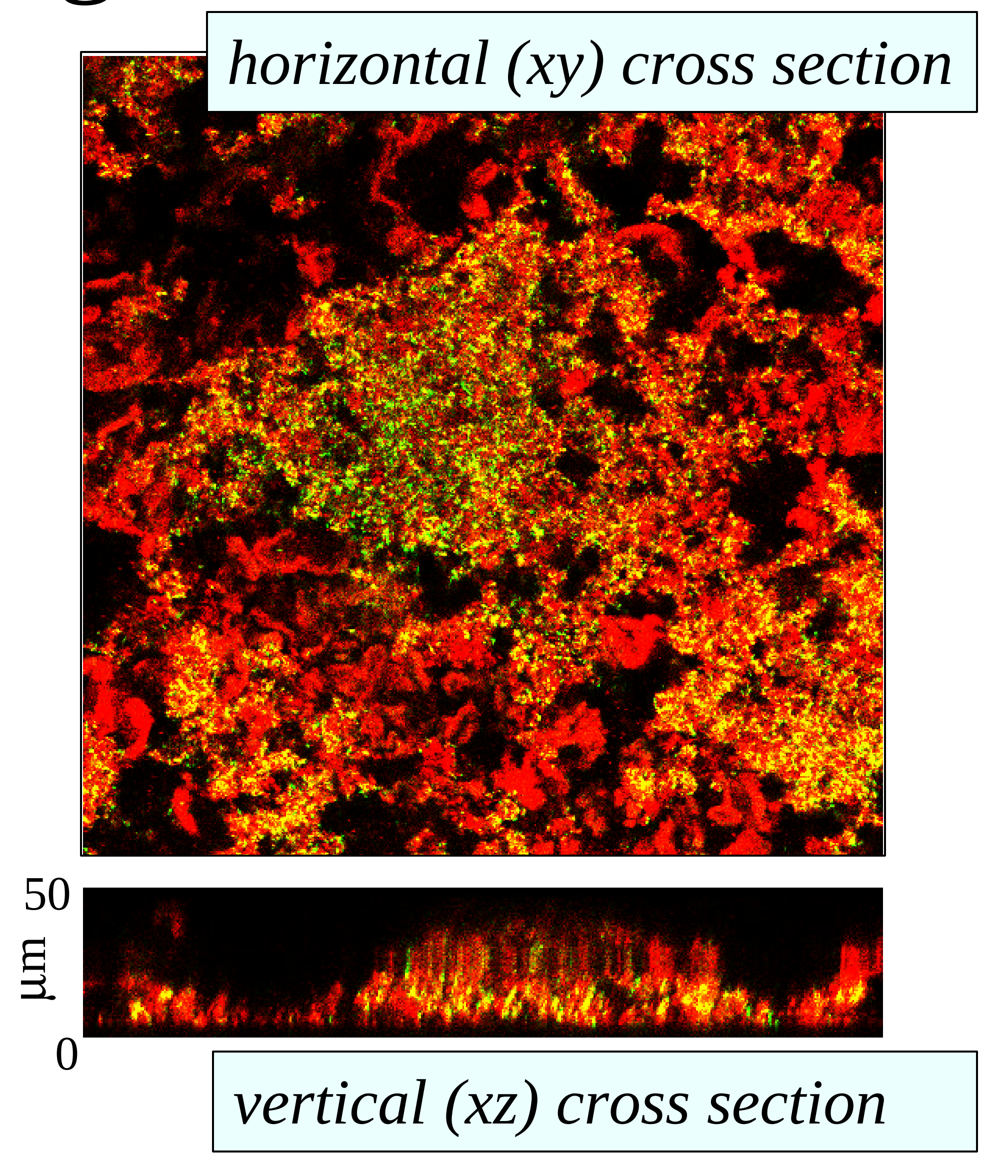
red: *P. putida* KT2440

yellow/green: *E. coli* SD2

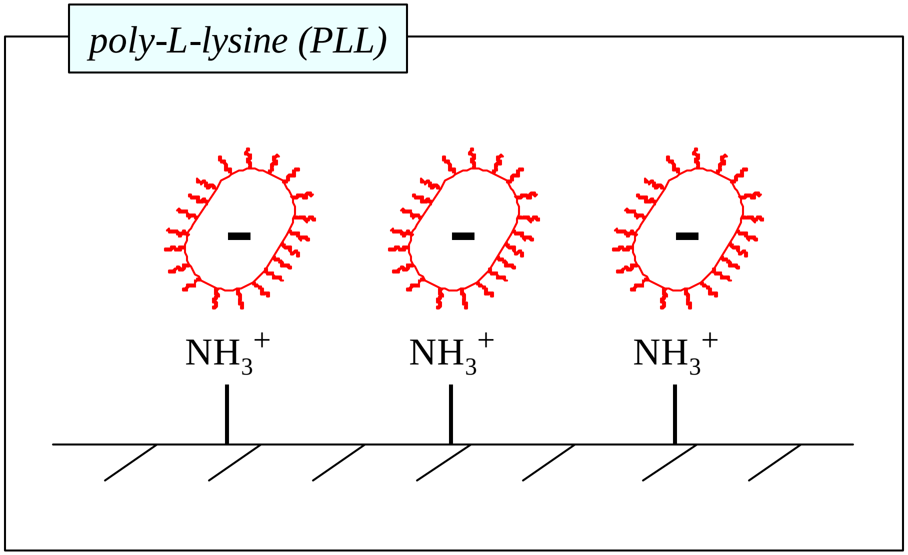
black: voids within the biofilm



orientation



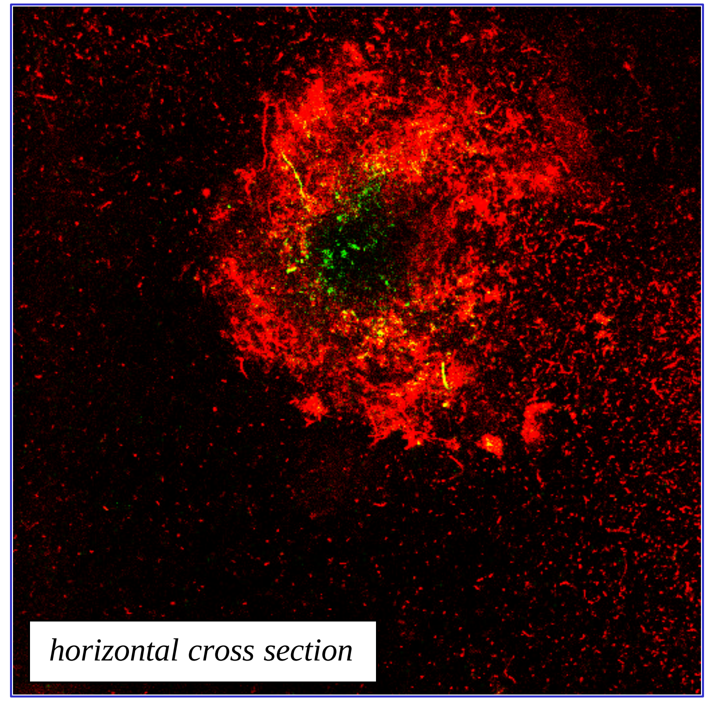
Biofilm engineering

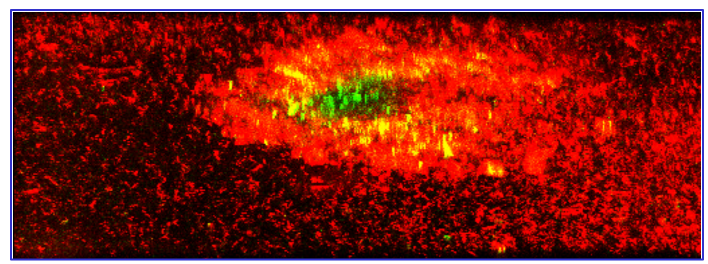


yellow/green: *E. coli* SD2 attached to glass sphere with PLL

red: *P. putida* KT2440

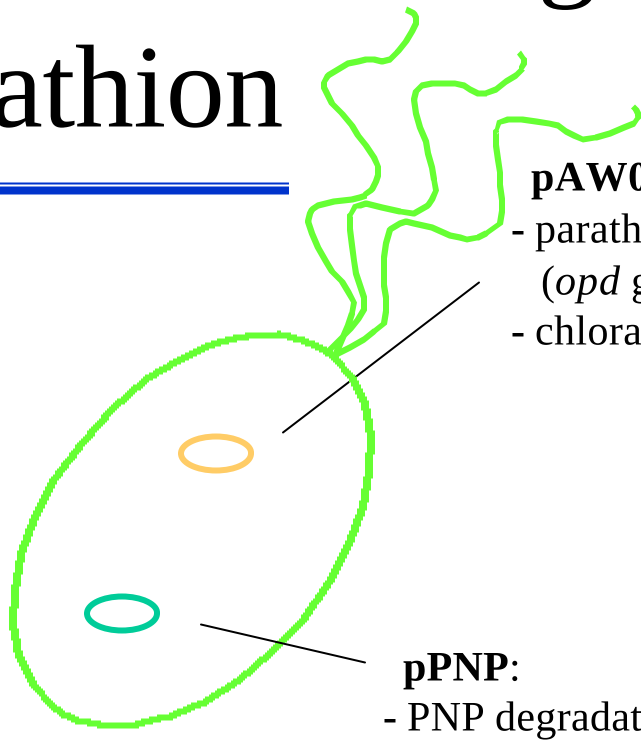
- strains were sequentially applied





70 degree tilted projection

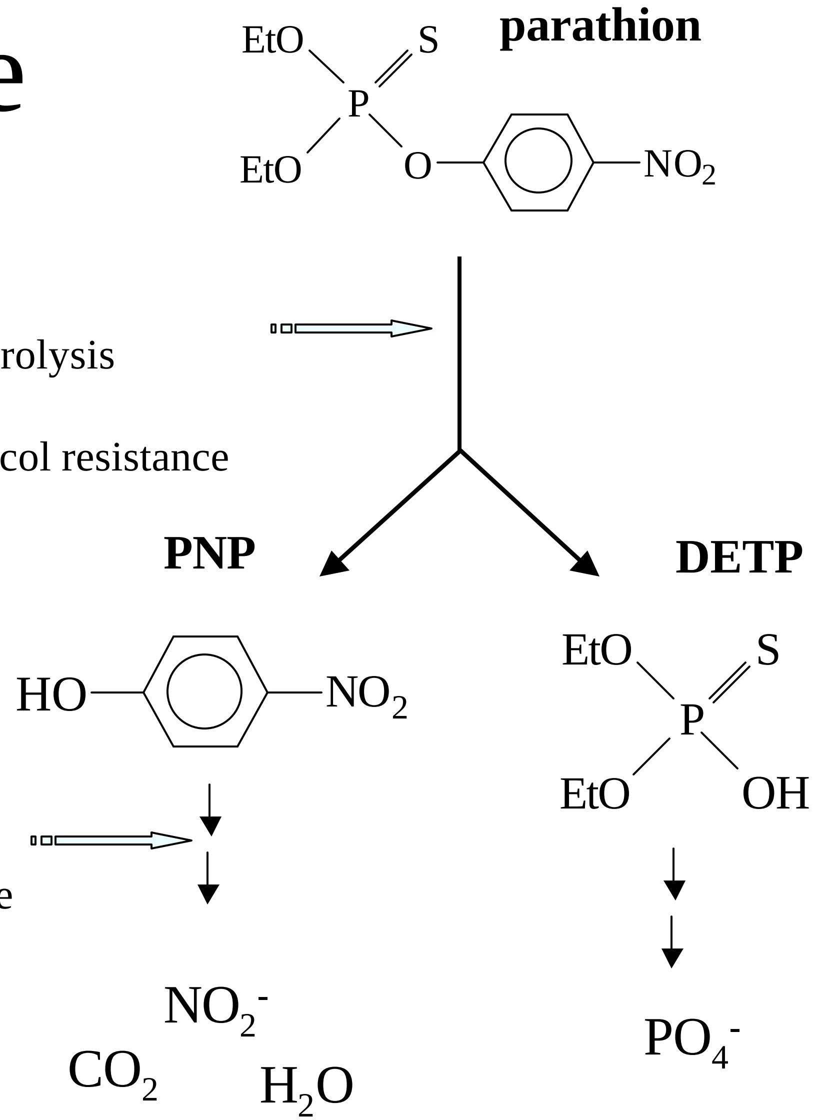
Engineering a single organism to degrade parathion



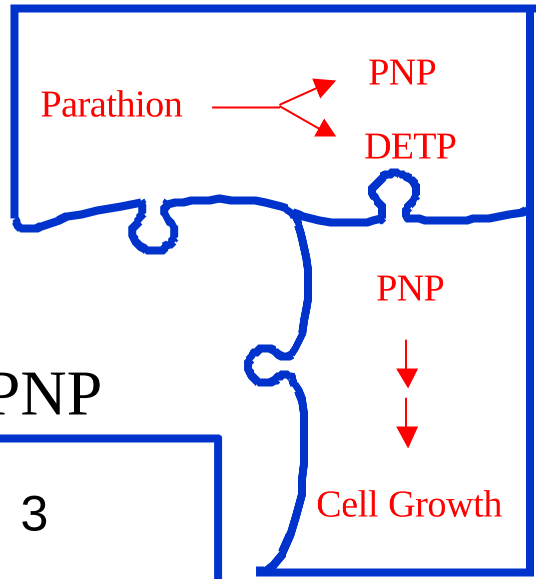
pAW02:
- parathion hydrolysis
  (*opd* genes)
- chloramphenicol resistance

pPNP:
- PNP degradation
- tetracycline resistance

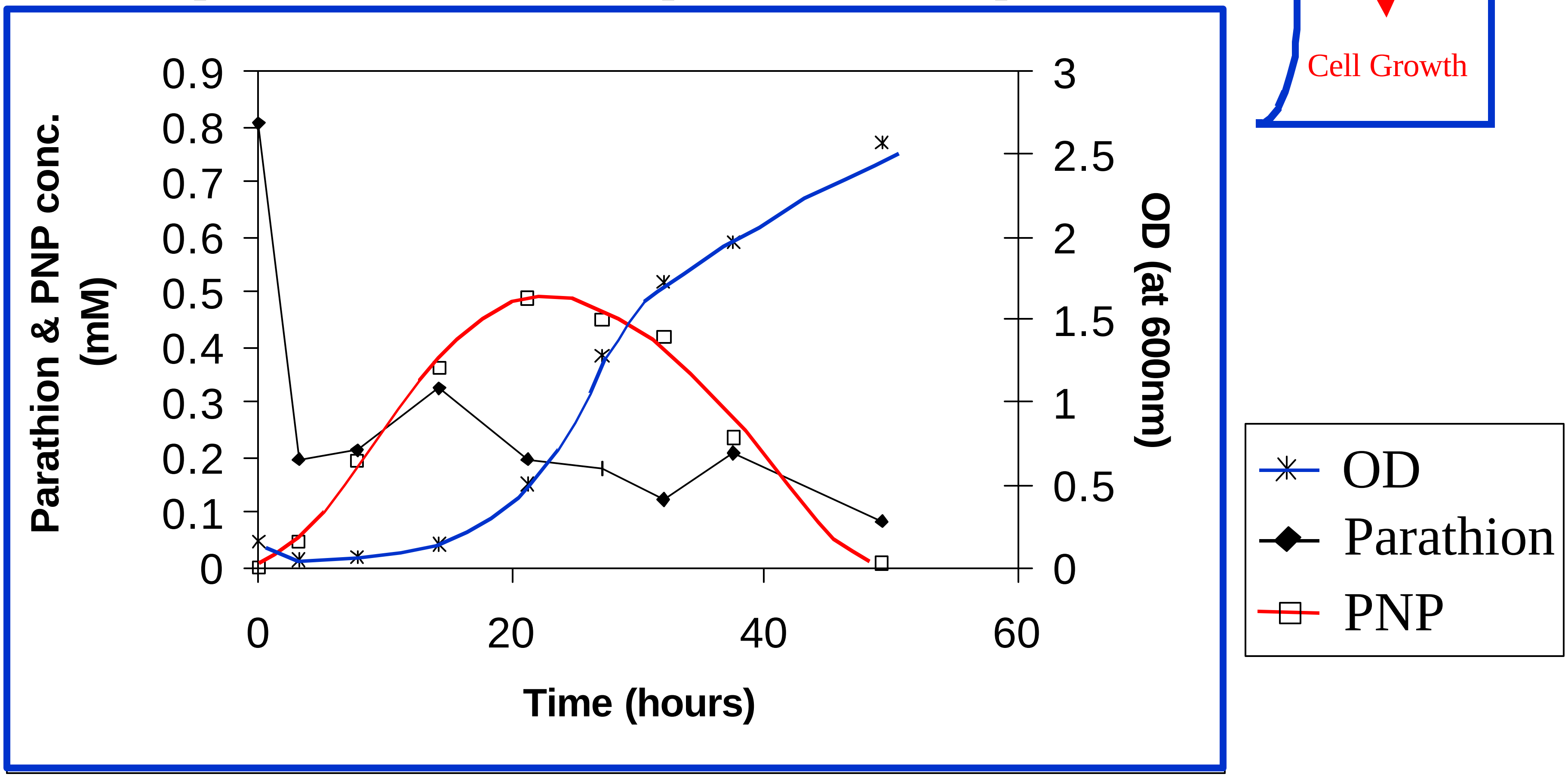
***Pseudomonas* KT2440**
mineralizes *p*-nitrophenol (PNP)



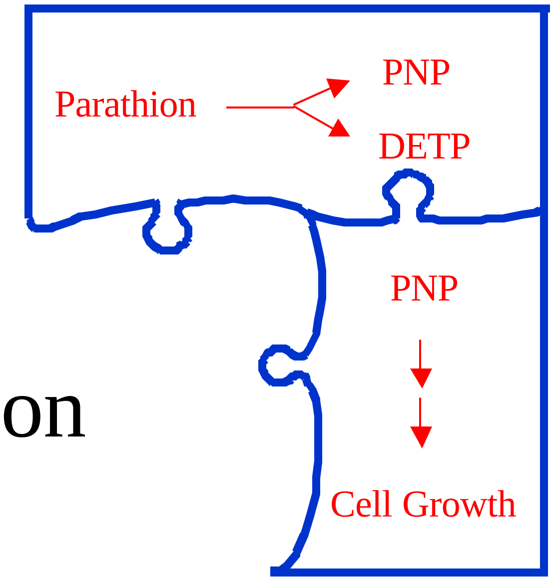
Parathion Utilization as a Carbon Source



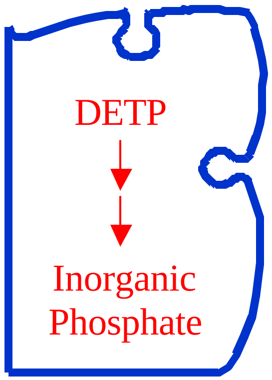
Parathion Degradation by
P. putida KT2442 with pAWW04 and pPNP



Parathion Utilization as a Carbon Source

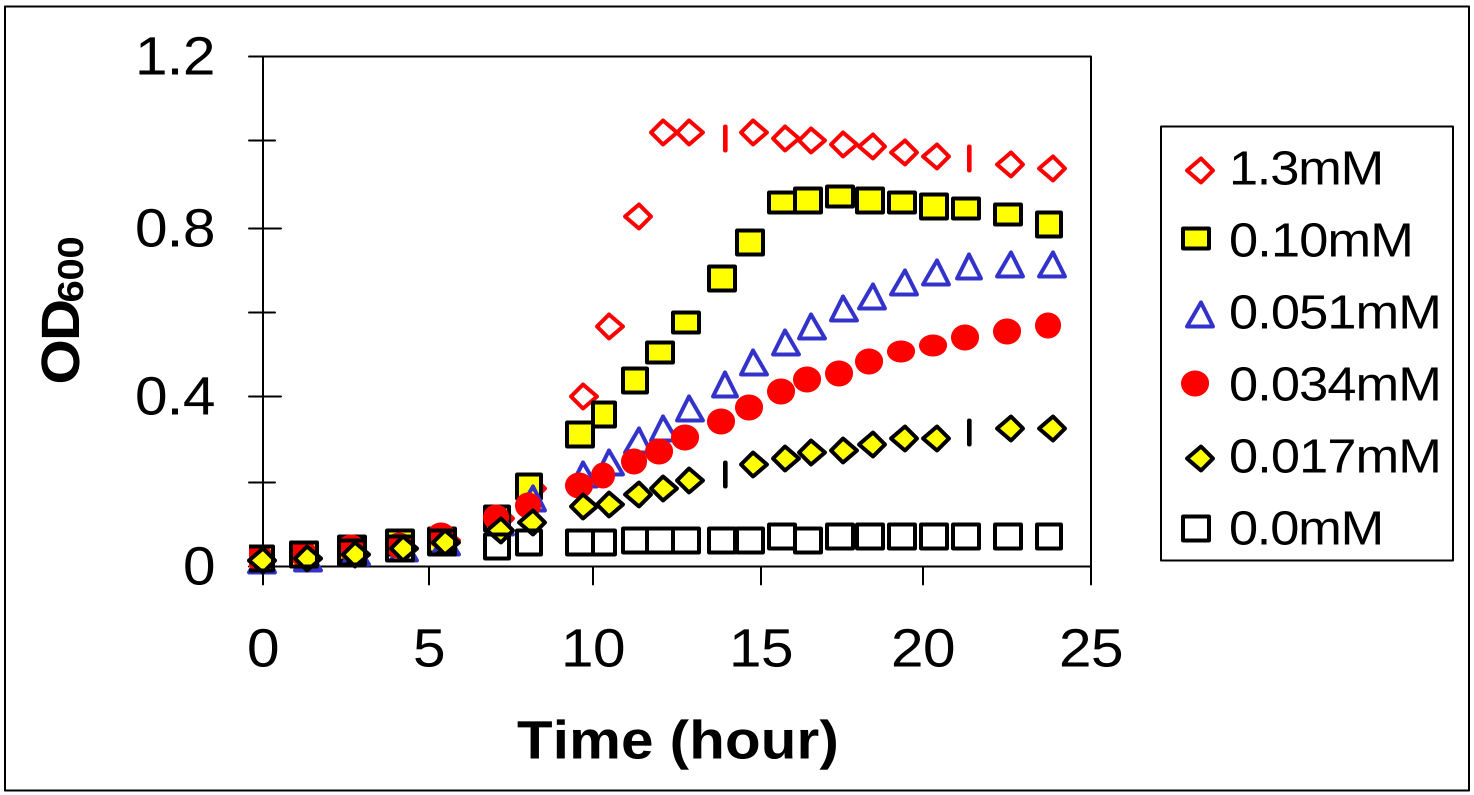


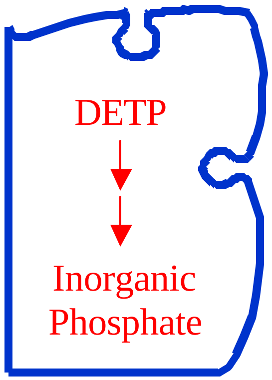
- Parathion is utilized as a carbon and energy source
- Parathion forms DNAPL, but is still bioavailable
- Measurement of aqueous phase parathion concentration is not a good indicator as to whether parathion degradation is occurring



DETP Degradation

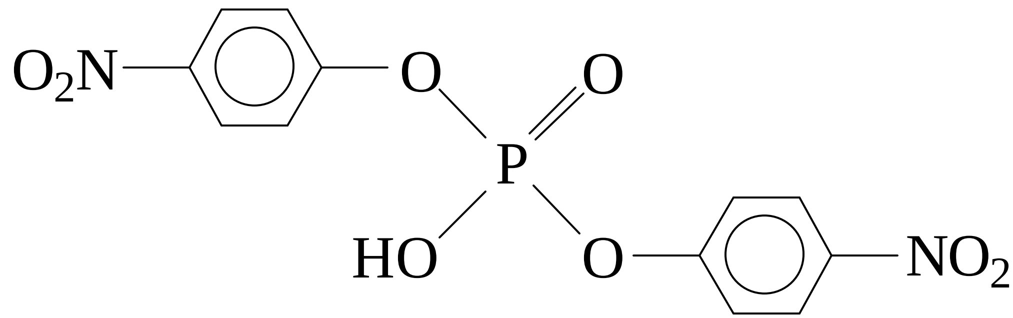
- *Comamonas acidovorans* is capable of utilizing DETP as a P-source:



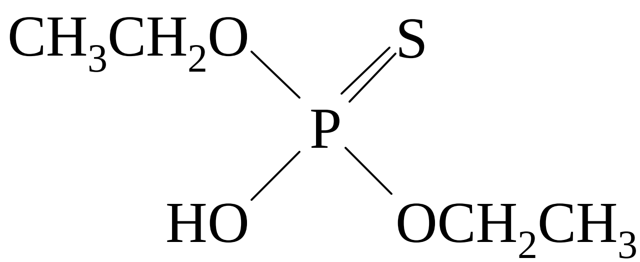


DETP Degradation

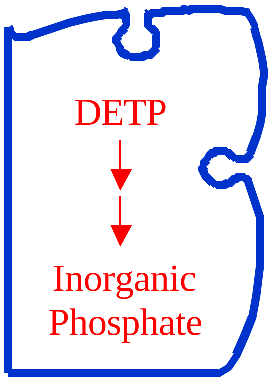
DETP degradation rates were estimated using a DETP analog, bis-(*p*-nitrophenol) phosphate (BNP).



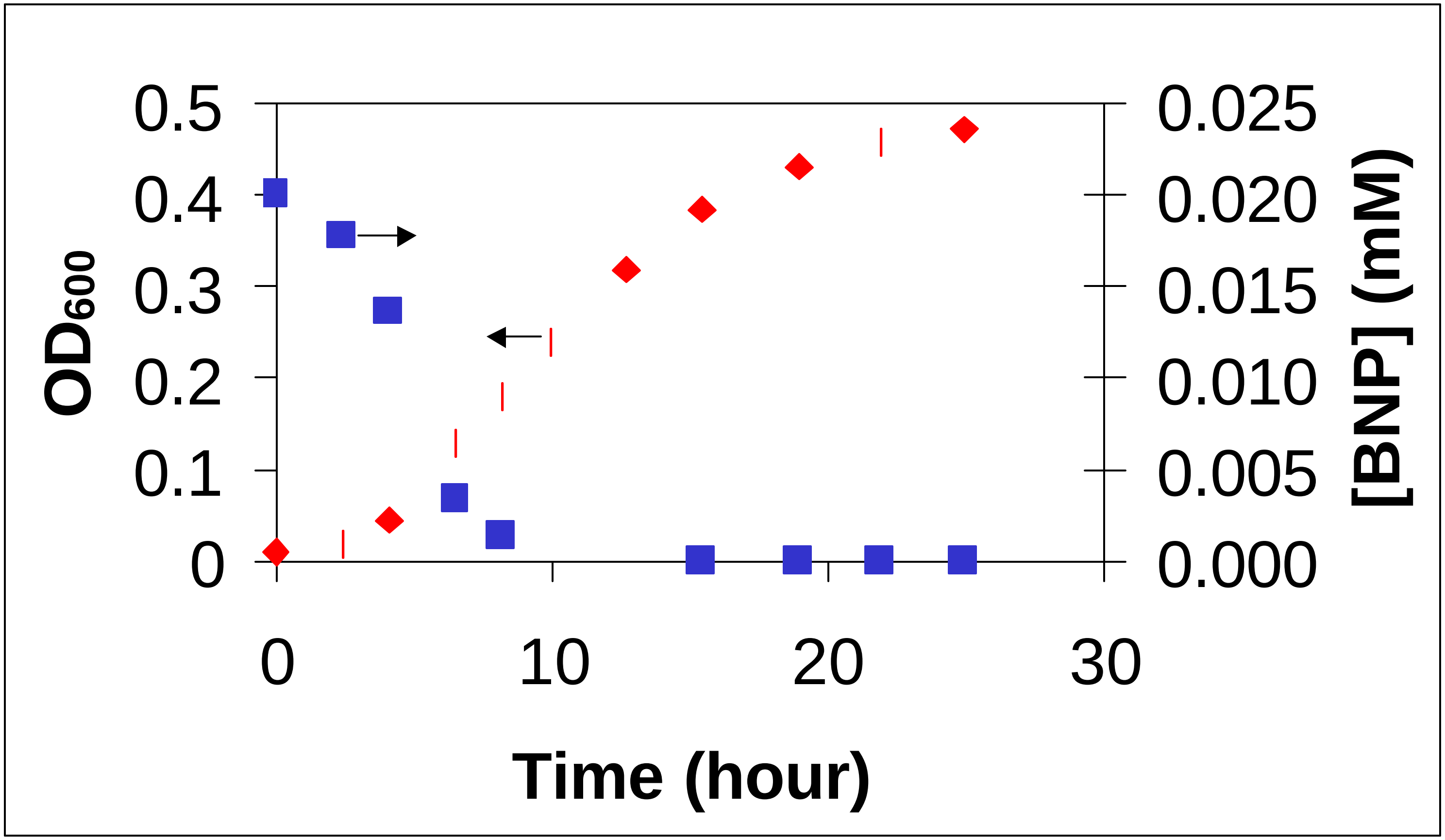
BNP



DETP



C. acidovorans growth and BNP disappearance

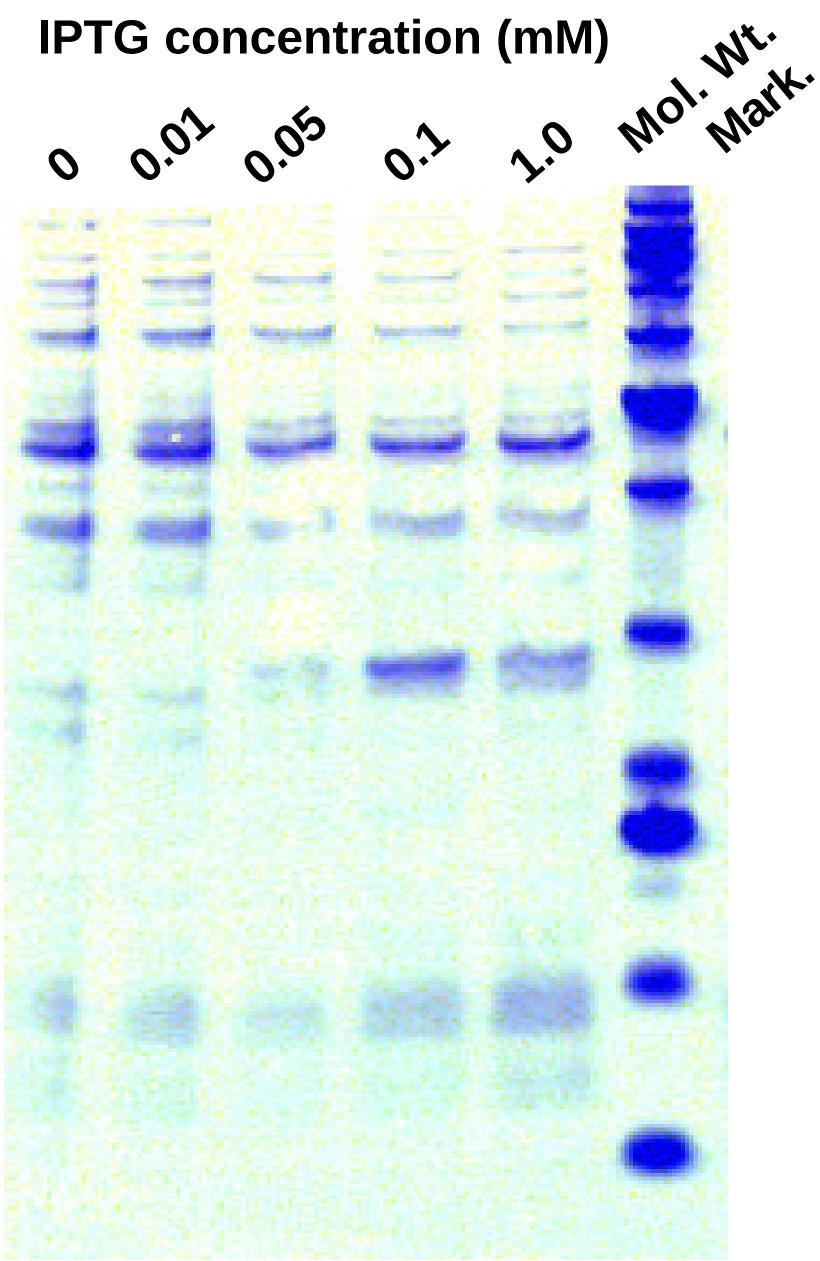


Purification and characterization of phosphodiesterase

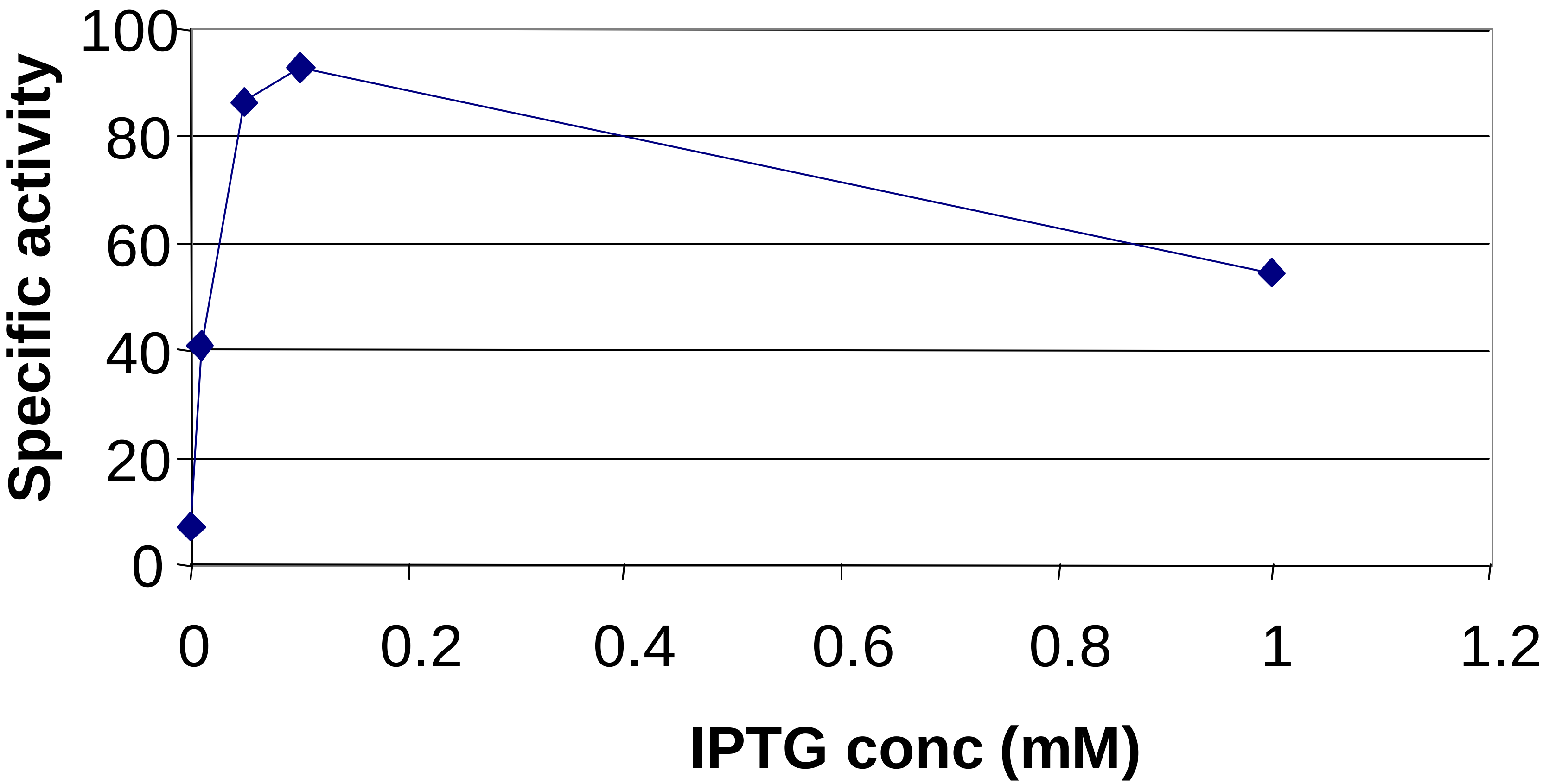
- The phosphodiesterase was purified to homogeneity
  - Monomer of 65 Kda
  - Most active toward phosphodiesters, less activity on phosphomonoesters and phosphotriesters
- N-terminal sequenced
- Degenerate primers synthesized
- Gene cloned
  - Low homology to nucleotide phosphodiesterases
- Overexpression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

Protein production

Recombinant PDE



Induction studies

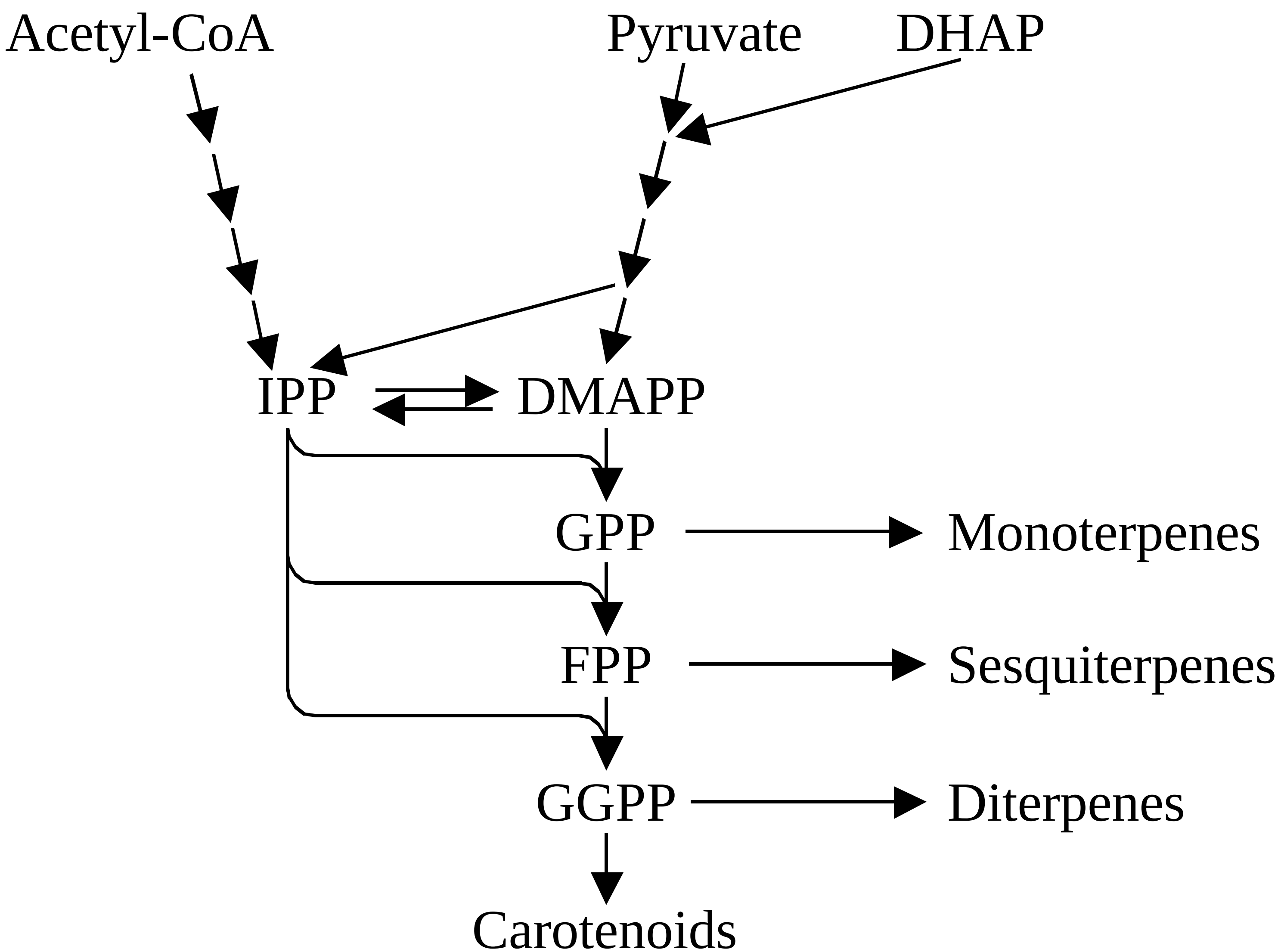


Synthesis of Isoprenoids

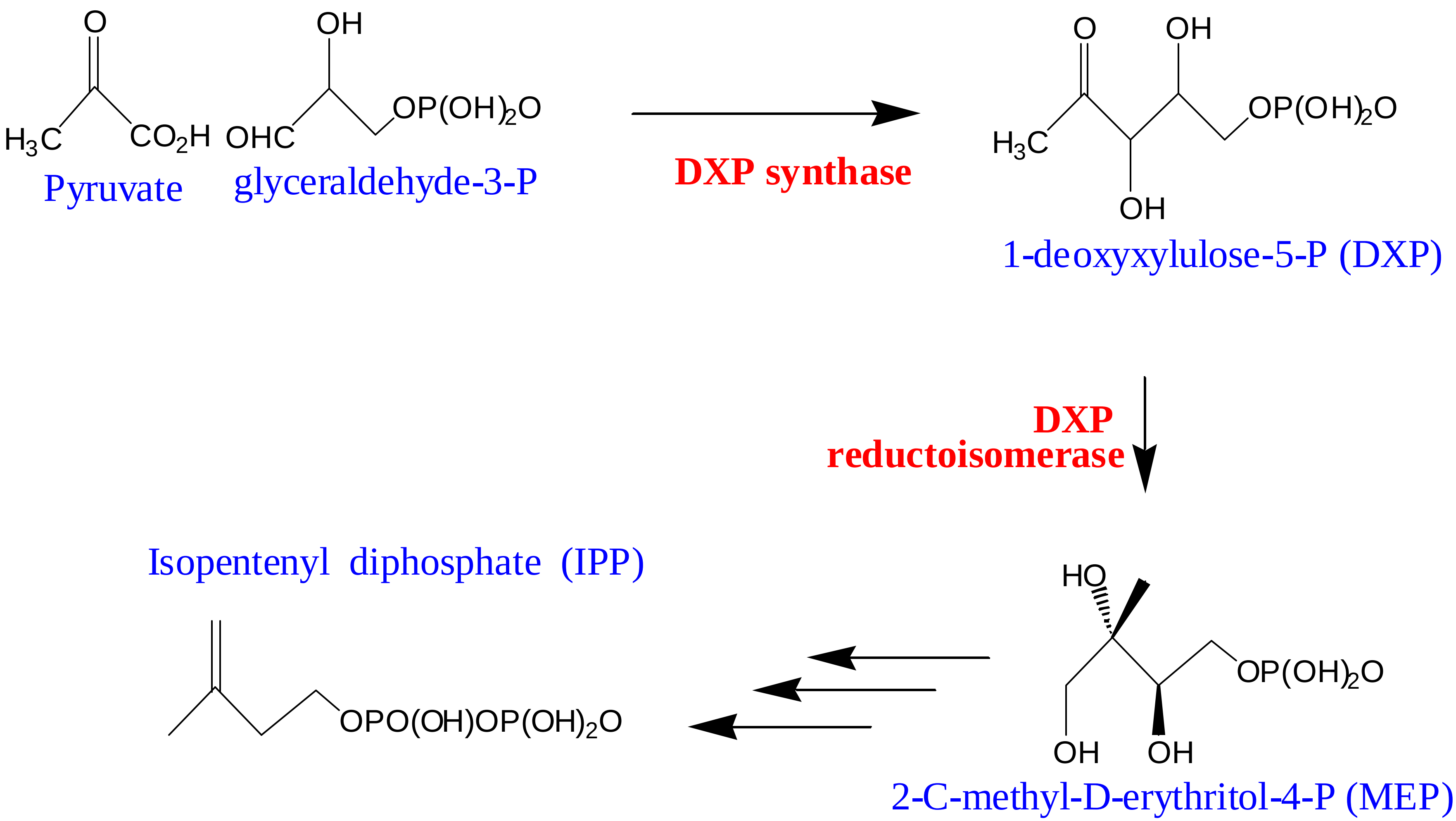
Goal -

- 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

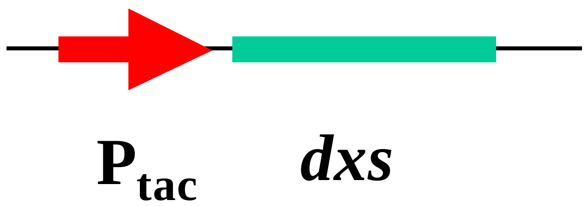
Isoprenoid biosynthetic pathways



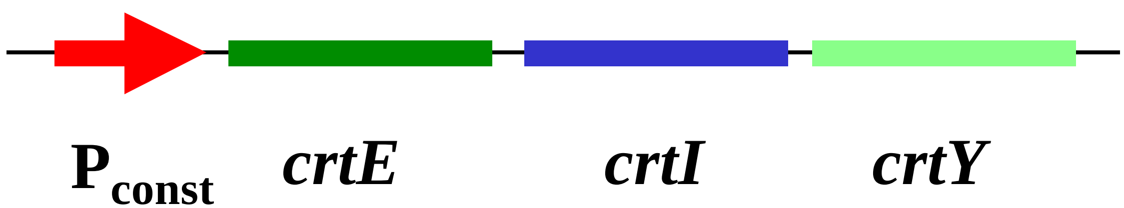
In bacteria, IPP is produced via the non-mevalonate pathway



dxs under P_{tac} control on high-copy cloning vectors

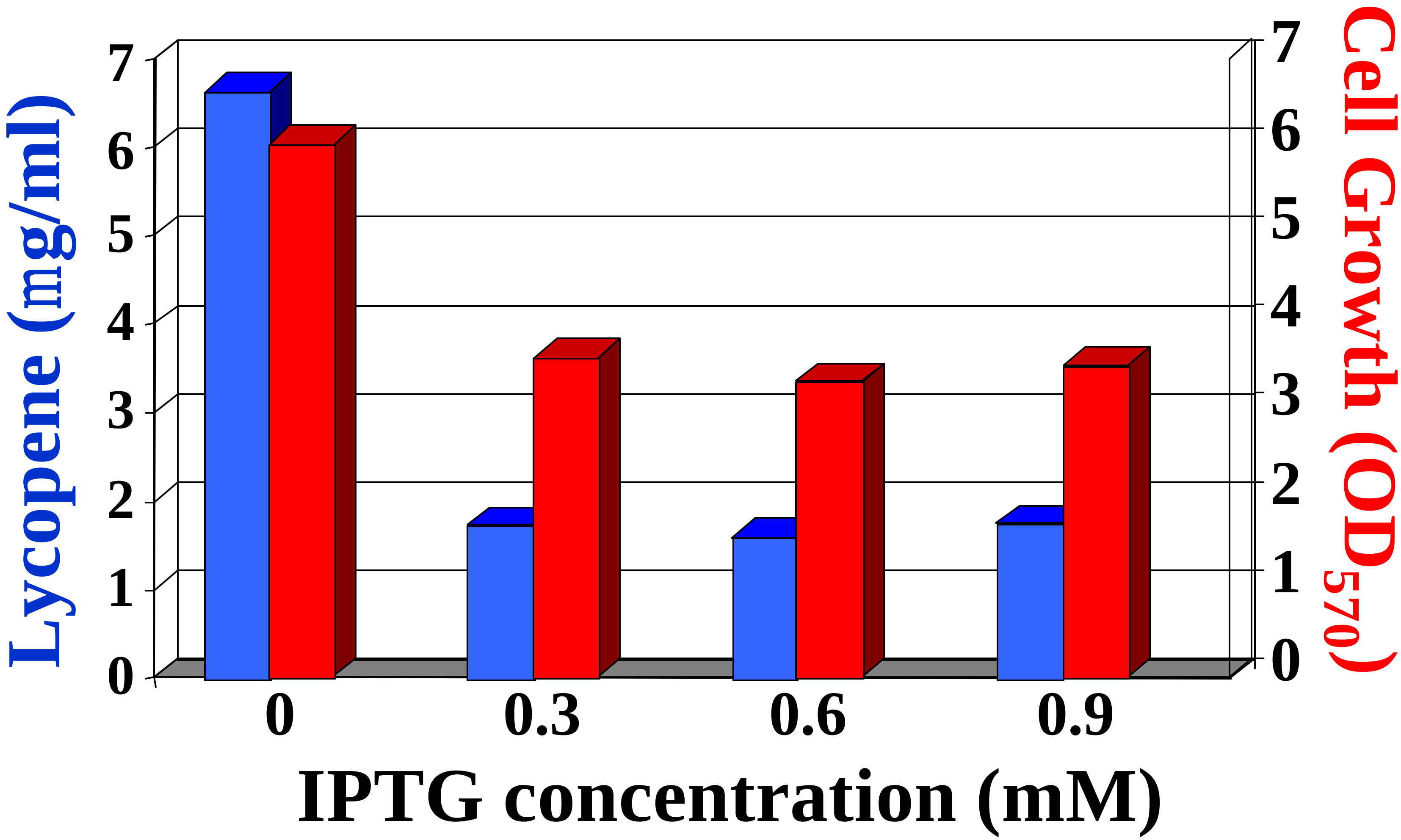


High-copy plasmid

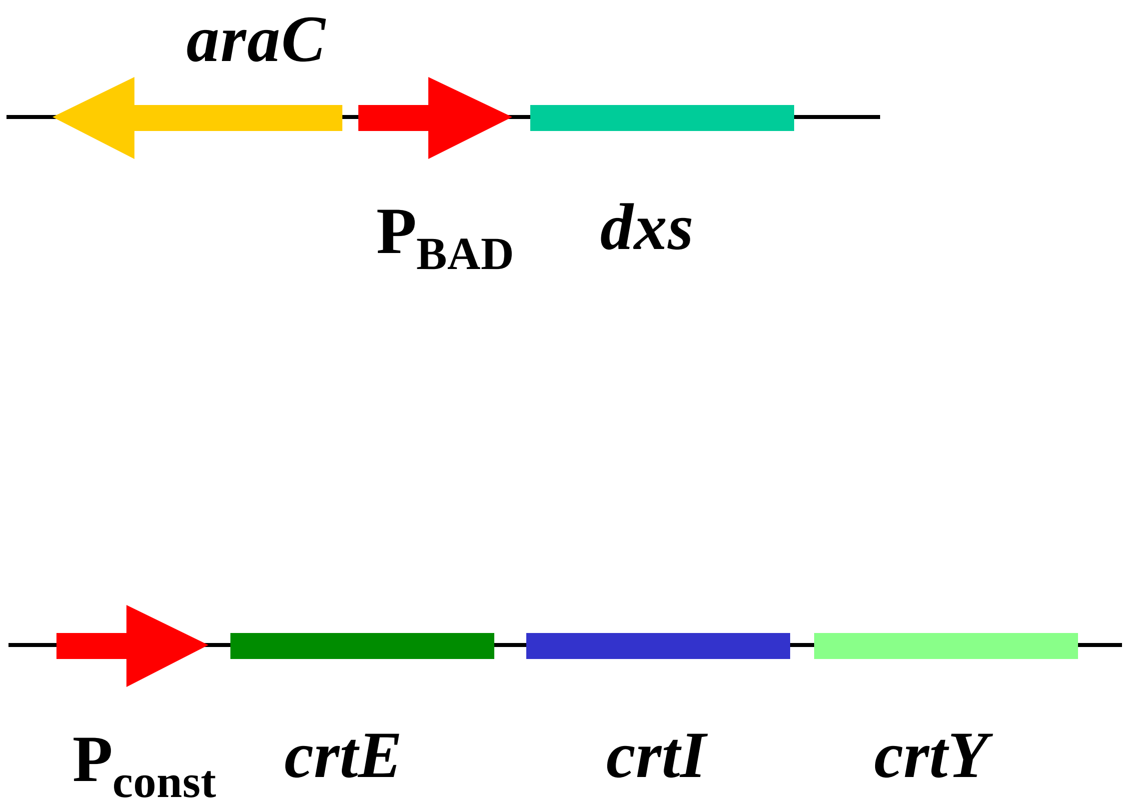


Medium-copy plasmid

Overexpression of *dxs* from a high-copy plasmid with a strong promoter



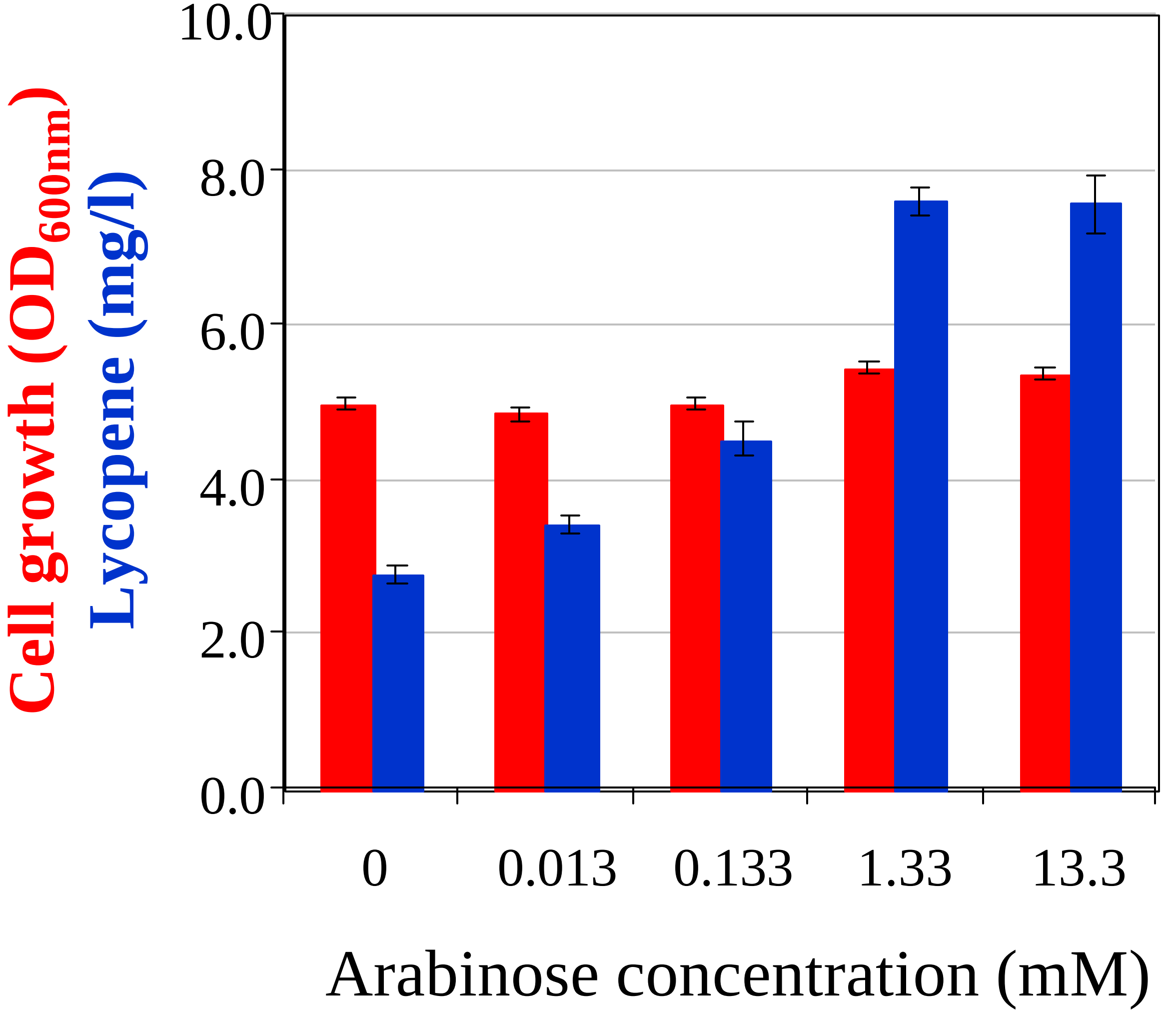
dxs under P_{BAD} control on bacterial artificial chromosome



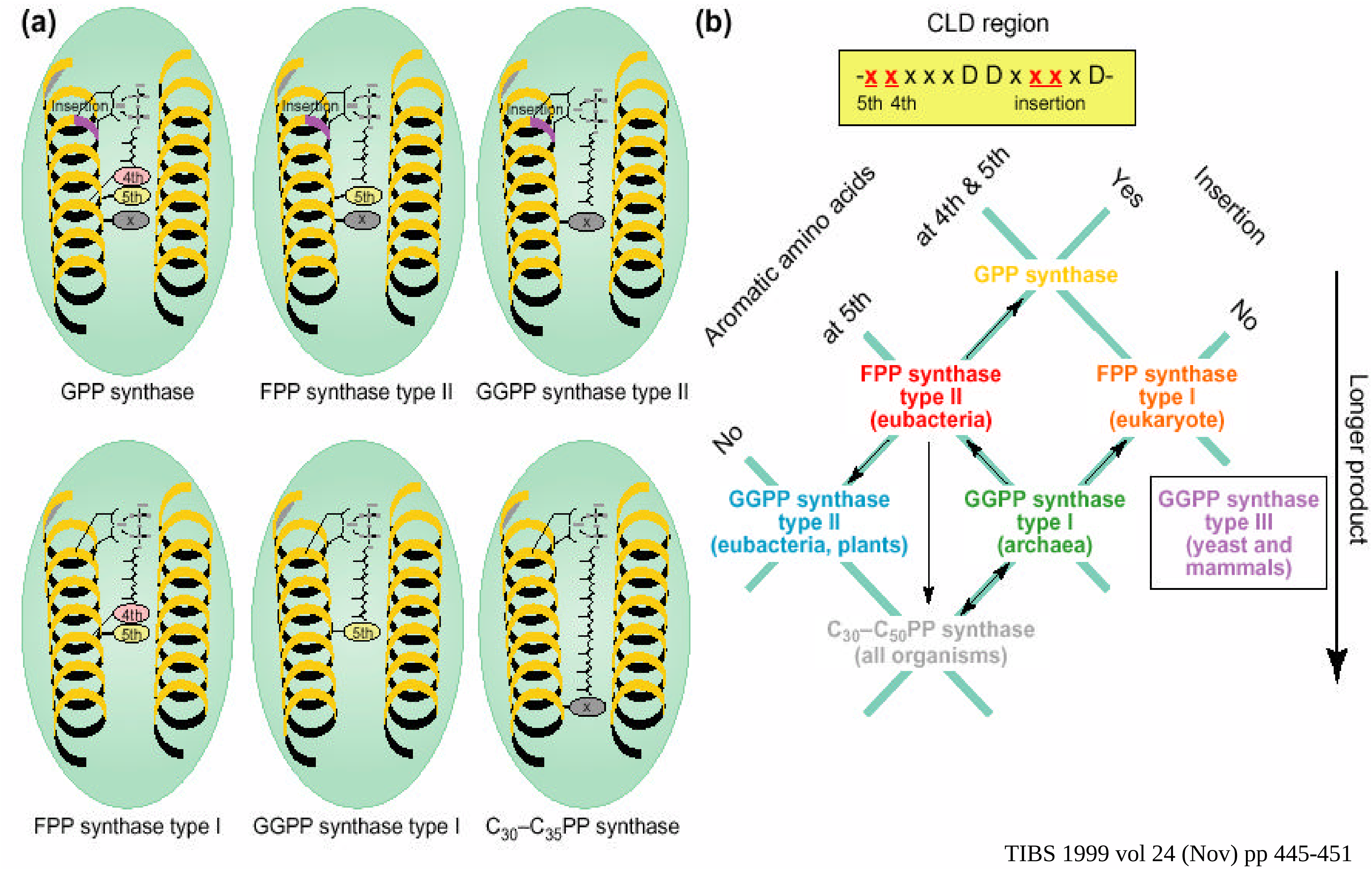
Bacterial artificial chromosome

Medium-copy plasmid

Production of lycopene: *dxs* under control of P_{BAD} on low-copy plasmid



Synthesis of GPP, FPP, and GGPP



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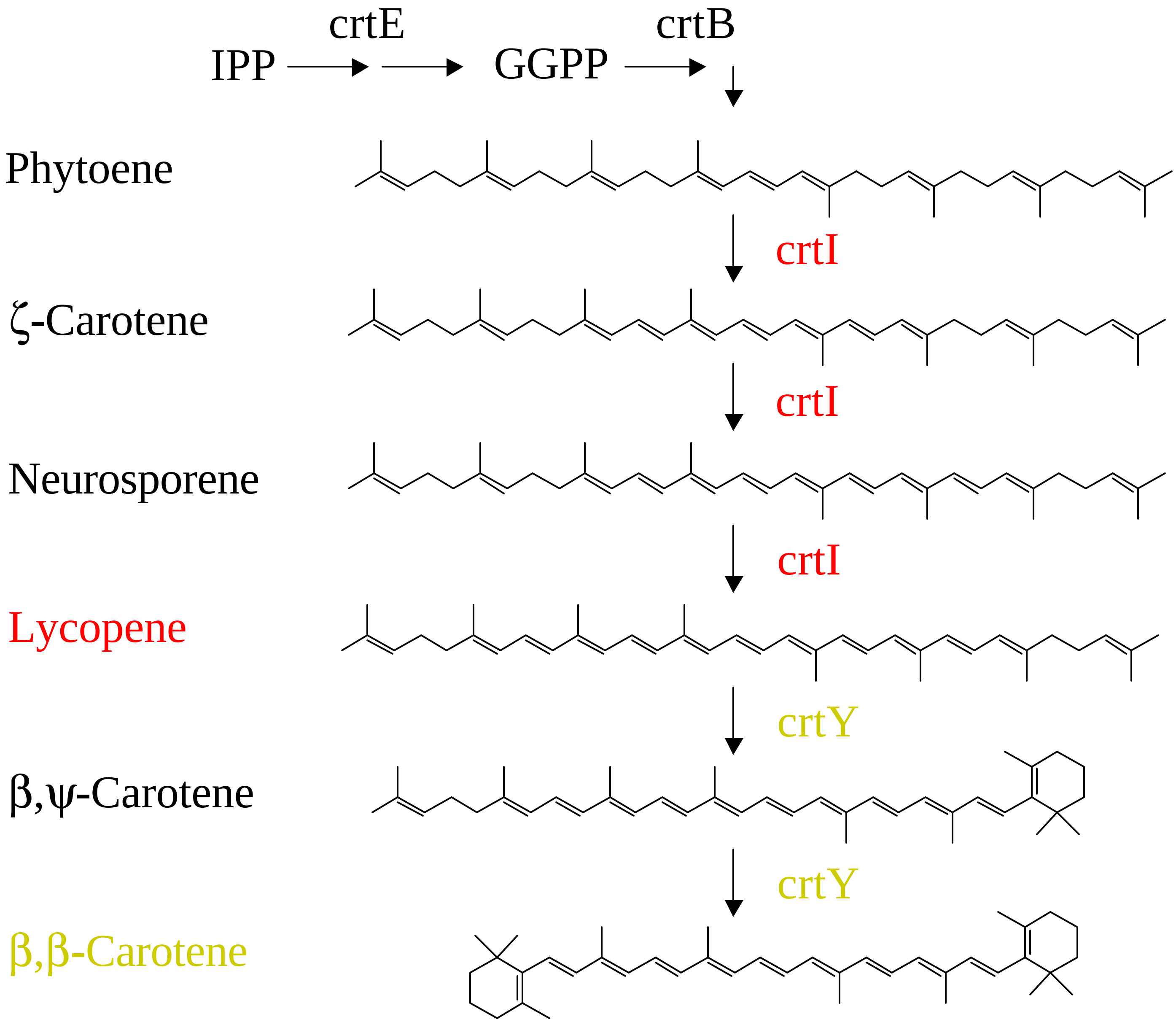
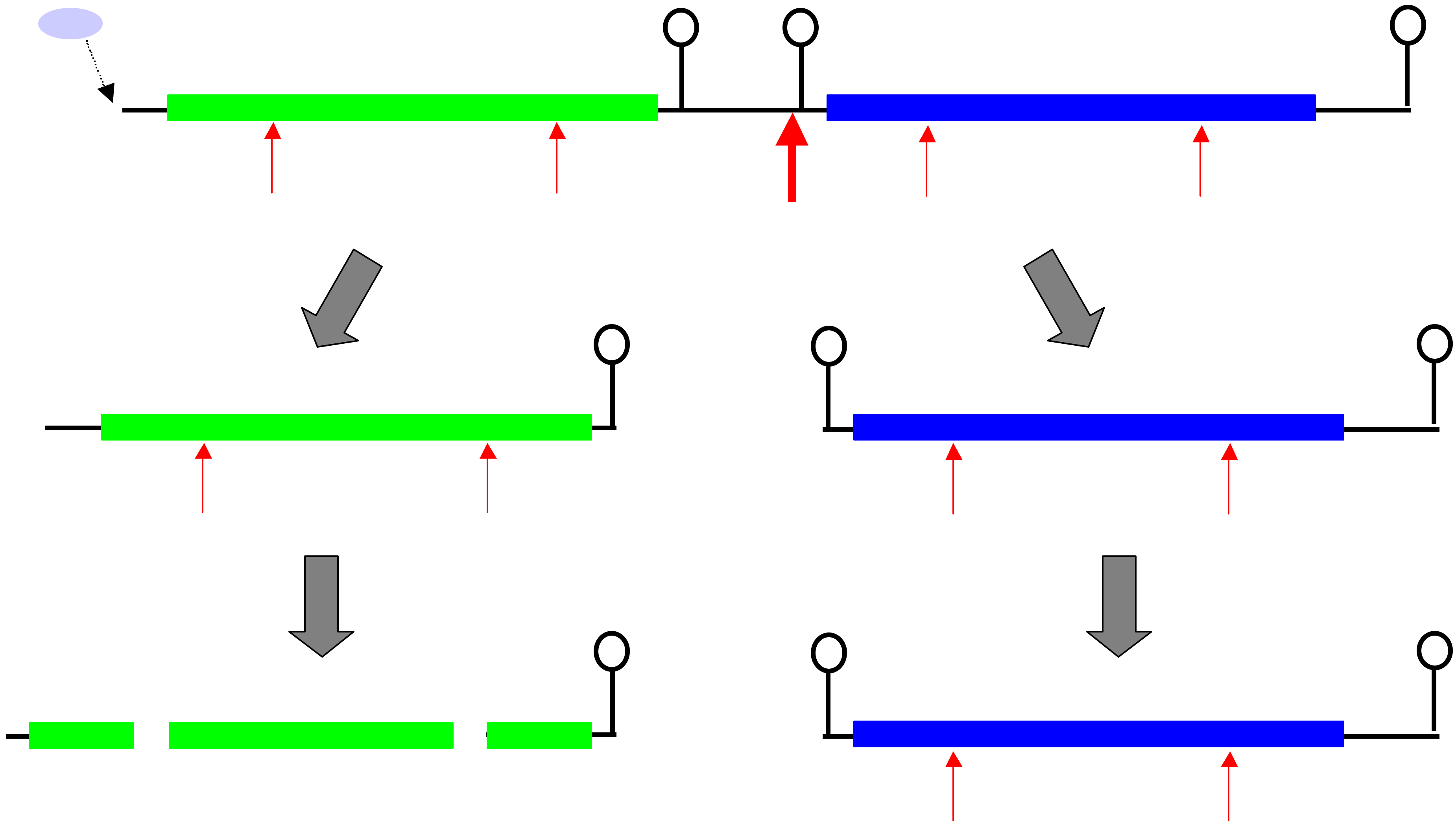
Carotenoid Pathway

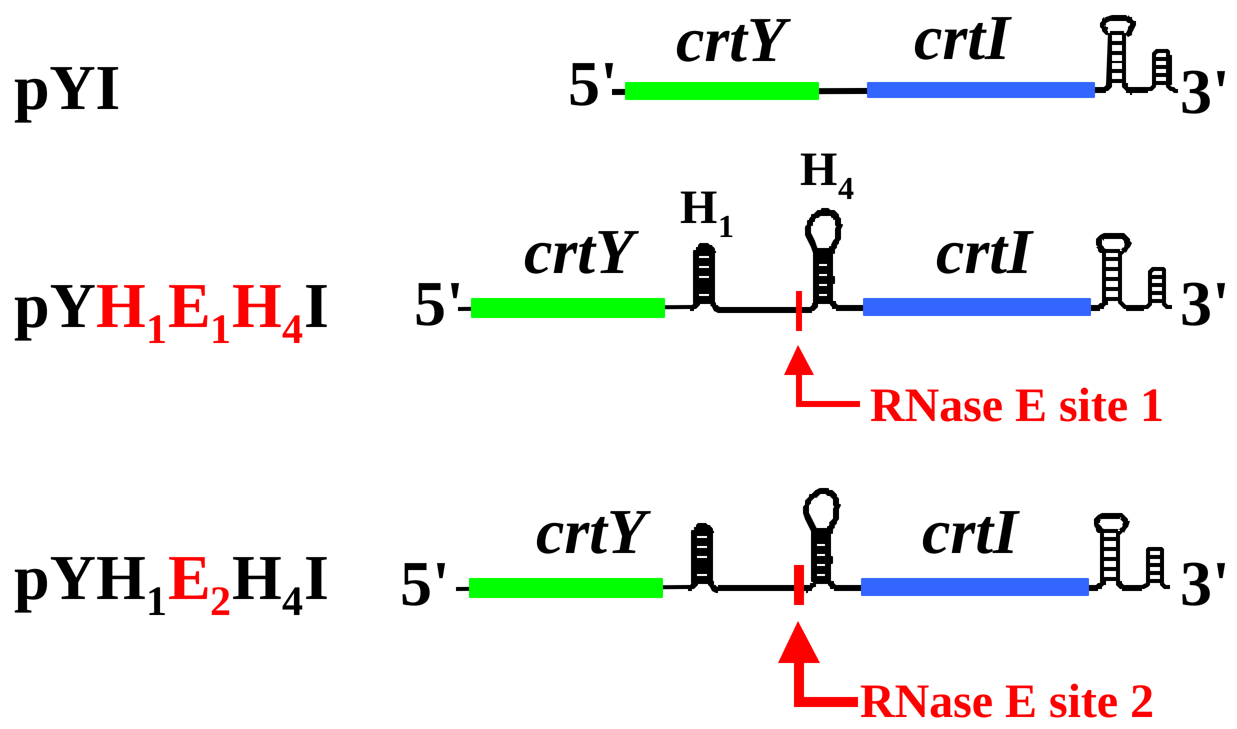


Degradation of multicistronic mRNA

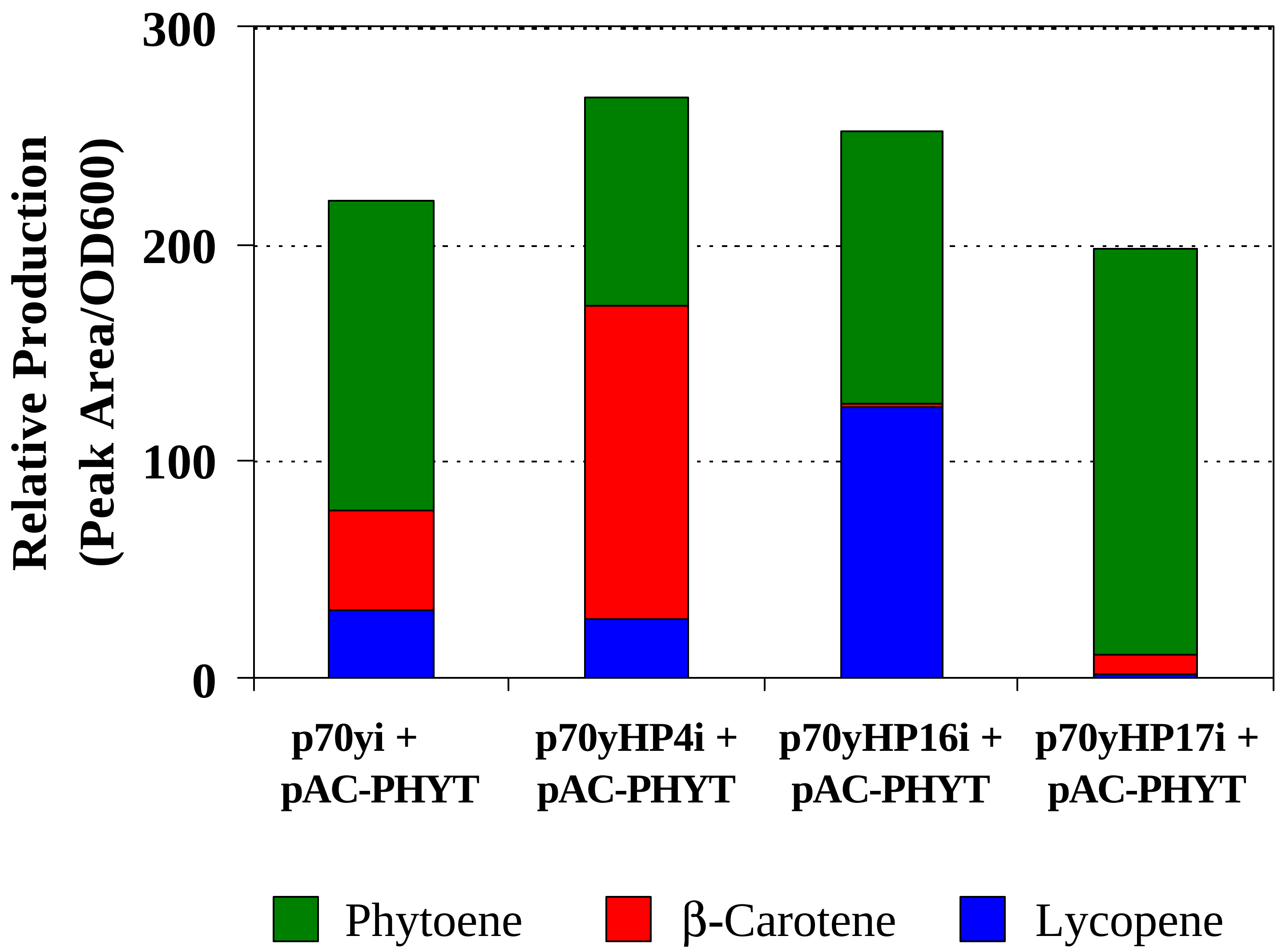


A synthetic operon for carotenoid production

CrtE → Phytoene **CrtI** → Lycopene **CrtY** → β-Carotene

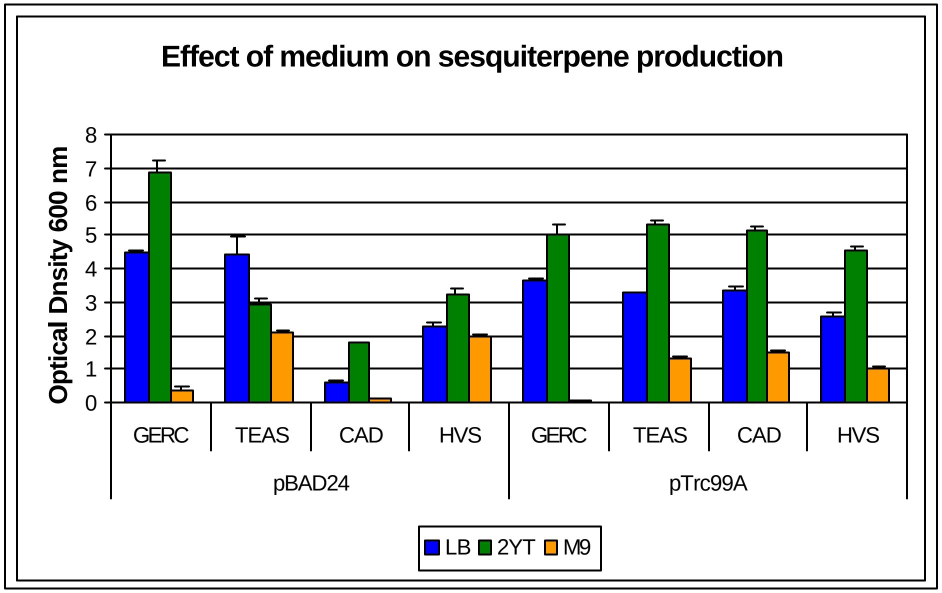


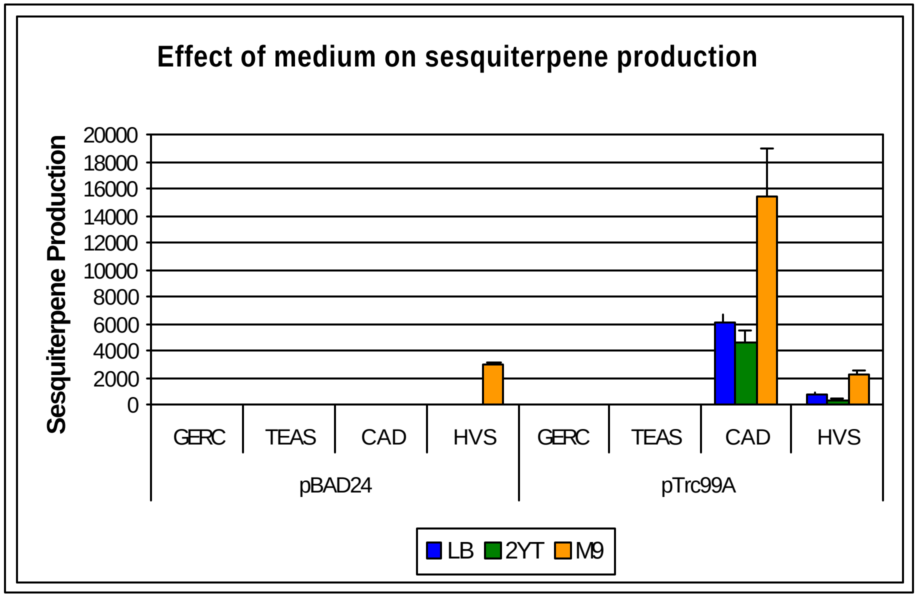
Control of Carotenoid Production

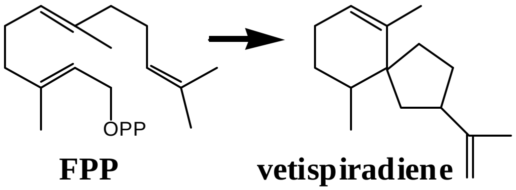


Production of plant sesquiterpenes in *E. coli*

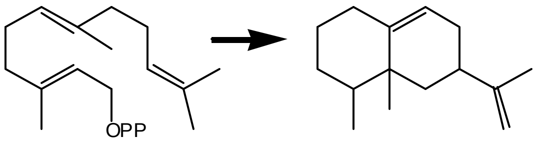
Optimization of culture medium and expression vector



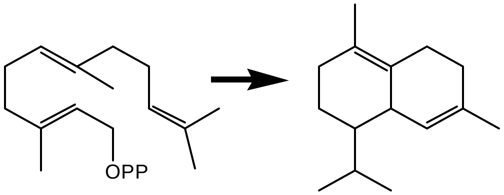




FPP **vetispiradiene**



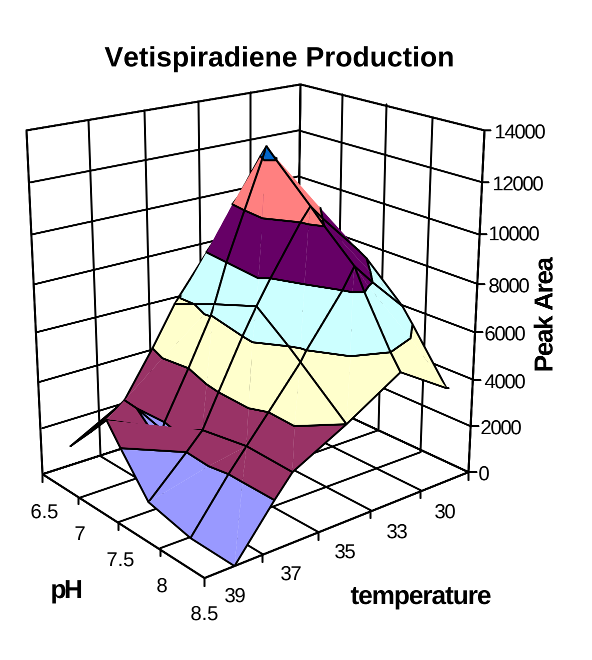
FPP **5-*epi*-aristolochene**

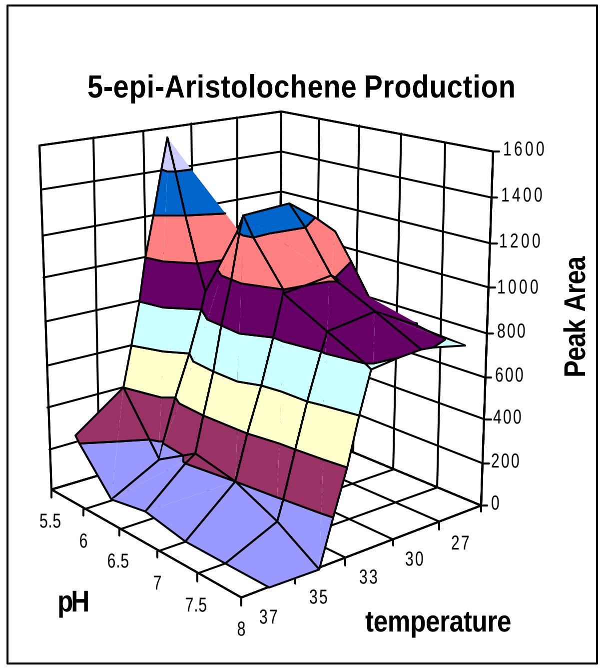


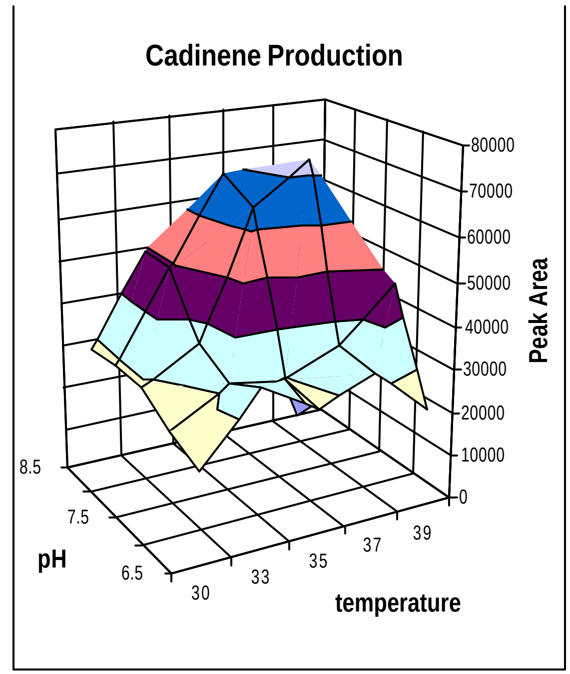
FPP **cadinene**

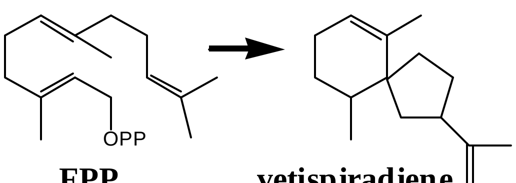
Production of plant sesquiterpenes in *E. coli*

Optimization of pH and Temperature

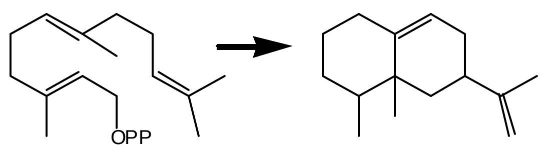




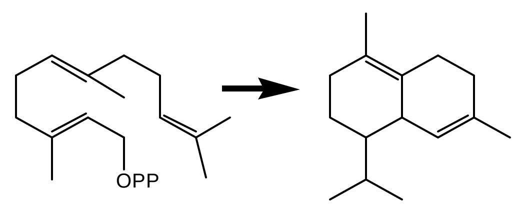




FPP **vetispiradiene**



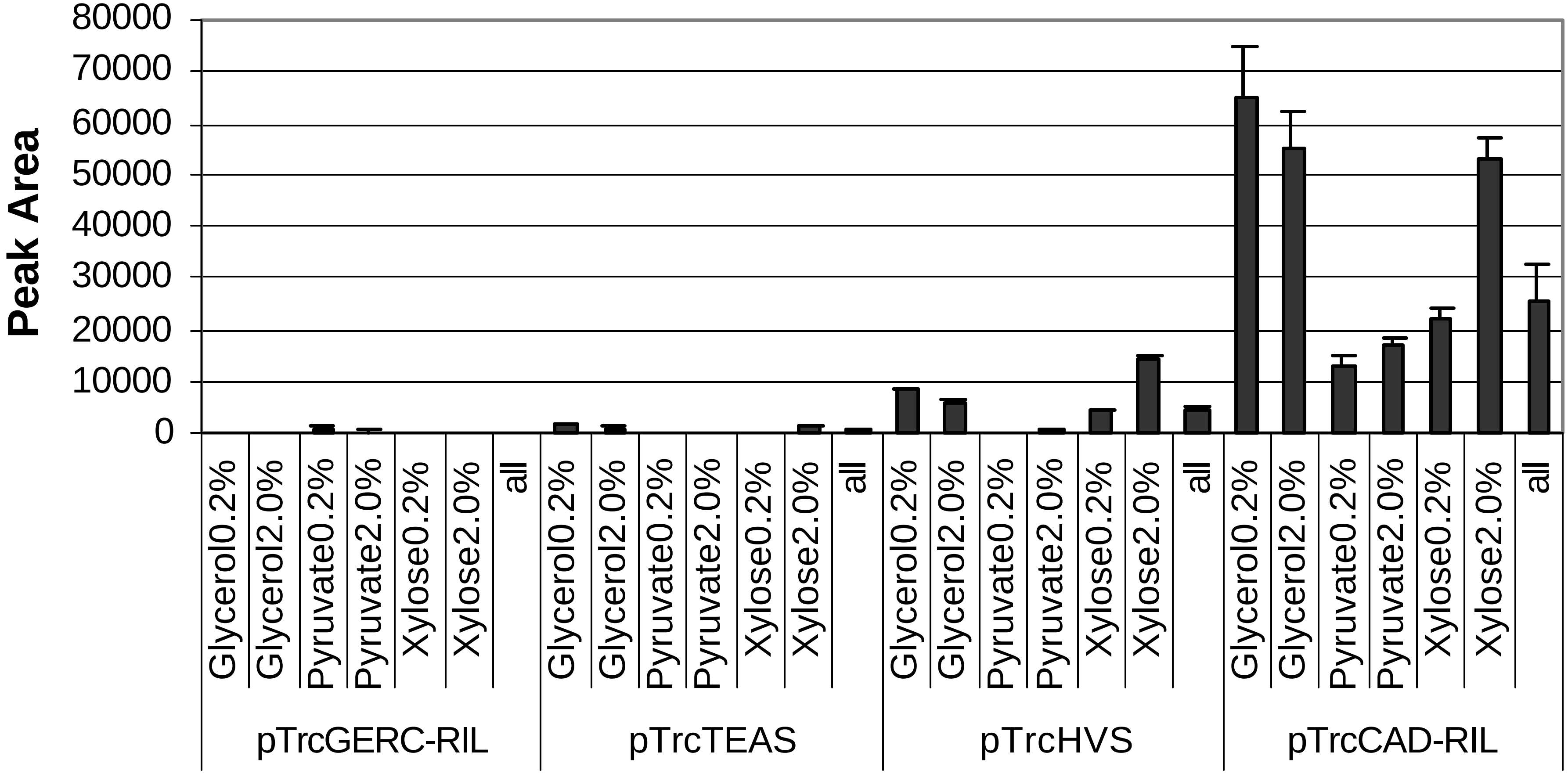
FPP **5-*epi*-aristolochene**



FPP **cadinene**

Production of plant sesquiterpenes in *E. coli*

Optimization of Carbon Source



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