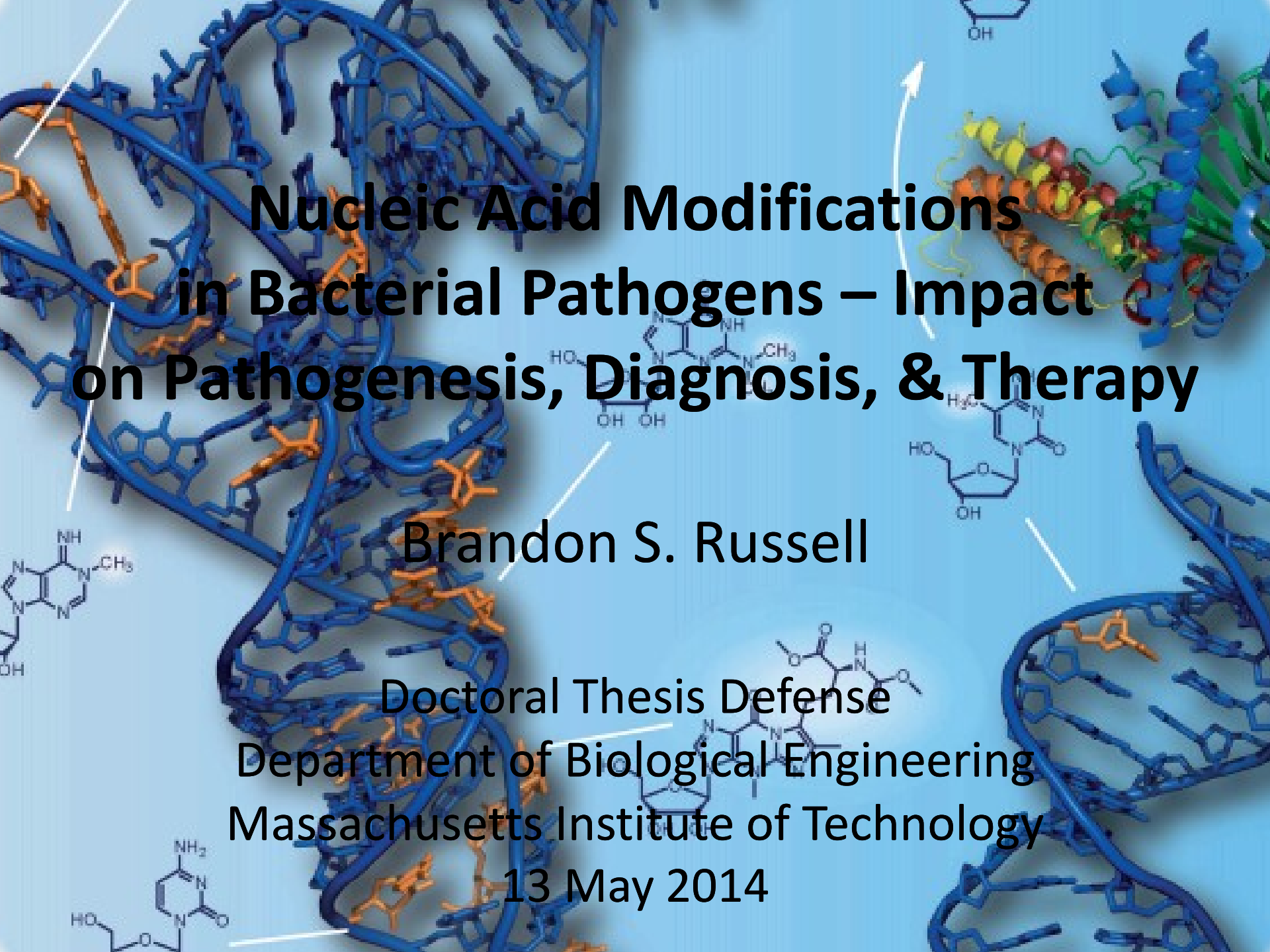

Nucleic Acid Modifications in Bacterial Pathogens – Impact on Pathogenesis, Diagnosis, & Therapy

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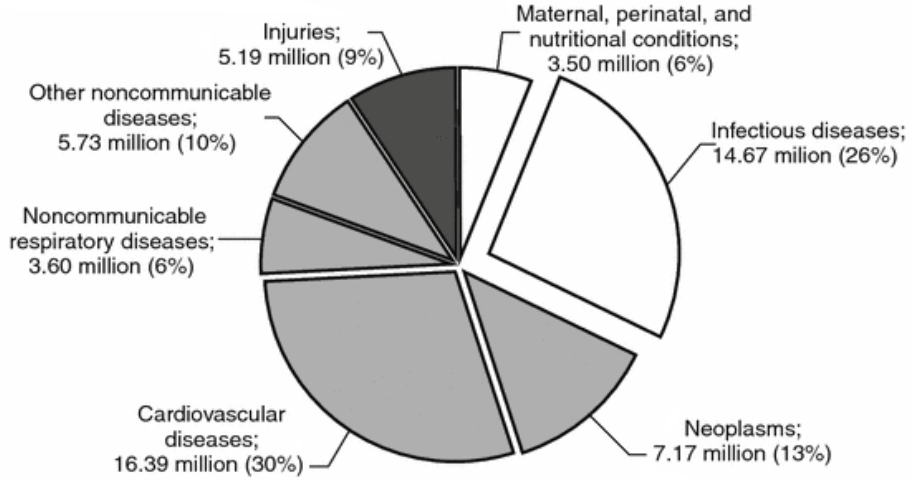
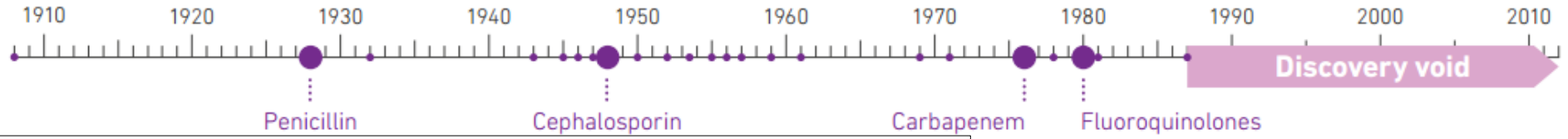
13 May 2014

Bacteria are killing us

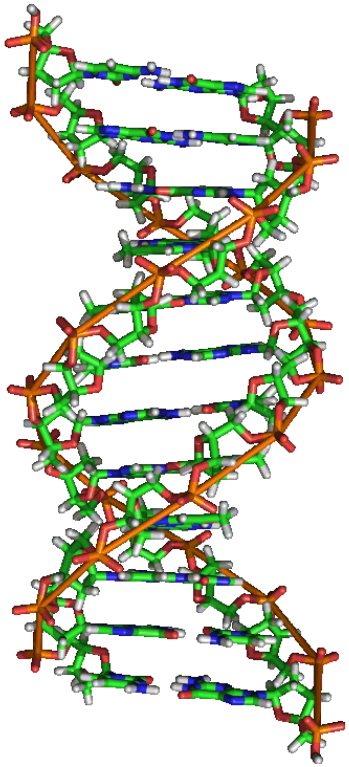


Over the last 30 years, no major new types of antibiotics have been developed



Pinheiro, Mathers, Kramer 2010 *Modern Infectious Disease Epidemiology* | CDC 2013 | WHO 2014

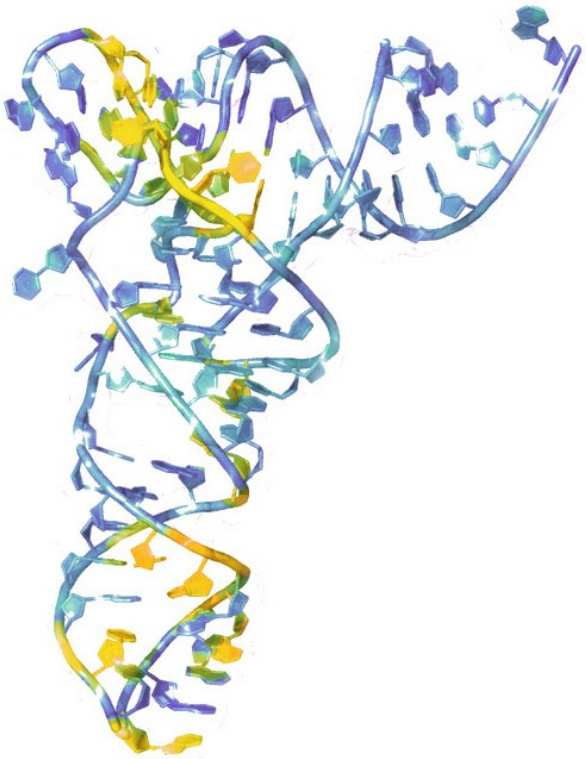
Bacterial DNA and RNA are complicated



DNA

A, C, G, T, U...

Why?



RNA

...m1A, m2A, m6A, Am, ms2m6A, i6A, ms2i6A, io6A, ms2io6A, g6A, t6A, ms2t6A, m6t6A, hn6A, ms2hn6A, Ar(p), I, m1I, m1Im, m3C, m5C, Cm, s2C, ac4C, f5C, m5Cm, ac4Cm, k2C, m1G, m2G, m7G, Gm, m22G, m2Gm, m22Gm, Gr(p), yW, o2yW, OHyW, OHyW*, imG, mimG, Q, oQ, galQ, manQ, preQ0, preQ1, G+, Y, D, m5U, Um, m5Um, m1Y, Ym, s2U, s4U, m5s2U, s2Um, acp3U, ho5U, mo5U, cmo5U, mcmo5U, chm5U, mchm5U, mcm5U, mcm5Um, mcm5s2U, nm5s2U, mnm5U, mnm5s2U, mnm5se2U, ncm5U, ncm5Um, cmnm5U, cmnm5Um, cmnm5s2U, m62A, Im, m4C, m4Cm, hm5C, m3U, m1acp3Y, cm5U, m6Am, m62Am, m2,7G, m2,2,7G, m3Um, m5D, m3Y, f5Cm, m1Gm, m1Am, tm5U, tm5s2U, imG-14, imG2, ac6A, inm5U, inm5s2U, inm5Um, m2,7Gm, m42Cm, C+, m8A

Central question for this research

What role are DNA and RNA modifications playing in bacterial pathogens, and do these modifications have utility as therapeutic targets?

Outline for today's talk

The role of tRNA modifications in *Helicobacter pylori* stress response and pathogenesis

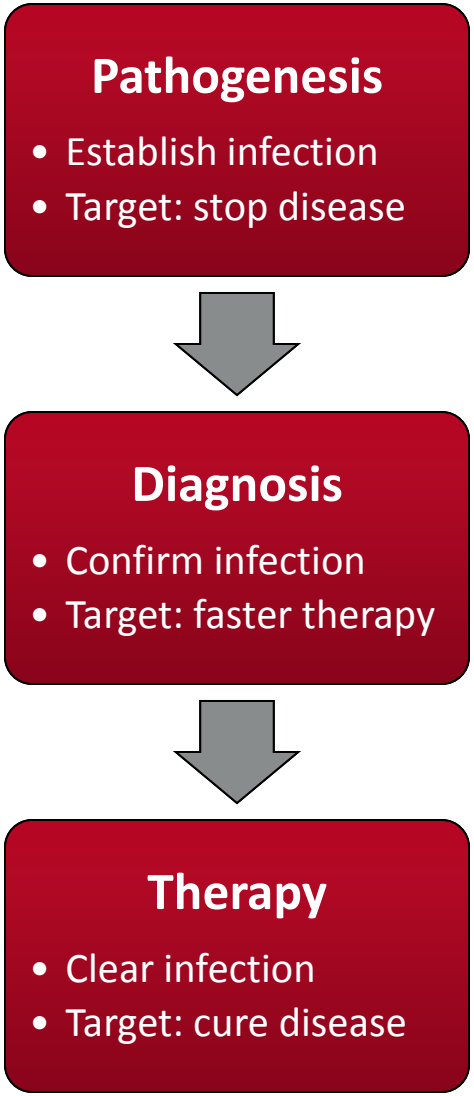
- Epidemiology and burden of disease
- RNA modifications as drivers of pathogenesis

Development of a novel BSL-2 animal model of mycobacterial lung infection and assessment of RNA modifications as urinary biomarkers

- Epidemiology and burden of disease
- RNA modifications as biomarkers

The role of bacterial DNA phosphorothioation in resistance to oxidative and antibiotic stresses

- DNA modifications as resistance determinants
- Relationship between oxidative and antibiotic stresses



Specific Aim 1: Pathogenesis

The role of tRNA modifications in *Helicobacter pylori* stress response and pathogenesis

In collaboration with Kok Seong Lim and Sebastian Smick

Helicobacter pylori is a major health concern

Pervasive human pathogen

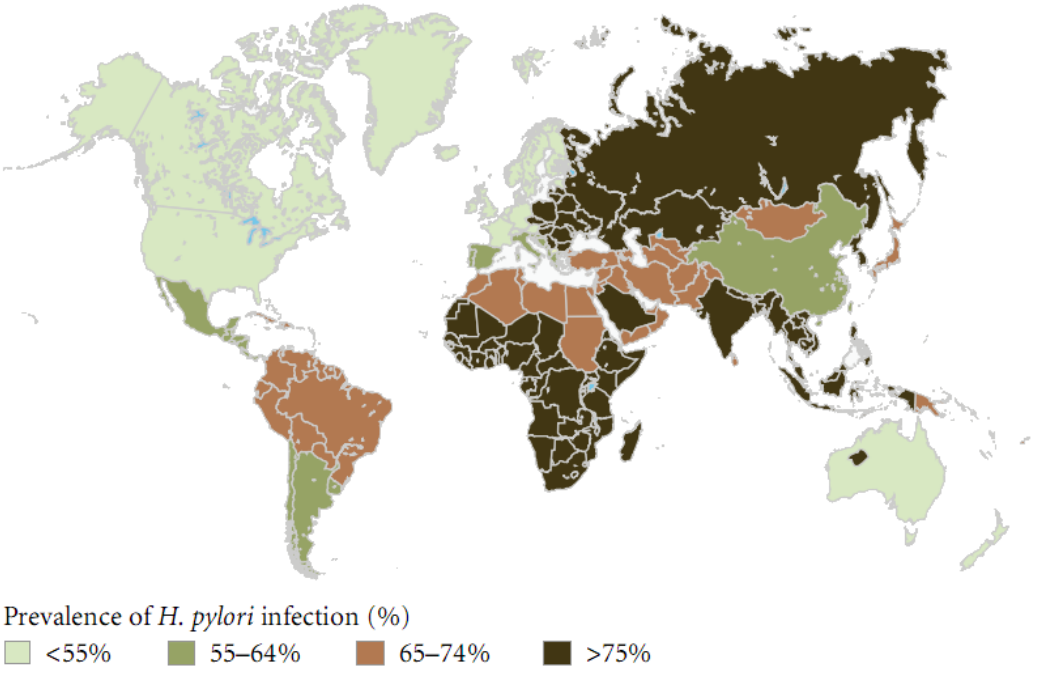
- Infects stomach, causes ulcers
- Undiagnosed in 90% of cases
- Usually persists for life

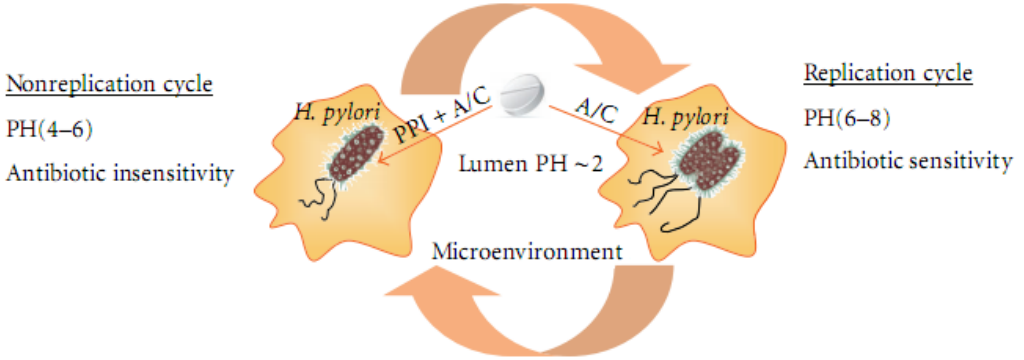
Huge public health burden

- 50% of the world infected
- Disproportionately affects underdeveloped regions
- Group 1 carcinogen for gastric cancer: 4th most deadly

Difficult to eradicate

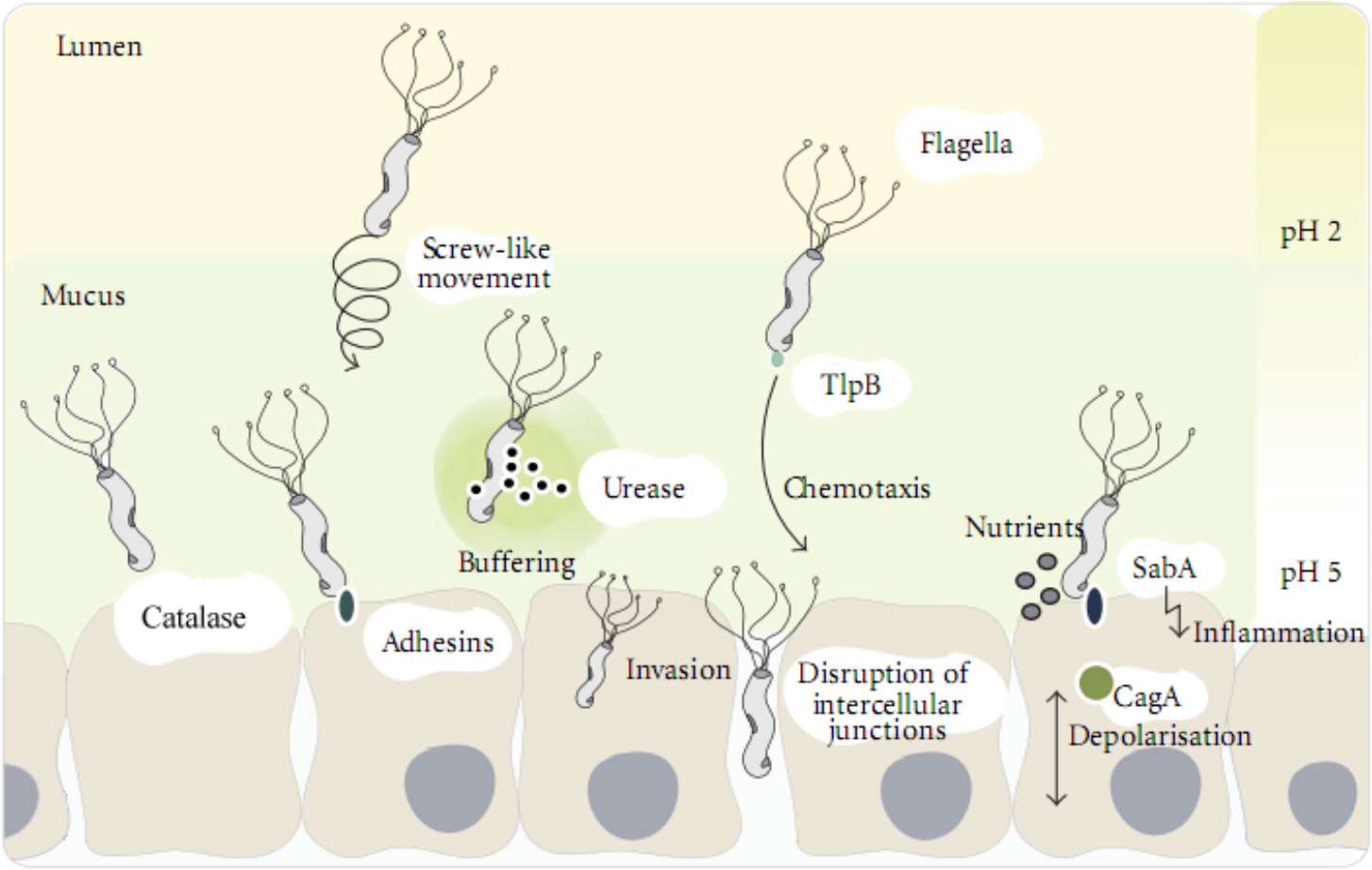
- Therapy synergizes resistance
- Extremely adapted to gut niche
- Need new drug targets





Bauer, Meyer 2011 *Ulcers* | Wu, Yang, Sun 2012 *Gastroenterol Res Pract*

H. pylori is well adapted to its niche

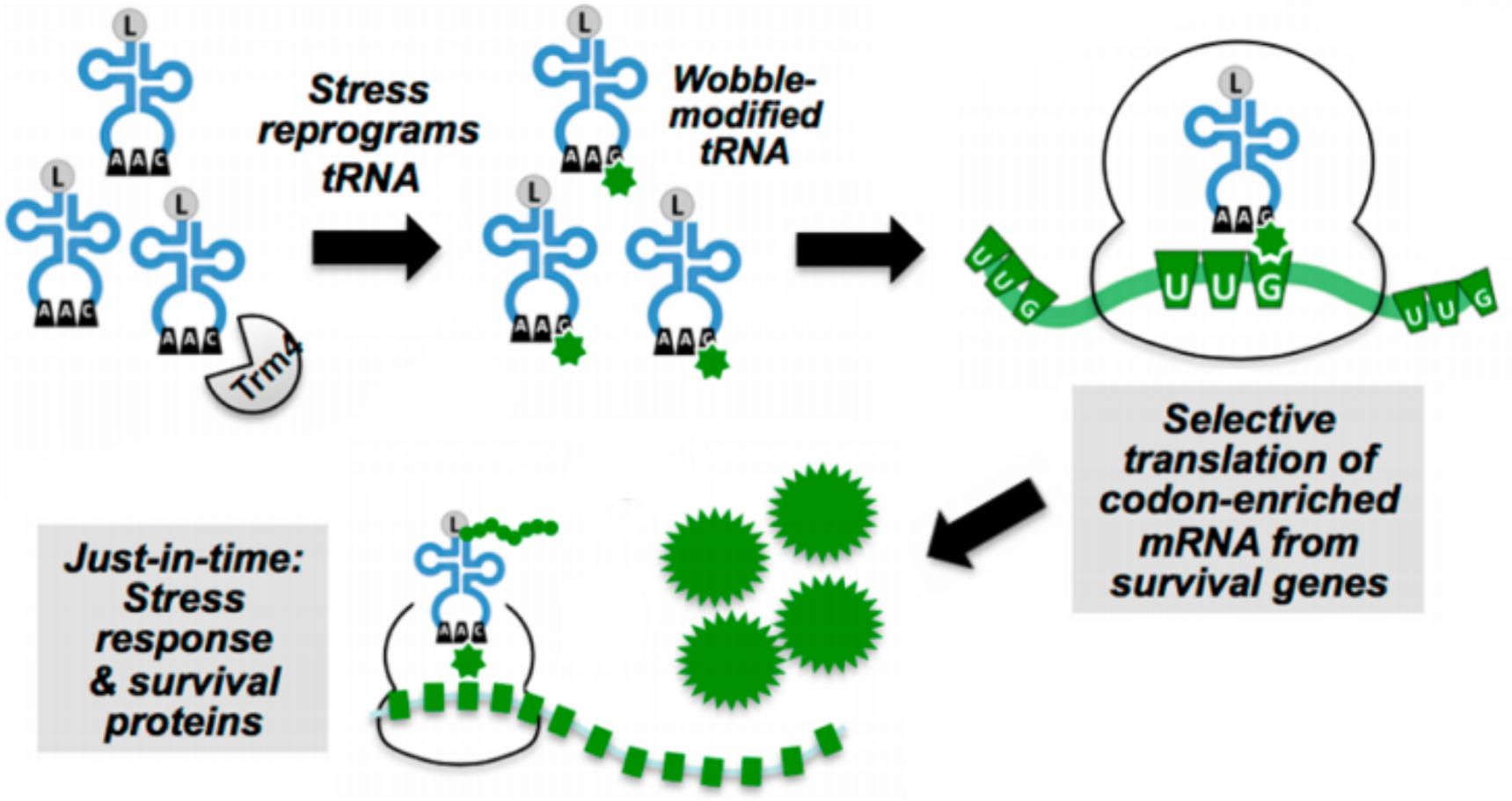


Molecular Microbiology (2004) **51**(1), 15–32
doi:10.1046/j.1365-2958.2003.03788.x

Faye M. Barnard, Michael F. Loughlin, Hernan P. Fainberg, Michael P. Messenger, David W. Ussery, Paul Williams and Peter J. Jenks

Although successful and persistent colonization of the gastric mucosa depends on the ability to respond to changing environmental conditions and co-ordinate the expression of virulence factors during the course of infection, *Helicobacter pylori* possesses relatively few transcriptional regulators.

Translational control by tRNA modifications



Well established in yeast and humans, but never shown in bacteria

H. pylori is a candidate due to its lack of transcriptional regulation

Dedon, Begley 2014 *Chem Res Toxicol*

Workflow for identifying critical modifications

Stress

RNA purification & processing

Global response profiling
- mRNA
- Proteins
- Translatome
- Ribosomal mRNA

Ribonucleoside chromatography

Ribonucleoside mapping in RNA & tRNA quantif.

Mechanistic modeling & pathways
- Genomic analysis
- Biosynthesis
- Bioinformatics

Qualitative mass spectrometry (QTOF, MS^n)

Quantitative mass spectrometry (QQQ)

Organism-centric rN database

Data analysis MVS

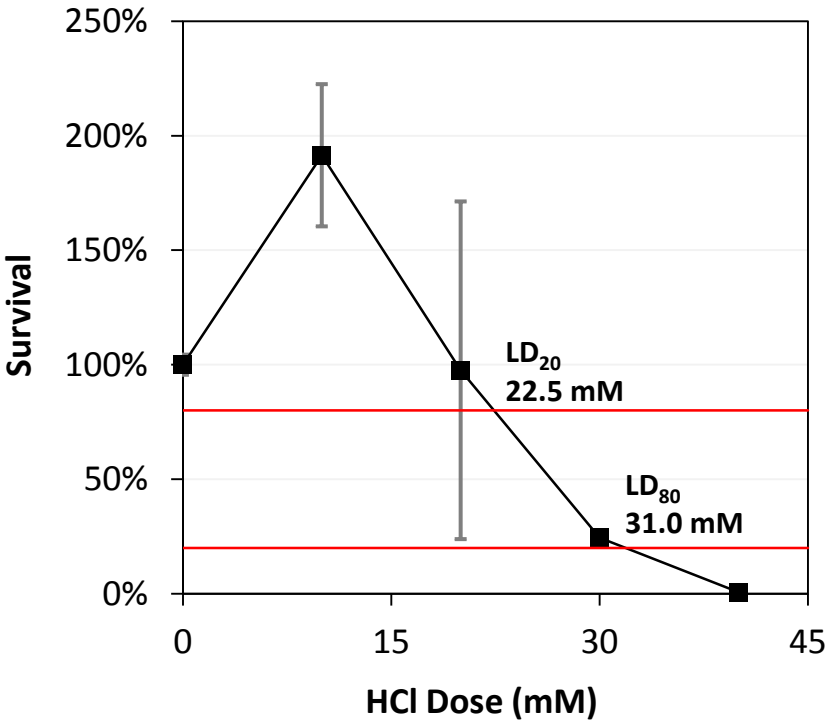
Translation & hypothesis testing

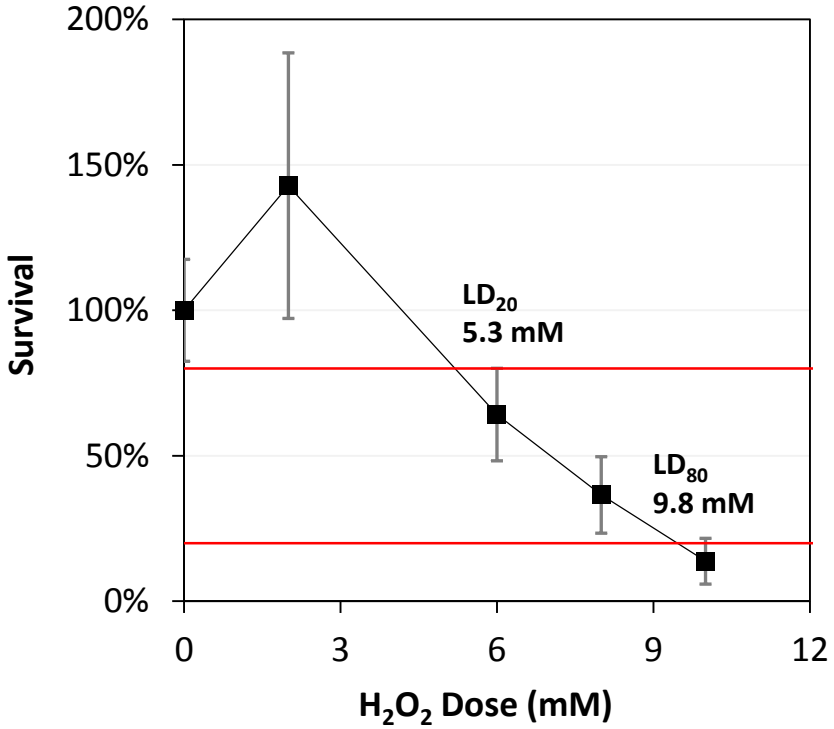
Dose-response curves standardize the stresses

H. pylori strain 26695 (virulent, sequenced) exposed to key stresses

- HCl is the first barrier to infection, synergizes with antibiotic resistance
- H_2O_2 is a key feature of the innate immune response

Hormesis seen at low toxicity doses for both stresses



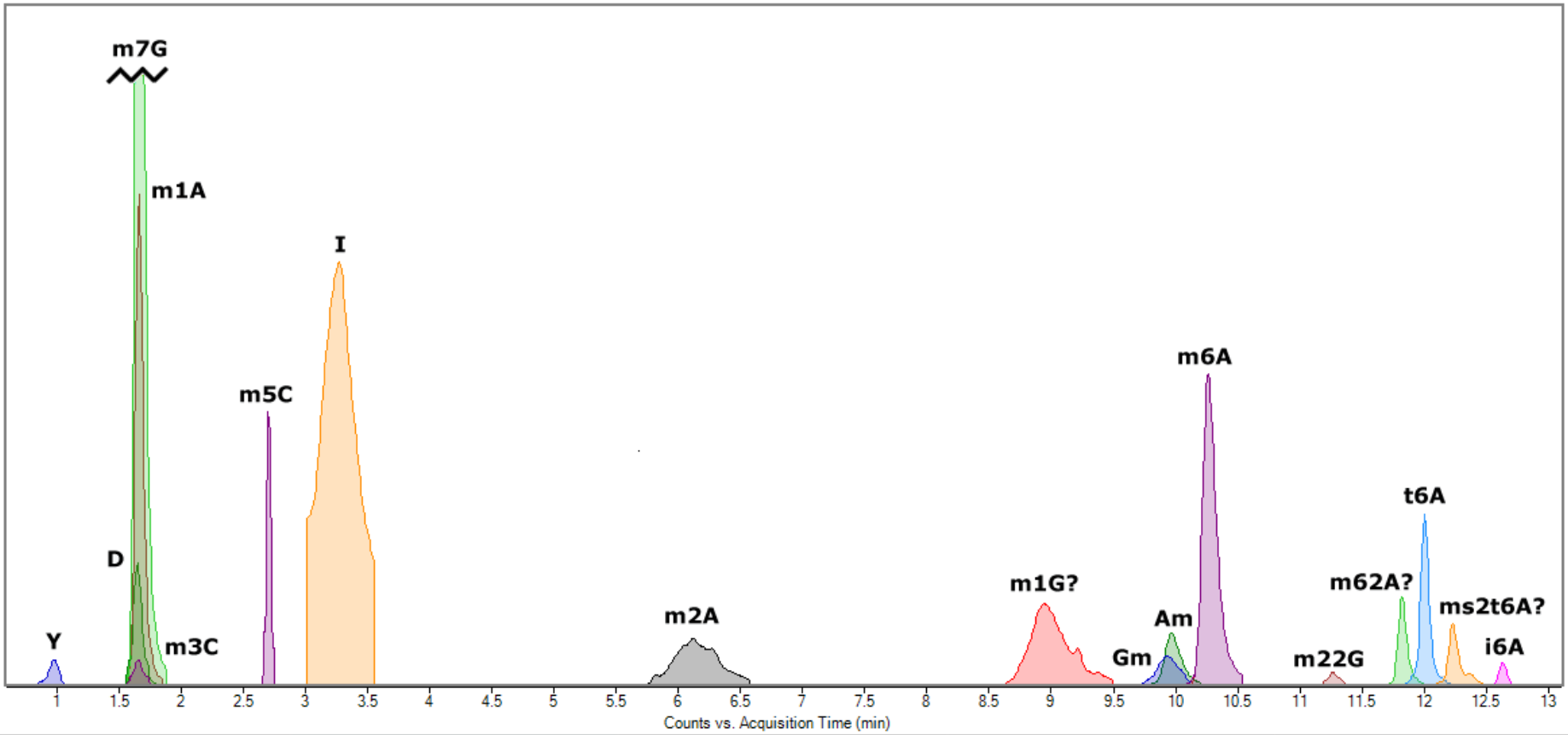


H. pylori modified nucleoside spectrum

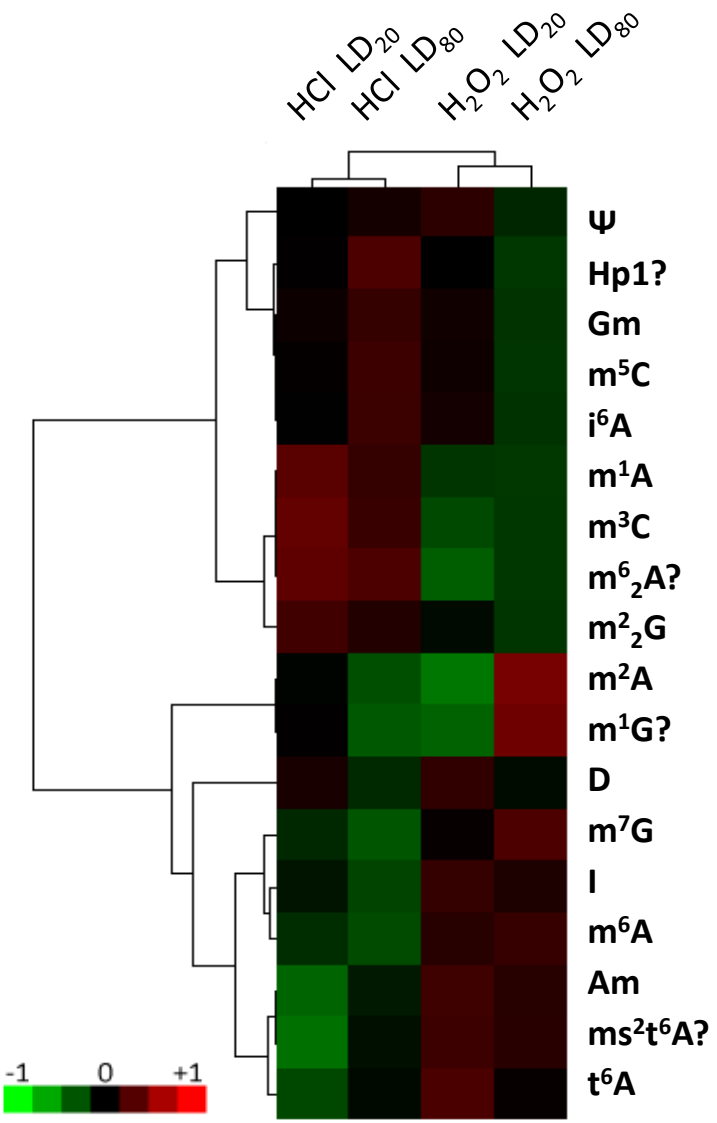
LC-MS/MS method to resolve and quantify 17 ribonucleoside species

14 species confirmed with standards; 3 (?) tentatively identified

Incomplete list, but sufficient to identify stress-induced patterns



Stress induces large changes in nucleoside pattern



Three biological replicates run in technical triplicate at each dose

Normalized for sample loading by UV signal against a calibration curve

Normalized signals log transformed, mean centered, plotted as a heatmap

First report of stress-induced tRNA modification reprogramming in a human pathogen

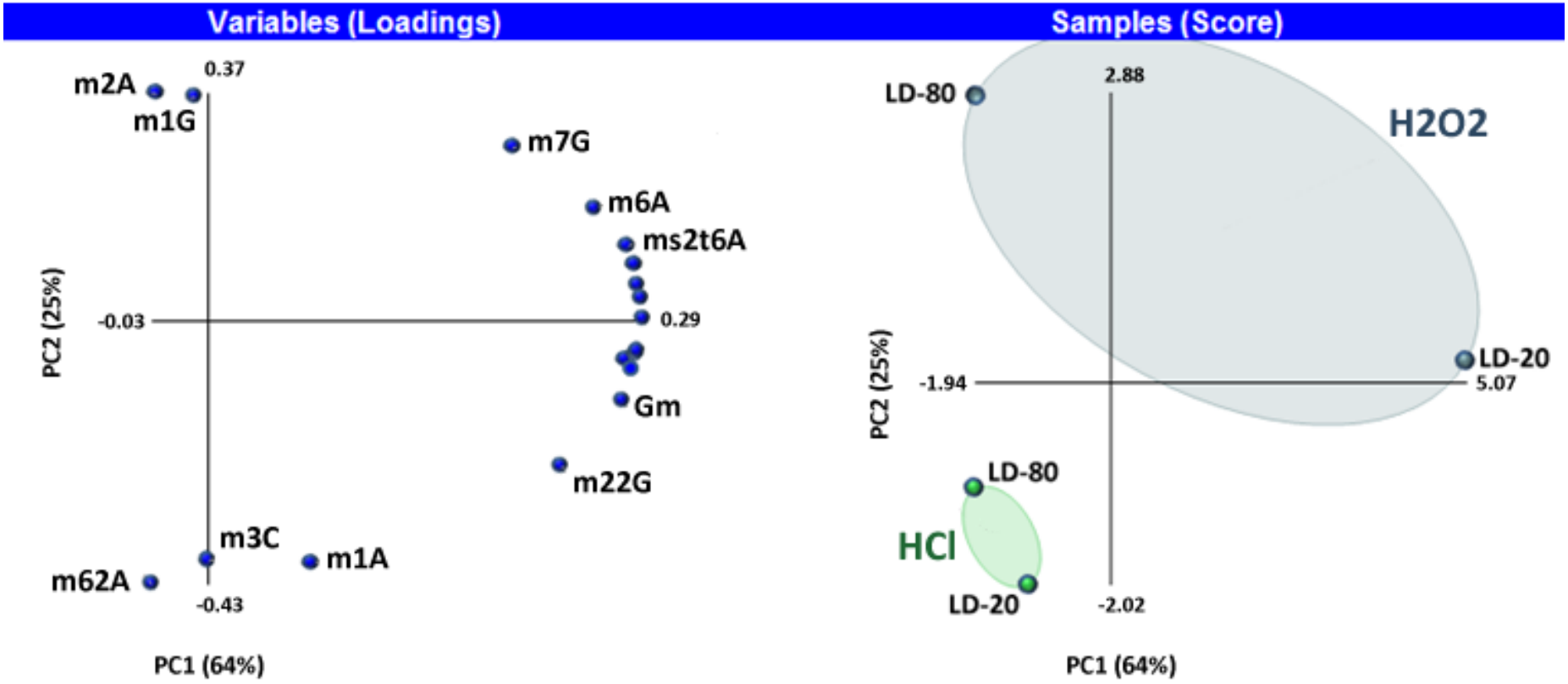
Are any of these modifications essential?

Key nucleosides distinguish stresses

Principal component analysis to reveal hidden associations

First two PCs capture 89% of the variance – highly predictive

Key modifications separate stresses into distinct classes



Summary and future directions

First example of tRNA modification profiling in *H. pylori*

First report of tRNA modification reprogramming in a pathogen

- Distinct patterns of modifications by stress
- Clear biomarker signature predicts exposure mechanism of action

First report of specific modifications associated with stress

- $m^6{}_2A$ also found in *Mycobacterium* tRNA – another harsh niche pathogen
- m^7G conserved across species and critical for stress response in yeast
- ms^2t^6A occurs adjacent to the wobble position, may stabilize translation

Modifying enzymes may represent new antibiotic targets

- Use sequence alignment to identify key enzymes
- Use targeted knockouts to identify critical modifications

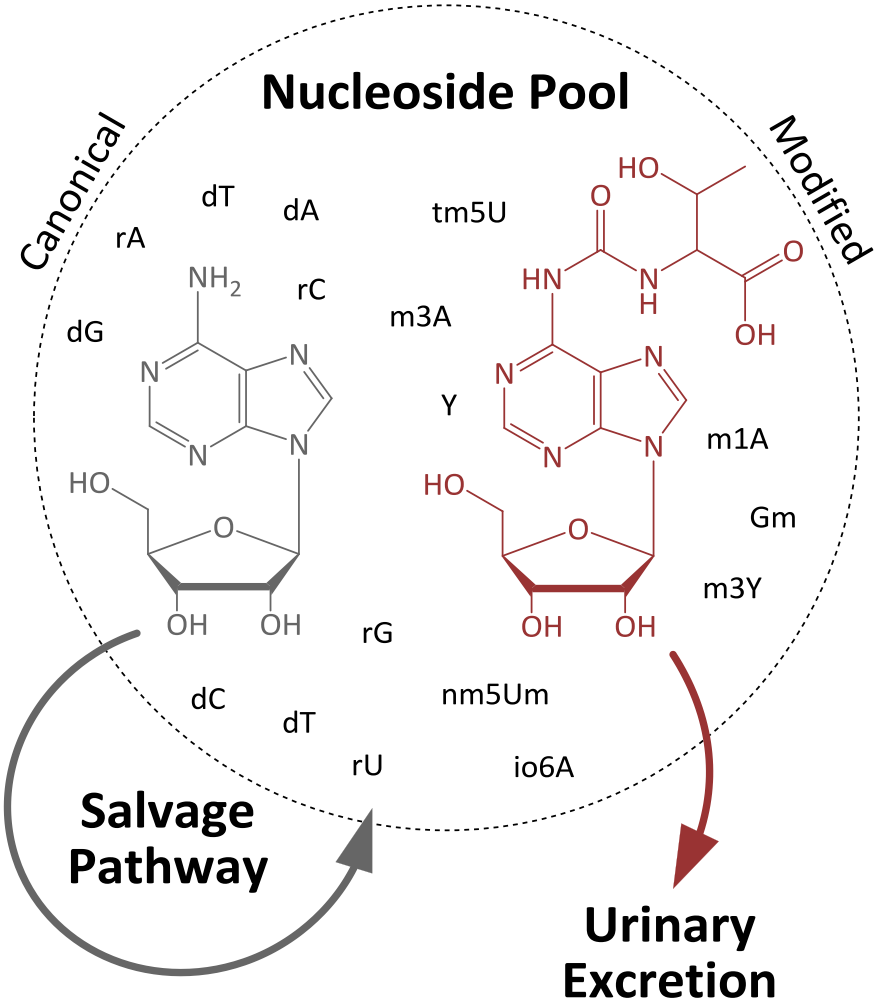
tRNA modifications appear to play a key role in pathogenesis

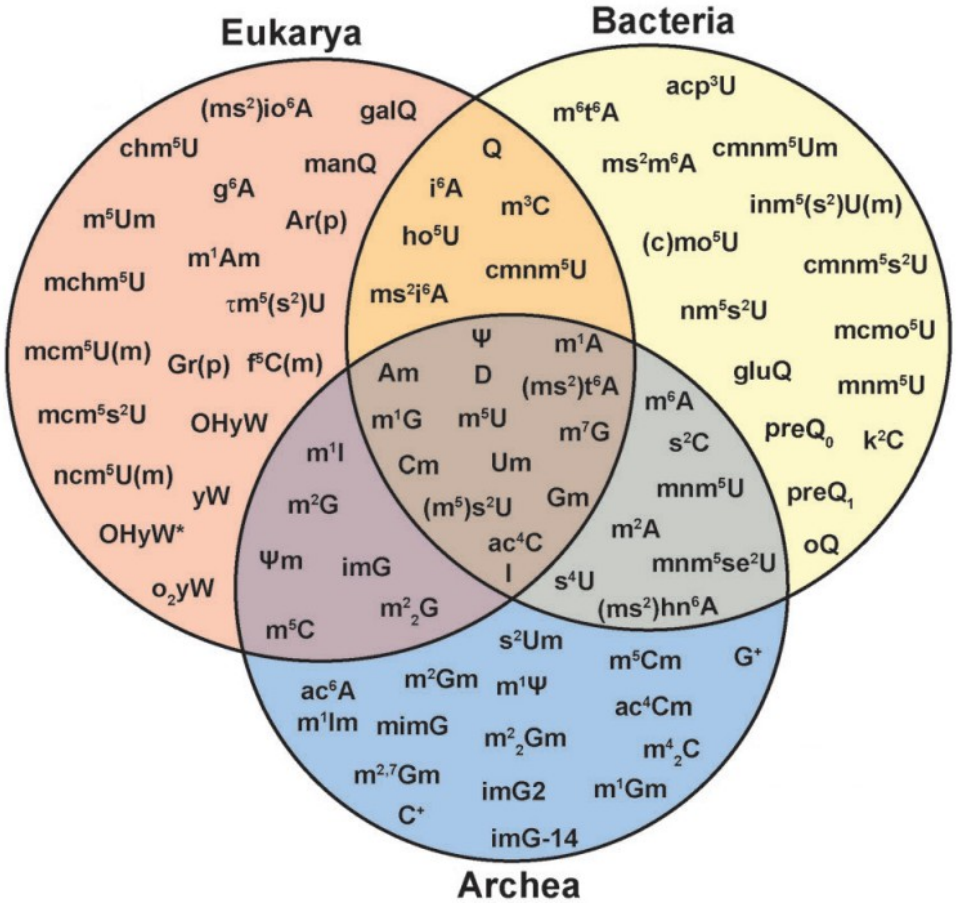
Specific Aim 2: Diagnosis

Development of a novel BSL-2 animal model of mycobacterial lung infection and assessment of RNA modifications as urinary biomarkers

In collaboration with Megan McBee, Sasilada Sirirungruang, Nicola Parry, and Sureshkumar Muthupalani

RNA modifications as urinary biomarkers





Carell *et al.* 2012 *Angew Chem Int Ed*

Tuberculosis (TB) is a major global health threat

Widespread disease

- 33% of the world infected
- 10 million new cases annually
- 2 million deaths annually

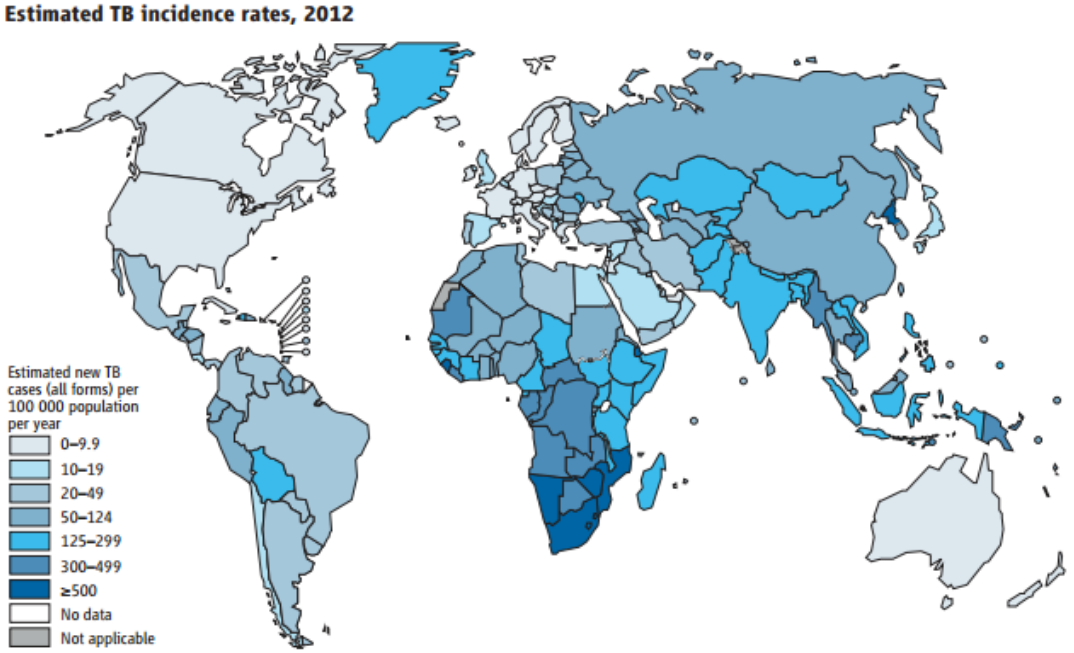
Complicated treatment

- Antibiotic therapy lasts years
- Same drugs for decades
- Resistance very common

Diagnosis shortcomings

- Sputum smear is insensitive
- Sputum culture is slow

Problem: no BSL-3 facility



WHO 2013

BSL-2 models of TB are important

Relatively small number of BSL-3 laboratories in the USA

- Majority are in public research facilities
- Building and operating costs prohibitive for many
- In contrast, virtually any laboratory can operate at BSL-2

BSL-2 models of TB are commonly used in research

- Zebrafish + *M. marinum*
- "Fish tuberculosis"
- Utility limited by biology

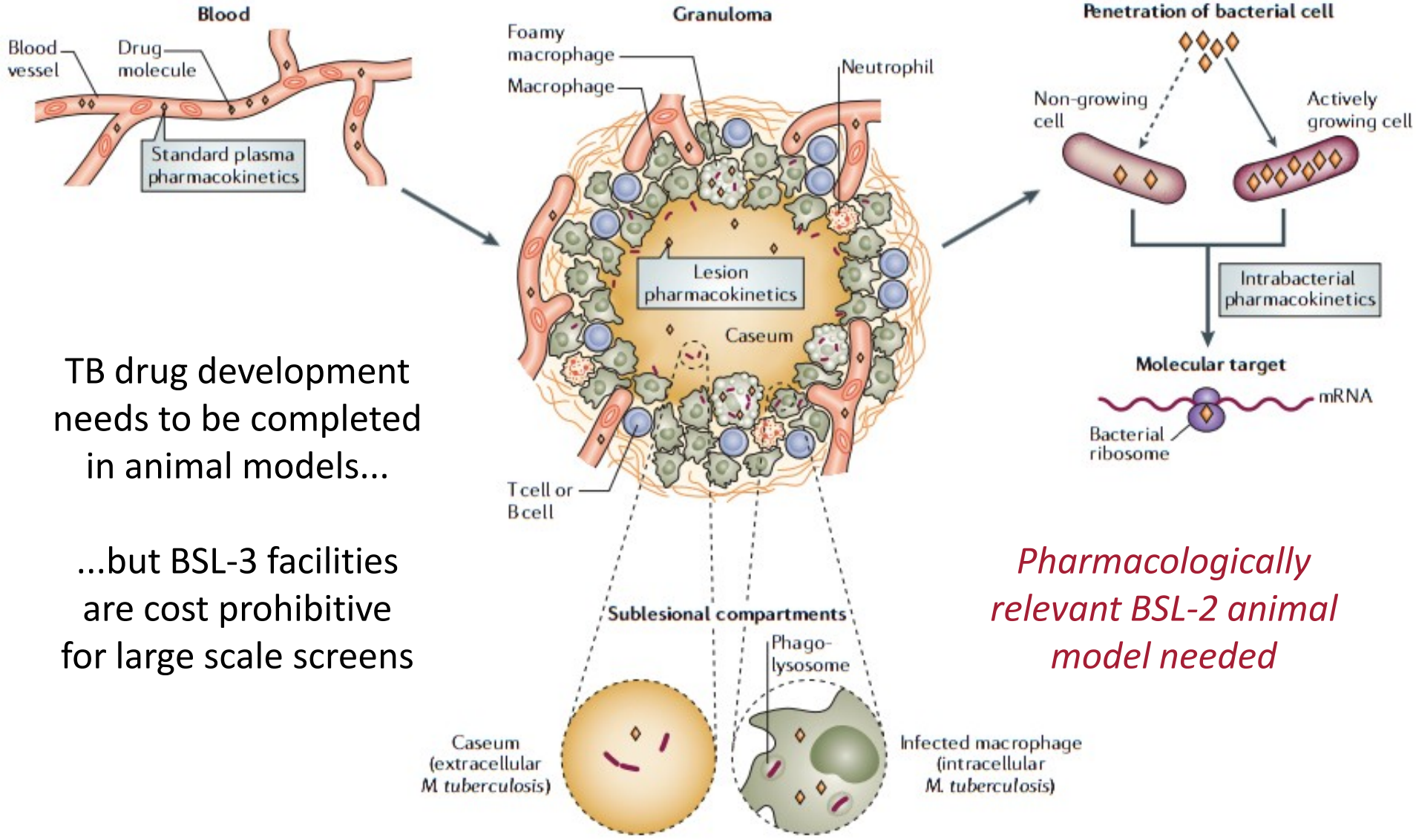
No current BSL-2 model is amenable to urine biomarker or drug development studies

Table 2: BSL-3 Labs Registered with the CDC and USDA

Sector	CDC-registered labs	USDA- registered labs	Total
	Number	Number	Number
Federal	291	167	458
Academic	429	58	487
State	248	20	268
Private	74	69	143
Total	**1042**	**314**	**1356**

GAO-08-108T

Animal models are essential to TB drugs



TB drug development needs to be completed in animal models...

...but BSL-3 facilities are cost prohibitive for large scale screens

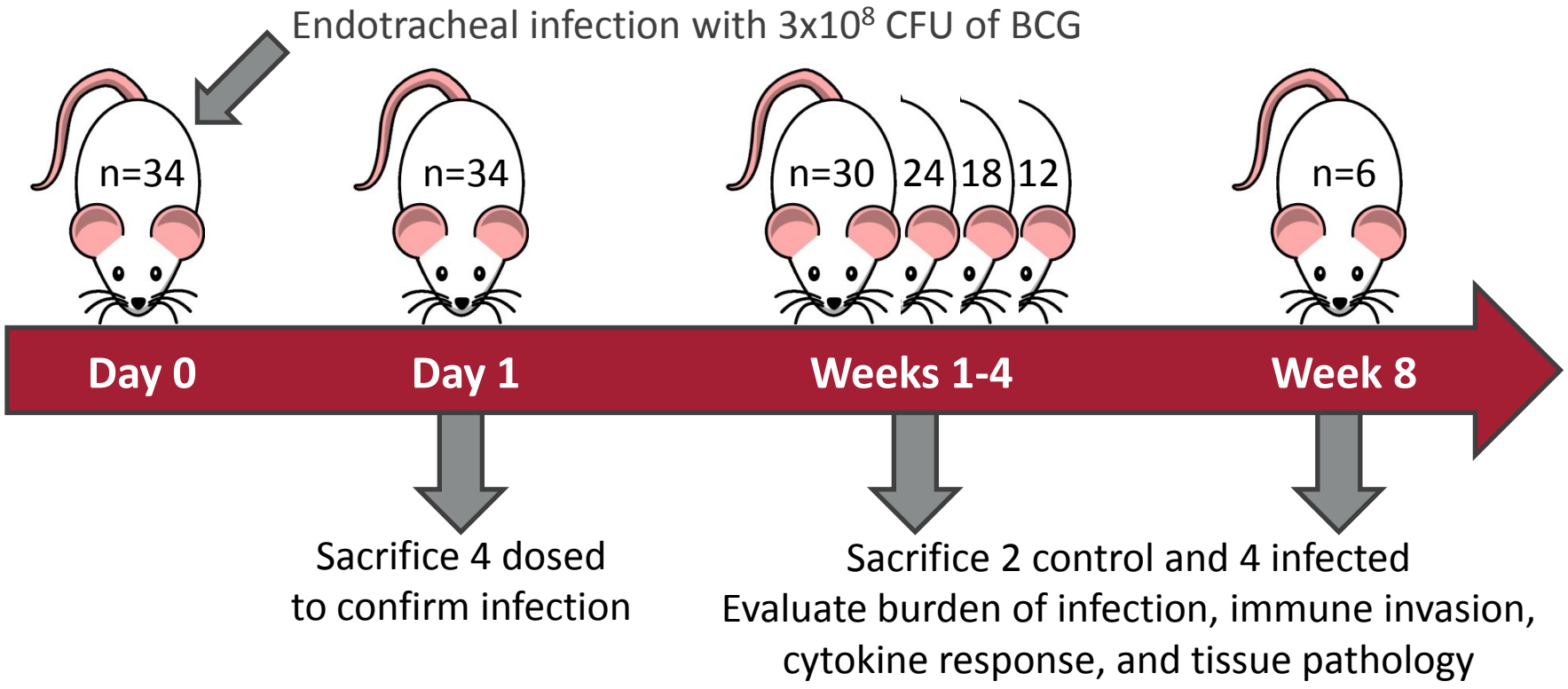
Pharmacologically relevant BSL-2 animal model needed

Study design and goals

M. bovis BCG: BSL-2 vaccine strain, 99.9% sequence identity to Mtb

Endotracheal infection: reproducible, controllable lung infection

Wistar rats: pharma workhorse, outbred for genetic variability



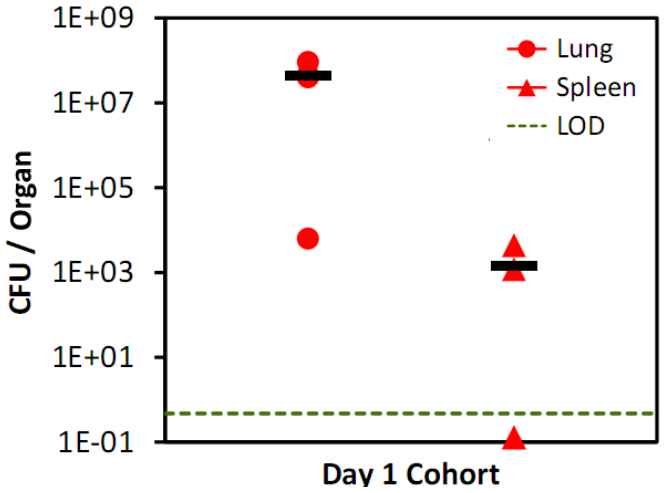
Infection is robust and sustained

Infection confirmed at day 1: all CFU+

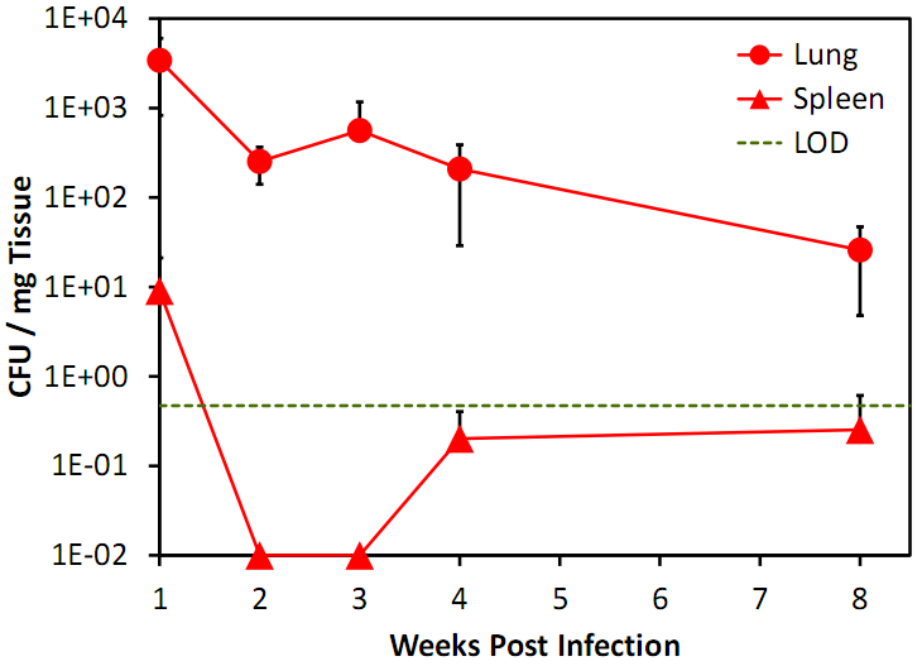
Infection success rate of at least 85%

- 20 of 24 dosed animals were CFU+
- 6 of 26 dosed animals were spleen CFU+
- One CFU- animal confirmed infected by qPCR

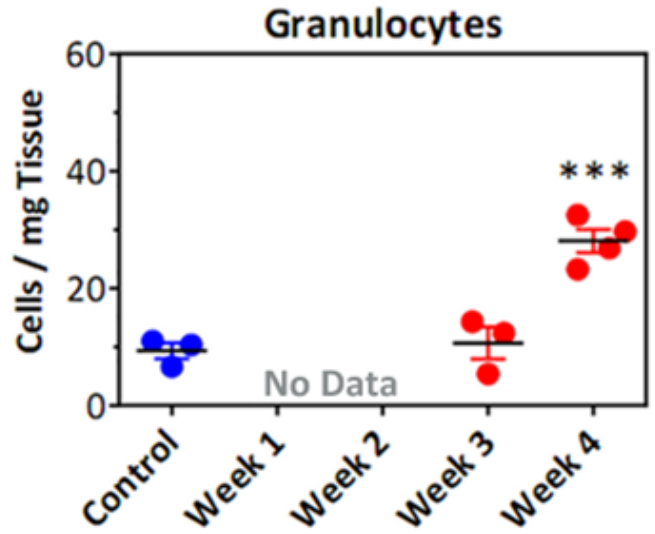
Sustained CFU load through week 8

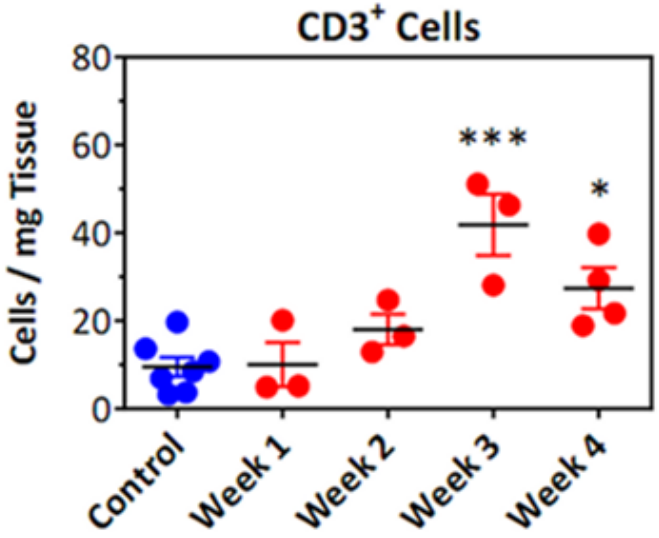


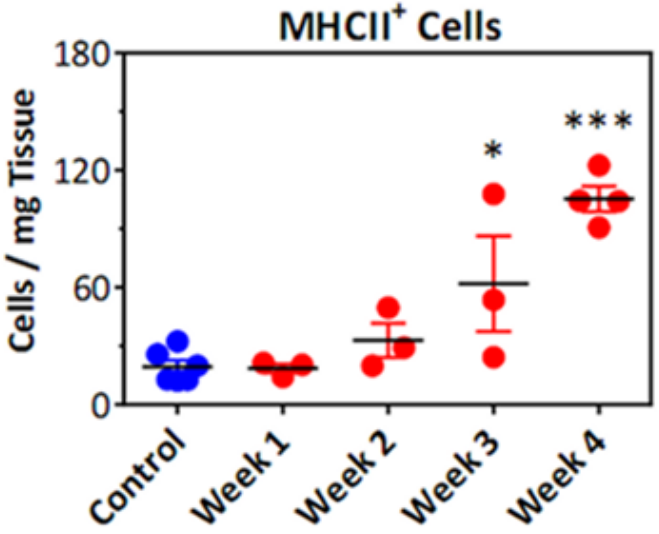
Cohort	Dosed	CFU +	CFU -	Success
Day 1	4	4	0	100%
Week 1	4	3	1	75%
Week 2	4	3	1	75%
Week 3	4	4*	0	75%
Week 4	4	4	0	100%
Week 8	4	3	1	75%
Total	**24**	**21**	**3**	**87.5%**

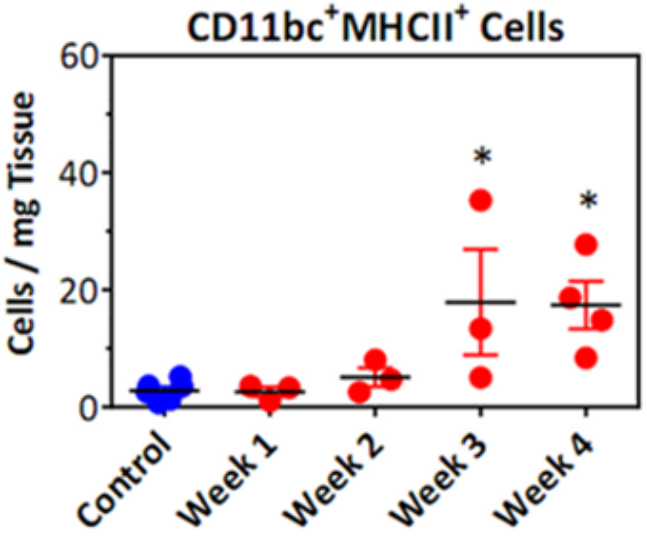


Lung immune infiltration is significant









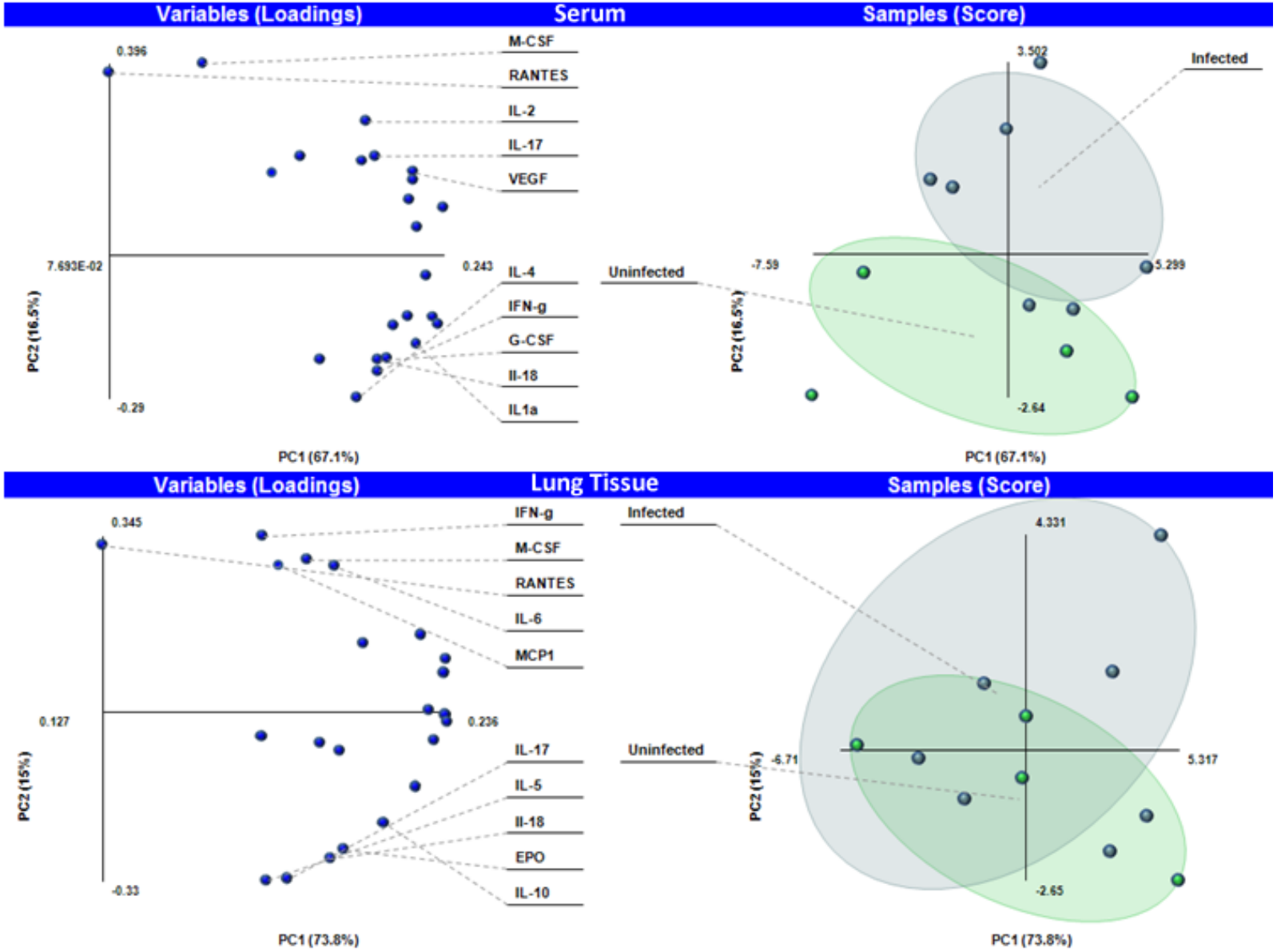
Granulocytes: innate immune cells (neutrophils)

CD3$^+$: adaptive immune T cells

MHCII$^+$: antigen presenting cells

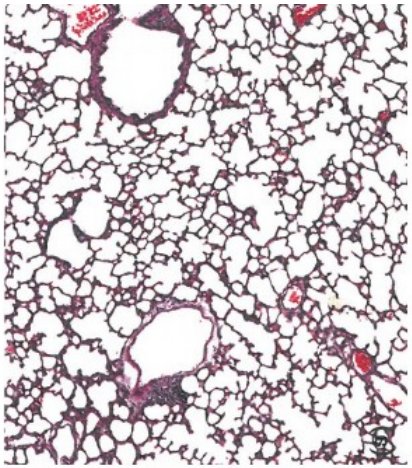
CD11bc$^+$MHCII$^+$: lung-derived antigen presenting cells

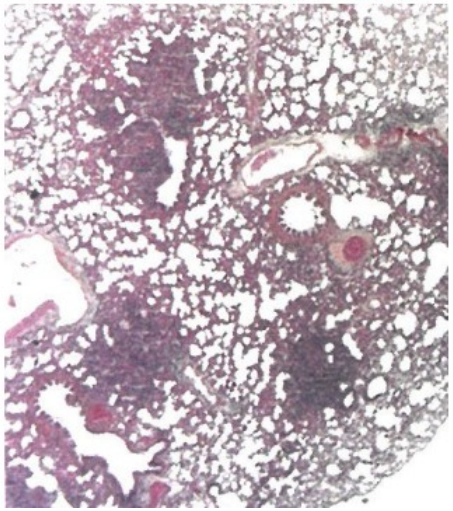
Cytokine response peaks late



Significant lung inflammation and pathology

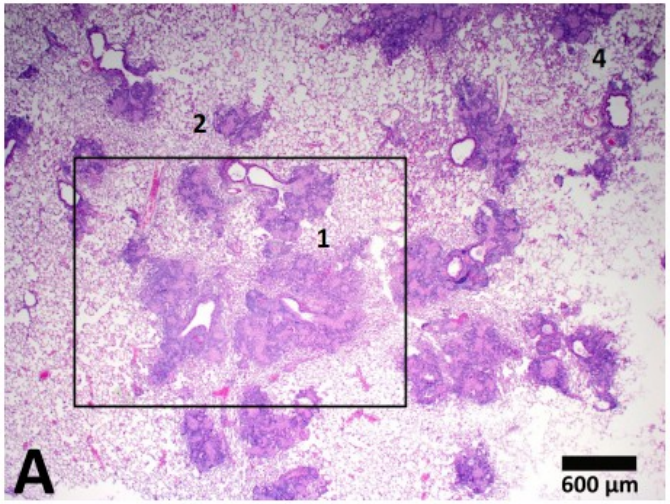
Control rat lung

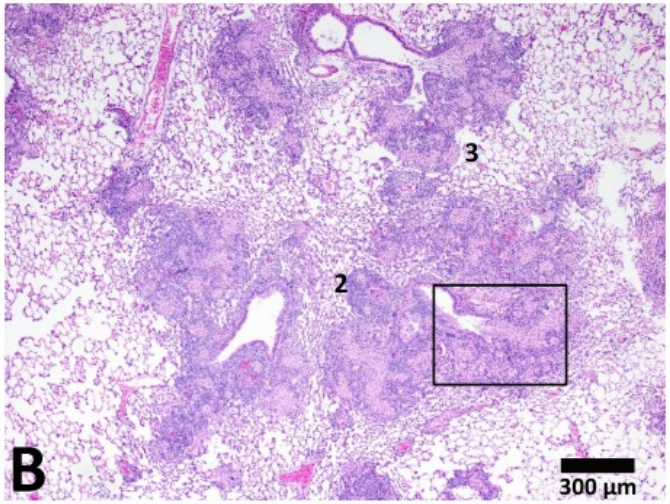




Mtb infection week 2

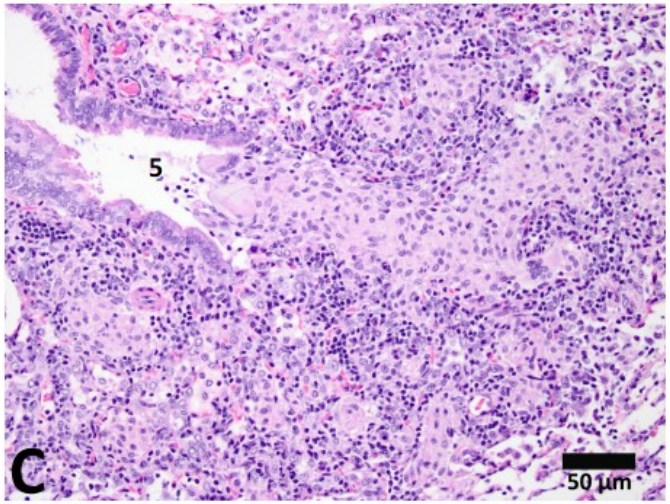
1 week post infection – representative micrographs





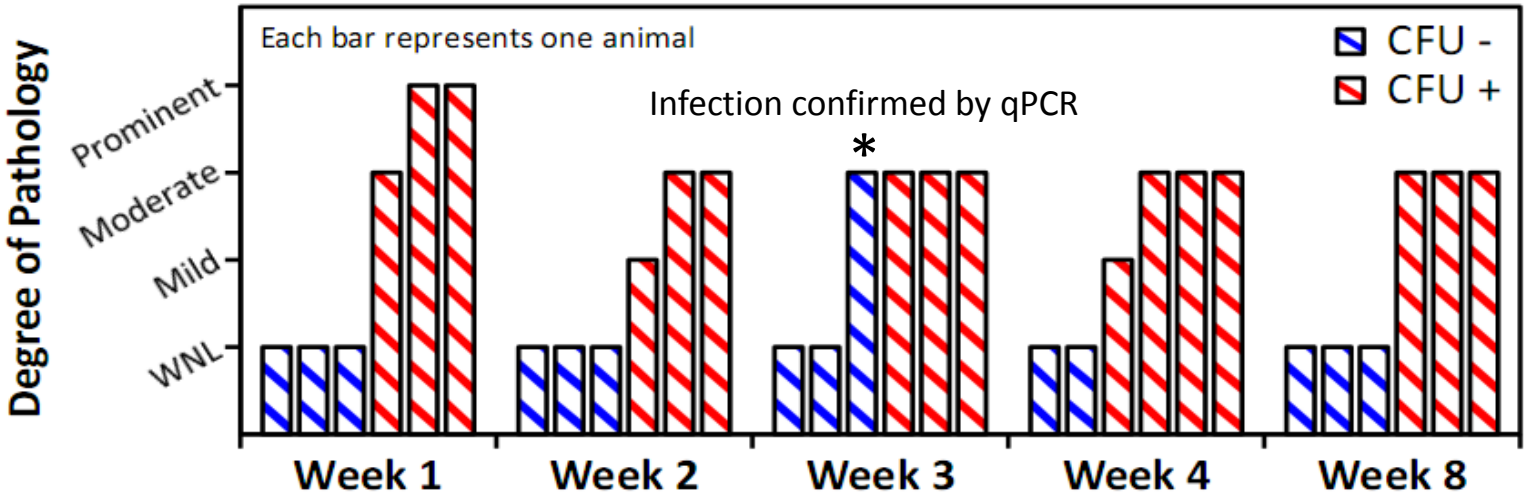
(A) 2x **(B)** 4x **(C)** 20x

1. Increased cellularity
2. Coalescing granulomas
3. Peribronchiolar location
4. Scattered small granulomas
5. Bronchiolar attenuation



Singhal *et al.* 2010 *Pulmonary Tuberculosis in the Rat*

Pathology is prominent throughout infection



Pathology remains high even as bacterial burden drops off

Several key features recapitulate the human response to Mtb

- Presence of multinucleated giant cells
- Formation of well organized granuloma and mineralization foci
- Breakdown of bronchiolar wall and invasion into the air space

Model validated – do modified nucleosides serve as biomarkers?

Urine collection and processing

Modified metabolic cage

- Mesh screen under floor (0.2 mm)
- Collection tubes suspended in dry ice
- Flash frozen urine

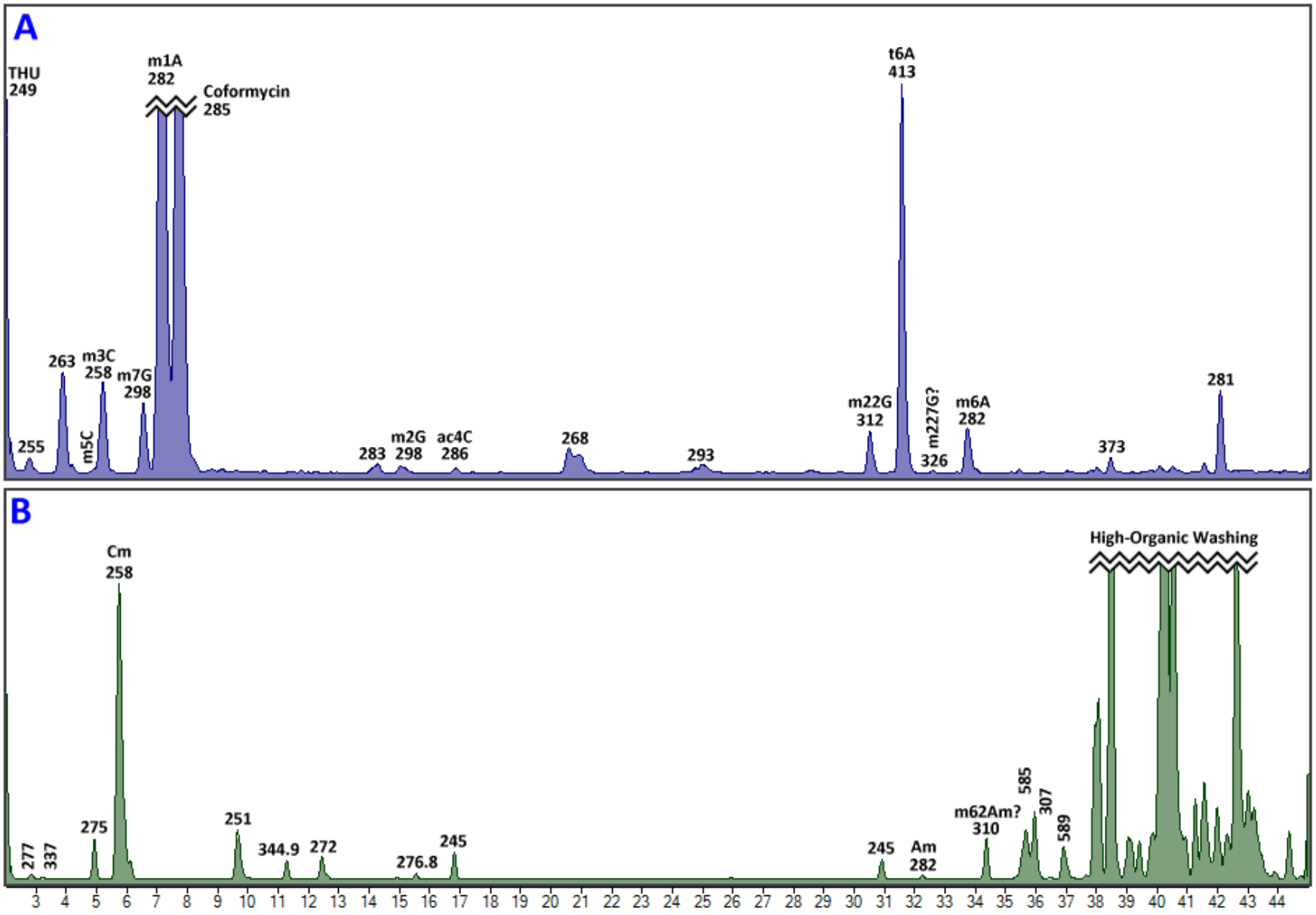
Overnight (8 – 12 hour) collection

- Fasting, unrestricted water
- Volumes from 4 to 12 mL

Minimal sample processing

- Thaw on ice
- Antioxidant cocktail
- Centrifuge and aliquot
- Benzonase digest
- 10 kD MW filter

Nucleosides are common in urine



Nucleoside profile does not predict infection

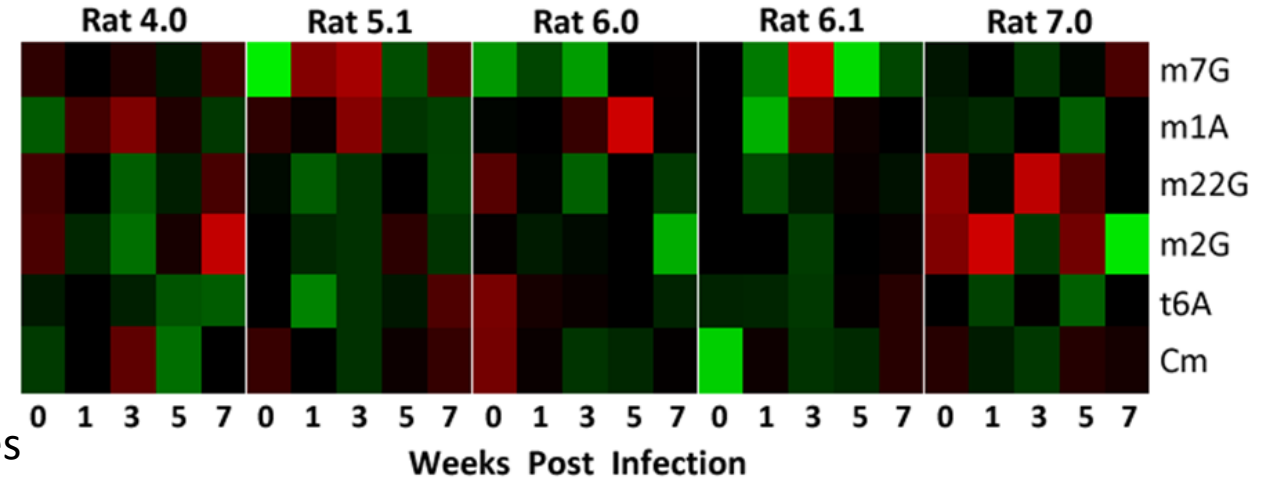
No species unique to infected

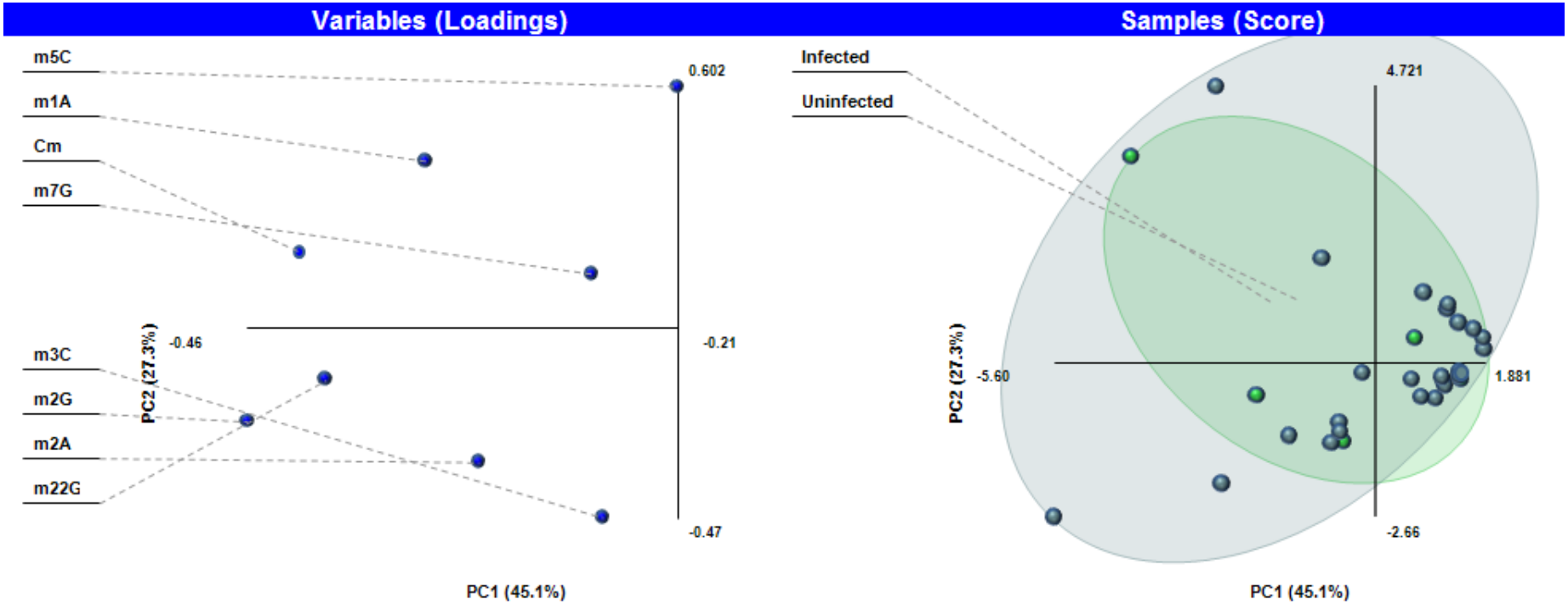
No species unique to control

No BCG-specific species

Verified species do not form a consistent pattern

Individual variability dominates





Summary and future directions

First pharmacologically useful BSL-2 animal model of Mtb

- Rat is ideal for size, ease of use, cost, and availability of reagents
- Disease is consistent even in outbred animals – human relevance

New insights into granuloma formation

- BCG lacks ESAT-6, which some reports have implicated in formation
- Most mice do not form granulomas, even with ESAT-6
- Mice lack MMP-1, but Wistar rats have it – further evidence for importance

First report of modified nucleoside profiling for infection biomarkers

- Large number of species present; patterns may emerge
- Sensitivity advances may reveal species below our limit of detection

New platform for testing RNA modifications as therapeutic targets

Our model will accelerate TB drug and diagnostic development

Specific Aim 3: Therapy

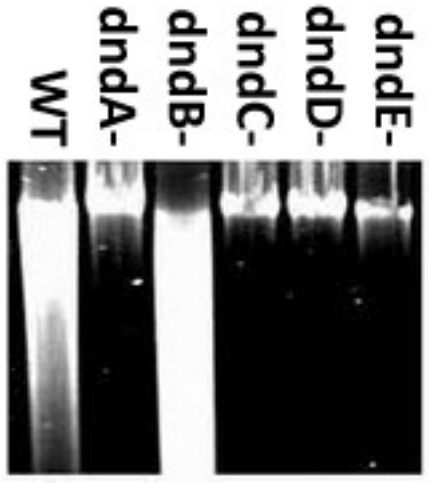
The role of bacterial DNA phosphorothioation in resistance to oxidative and antibiotic stresses

In collaboration with Michael DeMott, Bo Cao, Stefanie Kellner, Megan McBee, Emily Kolenbrander, and Aislyn Schlack

DNA degradation, sulfur, and *dnd* genes

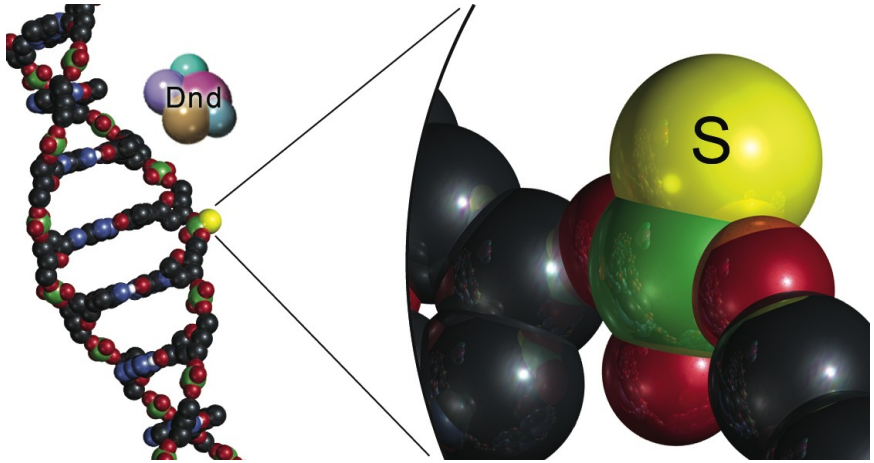
DNA degradation phenotype in *S. lividans*

- Genomic DNA subjected to Tris electrophoresis
- Knockouts of an uncharacterized gene cluster
- Phenotype could be ablated or enhanced



Caused by DNA phosphorothioation (PT)

- Phosphate oxygen replaced by sulfur
- Previously known synthetically
- Two diastereomers, *S*p and *R*p



Found in >200 bacterial species

- 5-gene cluster *dnd*
- Distantly related organisms
- Mobile genetic elements
- Many clinical isolates

Xu *et al.* 2009 | Wang *et al.* 2007 | Makarova *et al.* 2013

PT as a restriction-modification system

PT serves as a restriction system

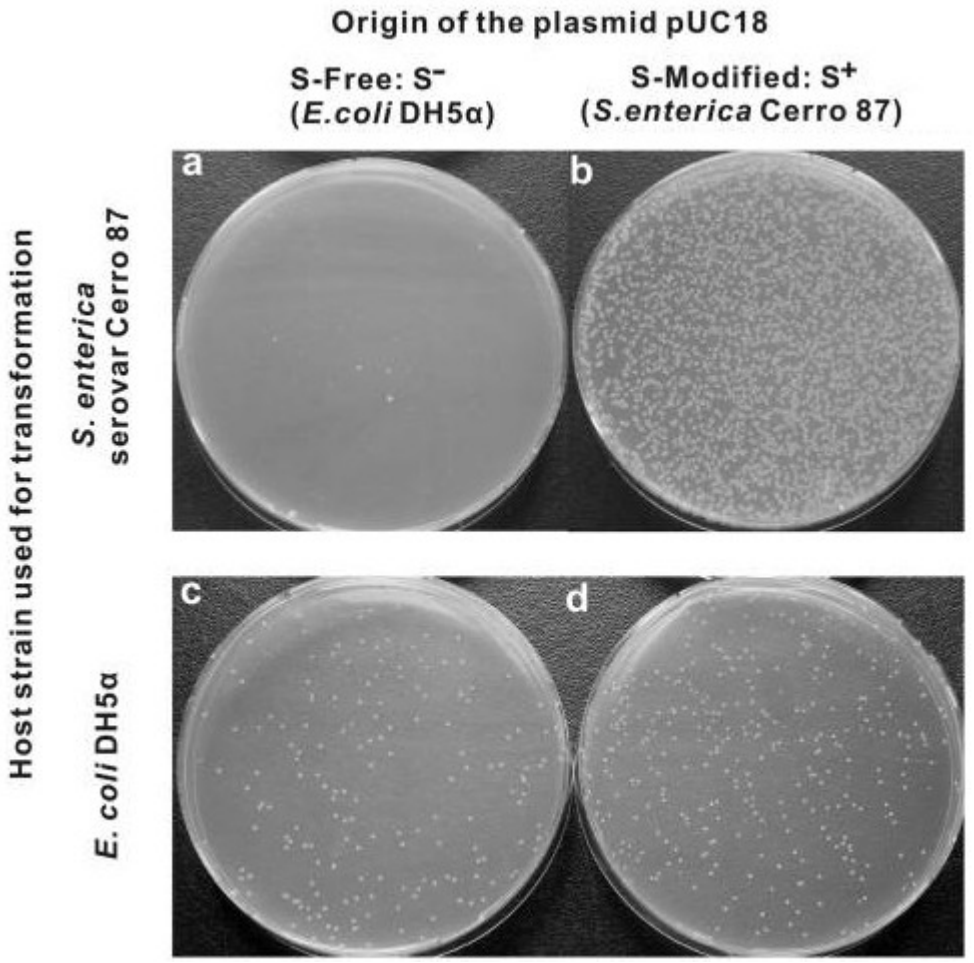
- DNA lacking PT is not taken up
- Cells lacking PT take up DNA

Additional *dnd* genes

- 3 gene cluster *dnd*F-H
- Clustered with *dnd*A-E
- Located on the same elements

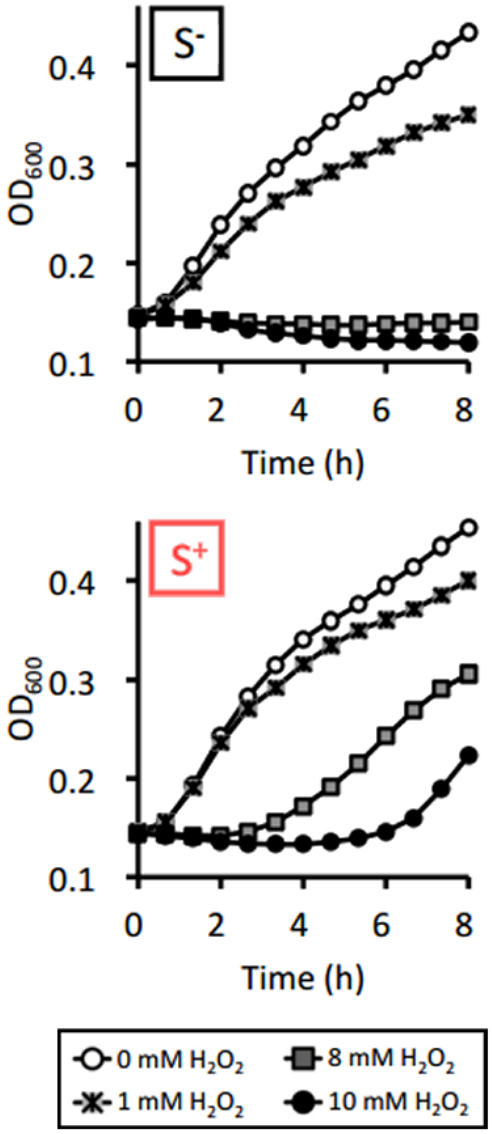
Most species lack restriction

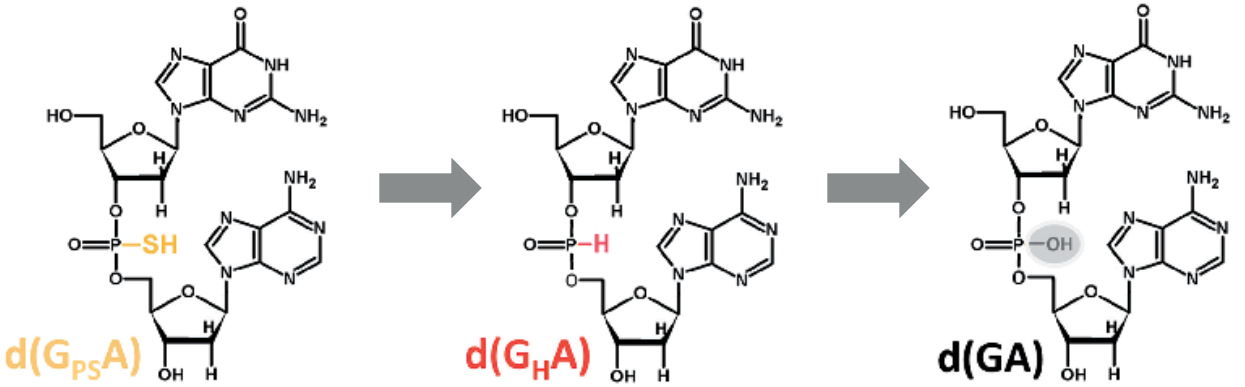
- More than half lack dndF-H
- dndF-H toxic without PT
- Other function for PT alone?

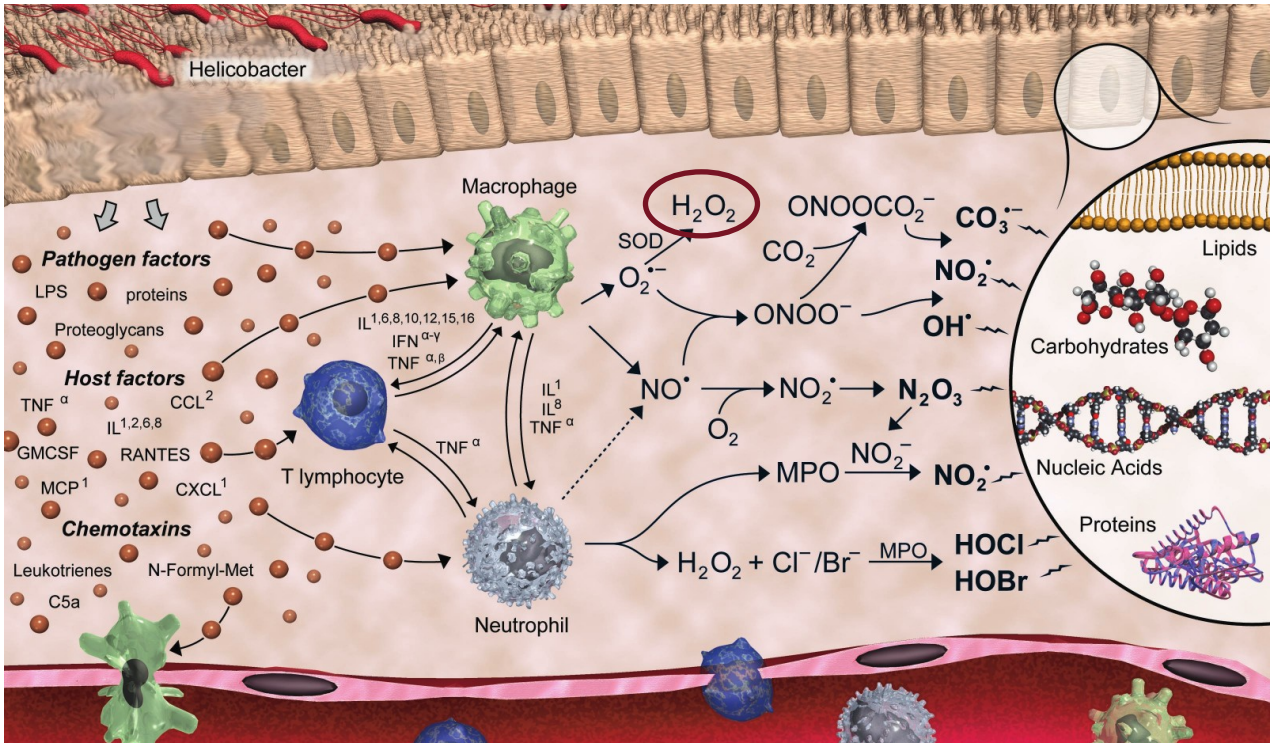


Xu *et al.* 2010 *Nucleic Acids Res*

PT and oxidative stress resistance







Xie *et al.* 2012 *Nucleci Acids Res* | Jeff Dixon

Organisms and assays

Organism	PT Source	Restriction
Citrobacter rodentium	Artificial	No
Salmonella enterica	Native	Yes
Streptomyces lividans	Native	No

C. rodentium Agents

- H_2O_2
- Streptomycin
- Kanamycin
- Gentamycin

S. enterica Agents

- H_2O_2
- Kanamycin
- Gentamycin
- Spectinomycin

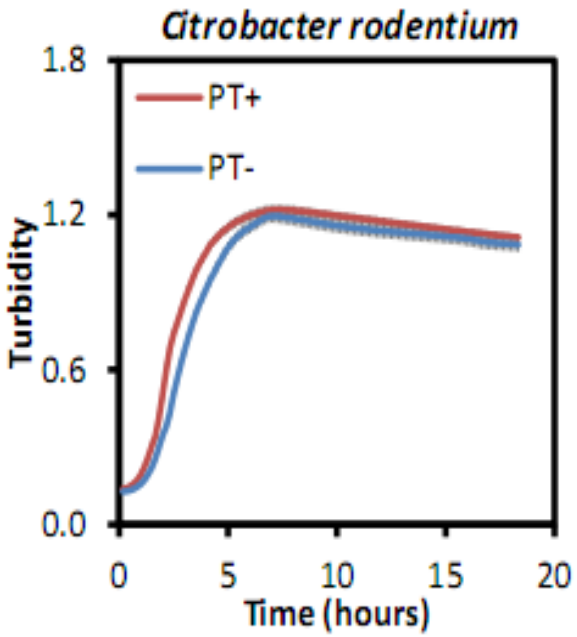
S. lividans Agents

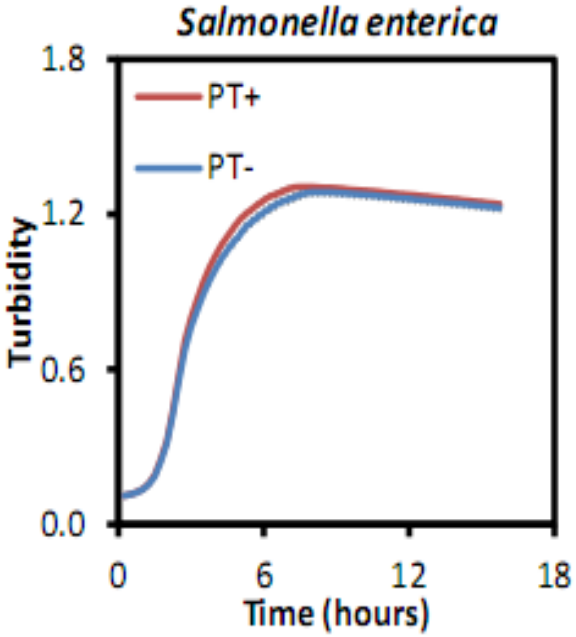
- H_2O_2
- Ampicillin
- Penicillin

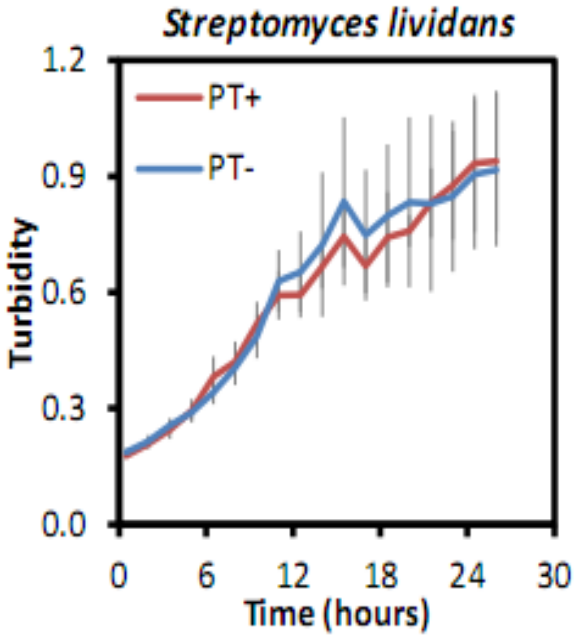
Growth curves show inhibition; platings show active killing

Representative data are shown for each organism; highly reproducible

PT genotypes do not alter growth rate



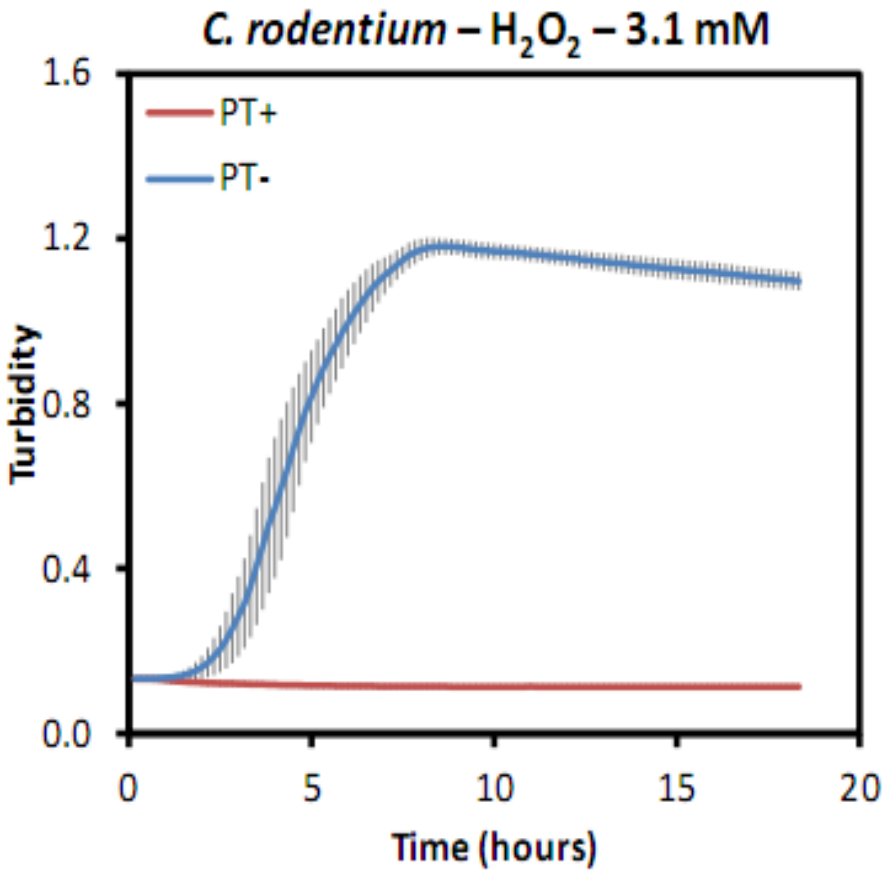


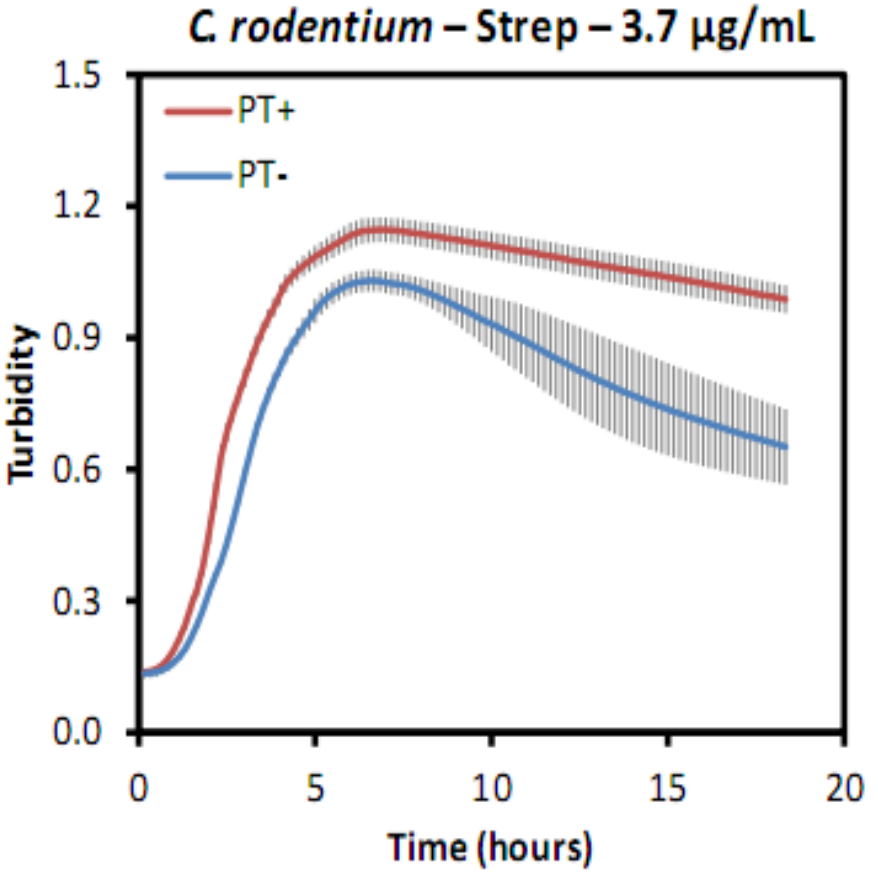


Artificial PT confers only antibiotic resistance

PT+ strain is much more sensitive to H_2O_2 stress

PT+ strain is more resistant to antibiotic stress

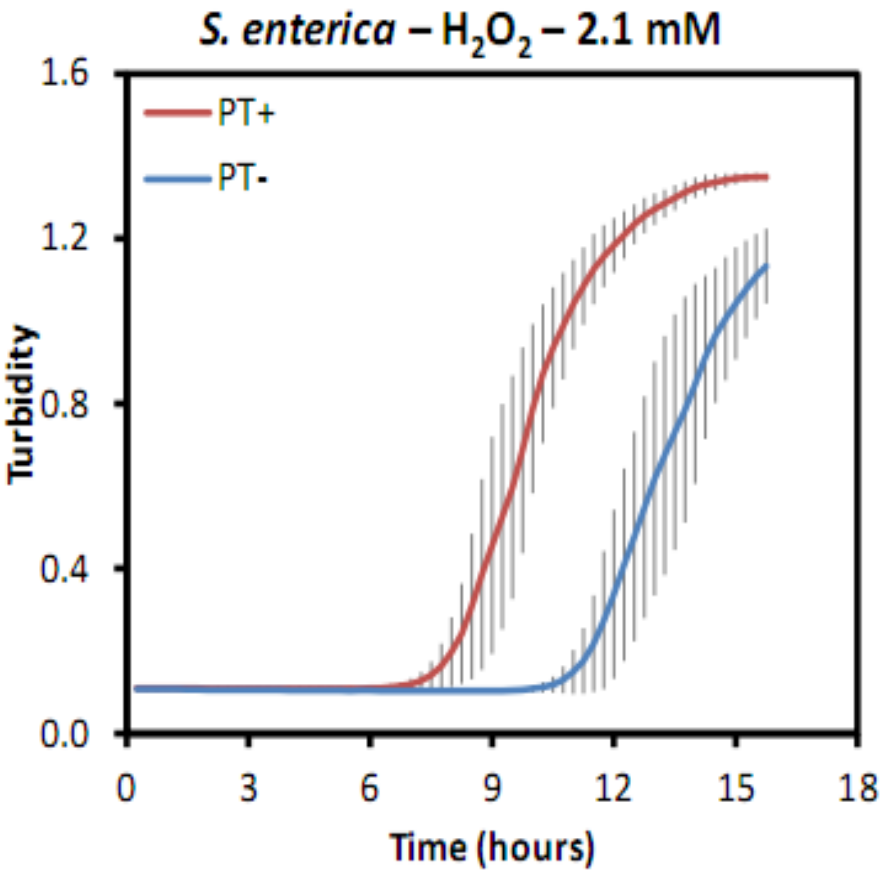


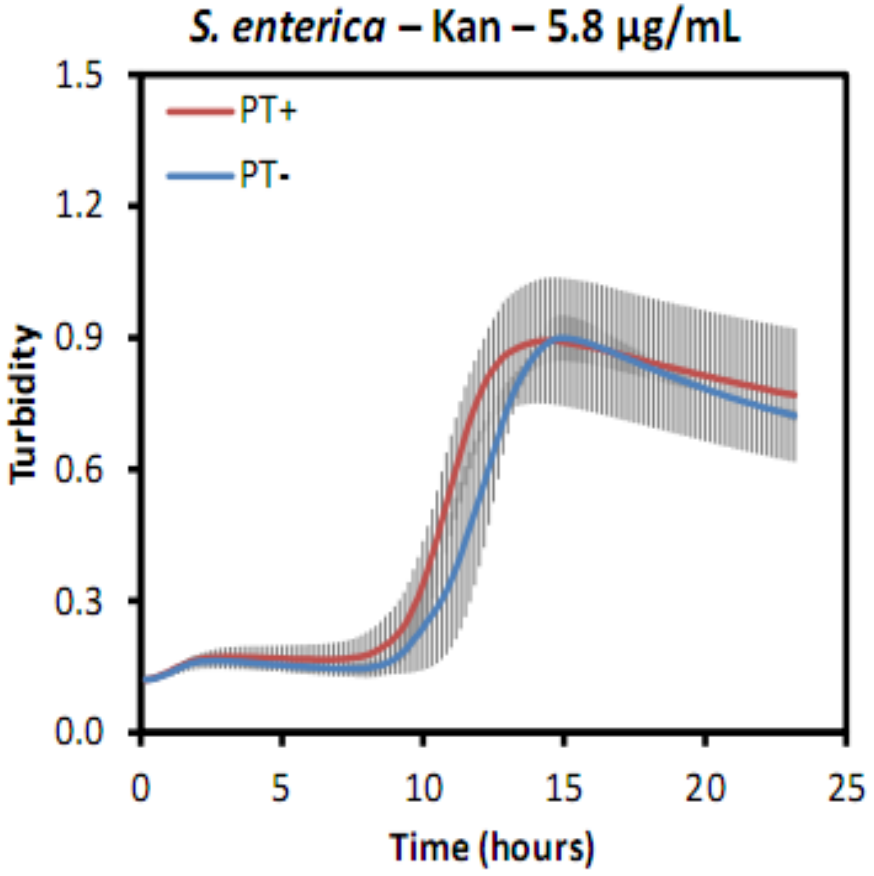


Native PT and restriction: only oxidative resistance

PT+ strain is more resistant to H_2O_2 stress

Strains are equivalent in their response to antibiotic stress

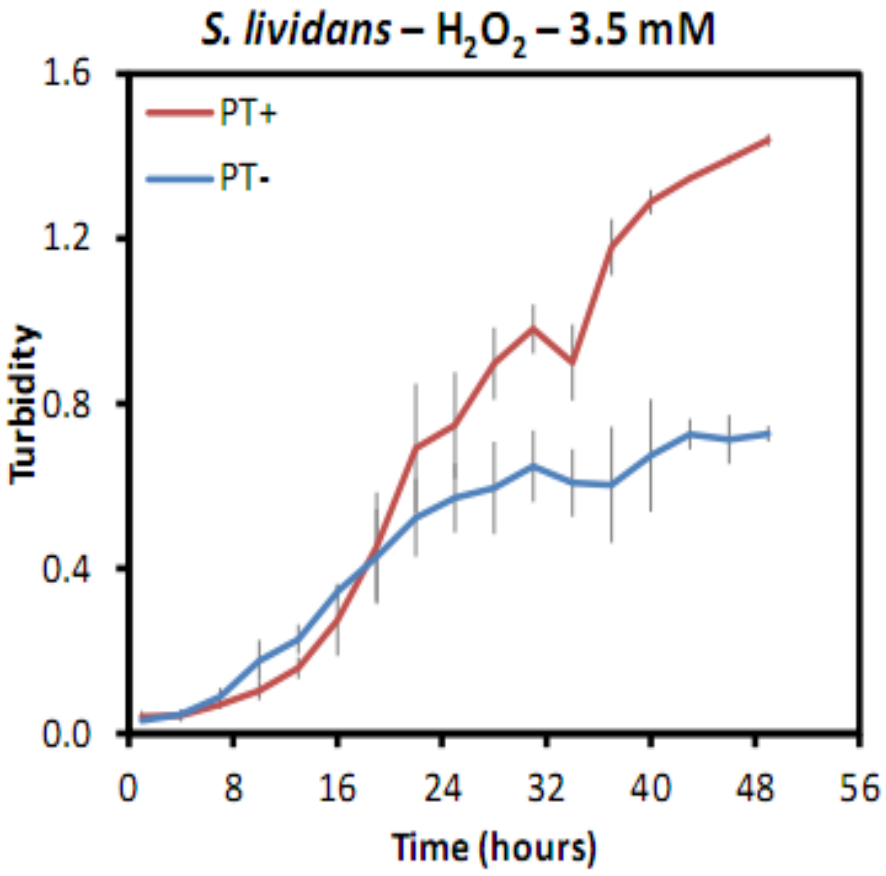


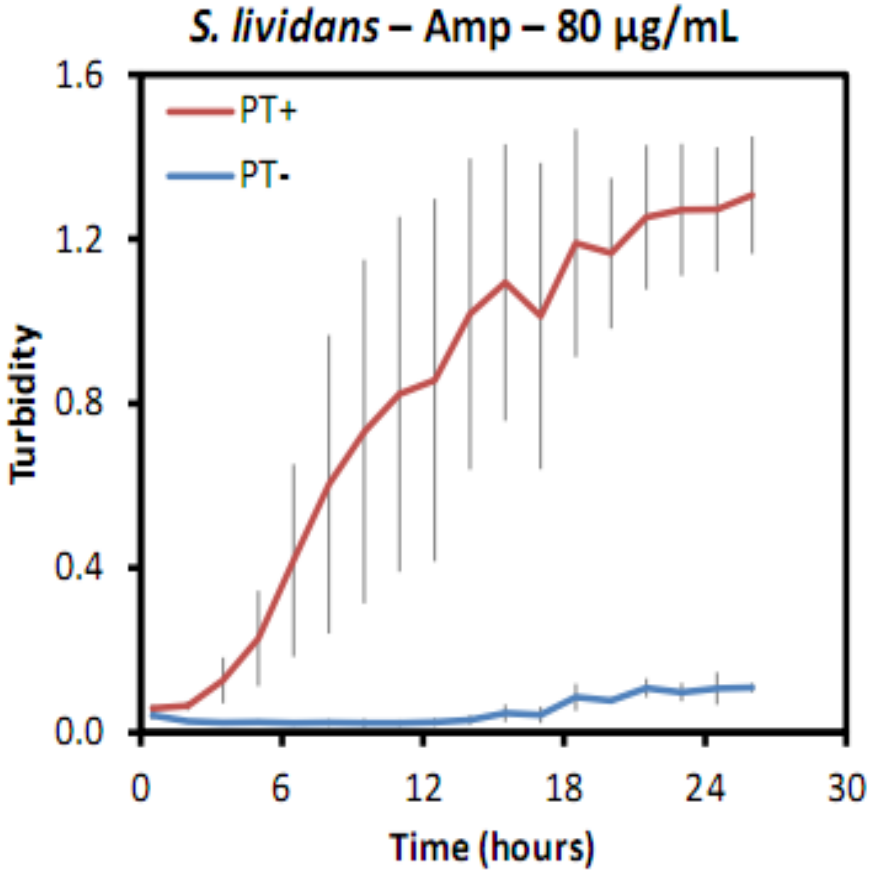


Native PT lacking restriction: complete resistance

PT+ strain is more resistant to H_2O_2 stress

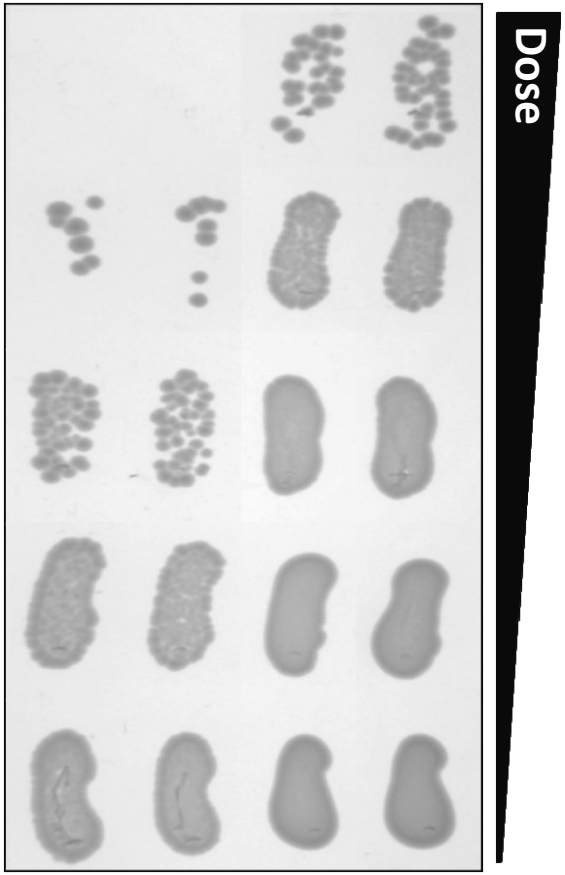
PT+ strain is much more resistant to antibiotic stress





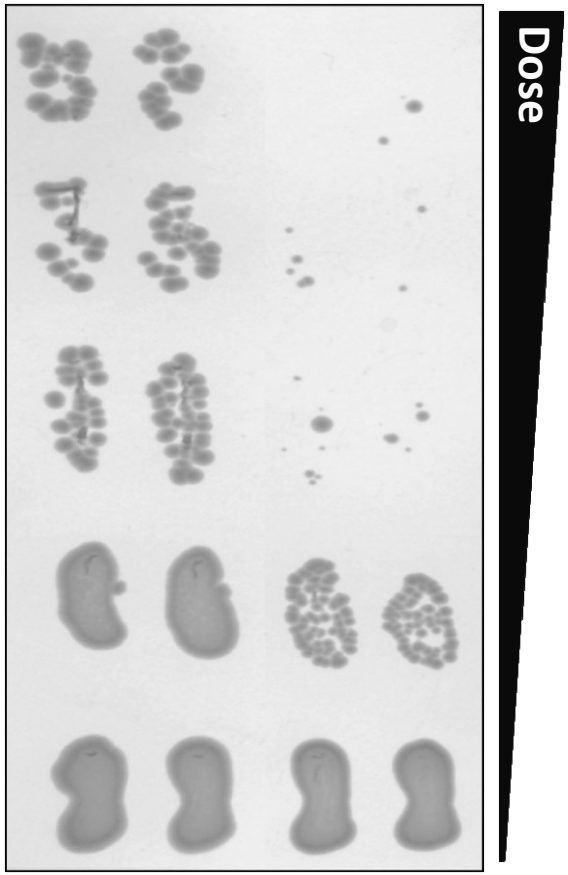
PT effects are mediated by cell death

C. rodentium – H_2O_2



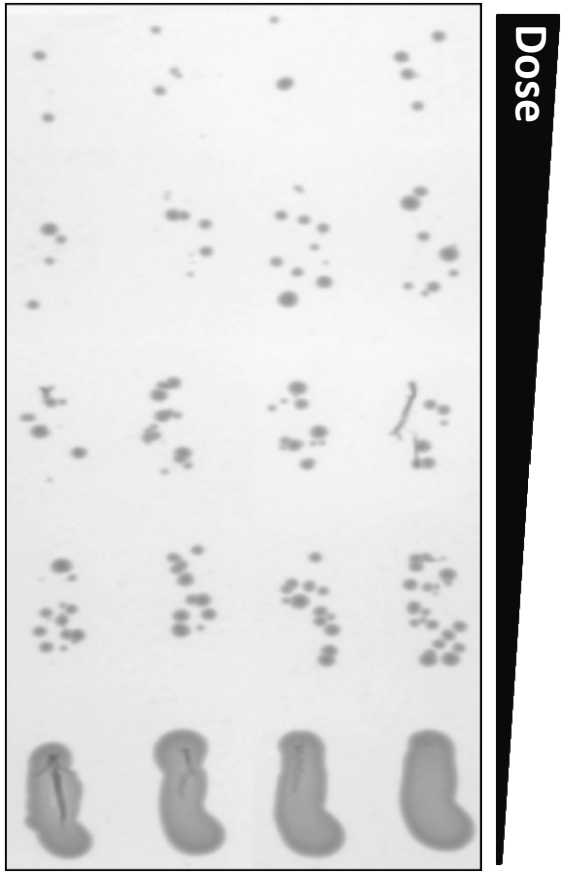
PT+ PT-

C. rodentium – Strep



PT+ PT-

S. enterica – Kan



PT+ PT-

Summary and future directions

First systematic profiling of the effect of PT

First report of PT conferring antibiotic resistance

- Significant effect: more than doubles the minimum inhibitory concentration
- Substantial growth advantage might allow PT-positives to take over

First report of organism- and toxicant-dependent PT effect

- Not all antibiotics causing oxidative stress?
- H_2O_2 not causing oxidative stress?

Many potential human health impacts

- PT widespread in clinical isolates – should we be testing for it?
- Can targeting PT synergize with antibiotics or immune system?
- Gut microbes known to harbor PT – role in disease?

Phosphorothioation may serve as a novel therapeutic target

Summary of contributions

First report of RNA modifications involved in bacterial pathogenesis

- New targets for antibiotic development
- Possible prophylaxis to prevent infection

First BSL-2 model of TB suitable for drug and diagnostic development

- Proof-of-concept biomarker study
- Suitable for determining granuloma targeting

First report of PT DNA conferring transferable antibiotic resistance

- Possibly important clinical parameter
- New target for antibiotic adjuvant

Pathogenesis
- Establish infection
- Target: stop disease

Diagnosis
- Confirm infection
- Target: faster therapy

Therapy
- Clear infection
- Target: cure disease

RNA and DNA modifications are critical in fighting bacterial disease

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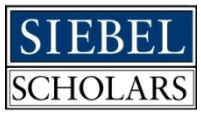

Final thoughts and words

Graduate school is hard [citation needed]

Kimberly Russell

1964 – 2009

Shirley Russell

1942 – 2012

Alexander McAdams

Peter Dedon

Thank you all from the bottom of my heart!