

**Molecular characterization of cytochrome P450
genes in the polycyclic aromatic hydrocarbon
degrading *Mycobacterium vanbaalenii* PYR-1**




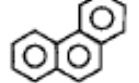
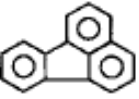
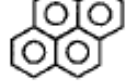
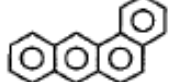
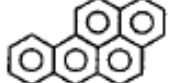
Barbara Brezna, Ohgew Kweon, Robin L. Stigley, James P. Freeman, Ashraf A. Khan, Bystrik Polek, Richard C. Jones and Carl E. Cerniglia

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**Samanthi Kottegoda
Microbiology Journal Club
April 26, 2010**

PAHs....

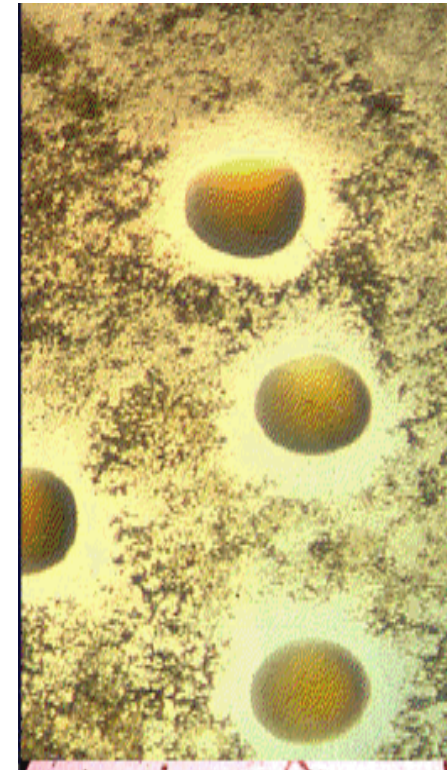
- Organic compounds containing two or more fused benzene rings
- Ubiquitous environmental pollutants
- Can form in a variety of ways . e.g. fossil fuel combustion, coal tar production
- Less soluble in water, but are highly lipophilic
- Recalcitrance increases with molecular weight
- High molecular weight PAHs are genotoxic and carcinogenic

PAH	Solubility mg l ⁻¹	Carcinogenicity
 Naphthalene	31.7	Non-carcinogen
 Acenaphthene	3.9	Non-carcinogen
 Anthracene	0.07	Non-carcinogen
 Phenanthrene	1.3	Non-carcinogen
 Fluoranthene	0.26	Weak carcinogen
 Pyrene	0.14	Non-carcinogen
 Benz[a]anthracene	0.002	Carcinogen
 Benzo[a]pyrene	0.003	Carcinogen

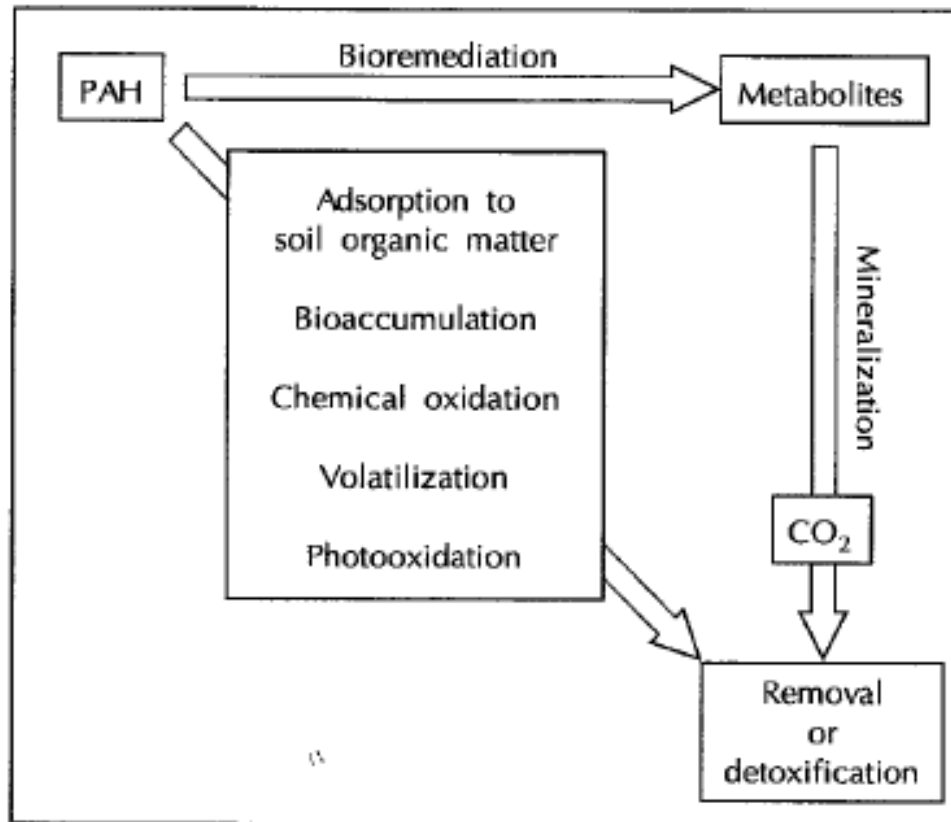
Recalcitrance ↓

Mycobacterium vanbaalenii PYR-1

- Isolated from oil contaminated marine sediment
- Yellow pigmented colonies
- Gram positive and Acid-alcohol fast
- G+C content = 66.7%
- Can utilize a wide range of PAHs
- First bacterium isolated by virtue of its ability to metabolize the PAH pyrene
- Closely related to *M.aurum* and *M.vaccae* (16S rRNA gene sequencing)
- Etymology: vanbaalenii of Van Baalen, in memory of Dr. Chase Van Baalen, late Professor at The University of Texas, Marine Science Institute.



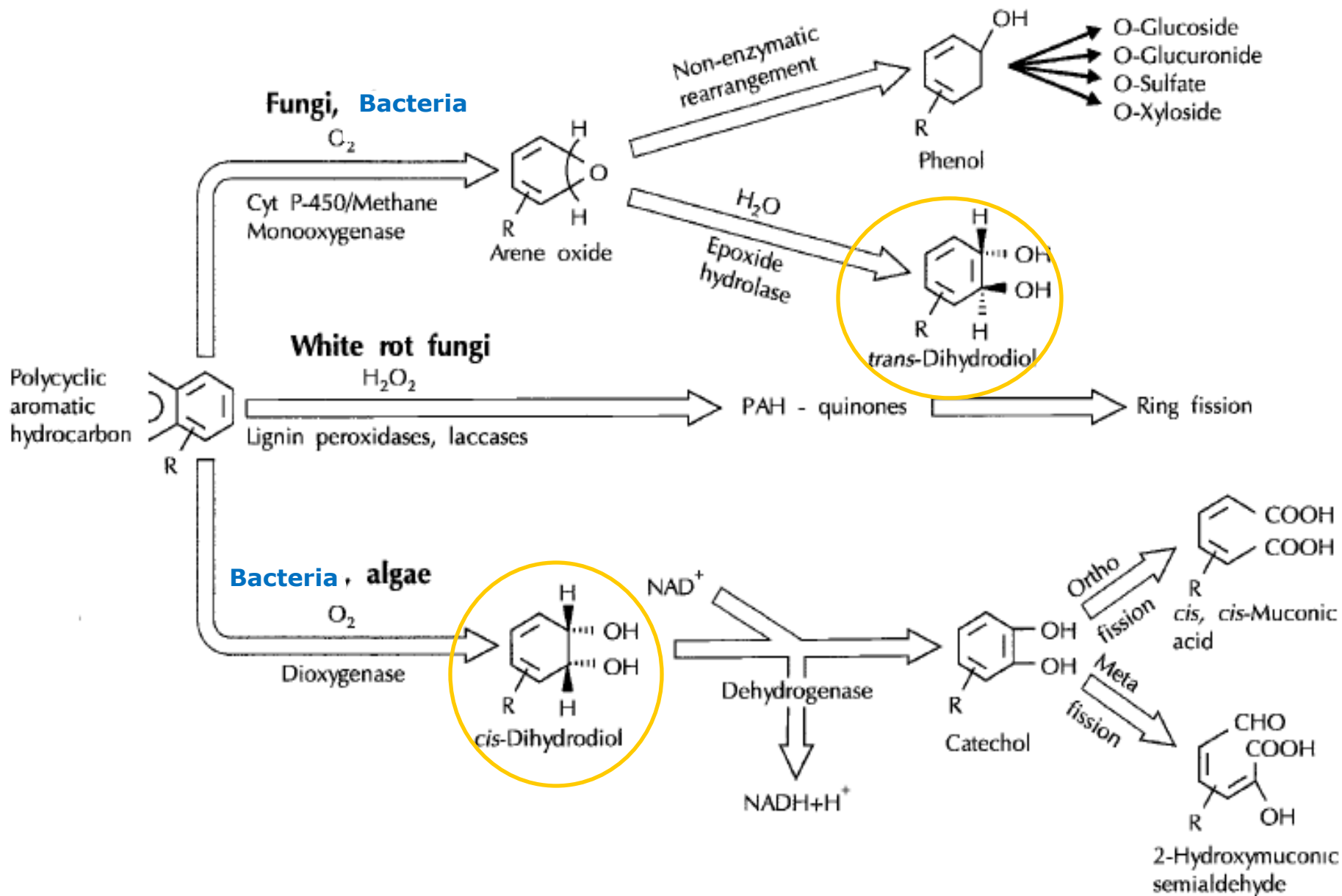
Environmental fate of PAHs



- Many bacterial and fungal species can degrade PAHs including benzo[*a*]pyrene
- High molecular weight PAH degrading bacteria are mostly Gram positive including Mycobacteria

Bacteria Oxidizing PAHs

Bacteria	Naph	Phen	Anth	Pyr	Flu	BA	B[a]P
Acinetobacter	X	X					
Acromobacter	X						
Aeromonas	X	X					
Alcaligenes	X	X	X	X	X		
Arthrobacter		X			X		
Bacillus	X						
Brevibacterium		X					
Comammonas		X	X				
Cycloclasticis	X	X	X				
Flavobacterium		X	X				
Micrococcus		X					
Moraxella	X						
Mycobacterium	X	X	X	X	X	X	X
Norcardia		X	X				
Pasteurella					X		
Pseudomonas	X	X	X	X	X	X	X
Rhodanobacter							X
Rhodococcus	X	X	X		X		
Sphingomonas	X	X	X			X	X
Stenrophomonas		X		X		X	X
Vibrio		X					



Proposed pathway for microbial catabolism of PAHs

Introduction

- *M. vanbaalenii* PYR-1 was the first organism known to produce both *cis*-dihydrodiol and *trans*-dihydrodiol metabolites of high-molecular-weight PAHs such as pyrene.
- Uses both **dioxygenase(s)** and **cytochrome P450 monooxygenase(s)** to metabolize PAHs
- Dioxygenase is encoded by ***nidA*** and ***nidB*** genes (cloned and characterized Previously)
- This study complements the previous information by identifying three genes encoding alternative PAH-oxidative enzymes, cytochromes P450, in this organism

Objectives

- Detect three cytochrome P450 genes, *cyp151 (pipA)*, *cyp150*, and *cyp51*
- Determine the complete sequence of these genes
- Clone and express two of these genes (*pipA* and *cyp150*) in *E.coli* and assess for their ability to oxygenate PAHs
- Screen several other *Mycobacterium* strains for the presence of cytochrome P450 and dioxygenase genes

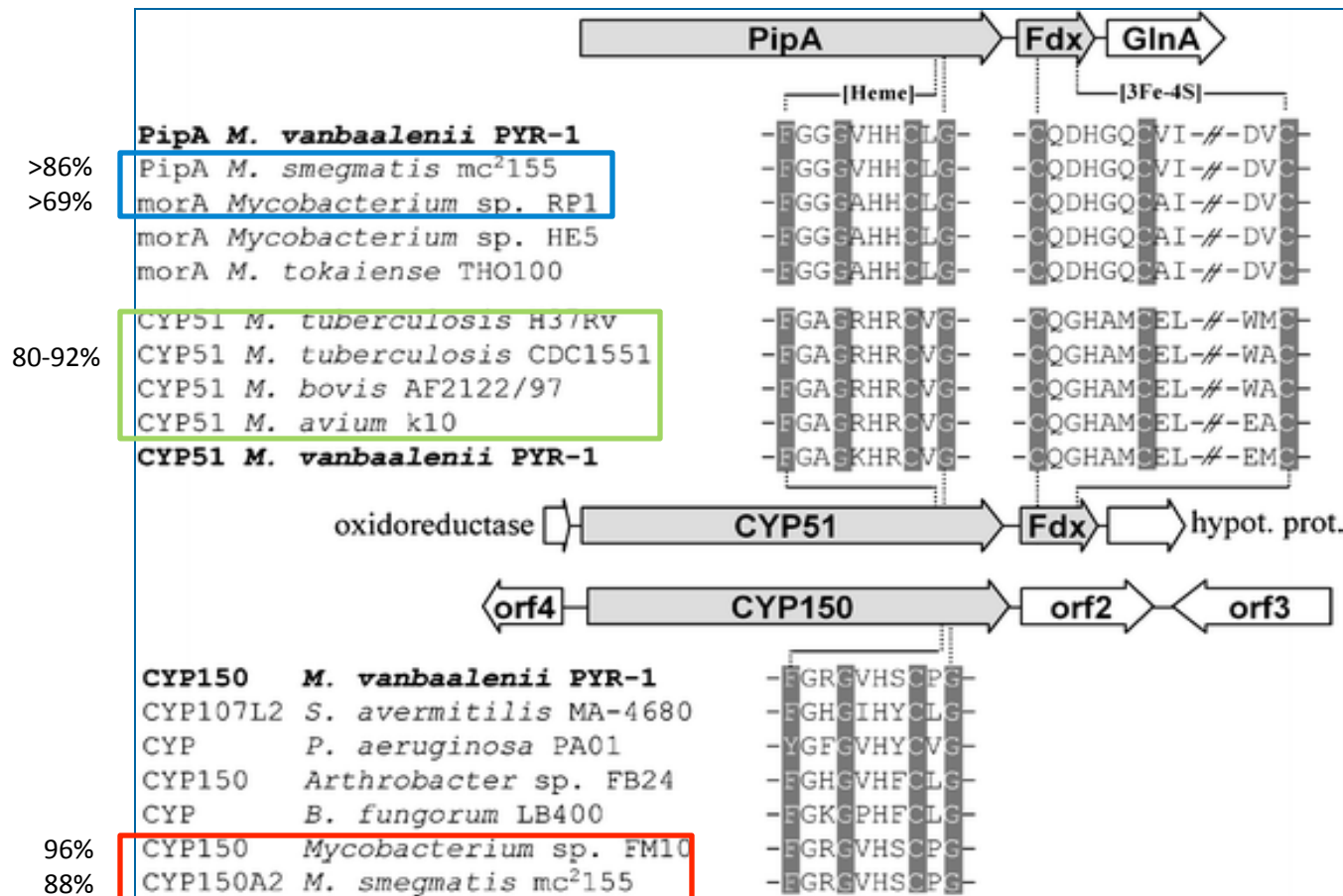
Detection & sequence analysis of cyt. P450 genes

Table 2 PCR primers used in this study

Primer name	Primer sequence	Reference microorganism	Reference sequence	Position	
<i>cyp151 (pipA)</i>	RP1F1	agctggatcctcaacaag	<i>Mycobacterium</i> sp. RP1	AJ310142	1,161–1,178
	RP1F2	tcategcgatcatgctc		1,354–1,370	
<i>cyp150</i>	Fm10F1	ccctacttcgatcacctgcgc	<i>Mycobacterium</i> sp. FM10	AF107046	489–509
	Fm10R2	ccgaacgcgatgtgctcgcg			1,504–1,485
	MSCYP51F1	gggccgatgttccagccg	<i>M. smegmatis</i> mc ² 155	contig3312	1,289,527–1,289,544
	MSCYP51R2	tcgccgagacgcgcgcg			1,291,356–1,291,339
	PipAclonF	<u>acgccatatgtcgtcggccactgtcggttctctgc</u> ^{a,b}	<i>M. vanbaalenii</i> PYR-1	AY485998	1–27
	PipAclonR	<u>agctaagcttcaatggtgatggtgatggtggaa</u> <u>gcgggcgtgaagccga</u> ^{a,c}			1,200–1,181
Cyp150clonF	<u>acgccatatgagcgcacttcgacacgatcgactac</u> ^{a,b}	<i>M. vanbaalenii</i> PYR-1	AY496703	322–348	
Cyp150clonR	<u>agctaagcttcaatggtgatggtgatggtgtcga</u> <u>accggggtgaacgtga</u> ^{a,c}			1,589–1,608	
<i>cyp51</i>	Cyp51F	cgacggcctgcctgatcg	<i>M. vanbaalenii</i> PYR-1	AY575951	507–524
	Cyp51R	tcctcggggatccggttg			1,137–1,120

- PCR screening of *M. vanbaalenii* PYR-1 genomic DNA for *pipA*, *cyp151* and *cyp51* genes gave expected PCR products sizes, 0.25, 1.0 and 1.8 kb, respectively.
- Screening of *M. vanbaalenii* PYR-1 genomic library with DIG-labeled versions of these PCR products confirmed the results.
- Preliminary sequencing of PCR products confirmed that they were indeed parts of the targeted cyp isogenes

Detection & sequence analysis of cyt. P450 genes

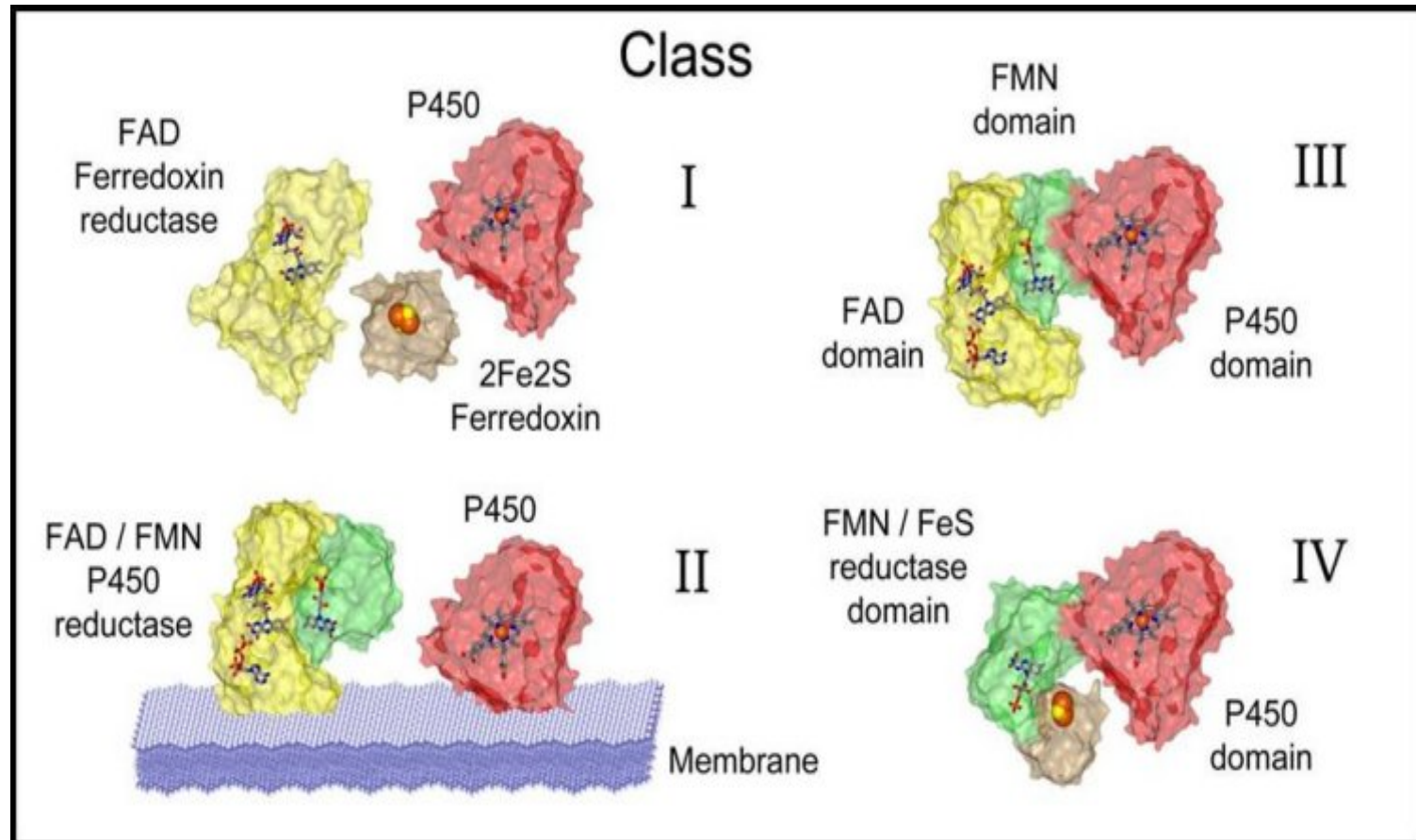


Physical maps and conserved sequence alignments of the cytochrome P450 monooxygenases and ferredoxins from *M. vanbaalenii* PYR-1 with those from other sources

Designations *CYP151* (*PipA*), *CYP150*, and *CYP51*—cytochromes P450
GlnA—putative glutamine synthetase
orf4—probable regulatory protein from TetR-family

Fdx—ferredoxins
orf2 and *orf3*—hypothetical proteins

Classes of the P450 Superfamily

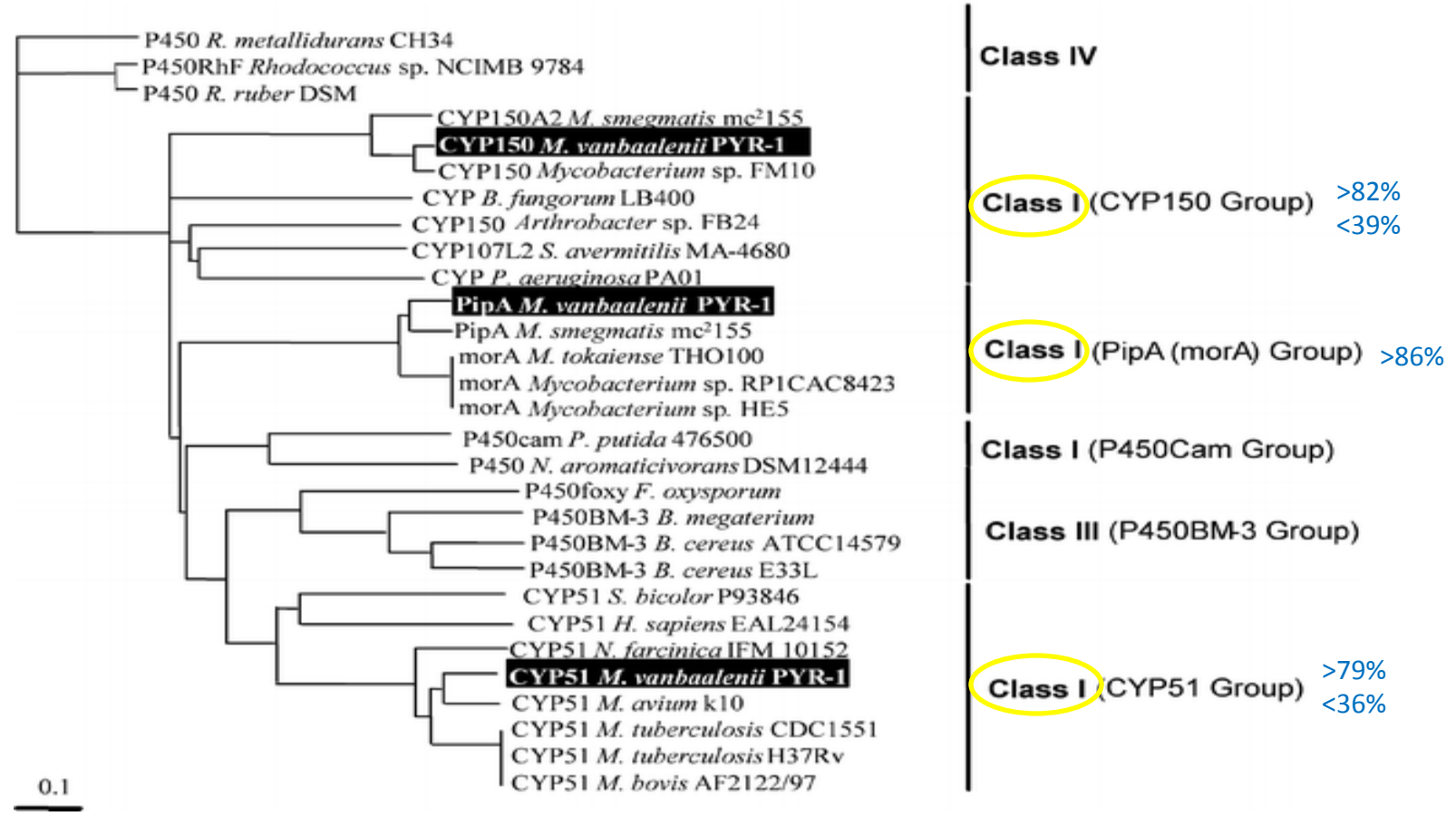


Class I - three-component systems

Class II - two-component systems

Class III and IV - single polypeptides

Phylogenetic tree obtained from the alignment of three cytochrome P450s from *M. vanbaalenii* PYR-1 with related proteins

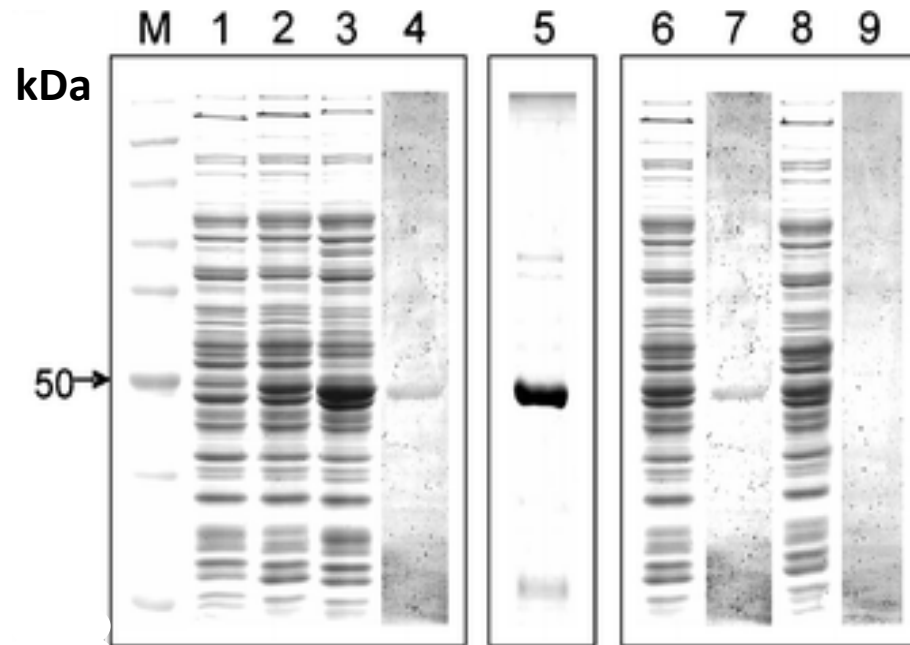


Protein sequences of the 29 cytochrome P450s are classified

Expression, purification, and identification of PipA and CYP150

- Previously constructed fosmid genomic library of *M. vanbaalenii* PYR-1 was used.
- Colonies of library clones were transferred to nylon membranes
- *pipA*- or *cyp150*-containing clones were identified by colony hybridization with *pipA*- or *cyp150*-specific DIG-labeled DNA probes, respectively
- One positive fosmid clone (for each cytochrome gene) was selected and digested
- Restriction fragments were subcloned into pGEM-11zf(+), and the resulting subclones were rescreened by colony hybridization
- *pipA*- and *cyp150*-containing subclones were named pGEM-PIP and pGEM-CYP, respectively, and were sequenced.
- Amplified genes with proofreading PCR (6-His-tag codon was incorporated in one of the primers)
- Subcloned into expression vector pET-17b, resulting in plasmids **pET-17b-PIP** and **pET-17b-CYP**
- Transformed into NovaBlue *E.coli* host strain and subsequently retransformed into BL21(DE3)pLysS host strain and expressed proteins
- 6xHis-tagged proteins were purified from the total soluble protein fractions using Ni-NTA resin

Expression and purification of recombinant PipA of *M. vanbaalenii* PYR-1



Lane 1 - cell extract from *E. coli* (BL21)(pET-17b)

Lane 2 - cell extract from *E. coli* (BL21)(pET-17b-PIP) - glass bead cell disruption

Lane 3 - cell extract from *E. coli* (BL21)(pET-17b-PIP) - boiling

Lane 4 - heme-stain of the same cell extract as *lane 2*

Lane 5 - partial purification of 6xHis-tagged PipA on Ni-NTA resin

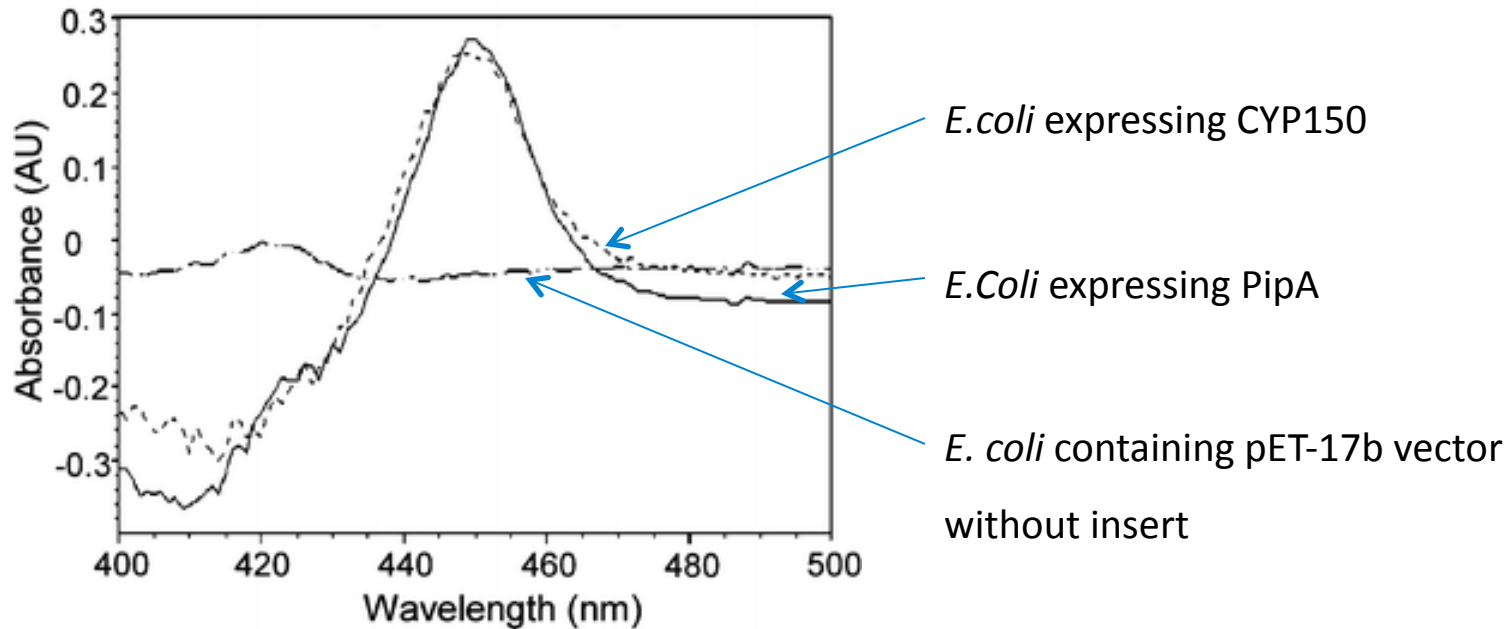
Lane 6 - Coomassie blue stained cell extract from *E. coli* (BL21)(pET-17b-PIP) -in the presence of ALA(aminolevulinic acid) and FeCl₃

Lane 7 - heme-stain of *lane 6*

Lane 8 - Coomassie-blue-stained cell extract from *E. coli* (BL21)(pET-17b-PIP) - in the absence of ALA and FeCl₃,

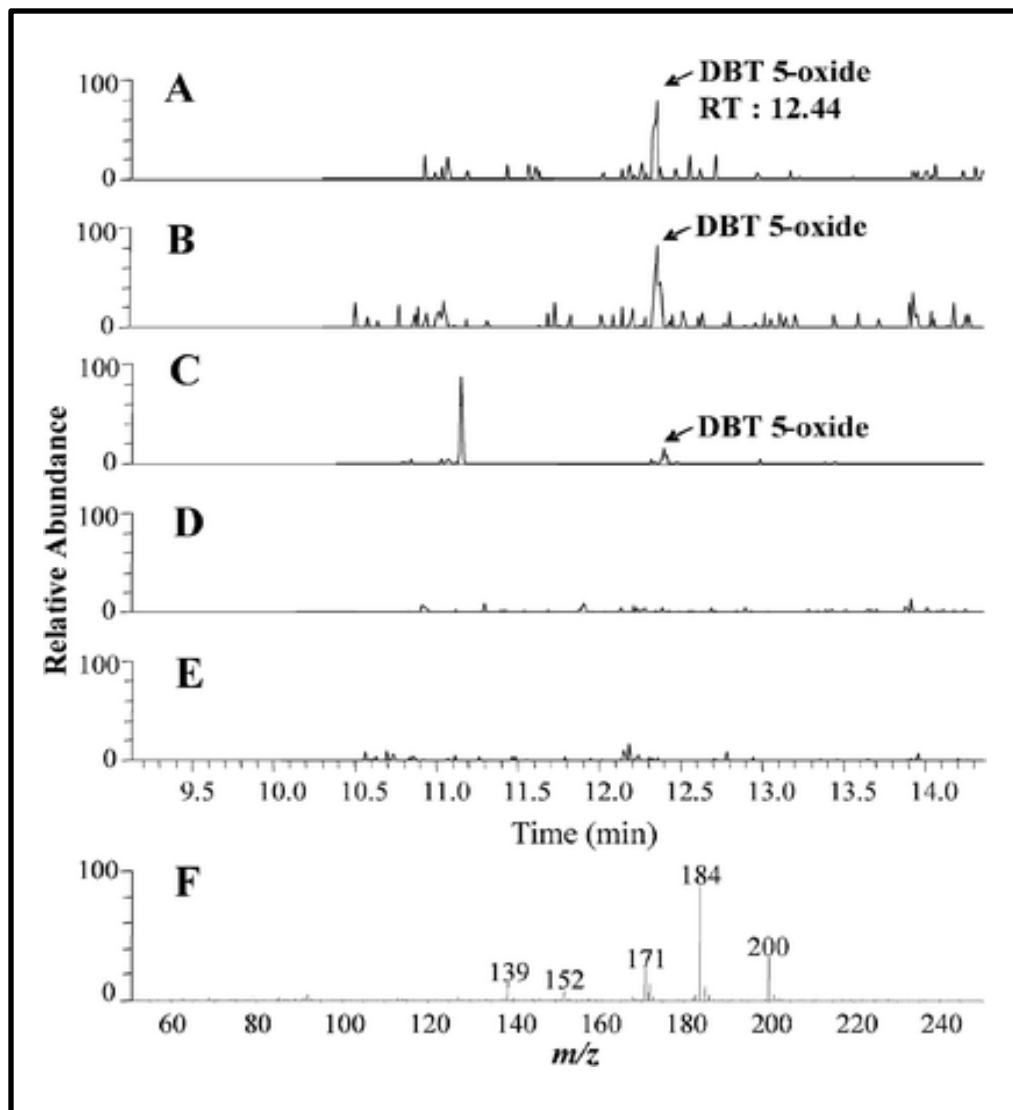
Lane 9 - heme-stain of *lane 8*

Spectrophotometric analysis of cytochrome P450



Reduced CO-difference spectra of soluble protein cell extracts from *E. coli* expressing PipA and CYP150 showed a typical peak at **450 nm**, confirming the cytochrome P450-like character of these proteins

Biotransformation Experiments



(A)CYP150

(C)PipA

(E)PipA + metyrapone (inhibits P450)

(G)apo-PipA (cells were grown in the absence of ALA & FeCl₃)

(E) negative control

and

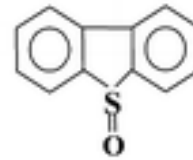
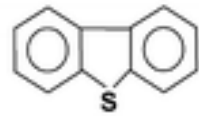
(F) mass spectrum of DBT 5-oxide

Cytochrome P450s involve in PAH metabolism in *M. vanbaalenii* PYR-1

GC/EI-MS extracted ion chromatograms of DBT extracts

Biotransformations

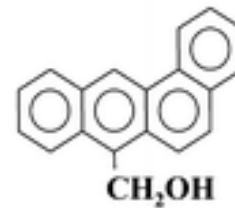
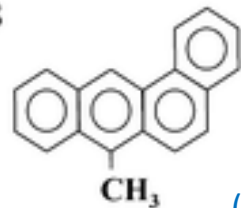
A



dibenzothiophene (DBT)

5-oxide dibenzothiophene

B



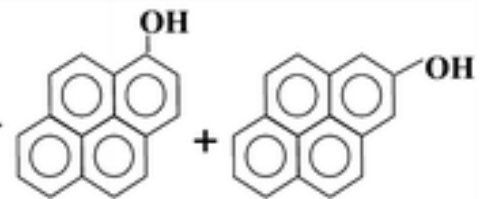
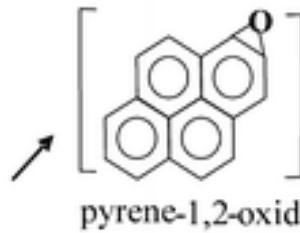
7-methylbenz[a]anthracene

7-hydroxymethylbenz[a]anthracene

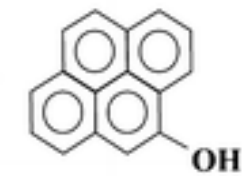
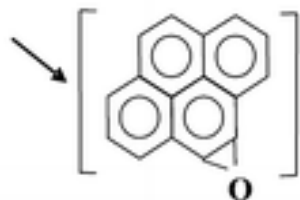
C



pyrene



1-hydroxypyrene 2-hydroxypyrene



4-hydroxypyrene

Table 1 Mycobacterium strains used in this study that were screened for the presence of cytochrome P450 genes and *nidA* and *nidB* genes

Strain	Substrate or other characteristic	Detection of				
		<i>nidA</i> ^c	<i>nidB</i> ^d	<i>pipA</i> ^e	<i>cyp150</i> ^f	<i>cyp51</i> ^g
<i>M. aurum</i> (ATCC 23366)	Type strain	(-)	(-)	-	+	+
<i>M. austroafricanum</i> (ATCC 33464)	Type strain, related to <i>M. vanbaalenii</i>	(-)	(-)	-	+	+
<i>M. austroafricanum</i> GTI-23 ^a	PAHs	+	+	+	-	-
<i>M. chlorophenicum</i> PCP-1 (ATCC 49826)	Polychlorinated phenols	(-)	(-)	-	-	+
<i>M. flavescens</i> PYR-GCK (ATCC 700033)	PAHs	(-)	(+)	+	+	-
<i>M. frederiksbergense</i> FAn9T (DSM 44346)	PAHs	(-)	(+)	+	+	+
<i>M. gilvum</i> (ATCC 43909)	Type strain	-	-	-	+	-
<i>M. gilvum</i> BB1 (DSM 9487)	PAHs	(+)	(+)	+	+	-
<i>M. petroleophilum</i> (ATCC 21497)	n-Paraffins	(-)	(-)	-	+	+
<i>M. smegmatis</i> mc ² 155 (ATCC 700084)	Transformation host	-	-	-	+	+
<i>M. vaccae</i> JOB-5 (ATCC 29678)	Gaseous, long chain, cycloparaffinic and monoaromatic hydrocarbons	(-)	(-)	-	+	+
<i>M. vanbaalenii</i> PYR-1(DSM 7251)	PAHs	(+)	(+)	+	+	+
<i>Mycobacterium</i> sp. 7E1B1W (ATCC 29676)	Gaseous and long chain hydrocarbons	(-)	(-)	-	+	-
<i>Mycobacterium</i> sp. PAH 2.135 (RJGII-135) ^b	PAHs	(+)	(+)	+	-	+

- According to PCR results, *pipA*, *cyp150*, and *cyp51* detection varied among the strains and exhibited no correlation with the strains' PAH-degrading ability.
- In contrast, the alternative PAH-oxygenation enzyme, dioxygenase encoded by *nidA* and *nidB* genes, was consistently present in PAH-utilizing mycobacteria and absent in PAH nonutilizers
- ❖ It seems that the genes *nidA* and *nidB* are specialized for the degradation of PAHs, whereas the primary role of *pipA*, *cyp150*, and *cyp51* is different and the PAH monooxygenation is only a fortuitous or nonspecific reaction.

Conclusions

- The three CYPs detected in *M.vanbaalenii* PYR-1 were >80% identical to other mycobacterial CYP151, CYP150, and CYP51, respectively. (PCR results)
- Cytochrome P450s involve in PAH metabolism(biotransformations)
- Dioxygenases are specialized in the degradation of PAHs(PCR screening)
- *M.vanbaalenii* PYR-1 uses both dioxygenases and monooxygenases to utilize PAHs.
- All of the strains that contained *nidAB* genes possessed at least one of the studied CYP isogenes.(PCR screening)
Thus, several mycobacteria have a potential to use both dioxygenation and monooxygenation reactions for initial biotransformation of PAHs.