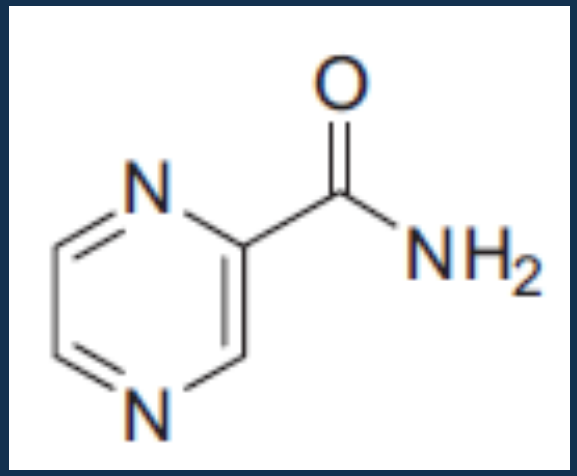
The Essentiality and Mystery of Pyrazinamide



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Content

- Historic notes
- The PZA paradox, the difficulties
- Simulations in vivo by in vitro conditions?
- PZA resistance and susceptibility
- The unique susceptibility of TB to PZA
- PZA analogs, what did we learn?
- In search of the actual site of action
- Summary

PZA history, the beginning

- 1936 Dalmer O& Walter E — Synthesis of PZA
- 1945 Chorine V — Nicotinamide efficacy TB in mice
- 1952 Kushner S — Testing PZA in mice
- 1952 Yeager et al. — First Clinical Use

The Cornell group contribution

- 1954 McDermott W& Tompsett R — Activation of PZA in acidic environment
- 1956 Mackaness GB — Activity of PZA in macrophages
- 1956 McCune RM — The model of TB latency and sterilization.
- 1967 Konno K et al. — Resistance to PZA loss of PZAse NAMase activity

Introduction of PZA into clinical studies and practice

- 1970s-1980s British Medical Research Council Mitchison D — PZA allows short course chemotherapy

Formal guidelines (ATS) for TB therapy include PZA for first two month with INH and RIF

- 1988-1991 Salfinger M Heifets LB — Standardized broth test for PZA

PZA resistance, unique activity and prospect

- 1991-1995 Welch J, Cynamon M H — Synthesis and testing of several PZA analogs
- 1996 Scorpio A, Zhang Y — Identification of *pncA* gene encoding pyrazinamidase
- 1999 Zhang Y Scorpio A — Role of acidic pH PZA unique activity in MTB

The mystery of PZA?

Why PZA is so Mysterious or what makes its study so difficult?

- Discrepancy between the sterilizing activity in animal models and human and in–vitro activity. "The PZA paradox"
- Poor activity: Inoculum effect, lack of bactericidal activity
- Acid medium dependent activity
- Difficult to assess susceptibility/resistance

The Implication of poor activity for PZA Studies.

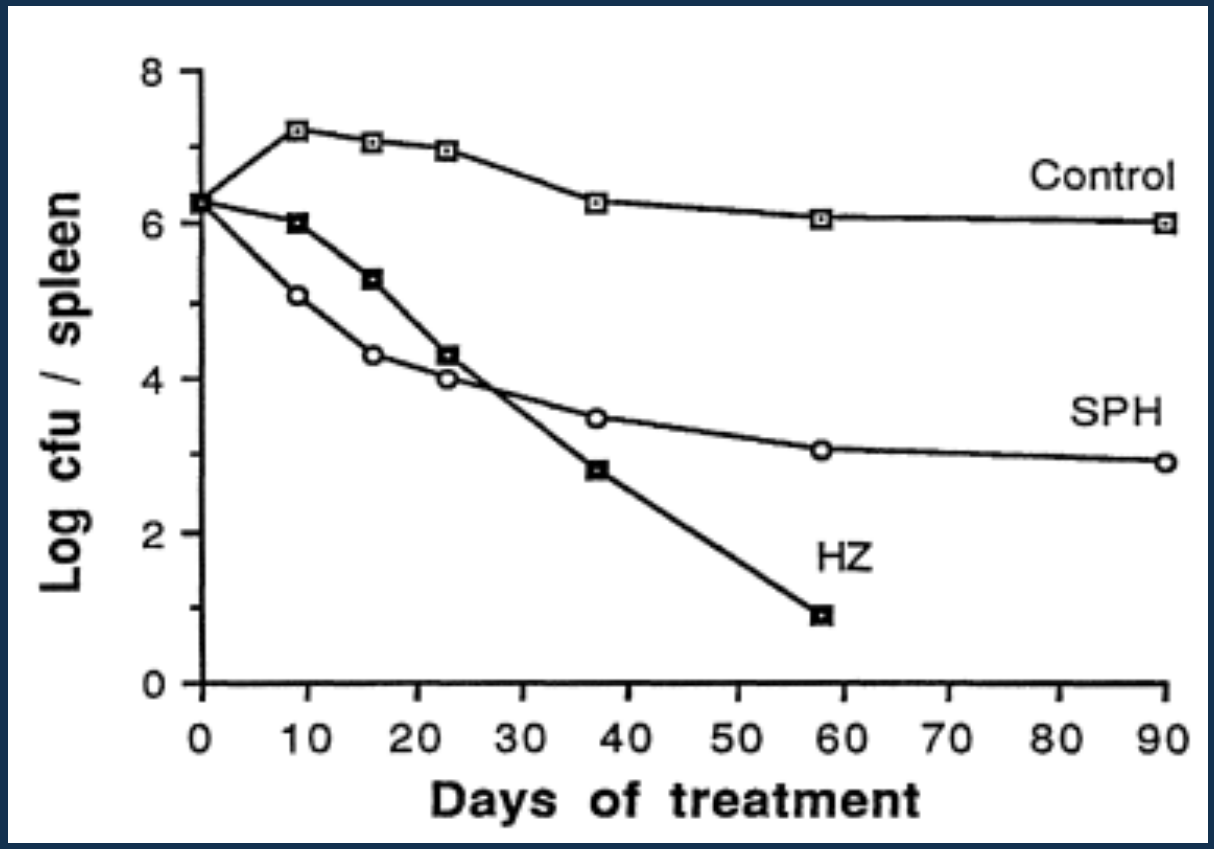
Does PZA is given and is reaching sufficient c% for an expected effect ?

In drug susceptibility tests

In physiologic/ biochemical tests

Animal and clinical studies

Sterilizing Effect of PZA in Murine Model (McCune R M, et al. JEM 1956)



"The fate of Mycobacterium tuberculosis in mouse tissues as determined …"
H isoniazid S streptomycin , P Para amino salicylic acid, Z pyrazinamide

The Cornell Model Studies(McCune RM , McDermott W 1956-1965).

- PZA is indispensable for tissue sterilization
- Sterilized/cured mice relapsed
- The remaining bacilli " Persisters" remained fully susceptible to the drugs.
- Extension of drug therapy from 12w to 26w resulted in lasting cure.

Interpretation of the Cornell Model Results

- "This complete disappearance of TB bacilli meets the definition of a truly *latent* infection … is hidden beyond the limits of diagnostic reach"

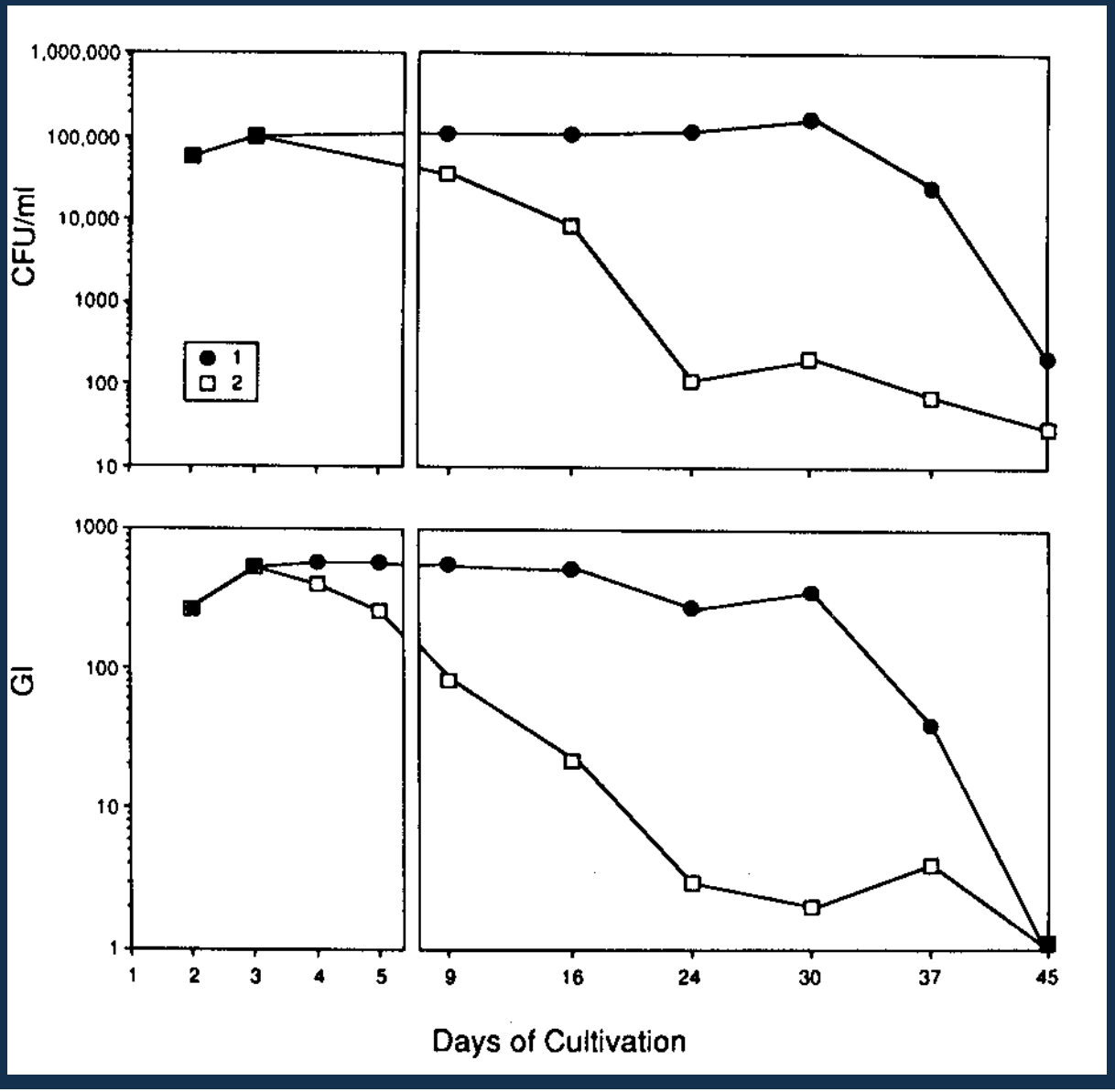
- Does the state of latency implies "dormancy " "semi dormancy"?

- What is meant by dormancy in vitro?

How relevant are in-vitro Conditions and Simulation to Explain the PZA Paradox ?

- Where do we find acidic pH in the context of TB , the phagosome ? caseating granuloma?
- Does causing latency through PZA imply activity against “ dormant” or semi dormant “ bacilli
- Does treating latent TB(clinical setting) imply activity against semi/ dormant bacilli?

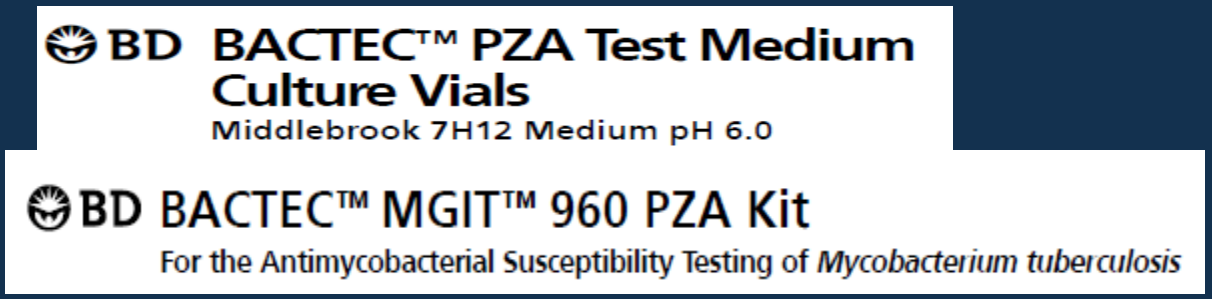
Activity of PZA Against Replicating vs. Non Replicating Bacilli "Semi-Dormant"? .



In favor of PZA Effect against Replicating Bacilli

- PZA is effective therapeutically when given early.
- In Ex-vivo model : A window of drug susceptibility that coincides with the onset of the T-cell-mediated immune response
- ALL Susceptibility tests for PZA recommend usage of freshly diluted replicating bacilli.

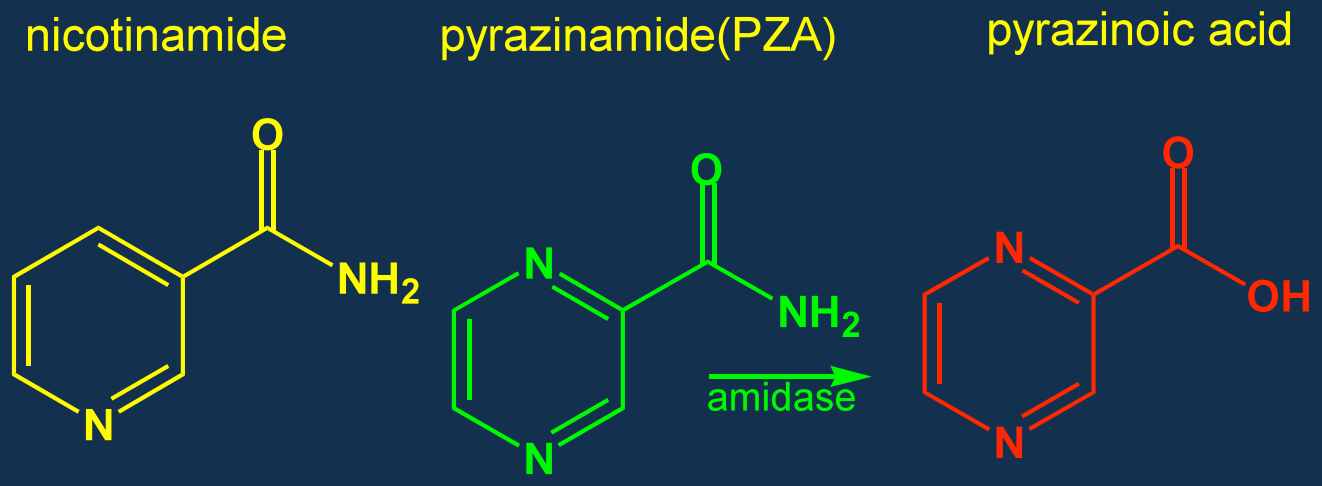
Susceptibility Tests, replicating Bacilli at the right Inoculum



Medium and specimen preparation: "cultures of *M. tuberculosis should be freshly grown in …..should be used for this test* when they are in an active growth phase. Do not use old, refrigerated cultures or cultures which have shown peak GI readings for more than one day."

"Scrape with a sterile loop as many colonies as possible from growth no more than fourteen days old"

Susceptibility and Resistance to PZA .



- Principle mechanism of resistance lack of PZAase activity due to *pncA* loss of function mutations
- Reliable / reproducible test for R/S is challenging

  False resistance, a major problem

Susceptibility and Resistance to PZA(2)

- Tests for PZA susceptibility/ resistance:

  1.Culture methods plates and broth tests

  2. Nicotinamide susceptibility to NAM at 5000µg/ml pH 7 !!! Tan Thiam Hok's test (1962)

  3. Enzymatic tests for PZAse activity, Wayne test(1974)

Susceptibility and Resistance to PZA(3)

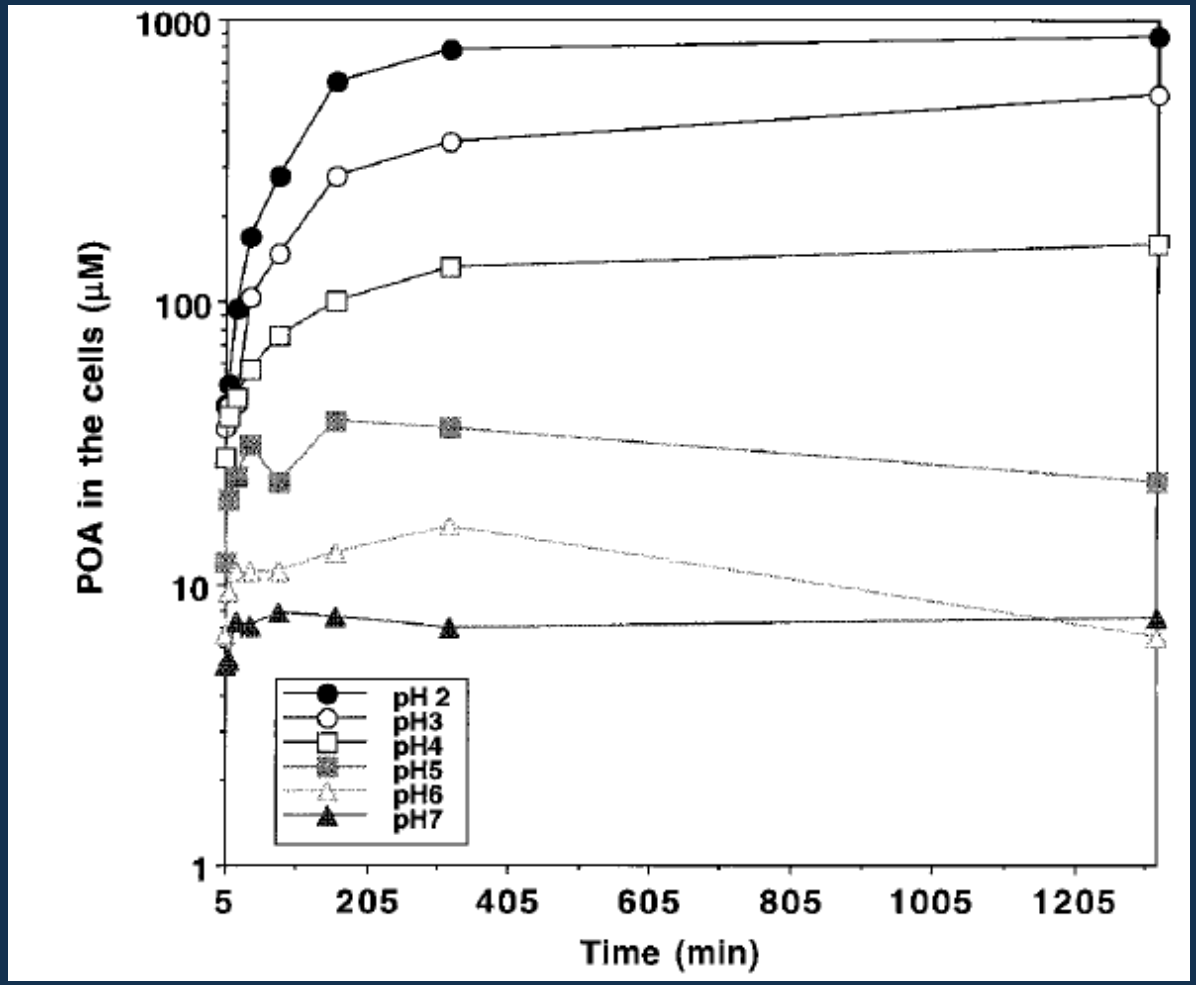
4. Sequence of *pncA*

- Discrepancy in *pncA* mutations rate between studies
- PZAse -, no *pncA* mutations
- Alternative mechanism? PZAase +
  Not excluded rare

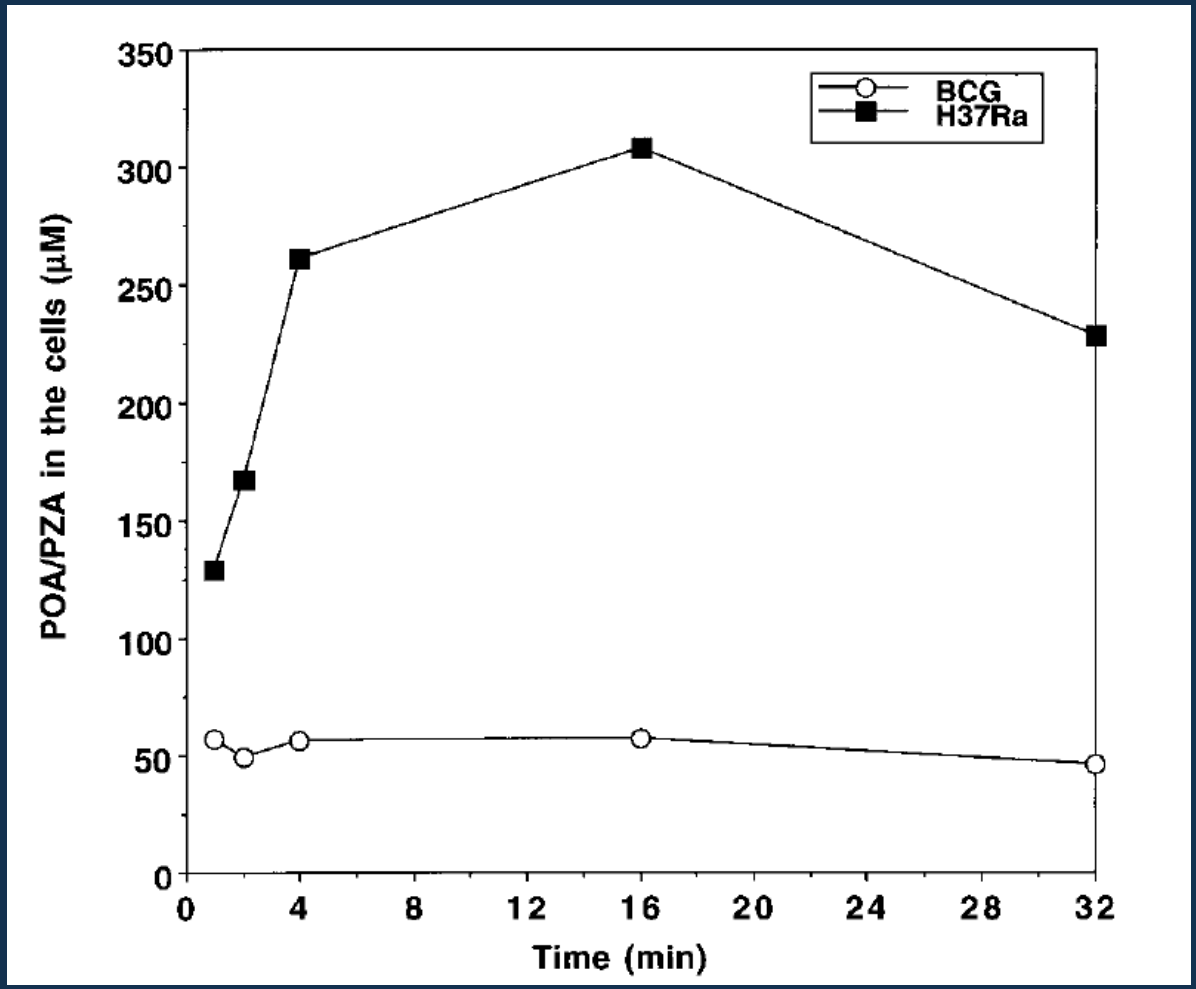
PZA Conversion and Accumulation.

Studies using ^{14}C PZA

Effect of pH on [14^{C}]POA accumulation in *M. tuberculosis.*



The Role of PncA in POA Accumulation

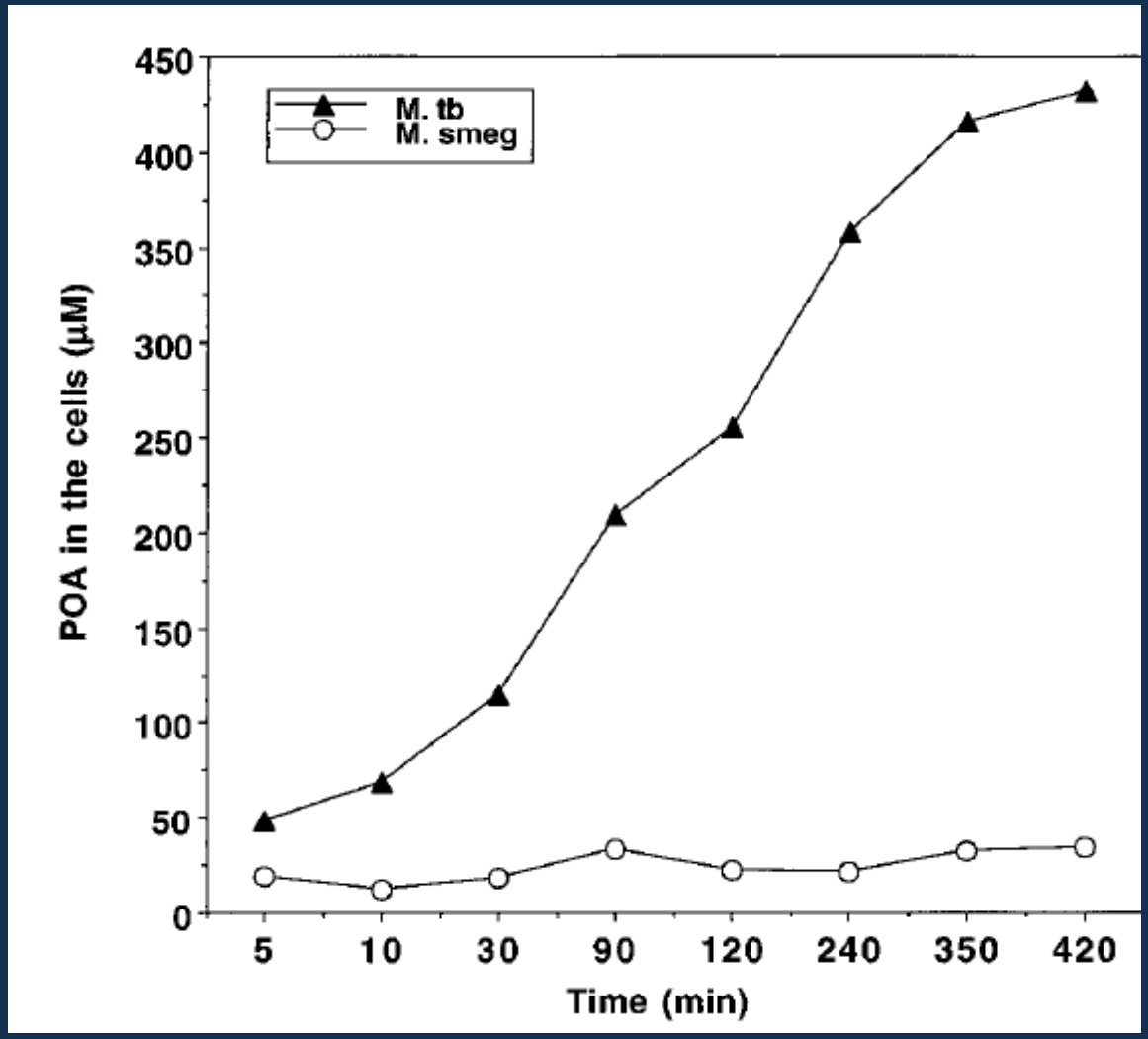


What Underlies the Unique Susceptibility of TB to PZA?

- Mycobacteria species that are proficient in PZAse yet PZA resistant.

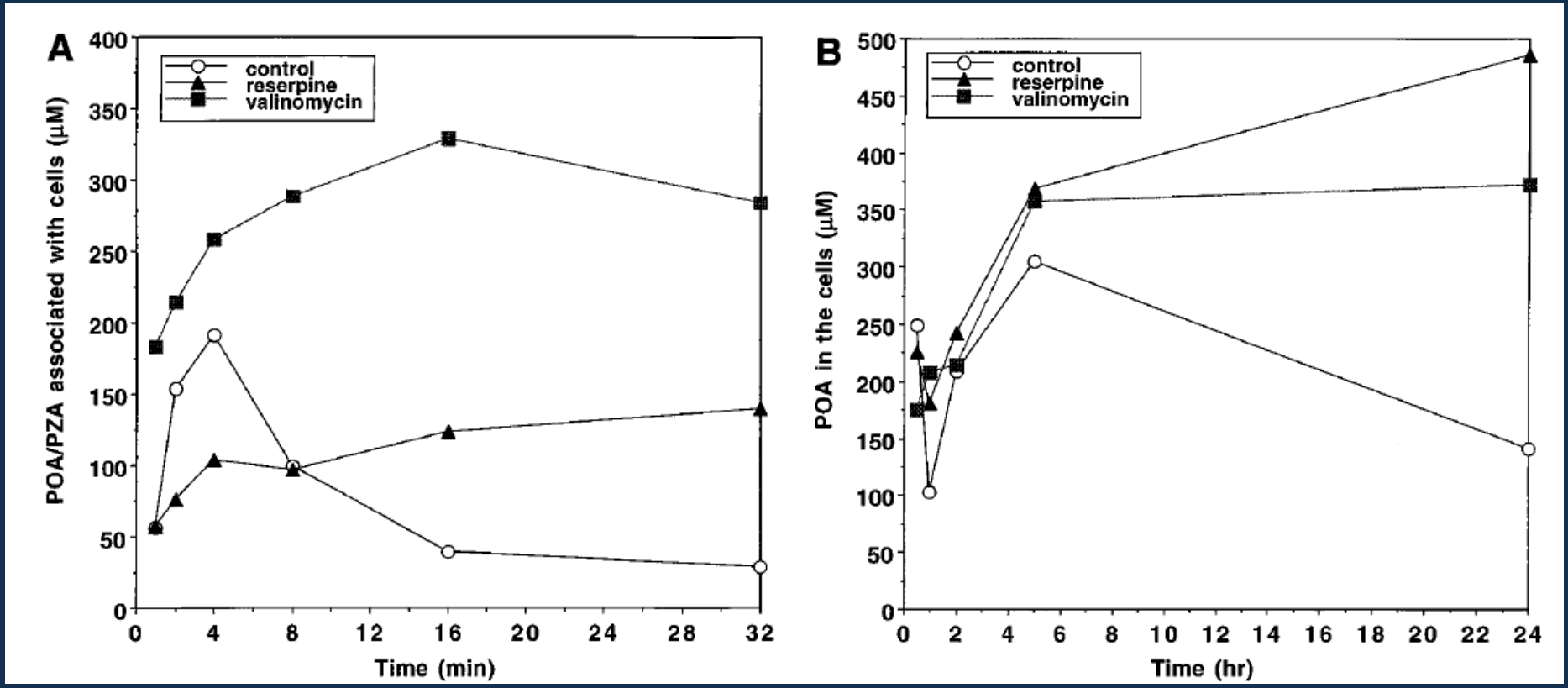
- MSMG possess two PZAses PncA and PzaA and is PZA R, MIC >2000 µg.

The Unique Susceptibility of TB, more than PZAse



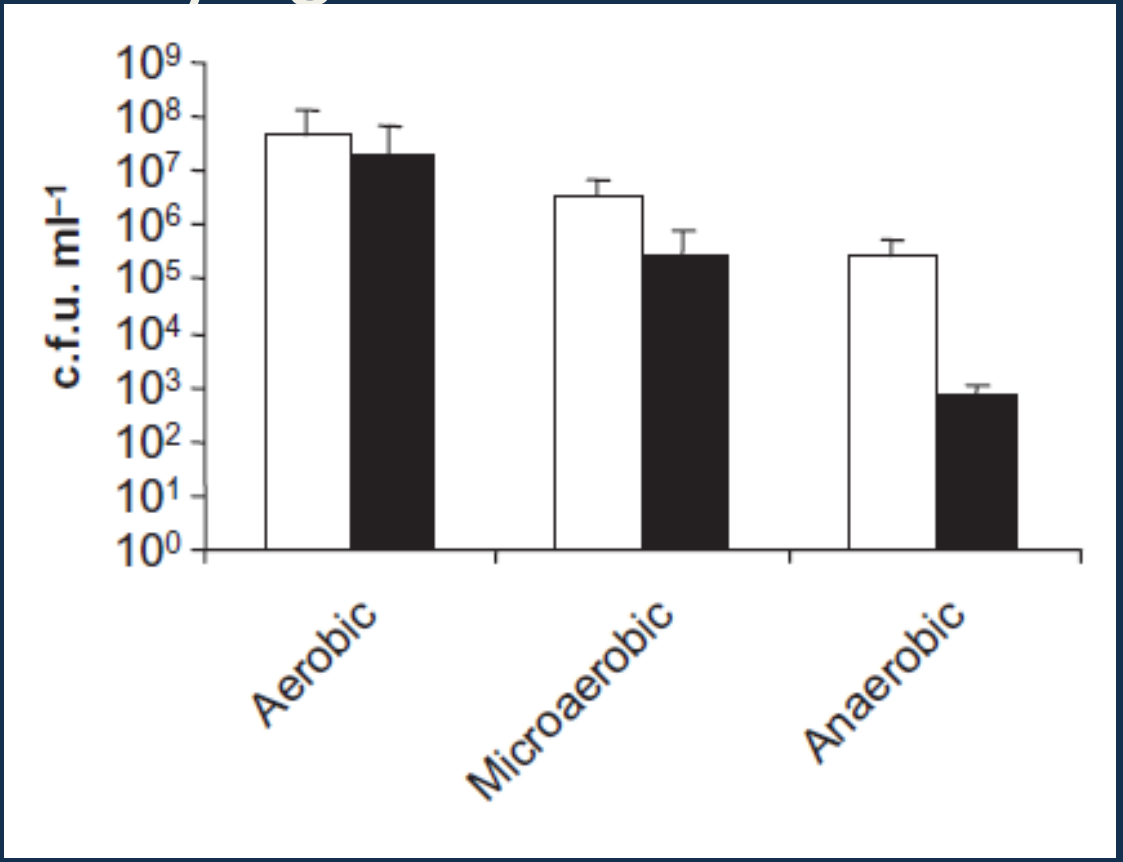
Differences in POA accumulation between *M. tuberculosis (M. tb* and *M. smegmatis (M. smeg*.

The Unique Susceptibility of TB amongst Mycobacteria to PZA



Effect of reserpine and valinomycin on accumulation of POA in *M. smegmatis (A)* and *M. tuberculosis (B).*

Other factors? Effect of Aeration on PZA Activity against *M. tuberculosis*



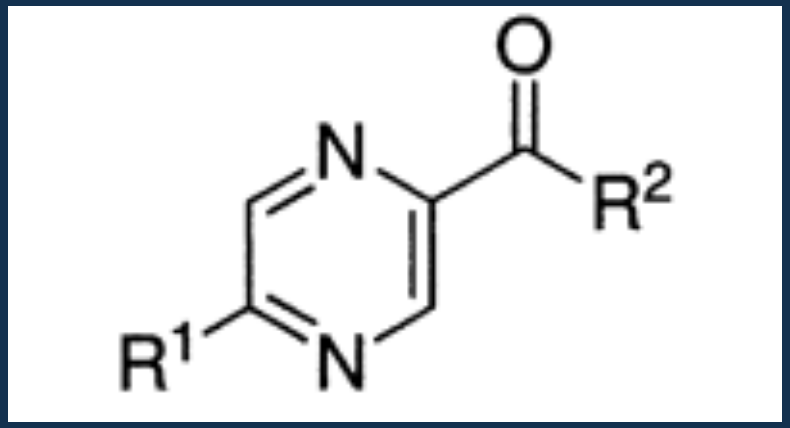
H37Ra cells were treated with 100 g PZA ml1 (filled bars) or not (open bars), under aerobic, microaerobic or anaerobic conditions for 5 days prior to c.f.u. determination.

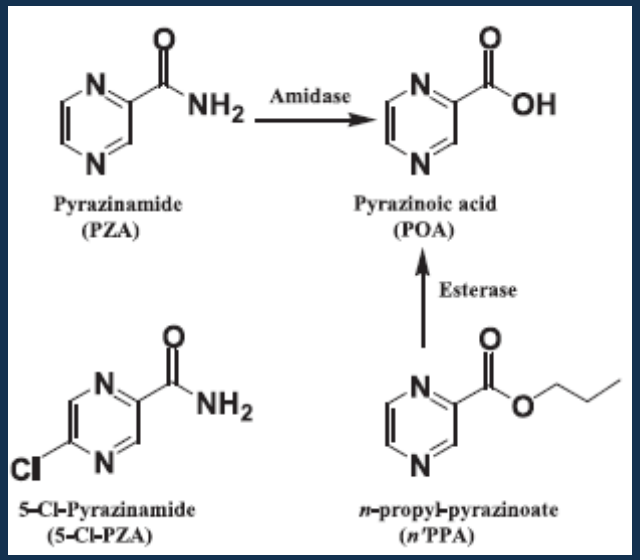
Can Increased PZAse Activity Affect the Susceptibility to PZA?

- PZA deamidation can be catalyzed by PncA and by PzaA
- Increased PZAse activity leads to MSMG susceptibility to PZA
- Increases MTB susceptibility

PZA Analogs

- The rationale? Circumvent PZAse
  Increase potency
  Expand activity to other species





- Pyrazinoates(POE), 5-Cl-PZA analogs, 5-Cl-POE, 5-fluoropyrazinoates (5-F-POE).

Summary and Conclusion from PZA Analogs Activity

- Insoluble in water.
- Broader spectrum activity that include *M. avium ,M. kansasii, M. smegamatis*
- Higher potency (up to 100 folds) for MTB.
- Ester hydrolysis of PAEs is possible but is not required.

Summary and conclusion from PZA Analogs Activity(2)

- 5-Cl-PZA does not require conversion to 5-Cl-POA(active in *M. bovis* lacking it).
- 5-Cl-POA, a stronger acid than POA, is much less active than either 5-Cl-PZA or POA.

TABLE 1. MICs of pyrazinamide analogs for various mycobacteria

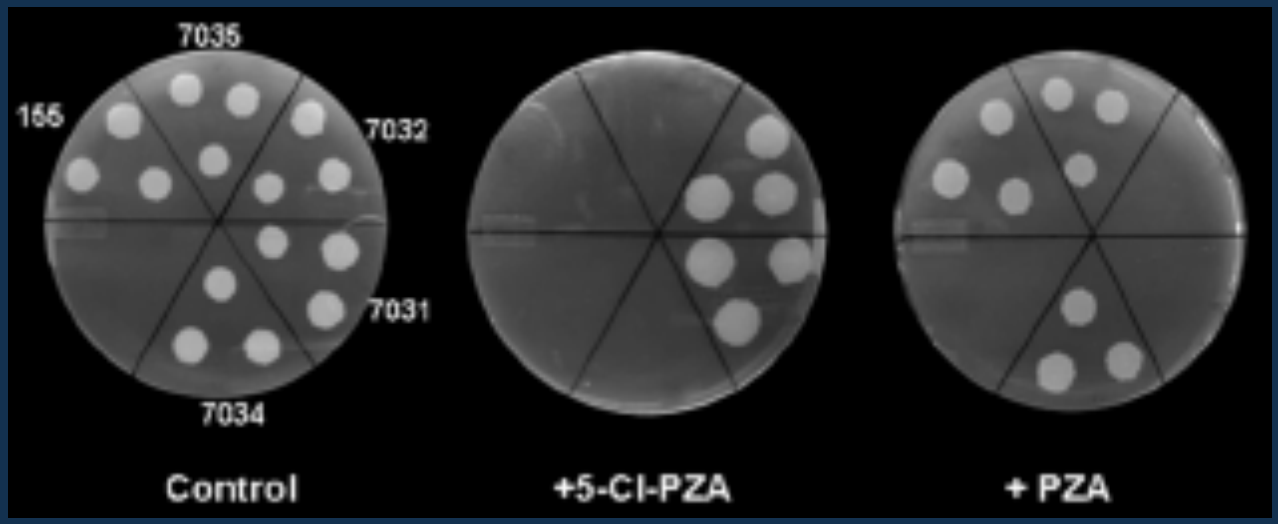
Organism	MIC (μg/ml) of:			
	PZA	5-Cl PZA	PA	5-Cl PA
M. tuberculosis strain				
ATCC 27294	64	16	32	128
ATCC 35801	32	16	32	64
ATCC 35828	>2,048	32	32	256

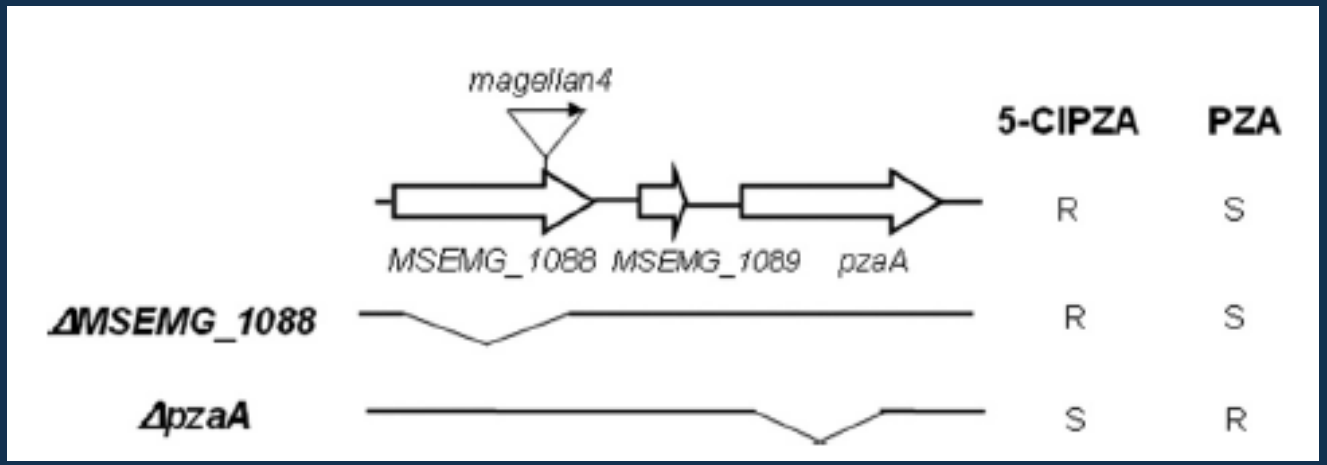
- Neither 5-ClPZA or *n*'PPA require acidic pH, yet suffer from inoculum effect.

Mutually Exclusive Genotypes for Pyrazinamide and 5-Chloro-pyrazinamide Resistance

Baughn A, Deng J et al. AAC 2010

Mutually Exclusive Genotypes for PZA and 5-Cl-PZA resistance

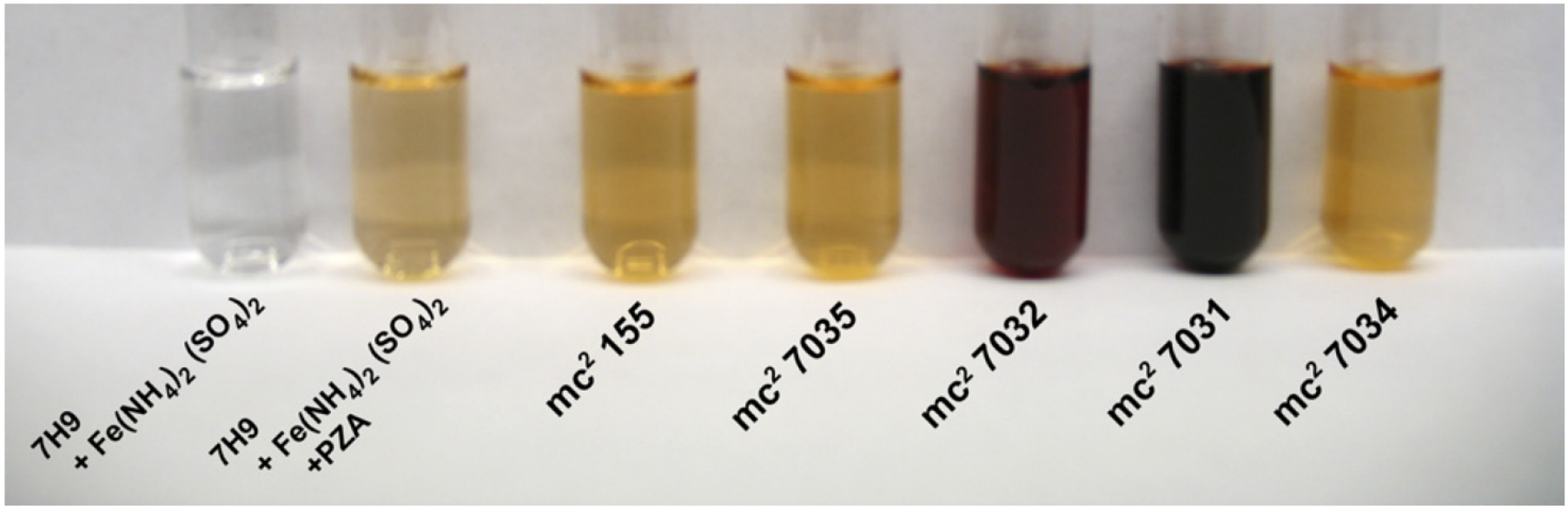




PZAse activity in 5-Cl-PZA resistant strains MSMEG

TABLE 1. PZA/5-ClPZA turnover and MICs in *M. smegmatis* mutants

M. smegmatis strain	Description	PZA/5-Cl PZA turnover (nmol/min/ml of cells [OD_{600}])	MIC (μg/ml)		Reference(s)
			5-Cl PZA	PZA	
mc^2155	Wild-type strain	0.85 ± 0.1	25	>4,000	19, 24
$mc^2$2612	Spontaneous 5-Cl PZA^r mutant	32 ± 3	125	>4,000	24
$mc^2$7031	mc^2155 *MSMEG_1088::magellan4*	100 ± 15	125	150	This work
$mc^2$7032	mc^2155 Δ*MSMEG_1088*	65 ± 5	125	150	This work
$mc^2$7034	$mc^2$7031 Δ*MSMEG_1090*	1.2 ± 0.1	25	>4,000	This work
$mc^2$7035	mc^2155 Δ*MSMEG_1090*	0.95 ± 0.15	25	>4,000	This work
$mc^2$7036	$mc^2$2612 Δ*MSMEG_1090*	1.2 ± 0.2	25	>4,000	This work
$mc^2$7037	Spontaneous 5-Cl PZA^r mutant	100 ± 15	125	150	This work
$mc^2$7038	mc^2155 $attB_{L5}$::P_{Tc}::$pzaA_{Msmeg}$	34 ± 1	125	>4,000	This work

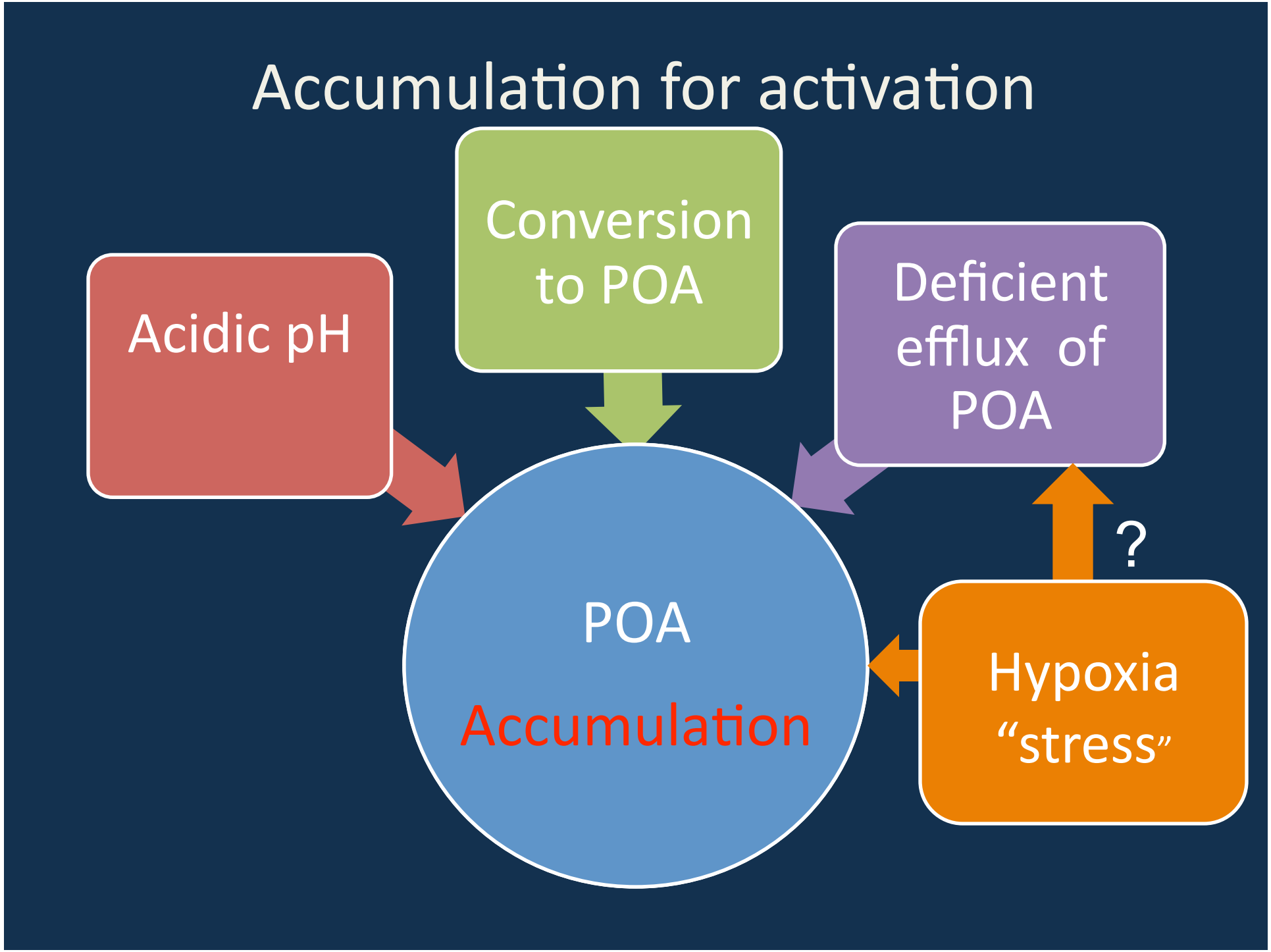


Increased conversion of PZA diminish the Requirement for Acidic pH

TABLE 2. Effect of PncA and PzaA expression on PZA and 5-Cl PZA turnover and MIC in tuberculous bacilli

Strain	Characteristic[a]	5-Cl PZA			PZA			Reference
		Turnover (nmol/min/ml of cells [OD_{600}])	MIC µg/ml		Turnover (nmol/min/ml of cells [OD_{600}])	MIC µg/ml		
			pH 6.8	pH 6		pH 6.8	pH 6	
M. tuberculosis H37Ra	Attenuated mutant of H37Rv	0.15 ± 0.01	25	25	0.23 ± 0.01	>1,000	50	
$mc^2$7092	H37Ra $attB_{L5}$::P_{Tc}::$pzaA_{Msmeg}$	27 ± 1	200	100	20 ± 1	62.5	25	This work
$mc^2$7093	H37Ra $attB_{L5}$::P_{Tc}::$pncA_{Mtb}$	0.096 ± 0.002	25	25	2.9 ± 1	62.5	50	This work
M. bovis BCG-Pasteur	Attenuated mutant of *M. bovis*	0.11 ± 0.01	12.5	12.5	0.015 ± 0.001	>1,000	>1,000	
$mc^2$7091	BCG-Pasteur $attB_{L5}$::P_{Tc}::$pzaA_{Msmeg}$	24 ± 1	200	50	16 ± 1	62.5	12.5	This work
$mc^2$7099	BCG-Pasteur $attB_{L5}$::P_{Tc}::$pncA_{Mtb}$	0.080 ± 0.01	12.5	12.5	3.5 ± 0.1	62.5	12.5	This work

[a] *Msmeg*, *M. smegmatis*; *Mtb*, *M. tuberculosis*.


Accumulation for activation
Acidic pH
Conversion to POA
Deficient efflux of POA
POA
Accumulation
?
Hypoxia "stress"

How PZA works? Inherent Difficulties in Identifying the Actual Mechanism

- No Bona-fide POA resistant mutant

Absence of POA R??

More than one mechanism or intolerable mutation.

- Need to correlate antimycobacterial activity with biochemical effect

Identification of the Target of 5-Cl-PZA

- Selection of 5-Cl- PZA resistant mutants in MSMEG
- The range of resistance of the mutants (5-Cl-PZA ^{R}) is narrow
- Fatty acid synthase 1 (*fas1*) is the gene that confers this phenotype (a 9.3 kb ORF)
- MTB does not tolerate multicopy *fas1* or even a single copy from MSMEG.

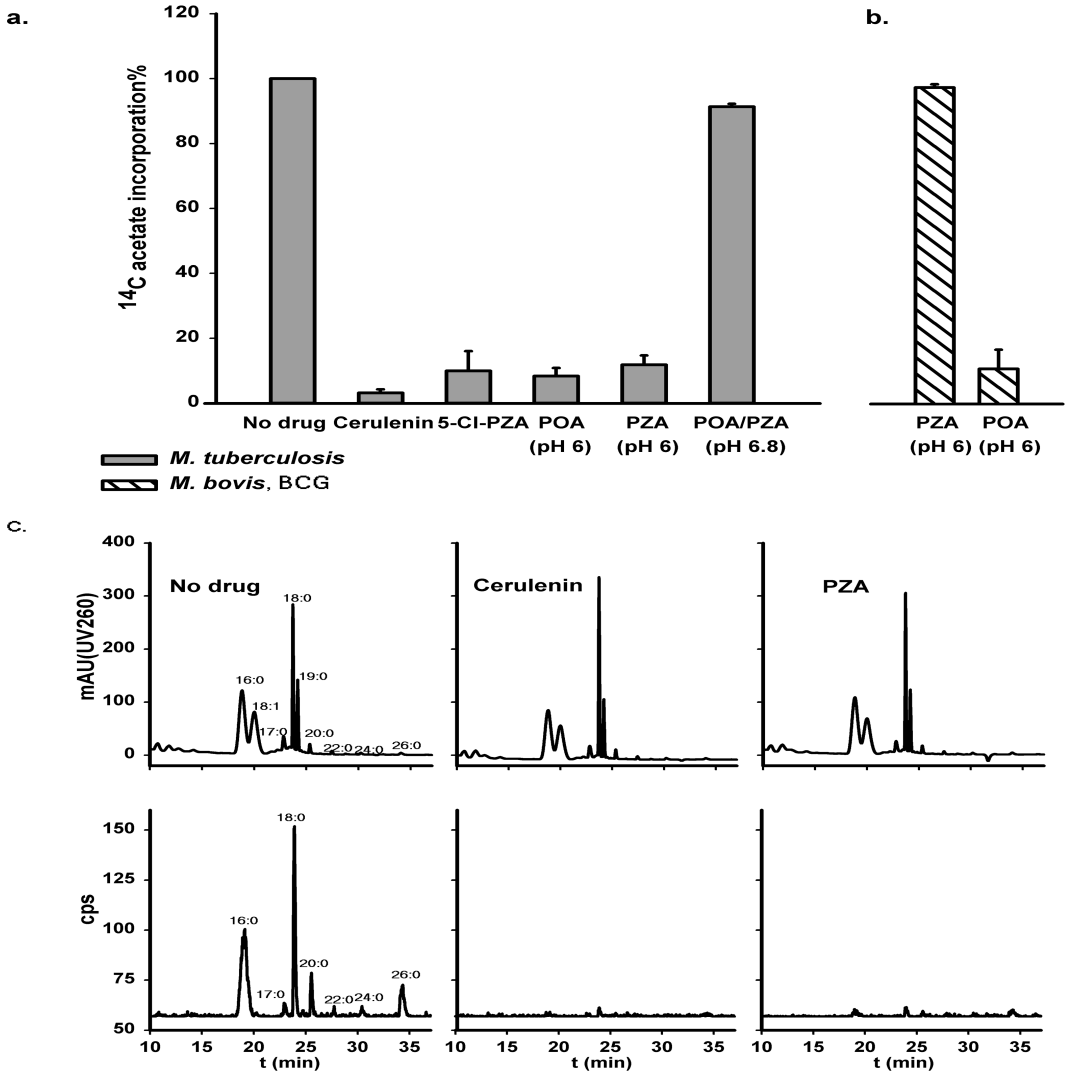
Fatty acid synthesis in mycobacteria and related species

- **FAS I** system **All** non-plant eukaryotes and certain prokaryotes

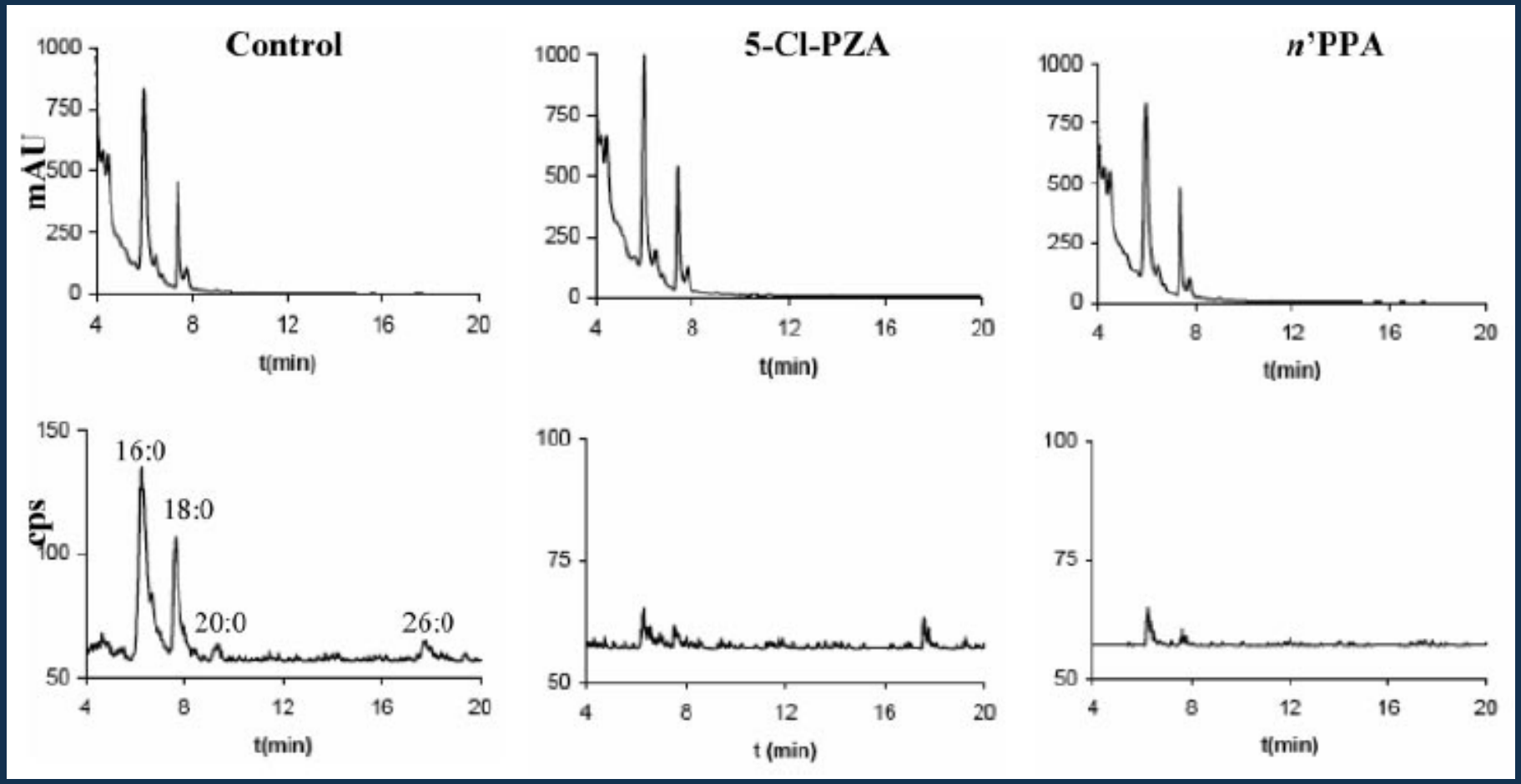
  Multi-functional multi-domain protein catalyzes the synthesis of long chain fatty acids from C_2 units

- **FAS II** Most prokaryotes, individual enzymes
- Mycobacteria: Both FAS I and FAS II, FAS II generates mycolic acid precursors

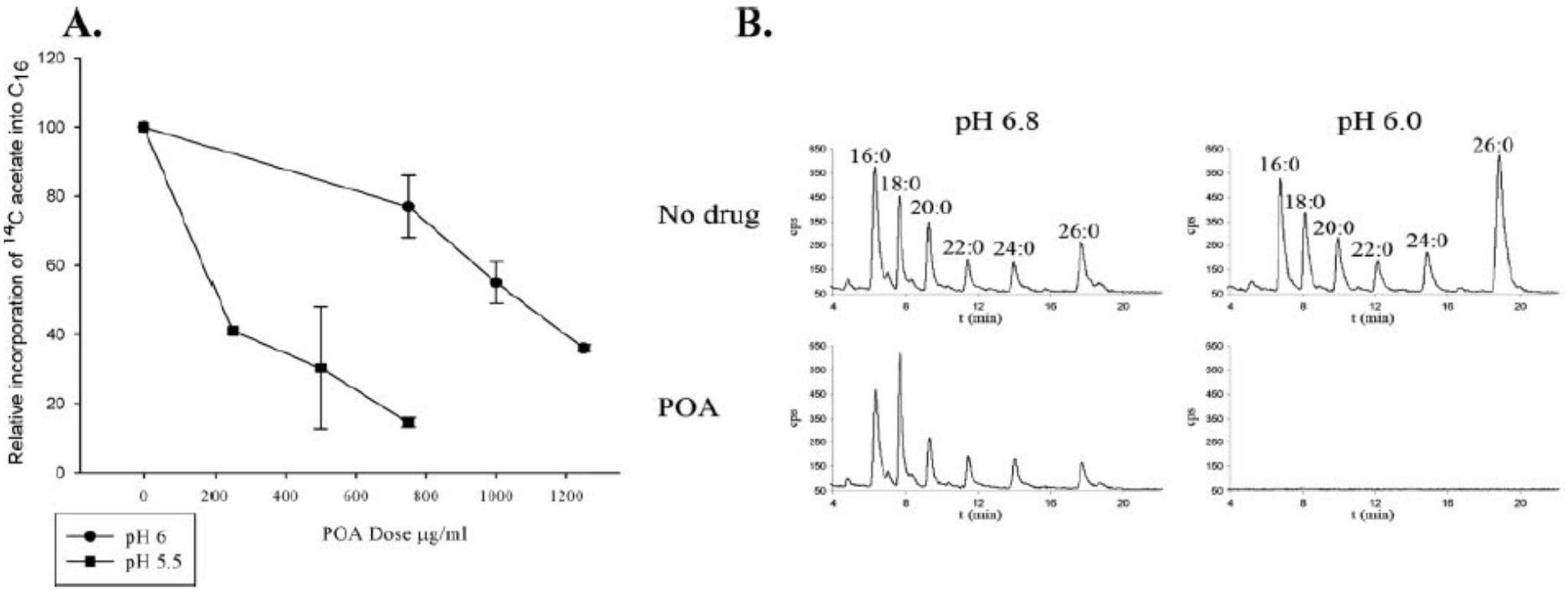
PZA/POA Activity Correlates with Inhibition of FA Biosynthesis



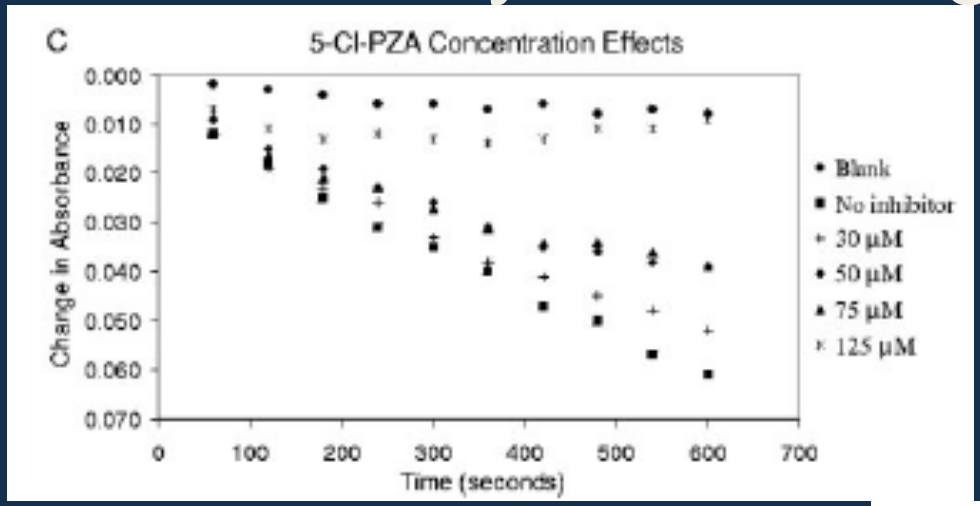
A Pyrazinoate Ester, *n*'PPA Inhibits Fatty Acid Synthesis in *M. tuberculosis*

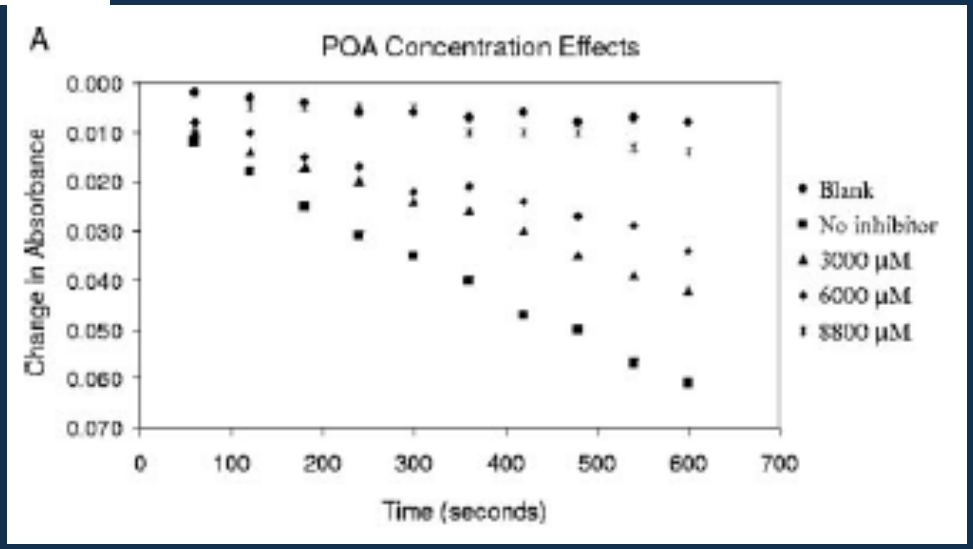


POA Inhibition of Fatty Acid Biosynthesis in TB Complex Bacilli Correlates to Antimycobacterial Activity



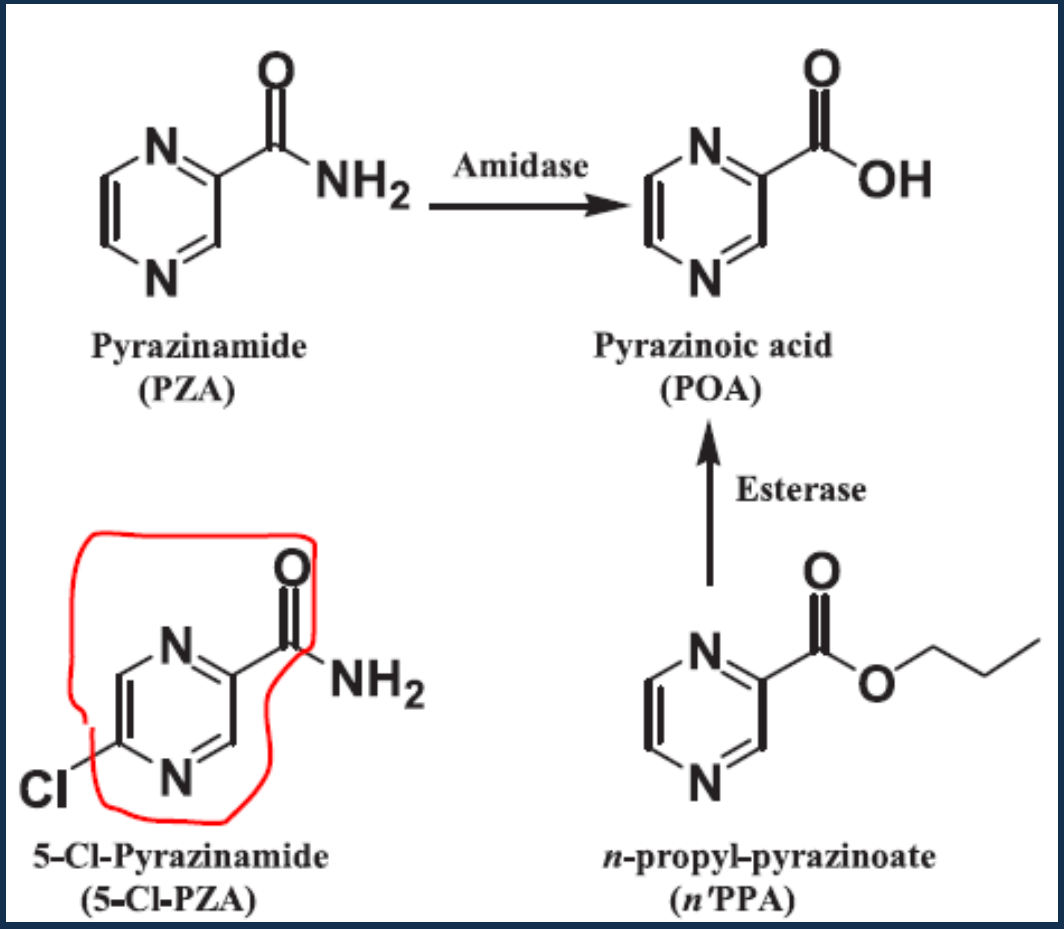
M. tuberculosis FAS I Inhibition in Cell Free System using NADPH Oxidation



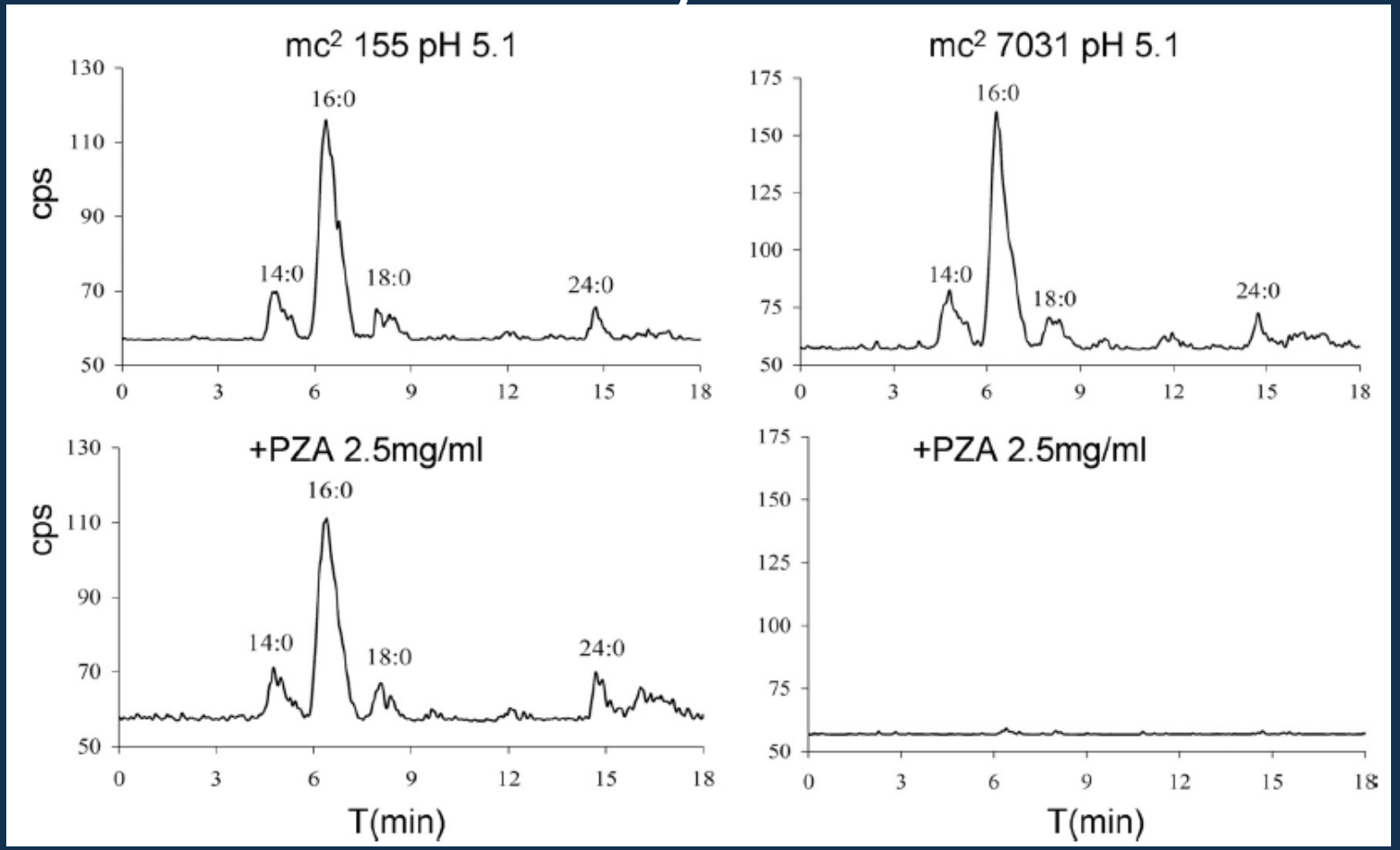


Effect of increasing inhibitor concentration on NADPH oxidation. Blank runs with no enzyme are included for reference.

Minimal Structure of Pyrazine Ring with an Acyl Group.



Susceptibility to PZA Translates to Inhibition of FA Synthesis in MSMEG



HPLC analysis of C16:0 to C26:0 fatty acids from PZA-treated MSMEG **mc²155** and mc²7031, treated with 2.5 mg/ml PZA for 2 h, and then pulsed with [1-^{14}C]acetate for an additional 2 h.

Similarities between POA and 5-Cl-PZA, *n'*PPA

- Relatively poor similar bacteriostatic activity, and killing curve in-vitro
- No resistant mutants in MTB
- Correlation of anti-mycobacterial activity to palmitate biosynthesis inhibition

Similarities between POA and 5-Cl-PZA *n'* PPA(2)

- In vitro inhibition albeit marked difference in potency

- Binding to FAS I NMR studies

Dissimilarities between 5-Cl-PZA and POA

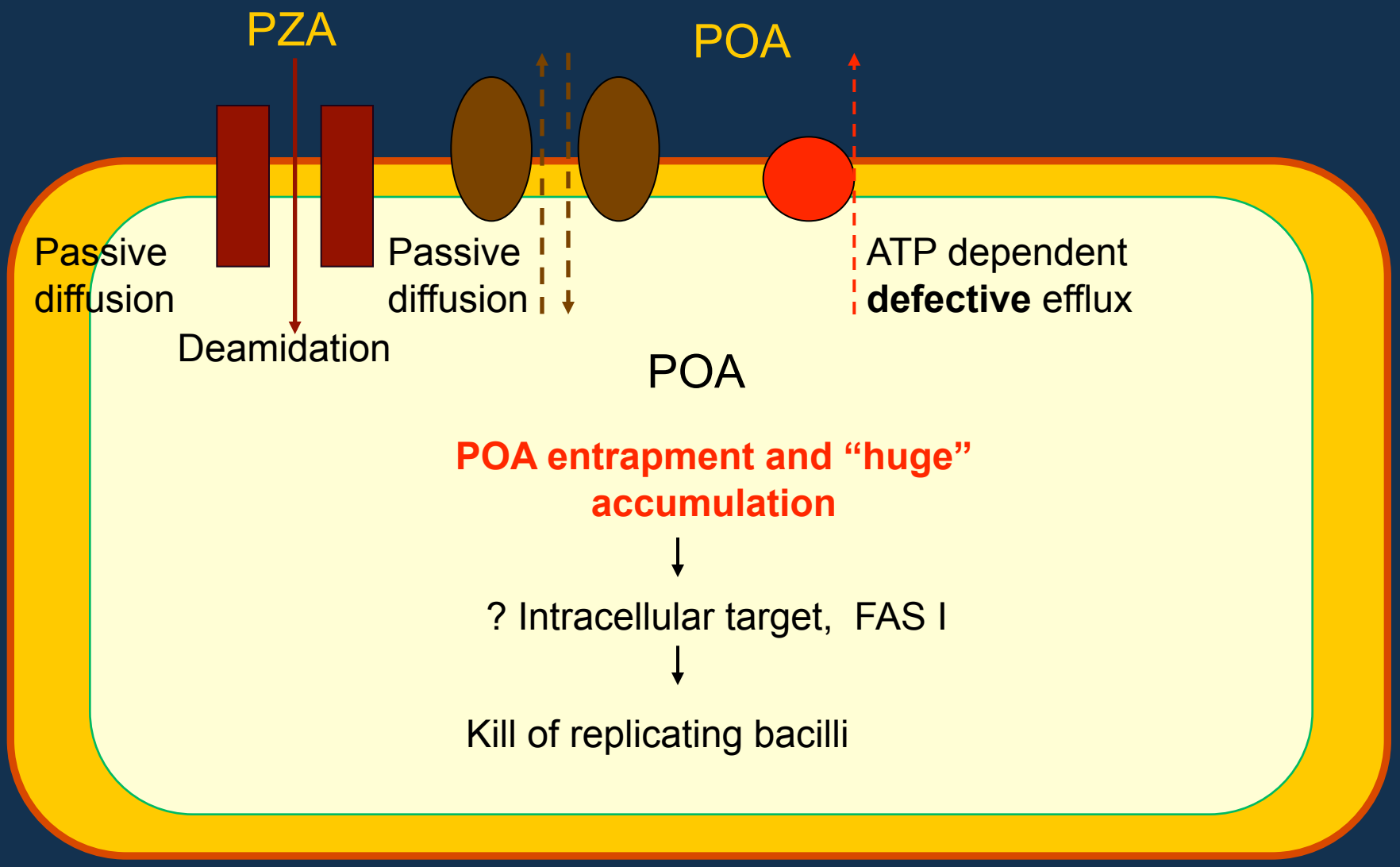
Need for acidic pH

Effect of FAS I overexpression on PZA resistance in MSMEG

Conclusions

- Sufficiency of PZA/POA is essential for correct interpretation and reproducibility
- PZA studies should be conducted on replicating bacilli(various conditions, ex vivo models etc)
- Acidic pH is a condition for POA accumulation can be partially substituted, no “mechanistic” role
- An accumulated dependent agent affects an intracellular site

PZA activity against MTB bacilli in media anoxic/acidic



Further Studies?

- How does the poor activity in vitro translate into sterilizing effect in-vivo?

  Tissue levels ?

  Synergism with inflammatory response?
  Promoting accumulation into the bacilli and or improved killing?

  Better induction of cellular immunity