Identification of *M. tuberculosis pncA* gene single nucleotide polymorphisms conferring resistance to pyrazinamide.

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Declaration

I, **Kashmeel Maharaj**, hereby declare the following:

I. Research presented in this dissertation is my original work, except where otherwise indicated, and has been carried out under the guidance and supervision of Dr. Alexander Pym at K-RITH, Nelson R Mandela School of Medicine, KwaZulu-Natal.

II. This dissertation has not been submitted for any degree or examination at any other university.

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V. This research is part of a study which is in the process of being submitted for publication under the title of "Comprehensive Genotypic-Phenotypic Characterization of *pncA* Polymorphisms Conferring Resistance to Pyrazinamide Using *in vivo* Selection", with myself as the co-prime author. As such components of research not done by myself will be clearly indicated and acknowledgement given to the relevant contributors.

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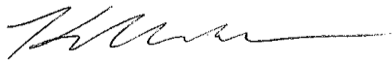

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List of abbreviations

ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BSA	Bovine Serum Albumin
cDNA	complementary Deoxyribonucleic Acid
CFU	Colony Forming Units
CoA	Co-enzyme A
Comp	Complement
DMSO	Dimethyl Sulphoxide
DnaK	Chaperone protein DnaK
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
DST	Drug Susceptibility Testing
EBA	Early Bactericidal Activity
FAS-I	Fatty Acid Sythase-I protein
HDB	Hartmans-de-Bont
HPOA	Protonated pyrazinoic acid
Indel	Insertion/deletion mutations
LB	Luria Bertoni
M. tuberculosis	*Mycobacterium tuberculosis*
M. bovis	*Mycobacterium bovis*
M. bovis BCG	*Mycobacterium bovis* Bacillus Calmette-Guerin
M. canetti	*Mycobacterium canetti*
M. smegmatis	*Mycobacterium smegmatis*
MBC	Minimum Bactericidal Concentration
MDR-TB	Multi drug resistant tuberculosis
MGIT	Mycobacteria Growth Indicator tube
MIC	Minimum Inhibitory Concentration

MODS	Microscopic Observation Drug Susceptibility
MOP	Mycobacterial Optimized Promoter
NAAT	Nucleic Acid Amplification Test
NAD^+	Nicotinamide adenine dinucleotide
MaMN	Nicotinic acid mononucleotide
PACT	Polymixin, Amphotericin, Carbenicillin, Trimethoprim
PanD	Aspartate Decarboxylase
PCR	Polymerase Chain Reaction
PDIM	Phthiocerol dimycoserate
PMF	Proton Motive Force
PncA	Pyrazinamidase protein
POA	Pyrazinoic Acid
POES	Polyoxyethylene Stearate
PZA	Pyrazinamide
PZase	Pyrazinamidase
QAPRTase	Quinolic acid phosphoribosyltransferase
RNA	Ribonucleic acid
RNase	Ribonulcease
RpsA	Ribosomal protein S1
RpoB	DNA-directed RNA polymerase subunit *beta*
RT-qPCR	Real Time-quantitative Polymerase Chain reaction
SmpB	Small basic protein B
SNP	Single Nucleotide Polymorphisms
SOC	Super Optimum broth with Catabolic susppression
TB	Tuberculosis
TE	Tris-EDTA
TTP	Time-to-positivity
VC	Vector control
WHO	World Health Organization
WT	Wild type
XDR-TB	Extensively drug resistant tuberculosis
OD_{600}	Optical density at 600 nanometers

L	Liter/s
M	Molar
mM	Millimolar
mL	Milliliter/s
ng	Nanogram/s
nm	Nanometer/s
µg	Microgram/s
µM	Micromolar
µm	Micron
µL	Microliter
x g	Relative centrifugal force

Abstract

Introduction: Pyrazinamide is an integral component of anti-tuberculosis therapy as it has contributed to the shortening of treatment. Pyrazinamidase, encoded by *pncA*, activates pyrazinamide, with mutations in *pncA* being the primary mechanism of resistance topyrazinamide. Defining the full repertoire of resistance conferring mutations, which is still unknown, is critical to developing a genotypic diagnostic assay and optimizing individualised regimens for more favourable treatment outcomes.

Methodology: Random PCR mutagenesis and cloning were used to generate and express a*pncA* mutant library harbouring single nucleotide polymorphisms in a *pncA*-null strain of *M. tuberculosis*. We screened our library *in vitro* and *in vivo* against pyrazinamide,and sequencedrecovered libraries to determine the enrichment of mutant clones during selection to predict pyrazinamide susceptibility. Isogenic *pncA* mutants underwent phenotypic drug susceptibility testing to validate our screen predictions. Proteomics and a modified Wayne's enzymatic assay were utilized to elucidate the molecular basis of pyrazinamide resistance in selected isogenic *pncA* mutants.

Results: After provisional analysis, predictions for >85% of all possible mutations at the amino acid level were made, with 411 and 357 mutations predicted to result in pyrazinamide resistant and susceptible phenotypes respectively, and 374 mutants being neither enriched nor depleted. Drug susceptibility testing of isogenic *pncA* mutants showed 100% and 92.86% agreement with provisional screen predictions for resistance and susceptibility respectively, with unselected mutants being classified as intermediate phenotype and concluded to be susceptible. Proteomics and an enzyme assay on selected isogenic mutants revealed reduction of pyrazinamidase and/or its enzymatic function were mechanisms of conferring resistance to pyrazinamide.

Conclusion: Random mutagenesis combined with deep-sequencing of isogenic libraries is a promising method of simultaneously evaluating multiple SNPs for their ability to confer drug resistant phenotypes, and allowed us to provisionally predict phenotypes for 1142 *pncA* mutants. Subject to completion of data analysis, this list may serve as a reference for interpreting sequences/mutations in a genotypic based diagnostic assay. In addition to contributing knowledge to the understanding of pyrazinamide activity and confirming disruption of the enzymatic activity of pyrazinamidase by selected mutations in *pncA*, reduction in pyrazinamidase abundance through intragenic *pncA* mutations was identified as a mechanism of pyrazinamide resistance.

Chapter 1: Introduction

Tuberculosis (TB), caused by the bacterial organism *Mycobacterium tuberculosis* (*M. tuberculosis*), remains a massive public health threat causing annually as estimated 9.6 million new cases and 1.5 million deaths[1]. Pyrazinamide remains a cornerstone of not only current, but potential future treatment regimens [2-4], particularly for drug susceptible tuberculosis, as in combination with rifampicin, it allowed for the shortening of treatment duration down to six months[5-9]. In light of the emergence of drug resistant tuberculosis, early detection of this resistance would allow for the customization of treatment regimens and more favourable patient outcomes.

With developments in medical research and technology, there has been a drive towards faster, genetic based diagnostic assays for the detection of drug resistance. This advancement has led to the endorsement of Nucleic Acid Amplification Tests (NAAT) for the detection of TB infection and drug resistance[10]. Examples of such are the GeneXpert MTB/RIF (Cepheid, USA) which allows for detection of rifampicin resistance, and the Hain MDRplus line probe assay (Hain Lifescience, Germany) which allows for the simultaneous detection of rifampicin and isoniazid resistance. The basis of these tests however, is that they target specific mutations in defined genes that have been well characterized and shown to confer resistance[11-18]. These tests generate drug susceptibility results within 24-48 hours, whereas the conventional phenotypic drug susceptibility tests (dependent on isolating and growing *M.tuberculosis*) can take six to eight weeks.

Phenotypic drug susceptibility testing to pyrazinamide is notoriously challenging and unreliable [19-24]. These factors coupled with the high commercial cost, need for specialised biosafety facilities and trained laboratory staff, means that phenotypic drug susceptibility testing to pyrazinamide is rarely performed in many settings[24] nor recommended unless in the management of drug resistant tuberculosis [25-27]. Given the essentiality of pyrazinamide in TB chemotherapy, and the alarming emergence and prevalence of drug resistant TB, the introduction and implementation of a rapid, genotypic based assay for detecting pyrazinamide resistance is a priority.

Mutations in the *pncA* gene of *M. tuberculosis* have been shown to be the primary driver of genetic resistance to pyrazinamide [24,28-33], however it is still unclear which specific mutations actually confer resistance as mutations have previously been identified along the full length of the 561 nucleotide*pncA* gene[24,31-33]. Initially it was thought that any non-synonymous mutation in *pncA* must have been selected by pyrazinamide treatment and would therefore confer resistance, but some mutations have been shown to have no effect on drug resistance [34]. In addition there are a substantial number of mutations that have been associated with both a resistant and susceptible phenotype. A comprehensive list of mutations

conferring resistance to pyrazinamide is crucial to the implementation of any genotypic diagnostic assay, and various approaches have been proposed[35-37].

Molecular biology tools such as site directed mutagenesis[38]and single-strand recombineering [39-41] have been developed, which have allowed for introduction of single mutations into genes in an isogenic background of the bacteria. These tools allowed for more rigorous and reliable testing of associations between specific mutations and drug resistance. In the case of *pncA* however, these techniques would be laborious and time consuming as it require the generation of 1683 separate mutant strains, thus a more efficient and feasible approach is required to comprehensively study the phenotypic consequences of mutations in*pncA*.

This thesis details our unbiased approach to generate a complex library of *pncA* single point mutations in an isogenic strain of *M. tuberculosis* where the endogenous copy of *pncA* has been disrupted by the insertion of a hygromycin resistance cassette, and then to screen this library to fully characterize the full repertoire of resistance conferring mutations.

The remainder of this first introductory chapter serves to highlight the history and importance of pyrazinamide in tuberculosis chemotherapy, our current knowledge on the action of pyrazinamide in tuberculosis chemotherapy as well as the gaps in current knowledge, and the difficulties associated with drug susceptibility testing for pyrazinamide. This chapter includes a problem statement, research rationale, and the specific aims and objectives of our work. Chapter two encompasses the methodologies used in generating our *pncA* mutant library, screening assays to determine mutations conferring pyrazinamide resistance, and in elucidating the mechanisms of resistance. Chapter three is a representation of the results obtained and is followed by a discussion of our findings, limitations of our work, and concluding remarks.

1.1. Role of pyrazinamide in tuberculosis chemotherapy

Although pyrazinamide is an essential cornerstone of the current treatment regimens, particularly drug susceptible tuberculosis, prior to the 1970's it was mainly utilized as a second-line TB agent for the treatment of drug resistant TB or in cases of relapse, due to hepatotoxic side-effects associated with a high dose and combination with isoniazid [42,43,44,45,46]. The discovery of pyrazinamide as an anti-tuberculosis agent was different from many other currently used tuberculosis drugs. Soon after its discovery it was tested *in vivo* and shown to have activity. When subsequent *in vitro* testing was carried out, no activity was detected, meaning it would not have been identified in the contemporary approach of using *in vitro* screens as a triage for taking compounds into animal models [9].

1.1.1. Early observations of pyrazinamide in tuberculosis chemotherapy

First synthesized in 1936 [48], pyrazinamide's use for the treatment of tuberculosis was discovered after the initial observation of the anti-tuberculosis effects of nicotinamide [49,50]. This work was followed up by the testing of nicotinamide analoguesin mouse and guinea pig models of tuberculosis treatment, where pyrazinamide was found to be a highly active analogue of nicotinamide against TB[51-54]. Pyrazinamide was tested as a single treatment agent in human trials where it showed sputum conversion after four months, however this finding was overshadowed by the later reactivation of the disease termed as relapse[55]. It showed more promising results as a sterilizing agent (the ability to kill "semi-dormant" or non/slow-replicating persister bacterial populations which are though to lead to relapse) when combined with isoniazid[42,56-58]. Combinations with streptomycin and *para*-aminosalicylic acid were less successful which could be due to isoniazid being more potent in targeting actively replicating bacteria[7,59].

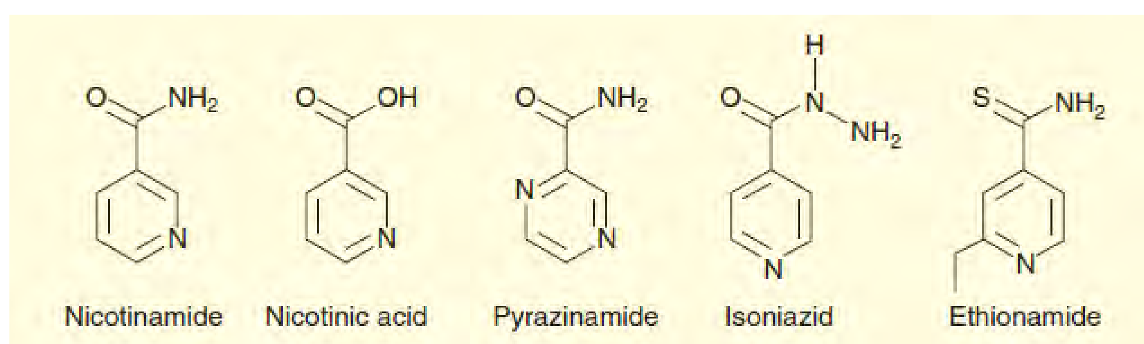

Figure 1: Structures of nicotinamide and nicotinamide analogues[60].

This result was mirrored when it was shown that almost all other singular or paired combination treatment regimens, containing streptomycin, isoniazid, *para*-aminosalicylic acid, and oxytetracycline, tested in a mouse model failed to eradicate TB infection, whereas a longer term "repression" of the infection (long period of non-culturability of bacteria after cessation of treatment, now known as the

Cornell model of TB dormancy) was observed when isoniazid was used in combination with pyrazinamide [61-64]. This finding supported the idea that pyrazinamide was able to target a so-called "persistent" population of bacteria, unaffected by other drugs. Although the findings of regimens combining streptomycin with pyrazinamide in retreatment patients were unsatisfactory due to relapses, it was still concluded that the addition of pyrazinamide was beneficial [44]. Later *in vitro* experimentation suggested that intermittent use of pyrazinamide produced a lag in growth of the bacteria making it suitable for tuberculosis chemotherapy [65].

1.1.2. Pyrazinamide in shortening treatment duration for drug susceptible tuberculosis

Initial findings in further human trials testing combination treatments with isoniazid, streptomycin, rifampicin and thiacetazone [66-69] showed that inclusion of pyrazinamide in experimental regimens proved superior for drug susceptible TB, reducing the time to negative culture conversion and relapse rates. This finding was corroborated with the findings that the addition of rifampicin or pyrazinamide to regimens drastically reduced the necessary treatment duration and rate of relapse in a mouse model [5]. Pyrazinamide and rifampicin were then classified as strong "sterilizing" agents with potential for TB treatment[5,45].

Pyrazinamide's use in the treatment of tuberculosis was cemented following several human clinical trials specifically testing short-course treatment regimens carried out over the course of three decades [8,70-80]. It was demonstrated that when either rifampicin or pyrazinamide was added to chemotherapy regimens, curative treatment duration could be shortened from over twelve months down to nine months. If rifampicin and pyrazinamide were utilized together in combinatory regimens, treatment could be further reduced to six months, with equivalent or lower rates of relapse[6,81-85]. This effect was attributed to their synergisticrelationship and ability to target slow or non-replicating bacteria, which would be unaffected by isoniazid [7,59].

Strangely it was also found that there was no additive benefit to retaining pyrazinamide through the full six months of the short-course treatmentin human trials and murine treatment models [73,77,80,86,87], despite its potency in sterilization. The combination of all the above mentioned historical findings was pivotal in leading to the currently recommended short-course regimen for drug-susceptible TB of a two month intensive phase consisting of rifampicin, isoniazid, pyrazinamide and ethambutol for two months, followed by a continuation phase of isoniazid and rifampicin for four months [88,89].In the cases of central nervous system or bone and joint infections longer therapy (including pyrazinamide) is recommended.

1.1.3. Pyrazinamide in the treatment of drug resistant tuberculosis

The crucial activity of pyrazinamide in the treatment of drug-susceptible TB means it has traditionally been retained in regimens for the treatment of multi drug resistant (MDR) and extensively drug resistant

(XDR) TB, even in the absence of drug susceptibility testing. Although direct evidence of the role of pyrazinamide in MDR- and XDR-TB treatment was initially scarce, results from a community-based therapy for the treatment of MDR-TB highlighted the inclusion of pyrazinamide in treatment regimens as a factor strongly associated with more favourable treatment outcomes [90]. This was further substantiated by a study in Hong-Kong where it was found that retention of pyrazinamide in fluoroquinolone-based MDR-TB regimens resulted in higher cure or treatment completion, and early culture conversion [4]. Separate trials carried out in Bangladesh [91-93] and Cameroon [94]showed highly encouraging results in the shortening of MDR-TB treatment duration down to nine to twelve months, with both trials including pyrazinamide in the treatment regimens. The WHO now recommends that in addition to using four core second line drugs, pyrazinamide should also be routinely utilized as an add-on agent unless resistance is confirmed from a reliable drug susceptibility test [26,27,89]. This applies to both the shorter nine to twelve month, and conventional eighteen to twenty-four month regimens for the treatment of MDR-TB.

1.1.4. Pyrazinamide in new experimental tuberculosis treatment regimens

In addition to its use in the treatment of drug-susceptible and drug-resistant TB, pyrazinamide is also a crucial component of new experimental regimens, aimed at increasing the efficacy and shortening the duration of treatment. Over the last decade, pyrazinamide has been found to have an additive or synergistic effect in both animal and human studies when combined with the diarylquinolone, bedaquiline [95-101] or the nitroimidazo-oxazine, pretomanid [2,99-103]. Pyrazinamide also showed effectiveness in reducing the time required for culture negativity and relapse rates in murine treatment models when used in combined with rifapentine and moxifloxacin [104]. It also displayed efficacy in reducing bacterial CFU burden in human [101] and murine [105] EBA studies when included in clofazimine containing regimens. In general experimental regimens testing new and/or repurposed drugs in different combinations prove less effective in murine and human treatment models when pyrazinamide is excluded [95,96,98,99,101-103,106]. These experimental findings highlights the importance of pyrazinamide as a sterilizing drug and the need to include it in experimental regimens to shorten the duration of TB treatment below six months. It is likely a rapid pyrazinamide susceptibility test will be needed to screen patients before they start these novel regimens.

1.2. The idiosyncratic nature of pyrazinamide

Despite its unequivocal essentiality in tuberculosis chemotherapy, pyrazinamide remains a strange and mysterious drug with controversy and debate following almost every discovery and gaps still remaining in our knowledge and understanding of its mechanism of action.

1.2.1. Entry of pyrazinamide into bacterial cells

Pyrazinamide is believed to enter the cell through passive diffusion [107,108]. Experimental evidence has suggested that the entry may be facilitated through an adenosine triphosphate (ATP) dependent transport system [109] as shown through competitive inhibition of uptake of [^{14}C]-pyrazinamide by unlabelled pyrazinamide and reduction in the uptake of [^{14}C]-pyrazinamide under low temperature conditions which abolish active uptake systems. In addition, the authors suggested that the proposed transporter was located in the plasma membrane since the addition of arsenate had no effect on pyrazinamidase activity but resulted in a reduction of the initial uptake of pyrazinamide, and that a mechanism of resistance to pyrazinamide was the failure of strains to take up the drug. The proposed transporter however remains unidentified.

1.2.2. Activation of pyrazinamide and biological role of pyrazinamidase

It was originally though that pyrazinamide was the active drug moiety, which was hydrolysed and rendered ineffective [110]. We now know pyrazinamide is a pro-drug, which requires conversion to its active form, pyrazinoic acid (POA) by the enzyme, pyrazinamidase (PZase or PncA) within the bacterial cytoplasm [107]. The unique susceptibility of *M.tuberculosis* is believed to be due to a functional pyrazinamidase and inefficient efflux of pyrazinamide/POA [9,107]. There has also been evidence produced that there is host-mediated activation of pyrazinamide to POA [111,112].

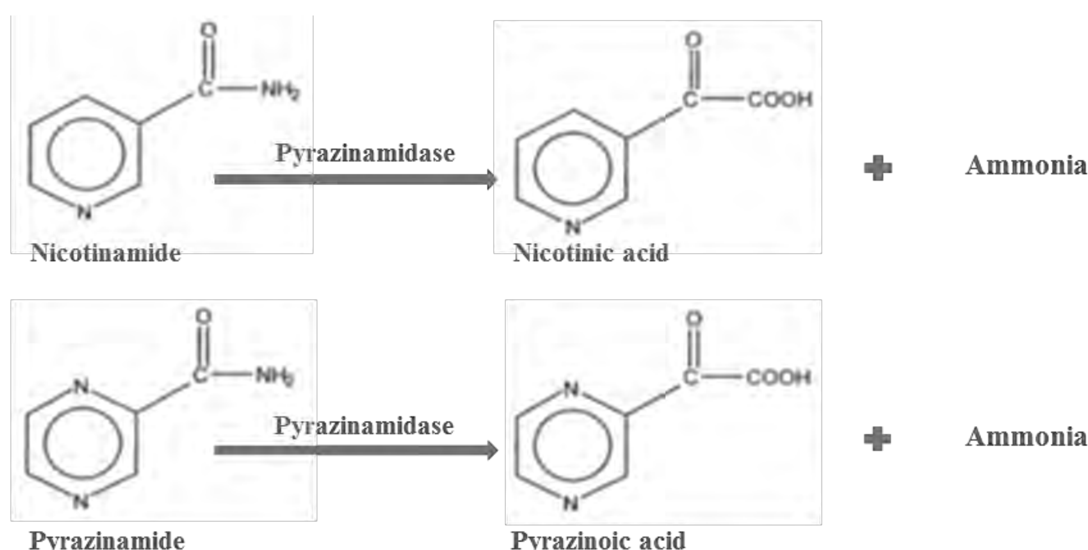

Figure 2: Conversion of nicotinamide/pyrazinamide to respective acid derivatives.

Pyrazinamidase, or nicotinamidase as it is also known, is responsible for the hydrolysis of nicotinamide to nicotinic acid which can then be utilized in the generation of nicotinamide adenine dinucleotide (NAD^+) through the salvage or recycling Preiss-Handler pathway [113]. This was initially believed not to be the case in *M. tuberculosis* due to the deficiency of a nicotinic acid phosphoribosyltransferase enzyme [114]. Two putative phosphoribosyltransferase enzymes (PncB1 and PncB2) have since been

identified [115], leading to the conclusion that *M. tuberculosis* can utilize the scavenging/recycling pathway [287].

1.2.3. Activity of pyrazinamide against replicating and non-replicating bacteria

Despite its potent sterilizing activity *in vivo*, pyrazinamide is considered a poor bactericidal drug *in vitro* as it was shown that even concentrations 50x higher than the minimum inhibitory concentration (MIC), were unable to kill more than 76% of the bacterial population [116]. It therefore cannot be assigned a minimum bactericidal concentration (MBC). Pyrazinamide has more potent activity against semi-dormant or slow/non-replicating persisters than actively replicating bacteria. It has been shown that pyrazinamide was more bactericidal against stationary-phase cultures than log-phase cultures [21,117] and against rifampicin-tolerant cultures [117]. In the latter metabolic activity of the bacteria was shown to be low, by lower incorporation of [^{3}H] uridine into ribonucleic acid (RNA) by viable cells. More recently, a group showed that pyrazinamide may have equivalent *in vitro* bactericidal activity against fast and slow growing bacilli when pyrazinamide concentration is higher than 250µg/mL [118]. Pyrazinamide's MIC against susceptible *M. tuberculosis in vitro* has been shown to be between 6.25µg/mL and 50µg/mL at a pH of 5.5 [9,119-121].

1.2.4. Pyrazinamide and external acidic environments

It is widely believed that pyrazinamide requires an acidic environment to be active against susceptible *Mycobacterial* species. This has been demonstrated *in vitro* where culture conditions require acidification to between pH 4.8 and pH 6.0 in order for pyrazinamide to be active against *M. tuberculosis* [65,116,119,122,123]. There islittle to no activity at near-neutral pH in standard culture conditions[124-126]. It is worth noting that lower pH (pH4.8 to 5.5) culture conditions may inhibit the growth of the bacteria [65,120,122,123] which has made it difficult to precisely define the role of acid in the action of pyrazinamide. The MIC of pyrazinamide is however dependent on the pH of the media being utilized, with increases in MIC values being observed as the pH moves from acidic closer to neutral [21,116,127]. Interestingly, the expression of pyrazinamidase does not appear to induced by acid stress [128] with the optimal range for enzyme function being between pH 6 and pH 8 when utilizing nicotinamide as a substrate with purified *M. tuberculosis* pyrazinamidase [129].

1.2.5. Factors affecting pyrazinamide activity

It is less appreciated that many external environmental factors other than acid can also enhance the activity of pyrazinamide. *M. tuberculosis* subjected to anaerobic or microaerobic conditions [130], nutrient-starvation [128,131], low incubation temperature [132,133], inhibitors of the *M. tuberculosis* respiratory machinery and compounds which disrupt membrane potential and energetics [108,130,134,135], the efflux pump inhibitor reserpine [21,107], ultraviolet light and some weak acids [131,134], as well as aspirin and

ibuprofen [136] is more susceptible to pyrazinamide. Pyrazinamidase is a metalloenzyme with various metal co-factors influencing activity [137], with iron in particular being shown to magnify the effect of pyrazinamide and pyrazinoic acid *in vitro*[138]. It is interesting that certain conditions such as lower incubation temperature [133], efflux pump inhibitors [21,107] and ectopic over-expression of the pyrazinamidase enzyme [128,139] appear to nullify pyrazinamide reliance on an external acidic environment for *in vitro*activity. This is highly suggestive that an acidic external environment may not influence pyrazinamide activity directly, but along with other external stress conditions, it induce metabolic changes which renders pyrazinamide more active. One possibility is that acid increasesthe accumulation of POA.

1.2.6. Intracellular activity of pyrazinamide

It has been proposed that pyrazinamide is active within macrophages due to an internal acidic environment [5,7]. However there have been mixed reports on the activity of pyrazinamide within macrophages obtained from humans or animals using *in vitro* culture systems. Some authors report slow bactericidal activity or mostly bacteriostatic (inhibition of growth of bacteria) activity against intracellular bacteria [28,122,126,140-142], while other groups have demonstrated no activity of pyrazinamide in macrophage treatment models [143-146]. It is interesting that chloroquine, an anti-malarial agent which raises phagolysosome pH, had no detrimental effect on pyrazinamide activity in macrophages, although chloroquine did itself show activity against *M. tuberculosis*[147]. In addition, it is well known that *M. tuberculosis* can inhibitphagosome-lysosome fusion which is required for acidification [148,149], and that organelles within macrophages may only be neutral or slightly acidic [150,151].

1.2.7. Pyrazinamide activity *in vivo*

If pyrazinamide is predominantly active against semi-dormant or non-replicating persister bacilli [7,123], it would be expected that pyrazinamide should be more active during the continuation phase of treatment rather than the intensive phase. Early bactericidal activity (EBA) and extended EBA studies in human as a measure of the effectiveness of anti-tuberculosis drugs in chemotherapy [59,100,101,152-157] have shown that pyrazinamide has very little activity within the first two weeks of treatment. Furthermore Phase 3 studies have demonstrated activity is not extended beyond 2 months [73,77,80]. This is supported by similar findings from murine treatment models [5,86,87,158,159]. This anomaly has been put down to a reduction in acidic environments as inflammation due to the infection subsides [5,7].

1.3. Proposed targets/mechanisms of action of pyrazinamide/POA

Despite extensive research, the precise mechanism/s of action of pyrazinamide remains elusive [9,46,60]. Researchers have proposed different cellular targets and mechanisms of action for pyrazinamide, and

there is some suggestion that the drug may have pleiotropic activity, exerting it effect/s on multiple pathways or systems [9,60,128,160-162] rather than a single cellular target, as is the case with many other anti-tuberculosis agents.

1.3.1. Panthothenate and coenzyme A synthesis

Pyrazinamide has recently been implicated in the inhibition of panthothenate and coenzyme A (CoA) biosynthesis [163]. *panD* encodes for aspartate decarboxylase, which is required for the synthesis of β-alanine (a pre-cursor to panthothenate, which itself is a pre-cursor for CoA) from L-aspartate [164]. It was subsequently shown that POA but not pyrazinamide had an inhibitory effect on aspartate decarboxylase activity, overexpression of *panD* conferred resistance to pyrazinamide [165], andsupplementation with β-alanine [165], panthothenate [162,165] or pantetheine [162] reduced susceptibility to POA. In addition, POA was shown to deplete CoA, with mutations in *panD* abrogating this depletion [166].Molecular docking analysis of crystal structures have confirmed potential interaction between a PanD and POA [167]. There has however been some suggestion that PanD and therefore panthothenate synthesis itself may not be the direct target after it was found that pantetheine supplementation of a *panCD* auxotrophic strain did not abrogate susceptibility to pyrazinamide as would be expected [162].

1.3.2. *trans*-Translation

It was also proposed that POA inhibits *trans*-translation [168] via binding to the C-terminal end of ribosomal protein S1 (RpsA). *trans*-Translation is an essential process mediated by transfer-messenger ribonucleic acid (tmRNA) and a small basic protein B that rescues and recycles stalled ribosomes during the process of translation. This is achieved by tagging the stalled protein and releasing messenger RNA (mRNA) for degradation [169]. The process is believed to be dispensable during normal growth, but critical during stress conditions [169,170]. Inhibition of *trans*-translation may result in an accumulation of toxic peptide waste and a shortage in free ribosomes leading to an arrest in translation and eventually cell death. Crystal structure analysis supported POA binding to the C-terminus residues of RpsA, which are believed to be the binding sites of the tmRNA [171]. This hypothesis has been challenged because mutations of *ssrA*or a deletion of SmpB (which encode the tmRNA and small basic protein B respectively in *M. tuberculosis*), which result in a defective *trans*-translational response, have no effect on pyrazinamide susceptibility [172].

1.3.3. Intracellular acidification, membrane potential and cellular energy state

One of the earliest proposed mechanisms of action for pyrazinamide was based on the drug's requirement for an external acidic environment for *in vitro* activity. It was proposed that POA produced through catalytic hydrolysis of pyrazinamide exited bacterial cells through diffusion and an as yet unidentified efflux mechanism. In an externally acidic environment, negatively charged POA would

become protonated to form an uncharged conjugate, HPOA, which could then easily permeate back into the bacterial cell where the conjugated form would dissociate to release a proton and POA. This cycle would continue until an equilibrium is reached, which is believed to result in accumulation of POA and intracellular acidification, ultimately inhibiting vital enzymatic functions leading to death [9,107]. Indeed it has been shown that an external acidic environment does facilitate intracellular accumulation of POA [107], but it has been difficult to demonstratea decrease in intracellular pH [107,128].

In keeping with the hypotheses that POA is cyclically shuttled and eventually accumulated intracellularly, it was suggested that protonated POA re-entering the cells potentially impaired membrane transport (as demonstrated by the reduced uptake of radiolabelled precursors of RNA and protein synthesis) and disrupted membrane potential [9,108]. Lower membrane potential was reported in old non-replicating cells versus replicating cells, and in cells exposed to an acidic environment, with POA causing a further reduction in membrane potential in older bacteria. POA-mediated lowering of membrane potential could cause a reduction in ATP synthesis leading to a depletion of cellular energy reserves and reduced viability. Cells with diminished membrane potential as in the case of non-replicating and acid-exposed cells would therefore be particularly vulnerable to pyrazinamide.

The observation of a reduction in proton motive force (PMF) and inhibition of ATP synthesis using membrane vesicles, and depletion of cellular ATP reserves in a whole cell assay by POA in a dose dependent manner supported the idea that a reduction in membrane potential was central to the mechanism of action of pyrazinamide[173]. It would also explain the reduced uptake of essential metabolites through impaired membrane transport[108], and is consistent with the observations that pyrazinamide's activity is potentiated by cellular respiratory inhibitors. However a recent report found no reduction in membrane potential in a whole cell assay at an acidic pH of 5.5 with POA treatment [128].

1.3.4. NAD^+ biosynthesis

Another proposed mechanism of action of pyrazinamide was the inhibition of *de novo* NAD^+ biosynthesis. By assaying for production of nicotinic acid mononucleotide (NaMN), it was found that both pyrazinamide and POA inhibited the catalytic capacity of quinolic acid phosphoribosyltransferase (QAPRTase encoded for by *nadC*). Molecular docking analysis revealed that pyrazinamide could potentially competitively bind to the QAPRTase enzyme thus preventing the synthesis of nicotinic acid mononucleotide from quinolic acid (a structural analogue of pyrazinamide and the substrate for QAPRTase) and 5-phosphoribosyl-1-pyrophosphate[174]. It is unlikely that NAD^+ biosynthesis is the unique intracellular target of pyrazinamide given the discovery of the two putative phosphoribosyltransferase enzymes (PncB1 and PncB2) which suggests that the organism can switch between the *de novo* synthesis and the recycling/scavenging Preiss-Handler pathways for the generation of NAD^+[115]. However this mechanism cannot be ruled out as we do not fully understand the interactions

between these pathways in an *in vivo* setting. Indeed it is plausible that in a nutrient starved environment where scavenging and recycling is limited or non-existent, obstruction of the *de novo* synthesis pathway through POA inhibition of the QAPRTase catalytic function may be detrimental to the organism. Competitive binding and inhibition of the QAPRTase enzyme by pyrazinamide itself however would be highly unlikely as strains lacking a functional pyrazinamidase should remain susceptible to pyrazinamide.

1.3.5. Fatty acid synthesis

Fatty acid synthase I (FAS-I) of *M. tuberculosis*has also been proposed as a target, after it was found that over-expression of the *fasI* gene from *Mycobacterium avium, M. tuberculosis* and *Mycobacterium bovis* BCG all resulted in resistance to the pyrazinamide analogue 5-Cl-PZA in *Mycobacterium smegmatis* [175]. The authors went on to report inhibition of FAS-I activity in *Mycobacterium smegmatis and M. tuberculosis* by 5-Cl-PZA, as well by pyrazinamide and POA in *M. tuberculosis* at an acidic pH (pH 6.0), but no effect on FAS-I by pyrazinamide and POA at pH 6.8. In addition they showed no effect on FAS-I in *M. bovis* BCG with pyrazinamide but inhibition with POA, consistent with previous findings that *M. bovis* BCG is inherently resistant to pyrazinamide as it lacks a functional pyrazinamidase enzyme [176,177]. The author's findings on inhibition of FAS-I was corroborated by similar observations using pyrazinamide, POA or closely related structural derivatives [178,179]. It was proposed that pyrazinamide and other analogues reversibly bound to *M. tuberculosis* FAS-I, with pyrazinamide competitively inhibiting the binding of dihydronicotinamide-adenine dinucleotide phosphate (NADPH). POA was thought to have a different biological effect as it did not competitively displace pyrazinamide [180,181].

As in the case of the hypothesis of pyrazinamide binding the QAPRTase enzyme, it would be unlikely that FAS-I is the single, direct target of pyrazinamide, as susceptibility to pyrazinamide would be retained in strains lacking a functional pyrazinamidase enzyme. It is worth noting that the concentrations of pyrazinamide and POA utilized in the abovementioned experiments were well above the MIC for drug susceptible *M. tuberculosis*, and concentrations observed in a pharmocokinetic study [9,182]. The proposed mechanism of direct FAS-I inhibition by pyrazinamide and POA has been questioned after findings that pyrazinamide and POA showed no inhibitory effect on fatty acid synthesis mediated through FAS-I in experiments using whole cell and whole cell lysates of*M. tuberculosis* and *M. smegmatis*, and purified *M. smegmatis* FAS-I [160]. The authors suggested that any effect on fatty acid synthesis was indirect and rather a response to inorganic acid stress. Indeed it is plausible that fatty acid synthesis is affected in an indirect manner given that fatty acid synthesis is dependent on CoA, which may itself be depleted by POA [166].

1.3.6. Immune modulation

Pyrazinamide has also been implicated in potentially having a role in host immune modulation during the course of treatment. It has been shown that pyrazinamide treatment significantly reduced the release of pro-inflammatory cytokines and chemokines in a murine model of infection [183], and that pyrazinamide promoted autophagy and phagosomal maturation [184]. It is also interesting to note that pyrazinamide induced a pro-inflammatory in response to *Leishmania major* infection *in vivo*[47]. More recently it has been proposed that pyrazinamide may act as an anti-virulence agent by affecting phthiocerol dimycoserate (PDIM) synthesis[166]. Further investigation is however required given the findings of Grosset and colleagues in immune-competent and immune-deficient murine treatment models which suggests no direct significant host immune modulation by pyrazinamide [159].

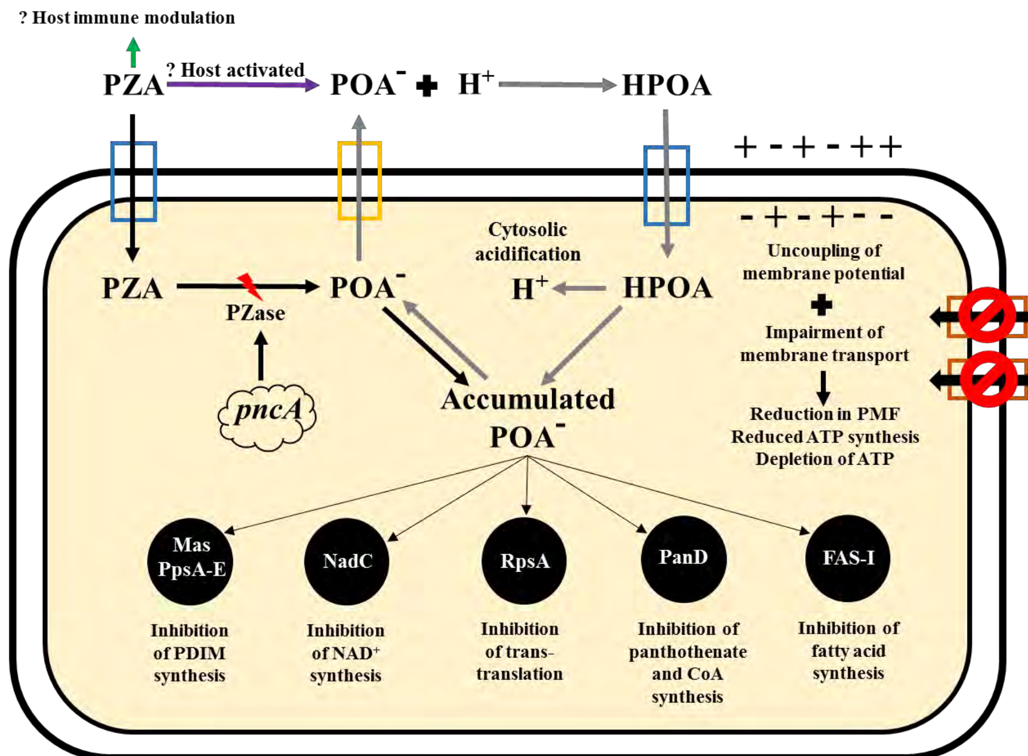

Figure 3: Summary of proposed targets/mechanisms of action of pyrazinamide. Blue boxes highlight potentially passive import through diffusion. Yellow box indicates potentially passive export through diffusion. Copper boxes highlight potentially energy dependant transporters. Grey arrows indicate proposed cycling hypothesis [9,107]. Purple arrow indicate potential host activiation of pyrazinamide [111,112]. Green arrow indicates potential host immune modulation by pyrazinamide [184]. Solid black circles indicate postulated targets of pyrazinoic acid. PZA = pyrazinamide, POA^- = negatively charged pyrazinoic acid, PZase = pyrazinamidase, H^+ = proton, HPOA = protonated pyrazinoic acid, PMF = Proton Motive Force, ATP = Adenosine Tri-Phosphate, Mas = Mycocerosic acid synthase, Ppsa-E = Phenolphthiocerol synthesis type-I polyketidesynthase, PDIM = Phthiocerol dimycoserate, NadC = Quinolic acid phosphoribosyltransferase, NAD^+ = Nicotinamide Adenine Dinucleotide, RpsA = Ribosmal Protein S1, PanD = Aspartate Decarboxylase, CoA = Co-enzyme A, FAS-I = Fatty Acid Synthase-I.

1.4. Genetic mechanisms of resistance to pyrazinamide

It is estimated that 16.2% of all TB cases harbour resistance to pyrazinamide with the pooled prevalence of pyrazinamide resistance in MDR-TB cases being around 60.5% [24], although this number may be as high as 85% in certain regions [185,186], and as high as 93% in fluoroquinolone resistant MDR-TB cases [187]. Although multiple mechanisms of resistance to pyrazinamide have been suggested, related to recently proposed modes of action, the primary mechanism of resistance is analogous to that of the drug isoniazid, which is also an analogue of nicotinamide. Isoniazid is a pro-drug which requires intracellular conversion to its active form, by the catalase-peroxidase (encoded by the *katG* gene) of *M. tuberculosis*. Mutations in *katG* which cause loss of enzymatic conversion of isoniazid are the primary mechanism

of high level resistance in clinical isolates [12,17,18,188-191]. Similarly mutations within *pncA*, encoding for the pyrazinamidase, that result in loss or reduction of pyrazinamide activation are associated with primary high level resistance [20,177,192-195].

1.4.1. Mutations in *M. tuberculosis pncA*

A breakthrough finding in understanding the molecular basis of resistance to pyrazinamide was the identification of the 561 nucleotide *pncA* gene in *M. tuberculosis*. It was discovered that pyrazinamidase activity and susceptibility to pyrazinamide could be restored in *M. bovis* BCG *and* pyrazinamide resistant strains of *M. tuberculosis* by complementation with wild type *pncA* from *M. tuberculosis*[28]. Subsequently it was shown that resistance to pyrazinamide could be conferred via intragenic non-synonymous single nucleotide polymorphisms and frameshift mutations or mutations in the putative promoter region of *pncA* resulting in a decrease or loss of pyrazinamidase activity [28,29,195-201]. *M. bovis* and *M. bovis* BCGboth carry a non-synonymous single nucleotide polymorphism (histidine to aspartic acid substitution at codon 57 - H57D) in the *pncA* gene and are intrinsically resistant to pyrazinamide[28].

Given that pyrazinamidase is part of the NAD^+ salvage pathway in TB which may be dispensable due to the presence of a *de novo* synthesis pathway for NAD^+, it is likely that loss of function mutations in *pncA* do not result in loss of fitness [162]. This idea is supported by the fact that *pncA* is a non-essential gene in *M. tuberculosis*[202-206]and loss of pyrazinamidase activity in *M. bovis* does not affect virulence in animal hosts. The diversity of mutations which can occur along the full length of the gene [207] is unlike resistance to isoniazid where a single substitution in *katG* (S315T) accounts for the vast majority of mutations [191]. This is because the S315T mutation results in an enzyme that retains some catalase activity whilst losing the ability to activate isoniazid [208]. The absence of a similar dominant substitution that retains enzymatic activity with loss of drug activation also suggests that pyrazinamidase is not essential for full virulence in humans. There is some noticeable clustering of mutations at residues 3-17, 61-85, and 132-143 [29,32,33] believed to be residues related to the catalytic site of the enzyme, the metal co-ordinating sites, or essential to the structure of the enzymatic core [31,129,209-213]. Mutations also occur outside of these regions indicating a more complex relationship between mutations and enzymatic function.

Investigations into associating specific *pncA* genotypes to resistant phenotypes have so far been carried mainly by sequencing of clinical isolates of *M. tuberculosis* with resistance to pyrazinamide (identified by phenotypic drug susceptibility testing or measurement of pyrazinamidase activity as a proxy for pyrazinamide resistance). Some studies have alsoderived spontaneous resistant mutants to pyrazinamide or POA. The proportion of pyrazinamide resistance based on intragenic mutations in *pncA* or in its promoter is between 70% and 97% depending on geographic location [29,31,32,195-198,200,214-220]. Unexplained resistance (pyrazinamide resistance without the occurrence of mutations in *pncA* or its

promoter) is often attributed to errors in pyrazinamide drug susceptibility testing [19], although alternate resistance conferring mutations may occur. In addition polymorphisms in *pncA* which do not confer resistance to pyrazinamide have been identified[34,220].

To date, the most comprehensive attempt to associate *pncA* mutant genotypes to pyrazinamide resistant phenotypes was a multi-centre study which sequenced the *pncA* gene and promoter from 843 pyrazinamide resistant and 1107 pyrazinamide susceptible clinical isolates resulting in the identification of 239 mutations believed to confer resistance to pyrazinamide [32]. In addition, two systematic reviews were recently undertaken in an attempt to correlate the association between mutations in *pncA* and clinical pyrazinamide resistance [24,33], and identified 641 and 608 unique mutations in *pncA* and/or its promoter region respectively, which are believed to confer resistance to pyrazinamide.These mutations include single nucleotide polymorphisms, insertions and deletions. Indeed the wide body of data that currently exists is useful in our attempts to do genotype-phenotype associations, however these data-sets may be skewed through the identification of pyrazinamide resistant clinical isolates, which is drastically influenced by drug susceptibility testing and by the inherent genetic diversity of the isolates. There has been no literature produced where *pncA* mutants have been introduced and tested in an isogenic mycobacterial background to concretely associate the genotypes with pyrazinamide resistance, with the only expression of mutants (albeit a small subset) being undertaken in *Escherichia coli* (*E. coli*) to purify the pyrazinamidase in order to study enzyme activity in relation to resistance [201,221].

1.4.2. Mutations in *M. tuberculosis rpsA*

Mutations in *rpsA* have been suggested as an alternate mechanism for pyrazinamide resistance in *M. tuberculosis* as RpsA has been proposed as a target for the drug [168]. It has been shown that ectopic over-expression of *rpsA* does confer resistance to pyrazinamide, and POA binding to the C-terminus of RpsA was abolished in a “low-level” pyrazinamide resistant clinical isolate (MIC = 200-300µg/mL) with no mutations in *pncA*, but a deletion of codon 438 of *rpsA*[168]. Subsequent sequencing and analysis of clinical isolates has however yielded mixed results. While some groups have identified non-synonymous *rpsA* mutations in pyrazinamide resistant, wild type*pncA* (*pncA*^{WT}) clinical isolates [222], there have also been non-synonymous *rpsA* mutations identified in pyrazinamide susceptible *pncA*^{WT} clinical isolates [223]. Additionally there still remain pyrazinamide resistant *pncA*^{WT} clinical isolates with no *rpsA* mutations [220,223-226]. It is worth noting that Gu and colleagues identified 26 pyrazinamide resistant and 6 pyrazinamide susceptible isolates containing mutations diversely located throughout *rpsA* (and not restricted the the C-terminal end as suggested by Shi and colleagues). However it was not clear if the isolates also contained mutations in *pncA* or not. It is also interesting that a variety of *rpsA* mutations have also been identified in the intrinsically pyrazinamide resistant *Mycobacterium canetti* (*M. canetti*), which has a synonymous *pncA* mutation believed not to affect pyrazinamidase activity, as

well as a *panD* mutation[227,228]. It is therefore unclear whether mutations in *rpsA* plays any role in conferring resistance in a clinical setting.

1.4.3. Mutations in *M. tuberculosispanD*

The role of *panD* in *M. tuberculosis* pyrazinamide resistance was proposed after Zhang and colleagues isolated and whole genome sequenced five *in vitro* selected pyrazinamide resistant mutants lacking mutations in *pncA* and *rpsA*, which resulted in the identification of mutations in the *panD* gene of *M. tuberculosis*[163].It was then shown that a pyrazinamide resistant clinical isolate as well as *M. canetti* also harboured mutations in *panD*. Subsequent work derived 30 *in vitro* selected pyrazinoic acid resistant mutants with seven different *panD* mutations, and showed that ectopic over-expression of either wild type or mutated *panD* conferred resistance to POA and resulted in a 3-fold increase in pyrazinamide MIC [165]. Apart from the isolate tested by Zhang and colleagues, there have been no further clinical isolates identified with *panD* mutations so it is unclear if this mechanism of resistance is important clinically[220].

1.4.4. Potential alternate resistance conferring mechanisms

Loss of function frameshift mutations within the *mas* and *ppsA-E* genes have also been proposed as a potential resistance conferring mechanism to pyrazinamide and POA.These mutants were only derived in the absence of glycerol *in vitro* [166]. These genes are required for PDIM synthesis which has been shown to be dispensable *in vitro* [203,205,229,230] but results in attenuation when lost *in vivo* [204,231,232]. Loss of PDIM synthesis has occurred spontaneously during *in vitro* passage and no mutations in these two genes have yet been identified in clinical isolates [233,234], so it seems unlikely that this pathway constitutes a clinically relevant resistance mechanism.

M. smegmatis has two functional pyrazinamidase enzymes, PncA and PzaA, which can confer susceptibility to pyrazinamide [202,235,236], but this organism is intrinsically resistant to pyrazinamide, which is thought to be due to an as yet unidentified efflux transporter of POA[107]. Given that pyrazinamide and POA activity are enhanced by efflux pump inhibitors, investigation into drug efflux in confirmed pyrazinamide resistant strains lacking mutations in *pncA*, *rpsA* and *panD* may be warranted.

1.5. Drug susceptibility testing

Drug susceptibility testing (DST) for *M. tuberculosis* can be broadly divided into phenotypic and genotypic based tests, and is essential in clinical settings to identify resistance to antibiotics utilized in the treatment of TB in order to ensure the curing of patients, as well as to reduce or prevent the

transmission of TB and further emergence of drug resistance. Phenotypic drug susceptibility testing is based on measurements of viability such as growth inhibition on solid or in liquid culture media, or the production of metabolites or substrates, in response to drug treatment. Genotypic assays are based on the detection or identification of genetic mutations within genes known to result in a drug resistant phenotype [237].

1.5.1. Phenotypic drug susceptibility testing for pyrazinamide resistance

Phenotypic drug susceptibility testing for pyrazinamide is complicated, technically challenging and at times unreliable [19,21,23,238,239]. Initial attempts at pyrazinamide susceptibility testing on solid media proved difficult primarily due to the requirement of acidity and the presence of culture supplements which impaired the growth of mycobacteria. A lack of growth inhibition when a dense inoculum was used was also observed and believed to be due to localized neutralization of pyrazinamide in the media [119,240-245]. Also the need to prepare the media in house as no commercially available alternate options has made reproducibility a problem[120,245,246].

Adaptations and modifications to solid agar testing for improved detection of pyrazinamide resistance have been tested [120,193,241,242,245,247]. Solid media testing is currently not recommended by the WHO with susceptibility testing in liquid media being the preferred method [25-27].

Liquid based pyrazinamide susceptibility testing has mainly been carried out in the discontinued radiometric Bactec 460TB system and the currently available Bactec MGIT 960 PZA system (Becton Dickinson and company, USA) at pH 6.0 and pH 5.9 respectively using a 100µg/mL critical concentration. These commercial assays are expensive, and require trained laboratory personnel and specialized biosafety level III (BSL-III) facilities [248-250] so they have not been widely used in resource poor settings. Although standardized, these systems have a significant false-resistance rate and suffer from lack of reproducibility[19-23,238,239,251-258]. Testing outcomes have been shown to be influenced by conditions such as acidification of the media, inoculum size, growth phase dependency and additives such bovine serum albumin (BSA) and polyoxyethylene stearate (POES) [21,65,119,259,260]. It is worth noting that susceptibility testing to pyrazinamide is not included in the WHO yearly proficiency testing [23] and there has been some suggestion that the critical concentration for pyrazinamide susceptibility testing should be increased [21,121,198,261].

1.5.2. Alternate methods for detection of pyrazinamide resistance

Methods have also been developed to detect the loss of pyrazinamidase activity as a marker for loss of function mutations in *pncA*. The Wayne assay [262] is a colorometric assay based on the reaction of POA with ferrous ammonium sulphate. Mutations in *pncA* causing loss of pyrazinamidase activity result in no colour change of the ferrous ammonium sulphate (negative result) since no POA is available to

complex with the ferrous iron, whereas, a functional pyrazinamidase converting pyrazinamide to POA causes the development of a pink/red colour upon addition of ferrous ammonium sulphate due to the formation of a complex between POA and the ferrous iron.

The method was originally carried out by adding ferrous ammonium sulphate to LJ media on which the bacteria was grown in the presence of pyrazinamide and looking for the development of a pink/red coloured band on the media. Despite being a good indicator of loss of pyrazinamidase function and therefore pyrazinamide resistance, the assay is highly subjective, has poor sensitivity and may also predict false resistance and susceptibility [22,193,260]. The method has however been adapted over the years to be used with liquid culture systems leading to the development of a more quantitative assay which can be combined with spectrophotometry [263].

Additional methods such as microscopic observation drug susceptibility or MODS [264] flow cytometry [265] viability indicators [266,267], a PCR amplified and *in vitro* synthesized pyrazinamidase assay [268,269] and colometric methods substituting pyrazinamide with nicotinamide [270,271] have also been developed for use in pyrazinamide susceptibility testing but have not gained much traction.

1.5.3. Genotypic susceptibility testing for pyrazinamide resistance

The use of genotypic based methods or nucleic acid amplification tests (NAAT such as the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) and MTBDRplus (Hain Lifescience, Nehren, Germany) have been endorsed for use by the WHO as these tests have allowed more rapid time-to-diagnosis of *M. tuberculosis* and potential drug resistance to rifampicin and isoniazid which allows for more timely intervention with regards to selection of treatment regimens [10]. The GeneXpert MTB/RIF relies on the polymerase chain reaction (PCR) amplification and overlapping molecular beacon detection of mutations within an 81 base pair region of the *M. tuberculosis rpoB* gene known as the rifampicin resistance determining region (RRDR) for identification of rifampicin resistance [11] in under two hours excluding sample preparation [272], while the MTBDRplus is based on PCR amplification and hybridization of complementary fragments to a line probe strip for the detection of mutations within the RRDR for identification of rifampicin resistance, as well as specific mutations within *katG* and the promoter region of *inhA* [188,273-275] for determination of isoniazid resistance, in 6 to 48 hours [276-278]. A line-probe assay for pyrazinamide has been developed [36,279,280] but has not yet been fully evaluated clinically. However such tests will only be able to determine if a mutation is present within *pncA* and will not be able to distinguish between resistance conferring and non-conferring mutations.

Genotypic susceptibility testing for pyrazinamidebased on PCR amplification and sequencing of the full length of *pncA* and its promoter [29,32,197,225,281] or through whole genome sequencing [282] is an alternate approach that has the advantage of being able to detect specific mutations. For example, it is possible

to detect mutations in *pncA* directly from clinical specimens (sputum samples) in under 48 hours using PCR and sequencing [37].

Genotypic testing approaches require that the genotype-phenotype relationship of all *pncA* mutations is known, and limitations in susceptibility testing has made this challenging. Genotypic methods could be expanded to include *rpsA* and *panD*, however their role in improving susceptibility testing to pyrazinamide requires further evaluation [220,222,223,225,283].

1.6. Problem Statement

Emergence of drug resistant tuberculosis has compromised TB control. Rapid genetic based assays for detection of drug resistance will allow for individualized treatment regimens and improve patient outcomes. The development of a genetic based test requires detailed knowledge of all the mutations that can confer drug resistance. In the case of pyrazinamide, this knowledge is lacking.

1.7. Research Rationale

Mutations within *M. tuberculosis pncA*are the primary mechanism of resistance to pyrazinamide and need to be analysed in a rapid genetic test for pyrazinamide resistance. The diversity of *pncA* mutations, the occurrence of mutations in *pncA* that do not confer resistance, and the variability of phenotypic drug susceptibility testing means that the genotype-to-phenotype associations of *pncA* mutations need to be accurately defined to develop a genetic based diagnostic assay for detection of pyrazinamide resistance. Current knowledge of resistance conferring mutations is incomplete and based on sequencing of clinical isolates. A comprehensive screen of the full repertoire of *pncA* mutants in an isogenic background would fill this knowledge gap.

1.8. Aims

Our study aims to generate a pool of isogenic *M. tuberculosis* clones, each harbouring a single nucleotide polymorphism representing every possible PncA mutation. We then intend to use *in vitro* and *in vivo* selection to define the full repertoire of drug resistance conferring mutations in *pncA*.

1.9. Objectives

- Using PCR random mutagenesis to generate a mutant library of the *M. tuberculosispncA* gene containing every possible single nucleotide polymorphism (SNP).

- Transform the *pncA* mutant library into an isogenic *M. tuberculosis* strain which has an insertionally inactivated *pncA*.
- Develop and optimize an *in vitro* phenotypic drug screen using pyrazinamide to select for bacterial clones containing *pncA* mutations which confer resistance.
- Phenotypically screen the*pncA* mutant library in a murine model using pyrazinamide monotherapy to select for bacterial clones containing *pncA* mutations which confer resistance.
- Use conventional phenotypic drug susceptibility testing to validate if selected *pncA* mutants confer resistance to pyrazinamide.
- Use quantitative mass spectrometry and enzymatic assays to investigate the structural basis by which *pncA* mutations confer resistance.

Chapter 2: Materials and Methods

2.1. Bacterial strains, media and materials

We used *E. coli* DH5α (a gift from the Rubin lab at the Harvard School of Public Health, and *M. tuberculosis* H37Rv (sAY101) and H37RvΔ*pncA* (a gift from Dr. Valerie Mizrahi and Dr. Helena Boshoff [202]) in this study. All plasmids and strains utilized and generated through the course of this work are listed in supplementary table 1 and supplementary table 2 respectively. Unless otherwise specified, *M. tuberculosis* were grown either in 7H9 broth or on 7H10/7H11 agar while *E. coli* DH5α was grown in LB broth or on LB agar. All media formulations are provided in the Appendix section of this dissertation. Zeocin (R25001, Thermo Fisher Scientific, USA) was used at 50 µg/mL and 20 µg/mL for *E. coli* and *M. tuberculosis,* respectively. Hygromcyin B (H0654, Sigma Aldrich, USA) was used at 50 µg/mL for *M. tuberculosis*. For storage of strains as stocks or titres, *E. coli*and *M. tuberculosis* strains were grown to $O.D._{600} \approx 1.2$ and stored in 1mL aliquots at -80°C. Pyrazinamide (P7136, Sigma Aldrich, USA) and pyrazinoic acid (P56100, Sigma Aldrich, USA) stocks of 40mg/mL and 100mg/mL were prepared in water and DMSO (D8418, Sigma Aldrich, USA) respectively. All strains were grown at 37°C. All DNA oligonucleotides for PCR amplification were purchased from Integrated DNA Technologies (USA) and are listed in supplementary table 3. Other reagents and materials catalogue numbers and suppliers will be specified where required.

2.2. Basic methodologies

Due to the standardised nature of the primary molecular biology tools utilized in generating our control strains, as well as our *pncA* mutant library, the principal methodologies, standard reaction concentrations and conditions will be detailed initially in the section below, with any specific changes to protocol being detailed where relevant.Examples of reaction volumes and cycling conditions are represented below the relevant methods.

2.2.1 PCR amplification

All PCR amplification reactions with the exception of the error-prone mutagenesis PCR (which will be detailed in section 2.4.) were carried out in a standardized manner using KOD Hot Start DNAPolymerase (71086-3, Merck-Millipore, USA). The volume of DNA template for added to reactions for PCR amplification varied between 0.4 uL for amplification from plasmid DNA to 2.5 µL for amplification from Hain GenoLyse DNA extractions. Reactions were carried out in 50µL volumes with reagents at the following final concentrations: 1x PCR Buffer, 5% DMSO, 0.4mM each dNTP, 0.5µM forward primer, 0.5µM reverse primer, 1 unit of KOD *Taq* polymerase and nuclease free water (129114, Qiagen, Germany) up to the final 50 µL reaction volume. PCR reaction started with a 2 minute incubation at 94°C for denaturation of DNA and activation of the polymerase. Cycling started with a

15 second incubation at 98°C, followed by 30 second annealing and extension at 68°C. Cycling annealing temperatures and cycling extension times are unique to the primer pairs utilized and size of the PCR product being amplified respectively and will be specified where necessary. Final extension was carried out at 68°C for 5 minutes. Cycling extension time was kept standard at 1 minute for PCR products less than 1kb, with 30 second added on to the extension time for every additional 500 base pairs to be amplified.

DNA template	Variable
2x KOD PCR buffer	25 µL
dNTPs (2mM each)	10 µL
DMSO	2.5 µL
Forward primer (10µM)	2.5 µL
Reverse primer (10µM)	2.5 µL
KOD Hot Start DNA polymerase (1u/µL)	1 µL
Nuclease free water	Up to 50 µL
Total	**50 µL**

94°C	2 minutes	
98°C	15 seconds	x 40 cycles
*°C	30 seconds	
68°C	1minute/kb, 30 sec/0.5kb thereafter	
68°C	5 minutes	
4°C	Hold	

2.2.2. Restriction Digests and dephosphorylation

All restriction digests were carried out in a standardized manner using restriction enzymes purchased from New England Biolabs. Reactions were carried out in a 50 µL reaction volume containing the following at their final concentrations: 2µg of vector/PCR product, 1x digest buffer (NEB supplied buffer specific to enzymes), 1x BSA (NEB supplied 100x stock), 50-100 units of Restriction Enzyme/s (no more than 5 µL or 10% of total reaction volume), and nuclease free water up to 50 µL. In the case of double digests, an appropriate digest buffer was selected using the NEB guide and concentration of enzyme was adjusted according to the compatibility of restriction enzymes to the digest buffer. Restriction digests were carried out at 37°C over-night. Dephosphorylation was carried out by adding 2 units of Alkaline Phosphatase (EF0651, Thermo Fisher Scientific, USA) to post-digested reactions and incubating at 37°C for 30 minutes followed by enzyme inactivation at 75°C for 10 minutes.

DNA (2 µg)	Variable
10x Digest Buffer	5 µL
100x BSA	0.5 µL
Enzyme 1	2.4 µL
Enzyme 2	2.4 µL
Nuclease free water	Up to 50 µL
Total	**50 µL**

2.2.3. Purification of PCR and restriction digest products

All purification of PCR and restriction digest products was performed by spin-column purification following gel extraction. Briefly, all products were run on a 0.9% agarose gel prepared with 1x TAE buffer. Gel electrophoresis was carried out at a constant 150V for 30 minutes at 400mA. Products of appropriate sizes were visualized and excised using the Bio-Rad ChemiDoc MP Imaging system for UV translumination. Gel slices were then dissolved and DNA purified using the QIAquick Gel Extraction Kit (28706, Qiagen, Germany), as per manufacturers recommended protocol with the exception that purified products were eluted in nuclease free water rather than the provide elution buffer.

2.2.4. Ligation and dialyzation

Ligation reactions were performed under standardized conditions using T4 DNA Ligase (Thermo Scientific, EL0011). Reactions were carried out in a 20 µL total volume containing 50ng of vector DNA, 1x supplied Ligase Buffer, 1 Weiss unit of T4 DNA ligase, 10:1 molar ratio of insert DNA (calculated using online resource: http://www.insilico.uni-duesseldorf.de/Lig_Input.html) and nuclease free water up to 20 µL. Ligation reactions were incubated at 22°C for 4 hours. Ligation reactions were dialyzed on 0.025µM nitrocellulose membranes (Merck Millipore, VSWP02500) on de-ionized water for 30 minutes prior to transformations.

Vector (50ng)	Variable
Insert (10:1 molar ratio to vector	Variable
10x T4 Ligase Buffer	2 µL
T4 DNA Ligase (5 Weiss units/µL)	0.2 µL
Nuclease free water	Up to 20 µL
Total	**50 µL**

2.2.5. *E. coli* transformations

E. coli transformations were performed using in-house prepared *E. coli* DH5α electro-competent cells. All steps of preparation of electro-competent cells were carried out on ice. Cultures of *E. coli* DH5α

were grown to O.D.$_{600}$ ≈ 0.8 in LB broth overnight at 37°C and kept on ice for 10 minutes. Cells were harvested by centrifugation at 3000x g for 15 minutes at 4°C and washed by resuspension in a half-volume of ice-cold water. The suspension was centrifuged at 3000x g for 15 minutes at 4°C following which the supernatant was discarded. Washing of cells was carried out three times with the resuspension volume being halved with each successive wash. Washed cells were re-suspended in 1/100th the initial starting culture volume of ice-cold water. Competent cell aliquots (50 µL) were snap frozen in bath containing pure ethanol with dry-ice, and stored at -80°C until required. Transformations were carried out as follows. *E. coli* DH5α electro-competent cells were thawed on ice prior to the addition of 5µL dialyzed ligation product, following which the mixture was added to a pre-chilled 0.2cm Gene Pulser cuvette (165-2086, Bio-Rad,) and allowed to sit on ice for 30 minutes. Electroporation was carried out at 2500V, 25µF and 1000Ω using the Bio-Rad GenePulser Xcell electroporator. Electroporation cuvettes were incubated on ice for 2 minutes prior to the addition of 1mL of SOC media for the recovery of transformants. The suspension was transferred to a 2mL micro-centrifuge tube and incubated shaking at 37°C for 1 hour. A 1:10 dilution and the remaining suspension was then plated LB agar containing appropriate selective antibiotic and incubated overnight for growth of transformant colonies. In the case of transformation of the candidate *pncA* mutant library, 500 µL of recovered cells in SOC medium was plated on LB agar containing zeocin at the appropriate concentration in 120mm large plates.

2.2.6 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* by cell-lysis and spin-column isolation and purification using the QIAprep Spin Miniprep Kit (27106, Qiagen, Germany) as per manufacturer's protocol. Plasmid DNA from our *E. coli pncA* mutant library (pAY230) was isolated using the EndoFree Plasmid Maxi Kit (12362, Qiagen, Germany) as per manufacturer's protocol.

2.2.7. *M. tuberculosis* transformation

M. tuberculosis transformations were performed using in-house prepared electro-competent cells which were prepared fresh for each transformation. Cultures of *M. tuberculosis* were grown to O.D.$_{600}$ ≈ 0.8 in 7H9 broth at 37°C. Cells were harvested by centrifugation at 3000x g for 15 minutes at 4°C and washed by resuspension in a half-volume of 10% glycerol. The suspension was centrifuged at 3000x g for 15 minutes at 4°C following which the supernatant was discarded. Washing of cells was carried out three times with the resuspension volume being halved with each successive wash. Washed cells were re-suspended in 1/10th the initial starting culture volume of 10% glycerol. Transformations were carried out as follows. Plasmid DNA (100ng in a 5µL volume) was added to 450µL of freshly prepared electrocompetent cells, following which the mixture was added to a 0.2cm Gene Pulser cuvette (Bio-Rad, 165-2086) and allowed rest for 15 to 30 minutes. Electroporation was carried out at 2500V, 25µF and 1000Ω using the Bio-Rad GenePulser Xcell electroporator. 1mL of 7H9 broth was added immediately to the cuvette for the recovery of transformants. The suspension was transferred to a 30mL

inkwell culture vessel and incubated shaking at 37°C overnight. A 1:10 dilution and the remaining suspension was then plated 7H10 agar containing appropriate selective antibiotic and incubated for anywhere between two and four weeks for growth of transformant colonies. In the case of transformation of the *pncA* mutant library, 500 µL of recovered cells in 7H9 broth was plated on 7H10 agar containing hygromycin and zeocin at the appropriate concentrations in 120mm large plates.

2.2.8. Sanger sequencing

All Sanger sequencing reactions were carried out using standardized conditions using the BigDye Terminator v3.1 Cycle Sequencing Kit (4337455, Thermo Fisher Scientific, USA). Reactions were set up in 10 µL reactions as follows: 20ng template DNA in the form of purified PCR product, 1µL BigDye Terminator 3.1 Ready Reaction Mix, 1x BigDye™ Terminator Sequencing Buffer, 0.32µM sequencing primer and water to the 10µL reaction volume. Reactions were incubated at 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes using a Bio-Rad T100 Thermocycler. Samples were then held at 4°C until removed.

DNA (5ng/µL)	4 µL
Ready Reaction Mix	1 µL
5x Sequencing buffer	2 µL
Primer (3.2 µM)	1 µL
Nuclease free water	2 µL
Total	**50 µL**

Sequencing reactions were then purified. To the 10 µL reaction, we added 5 µL of pH 5.2 sodium acetate (S7899, Sigma Aldrich, USA) and 50 µL of 100% ethanol. Samples were vortexed vigorously and centrifuged at 3500x g for 15 minutes. Following centrifugation, all liquid was removed by turning tubes/plates upside down on absorbent paper and centrifuging at 100x g for 1 minute. Immediately, 150 µL of ice cold 70% ethanol was added to samples, vortexed and 3500x g for 5 minutes. Once again, all liquid was removed by turning tubes/plates upside down on absorbent paper and centrifuging at 100x g for 1 minute. Samples were then placed uncovered in a Bio-Rad T100 thermocycler at 50°C for 5 minutes to dry products. Products were then submitted to the Africa Centre Sequencing Core following which samples were reconstituted with formamide and run on an ABI Prism 3130 sequencer (Thermo Fisher Scientific, USA). Sequence results in the form of ab1 files were imported into Geneious v6 software (Biomatters Ltd.). Reads were aligned to a reference wild type *pncA* sequence and mutations identified using the variant/SNP identification tool.

2.3. Construction of *pncA* expression vector

Wild type *pncA* was PCR amplified as previously described from pAY29 using ANY_P55 and ANY_P57 at an annealing temperature of 56°C with a cycling extension time of 1 minute. The Mycobacterial Optimized Promoter (MOP) [284] was PCR amplified from pAY1 using ANY_P149 and ANY_P150 at an annealing temperature of 58°C with a cycling extension time of 1 minute. Wild type *pncA* was cloned into our Zeocin resistant L5 integrating vector, pAY59 using the *ClaI* and *PciI* restriction sites to create pAY107. MOP was then cloned into pAY107 using the *ClaI* and *Not I* restriction sites to create pAY108 (*pncA* expression vector for complementation). Emerald fluorescent protein was PCR amplified from pAY21 using ANY_P147 and ANY_P148 an annealing temperature of 58°C with a cycling extension time of 1 minute, and cloned into pAY107 and pAY108 using the *Not I* and *Xba I* restriction sites to create pAY111 and pAY112 respectively. Expression plasmids pAY108 and pAY111 were transformed into sAY251 (Δ*pncA*) to created sAY245 (*pncA* complement referred to as complement) and sAY257 (Vector control) respectively.

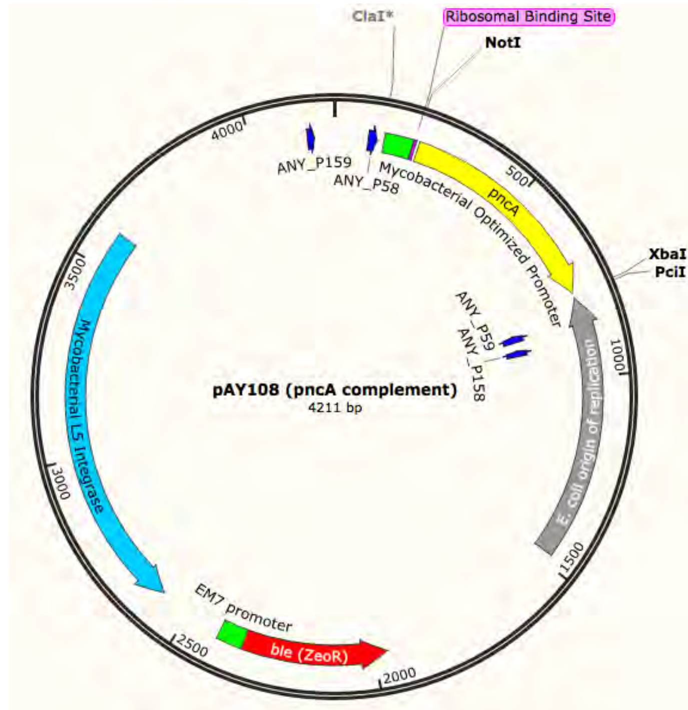

Figure 4: Expression vector for *pncA* expression. Graphical representation of theL5 integrating, zeocin-marked mycobacterial expression vector for *pncA* complementation and generation *of pncA* mutant library. Full lenght *pncA* was cloned in using *ClaI and PciI restriction* sites, with the Mycobacterial Optimized Promoter (MOP) being cloned using the *ClaI* and *NotI* restriction sites to drive *pncA* expression. Four key sequencing primers have also been highlighted. Emerald fluorescent protein was cloned in using the *ClaI* and *PciI* restriction sites to generate our vector control.

2.4. Generation of *M. tuberculosis pncA* mutant library

The GeneMorph II Random Mutagenesis Kit (200550, Agilent Technologies, USA) was used for random PCR mutagenesis with the ANY_P153 and ANY_P154 primers. Reactions were carried out in 50 µL reactions with either 100, 500 or 1000ng of target template (wild type*pncA*) added for generation

of mutagenesis products. It should be noted that the DNA amount stated is only for the target templateto be amplified. The quantity of DNA added to reactions was calculated based on the size of the plasmid used for amplification. Reagents were used at the following final concentrations: 1x Mutazyme II reaction buffer, 200µM each dNTP, 2.5 ng/µL primer mix, 2.5 units of Mutazyme II DNA polymerase and nuclease free water up to the final 50 µL reaction volume. PCR cycling was carried out as follows: 95°C for 2 minutes as initial denaturation, followed either 10, 15, 20, 25 or 30 cycles (depending on input concentration of DNA) of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. Final extension was carried out at 72°C for 10 minutes. Specific template DNA concentrations and PCR cycles for mutagenesis are listed in supplementary table 5.

DNA template	Variable
10x Mutazyme II reaction buffer	5 µL
dNTPs mix (40mM)	1 µL
250ng/µL primer mix	0.5 µL
Mutazyme II DNA polymerase	1 µL
Nuclease free water	Up to 50 µL
Total	**50 µL**

95°C	2 minutes	
95°C	15 seconds	x * cycles *Variable
55°C	30 seconds	
72°C	1minute	
72°C	10 minutes	
4°C	Hold	

Selected mutagenesis products were purified by gel extraction and cloned into pAY112 using the *NotI* and *XbaI* restriction sites to generate individual libraries of *pncA* SNPs. Single colonies from libraries were picked for DNA extraction. DNA was then PCR amplified using ANY_P58 and ANY_P59 at a cycling annealing temperature of 55°C with a 1 minute cycling extension time, and sequenced (as detailed in section 2.2.8.) in order to determine the frequency of mutations within the libraries. Approximately 50,000 *E. coli* clones from the candidate library were harvested by scraping to create our pooled *pncA* SNP library (pAY230). Pooled library plasmid DNA was extracted using EndoFree Plasmid Maxi Kit and transformed into sAY251 (Δ*pncA*). Approximately 34,000 *M. tuberculosis* clones were scraped and pooled to constitute sAY260 (*M. tuberculosis pncA* mutant library. It is worth noting that that if any single SNPs resulted in the formation of a *NotI* or *XbaI* site within an amplicon, it is

likely that those particular SNPs would be under-represented in our library as any amplicons carrying the restriction sites would be truncated during the restriction digest process and lost during the process of gel extraction as amplicons of specific size were excised and purified for cloning. Further bioinformatics analysis would be required to confirm if any single SNP generated during random mutagenesis could result in the formation of either a *NotI* or *XbaI* site within a PCR amplicon.

2.5. Pyrazinamide and pyrazinoic acid *in vitro* growth inhibition assay

Three titred stocks of each sAYs101 (H37Rv wild type), sAY251 (Δ*pncA*), sAY245 (*pncA* complement), and sAY257 (vector control), and sAY260 (*M. tuberculosis pncA* mutant library) were thawed and diluted to 100 000 CFU/mL in 7H9 broth. BACTEC MGIT 960 supplemented PZA medium (245115 and 245128, Becton Dickonson and company, USA) and BBL MGIT supplemented medium (245122 and 245124, Becton Dickonson and company, USA) containing either 0, 1, 4, 20, 100, 500 or 1000µg/mL pyrazinamide or pyrazinoic acid were inoculated with approximately 50 000 CFU. MGITs were loaded in the BACTEC MGIT 960 automated culture system (Becton Dickonson and company, USA) and incubated until the predetermined growth threshold was reached or until 42 days had elapsed upon which MGITs showing no growth were removed as culture negative and recorded. Time-To-Positivity (TTP) for each MGIT was recorded and the ratio of TTP of drug containing MGITs over the no-drug control were calculated and expressed as mean ± standard deviation in order to determine growth inhibition at the various concentrations of drug tested.

2.6. Pyrazinamide and pyrazinoic acid drug susceptibility testing

Conventional phenotypic drug susceptibility testing was done using the Bactec MGIT 960 PZA medium and BBL MGIT medium with slight modifications in order to determine the MIC (minimum inhibitory concentration) of pyrazinamide and pyrazinoic acid. Three titred stocks of each sAY101 (H37Rv wild type), sAY251 (ΔpncA) and sAY245 (*pncA* complement) were thawed, diluted to approximately 50 000 CFU/mL and 5000 CFU/mL respectively. BACTEC MGIT 960 supplemented PZA medium and BBL MGIT supplemented medium containing either 0.04, 0.2, 1, 4, 20, 100 or 1000µg/mL pyrazinamide depending on the strain were inoculated with approximately 25 000 CFU (500µL of 50 000 CFU/mL dilution). BACTEC MGIT 960 supplemented PZA medium and BBL MGIT supplemented medium containing no pyrazinamide (AST control) were inoculated with approximately 2500 CFU (500µL of 5000 CFU/mL). MGITs were appropriately loaded into 5 place AST carriers and incubated in the BACTEC MGIT 960 automated culture system until the AST control reached predetermined growth threshold. MGITs were then removed and the growth of each drug containing tube recorded. The lowest drug containing tube showing a growth index <100 was considered the MIC. Drug susceptibility testing was also carried out against pyrazinoic acid at 4, 20, 100 and 1000µg/mL.

2.7. Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)

RNA extraction, DNase treatment, purification and cDNA synthesis

Control strains were grown up in triplicate from separate glycerol stocks. RNA was extracted from 25 ml, mid-log phase ($OD_{600} \approx 0.5$-0.6) cultures as follows. Cultures were centrifuged at 4000x g for 15 minutes at room temperature. Pelleted cells were suspended in 1 ml of TRIzol (15596026, Thermo Fisher Scientific, USA), transferred into 2mL screw-cap micro-centrifuge tubes containing 100 µL of 0.1 mm sterile zirconia/silica beads (11079101z, Biospec, USA), and incubated for 15 minutes at room temperature. Five cycles of 1 min bead-beatingusing a MagnaLyser (Roche, Switzerland) and 1 min incubation at -20°C was performed. Phase separation was carried out by the addition of 200 µL of chloroform (650498, Sigma Aldrich, USA) followed by vigorous shaking by hand for 15 seconds, 2 minute incubation at room temperature, and centrifugation at 15000x g for 10 minutes at 4°C. The top aqueous layer was transferred to a fresh micro-centrifuge tube and phase separation repeated s above. The top aqueous layer was again recovered and transferred to a fresh micro-centrifuge tube, followed by the addition of 500 µL ice-cold isopropanol (I9516, Sigma Aldrich, USA). The solution was mixed well by inversion and incubated at -20°C for 30 minutes, prior to centrifugation at 15000x g for 10 minutes at 4°C. After removing the supernatant, 1 mL of ice-cold 75% ethanol was added to the resulting pellet, followed by mild vortexing and centrifugation at 15000x g for 10 minutes at 4°C. This process was repeated following which the resulting RNA pellet was air-dried at room temperature for 10 minutes. RNA was eluted in a 50µL DEPC-treated water (750024, Thermo Fisher Scientific, USA).Eluted RNA was quantified using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, USA) and stored at -80°C in three equal aliquots. A single aliquot for each replicate of each strain was thawed on ice and DNase treated as follows. DNase treatment was carried out in a 50 µL volume using RNase-free DNase (AM2222, Thermo Fisher Scientific, USA).

RNA	5 µg
10x reaction buffer	5 µL
DNase I	5 units (5 µL)
DEPC-treated water	Up to 50 µL
Total	**50 µL**

Reactions were incubated for 30 minutes at 37°C followed by the addition of 5 µL of 50mM EDTA and incubation at 65°C for 10 minutes. DNase treated RNA was purified using the RNeasy Mini Kit (74104, Qiagen, Germany) as per manufacturer’s protocol and quantified using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, USA).

RNA was reverse transcribed to cDNA in 20µL reaction volumes using the iScript cDNA Synthesis Kit (1708891, Bio-Rad, USA) as per manufacturer's instructions. Reactions were incubated at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. The resulting cDNA was quantified as above and stored at -80°C in three separate aliquots until ready for Real-Time quantitative PCR.

Purified DNase treated RNA	250ng
5x iScript Reaction mix	4 µL
iScript Reverse Transcriptase	1 µL
Nuclease free water	Up to 20 µL
Total	**20 µL**

Real-Time quantitative PCR

RT-qPCR was performed on CFX 96 Real Time PCR detection system (Bio-Rad, USA) using iTaq Universal SYBR Green Supermix (172-5121, Bio-Rad, USA) and KM_P35/KM_P36 and sigA_F/sigA_R primers for *pncA* and *sigA*, respectively.

2x iTaq universal SYBR green supermix	5 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
DNA template	1 µL (≈100ng)
Nuclease free water	2 µL
Total	**10**

RT-qPCR cycling conditions were as follows:

95°C	2 minutes	
95°C	5 seconds	x 40 cycles
60°C	30 seconds	
65-95°C High Resolution Melt	5 seconds per temperature	0.5°C increments

All samples were performed in triplicate, internally normalized to *sigA*, and the mean fold-change and standard deviations evaluated relative to wild type (sAY101). Three biological replicates for each strain was performed.

2.8. Sequential *in vitro* screening of *M. tuberculosis pncA* mutant library

Three stocks of sAY260 were thawed and diluted to 100 000 CFU/mL in 7H9 broth. Approximately 50000 CFU was then inoculated into BACTEC MGIT 960 PZA supplemented medium, containing either 0µg/mL PZA (unselected library) or 4, 20, 100 or 5000µg/mL PZA (PZA selected libraries), and incubated in the Bactec MGIT 960 automated culture system. Within 24 hours of a MGIT being flagged as positive, it was removed and processed with the time-to-positivity (TTP) being recorded. The entire 8mL culture was centrifuged at 4500x g for 10 minutes. After discarding the supernatant, the bacterial pellet was re-suspended in 1mL 7H9 broth, from which 30µL was used to inoculate a new MGIT containing the same concentration of PZA. The remaining suspension was transferred to a new micro-centrifuge tube and centrifuged at 12000x g for 5 minutes. The supernatant was discarded and the bacterial pellet stored at -20°C. This process was repeated for 6 rounds of PZA selection. DNA extractions of samples from the sequential *in vitro*library screen were done using the Hain GenoLyse kit (51610, Hain Lifesciences, Germany). Briefly, frozen bacterial pellets were thawed out, resuspended in 100µL of lysis buffer, and incubated at 95°C for 30 minutes before the addition of 100µL neutralization buffer. Samples were centrifuged at 12000x g for 5 minutes and the supernatants containing DNA recovered in a fresh 1.5mL micro-centrifuge tube for use as PCR template.

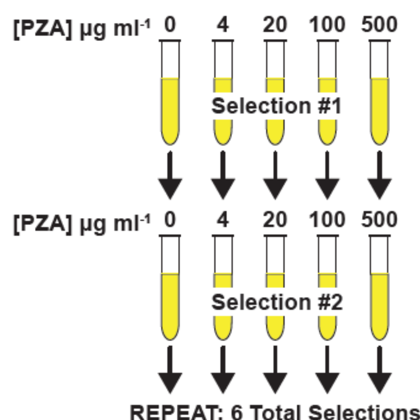

Figure 5: Schematic representation of sequential *in vitro* selection procedure.Our *pncA* mutant library was used to inoculate BACTEC MGIT 960 PZA supplemented medium, containing either 0µg/mL PZA (unselected library) or 4, 20, 100 or 5000µg/mL PZA (PZA selected libraries). Upon culture positivity, libraries were recovered for sequencing, as well as to inoculate fresh MGITs containing the same concentration of drug to allow for sequential enrichment of mutant clone. Selection was repeated for 6 rounds.

2.9. Screening of *M. tuberculosis pncA* mutant library*in vivo*

A culture of sAY260 was grown to an OD_{600} = 1.071 (approximately $1x10^8$cells/mL). 750µL of culture was centrifuged at 12000x g for five minutes, washed by resuspension in 5mL of PBS and centrifugation, and finally re-suspended in 15mL of PBS for the tail-vein infections of thirty 6-8 week old (≈20g) female BALB/C mice with an expected implantation of approximately 1 000 000 CFU per mouse (≈ 100 000 CFU in the spleen). Five mice each were sacrificed on the day after infection (Day -

3) and the day of treatment initiation (Day 0) to ensure adequate implantation of sufficient bacteria to represent the complexity of our library. Upon treatment initiation, mice were randomized into two groups with one group of mice receiving 150 mg/kg pyrazinamide (treatment arm), while the second group received PBS (mock arm), once daily for five days per week by oral gavage.

Mice were then sacrificed at Day 21 and Day 42 post treatment initiation. At all sacrifice time-points sacrifice, spleens of the mice were placed in 2mL of PBS and homogenized using a pestle and mortar. The resulting homogenate serial diluted and plated on 7H11 agar containing hygromycin and zeocin, with and without200,000 iu/L polymixin B, 10 mg/L amphotericin B, 50 mg/L carbenicillin, and 20 mg/L trimethoprim (PACT antimicrobial cocktail). Upon growth of colonies (three to four weeks), colony counts were carried out for all sacrificial time-points and colonies from plates with adequate representation of our library (>100 000 colonies) of the were scraped into 50mL conical tubes and stored at -20°C. DNA was extracted using the GTC genomic DNA extraction method (Larsen et al., 2007), with the only modifications being a 2nd chloroform separation after separation with GTC solution to ensure the removal of phenol from the mixture, and a 2nd ethanol wash prior to air-drying and resuspension of the DNA pellet in 1x TE buffer.

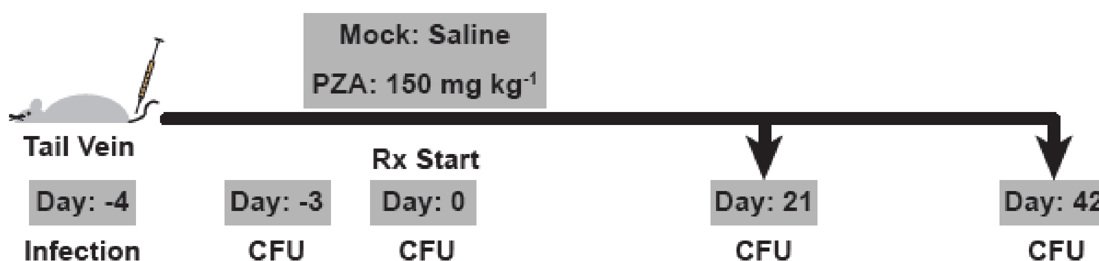

Figure 6: Schematic representation of time-line of murine selection model. Mice were infected with our *pncA* mutant library at Day -4 by tail vein injection and sacrificed at Day -3 (day after infection) and Day 0 (treatment initiation) to enumerate bacterial burden in the spleens to ensure adequate library representation and to recover baseline input libraries. Mice were randomized into two groups, receiving either 150mg/kg pyrazinamide (treatment arm) or saline (mock arm). Mice were then sacrificed at Day 21 or Day 42 after treatment to enumerate bacterial burden and recover selected/unselected libraries for sequencing. CFU = colony forming units, Rx Start = Treatment initiation.

2.10. Library preparation and sequencing

DNA from screen samples was PCR amplified using ANY_P158 and ANY_P159 with an annealing temperature of 60°C and a cycling extension period of 90 seconds, and purified by gel extraction. A total of 1ng of DNA from each *in vivo* sample was utilized for PCR amplification. Since the DNA concentration from GenoLyse extracts could not be quantified, 2.5 µL of extract was utilized as DNA template for amplification.A total of 200ng of each purified product were vacuumed-dried and submitted for sequencing. Sequencing library preparation and sequencing were carried out by Dr.

Thomas Ioerger and the Sacchettini lab at Texas A&M University (Texas, USA). Libraries for next generation sequencing (NGS) were prepared using standard Illlumina genomic DNA sequencing sample preparation protocol (Illumina, Inc.), with barcodes for multiplexing. Shearing was performed using a Covaris E220 (Covaris, Inc.). Samples were sequenced in paired-end mode (125+125 bp) on an Illumina 2500 sequencer.

2.11. Bioinformatics

Bioinformatics on sequence libraries and subsequent data analysis resulting in the generation of the list of mutations with associated susceptibility predictions were carried out by Dr. Thomas Ioerger and by Dr. Adam Yadon. On average 2.9 million pairs of reads were collected for each sample and mapped to the *M. tuberculosis* H37Rv *pncA* gene the Burroughs-Wheeler Aligner program[285]. Pairs of reads with 2 or more mismatches or containing indels (insertions or deletions) were discarded. The number of single-nucleotide polymorphisms at each position was tabulated and individual single-nucleotide polymorphism frequencies were calculated by dividing the count by the total number of reads covering that site in each dataset. The frequency of single-nucleotide polymorphisms from an unselected wild type *pncA* was subtracted from the observed frequencies in each sample. Enrichment or depletion was calculated as the log2 mean fold-change for each pyrazinamide selection relative to the no-drug selection of the same round or at implantation (day -3) for the sequential *in vitro* selection and murine selection model respectively. Statistical significance was determined using a t-test (two-sided, unequal variance).

Single-nucleotide polymorphisms exhibiting ≥2 fold-change (p-value≤0.05, t-test) in two or more *in vitro* selections (any round of drug selection or drug concentrations) or after either 21 or 42 days of pyrazinamide treatment in our murine selection model were identified. Single-nucleotide polymorphisms identified as both enriched and depleted in a screen were discarded. Mutations were then converted to the respective amino acid form and predicted to be either resistance-conferring or susceptible-conferringmutations identified either *in vitro* or during infection in mice. Data from both screens were combined after conversion to the amino acid level, with mutations being identified as both enriched and depleted in both screens being discarded to provide us with a provisional list of mutations with associated predictions.

2.12. Validation of screen using selected isogenic *pncA* mutants

Single colony picks for validation were obtained by streaking out our *pncA* mutant library onto 7H10 plates containing selective antibiotics. Two hundred and eleven single colonies were picked and sub-cultured into 1mL 7H9 broth and 7H10 plates containing selective antibiotics, as well as re-suspended in 100µL nuclease free water. Plates and broth were incubated at 37°C to allow outgrowth of the mutants, while colonies re-suspended in nuclease-free water were boiled at 95°C for 30 minutes and 2.5 µL lysate used as template for PCR amplification using primers ANY_P158 and ANY_P159 with

an annealing temperature of 60°C and a cycling extension period of 90 seconds. Products were purified using the gel extraction and sequenced using ABI Big Dye Terminator Kit and the ABI Prism 3130 sequencer with primers ANY_P58 and ANY_P59 in order to identify the polymorphism/s carried within each mutant clone. After analysis of sequence results, 48 isogenic isolates containing mutations of interest were further outgrown from the initial cultures and used to create titres. These 48 isogenic mutant isolates then underwent drug susceptibility testing using Bactec MGIT 960 PZA kit as previously done for control strains.

2.13. Preliminary comparison of clinical data to screen predictions

Genotype-to-phenotype data was extracted from the supplementary data from a multicentre study which sequenced *pncA* from pyrazinamide resistant and susceptible isolates whose susceptibility testing was carried out using the Bactec MGIT 960 PZA system[32]. Mutation data for *pncA* mutations conferring pyrazinamide resistance at very high confidence was condensed to amino acid form with the associated phenotypes, after removing all indels (insertions and deletions). Mutations associated with both a resistant and susceptible phenotype were also removed. Respective *pncA* mutant genotypes associated with pyrazinamide resistance from our provisional screen prediction data and the single clinical dataset were then compared to look for overlap.

2.14. Determining mechanisms of resistance to pyrazinamide

In order to determine the molecular basis of mechanisms of resistance to pyrazinamide, we sought quantify PncA and its activity.

2.14.1. Quantitation of PncA

Due to the lack of an anti-PncA antibody, we utilized a stable isotope labelling and targeted mass-spectrometry approach in order to carry out relative quantitation of the PncA. For initial development and optimization of our method, ^{13}C-labelled lysate alone, as well as ^{13}C-labelled lysates plus lysates from our complement and Δ*pncA* respectively, were digested, purified, run on the mass spectrophotometer and analyzed using the methodology described below.

2.14.1.1. Growth of isotopically labelled control and isogenic mutants

The proteome of sAY245 (Comp) was grown to OD_{600} = 1 in a modified Hartmans-de Bont (HDB) medium (recipe provided in appendix) in order to uniformly label the proteome with [^{13}C]. Selected isogenic mutants as well as the complement and Δ*pncA* controls were grown in triplicate to an OD_{600} = 0.6 ~ 0.8 in a 30mL volume of standard 7H9 broth, with the addition of the appropriate concentrations of selective antibiotics.

2.14.1.2. Extraction of cell lysates

Cell lysates were prepared as follows. Cultures were centrifuged at 3500x g for 15 minutes at 4°C, following which pellets were washed three times by resuspension in 10mL of 10% glycerol and centrifugation at 3500x g for 15 minutes at 4°C in order to remove Tween 80 and BSA. Washed pellets were resuspended in 1mL sterile phosphate buffered saline (PBS) containing Protease inhibitor cocktail (11873580001, Roche, Switzerland), and transferred to fresh screw-cap tubes containing 100µL of 0.1mm sterile zirconia/silica beads (11079101z, Biospec, USA) beads, vortexed and incubated at -20°C for 5 minutes. Cells were ruptured by repeated bead-beating at 7000rpm for one minute using a MagnaLyser (Roche, Switzerland) and a one minute incubation at -20°C, a total of four times. Sodium dodecyl sulphate (SDS) (L4390, Sigma Aldrich, USA) was added to a final concentration of 2%, with the samples being vortexed and incubated at 65°C for 30 minutes. Lysates were recovered by twice recovering the supernatant of samples after centrifugation at 15000x g for 5 minutes. Lysates were then passed 0.2micron Corning Costar SpinX columns (CLS8160, Sigma Aldrich, USA), removed from the BSL3 and stored at -80°C.

2.14.1.3. Protein isolation, peptide digest and purification

^{13}C heavy labelled lysate and lysates from *pncA* mutants and control strains were mixed 50:50 in a 200µL volume prior to the addition of 600µL of methanol (34860Sigma Aldrich, USA) and 150µL chloroform (650498, Sigma Aldrich, USA).This was briefly vortexed before adding 450µL MilliQ water and vortexing again before centrifugation for 1 minute at 16000x g. The upper aqueous layer was discarded and 450µL methanol added to the organic phase, vortexed and centrifuged for 2 minutes at 16000x g. The resulting supernatant was discarded and 100µL of 6M urea buffer (U5378, Sigma Aldrich, USA) was added to the precipitate, prior to sonication for 2 minutes. 5µL of 200mM DL-Dithiothreitol (DTT) (646563, Sigma Aldrich, USA), 20µL of 200mM iodoacetamide (I6125, Sigma Aldrich, USA), and 20µL 200mM DTT were added with 30 minute incubation periods at room temperature after the addition of each reagent. 775µL MilliQ water was then added to the samples, followed by the addition of 20µL of 0.2µg/mL Promega sequencing grade modified Trypsin (V5117, Promega, USA) and overnight incubation at room temperature. Reactions were stopped by the addition of 10µL concentrated acetic acid (A6283,Sigma Aldrich, USA) to reduce the sample pH <6. Samples then underwent column purification using Sep-Pak Vac 6cc 500mg C18 cartridges (WAT043395, Waters,USA). Briefly, columns were cleaned and equilibrated by passing through 5mL Acetonitrile (34967,Sigma Aldrich, USA), 5mL 65% Acetonitrile with 0.1% Formic Acid (64-18-6, Merck Millipore, USA), and 10mL 2% Acetonitrile with 0.1% Formic Acid. Peptide digested samples were then added to the column, and washed with 10mL 2% Acetonitrile with 0.1% Formic Acid, before being eluted in 2mL 65% Acetonitrile with 0.1% Formic Acid. Samples were completely dried down by vacuum concentration, resuspended in 50µL 2% Acetonitrile with 0.1% Formic Acid and stored at 4°C.

2.14.1.4. Mass spectrometry and data analysis

Unique PncA, RpoB, and DnaK peptides (supplemental table 4) were targeted using a Q-Exactive Mass Spectrometer coupled to a Dionex RSLC 3000 nano Liquid Chromotograph system (Thermo Fisher Scientific, USA), using a 30 minute gradient and mobile phase from 1% acetonitrile and 0.1% formic acid to 50% acetonitrile and 0.1% formic acid, with a regeneration phase to clean up and equilibrate the column. Separation was carried out on a 15 cm by 75 uM ID fused silica column, backed with 3 uM C18 stationary phase, and a flow rate of 0.300 ul min^{-1}. The nano-spray source was operated at a capillary temperature of 275°C, an emitter voltage of 1.6 Kv and S-lens voltage of -55 V. A targeted-MS2 method was employed with an AGC target of $1.0e^5$ ions, an isolation width of 2 m z^{-1} and an HCD setting of 27. Sample were run on the Mass Spectrophotometer by Dr. John Adamson of the Africa Health Research Institute Pharmacology Core.

Spectral traces were quantified using Skyline proteomics software. The area-under-the-curve (AUC) for each peptide transition (peptide breakdown product)was exported using the MS1 probe plug-in. Any transitions containing a mutation of interest or with an AUC = 0 were discarded. For each sample, the quantified light/unlabelled AUC was normalized to the ^{13}C-heavy AUC for each transition. The mean ratio of unlabeled to heavy labelled AUC for all PncA peptides was then normalized to the mean ratio of unlabeled to heavy labelled AUC for all RpoB or DnaK peptides across all biological replicates. Three biological replicates each per mutant and 10 biological replicates for sAY254 (Comp) was performed. The fold-change and standard deviations of protein abundance for each mutant is reported relative to the isogenic complement control.

2.14.2. Quantitation of pyrazinamidase activity

A modified quantitative Wayne assay[262] was used to determine the enzymatic activity of pyrazinamidase by assaying for the production of pyrazinoic acid.

2.14.2.1. POA assay and data analysis

Cultures for selected mutants of interest as well as our complement and Δ*pncA* strains were grown in triplicate to OD_{600} = 0.6~0.8, and treated with 500µg/mL pyrazinamide. Aliquots of 500µL were taken in sterile screw-cap tubes containing 100µL of 0.1mm sterile zirconia/silica beads beads, immediately prior to the addition of pyrazinamide and after 48 hours pyrazinamide treatment. Samples were bead-beaten 4 times at 7000rpm for 1 minute with 1 minute incubations at -20°C between bead-beating to allow for the release of intracellular POA. Samples were then centrifuged at 15000x g for 5 minutes. Whole cell lysate (100µL) was then aliquoted into two fresh tubes for each sample, with 900µL of 0.1M pH3.4 Glycine-HCl (G2879, Sigma Aldrich, USA) being added to the first tube as a blank. 10µL of 20% ferrous ammonium sulphate(215406, Sigma Aldrich, USA) was added to the second tube, followed by the addition of 890µL 0.1M pH3.4 Glycine-HCl. Absorbances were then read at 460nm [288] (Bio-Rad

SmartSpec Plus) with the tube not containing ferrous ammonium sulphate being used to blank the spectrophotometer for each sample. Individual absorbance values were normalized to absorbance values prior to pyrazinamide treatment and scaled by bulk protein content (described in section 2.14.2.2), and pyrazinamidase enzyme levels determined by mass spectrometry. Relative enzymatic activity is reported as the mean and standard deviation relative to the isogenic complement control.

2.14.2.2. Bulk protein quantification

Protein quantification was carried out on whole cell lysates to correct for cell breakage, as per the "test-tube room temperature" protocol of the Pierce BCA Protein Assay kit (23225, Thermo Fisher Scientific, USA), with a minor modification in that a reaction volume of 1mL was used. A standard curve was set up using BSA provided with 1x PBS containing 2% SDS as a diluent. Whole cell lysates were diluted 1 in 50 in 1x PBS containing 2% SDS.Absorbances were then read at 562nm on a Bio-Rad SmartSpec Plus spectrophotometer.

Chapter 3: Results

3.1. Generation and characterization of *pncA* mutant library

In order to comprehensively and systematically screen all potential *pncA* single nucleotide polymorphisms (SNPs), multiple libraries of *pncA* mutants were constructed using random error-prone PCR mutagenesis under different PCR conditions varying the template concentration and PCR cycle number in order to maximize the introduction of single SNPs.The technique requires a high template concentration which results in all libraries having a significant proportion of wild type sequences. Initially the products of five mutagenesis conditions were cloned into our *pncA* expression vector and expressed in *E. coli*. We then classified the single colony sequences as wild type, containing a single SNP, or containing more than one SNP. Results of one round of mutagenesis of these five libraries is shown in Figure 7, from which we selected one candidate library.

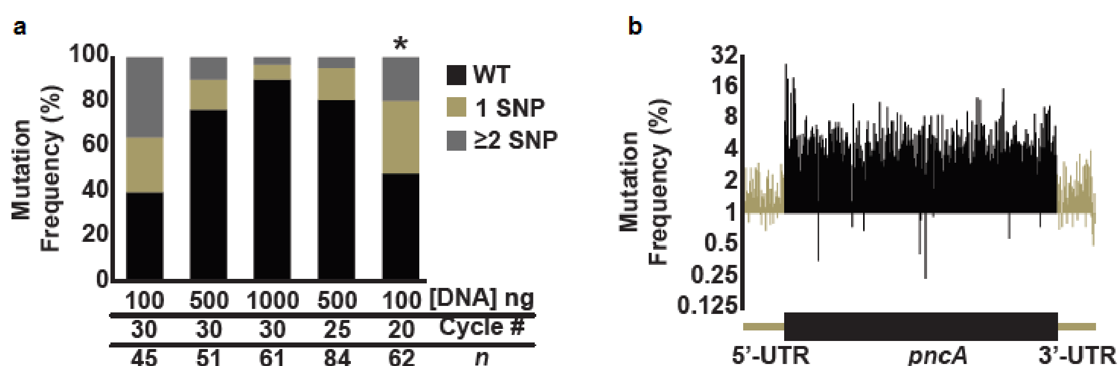

Figure 7: Characterization of *pncA* mutant library. (a) Distribution of wild type (WT), single SNPs and 2 or more SNPs within individual mutant libraries constructed using random error-prone PCR mutagenesis. * indicates candidate library selected **(b)** Mean *pncA* single nucleotide polymorphism frequency (%) at each nucleotide of our *M. tuberculosis pncA* mutant library in relation to an unmutagenized control

The library with the greatest proportion of single nucleotide mutations, which contained approximately 48.39% wild type*pncA*, 32.25% single *pncA* mutations, and 19.36% *pncA* containing 2 or more mutations (indicated by * in Figure 7a), was then transformed into an H37Rv *M. tuberculosis*strain where the endogenous *pncA* locus had been inactivated through insertion of a hygromycin resistance cassette (Δ*pncA*). Additional mutagenesis conditions using different template concentrations and cycle numbers (supplementary table 5) were sequenced using PacBio Single Molecule Real Time Sequencing, however no other mutagenesis condition produced a more favourable mutation breakdown (data not shown) than observed in our candidate library. Transformants were pooled in order to constitute the library which then underwent confirmatory deep sequencing which revealed roughly 4.5 fold higher intragenic *pncA* SNPs at each nucleotide relative to our unmutagenized wild type control strain (Figure 7b).

3.2. Development of *in vitro*screen

Due to the challenging nature of pyrazinamide susceptibility testing, we sought to develop and optimize a selection screen based on the BD Bactec MGIT 960 PZA system, as this would allow for standardized screening using fixed pre-adjusted pH media and culture conditions.

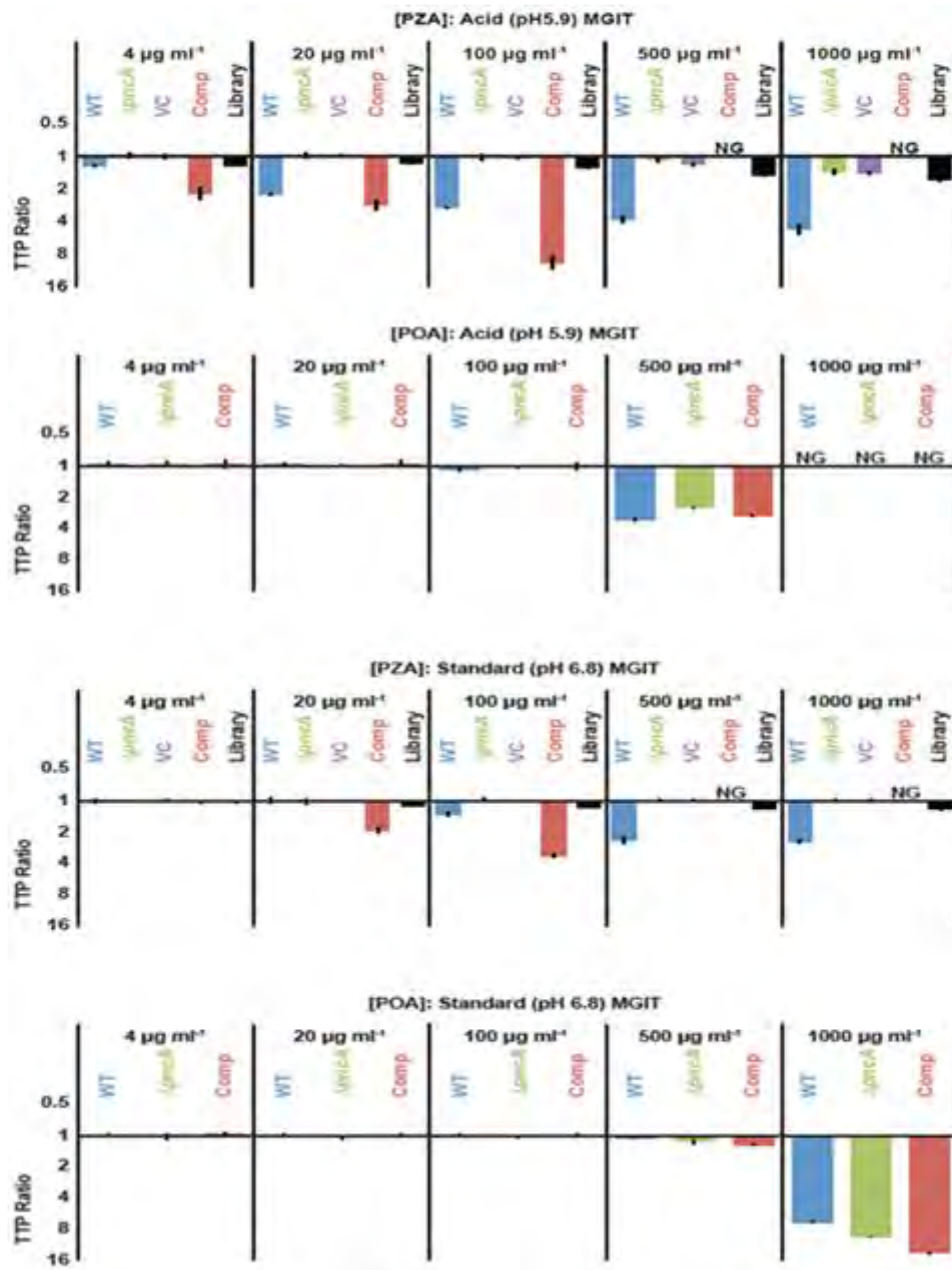

Figure 8: Phenotypic assaying against pyrazinamide and pyrazinoic acid under acid (pH5.9) and standard culture conditions to determine growth inhibition.MGITs inoculated with respective strains were assayed against varying concentrations of pyrazinamide and pyrazinoic acid under two different culture conditions. Results are represented as an average with standard deviation of TTP ratio (time to positivity in drug treated culture compared to untreated control) as a measure of growth inhibition. PZA = pyrazinamide, POA = pyrazinoic acid, WT = H37Rv wild type control, Δ*pncA* = H37Rv with *pncA* insertionally inactivated, Comp = Δ*pncA* complemented with wild type *pncA*, VC = Δ*pncA* carrying Emerald fluorescent protein as a vector control, Library = Δ*pncA* carrying our *pncA* mutant library.

As expected our Δ*pncA* and vector control strains showed high level resistance to pyrazinamide, demonstrated by lack of growth inhibition in both acid and standard culture media at all tested concentrations (Figure 8). Our complement control showed a greater degree of growth inhibition to pyrazinamide compared to the H37Rv wild type strain, with the growth inhibition being more pronounced in acidified media than standard culture media for both strains. Complete inhibition of growth was observed in the complement strain at both 500µg/mL and 1000 µg/mL in both forms of media tested. Growth inhibition to pyrazinoic acid was almost identical across strains with the inhibition again being more pronounced in acidic culture media than in standard culture media. Complete inhibition of growth was observed at 1000 µg/mL in acidic media. It was interesting to note that our *M. tuberculosis pncA* mutant library showed mild growth inhibition when treated with pyrazinamide at higher concentrations in acidified media compatible with it containing a proportion of wild type *pncA* sequences.

Table 1: Drug susceptibility testing results of control strains. Minimum inhibitory concentrations (µg/mL) of pyrazinamide and pyrazinoic acid determined under standard pH (pH6.8) and acidic pH (pH 5.9) culture conditions using the the Bactec MGIT 960 PZA medium and BBL MGIT medium. PZA = pyrazinamide, POA = pyrazinoic acid, WT = H37Rv wild type control, Δ*pncA* = H37Rv with *pncA* insertionally inactivated, Comp = Δ*pncA* complemented with wild type *pncA*.

Drug	Media	WT	Δ*pncA*	Comp
PZA	Acid	20	>1000	1 – 4
PZA	Standard	100	>1000	20
POA	Acid	100	100	100
POA	Standard	500	500	500

The observations of our growth inhibition assays was confirmed through susceptibility testing of our wild type and complement controls with MIC's of 20 µg/mL and 1-4 µg/mL respectively in acidified media compared to MIC's of 100 µg/mL and 20 µg/mL respectively in standard culture media (Table 1), indicating hyper-susceptibility of our complement to pyrazinamide. The Δ*pncA* strain was highly resistant to pyrazinamide with an MIC of >1000µg/mL in both tested media conditions. Pyrazinoic

acid MICs were identical across all strains confirming the observations of our growth inhibition assays, with a 5-fold lower MIC when tested in acidified media compared to standard culture media.

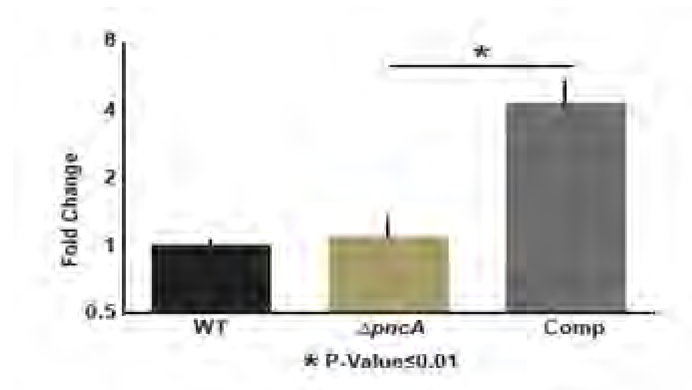

Figure 9: Expression of *pncA* in wild type H37Rv, Δ*pncA* and complement. RT-qPCR determination of *pncA* expression levels across control strains reveals 4-fold over-expression of *pncA* in our complement. Data represented as mean with standard deviation (* P-value ≤0.01).WT = H37Rv wild type control, Δ*pncA* = H37Rv with *pncA* insertionally inactivated, Comp = Δ*pncA* complemented with wild type*pncA*.

Real-Time qualitative PCR revealed 4-fold over-expression of *pncA* in our complement strain relative to our H37Rv wild type control and Δ*pncA* (Figure 9). This explains why we saw a hyper-susceptibility phenotype with the complemented stain, since higher expression of *pncA* would lead to more activation of pyrazinamide.

Varying growth inhibition across the full ranges of pyrazinamide tested, as well as the respective MIC's of our complement and Δ*pncA*, suggested that we could select for resistance conferring mutant clones across a wide dynamic range of susceptibilities. As growth inhibition or lack thereof was more pronounced in acidified media, we concluded that acidified medium would give us greater enrichment of resistance conferring mutants and hence was the ideal medium for *in vitro* selection.

3.3. Phenotypic screening of *pncA* mutant library*in vitro*

Next we sought to enrich for resistance conferring mutants by exposing our *pncA* mutant library to sequential *in vitro* selection using the Bactec MGIT 960 PZA medium with a range of pyrazinamide concentrations. MGIT tubes were inoculate from titred frozen stocks and grown to positivity under one of several concentrations of pyrazinamide. Upon positivity, pyrazinamide-selected libraries were harvested and transferred to a second tube containing the same concentration of pyrazinamide and the cycle repeated to six rounds of selection.

As expected, cultures showed a progressive reduction in time-to-positivity (TTP) with each subsequent selection at all concentrations of pyrazinamide tested (Figure 10). Since TTP is a surrogate read-out for

rate of growth of bacteria as MGIT positivity is based on a set-threshold, we believe this to be indicative of enrichment for resistance conferring mutant clones, as well as some bacterial adaptation to the acidic culture condition which also showed a successive reduction in TTP in the untreated control library. Recovered libraries for each selection were then PCR amplified and deep-sequenced using Illumina sequencing.

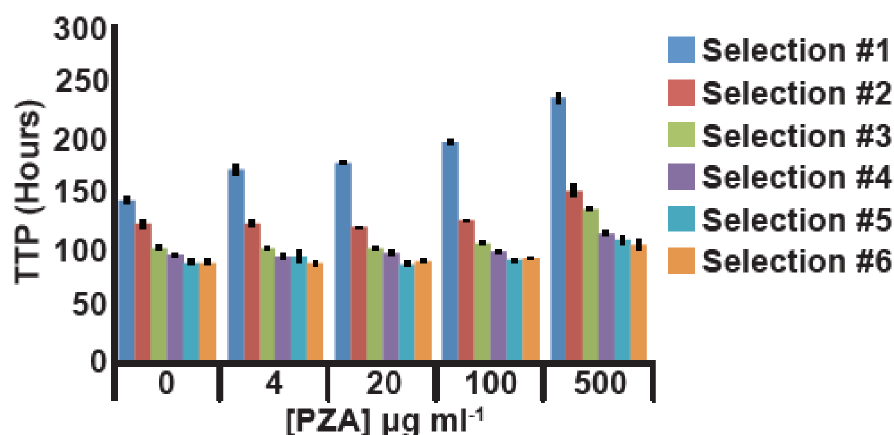

Figure 10: Change in time-to-positivity (TTP) with sequential *in vitro* selection exposed to different concentrations of pyrazinamide. Average time to positivity (TTP) of *in vitro* selection indicating reduction of growth inhibition with sequential selection. Data represented as mean with standard deviation.

3.4. Phenotypic screening of *pncA* mutant library *in vivo*

As *in vitro* susceptibility to pyrazinamide may not faithfully recapitulate susceptibility *in vivo*, we further screened our *pncA* library using a murine treatment model. At day -3 (day after infection), we observed approximately 5.9 x10^5 CFU in the spleen of infected mice, which represents over 300-fold coverage of the library (Figure 11). Bacterial burden did increase over the course of 42 days after the initiation of treatment in the mock (PBS-treated) arm, while showing a moderate decline from day 21 to day 42 after treatment initiation in the pyrazinamide treated arm, although the difference in bacterial burden at day 42 between the pyrazinamide treated and untreated mice was not statistically significant. Organ DNA was extracted at each time-point and used as template for PCR amplification of *pncA* which was then sequenced to determine changes in library complexity.

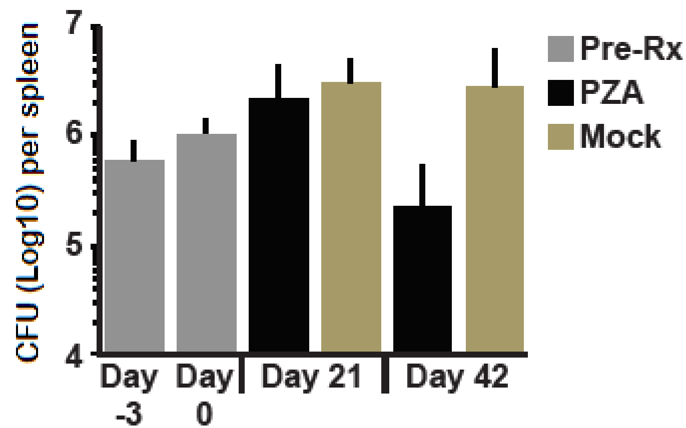

Figure 11: Bacterial burden observed in the spleens of mice infected with our *M. tuberculosis pncA* mutant library.Averagebacterial burden in colony forming units (CFU) observed in spleens of infected mice at pre-treatment Day -3 (implantation control), Day 0 (treatment initiation), and Day 21 and Day 42 (selection timepoints) of either pyrazinamide or mock treatment. Data represented as mean with standard deviation.

3.5. Provisional list of predicted susceptibilities of mutations

The analysis of the *in vitro* and *in vivo* pyrazinamide selected libraries (detailed in the methodology) is beyond the scope of this dissertation and was carried out by Dr. Thomas Ioerger and Dr. Adam Yadon. In brief, reads from sequenced libraries were aligned to a reference *pncA* sequence with reads containing multiple SNPs or indels (insertions or deletions) being discarded. SNPs at each nucleotide were tabulated and used to calculate the mutation frequency by normalizing to total read count at that given nucleotide. Mutation frequency from an unmutagenized control was subtracted from each library prior to calculating the enrichment or depletion of mutations by comparing fold change in mutation frequency of drug selected libraries to respective controls.Mutations were converted to amino acid form with phenotype predictions being associated for *in vitro* and *in vivo* selections, with data from both screens being eventually combined. It is worth noting that fold change comparison

The output of this analysis was a provisional list of mutations at the amino acid level (supplementary table 7) which were either enriched (predicted pyrazinamide resistant), depleted (predicted pyrazinamide susceptible), or neither enriched nor depleted (unselected or intermediate) during either one or both of the phenotypic screens when comparing libraries selected with pyrazinamide treatment versus unselected libraries. Of the 1332 possible mutations (including amino acid substitutions, non-sense mutations resulting in stop codons, and mutations not resulting in an amino acid substitution) in *pncA*, 1197 (89.86%) were adequately represented in our *M. tuberculosis pncA* mutant library. In total we identified 411 mutations that were enriched in either or both of our screens. We also found 357 mutations that were depleted, and 374 mutations which were neither enriched nor depleted. This meant

that we could potentially predict phenotypes for 1142 or 85.74% of all possible mutations at the amino acid level.

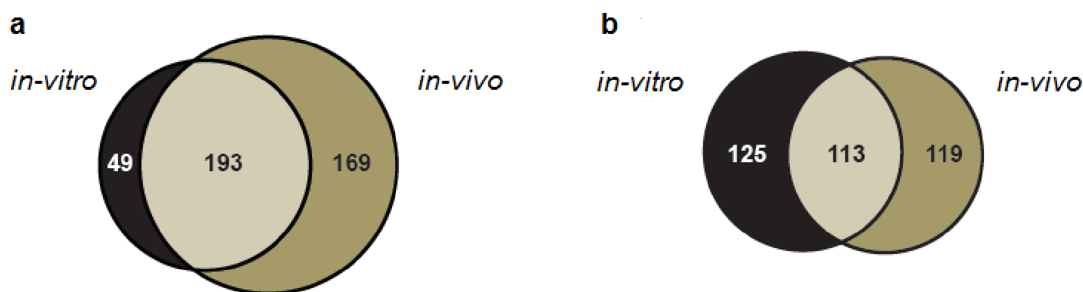

Figure 12: Breakdown of mutations identified. Venn diagrams comparing overlap of **(a)** all enriched mutations, and **(b)** all depleted mutations identified through *in vitro* and *in vivo* screening.

Of the 411 mutationswhich were enriched in our phenotypic screens, (46.96%) were identified by both screens, while 49 (11.92%) and 169 (41.11%) predicted mutations were unique to the *in vitro* and *in vivo* screens respectively. When looking at mutations that were depleted, 113 (31.66%) were identified in both screens, while 125 (35.01%) and 119 (33.33%) were identified uniquely in the *in vitro* and *in vivo* screens respectively.

When we examine the distribution mutations that were enriched, we observed that these occurred along the full length of the gene (Figure 13). When we looked at both the distribution and frequency of mutations enriched in our screens (and therefore predicted to confer pyrazinamide resistance) at each codon, we found some clustering of mutations around resides predicted to be involved in the enzymatic function of PncA.

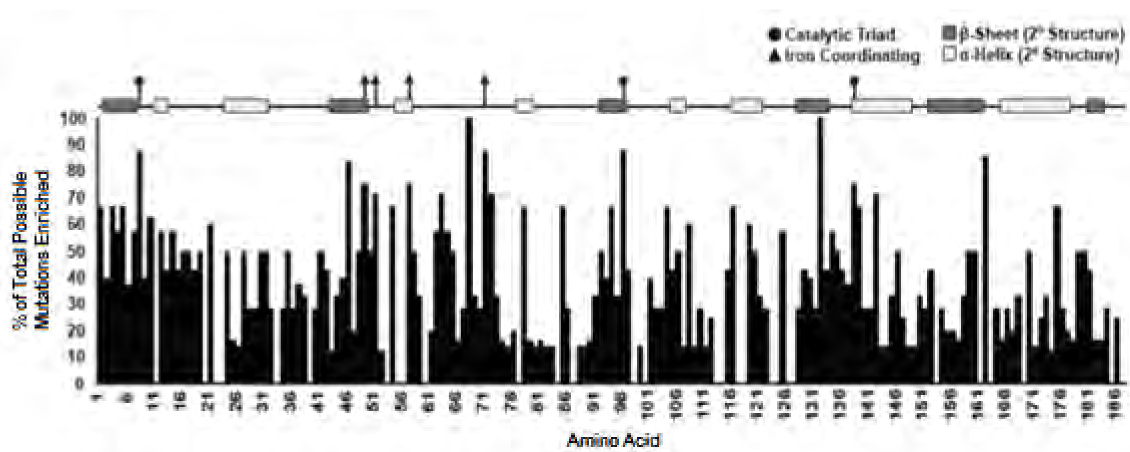

Figure 13: Distribution of resistance conferring mutations across the *pncA* gene. Proportion (%) of all mutations represented in our library that were enriched in either one or both of our screens for each codon of PncA. Predicted linearized structure of PncA is shown above the frequency and distribution graph with key functional and structural regions indicated. Mutations predicted to confer resistance are more frequent and cluster around residues believed to make up the catalytic triad.

3.6. Validation of screen results

In order to determine if our enriched mutations conferred resistance to pyrazinamide, we randomly selected clones from our unselected library, sequence confirmed the isogenic *pncA* mutation they harboured and subjected them to drug susceptibility testing using the Bactec MGIT 960 PZA kit in order to determine MIC's. Of the two hundred and eleven colonies picked and sequenced, we identified 48 unique SNP containing clones.

All 23(100%) of mutant clones that were classified as enriched by our screens showedan elevated MIC atleast five-fold greater (≥ 20 µg/mL) than the isogenic complement control stain which had an MIC between 1 and 4 µg/mL, whereas 13 of 14 (92.86%) of clones classified as depleted retained an MIC identical to the isogenic complement control stain (supplementary table 6). Strikingly all 11 clones that were neither enriched nor depleted in our screens retained an MIC identical to the isogenic complement. From these results we concluded that mutations enriched in our screens could be classified as resistance conferring mutations and mutations that were depleted classified as susceptible conferring. Mutations that were neither enriched nor depleted, we classified as intermediate and concluded that they conferred a susceptible phenotype.

3.7. Preliminary comparison to clinical data

Since our list of genotype to phenotype association is provisional, a fully comprehensive comparison between our catalogue of predicted resistance conferring mutations to association data available for mutations from pyrazinamide resistant clinical isolates is yet to be done. We did however undertake a smaller preliminary comparison to test our provisional prediction of associated phenotypes-to-genotypes for pyrazinamide resistance to that from a single clinical dataset. After extracting data from the clinical dataset we had selected, we compared the 120*pncA* mutations (at the amino acid level) associated with pyrazinamide resistance at a very high confidence with the 411 predicted resistance conferring *pncA* mutations within our provisional list of predictions.

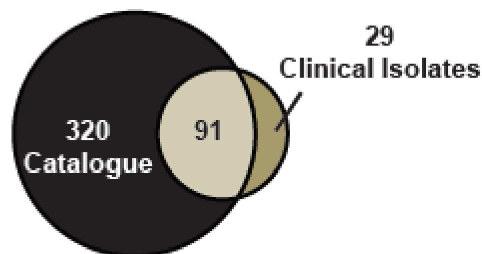

Figure 14: Preliminary comparison of screen data to mutations associated with pyrazinamide resistance from clinical isolates. The 411 predicted resistant conferring *pncA* mutations from our provisional list of screen predictions were compared to a list of 120 *pncA*mutations associated with pyrazinamide resistance from pyrazinamide resistant clinical isolates.

Of these 120 mutations, we found 91 mutations (75.83%) in our provisional catalogue of resistance conferring mutations (Figure 14), while the remaining 29 mutations associated with resistance in the clinical dataset were predicted to be susceptible by our screening methods.On the converse side, 320 predicted resistance conferring mutations from our provisional catalogue where not identified within the pyrazinamide resistant clinical isolates sequenced in the compared dataset.

3.8. Mechanisms of resistance

Of particular interest to us were mutations predicted to confer resistance which are located outside of the regions believed to be essential in the enzymatic activity, leading us to hypothesize two mechanisms for pyrazinamide resistance mediated through *pncA* mutations – abrogation of enzyme function through disruption of catalytic domains, and changes in the conformation/structure of the protein which lead to reduced abundance or affect functionality without being directly involved in catalytic activity. In order to test our hypotheses, we sought to quantify protein abundance and enzyme activity of selected mutants.

Since there is no available anti-PncA antibody, quantification by western blotting would require the time consuming endeavour of immunization and generation of an antibody. As isogenic mutants were already isolated for the validation of our screen and had known phenotypes associated to the mutant genotypes, we decided to develop a mass quantitative spectrometry assay for protein quantification.

To accomplish our aim and confirm our hypothesis, we developed a method for the detection of unlabelled and ^{13}C stable isotope labelled proteins which would allow for quantification of PncA relative to two house-keeping proteins, RpoB and DnaK. First we grew our isogenic complement control in the presence of ^{13}C-labelled glycerol as the sole carbon source to ensure heavy labelling of the entire proteome. Using an *in silico* trypsin digest we selected 3 peptides from PncA and single peptides from RpoB and DnaK. We then determined if we could simultaneously detect both unlabelled as well as ^{13}C-labelled peptides on the Q-Exactive mass spectrometer using our protocol (Figure 15). Preliminary experiments showed that the detection of RpoB was more reproducible than DnaK, so we then continued only with RpoB as the control protein.

Saturated labelling of protein was clearly shown by the absence of unlabelled PncA and RpoB peptides when lysate from our ^{13}C-labelled complement strain was run alone, with ^{13}C-labelled PncA and RpoB peptides clearly detectable. Both unlabelled and ^{13}C-labelled RpoB were detected in equivalent amounts when lysates from either our complement or Δ*pncA* strains were combined with ^{13}C-labelled complement lysate indicating the suitability of RpoB as a house-keeping control peptide for relative

quantification of PncA. As expected unlabelled PncA peptides were detected only in our complement strain and not in Δ*pncA*, confirming no residual PncA after insertional inactivation.

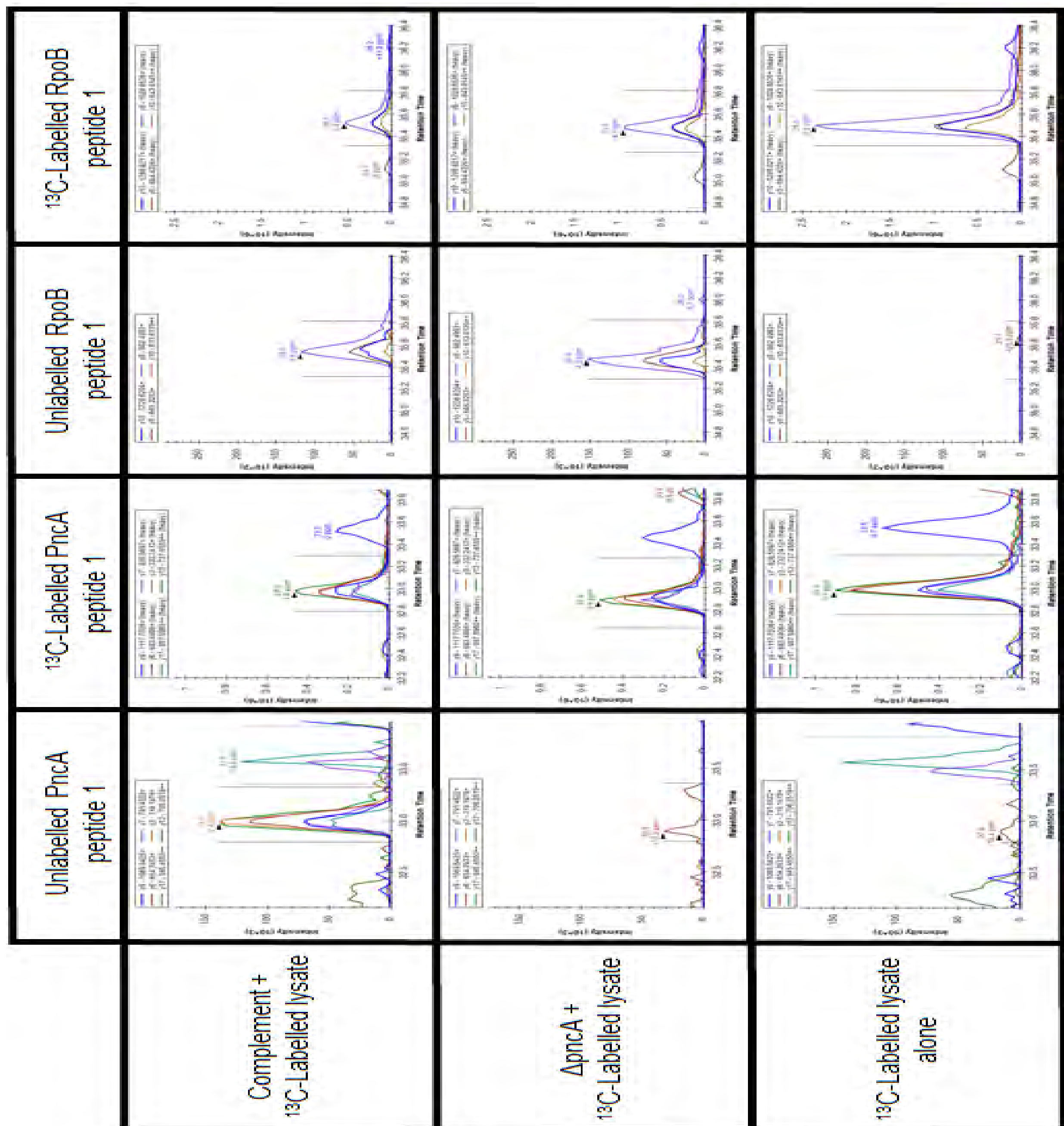

Figure 15: Detection of PncA and RpoB peptides through mass spectrophotometry. Spectral tracesof unlabelled and ^{13}C-labelled PncA and RpoB peptides in Skyline software. X-axis indicates the peptide retention time while Y-axis represents total count. Each coloured line peak represent the total ion count within a retention time window for a particular peptide transition (breakdown fragment) of the parent peptide detected by mass spectrometry. Area under the curve (AUC) for each transition was used for relative quantification of PncA.

Relative PncA abundance for isogenic mutants with either confirmed resistant or susceptible phenotypes was then determined using the method we had developed using the area under the curve (AUC) for each peptide transition for relative quantification of peptides. In brief, unlabelled peptide transitions were normalized to ^{13}C-labelled peptide transitions for each peptide. The mean ratio of AUC for light and heavy labelled peptides for PncA were then normalized to the mean ratio of AUC for RpoB light and heavy labelled peptides. Protein abundance was calculated relative to our isogenic complement.

In strong support of our hypothesis that *pncA* mutations not affecting residues linked to the active site of PncA resulted in reduced abundance of the enzyme, 16 of the 22 pyrazinamide resistant isogenic *pncA* mutants we testedhad significantly lower pyrazinamidase levels (Figure 16) relative to our complement strain. In contrast all but one of our mutants susceptible to pyrazinamide showed PncA levels comparable to our complement control. It is intersting to note that three additional pyrazinamide resistant isogenic mutants (V7L, D8E, and G97D) which showed significantly lower pyrazinamidase levels are in close proximity to proposed catalytic sites suggesting that active site mutations may have a destabilizing effect on the protein structure.

When we looked at the location of the remaining three pyrazinamide resistant isogenic mutants with wild type protein abundance (D8N, C138G, and C138S) on PncA, we found they were all located at the catalytic triad, suggesting they may cause resistance by disrupting the active site rather than disrupting protein stability and abundance. To test this hypothesis we used a modified Wayne's assay for the detection of pyrazinoic acid produced through the activation of pyrazinamide, which is a reliable proxy for enzymatic activity.

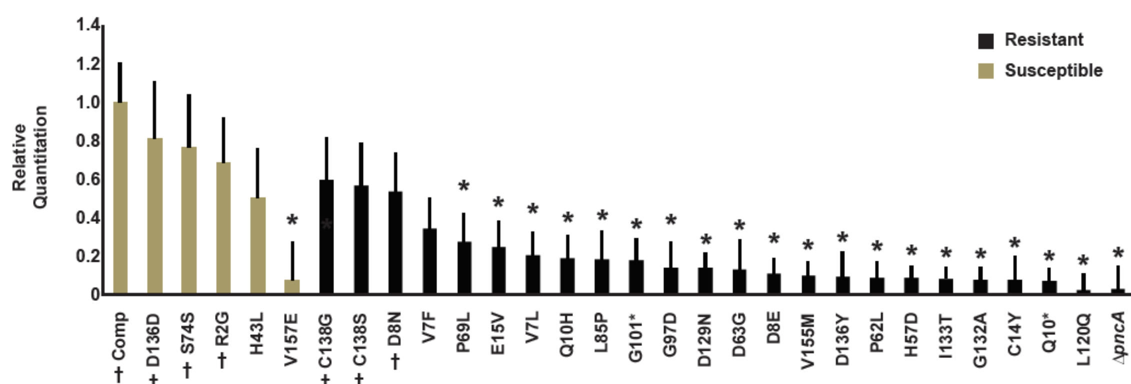

Figure 16: Relative abundance of PncA in clones harbouring mutations in *pncA*.PncA abundance normalized to RpoB and relative to our isogenic complement strain was determined using mass spectrometry in selected isogenic isolates harbouring single mutations in *pncA* and having varying susceptibility phenotypes. Data

represented as mean abundance and standard deviation relative to complement. * P-value ≤ 0.05 (t-test, two-sided). † corresponds to clones tested for enzymatic activity (Figure 17).

We found all three of the phenotypically confirmed pyrazinamide resistant mutants with complement-equivalent PncA levels showed significant reduction in the catalytic conversion of pyrazinamide to pyrazinoic acid (Figure 17) and therefore reduced enzymatic activity. Three pyrazinamide susceptible mutants with complement-equivalent PncA abundance also had complement-equivalent enzyme activity as measure by pyrazinoic acid production.

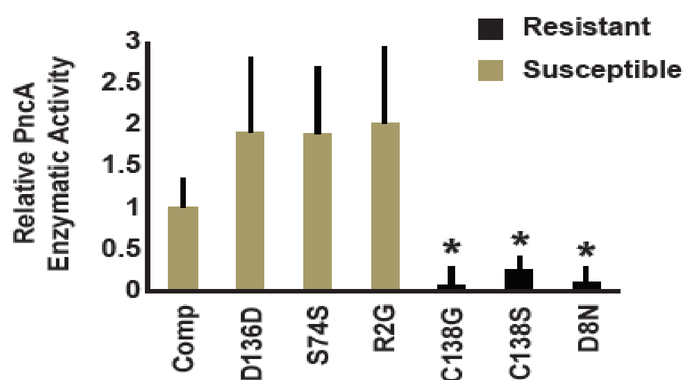

Figure 17: Relative pyrazinamidase (PncA) activity. PncA enzyme activity of selected isogenic mutants was carried out using a modified Wayne's assay. RelativeActivity refers to the amount of POA generated in a 48 hour time-period as measured by the absorbance at 460nm after the addition of ferrous ammonium sulphate in a respective *pncA* mutant clone compared to that of the isogenic complement strain. Individual absorbance values were normalized to absorbance values prior to pyrazinamide treatment and scaled by bulk protein content (described in section 2.14.2.2), and pyrazinamidase enzyme levels determined by mass spectrometry. Relative enzymatic activity is reported as the mean and standard deviation relative to the isogenic complement control. * P-value ≤ 0.05 (t-test, two-sided).

Chapter 4: Discussion

The emergence of resistance to pyrazinamide jeopardizes its use both in current and future treatment regimens for tuberculosis. Given the pitfalls of current phenotypic drug diagnostic testing as well the vital role of pyrazinamide in tuberculosis chemotherapy, the development and introduction of a genotypic assay for detection of pyrazinamide resistance is a high priority for TB control programmes. It has been previously illustrated that mutations in *pncA*, which codes for the activator of pyrazinamide, can occur along the full length of the gene and is the primary mechanism of resistance to pyrazinamide [28,29,207]. The development of a genotypic based assay for detection of pyrazinamide resistance is hindered by the lack of knowledge of the full spectrum of the specific resistance conferring mutations, since mutations occur in *pncA* which do not confer pyrazinamide resistance by standard drug susceptibility methods[34,220].

Current knowledge of the mutations believed to confer resistance have been reliant on identifying pyrazinamide resistant isolates and deducing the specific mutations within *pncA* associated with the resistant phenotype by sequencing [29,32]. The identification of these pyrazinamide resistant isolates is based on conventional drug susceptibility testing and it has been observed that some mutations associate with both pyrazinamide resistant and susceptible phenotypes in clinical isolates [24,32,33]. This observation is probably due to the shortcomings associated with testing methods, and may be complicated by the genetic background of these isolates

Our work details a screen to define the full repertoire of *pncA* mutations that confer resistance to pyrazinamide in an isogenic background which could be utilized as a reference for genotypic based diagnostic tools. Using two independent screening methods, we generated a provisional list of 411 mutations predicted to confer resistance. In addition we have provisionally identified 357 mutations predicted to be susceptible to pyrazinamide with a further 374 mutations being classified as intermediate. It is plausible that these mutations which were neither enriched nor depleted may result in phenotype that sits on a borderline of the critical concentration used for susceptibility testing, however we conclude that they confer a susceptible phenotype. It is worth reiterating that additional data analysis is currently underway which may alter the final predictions of mutations represented in our mutant library.

The use of an isogenic background is major strength in our study in that it allows us to isolate and specifically predict phenotypes of *pncA* mutations, while eliminating the inherent variability in genetic background which may be a confounding factor in clinical isolates. It should be noted that sequencing of *pncA* may not predict all clinical pyrazinamide resistance, as mutations in *rpsA*[168] and *panD*[163,165]

have also been implicated in conferring resistance. However variants in these genes likely account for a minority of pyrazinamide resistant cases since few mutations have been reported clinically [168,220,222]. This is in stark contrast to mutations in *pncA* which are reported to be harboured in between 70 to 97% of pyrazinamide resistant cases [24,29,31-33,191,195-197,200,214-218,220], however these may be underestimates when taking into account the limited reported sequence data and the pitfalls of drug susceptibility testing.

Given the random nature of the error-prone mutagenesis, our library did contain clones with wild type*pncA* and multiple mutations in *pncA*. Thus predictions of the resistance or susceptibility to pyrazinamide of *pncA* mutants was based on statistical analysis of pooled mutation abundance under the different screening conditions, rather than direct testing of individual mutants which would be extremely cumbersome and laborious. As such, there certainly will be false negative and, and to a lesser extent, false positive predictions though we do not have enough data to estimate this at present. Despite this, the high correlation between screen predictions and susceptibility results carried out through conventional drug susceptibility testing of isogenic *pncA* mutants (100% and 92.86% for resistant and susceptible predictions respectively) would suggest that our results are robust and reliable, particularly for resistance predictions.

A striking observation in our data analysis were the differences in resistance conferring mutations identified *in vitro* vs *in vivo*. The majority of mutations found to be resistant *in vitro* were also found to be resistant in our *in vivo* murine treatment model while the converse was not true in that a high proportion of mutations identified as resistant *in vivo* were not selected through *in vitro* screening. These results suggests that selection was stronger *in vivo* that *in vitro*. This was surprising as the duration of exposure to pyrazinamide was similar, but *in vitro*, bacteria would have been in the presence of higher and continuous concentrations of the drug. Our *in vitro* screening methodology is based on an artificial sequential selection in the MGIT system in which bacterial growth is logarithmic and pyrazinamide is believed to have only modest *in vitro* killing efficacy against actively replicating *M. tuberculosis* [21,117]. Although the mechanism of action of pyrazinamide has not been unequivocally defined, *in vivo* selection may reproduce conditions that enhance drug efficacy which would account for greater selection of enriched mutants. The role of the immune system in our *in vivo* screening is unknown but may have also contributed to stronger enrichment of resistance conferring mutant clones.

Our murine treatment model does not perfectly replicate infection in a human model. For our *in vivo* screen, we utilized a high-dose infection in order to adequately represent our mutant library which is dissimilar to human infection.Lesions are markedly different from those that affect drug penetration [286]. In addition, treatment of tuberculosis in humans is based on combinatory therapy [88,89] rather than monotherapy used in our screen. Mutations selected using monotherapy might not affect treatment

outcome in combination therapy. Nevertheless, conclusions derived from pyrazinamide treatment in a murine model may be closer to those observed in the treatment of human tuberculosis compared to *in vitro* models.

We are however confident of the outcome of our screens due to the high degree of selection of stop codons which result in loss of function observed in both screens, the substantial overlap in mutations observed in our independent screening, as well as the results of phenotypic drug susceptibility testing of isogenic mutants. Our findings highlight the inherent limitations of clinical drug susceptibility testing. Understanding the mechanism of action of pyrazinamide *in vivo* would enable the development of a more reliable phenotypic test that more faithfully replicates the critical factors that account for drug efficacy *in vivo.*

The role of an acidic extracellular environment and its impact on the efficacy of pyrazinamide has been greatly debated over the course of many decades. Although it was initially suggested that pyrazinamide required an acid environment to be active anti-tuberculosis agent [65,107,116,119,122,123], recent evidence has begun to uncouple this relationship. Consisting with previous findings [128,139], we observed that acidified media was not a prerequisite for pyrazinamide activity *in vitro*. Although susceptibility to pyrazinamide and growth inhibition were more pronounced when media was acidic, we observed that over-expression of *pncA* negated the requirement for acidity. We found our complement strain in standard culture media was equally susceptible to pyrazinamide as our H37Rv wild type control in acidified media. This overexpression of *pncA* in our complement strain resulted in hyper-susceptibility in acidified media. Taken together the results suggests that the *in vitro* activity of pyrazinamide is limited by drug activation. The hyper-susceptibility phenotype is both a strength and a limitation. While it widens the dynamic range of susceptibility within which we can select mutant, MICs obtained for our isogenic mutants cannot be directly compared to any available data from clinical isolates.

We observed equivalent susceptibility to pyrazinoic acid across our H37Rv, complement and Δ*pncA* strains, with a fall in MIC in acidic media, indicating that the role of acid in the action of pyrazinamide is independent of its activation by pyrazinamidase. It could be that susceptibility to pyrazinamide is not directly dependent on an external acidic environment environment, but rather that an acidic environment may induce a stress response which in turn renders *M. tuberculosis* more susceptible to pyrazinamide. More experiments are needed to understand the optimal conditions for pyrazinamide killing *in vitro* which will help in improving phenotypic drug susceptibility testing.

In our study we were able to show that reduction of pyrazinamidase abundance is a molecular basis for pyrazinamide resistance for many mutations and probably explains the widespread distribution of drug resistance conferring mutations throughout *pncA*. We were also able to show selective disruption of

enzymatic function[212] without significant reductions in protein levels is a mechanism of resistance for substitutions at certain codons. The precise biological mechanisms by which these mutations may cause reduced abundance of the protein is yet to be elucidated, however it remains plausible that these mutations may affect transcriptional or translational efficiency or result in post-translational degradation of the protein due to structural deformities. It has been shown previously that expression of *pncA* can vary in pyrazinamide resistant strains harbouring *pncA* intragenic or promoter mutations [201], however additional experimentation is required to concretely determine at what point in protein metabolism the low-abundance associated substitutions act.We did find a single mutation (V157E) with a discordant proteomic result relative to phenotypic susceptibility. However it was classified as intermediate and it may be an example of a resistance conferring mutation with an MIC close to the breakpoint that is not reliably identified using conventional susceptibility testing.

In keeping with aim of this study, we were successful in generating a comprehensive *pncA* mutant library, as observed by the representation of 89.86% of possible *pncA* intragenic mutations at the amino acid level. Through phenotypic screening, we are provisionally able to predict the susceptibilities of 85.74% of intragenic *pncA* mutations, with 411 mutations predicted to confer pyrazinamide resistance. As our list of resistance conferring mutations generated to date is provisional, we have not yet done a comprehensive formal comparison with clinically selected mutations from previous studies. This type of comparison is problematic because studies from different laboratories have used different phenotypic methods to evaluate susceptibilities and the differing backgrounds of clinical isolates. Nevertheless, a preliminary comparison of the results from a single prospective multicentre study from WHO reference laboratories [32] to our list of enriched mutations, showed a reasonable degree of agreement (75.83%) for mutations associated with pyrazinamide resistance. We anticipate that this congruence will improve with the conclusion of our additional data analysis and a more comprehensive comparative analysis against representative data sets.

Random PCR mutagenesis and phenotypic screening of isogenic mutant libraries represents a powerful tool for defining novel resistance conferring mutations yet to be determined for other anti-tuberculosis drugs. In addition to validating the strengths of PCR mutagenesis and phenotypic screening of isogenic mutant libraries, we have also contributed further knowledge to our understanding of pyrazinamide activity. The serendipitous observation of hyper-susceptibility to pyrazinamide and the abrogation of an extracellular acidic through over-expression of *pncA* suggests that this over-expression could be a useful tool to study the elusive mode of action of pyrazinamide as it would allow for the assessment of mode of action independent of extracellular acidic conditions. We have also optimized a technique that could be used to quantitatively evaluate protein abundance for any protein for which there is no antibody.

In closing, our provisional list of mutations and associated predicted phenotypes represents a step towards a clinically applicable algorithm for interpreting *pncA* sequences. With the conclusion of the additional data analysis of our phenotypic screens we anticipate we will be able to rule in and rule out patients for successful pyrazinamide therapy. We believe our screens were close to saturation as the vast majority of stop codons were enriched but ongoing analysis and comparison with clinical studies should be able to refine our predictions and lead ultimately to a reference list and rapid genotypic based assay for detecting pyrazinamide resistance.

Appendix

M. tuberculosis cultivation media

7H9 Broth (1 Liter)

4.9g Middlebrook 7H9 powder (271310, Difco, BD, USA)

0.2% Glycerol

0.05% Tween 80

Water up to 900 mL

100 mL Middlebrook OADC Enrichment supplement (212240, BBL, BD, USA)

Filter sterilized using Corning 0.22µm filter sterilization units and stored at 2-8°C.

7H10 Agar (500 mL)

9.5g Middlebrook 7H10 powder (262710, Difco, BD, USA)

0.5% Glycerol

Water up to 450 mL

Autoclave sterilized at 121°C for 15 minutes.

Cooled to 55°C in waterbath.

50 mL Middlebrook OADC Enrichment supplement (212240, BBL, BD, USA).

Appropriate antibiotic added if necessary.

Poured and allowed to set at room temperature until solid, then stored at 2-8°C.

7H11 Agar

10.5g Middlebrook 7H10 powder (283810, Difco, BD, USA)

0.5% Glycerol

Water up to 450 mL

Autoclave sterilized at 121°C for 15 minutes.

Cooled to 55°C in waterbath.

50 mL Middlebrook OADC Enrichment supplement (212240, BBL, BD, USA).

Appropriate antibiotic added if necessary.

Poured and allowed to set at room temperature until solid, then stored at 2-8°C.

Modified Hartmans-de Bont (HDB) medium

3.4 μM EDTA

49 μM $MgCl_2•6H_2O$

0.7 μM $CaCl_2•2H_2O$

0.08 μM $NaMoO_4•2H_2O$

0.17 μM $CoCl_2•6H_2O$

0.62 μM $MnCl_2•2H_2O$

0.7 μM $ZnSO_4•7H_2O$

1.8 μM $FeSO_4•7H_2O$, 0.08 μM $CuSO_4•5H_2O$

890 μM K_2HPO_4

708 μM NaH_2PO_4

15 mM $(NH_4)_2SO_4$

27.4 mM glycerol-$^{13}C_3$

0.05% tyloxapol,

E. coli **cultivation media**

LB Broth (500 mL)

10g LB Lennox broth (L3022, Sigma Aldrich, USA)

- 10g/L Tryptone
- 5g/L Yeast Extract
- 5g/L NaCl

500 mL water

Autoclave sterilized at 121°C for 15 minutes.

Allow to cool and store at room temperature.

LB Agar (500 mL)

10g LB Lennox broth (L3022, Sigma Aldrich, USA)

- 10g/L Tryptone
- 5g/L Yeast Extract
- 5g/L NaCl

7.5g Agar (A1296, Sigma Aldrich, USA)

500 mL water

Autoclave sterilized at 121°C for 15 minutes.

Cooled to 55°C in waterbath.

Appropriate antibiotic added if necessary.

Poured and allowed to set at room temperature until solid, then stored at 2-8°C.

S.O.C. Medium

2% tryptone

0.5% yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM glucose.

Supplementary table 1: Strains

Strain	Lab Number	Description	Resistance marker	Source
E. coli DH5α	sAY97	*E. coli* strain used for propagation of plasmids		Rubin Lab
H37Rv	sAY101	ATCC 27294 laboratory reference strain		Our lab
H37RvΔ*pncA*	sAY251	H37Rv with *pncA* insertionally inactivated by a Hygromycin resistance cassette	Hygromycin	[202]
Complement	sAY245	Δ*pncA* containing L5 integrated wild type*pncA*constitutively expressed of the MOP (pAY108)	Hygromycin, Zeocin	Our work
Vector control	sAY257	Δ*pncA* containing L5 integrated vector control (pAY111)	Hygromycin, Zeocin	Our work
*M. tuberculosis**pncA*mutant library	sAY260	Δ*pncA* expressing L5 integrated pooled *pncA* SNP library (pAY230)	Hygromycin, Zeocin	Our work

<u>Supplementary table 2: Plasmids</u>

Plasmid	Description	Resistance marker	Source
pAY1	pJEB402 L5 integrating plasmid containing constitutive mycobacterial optimizted promoter (MOP)	Kanamycin	Rubin Lab
pAY21	pUC57 plasmid containing Emerald fluorescent protein	Ampicillin	Rubin Lab
pAY29	pBlueScriptSK plasmid containing wild type*pncA*	Ampicillin	Rubin Lab
pAY59	L5 integrating Gateway expression vector with Zeocin resistance marker	Zeocin	Rubin Lab
pAY107	ZeoR, L5 integrating vector containing wild type*pncA*	Zeocin	Our work
pAY108	ZeoR, L5 integrating vector with wild type*pncA* constitutively expressed from the MOP	Zeocin	Our work
pAY111	ZeoR, L5 integrating vector with Emerald fluorescent protein	Zeocin	Our work
pAY112	ZeoR, L5 integrating vector with Emerald fluorescent protein constitutively expressed by the MOP	Zeocin	Our work
pAY230	ZeoR, L5 integrating vector with MOP expressed *pncA* mutagenesis library	Zeocin	Our work

Supplementary table3: DNA oligonucleotide primers

Primer ID	**Sequence 5' --> 3'**
ANY_P55	AAATTTACATGTTCTAGAGCTAGGAGCTGCAAACCAACTCGACGCT
ANY_P57	AAATTTATCGATGCGGCCGCATGCGGGCGTTGATCATCGTCGA
ANY_P58	TGGCAGTCGATCGTACGCTAGTT
ANY_P59	GAGCCTATGGAAAAACGCCAGCA
ANY_P147	AAATTTGCGGCCGCATGAGCAAGGGCGAGGAGCT
ANY_P148	GGTGGTACATGTTCTAGACTTGTAGAGCTCGTCCATGCC
ANY_P149	AAATTTATCGATCCCCAGGCTTGACACTTTATG
ANY_P150	GGTGGTGCGGCCGCTCTCCTGCTGGATCCGAATTGTGAGC
ANY_P153	GTGGACGTGCGGCCGCATG
ANY_P154	GGCCTTTTGCTCACATGTTCTAGAGCTA
KM_P35	GGCACACCGGACTATTCCTC
KM_P36	CCCTTGTAGAACACCGCCTC
sigA_F	CCTACGCTACGTGGTGGATT
sigA_R	TGGATTTCCAGCACCTTCTC

Supplementary table 4: Proteomic peptides for mass spectrometry

Peptide Name	Peptide Sequence
PncA-1	AISDYLAEAADYHHVVATK
PncA-2	GAYTGAYSGFEGVDENGTPLLNWLR
PncA-3	GVDEVDVVGIATDHCVR
RpoB	VNPFGFIETPYR
DnaK	LLGSFELTGIPPAPR

Supplementary table 5: Summary of PCR mutagenesis conditions

Library ID	DNA template (ng)	PCR cycles
A	100	20
B	100	25
C	100	30
D	500	20
E	500	25
F	500	30
G	1000	20
H	1000	30
I	25	15
J	50	10
K	50	15
L	75	15
M	100	15
N	150	20
O	150	25

<u>Supplementary Table 6: Drug susceptibility testing results of isogenic *pncA* mutants.</u>

Minimum inhibitory concentration (MIC in µg/mL) were determined using the Bactec MGIT 960 PZA medium. Provisional *in vitro* and *in vivo* screen, as well as combined classifications are presented. E = Enriched, D = Depleted, U = Unselected

Mutation	*in vitro*	*in vivo*	Combined	MIC (µg/mL)
Comp (WT)	NA	NA		1 - 4
Δ*pncA*	NA	NA		>500
S74S	U	U	U	1
D136D	D	D	D	1
V109V	D	D	D	1
S18S	D	D	D	4
A134A	D	D	D	4
G108G	D	U	D	4
V93V	D	D	D	4
I6I	U	U	U	4
V131V	U	U	U	4
D8D	U	U	U	4
H43L	U	D	D	1
G55R	U	U	U	1
S104N	U	U	U	1
C72S	U	D	D	4
V7I	D	U	D	4
D40E	D	U	D	4
V147L	U	D	D	4
E144K	D	U	D	4
V9M	D	U	D	4
V157E	U	U	U	4
F81I	U	U	U	4

Mutation	*in vitro*	*in vivo*	Combined	MIC (µg/mL)
S67W	U	U	U	4
Q122R	U	U	U	4
R2G	U	U	U	4
I133T	D	U	D	20
D63G	E	E	E	20
E15V	E	U	E	20
R2W	E	E	E	20
V155M	U	E	E	20
D129N	U	E	E	20
P62L	U	E	E	20
G97D	E	E	E	100
V7L	E	E	E	100
D8E	E	E	E	500
C14Y	U	E	E	500
L85P	E	E	E	500
D136Y	E	E	E	>500
D8N	E	E	E	>500
V7F	E	E	E	>500
Q10H	E	E	E	>500
G101*	E	E	E	>500
Q10*	E	E	E	>500
C138G	E	E	E	>500
G132A	E	E	E	>500
H57D	E	E	E	>500
C138S	E	E	E	>500
L120Q	U	E	E	>500
P69L	U	E	E	>500

Supplementary Table 7: Provisional catalogue of associated phenotypes to mutations represented in our *M. tuberculosispncA* mutant library

Resulting mutation	*in vitro* prediction	*in vivo* prediction	Catalogue
M1I	Resistant	Resistant	Resistant
M1L	Resistant	Resistant	Resistant
M1V	Neutral	Neutral	Cautiously Susceptible
R2G	Neutral	Neutral	Cautiously Susceptible
R2L	Neutral	Resistant	Resistant
R2Q	Susceptible	Susceptible	Susceptible
R2R	Susceptible	Susceptible	Susceptible
R2W	Resistant	Resistant	Resistant
A3A	Resistant	Resistant	Resistant
A3E	Resistant	Resistant	Resistant
A3G	Resistant	Neutral	Resistant
A3S	Neutral	Neutral	Cautiously Susceptible
A3T	Neutral	Resistant	Resistant
A3V	Susceptible	Susceptible	Susceptible
L4*	Resistant	Resistant	Resistant
L4F	Neutral	Resistant	Resistant
L4L	Neutral	Susceptible	Susceptible
L4M	Susceptible	Neutral	Susceptible
L4S	Neutral	Resistant	Resistant
L4V	Neutral	Neutral	Cautiously Susceptible
L4W	Resistant	Neutral	Resistant
I5F	Resistant	Resistant	Resistant
I5I	Susceptible	Susceptible	Susceptible
I5M	Neutral	Resistant	Resistant
I5N	Resistant	Resistant	Resistant
I5T	Resistant	Resistant	Resistant
I5V	Susceptible	Neutral	Susceptible
I6F	Resistant	Resistant	Resistant
I6I	Neutral	Neutral	Cautiously Susceptible
I6L	Neutral	Neutral	Cautiously Susceptible
I6M	Neutral	Resistant	Resistant
I6N	Neutral	Neutral	Cautiously Susceptible
I6S	Neutral	Neutral	Cautiously Susceptible
I6T	Neutral	Resistant	Resistant

I6V	Neutral	Neutral	Cautiously Susceptible
V7A	Neutral	Resistant	Resistant
V7D	Resistant	Resistant	Resistant
V7F	Resistant	Resistant	Resistant
V7G	Neutral	Neutral	Cautiously Susceptible
V7I	Susceptible	Neutral	Susceptible
V7L	Resistant	Resistant	Resistant
V7V	Neutral	Susceptible	Susceptible
D8A	Neutral	Resistant	Resistant
D8D	Neutral	Neutral	Cautiously Susceptible
D8E	Resistant	Resistant	Resistant
D8G	Resistant	Resistant	Resistant
D8H	Resistant	Resistant	Resistant
D8N	Resistant	Resistant	Resistant
D8V	Resistant	Resistant	Resistant
D8Y	Resistant	Resistant	Resistant
V9A	Neutral	Resistant	Resistant
V9M	Susceptible	Neutral	Susceptible
V9V	Resistant	Resistant	Resistant
Q10*	Resistant	Resistant	Resistant
Q10E	Neutral	Resistant	Resistant
Q10H	Resistant	Resistant	Resistant
Q10K	Neutral	Neutral	Cautiously Susceptible
Q10L	Resistant	Resistant	Resistant
Q10P	Neutral	Neutral	Cautiously Susceptible
Q10R	Resistant	Resistant	Resistant
N11D	Neutral	Neutral	Cautiously Susceptible
N11H	Neutral	Susceptible	Susceptible
N11I	Susceptible	Neutral	Susceptible
N11K	Neutral	Neutral	Cautiously Susceptible
N11N	Susceptible	Neutral	Susceptible
N11S	Susceptible	Neutral	Susceptible
N11T	Neutral	Neutral	Cautiously Susceptible
N11Y	Susceptible	Neutral	Susceptible
D12D	Susceptible	Neutral	Susceptible
D12E	Resistant	Resistant	Resistant
D12G	Neutral	Resistant	Resistant
D12H	Neutral	Neutral	Cautiously Susceptible

D12N	Resistant	Resistant	Resistant
D12V	Neutral	Neutral	Cautiously Susceptible
D12Y	Neutral	Resistant	Resistant
F13C	Neutral	Neutral	Cautiously Susceptible
F13F	Susceptible	Susceptible	Susceptible
F13I	Resistant	Neutral	Resistant
F13L	Neutral	Neutral	Cautiously Susceptible
F13S	Neutral	Neutral	Cautiously Susceptible
F13V	Resistant	Resistant	Resistant
F13Y	Neutral	Resistant	Resistant
C14*	Resistant	Resistant	Resistant
C14C	Neutral	Neutral	Cautiously Susceptible
C14F	Neutral	Susceptible	Susceptible
C14R	Resistant	Resistant	Resistant
C14S	Resistant	Neutral	Resistant
C14W	Neutral	Neutral	Cautiously Susceptible
C14Y	Neutral	Resistant	Resistant
E15*	Resistant	Resistant	Resistant
E15D	Susceptible	Neutral	Susceptible
E15E	Neutral	Neutral	Cautiously Susceptible
E15G	Neutral	Resistant	Resistant
E15K	Susceptible	Susceptible	Susceptible
E15Q	Neutral	Susceptible	Susceptible
E15V	Resistant	Neutral	Resistant
G16C	Neutral	Resistant	Resistant
G16D	Neutral	Neutral	Cautiously Susceptible
G16G	Neutral	Resistant	Resistant
G16R	Resistant	Resistant	Resistant
G16S	Neutral	Neutral	Cautiously Susceptible
G16V	Neutral	Neutral	Cautiously Susceptible
G17A	Neutral	Resistant	Resistant
G17C	Neutral	Resistant	Resistant
G17D	Neutral	Resistant	Resistant
G17G	Susceptible	Neutral	Susceptible
G17S	Neutral	Neutral	Cautiously Susceptible
S18*	Resistant	Resistant	Resistant
S18A	Neutral	Neutral	Cautiously Susceptible
S18L	Neutral	Resistant	Resistant

S18P	Neutral	Neutral	Cautiously Susceptible
S18S	Susceptible	Susceptible	Susceptible
S18T	Neutral	Susceptible	Susceptible
S18W	Neutral	Resistant	Resistant
L19L	Neutral	Neutral	Cautiously Susceptible
L19M	Neutral	Resistant	Resistant
L19P	Resistant	Resistant	Resistant
L19Q	Resistant	Neutral	Resistant
L19R	Neutral	Neutral	Cautiously Susceptible
L19V	Neutral	Susceptible	Susceptible
A20A	Susceptible	Susceptible	Susceptible
A20E	Neutral	Susceptible	Susceptible
A20G	Neutral	Neutral	Cautiously Susceptible
A20S	Susceptible	Susceptible	Susceptible
A20T	Susceptible	Neutral	Susceptible
A20V	Susceptible	Neutral	Susceptible
V21A	Neutral	Resistant	Resistant
V21E	Resistant	Resistant	Resistant
V21I	Neutral	Neutral	Cautiously Susceptible
V21L	Neutral	Susceptible	Susceptible
V21V	Resistant	Neutral	Resistant
T22A	Neutral	Susceptible	Susceptible
T22N	Neutral	Neutral	Cautiously Susceptible
T22S	Neutral	Neutral	Cautiously Susceptible
T22T	Neutral	Neutral	Cautiously Susceptible
G23A	Neutral	Susceptible	Susceptible
G23D	Neutral	Neutral	Cautiously Susceptible
G23G	Neutral	Susceptible	Susceptible
G23R	Neutral	Susceptible	Susceptible
G23S	Neutral	Neutral	Cautiously Susceptible
G23V	Neutral	Neutral	Cautiously Susceptible
G24C	Resistant	Neutral	Resistant
G24D	Resistant	Resistant	Resistant
G24G	Susceptible	Neutral	Susceptible
G24R	Neutral	Neutral	Cautiously Susceptible
G24S	Neutral	Neutral	Cautiously Susceptible
G24V	Neutral	Resistant	Resistant
A25A	Neutral	Resistant	Resistant

A25D	Neutral	Neutral	Cautiously Susceptible
A25P	Neutral	Neutral	Cautiously Susceptible
A25S	Neutral	Neutral	Cautiously Susceptible
A25T	Susceptible	Susceptible	Susceptible
A25V	Susceptible	Susceptible	Susceptible
A26A	Susceptible	Susceptible	Susceptible
A26G	Neutral	Neutral	Cautiously Susceptible
A26P	Susceptible	Neutral	Susceptible
A26S	Neutral	Susceptible	Susceptible
A26T	Susceptible	Susceptible	Susceptible
A26V	Resistant	Neutral	Resistant
L27L	Susceptible	Susceptible	Susceptible
L27M	Neutral	Resistant	Resistant
L27P	Resistant	Resistant	Resistant
L27Q	Neutral	Resistant	Resistant
L27R	Susceptible	Neutral	Susceptible
L27V	Neutral	Susceptible	Susceptible
A28A	Susceptible	Susceptible	Susceptible
A28D	Neutral	Neutral	Cautiously Susceptible
A28G	Neutral	Susceptible	Susceptible
A28P	Neutral	Resistant	Resistant
A28S	Neutral	Resistant	Resistant
A28T	Neutral	Neutral	Cautiously Susceptible
A28V	Neutral	Neutral	Cautiously Susceptible
R29C	Resistant	Neutral	Resistant
R29G	Neutral	Susceptible	Susceptible
R29H	Neutral	Neutral	Cautiously Susceptible
R29L	Neutral	Neutral	Cautiously Susceptible
R29P	Neutral	Resistant	Resistant
R29R	Susceptible	Susceptible	Susceptible
R29S	Neutral	Susceptible	Susceptible
A30A	Neutral	Resistant	Resistant
A30D	Susceptible	Susceptible	Susceptible
A30P	Resistant	Resistant	Resistant
A30T	Susceptible	Susceptible	Susceptible
A30V	Resistant	Resistant	Resistant
I31F	Resistant	Resistant	Resistant
I31I	Susceptible	Neutral	Susceptible

I31M	Neutral	Neutral	Cautiously Susceptible
I31N	Resistant	Resistant	Resistant
I31T	Neutral	Neutral	Cautiously Susceptible
I31V	Neutral	Resistant	Resistant
S32C	Neutral	Neutral	Cautiously Susceptible
S32G	Susceptible	Neutral	Susceptible
S32I	Neutral	Resistant	Resistant
S32N	Resistant	Neutral	Resistant
S32S	Neutral	Neutral	Cautiously Susceptible
S32T	Susceptible	Susceptible	Susceptible
D33A	Susceptible	Susceptible	Susceptible
D33D	Susceptible	Susceptible	Susceptible
D33E	Neutral	Neutral	Cautiously Susceptible
D33G	Neutral	Neutral	Cautiously Susceptible
D33H	Neutral	Neutral	Cautiously Susceptible
D33N	Susceptible	Neutral	Susceptible
D33V	Neutral	Neutral	Cautiously Susceptible
D33Y	Neutral	Neutral	Cautiously Susceptible
Y34*	Neutral	Neutral	Cautiously Susceptible
Y34C	Neutral	Resistant	Resistant
Y34F	Neutral	Susceptible	Susceptible
Y34H	Neutral	Susceptible	Susceptible
Y34N	Neutral	Neutral	Cautiously Susceptible
Y34S	Neutral	Neutral	Cautiously Susceptible
Y34Y	Resistant	Resistant	Resistant
L35L	Susceptible	Neutral	Susceptible
L35M	Neutral	Neutral	Cautiously Susceptible
L35P	Neutral	Resistant	Resistant
L35Q	Neutral	Resistant	Resistant
L35R	Neutral	Resistant	Resistant
L35V	Neutral	Susceptible	Susceptible
A36A	Susceptible	Susceptible	Susceptible
A36G	Neutral	Neutral	Cautiously Susceptible
A36P	Susceptible	Neutral	Susceptible
A36S	Resistant	Resistant	Resistant
A36T	Resistant	Neutral	Resistant
A36V	Susceptible	Susceptible	Susceptible
E37*	Resistant	Resistant	Resistant

E37A	Neutral	Neutral	Cautiously Susceptible
E37D	Susceptible	Neutral	Susceptible
E37E	Neutral	Neutral	Cautiously Susceptible
E37G	Neutral	Neutral	Cautiously Susceptible
E37K	Neutral	Resistant	Resistant
E37Q	Neutral	Neutral	Cautiously Susceptible
E37V	Resistant	Neutral	Resistant
A38A	Neutral	Neutral	Cautiously Susceptible
A38E	Neutral	Neutral	Cautiously Susceptible
A38G	Resistant	Neutral	Resistant
A38S	Resistant	Neutral	Resistant
A38T	Susceptible	Neutral	Susceptible
A38V	Susceptible	Susceptible	Susceptible
A39A	Neutral	Susceptible	Susceptible
A39E	Neutral	Neutral	Cautiously Susceptible
A39G	Susceptible	Susceptible	Susceptible
A39P	Neutral	Neutral	Cautiously Susceptible
A39S	Susceptible	Neutral	Susceptible
A39T	Neutral	Neutral	Cautiously Susceptible
A39V	Susceptible	Neutral	Susceptible
D40D	Susceptible	Neutral	Susceptible
D40E	Susceptible	Neutral	Susceptible
D40G	Neutral	Resistant	Resistant
D40H	Resistant	Resistant	Resistant
D40N	Neutral	Neutral	Cautiously Susceptible
D40V	Neutral	Neutral	Cautiously Susceptible
D40Y	Susceptible	Neutral	Susceptible
Y41*	Resistant	Resistant	Resistant
Y41F	Neutral	Neutral	Cautiously Susceptible
Y41H	Resistant	Neutral	Resistant
Y41N	Neutral	Neutral	Cautiously Susceptible
Y41S	Neutral	Neutral	Cautiously Susceptible
Y41Y	Neutral	Resistant	Resistant
H42D	Neutral	Resistant	Resistant
H42H	Neutral	Neutral	Cautiously Susceptible
H42L	Susceptible	Susceptible	Susceptible
H42N	Resistant	Resistant	Resistant
H42Q	Resistant	Resistant	Resistant

H42R	Neutral	Susceptible	Susceptible
H42Y	Neutral	Neutral	Cautiously Susceptible
H43H	Neutral	Neutral	Cautiously Susceptible
H43L	Neutral	Susceptible	Susceptible
H43N	Neutral	Neutral	Cautiously Susceptible
H43P	Neutral	Resistant	Resistant
H43R	Susceptible	Neutral	Susceptible
H43Y	Susceptible	Neutral	Susceptible
V44A	Neutral	Resistant	Resistant
V44D	Resistant	Resistant	Resistant
V44F	Neutral	Neutral	Cautiously Susceptible
V44I	Susceptible	Susceptible	Susceptible
V44L	Neutral	Susceptible	Susceptible
V45A	Neutral	Susceptible	Susceptible
V45E	Resistant	Resistant	Resistant
V45M	Neutral	Resistant	Resistant
V45V	Neutral	Neutral	Cautiously Susceptible
A46A	Resistant	Neutral	Resistant
A46E	Neutral	Resistant	Resistant
A46P	Resistant	Resistant	Resistant
A46S	Susceptible	Neutral	Susceptible
A46T	Neutral	Resistant	Resistant
A46V	Resistant	Resistant	Resistant
T47A	Neutral	Neutral	Cautiously Susceptible
T47I	Resistant	Resistant	Resistant
T47P	Neutral	Neutral	Cautiously Susceptible
T47T	Susceptible	Neutral	Susceptible
K48*	Resistant	Resistant	Resistant
K48E	Resistant	Resistant	Resistant
K48K	Neutral	Neutral	Cautiously Susceptible
K48N	Resistant	Resistant	Resistant
K48Q	Neutral	Susceptible	Susceptible
K48R	Neutral	Neutral	Cautiously Susceptible
K48T	Neutral	Resistant	Resistant
D49A	Neutral	Neutral	Cautiously Susceptible
D49D	Neutral	Susceptible	Susceptible
D49E	Neutral	Resistant	Resistant
D49G	Resistant	Resistant	Resistant

D49H	Resistant	Neutral	Resistant
D49N	Resistant	Resistant	Resistant
D49V	Resistant	Resistant	Resistant
D49Y	Neutral	Resistant	Resistant
F50F	Resistant	Resistant	Resistant
F50I	Neutral	Neutral	Cautiously Susceptible
F50L	Neutral	Resistant	Resistant
F50S	Resistant	Resistant	Resistant
F50V	Neutral	Neutral	Cautiously Susceptible
F50Y	Susceptible	Susceptible	Cautiously Susceptible
H51D	Neutral	Resistant	Resistant
H51H	Susceptible	Neutral	Susceptible
H51L	Neutral	Resistant	Resistant
H51N	Neutral	Resistant	Resistant
H51Q	Neutral	Neutral	Cautiously Susceptible
H51R	Neutral	Resistant	Resistant
H51Y	Resistant	Resistant	Resistant
I52F	Neutral	Neutral	Cautiously Susceptible
I52I	Neutral	Neutral	Cautiously Susceptible
I52L	Neutral	Neutral	Cautiously Susceptible
I52M	Neutral	Susceptible	Susceptible
I52N	Neutral	Susceptible	Susceptible
I52S	Neutral	Neutral	Cautiously Susceptible
I52T	Resistant	Neutral	Resistant
I52V	Susceptible	Neutral	Susceptible
D53D	Neutral	Susceptible	Susceptible
D53E	Neutral	Neutral	Cautiously Susceptible
D53N	Susceptible	Susceptible	Susceptible
D53V	Neutral	Neutral	Cautiously Susceptible
D53Y	Neutral	Neutral	Cautiously Susceptible
P54L	Resistant	Resistant	Resistant
P54P	Susceptible	Neutral	Susceptible
P54Q	Neutral	Resistant	Resistant
P54R	Neutral	Neutral	Cautiously Susceptible
P54S	Resistant	Resistant	Resistant
P54T	Resistant	Resistant	Resistant
G55C	Neutral	Neutral	Cautiously Susceptible
G55D	Neutral	Neutral	Cautiously Susceptible

G55G	Neutral	Susceptible	Susceptible
G55R	Neutral	Neutral	Cautiously Susceptible
G55S	Susceptible	Neutral	Susceptible
G55V	Neutral	Susceptible	Susceptible
D56A	Neutral	Susceptible	Susceptible
D56E	Neutral	Susceptible	Susceptible
D56G	Neutral	Neutral	Cautiously Susceptible
D56H	Neutral	Neutral	Cautiously Susceptible
D56N	Neutral	Susceptible	Susceptible
D56V	Neutral	Neutral	Cautiously Susceptible
D56Y	Neutral	Neutral	Cautiously Susceptible
H57D	Resistant	Resistant	Resistant
H57H	Susceptible	Susceptible	Susceptible
H57L	Neutral	Resistant	Resistant
H57N	Resistant	Resistant	Resistant
H57P	Neutral	Neutral	Cautiously Susceptible
H57Q	Resistant	Resistant	Resistant
H57R	Resistant	Resistant	Resistant
H57Y	Neutral	Resistant	Resistant
F58C	Neutral	Neutral	Cautiously Susceptible
F58F	Neutral	Resistant	Resistant
F58I	Neutral	Neutral	Cautiously Susceptible
F58L	Resistant	Resistant	Resistant
F58S	Resistant	Resistant	Resistant
F58Y	Neutral	Neutral	Cautiously Susceptible
S59C	Neutral	Neutral	Cautiously Susceptible
S59F	Neutral	Resistant	Resistant
S59P	Neutral	Resistant	Resistant
S59S	Susceptible	Neutral	Susceptible
S59T	Neutral	Neutral	Cautiously Susceptible
S59Y	Neutral	Neutral	Cautiously Susceptible
G60A	Neutral	Neutral	Cautiously Susceptible
G60C	Neutral	Susceptible	Susceptible
G60D	Susceptible	Susceptible	Susceptible
G60G	Susceptible	Neutral	Susceptible
G60R	Neutral	Neutral	Cautiously Susceptible
G60S	Susceptible	Neutral	Susceptible
G60V	Susceptible	Susceptible	Susceptible

T61A	Neutral	Susceptible	Susceptible
T61I	Susceptible	Neutral	Susceptible
T61K	Neutral	Susceptible	Susceptible
T61S	Neutral	Neutral	Cautiously Susceptible
T61T	Neutral	Resistant	Resistant
P62A	Neutral	Neutral	Cautiously Susceptible
P62L	Neutral	Resistant	Resistant
P62Q	Neutral	Resistant	Resistant
P62R	Neutral	Resistant	Resistant
P62S	Neutral	Resistant	Resistant
P62T	Neutral	Neutral	Cautiously Susceptible
D63D	Susceptible	Neutral	Susceptible
D63E	Neutral	Resistant	Resistant
D63G	Resistant	Resistant	Resistant
D63H	Neutral	Resistant	Resistant
D63N	Susceptible	Neutral	Susceptible
D63V	Neutral	Resistant	Resistant
D63Y	Neutral	Resistant	Resistant
Y64*	Resistant	Resistant	Resistant
Y64C	Neutral	Neutral	Cautiously Susceptible
Y64D	Neutral	Resistant	Resistant
Y64F	Neutral	Resistant	Resistant
Y64H	Resistant	Neutral	Resistant
Y64N	Neutral	Susceptible	Susceptible
Y64Y	Neutral	Neutral	Cautiously Susceptible
S65C	Susceptible	Susceptible	Susceptible
S65F	Resistant	Neutral	Resistant
S65P	Resistant	Resistant	Resistant
S65S	Neutral	Resistant	Resistant
S65T	Susceptible	Neutral	Susceptible
S65Y	Neutral	Neutral	Cautiously Susceptible
S66*	Resistant	Resistant	Resistant
S66L	Susceptible	Neutral	Susceptible
S66S	Susceptible	Susceptible	Susceptible
S66T	Neutral	Susceptible	Susceptible
S66W	Neutral	Neutral	Cautiously Susceptible
S67*	Neutral	Resistant	Resistant
S67A	Neutral	Neutral	Cautiously Susceptible

S67L	Neutral	Neutral	Cautiously Susceptible
S67P	Neutral	Resistant	Resistant
S67S	Neutral	Neutral	Cautiously Susceptible
S67T	Neutral	Susceptible	Susceptible
S67W	Neutral	Neutral	Cautiously Susceptible
W68*	Resistant	Resistant	Resistant
W68C	Resistant	Resistant	Resistant
W68G	Resistant	Neutral	Resistant
W68L	Resistant	Resistant	Resistant
W68R	Resistant	Resistant	Resistant
P69A	Neutral	Neutral	Cautiously Susceptible
P69L	Neutral	Resistant	Resistant
P69P	Susceptible	Susceptible	Susceptible
P69Q	Neutral	Neutral	Cautiously Susceptible
P69S	Resistant	Resistant	Resistant
P69T	Neutral	Neutral	Cautiously Susceptible
P70A	Neutral	Neutral	Cautiously Susceptible
P70L	Neutral	Neutral	Cautiously Susceptible
P70P	Neutral	Resistant	Resistant
P70Q	Neutral	Susceptible	Susceptible
P70R	Susceptible	Neutral	Susceptible
P70S	Resistant	Resistant	Resistant
P70T	Neutral	Neutral	Cautiously Susceptible
H71D	Neutral	Resistant	Resistant
H71H	Resistant	Neutral	Resistant
H71L	Resistant	Resistant	Resistant
H71N	Neutral	Neutral	Cautiously Susceptible
H71P	Resistant	Neutral	Resistant
H71Q	Resistant	Resistant	Resistant
H71R	Resistant	Resistant	Resistant
H71Y	Resistant	Resistant	Resistant
C72*	Resistant	Resistant	Resistant
C72C	Neutral	Susceptible	Susceptible
C72F	Resistant	Resistant	Resistant
C72R	Resistant	Resistant	Resistant
C72S	Neutral	Susceptible	Susceptible
C72W	Resistant	Resistant	Resistant
C72Y	Resistant	Resistant	Resistant

V73A	Neutral	Neutral	Cautiously Susceptible
V73D	Neutral	Resistant	Resistant
V73F	Neutral	Resistant	Resistant
V73I	Susceptible	Susceptible	Susceptible
V73L	Susceptible	Neutral	Susceptible
V73V	Susceptible	Neutral	Susceptible
S74C	Resistant	Neutral	Resistant
S74G	Susceptible	Susceptible	Susceptible
S74I	Neutral	Susceptible	Susceptible
S74N	Neutral	Neutral	Cautiously Susceptible
S74R	Susceptible	Neutral	Susceptible
S74S	Neutral	Neutral	Cautiously Susceptible
G75A	Neutral	Neutral	Cautiously Susceptible
G75C	Susceptible	Neutral	Susceptible
G75D	Neutral	Susceptible	Susceptible
G75G	Neutral	Susceptible	Susceptible
G75R	Neutral	Neutral	Cautiously Susceptible
G75V	Resistant	Resistant	Resistant
T76A	Neutral	Neutral	Cautiously Susceptible
T76I	Neutral	Resistant	Resistant
T76N	Susceptible	Neutral	Susceptible
T76S	Neutral	Neutral	Cautiously Susceptible
T76T	Neutral	Susceptible	Susceptible
P77H	Neutral	Neutral	Cautiously Susceptible
P77L	Neutral	Susceptible	Susceptible
P77P	Susceptible	Susceptible	Susceptible
P77S	Neutral	Neutral	Cautiously Susceptible
P77T	Neutral	Neutral	Cautiously Susceptible
G78C	Resistant	Resistant	Resistant
G78D	Resistant	Resistant	Resistant
G78G	Susceptible	Susceptible	Susceptible
G78R	Neutral	Resistant	Resistant
G78S	Neutral	Neutral	Cautiously Susceptible
G78V	Resistant	Resistant	Resistant
A79A	Susceptible	Neutral	Susceptible
A79E	Neutral	Neutral	Cautiously Susceptible
A79P	Resistant	Resistant	Resistant
A79S	Susceptible	Neutral	Susceptible

A79T	Neutral	Susceptible	Susceptible
A79V	Susceptible	Neutral	Susceptible
D80D	Susceptible	Susceptible	Susceptible
D80G	Neutral	Neutral	Cautiously Susceptible
D80H	Resistant	Resistant	Resistant
D80V	Susceptible	Neutral	Susceptible
D80Y	Neutral	Neutral	Cautiously Susceptible
F81F	Neutral	Neutral	Cautiously Susceptible
F81I	Neutral	Neutral	Cautiously Susceptible
F81L	Neutral	Neutral	Cautiously Susceptible
F81S	Resistant	Resistant	Resistant
F81V	Neutral	Neutral	Cautiously Susceptible
H82D	Neutral	Neutral	Cautiously Susceptible
H82H	Neutral	Neutral	Cautiously Susceptible
H82L	Neutral	Resistant	Resistant
H82N	Neutral	Neutral	Cautiously Susceptible
H82Q	Susceptible	Neutral	Susceptible
H82Y	Neutral	Neutral	Cautiously Susceptible
P83A	Neutral	Neutral	Cautiously Susceptible
P83H	Neutral	Neutral	Cautiously Susceptible
P83L	Neutral	Resistant	Resistant
P83P	Susceptible	Susceptible	Susceptible
P83R	Neutral	Neutral	Cautiously Susceptible
P83S	Neutral	Susceptible	Susceptible
P83T	Neutral	Neutral	Cautiously Susceptible
S84C	Susceptible	Neutral	Susceptible
S84G	Neutral	Susceptible	Susceptible
S84I	Susceptible	Susceptible	Susceptible
S84N	Neutral	Susceptible	Susceptible
S84R	Neutral	Susceptible	Susceptible
S84S	Susceptible	Susceptible	Susceptible
S84T	Neutral	Neutral	Cautiously Susceptible
L85L	Neutral	Susceptible	Susceptible
L85M	Neutral	Resistant	Resistant
L85P	Resistant	Resistant	Resistant
L85Q	Resistant	Resistant	Resistant
L85R	Resistant	Resistant	Resistant
L85V	Neutral	Susceptible	Susceptible

D86A	Susceptible	Neutral	Susceptible
D86D	Neutral	Neutral	Cautiously Susceptible
D86E	Susceptible	Susceptible	Susceptible
D86G	Susceptible	Susceptible	Susceptible
D86N	Neutral	Susceptible	Susceptible
D86V	Resistant	Resistant	Resistant
D86Y	Neutral	Resistant	Resistant
T87A	Susceptible	Neutral	Susceptible
T87K	Neutral	Neutral	Cautiously Susceptible
T87M	Neutral	Neutral	Cautiously Susceptible
T87R	Neutral	Susceptible	Susceptible
T87S	Neutral	Neutral	Cautiously Susceptible
S88*	Resistant	Resistant	Resistant
S88A	Susceptible	Neutral	Susceptible
S88L	Neutral	Neutral	Cautiously Susceptible
S88P	Neutral	Neutral	Cautiously Susceptible
S88S	Susceptible	Neutral	Susceptible
S88T	Neutral	Neutral	Cautiously Susceptible
A89A	Susceptible	Susceptible	Susceptible
A89E	Neutral	Neutral	Cautiously Susceptible
A89G	Neutral	Neutral	Cautiously Susceptible
A89P	Susceptible	Neutral	Susceptible
A89S	Neutral	Susceptible	Susceptible
A89T	Susceptible	Neutral	Susceptible
A89V	Resistant	Resistant	Resistant
I90I	Susceptible	Neutral	Susceptible
I90M	Neutral	Resistant	Resistant
I90N	Neutral	Neutral	Cautiously Susceptible
I90T	Neutral	Neutral	Cautiously Susceptible
I90V	Neutral	Neutral	Cautiously Susceptible
E91*	Resistant	Neutral	Resistant
E91D	Susceptible	Neutral	Susceptible
E91E	Susceptible	Neutral	Susceptible
E91G	Neutral	Neutral	Cautiously Susceptible
E91K	Neutral	Neutral	Cautiously Susceptible
E91V	Resistant	Resistant	Resistant
A92A	Resistant	Neutral	Resistant
A92E	Neutral	Resistant	Resistant

A92P	Neutral	Neutral	Cautiously Susceptible
A92S	Neutral	Susceptible	Susceptible
A92T	Neutral	Resistant	Resistant
A92V	Susceptible	Neutral	Susceptible
V93A	Neutral	Neutral	Cautiously Susceptible
V93E	Neutral	Resistant	Resistant
V93L	Neutral	Neutral	Cautiously Susceptible
V93M	Resistant	Neutral	Resistant
V93V	Susceptible	Susceptible	Susceptible
F94C	Resistant	Neutral	Resistant
F94F	Resistant	Resistant	Resistant
F94I	Neutral	Neutral	Cautiously Susceptible
F94L	Resistant	Resistant	Resistant
F94S	Resistant	Resistant	Resistant
F94Y	Neutral	Neutral	Cautiously Susceptible
Y95*	Resistant	Resistant	Resistant
Y95C	Neutral	Neutral	Cautiously Susceptible
Y95F	Neutral	Resistant	Resistant
Y95H	Susceptible	Neutral	Susceptible
Y95N	Neutral	Neutral	Cautiously Susceptible
Y95Y	Neutral	Neutral	Cautiously Susceptible
K96*	Resistant	Resistant	Resistant
K96E	Resistant	Resistant	Resistant
K96K	Susceptible	Susceptible	Susceptible
K96M	Resistant	Resistant	Resistant
K96N	Resistant	Resistant	Resistant
K96Q	Resistant	Resistant	Resistant
K96R	Resistant	Resistant	Resistant
K96T	Neutral	Resistant	Resistant
G97A	Neutral	Neutral	Cautiously Susceptible
G97C	Resistant	Resistant	Resistant
G97D	Resistant	Resistant	Resistant
G97G	Neutral	Neutral	Cautiously Susceptible
G97R	Neutral	Neutral	Cautiously Susceptible
G97S	Resistant	Resistant	Resistant
G97V	Neutral	Neutral	Cautiously Susceptible
A98A	Susceptible	Susceptible	Susceptible
A98D	Neutral	Neutral	Cautiously Susceptible

A98P	Neutral	Neutral	Cautiously Susceptible
A98S	Neutral	Neutral	Cautiously Susceptible
A98T	Neutral	Neutral	Cautiously Susceptible
A98V	Neutral	Neutral	Cautiously Susceptible
Y99*	Resistant	Resistant	Resistant
Y99C	Neutral	Neutral	Cautiously Susceptible
Y99D	Neutral	Susceptible	Susceptible
Y99F	Susceptible	Susceptible	Susceptible
Y99H	Neutral	Neutral	Cautiously Susceptible
Y99N	Neutral	Susceptible	Susceptible
Y99Y	Neutral	Neutral	Cautiously Susceptible
T100A	Neutral	Susceptible	Susceptible
T100I	Susceptible	Neutral	Susceptible
T100N	Neutral	Neutral	Cautiously Susceptible
T100P	Neutral	Neutral	Cautiously Susceptible
T100S	Neutral	Neutral	Cautiously Susceptible
T100T	Susceptible	Susceptible	Susceptible
G101*	Resistant	Resistant	Resistant
G101E	Resistant	Resistant	Resistant
G101G	Neutral	Susceptible	Susceptible
G101R	Susceptible	Neutral	Susceptible
G101V	Neutral	Neutral	Cautiously Susceptible
A102E	Susceptible	Neutral	Susceptible
A102G	Neutral	Neutral	Cautiously Susceptible
A102P	Neutral	Resistant	Resistant
A102S	Susceptible	Neutral	Susceptible
A102T	Resistant	Resistant	Resistant
Y103*	Neutral	Resistant	Resistant
Y103C	Susceptible	Neutral	Susceptible
Y103F	Neutral	Neutral	Cautiously Susceptible
Y103H	Neutral	Neutral	Cautiously Susceptible
Y103N	Neutral	Neutral	Cautiously Susceptible
Y103S	Neutral	Neutral	Cautiously Susceptible
Y103Y	Resistant	Neutral	Resistant
S104C	Resistant	Resistant	Resistant
S104G	Neutral	Resistant	Resistant
S104I	Neutral	Resistant	Resistant
S104N	Neutral	Neutral	Cautiously Susceptible

S104R	Resistant	Resistant	Resistant
S104S	Susceptible	Susceptible	Susceptible
G105A	Susceptible	Neutral	Susceptible
G105C	Susceptible	Neutral	Susceptible
G105D	Resistant	Resistant	Resistant
G105G	Susceptible	Susceptible	Susceptible
G105R	Neutral	Resistant	Resistant
G105S	Susceptible	Neutral	Susceptible
G105V	Neutral	Resistant	Resistant
F106F	Susceptible	Neutral	Susceptible
F106I	Resistant	Resistant	Resistant
F106S	Neutral	Resistant	Resistant
F106V	Neutral	Neutral	Cautiously Susceptible
F106Y	Resistant	Resistant	Resistant
E107*	Neutral	Resistant	Resistant
E107D	Susceptible	Susceptible	Susceptible
E107E	Neutral	Neutral	Cautiously Susceptible
E107G	Neutral	Susceptible	Susceptible
E107K	Susceptible	Neutral	Susceptible
E107Q	Susceptible	Neutral	Susceptible
E107V	Susceptible	Neutral	Susceptible
G108*	Resistant	Resistant	Resistant
G108E	Resistant	Resistant	Resistant
G108G	Susceptible	Neutral	Susceptible
G108R	Resistant	Resistant	Resistant
G108V	Neutral	Neutral	Cautiously Susceptible
V109A	Neutral	Neutral	Cautiously Susceptible
V109D	Neutral	Neutral	Cautiously Susceptible
V109F	Susceptible	Neutral	Susceptible
V109G	Neutral	Susceptible	Susceptible
V109L	Resistant	Neutral	Resistant
V109V	Susceptible	Susceptible	Susceptible
D110D	Neutral	Neutral	Cautiously Susceptible
D110G	Neutral	Susceptible	Susceptible
D110H	Neutral	Resistant	Resistant
D110N	Neutral	Resistant	Resistant
E111*	Resistant	Resistant	Resistant
E111A	Neutral	Susceptible	Susceptible

E111D	Susceptible	Neutral	Susceptible
E111E	Neutral	Neutral	Cautiously Susceptible
E111G	Neutral	Neutral	Cautiously Susceptible
E111K	Susceptible	Susceptible	Susceptible
E111Q	Neutral	Neutral	Cautiously Susceptible
N112D	Neutral	Susceptible	Susceptible
N112H	Neutral	Susceptible	Susceptible
N112I	Neutral	Resistant	Resistant
N112K	Neutral	Susceptible	Susceptible
N112N	Susceptible	Susceptible	Susceptible
N112S	Resistant	Neutral	Resistant
N112T	Neutral	Neutral	Cautiously Susceptible
N112Y	Neutral	Neutral	Cautiously Susceptible
G113A	Susceptible	Neutral	Susceptible
G113C	Neutral	Neutral	Cautiously Susceptible
G113D	Neutral	Neutral	Cautiously Susceptible
G113G	Susceptible	Neutral	Susceptible
G113R	Susceptible	Susceptible	Susceptible
G113S	Neutral	Neutral	Cautiously Susceptible
G113V	Susceptible	Susceptible	Susceptible
T114A	Neutral	Neutral	Cautiously Susceptible
T114M	Neutral	Neutral	Cautiously Susceptible
T114P	Neutral	Neutral	Cautiously Susceptible
T114S	Neutral	Neutral	Cautiously Susceptible
P115A	Resistant	Resistant	Resistant
P115L	Susceptible	Susceptible	Susceptible
P115P	Neutral	Resistant	Resistant
P115Q	Neutral	Neutral	Cautiously Susceptible
P115R	Neutral	Resistant	Resistant
P115S	Neutral	Neutral	Cautiously Susceptible
P115T	Neutral	Neutral	Cautiously Susceptible
L116L	Susceptible	Susceptible	Susceptible
L116M	Susceptible	Neutral	Susceptible
L116P	Neutral	Resistant	Resistant
L116Q	Resistant	Resistant	Resistant
L116R	Neutral	Resistant	Resistant
L116V	Neutral	Resistant	Resistant
L117L	Susceptible	Susceptible	Susceptible

L117M	Susceptible	Neutral	Susceptible
L117P	Susceptible	Neutral	Susceptible
L117Q	Neutral	Neutral	Cautiously Susceptible
L117R	Neutral	Neutral	Cautiously Susceptible
L117V	Susceptible	Susceptible	Susceptible
N118D	Neutral	Neutral	Cautiously Susceptible
N118I	Neutral	Neutral	Cautiously Susceptible
N118K	Neutral	Neutral	Cautiously Susceptible
N118N	Neutral	Neutral	Cautiously Susceptible
N118S	Neutral	Susceptible	Susceptible
N118Y	Neutral	Neutral	Cautiously Susceptible
W119*	Resistant	Resistant	Resistant
W119C	Neutral	Neutral	Cautiously Susceptible
W119G	Neutral	Neutral	Cautiously Susceptible
W119L	Neutral	Resistant	Resistant
W119R	Resistant	Resistant	Resistant
L120M	Susceptible	Neutral	Susceptible
L120P	Resistant	Resistant	Resistant
L120Q	Neutral	Resistant	Resistant
L120R	Neutral	Resistant	Resistant
L120V	Neutral	Neutral	Cautiously Susceptible
R121G	Neutral	Neutral	Cautiously Susceptible
R121L	Susceptible	Susceptible	Susceptible
R121P	Neutral	Resistant	Resistant
R121Q	Resistant	Neutral	Resistant
R121R	Susceptible	Susceptible	Susceptible
R121W	Neutral	Neutral	Cautiously Susceptible
Q122*	Resistant	Resistant	Resistant
Q122E	Neutral	Neutral	Cautiously Susceptible
Q122H	Neutral	Neutral	Cautiously Susceptible
Q122K	Resistant	Neutral	Resistant
Q122L	Neutral	Susceptible	Susceptible
Q122Q	Neutral	Neutral	Cautiously Susceptible
Q122R	Neutral	Neutral	Cautiously Susceptible
R123C	Neutral	Neutral	Cautiously Susceptible
R123G	Neutral	Susceptible	Susceptible
R123H	Susceptible	Susceptible	Susceptible
R123L	Susceptible	Susceptible	Susceptible

R123P	Neutral	Neutral	Cautiously Susceptible
R123R	Neutral	Neutral	Cautiously Susceptible
R123S	Neutral	Neutral	Cautiously Susceptible
G124A	Susceptible	Susceptible	Susceptible
G124C	Neutral	Susceptible	Susceptible
G124D	Neutral	Susceptible	Susceptible
G124G	Susceptible	Neutral	Susceptible
G124V	Susceptible	Neutral	Susceptible
V125A	Resistant	Resistant	Resistant
V125D	Neutral	Resistant	Resistant
V125F	Neutral	Resistant	Resistant
V125G	Neutral	Neutral	Cautiously Susceptible
V125I	Susceptible	Neutral	Susceptible
V125L	Neutral	Susceptible	Susceptible
V125V	Resistant	Neutral	Resistant
D126D	Susceptible	Neutral	Susceptible
D126E	Neutral	Neutral	Cautiously Susceptible
D126G	Neutral	Neutral	Cautiously Susceptible
D126H	Neutral	Susceptible	Susceptible
D126N	Susceptible	Susceptible	Susceptible
D126V	Susceptible	Neutral	Susceptible
D126Y	Neutral	Susceptible	Susceptible
E127*	Neutral	Neutral	Cautiously Susceptible
E127E	Neutral	Susceptible	Susceptible
E127G	Susceptible	Susceptible	Susceptible
E127K	Neutral	Susceptible	Susceptible
E127V	Neutral	Neutral	Cautiously Susceptible
V128A	Neutral	Resistant	Resistant
V128D	Neutral	Neutral	Cautiously Susceptible
V128F	Neutral	Neutral	Cautiously Susceptible
V128G	Neutral	Resistant	Resistant
V128I	Susceptible	Neutral	Susceptible
V128L	Neutral	Neutral	Cautiously Susceptible
V128V	Susceptible	Susceptible	Susceptible
D129D	Neutral	Susceptible	Susceptible
D129E	Neutral	Resistant	Resistant
D129G	Neutral	Neutral	Cautiously Susceptible
D129H	Susceptible	Neutral	Susceptible

D129N	Neutral	Resistant	Resistant
D129V	Susceptible	Neutral	Susceptible
D129Y	Neutral	Resistant	Resistant
V130A	Neutral	Resistant	Resistant
V130E	Resistant	Resistant	Resistant
V130L	Susceptible	Susceptible	Susceptible
V130V	Susceptible	Susceptible	Susceptible
V131A	Susceptible	Susceptible	Susceptible
V131D	Resistant	Resistant	Resistant
V131F	Resistant	Resistant	Resistant
V131G	Neutral	Neutral	Cautiously Susceptible
V131I	Neutral	Neutral	Cautiously Susceptible
V131L	Susceptible	Neutral	Susceptible
V131V	Neutral	Neutral	Cautiously Susceptible
G132A	Resistant	Resistant	Resistant
G132C	Resistant	Resistant	Resistant
G132D	Resistant	Resistant	Resistant
G132G	Neutral	Resistant	Resistant
G132R	Resistant	Resistant	Resistant
G132S	Resistant	Resistant	Resistant
G132V	Neutral	Resistant	Resistant
I133F	Resistant	Resistant	Resistant
I133I	Neutral	Neutral	Cautiously Susceptible
I133M	Susceptible	Susceptible	Susceptible
I133N	Resistant	Resistant	Resistant
I133S	Neutral	Resistant	Resistant
I133T	Susceptible	Neutral	Susceptible
I133V	Neutral	Susceptible	Susceptible
A134A	Susceptible	Susceptible	Susceptible
A134D	Neutral	Neutral	Cautiously Susceptible
A134G	Neutral	Resistant	Resistant
A134P	Resistant	Neutral	Resistant
A134S	Susceptible	Susceptible	Susceptible
A134T	Resistant	Resistant	Resistant
A134V	Resistant	Resistant	Resistant
T135A	Neutral	Neutral	Cautiously Susceptible
T135I	Resistant	Neutral	Resistant
T135N	Neutral	Resistant	Resistant

T135P	Neutral	Resistant	Resistant
T135S	Susceptible	Neutral	Susceptible
T135T	Neutral	Susceptible	Susceptible
D136D	Susceptible	Susceptible	Susceptible
D136E	Neutral	Neutral	Cautiously Susceptible
D136G	Neutral	Neutral	Cautiously Susceptible
D136H	Neutral	Resistant	Resistant
D136N	Susceptible	Neutral	Susceptible
D136V	Resistant	Resistant	Resistant
D136Y	Resistant	Resistant	Resistant
H137D	Neutral	Neutral	Cautiously Susceptible
H137H	Neutral	Susceptible	Susceptible
H137L	Neutral	Neutral	Cautiously Susceptible
H137N	Neutral	Resistant	Resistant
H137P	Resistant	Resistant	Resistant
H137Q	Neutral	Resistant	Resistant
H137R	Neutral	Neutral	Cautiously Susceptible
H137Y	Neutral	Neutral	Cautiously Susceptible
C138*	Resistant	Resistant	Resistant
C138C	Neutral	Neutral	Cautiously Susceptible
C138F	Neutral	Resistant	Resistant
C138G	Resistant	Resistant	Resistant
C138R	Resistant	Resistant	Resistant
C138S	Resistant	Resistant	Resistant
C138W	Neutral	Neutral	Cautiously Susceptible
C138Y	Resistant	Resistant	Resistant
V139A	Resistant	Resistant	Resistant
V139E	Resistant	Resistant	Resistant
V139G	Neutral	Resistant	Resistant
V139L	Susceptible	Neutral	Susceptible
V139M	Resistant	Resistant	Resistant
V139V	Susceptible	Neutral	Susceptible
R140C	Neutral	Neutral	Cautiously Susceptible
R140G	Neutral	Resistant	Resistant
R140L	Susceptible	Susceptible	Susceptible
R140P	Resistant	Resistant	Resistant
R140R	Susceptible	Susceptible	Susceptible
R140S	Susceptible	Susceptible	Susceptible

Q141*	Resistant	Resistant	Resistant
Q141E	Neutral	Susceptible	Susceptible
Q141H	Resistant	Neutral	Resistant
Q141K	Neutral	Neutral	Cautiously Susceptible
Q141L	Susceptible	Susceptible	Susceptible
Q141Q	Neutral	Neutral	Cautiously Susceptible
Q141R	Susceptible	Susceptible	Susceptible
T142A	Resistant	Resistant	Resistant
T142K	Resistant	Resistant	Resistant
T142M	Resistant	Resistant	Resistant
T142P	Neutral	Neutral	Cautiously Susceptible
T142R	Resistant	Resistant	Resistant
T142S	Neutral	Neutral	Cautiously Susceptible
T142T	Resistant	Neutral	Resistant
A143A	Neutral	Susceptible	Susceptible
A143D	Neutral	Neutral	Cautiously Susceptible
A143G	Susceptible	Neutral	Susceptible
A143P	Neutral	Neutral	Cautiously Susceptible
A143S	Neutral	Resistant	Resistant
A143T	Neutral	Neutral	Cautiously Susceptible
A143V	Neutral	Neutral	Cautiously Susceptible
E144*	Neutral	Resistant	Resistant
E144D	Susceptible	Susceptible	Susceptible
E144E	Neutral	Neutral	Cautiously Susceptible
E144G	Neutral	Susceptible	Susceptible
E144K	Susceptible	Neutral	Susceptible
E144Q	Neutral	Neutral	Cautiously Susceptible
D145D	Susceptible	Susceptible	Susceptible
D145E	Neutral	Resistant	Resistant
D145G	Susceptible	Susceptible	Susceptible
D145N	Neutral	Susceptible	Susceptible
D145V	Neutral	Resistant	Resistant
D145Y	Neutral	Neutral	Cautiously Susceptible
A146A	Neutral	Susceptible	Susceptible
A146E	Neutral	Neutral	Cautiously Susceptible
A146P	Neutral	Neutral	Cautiously Susceptible
A146S	Resistant	Neutral	Resistant
A146T	Resistant	Resistant	Resistant

A146V	Resistant	Resistant	Resistant
V147A	Resistant	Resistant	Resistant
V147I	Neutral	Neutral	Cautiously Susceptible
V147L	Neutral	Susceptible	Susceptible
V147V	Susceptible	Susceptible	Susceptible
R148C	Neutral	Neutral	Cautiously Susceptible
R148G	Susceptible	Neutral	Susceptible
R148H	Susceptible	Neutral	Susceptible
R148L	Neutral	Neutral	Cautiously Susceptible
R148P	Neutral	Resistant	Resistant
R148R	Susceptible	Susceptible	Susceptible
R148S	Susceptible	Susceptible	Susceptible
N149D	Susceptible	Neutral	Susceptible
N149H	Neutral	Susceptible	Susceptible
N149I	Neutral	Neutral	Cautiously Susceptible
N149N	Neutral	Susceptible	Susceptible
N149S	Susceptible	Neutral	Susceptible
N149Y	Resistant	Resistant	Resistant
G150A	Neutral	Neutral	Cautiously Susceptible
G150C	Neutral	Neutral	Cautiously Susceptible
G150D	Neutral	Resistant	Resistant
G150G	Resistant	Resistant	Resistant
G150S	Neutral	Neutral	Cautiously Susceptible
G150V	Neutral	Neutral	Cautiously Susceptible
L151*	Resistant	Resistant	Resistant
L151F	Neutral	Susceptible	Susceptible
L151M	Neutral	Neutral	Cautiously Susceptible
L151S	Neutral	Resistant	Resistant
L151V	Neutral	Neutral	Cautiously Susceptible
L151W	Neutral	Neutral	Cautiously Susceptible
A152A	Susceptible	Susceptible	Susceptible
A152D	Neutral	Resistant	Resistant
A152G	Susceptible	Neutral	Susceptible
A152P	Neutral	Susceptible	Susceptible
A152S	Neutral	Neutral	Cautiously Susceptible
A152T	Resistant	Neutral	Resistant
A152V	Resistant	Neutral	Resistant
T153A	Susceptible	Susceptible	Susceptible

T153I	Neutral	Neutral	Cautiously Susceptible
T153N	Neutral	Neutral	Cautiously Susceptible
T153T	Susceptible	Susceptible	Susceptible
R154G	Susceptible	Neutral	Susceptible
R154K	Neutral	Neutral	Cautiously Susceptible
R154M	Resistant	Resistant	Resistant
R154R	Susceptible	Susceptible	Susceptible
R154S	Neutral	Susceptible	Susceptible
R154T	Neutral	Susceptible	Susceptible
R154W	Neutral	Resistant	Resistant
V155A	Neutral	Neutral	Cautiously Susceptible
V155E	Neutral	Neutral	Cautiously Susceptible
V155L	Neutral	Neutral	Cautiously Susceptible
V155M	Neutral	Resistant	Resistant
V155V	Neutral	Neutral	Cautiously Susceptible
L156L	Susceptible	Susceptible	Susceptible
L156M	Neutral	Susceptible	Susceptible
L156P	Neutral	Neutral	Cautiously Susceptible
L156Q	Neutral	Neutral	Cautiously Susceptible
L156R	Resistant	Resistant	Resistant
V157A	Neutral	Resistant	Resistant
V157E	Neutral	Neutral	Cautiously Susceptible
V157G	Neutral	Neutral	Cautiously Susceptible
V157L	Susceptible	Susceptible	Susceptible
V157M	Susceptible	Neutral	Susceptible
V157V	Susceptible	Neutral	Susceptible
D158D	Neutral	Neutral	Cautiously Susceptible
D158E	Susceptible	Susceptible	Susceptible
D158G	Neutral	Resistant	Resistant
D158N	Susceptible	Susceptible	Susceptible
D158V	Resistant	Resistant	Resistant
L159L	Neutral	Susceptible	Susceptible
L159M	Susceptible	Neutral	Susceptible
L159P	Resistant	Resistant	Resistant
L159Q	Neutral	Resistant	Resistant
L159R	Susceptible	Neutral	Susceptible
L159V	Resistant	Resistant	Resistant
T160A	Neutral	Resistant	Resistant

T160I	Neutral	Neutral	Cautiously Susceptible
T160K	Resistant	Resistant	Resistant
T160R	Resistant	Resistant	Resistant
T160S	Neutral	Susceptible	Susceptible
T160T	Neutral	Susceptible	Susceptible
A161A	Neutral	Neutral	Cautiously Susceptible
A161E	Neutral	Neutral	Cautiously Susceptible
A161P	Neutral	Neutral	Cautiously Susceptible
A161S	Susceptible	Neutral	Susceptible
A161T	Neutral	Susceptible	Susceptible
A161V	Neutral	Neutral	Cautiously Susceptible
G162A	Resistant	Neutral	Resistant
G162C	Neutral	Susceptible	Susceptible
G162D	Resistant	Resistant	Resistant
G162G	Resistant	Resistant	Resistant
G162R	Neutral	Resistant	Resistant
G162S	Neutral	Resistant	Resistant
G162V	Neutral	Resistant	Resistant
V163A	Neutral	Neutral	Cautiously Susceptible
V163G	Neutral	Neutral	Cautiously Susceptible
V163L	Neutral	Neutral	Cautiously Susceptible
V163M	Susceptible	Susceptible	Susceptible
V163V	Neutral	Susceptible	Susceptible
S164*	Resistant	Resistant	Resistant
S164A	Neutral	Neutral	Cautiously Susceptible
S164L	Neutral	Neutral	Cautiously Susceptible
S164P	Resistant	Resistant	Resistant
S164S	Neutral	Susceptible	Susceptible
S164W	Neutral	Neutral	Cautiously Susceptible
A165A	Susceptible	Susceptible	Susceptible
A165D	Neutral	Resistant	Resistant
A165G	Susceptible	Neutral	Susceptible
A165S	Neutral	Neutral	Cautiously Susceptible
A165T	Susceptible	Neutral	Susceptible
A165V	Neutral	Neutral	Cautiously Susceptible
D166D	Susceptible	Susceptible	Susceptible
D166E	Resistant	Neutral	Resistant
D166G	Neutral	Neutral	Cautiously Susceptible

D166H	Susceptible	Neutral	Susceptible
D166N	Neutral	Neutral	Cautiously Susceptible
D166V	Neutral	Susceptible	Susceptible
D166Y	Neutral	Resistant	Resistant
T167A	Neutral	Neutral	Cautiously Susceptible
T167I	Neutral	Resistant	Resistant
T167N	Neutral	Neutral	Cautiously Susceptible
T167S	Neutral	Neutral	Cautiously Susceptible
T167T	Susceptible	Neutral	Susceptible
T168A	Neutral	Neutral	Cautiously Susceptible
T168N	Neutral	Resistant	Resistant
T168P	Neutral	Resistant	Resistant
T168S	Neutral	Neutral	Cautiously Susceptible
T168T	Susceptible	Susceptible	Susceptible
V169A	Neutral	Neutral	Cautiously Susceptible
V169D	Neutral	Neutral	Cautiously Susceptible
V169F	Susceptible	Neutral	Susceptible
V169I	Susceptible	Susceptible	Susceptible
V169L	Neutral	Susceptible	Susceptible
V169V	Susceptible	Neutral	Susceptible
A170A	Resistant	Resistant	Resistant
A170D	Neutral	Neutral	Cautiously Susceptible
A170P	Neutral	Neutral	Cautiously Susceptible
A170S	Resistant	Neutral	Resistant
A170T	Susceptible	Neutral	Susceptible
A170V	Resistant	Neutral	Resistant
A171A	Neutral	Neutral	Cautiously Susceptible
A171E	Neutral	Neutral	Cautiously Susceptible
A171G	Neutral	Neutral	Cautiously Susceptible
A171P	Neutral	Neutral	Cautiously Susceptible
A171S	Neutral	Neutral	Cautiously Susceptible
A171T	Resistant	Resistant	Resistant
L172L	Neutral	Neutral	Cautiously Susceptible
L172P	Resistant	Resistant	Resistant
L172Q	Neutral	Neutral	Cautiously Susceptible
E173*	Neutral	Resistant	Resistant
E173D	Neutral	Susceptible	Susceptible
E173E	Neutral	Neutral	Cautiously Susceptible

E173G	Resistant	Resistant	Resistant
E173K	Susceptible	Susceptible	Susceptible
E173V	Neutral	Susceptible	Susceptible
E174A	Neutral	Neutral	Cautiously Susceptible
E174D	Neutral	Neutral	Cautiously Susceptible
E174E	Neutral	Neutral	Cautiously Susceptible
E174G	Resistant	Resistant	Resistant
E174K	Neutral	Neutral	Cautiously Susceptible
E174Q	Neutral	Neutral	Cautiously Susceptible
E174V	Neutral	Neutral	Cautiously Susceptible
M175I	Resistant	Neutral	Resistant
M175K	Resistant	Resistant	Resistant
M175L	Susceptible	Susceptible	Susceptible
M175R	Neutral	Neutral	Cautiously Susceptible
M175T	Resistant	Resistant	Resistant
M175V	Resistant	Resistant	Resistant
R176C	Neutral	Resistant	Resistant
R176G	Neutral	Neutral	Cautiously Susceptible
R176H	Neutral	Resistant	Resistant
R176L	Neutral	Neutral	Cautiously Susceptible
R176P	Neutral	Neutral	Cautiously Susceptible
R176R	Susceptible	Neutral	Susceptible
R176S	Neutral	Neutral	Cautiously Susceptible
T177A	Neutral	Neutral	Cautiously Susceptible
T177I	Neutral	Susceptible	Susceptible
T177N	Neutral	Susceptible	Susceptible
T177S	Neutral	Susceptible	Susceptible
T177T	Resistant	Resistant	Resistant
A178A	Susceptible	Susceptible	Susceptible
A178D	Neutral	Susceptible	Susceptible
A178P	Neutral	Neutral	Cautiously Susceptible
A178S	Neutral	Neutral	Cautiously Susceptible
A178T	Neutral	Resistant	Resistant
A178V	Neutral	Susceptible	Susceptible
S179C	Susceptible	Susceptible	Susceptible
S179G	Neutral	Resistant	Resistant
S179I	Susceptible	Susceptible	Susceptible
S179N	Resistant	Resistant	Resistant

S179R	Susceptible	Susceptible	Susceptible
S179S	Resistant	Resistant	Resistant
V180A	Neutral	Resistant	Resistant
V180D	Neutral	Resistant	Resistant
V180F	Neutral	Resistant	Resistant
V180I	Neutral	Neutral	Cautiously Susceptible
V180L	Neutral	Neutral	Cautiously Susceptible
V180V	Susceptible	Susceptible	Susceptible
E181*	Neutral	Resistant	Resistant
E181A	Neutral	Neutral	Cautiously Susceptible
E181D	Susceptible	Susceptible	Susceptible
E181E	Neutral	Resistant	Resistant
E181G	Neutral	Neutral	Cautiously Susceptible
E181K	Resistant	Neutral	Resistant
E181V	Neutral	Neutral	Cautiously Susceptible
L182*	Neutral	Neutral	Cautiously Susceptible
L182F	Neutral	Neutral	Cautiously Susceptible
L182M	Susceptible	Neutral	Susceptible
L182S	Neutral	Resistant	Resistant
V183A	Neutral	Susceptible	Susceptible
V183D	Neutral	Neutral	Cautiously Susceptible
V183F	Neutral	Neutral	Cautiously Susceptible
V183I	Susceptible	Susceptible	Susceptible
V183L	Resistant	Neutral	Resistant
V183V	Neutral	Neutral	Cautiously Susceptible
C184*	Neutral	Neutral	Cautiously Susceptible
C184C	Neutral	Resistant	Resistant
C184F	Neutral	Neutral	Cautiously Susceptible
C184S	Neutral	Resistant	Resistant
C184W	Neutral	Neutral	Cautiously Susceptible
C184Y	Susceptible	Neutral	Susceptible
S185C	Neutral	Neutral	Cautiously Susceptible
S185G	Neutral	Susceptible	Susceptible
S185I	Neutral	Neutral	Cautiously Susceptible
S185N	Neutral	Neutral	Cautiously Susceptible
S185R	Susceptible	Susceptible	Susceptible
S185S	Neutral	Neutral	Cautiously Susceptible
S185T	Neutral	Neutral	Cautiously Susceptible

S186F	Neutral	Resistant	Resistant
S186P	Neutral	Susceptible	Susceptible
S186Y	Neutral	Neutral	Cautiously Susceptible
187	Neutral	Neutral	Cautiously Susceptible
*187W	Susceptible	Susceptible	Susceptible
*187Y	Neutral	Susceptible	Susceptible

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