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Molecular targets and mechanisms of action of pyrazinamide in *Mycobacterium tuberculosis*

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Declaration

This dissertation, "Molecular targets and mechanisms of action of pyrazinamide in *Mycobacterium tuberculosis*" is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. The work was done under the guidance of Professor Tianyu Zhang, at the Guangzhou Institutes of Biomedicine and Health, University of Chinese Academy of Sciences, and The Guangzhou Chest Hospital, China.

Moses Mucugi Njire

吡嗪酰胺作用于结核分枝杆菌的分子靶点和机制

摘要

由于耐多药和全耐药结核分枝杆菌(*Mycobacterium tuberculosis*) 的不断肆虐，全球结核病（TB）的防控正面临着极严峻的挑战. 吡嗪酰胺（PZA）是组成直接督导短程疗法（DOTS）的四个一线药物之一，可用于治疗敏感（DS-TB）和耐药结核病（DR-TB）. PZA 在酸性（低 pH）环境下对处于持留状态的 *M. tuberculosis* 具有杀菌活性，而其它药物难以在相同条件下发挥作用，因此，PZA 是组成治疗 TB 方案中不可或缺的成分. 虽然前药 PZA 通过由 *pncA* 基因编码的吡嗪酰胺酶（PZase）水解为活性的吡嗪酸（POA）的相关机制已被揭示，但其细胞靶点和抑制细胞功能的作用机制仍不清楚. 有研究显示，参与反式翻译过程的核糖体蛋白 S1（RpsA）可能是 POA 的靶点，天冬氨酸脱羧酶（panD）与 PZA 耐药有关.

然而在本研究中，我们通过基因测序发现两株对 PZA 耐药的临床 *M. tuberculosis* 菌株既没有 *pncA* 基因突变，也没有 *rpsA* 和 *panD* 基因突变. 在进一步研究中，我们发现这两株 PZA 耐药的临床菌株的 *Rv2783c* 基因有 C948A 基因无义突变和 G199A （Asp67Asn）错义突变. 同源性比对发现 *M. tuberculosis* 的 Rv2783c 可能具有双重功能，不仅具有不依赖模板的单链 DNA 和 RNA 合成及其磷酸解的聚核苷酸磷酸化酶（PNPase）活性，而且具有合成和水解 (p)ppGpp 的鸟苷五磷酸合成酶（GPSI) 活性. 为了更深入地了解 PZA 的潜在靶点，我们在 *M. tuberculosis* 毒力株 H37Rv 中分别过表达野生型和 *Rv2783c*$_{G199A}$ 突变基因. 研究证实，过表达突变的 *Rv2783c* 可导致重组菌对 PZA 耐药，而过表达野生型的 *Rv2783c* 的重组菌并没有产生耐药性. 利用等温滴定量热法（ITC)，我们进一步揭

示野生型的 Rv2783c 蛋白可以与 POA 结合但不与前药 PZA 结合，但是突变的 $Rv2783c_{G199A}$ 蛋白和对 PZA 天然耐药的耻垢分枝杆菌（*M. smegmatis*）的野生型 PNPase 蛋白均不能与 POA 和 PZA 结合. 此外，野生型和突变型的 *M. tuberculosis* Rv2783c 蛋白均有不依赖模板的单链 DNA 和 RNA 的聚合和磷酸解作用。然而有趣的是，POA 可明显抑制野生型 Rv2783c 蛋白的 DNA 和 RNA 催化活性，但 PZA 不能，突变的 $Rv2783c_{G199A}$ 蛋白的 DNA 和 RNA 催化活性均不易受 POA 和 PZA 的影响。另一方面，野生型和突变型的 Rv2783c 蛋白表现出很强的水解(p)ppGpp 的能力，但只有很低的 ppGpp 合成活性. 这种分子与细菌胁迫应答紧密相关. 与上述结论相似的是，POA 可显著抑制野生型 Rv2783c 蛋白的 ppGpp 水解活性，但对突变的 $Rv2783c_{G199A}$ 蛋白不抑制。总而言之，我们的研究成果证明了 Rv2783c 蛋白可能是 POA 的一个作用靶点. 我们的发现有助于进一步揭开 POA 是如何通过影响 Rv2783c 蛋白从而杀死宿主体内的持留 *M. tuberculosis* 的作用机理，并为设计新型抗 TB 药物提供新思路.

关键词: 结核分枝杆菌, 结核病, 吡嗪酰胺, (p)ppGpp, *Rv2783c* 基因.

Molecular targets and mechanisms of action of pyrazinamide in *Mycobacterium tuberculosis*

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Abstract

The global control and management of tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is faced with the formidable challenge of worsening scenarios of drug-resistant disease. Pyrazinamide (PZA) is one of four first-line drugs used in standard short-course combination therapy for the treatment of both drug-sensitive TB (DS-TB) and drug-resistant TB (DR-TB). It exhibits a preferential sterilizing activity against non-replicating persistent bacilli with low metabolism at acidic pH, and is thus anticipated to be an irreplaceable component of future first-line TB drug regimens. Although the mechanism of PZA activation by the enzyme pyrazinamidase (PZase), encoded by *pncA* gene, into its active moiety, pyrazinoic acid (POA), and resistance has been characterized, the precise cellular targets and physiological functions in *M. tuberculosis* that are inhibited by POA remain elusive. The ribosomal protein S1 (RpsA) and the aspartate decarboxylase (panD), involved in trans-translation and the synthesis of the essential metabolic cofactors pantothenate and coenzyme A respectively, have been suggested to be the targets of POA.

In this study however, sequencing analysis has identified the same G199A (Asp67Asn) non-synonymous substitution in *Rv2783c* of 2 PZA-resistant clinical strains lacking mutations in *pncA*, *rpsA* and *panD*. *M. tuberculosis Rv2783c* encode a probable bifunctional enzyme: polyribonucleotide nucleotidyltransferase (PNPase), involved in RNA and single stranded-DNA (ss-DNA) metabolism; and guanosine pentaphosphate synthetase (GpsI), involved in

the synthesis and degradation of the alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) implicated in the stringent response in bacteria. To gain more insight into a possible new target of PZA, we overexpressed the *Rv2783c*$_{G199A}$ mutant in *M. tuberculosis* H37Rv which resulted in PZA resistance *in vitro*, while overexpression of the wild type *Rv2783c* did not cause PZA resistance. Using isothermal titration calorimetry (ITC), purified wild type *M. tuberculosis* Rv2783 protein was found to bind to POA, and not to the prodrug PZA. However, purified *M. tuberculosis* Rv2783$_{D67N}$ protein and PNPase from naturally PZA-resistant *M. smegmatis* failed to bind either POA or PZA. In addition, both wild type and the mutant *M. tuberculosis* Rv2783 proteins catalyzed both template−independent RNA and ss-DNA polymerization and phosphorolysis activities. Interestingly however, the ss-DNA and RNA catalytic activities of the wild type and not the Rv2783$_{D67N}$ mutant protein were significantly inhibited by POA and not the pro-drug PZA. Moreover, both wild type and the mutant *M. tuberculosis* Rv2783 proteins demonstrated strong ppGpp hydrolysis but only weak ppGpp synthesis activities. Similarly, the ppGpp hydrolysis activity of the wild type but not the Rv2783$_{D67N}$ mutant protein was significantly inhibited by POA. Taken together, these results suggest *M. tuberculosis* Rv2783 as a possible cellular target of POA. Our findings thus have implications for a better understanding of this unique sterilizing drug and for the design of new drugs targeting *M. tuberculosis* persisters for improved treatment.

Key words: *M. tuberculosis,* tuberculosis, pyrazinamide, (p)ppGpp, *Rv2783c* gene

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1. Introduction

1.1. Disease

Tuberculosis (TB) is an 'ancient' disease in terms of its documentation throughout human history. It continues to be a burden on the health resources of many countries, even those that imagine themselves to be developed and to have effective programmes against TB. It is caused by infection with the acid-fast bacillus *Mycobacterium tuberculosis,* an actinomycete closely related to saprophytic bacteria such as *M. smegmatis*, which was first identified as a pathogen by Robert Koch in 1882 [1].

The pathogenesis of TB follows a relatively well-defined sequence of events [2]. The infectious bacilli are inhaled as droplets from the atmosphere (**Figure 1**). In the lungs, the bacteria are phagocytosed by alveolar macrophages and induce a localized pro-inflammatory response that leads to recruitment of mononuclear cells from neighbouring blood vessels. These cells are the building blocks for the granuloma, or tubercle, that defines the disease. The granuloma consists of a kernel of infected macrophages, surrounded by foamy giant cells and macrophages with a mantle of lymphocytes delineating the periphery of the structure. This tissue response typifies the 'containment' phase of the infection, during which there are no overt signs of disease and the host does not transmit the infection to others. Containment fails after a change in the immune status of the host, which is usually a consequence of old age, malnutrition, or HIV-coinfection [3]. Under such circumstances, the center of the granuloma undergoes caseation and spills viable, infectious bacilli into the airways. This leads to development of a productive cough that facilitates aerosol spread of infectious bacilli.

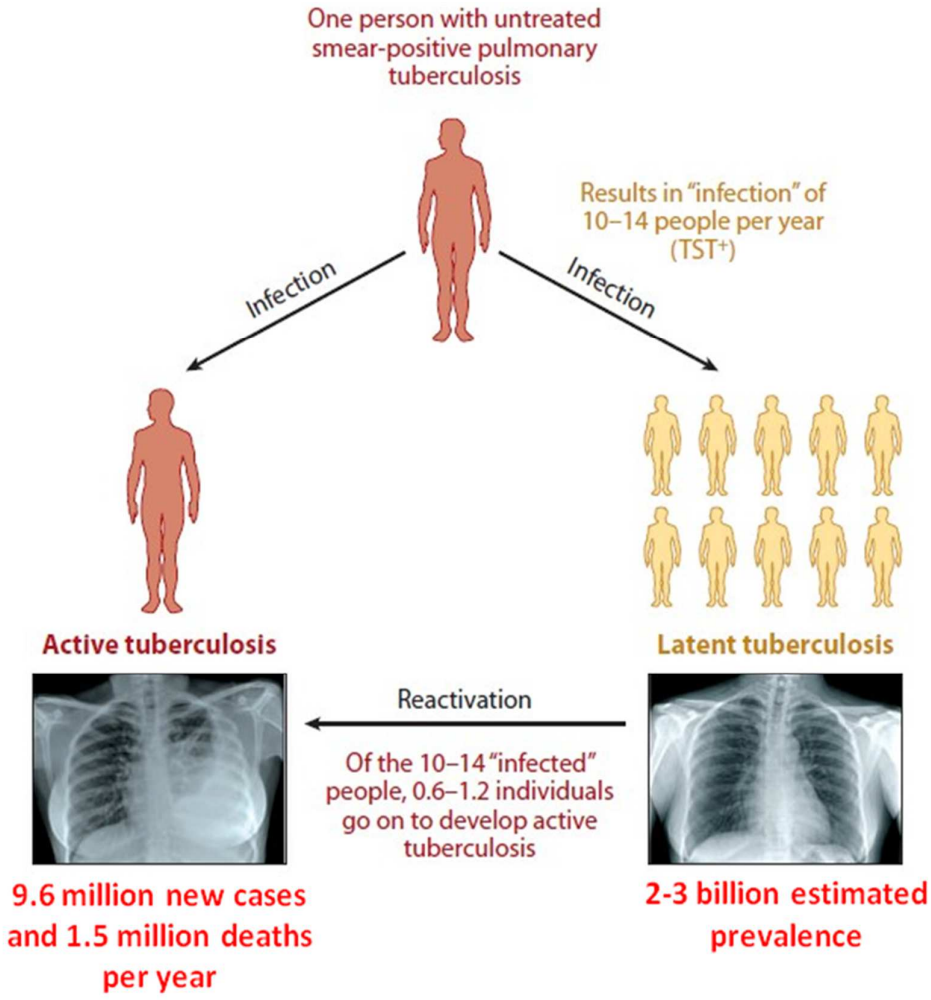

Figure 1: The epidemiology of *M. tuberculosis* infection indicating active and latent TB.

TB disease results from infection with the pathogen *M. tuberculosis*, which is spread by respiratory transmission. Although 9.6 million new cases of active TB are still reported annually, the majority of infected individuals do not develop active TB [4]. It is estimated that about 2-3 billion worldwide are infected with M. tuberculosis [4], yet they remain asymptomatic, defined as having latent TB [5]. Epidemiological studies and modeling suggest that the majority of these individuals will control this latent lifelong infection, with only 5 to 10% reactivating infection to develop active TB during their lifetime. Illustration adopted and modified from a previous report [6].

The characteristic features of the tubercle bacillus include its slow growth, dormancy/persistence, complex cell envelope, intracellular pathogenesis and genetic homogeneity [7]. The generation time of *M. tuberculosis*, in synthetic medium or infected animals, is about 24 hours. This contributes to the chronic nature of the disease, which

imposes lengthy treatment regimens and represents a formidable obstacle for researchers. The state of dormancy in which the bacillus remains quiescent within infected tissue may reflect metabolic shutdown resulting from the action of a cell-mediated immune response that can contain but not eradicate the infection. The molecular basis of dormancy and reactivation remains obscure but is expected to be genetically programmed and to involve intracellular signaling pathways. Moreover, the cell envelope of *M. tuberculosis*, a Gram-positive bacterium with a G + C-rich genome, contains an additional layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharides [8, 9]. Novel biosynthetic pathways generate cell-wall components such as mycolic acids, mycocerosic acid, phenolthiocerol, lipoarabinomannan and arabinogalactan, and several of these may contribute to mycobacterial longevity, trigger inflammatory host reactions and act in pathogenesis. The inordinate emphasis that the bacilli place on lipids is clear from the publication of the bacterium genome sequence, in which ~30% of the genes are devoted to lipid synthesis or metabolism [10].

TB is predominantly a disease of the lung, with pulmonary TB accounting for 70% of cases, although *M. tuberculosis* can disseminate to other organs, including lymph nodes, bone, and meninges, and cause extrapulmonary disease [11]. TB is transmitted by *M. tuberculosis*-containing aerosolized droplets generated by the cough of a person infected with bacilli. The primary infection involves replication of *M. tuberculosis* within alveolar macrophages of the terminal air spaces of the lung, spread to local lymph nodes within the lung, and eventual dissemination of infection to remote sites in the body within one or two years after an initial infection. Interestingly, although 9.6 million new cases of active TB are still reported annually, an estimated one-third of the world infected with *M. tuberculosis* remains asymptomatic, defined as having latent TB [5]. This is because the host immune response against *M. tuberculosis* is highly effective in controlling bacterial replication. Among those with latent TB, only 5–10% will develop active TB disease in their lifetimes [12] (**Figure 1**).

Post-primary TB, which is predominantly a pulmonary disease, develops later in life, and can be caused either by reactivation of bacilli remaining from the initial infection or by failure to control a subsequent infection. Post-primary TB involves extensive damage to the lungs and efficient aerosol transmission of bacilli. Active TB encompasses a heterogeneous range of presentations and forms of disease. It is characterized by systemic features such as

fever and weight loss, with localized symptoms of tissue destruction at the site of active infection and with actively replicating transmissible bacteria, diagnosed by detection of the pathogen in sputum or tissue. In latent TB, *M. tuberculosis* infection can be shown only by demonstrating the host's reactivity to *M. tuberculosis* antigens, typically using the tuberculin skin test (TST) [13]. The patient is intradermally challenged with an extract containing *M. tuberculosis* antigens, originally tuberculin [14], but now replaced with a commercially purified protein derivative (PPD) [15]. In latent infection, the TST is more frequently negative in those individuals most at risk of progression to active disease: the young, the elderly, and the immunosuppressed [16]. In addition, because PPD is prepared from culture filtrate of *M. tuberculosis*, it contains some antigens also found in the attenuated *M. bovis* BCG vaccine and in many environmental nontuberculous mycobacteria [17], and it therefore has limited specificity. Thus, false-positive TST reactions can occur both in those who have been vaccinated, and in those who have been sensitized to these common antigens through exposure to environmental nontuberculous mycobacteria [18, 19]. Development of TB disease results from interactions among the environment, the host, and the pathogen, and known risk factors include HIV coinfection, immunodeficiency, diabetes mellitus, overcrowding, malnutrition, and general poverty [20].

1.2. *M. tuberculosis* survival within macrophages

M. tuberculosis survives within the macrophages of their host, which contrasts sharply with the generally accepted view that these cells afford an effective initial barrier to bacterial infection [21]. The successful parasitization of the macrophage by *M. tuberculosis* involves modulating the normal progression of the phagosome into an acidic, hydrolytically active compartment **(Figure 2)** and avoiding the development of a localized, productive immune response that could activate the host cell. The modulation of host cell function is dynamic, requiring viable bacteria, and provides an interesting tool for the manipulation of normal phagosome biogenesis. For these reasons, research into the cell biology and genetics of infection by *M. tuberculosis* has attracted several new converts to the field and is generating information that is relevant to cell biology and immunology, as well as genetics and microbiology.

Phagosome biogenesis may be viewed as a legitimate maturation process because the 'cargo' is fixed and the vacuole remodeled by fusion and fission events [22]. During the

maturation process, phagosomes formed around inert particles show transient interactions with the endosomal network [23]. After internalization, the phagosome shows transient access to the rapid recycling pathway, as defined by the classic marker of this pathway, transferrin [24]. During these early stages, phagosomes have many of the markers found on early endosomes, such as early endosomal antigen 1 (EEA1) and Rab5.

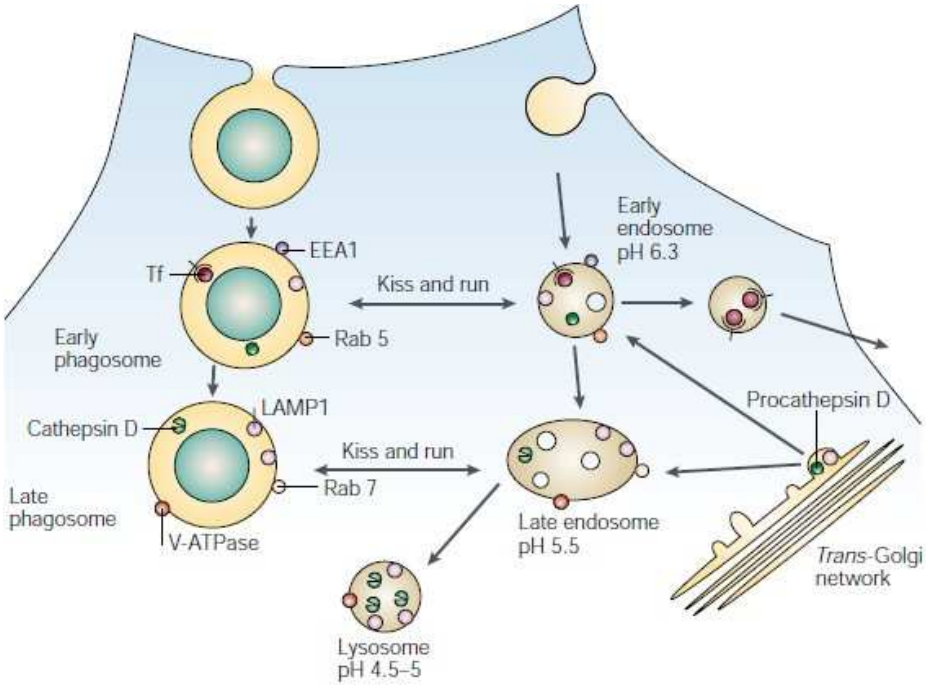

Figure 2: Illustration of phagosome maturation in *M. tuberculosis*.

Pathogenic mycobacteria have evolved a strategy to arrest the normal maturation process of phagosomes after uptake by macrophages. The successful parasitization of the macrophage by *M. tuberculosis* involves modulating the normal progression of the phagosome into an acidic, hydrolytically active compartment and avoiding the development of a localized, productive immune response that could activate the host cell [2]. Illustration adopted from a previous report [2].

Phagosomes do, however, rapidly acquire proteins associated with later compartments. Within three minutes after they are internalized, immunoglobulin-G-coated beads are in phagosomes that have lysosomes-associated membrane glycoprotein 1 (LAMP1) [23],which is transported from the *trans*-Golgi network to early endosomal–phagosomal compartments, and then to late endosomal and lysosomal compartments [25]. Pro-forms of lysosomal hydrolases such as pro-cathepsin D also appear in these early phagosomes. Lysosomal hydrolases are delivered to the endosomal network by both mannose 6-phosphate-dependent and -independent means [26]. The processing of cathepsin D to its

mature, two-chain form, seems to coincide with the rapid increase in numbers of proton-ATPases and a marked drop in pH.

1.3. Global TB disease burden

The treatment of TB was revolutionized in the 1940s with the discovery of streptomycin (STR) by Selman Waksman [27], for which he received a Nobel Prize in 1952. However, resistance to STR in *M. tuberculosis* was seen rapidly after the introduction of the drug [28]. Indeed, the British Medical Research Council (BMRC) trial that investigated the efficacy of STR showed that the majority of treated patients developed resistant strains [29]. The discovery of para-aminosalicylic acid (PAS) by Jorgen Lehman in the same year that Waksman discovered streptomycin allowed combination therapy to be trialled [30]. These trials showed that treatment with PAS and STR combined was more effective than either agent when used alone. This synergy greatly contributed to the reduction of TB incidence in the developed countries and, at the same time, suggested the possibility of eradicating *M. tuberculosis* as a major cause of morbidity and mortality [31]. Scanty historical data are available from the developing countries, although improved public welfare and accessibility to the poor people has been attributed to the declined incidence [31]. These gains have however been hampered by the onset of the HIV/AIDS pandemic, the lack of sensitive and rapid diagnostics, the breakdown of some national TB control programs, the lack of an effective vaccine and the emergence of multi-drug resistant TB (MDR-TB) [20, 32]. This prompted the WHO in 2006 to declare TB a global emergency [33]. TB ranks second after HIV/AIDS as the greatest killer worldwide [34], and in 2013, there were an estimated 9 million new cases of TB (13% co-infected with HIV) out of which 1.5 million people died [34]. Over 95% of TB mortalities occurred in low- and middle-income countries, and an estimated 480,000 people developed MDR-TB in 2013 globally. About 60% of TB cases and deaths occur among men, but the burden of disease among women is also high. In 2013, about 510,000 women died as a result of TB, more than one third of whom were HIV-positive. There were also 80,000 deaths from TB among HIV-negative children [34].

TB occurs in every part of the world and in 2013, the South-East Asia and Western Pacific Regions, accounted for 56% of new cases globally. India and China alone accounted for 24% and 11% of total cases, respectively [34]. However, Africa carried the greatest proportion of new cases per population with 280 cases per 100000 population in 2013 [34].

At the same time, about 80% of reported TB cases in 2013 occurred in 22 countries, some of which experienced a major decline, while in others the decline was very slow. Brazil and China for example, were among the 22 countries that showed a sustained decline in TB cases over the past 20 years. However, the emergence of multidrug-resistant (MDR-TB), with bacillary resistance to at least rifampin (RIF) and isoniazid (INH), extensively drug-resistant TB (XDR-TB), connoting MDR-TB with additional bacillary resistance to the fluoroquinolone(s) and the second-line injectable agent(s), and the ominous totally drug-resistant TB (TDR-TB), virtually in all countries worldwide is even more alarming. Globally, 3.5% of new and 20.5% of previously treated TB cases were estimated to have had MDR-TB in 2013 [34]. This translates into an estimated 480,000 people having developed MDR-TB in 2013. On average, an estimated 9.0% of patients with MDR-TB had XDR-TB, and by March 2013, 84 countries had reported at least one XDR-TB case. In 2007, the first case of the vaguely defined TDR-TB was reported, and has also been recently reported in 4 other countries (South Africa, India and Iran) [35-39], suggesting that human TB is joining the growing list of bacterial diseases entering the post-antibiotic era [40]. Although accounting for a small number of cases relative to drug-susceptible disease, drug-resistant TB imposes a disproportionate burden on public health systems - especially in endemic regions [41] - and, for this reason, MDR, XDR and TDR- *M. tuberculosis* strains are considered emerging pathogens in their own right [42]. Although for the most part, the social, programmatic and epidemiological factors involved in the spread of DS-TB are understood, insights into the bacterial drivers are only scanty. Most recent models suggest that the absolute number of TB cases has decreased since 2006 [43], however, the emergence and spread of the different variants of drug-resistant TB are threatening the disease control efforts throughout the globe [44]. And with increasing global burden of drug-resistant TB, the phenomenon is particularly alarming, and requires concerted efforts from all stakeholders.

1.4. Treatment of tuberculosis

The approach to chemotherapy for TB is very different from that of other bacterial infections. As earlier indicated, *M. tuberculosis* has a long generation time and a capacity for dormancy, when its low metabolic activity makes it a difficult therapeutic target [45, 46]. Moreover, some *M. tuberculosis* may be located in pulmonary cavities or empyema pus, where penetration of antibiotics is difficult or the pH is sufficiently low to inhibit the activity of most antibiotics [47, 48]. A series of animal and human clinical trials has led to the concept that there are different populations of bacteria present within the host [49, 50]. Organisms located within caseous foci are in an environment where the low pH is likely to inhibit the activity of agents such as aminoglycosides but provide the conditions necessary for pyrazinamide (PZA) activity. Bacteria found within macrophages probably only exhibit occasional spurts of metabolism and may be in relatively microaerophilic conditions, where mycobacterial latency can be induced [46].

The WHO guidelines for the treatment on Tuberculosis (4th edition) released in 2010 [51] divided TB cases into new cases (NE), previously treated cases (PT) and MDR-TB cases. The treatment regimen for the different TB cases depends upon the group the patient belongs. Treatment regimen consists of a standardized 6- or 8-month chemotherapy for new or previously treated cases, respectively **[51]** (**Table 1**).

Table 1: Recommended anti-TB drugs.

New TB cases:

- Two months of INH, RIF, PZA and EMB (intensive phase).
- Four months of INH and RIF (continuation phase).

Previously treated TB cases:

- Two months of INH, RIF, PZA, EMB and STR (intensive phase).
- One month of INH, RIF, PZA and EMB (intensive phase).
- Five months of INH, RIF and EMB (continuation phase).

MDR-TB cases:

- At least four drugs likely to be effective must be taken from each of the 5 groups of MDR-TB regimen:
- Any first-line oral agents likely to be effective should be included such as PZA or EMB.
- One effective injectable aminoglycoside or polypeptide drug should be included such as kanamycin (KAN), amikacin (AMK), capreomycin (CAP), or STR.
- One fluoroquinolone should be included.
- Intensive-phase therapy, including the injectable drug, should last for at least 6 months and the total duration of therapy should be at least 18 months.

In countries where drug susceptibility testing (DST) is routinely performed, MDR-TB treatment is individualized. Otherwise, MDR-TB is often treated using standardized drug regimens; however, these may vary between countries. Each of the anti-TB drugs has a major role in dealing with one of the bacteria populations. For example, PZA is only active at low pH, making it ideally suitable for killing the organisms inside caseous necrotic foci. This explains the finding that PZA appears to have no benefit after the second month of therapy [52].

1.5. Development of drug resistance in *M. tuberculosis*

1.5.1. Genetic drug resistance in *M. tuberculosis*

There are two types of drug resistance in *M. tuberculosis*, namely genetic resistance and phenotypic resistance. The tuberculosis bacilli is naturally resistant to many antibiotics, and only a few drugs are effective, making treatment difficult [53]. This intrinsic resistance is mainly due to the highly hydrophobic cell envelope acting as a permeability barrier for the penetration of antibiotics. Moreover, many potential resistance determinants are also encoded in the genome including hydrolytic or drug-modifying enzymes such as β-lactamases and aminoglycoside acetyl transferases, and many potential drug-efflux systems [54]. The *de novo* development of genetic resistance to the drugs that wild type *M. tuberculosis* is usually susceptible to arises through the acquisition of specific chromosomal mutations, although rarely movement of mobile genetic elements, such as the insertion sequence IS*6110*, has been associated with new resistance emerging through the inactivation of critical genes [55]. Chromosomal mutations occur through selection where in a selective environment (in the presence of a drug), the sensitive bacterial population is killed and the resistant mutants are allowed to grow [56]. Genetic resistance has resulted in the emergence of MDR-TB, XDR-TB and TDR-TB. The *de novo* emergence of drug resistance in an individual patient could arise from patient non-adherence [44], poor drug quality, and patient-dependent pharmacodynamic and kinetic properties of the drugs administered [57]. Additionally, treatments of comorbidities such as HIV can influence the pharmacodynamic properties of anti-TB drugs and increase the likelihood of genetic resistance development [58]. Importantly, resistance-conferring mutations to a given drug may confer cross-resistance to other drugs targeting related metabolic pathways [59], and the anti- TB drugs themselves can interact in ways that could promote drug resistance [60].

Genetic drug resistance-conferring mutations in *M. tuberculosis* have been described in genes (plus their regulatory regions) encoding enzymes directly targeted by the antibiotics, or in gene products involved in the activation of pro-drugs [61] **(Table 2).** The average rate of emergence of spontaneous resistance mutations to INH and RIF in *M. tuberculosis* was estimated at 10^{-8} and 10^{-9} mutations per bacterium per cell division, respectively [62]. This implies that, during monotherapy, the appearance of resistant bacteria will occur almost inevitably, given an average number of approximately 10^8 bacilli present in individual TB lesions [63]. Moreover, mutations in *M. tuberculosis* can occur during latent infections, when bacilli are thought to be replicating very slowly or not at all [64].

Table 2: Anti-TB drugs and their mechanisms of drug resistance.

Drug	Genes involved	Gene function	Resistance mechanism
Rifampicin	*rpoB*	β-subunit of RNA polymerase	Inhibition of transcription
Isoniazid	*KatG* *inhA,* *ahpC*	Catalase-peroxidase Enoyl-[acyl-carrier-protein] reductase Alkyl hyperperoxide reductase	Inhibition of mycolic acid Synthesis and other multiple effects
Pyrazinamide	*pncA* *rpsA* *panD*	Pyrazinamidase Ribosomal S1 protein Aspartate decarboxylase	Elimination of PZA conversion to POA Inhibition of trans-translation Inhibition of pantothenate and CoA synthesis
Ethambutol	*embA, embB, embC* *iniA*	Arabinosyl transferases Efflux pump associated	Inhibition of arabinogalactan synthesis Altered efflux pump activity
Streptomycin	*rpsL* *rrs* *gidB*	S12 ribosomal protein 16S rRNA protein 16S rRNA 7-methyltransferase	Alteration of drug target thereby inhibiting protein synthesis
Amikacin/ Kanamycin	*rrs* *eis* *whiB7*	16S rRNA protein, aminoglycoside acetyltransferase transcriptional regulator	Inhibition of protein synthesis
Fluoroquinolones	*gyrA, gyrB*	DNA gyrase subunit A and B	Inhibition of DNA synthesis
Capreomycin/ Viomycin	*rrs* *tlyA*	16S rRNA 2'-O-methyltransferase	Inhibition of protein synthesis
Ethionamide	*etaA/ethA,* *ethR,* *inhA*	Flavin monooxygenase Transcription repressor Enoyl ACP reductaase	Inhibition of mycolic acid synthesis

ACP, acyl-carrier-protein; CoA, coenzyme A.

These mutations are presumably the result of oxidative DNA damage rather than replication errors, and indicate that *M. tuberculosis* might have the capacity to acquire drug resistance

during latency [64]. This process could be further facilitated in bacteria with an intrinsically elevated mutation rate, such as ones with mutations in the mismatch repair system [65]. Therefore, TB treatment regimen must include at least four drugs (**Table 2**).

1.5.2. Phenotypic drug resistance in *M. tuberculosis*

Phenotypic resistance or drug tolerance has also been observed in *M. tuberculosis*. It arises due to epigenetic changes in gene expression and protein modification that cause tolerance to drugs in non-growing persister bacteria, and entails prolonged treatment and risk of post-treatment relapse [66, 67]. Persisters refer to subpopulations of cells that can phenotypically tolerate increased concentrations of drugs; however, they are not genetically resistant (i.e., drug resistance will not be inherited by daughter cells) [68]. Although drug tolerance in persisters is thought to be phenotypic, it is possible that under some conditions, antibiotic tolerant persisters may acquire mutations and develop genetic resistance. Similarly, a genetically antibiotic resistant mutant could also develop persisters with tolerance; thus, genetic resistance and tolerance may interconvert and overlap [66]. Persistence requires phenotypic differentiation into persister cells and is often linked to a state of slow growth or dormancy. It can be triggered by various factors, such as starvation, quorum sensing, intracellular signals, and antibiotic treatment itself [68, 69]. However, persistence is not strictly associated with dormancy; prior stress or sub-inhibitory concentration of drugs may induce efflux pump expression, which causes phenotypic resistance [70].

As pointed out that genetic drug resistance in *M. tuberculosis* is encoded on the chromosome, rapid detection of resistance by molecular methods is possible. These techniques overcome some of the limitations of the classical techniques of phenotypic drug susceptibility testing (DST), which may require standard mycobacterial culture for up to several months [71]. Hence, developing improved methods of molecular DST in TB is currently high on the agenda and, recently, important progress has been made [72]. However, for many anti-TB drugs, at least a proportion of drug resistance-conferring mutations remain unknown. This is especially so for PZA, which is a paradoxical first-line drug with unique sterilizing activity in anti-tuberculosis treatment. It has a preferential sterilizing activity against non-replicating persister bacilli with low metabolism at acid pH *in vitro* or *in vivo* during active inflammation where other drugs may not act so well. It is

on this account that this study "**Molecular targets and mechanisms of action of pyrazinamide in *Mycobacterium tuberculosis*'** was undertaken.

1.6. Pyrazinamide

Pyrazinamide is a structural analogue of nicotinamide (**Figure 3A**), which was chemically synthesized for the first time in 1936 [73]. However, its anti-tuberculosis activity was not recognized until 1952 [74]. The discovery of PZA as a TB drug was based on a serendipitous observation that nicotinamide exhibited antimycobacterial activity in animal models [75]. This led to a fast-tracked validation of PZA as a most active anti-TB agent through the synthesis of nicotinamide analogs and direct testing in the mouse model of TB infection bypassing in vitro testing [76, 77]. For a period of time, PZA found use as a second-line TB drug for the treatment of drug-resistant TB or treatment of relapsed TB because of the hepatic toxicity caused by higher PZA dosage (3.0 g) and longer treatment used in earlier clinical studies. However, following impressive mouse studies that demonstrated high sterilizing activity of PZA in combination with INH [78], the BMRC conducted clinical trials in East Africa with lower PZA doses (1.5-2.0 g daily) which were not significantly hepatotoxic. PZA was found almost as effective as RIF as a sterilizing drug as assessed by more frequent sputum conversion at 2 months and by the relapse rates. Subsequent clinical studies showed that the effects of RIF and PZA were synergistic [79]. These studies showed that treatment could be shortened from 12-9 months if either RIF or PZA was added to the regimen, and to 6 months if both were included. PZA has since been used as a first-line agent for treatment of drug susceptible TB with RIF, INH and EMB, which is the best TB therapy option currently. PZA is also a vital component of MDR-TB treatment regimens [80] and also of any new regimens in conjunction with new TB drug candidates in clinical trials [81].

Notably, PZA also inhibits the quiescent malaria parasite in the mouse model [82] and is also active against *E. coli* ampicillin-tolerant persisters [83]. Although there is considerable recent interest in developing antibiotics targeting persisters [84-86], PZA is the only prototype persister drug so far that has been shown to improve the treatment of a persistent infection. Nevertheless, PZA validates an important principle that drugs targeting dormant persisters, when used in combination with drugs that target growing organisms, are critical for shortening the treatment. Despite widespread recognition of its therapeutic importance,

the unique sterilizing and synergistic properties of PZA remain an enigma [87]. This knowledge gap stems from a very unusual *in vitro-in vivo* disconnect in which PZA exhibited activity in a murine model of TB rather than a culture of replicating bacilli [87]. Unlike all other TB drugs, PZA exhibits poor *in vitro* activity with the minimum inhibitory concentrations (MICs) varying widely depending on the pH, inoculum size, and other assay conditions [88, 89]. The clinical susceptibility breakpoint above which therapy fails is around 100 μg/mL (as determined by the BACTEC MGIT 960 method with an adjusted pH of 5.9) [90], whereas "normal" peak plasma concentrations are 20–60 μg/ mL 1 to 2 h postdose [91]. Unfortunately, despite PZA being one of the oldest anti-TB drugs, the biological mechanisms underlying this pharmacokinetic-pharmacodynamic puzzle have not yet been uncovered.

1.6.1. Importance of PZA in shortening TB Therapy

As aforementioned, PZA is a critical frontline TB drug that plays a unique role in shortening the treatment period from 9–12 months to 6 months [79, 92, 93]. The inclusion of PZA with INH and RIF forms the cornerstone of modern short course TB chemotherapy based on the work by McDermott and colleagues in a mouse model of TB infection [78, 94]. This powerful sterilizing activity is because PZA has the unique ability to sterilize semi-dormant bacilli that persist in acidic environments inside macropahges and other caseous foci where other drugs may not act so well [50]. PZA is only used during the first 2-month intensive phase of the 6 month therapy as giving it longer than 2 months does not appear to add additional benefit [79]. This is presumably because after 2 months of treatment, the inflammation that was leading to an acidified environment in the lesions have decreased. PZA has shown synergies with several drugs or drug candidates currently in clinical development for TB, and recent efforts to find optimal drug combinations with new drug candidates for shortening TB treatment in the mouse model suggest that PZA is the only drug that cannot be replaced without compromising treatment efficacy [95-97]. For example, PZA is being considered as part of future regimens in combination with bedaquiline (TMC207), the bicyclic nitroimidazole (PA-824) and moxifloxacin [81]. These new drug combinations are expected to shorten the treatment period for DS-TB, DR-TB as well as latent TB. Thus, PZA has a unique and an indispensable therapeutic potential across many TB patient populations.

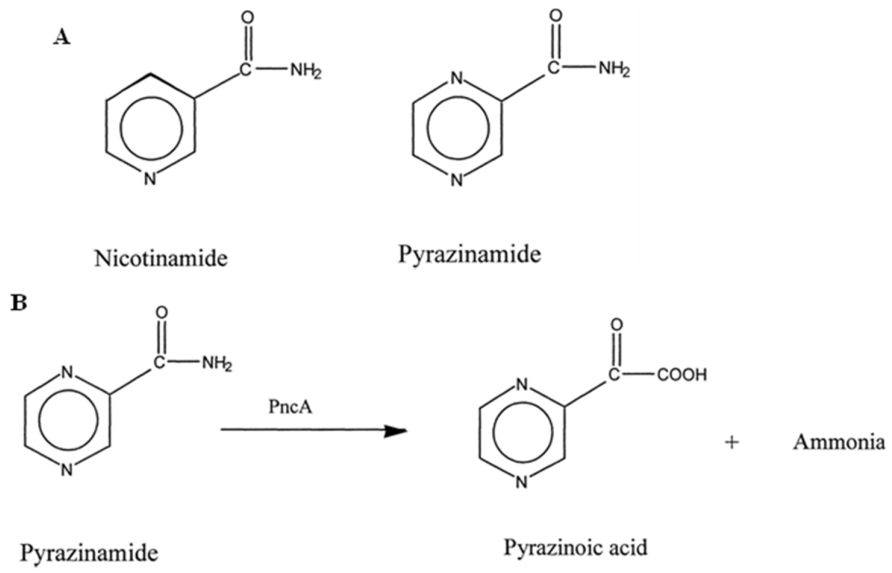

Figure 3: The structure of PZA and its conversion into POA.

A) Structure of PZA and its analogue nicotinamide [104]**; B)** Conversion of PZA into POA by enzyme PZase [99].

1.6.2. Mode of action of PZA in *M. tuberculosis*

PZA is a pro-drug that needs to be converted into its active form, pyrazinoic acid (POA) (**Figure 3**), by the enzyme pyrazinamidase (PZase) [98], encoded by the 561–nucleotide long *pncA* gene (*Rv2043c*) [99]. It is postulated that the mechanism of action of PZA is through POA, its active moiety, and this has been demonstrated by exposing *M. tuberculosis* cultures to extracellular POA and measuring growth inhibition at pH 5.5 to 6.0 against both laboratory and clinical strains. A surprisingly wide range of MICs has been reported for POA, from 8- to 16-fold less potent [100] to slightly more active [101] and up to 8-fold more potent than PZA [88]. The reported MICs of PZA itself vary between 4 and 400 µg/mL at acidic pH [88, 89]. Under the current model, bio-activation of PZA into POA primarily occurs inside the bacilli and is catalyzed by the *pncA*-encoded pyrazinamidase (PZase) [102, 103]. Accordingly, a large proportion of *M. tuberculosis* PZA-resistant strains have *pncA* mutations that reduce or abolish POA production [99].

Following the above, it was concluded that PZA undergoes intramycobacterial activation to POA and targets a subpopulation of non-replicating or slowly replicating bacilli that reside in an acidified niche. These observations have motivated a series of studies focusing on the

mechanism(s) of action of PZA and POA against *M. tuberculosis* and have identified a diverse range of potential targets, including fatty acid synthesis [105], trans-translation [101], membrane potential and integrity [83, 106], pantothenate biosynthesis [107, 108], and the host immune response [109, 110]. Collectively these studies indicate that PZA acts through a multiplicity of targets and mechanisms, many of which require biotransformation into POA.

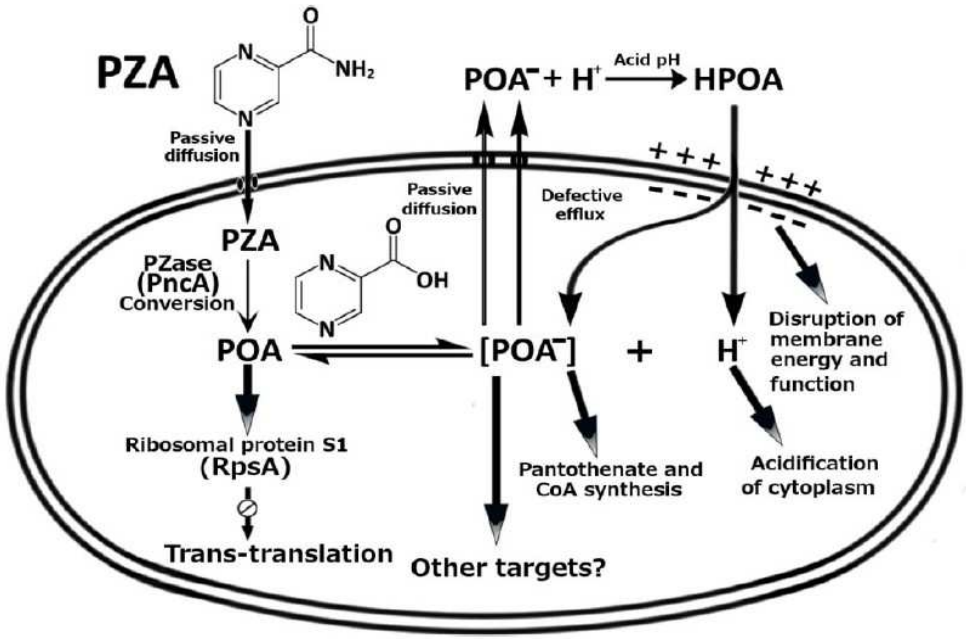

Figure 4: Proposed model for the mode of action of PZA

Based on the above studies, the model for the mode of action of PZA was proposed **(Figure 4)** [106, 111, 112]. PZA enters M. tuberculosis bacilli by passive diffusion and is converted into POA by the cytoplasmic PZase. POA is then excreted through passive diffusion and a weak efflux pump [111]. Under acid conditions, some POA become uncharged protonated acid HPOA which is readily reabsorbed inside the cell. The acid-facilitated POA influx can overcome the weak deficient POA efflux, which causes accumulation of POA inside the cell [111]. The HPOA brings protons into the cell and this could eventually cause cytoplasmic acidification such that vital enzymes could be inhibited. This is especially so for non-growing persisters with low metabolism at acidic pH. In addition, POA could de-energize the membrane by collapsing proton motive force and affect membrane transport inhibiting protein and RNA synthesis [106].

At neutral or alkaline pH, there is little POA found in *M. tuberculosis* bacilli [111], because over 99.9% of POA is in charged anion form [113] and does not get into cells easily and remains outside the cells [111]. This observation explains why PZA is active at acidic pH but not at neutral pH [102]. It is worth noting that acid pH not only allows POA to re-enter and accumulate in the bacilli [111] but also decreases the membrane potential and inhibits growth and metabolism required for the drug action. The unique activity of PZA against *M. tuberculosis* appears to be due to the deficient POA efflux mechanism that is unable to counteract the acid-facilitated POA influx, which could cause increased accumulation of POA and eventual acidification of the cytoplasm, de-energized membrane [106], inhibition of various targets and eventual cell death. The disruption of bacterial membrane energetics interferes with energy production, necessary for survival of *M. tuberculosis* at an acidic site of infection [114]. This correlates with the increased activity of PZA against non-replicating bacilli with lower membrane potential and its disruption by POA in acid environments [106]. Although PZA susceptible bacilli have been postulated to persist in the macrophage phagolysosomes [115], recent studies suggest that the interior pH of these organelles may be only slightly acidic [116], and PZA lacks even bacteriostatic activity against intracellular *M. tuberculosis* in human monocyte-derived macrophages [117]. However, activation of macrophages by gamma interferon leads to phagosome acidification to a pH range where PZA is highly active [118]. An alternative hypothesis is that PZA kills bacilli residing in acidified lung compartments present during the early inflammatory stages of infection [50], which is consistent with clinical observations that PZA activity is primarily limited to the first 2 months of therapy. This observation is also supported by Zhang et al. [119] in which the *in vivo* activity of PZA could be observed within 3 days of monotherapy with PZA, starting from the day after infection using an autoluminescent *M. tuberculosis* reporter strain.

Another theory suggests that POA and its N-propyl ester inhibit the enzyme fatty acid synthase (FAS) type I in replicating bacilli which is required by the bacterium to synthesize fatty acids, although this suggestion has been discounted [120]. PZA was also recently shown to markedly inhibit the catalytic activity of *M. tuberculosis* quinolinic acid phosphoribosyltransferase (MtQAPRTase) [121]. MtQAPRTase is a key enzyme in the *de novo* pathway of nicotinamide adenine dinucleotide (NAD) biosynthesis and a target for the development of new anti-tuberculosis drugs. Therefore, the structure of PZA may provide

the basis for the design of new inhibitors of MtQAPRTase and provide new insights into the catalytic properties of MtQAPRTase.

Recently, Via et al. [122] revisited the longstanding paradigms of PZA action and offered pharmacokinetic explanations for the apparent disconnect between its *in vitro* and *in vivo* activities. In line with earlier observations [123, 124], Via and colleagues measured substantial host-mediated conversion of PZA into POA, in both TB patients and animal species commonly used to model TB. In addition, they demonstrated favorable penetration of this pool of circulating POA from plasma into lung tissue and granulomas, where the pathogen resides. In standardized growth inhibition experiments, Via and colleagues reported that POA exhibits superior *in vitro* potency compared to PZA, indicating that the vascular supply of host-derived POA may contribute to the puzzling *in vivo* efficacy of PZA. They also reported the oral bioavailability and exposure of POA in mice, rabbits and guinea pigs, albeit with a short half-life which was significantly extended by pre-administration of the xanthine oxidase inhibitor and gout approved drug allopurinol [122]. These results pave some way for clinical exploration of oral POA as a therapeutic alternative or an add-on to overcome *pncA*-mediated PZA resistance and salvage this essential TB drug.

1.6.3. Targets of PZA

Given the alarming rise of resistance to TB drugs worldwide, and especially the emergence of MDR, XDR and TDR strains that are PZA-resistant, the identification of precise targets for PZA is critical for the future of TB control [125]. A promising target for drug development should be essential for survival of the human pathogen and absent from the human host, which should hopefully result in the development of non-toxic therapeutic agents to treat infectious diseases. Although the mechanism of PZA activation and resistance has been characterized, the precise targets and mycobacterial functions that are inhibited by POA remains elusive. Zimhony and colleagues [105] suggested the target of PZA to be fatty acid synthetase (FAS-I) in a study conducted using *M. smegmatis* and the PZA analog 5-chloro-pyrazinamide (5-Cl-PZA). They found that the eukaryotic-like *fas1*gene [126] from *M. avium, M. bovis* BCG or *M. tuberculosis* conferred resistance to 5-Cl-PZA when present on multi-copy vectors in *M. smegmatis.* 5-Cl-PZA and PZA markedly inhibited the activity of *M. tuberculosis* FASI, the biosynthesis of C_{16} to C_{24}/C_{26} fatty acids from acetyl-CoA [127]. They reported that PZA inhibited FASI in *M. tuberculosis* in

correlation with PZA susceptibility. However, no mutations in *fasI* have been found in PZA-resistant *M. tuberculosis* strains. In another study, Boshoff and colleagues showed that FASI was the target of 5-Cl-PZA but not the target of PZA [120]. In addition, other studies have shown that 5-Cl-PZA and PZA act very differently as 5-Cl-PZA is converted by PzaA, a second PZase enzyme not related to *pncA* and not present in *M. tuberculosis*, to less active 5-Cl-POA [128]. In *M. tuberculosis*, PZA is converted into POA by PZase encoded by *pncA* gene [99]. Despite some *in vitro* activity of 5-Cl-PZA, it exhibited no activity against *M. tuberculosis* or *M. bovis* in a mouse model [129]. In another study [130], the overexpression of FASI (target of 5-Cl-PZA) and PzaA (involved in inactivating 5-Cl-PZA) caused 5-Cl-PZA resistance in *M. smegmatis*. However, overexpression of FASI in *M. tuberculosis* was toxic. Further, studies on FASI as a possible target of PZA in cell-free assays or in whole cells were questionable as extremely high concentrations of PZA or POA above the physiological concentrations were used.

Recently, Shi et al. [101] identified a new target of POA as the ribosomal protein S1 (RpsA), a vital protein involved in protein translation and the ribosome-sparing process of trans-translation. Overexpression of RpsA caused resistance to PZA in *M. tuberculosis* as it caused a 5-fold increase in the MIC of PZA (MIC=500 μg/mL) compared with the vector control (MIC=100 μg/mL) at pH 5.5. Previously, this group [131] had identified a low level PZA-resistant *M. tuberculosis* clinical isolate DHM444 (MIC=200–300 μg/mL PZA) that lacked *pncA* mutations, but which later they found it contained a ΔA438 deletion at the C-terminus of RpsA protein [101]. They overexpressed and purified the mutant RpsAΔA438 protein, and found that unlike the wild type H37RV RpsA protein, it did not bind to POA using isothermal titration calorimetry. However, inconsistent with these results, strain DHMH444 had previously been shown to be fully susceptible to both POA [88] and PZA in a murine model of infection [132]. Moreover, trans-translation was more recently shown to be nonessential for growth under conditions that promote PZA susceptibility, fora strain defective for trans-translation was found to be fully susceptible to PZA [133]. Thus, inhibition of trans-translation is not likely to explain PZA mediated inhibition of *M. tuberculosis* growth. It thus means that further work is critical to resolve direct RpsA targeting by POA and its actual role in PZA resistance.

More recently Shi and colleagues [134], also identified a new gene, *panD* encoding aspartate decarboxylase as a new target of PZA in *M. tuberculosis*. They isolated 30 POA-resistant

mutants lacking mutations in *pncA* and *rpsA* from *M. tuberculosis in vitro*, and whole-genome sequencing of 3 mutants identified various mutations in the *panD* gene. Additionally, sequencing analysis revealed that the remaining 27 POA-resistant mutants all harbored *panD* mutations affecting the C-terminus of the PanD protein. PanD is involved in synthesis of β-alanine that is a precursor for pantothenate and co-enzyme A biosynthesis. Inducible PanD overexpression caused significant resistance to POA and PZA in *M. tuberculosis*. Shi and colleagues [134] found out that the activity of the *M. tuberculosis* PanD enzyme was inhibited by POA at therapeutically relevant concentrations in a concentration-dependent manner but was not inhibited by the prodrug PZA or the control compound nicotinamide. These finding suggests that *panD* mutations are closely associated with POA resistance and that the C-terminus of the PanD protein may be involved in POA binding. It is likely that PanD is a target of PZA and that POA binding to PanD could inhibit synthesis of pantothenate and co-enzyme A which may be critical for persister TB bacteria. Considering that FASI is pantothenate dependent and coenzyme A plays a crucial role in carbon and energy metabolism, the targeting of PanD by PZA provides a potential explanation for the pleiotropic effects of PZA on *M. tuberculosis*. However, additional genetic and biochemical studies are essential to delineate a mechanistic link between PanD and PZA action. While the targets described above are not mutually exclusive, many questions remain in refining our understanding of the PZA mode of action. This is especially so since some PZA-resistant clinical strains that do not have mutations in the *pncA, rpsA* or *panD* genes have been reported [107, 135, 136] indicating the presence of a possible new targets and resistance mechanisms of PZA.

Moreover, *M. tuberculosis* Rv2783 along with RpsA and two other proteins were previously reported to bind to a POA derivative, 5-hydroxyl-2-pyrazinecarboxylic acid and not the ethanolamine control column [101]. *M. tuberculosis* Rv2783 is a probable bifunctional enzyme: polyribonucleotide nucleotidyltransferase (PNPase), a component of the RNA degradosome involved in mRNA degradation [137], and guanosine pentaphosphate synthetase (GpsI), involved in the synthesis of the alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (collectively referred to as (p)ppGpp) [138] involved in regulating growth and several physiological processes commonly referred to as the stringent response [139] in several bacteria. *M. tuberculosis Rv2783c* gene is located within a cluster of co-oriented open reading frames and is deemed

essential for growth of *M. tuberculosis* under laboratory conditions as no transposon insertion mutants within the gene was recovered [140].

The alarmones ppGpp and pppGpp are formed by the addition of a pyrophosphate moiety to the 3′ position of GDP and GTP, respectively [141]. The stringent response and (p)ppGpp play important roles in the regulation of bacterial virulence [142], survival during host invasion [143], antibiotic resistance [144] and persistence [145]. In *E. coli*, (p)ppGpp is produced by the activity of the RelA protein and hydrolyzed by the SpoT protein [139]. *M. tuberculosis* contains a single homolog of these proteins, Rel_{Mtb}, encoded by *Rv2583c*, which responds to nutrient starvation by producing (p)ppGpp and thus facilitating long-term survival of non-replicating persister bacteria during chronic infection [146, 147]. Rel_{Mtb} is a dual-function enzyme carrying out ATP:GTP/GDP/ITP 3'-pyrophosphoryltransferase and (p)ppGpp 3'-pyrophosphohydrolase reactions [148]. In addition to $Rel_{Mtb,}$ *M. tuberculosis* Rv1366 protein has been implicated in catalyzing p)ppGpp synthesis, however its role in maintaining (p)ppGpp levels during stressful conditions and virulence has yet to be investigated [149]. To date, the probable ppGpp synthetase and hydrolase activities of *M. tuberculosis* Rv2783 have not been explored. We therefore focused our attention on investigating whether or not *M. tuberculosis* Rv2783 is involved in the regulation of intracellular concentrations of ppGpp, which is vital for long-term survival of the bacilli during dormancy.

Because of the known biochemical functions of PNPase and GPSI in bacteria such as *E. coli* and *S. antibioticus* and [137, 138, 150], we set out to investigate the same biochemical activities in *M. tuberculosis* Rv2783 and their role in in *M. tuberculosis* PZA resistance and the implications of POA binding to RV2783. To date, the genetics of mycobacterial PNPase has received little attention, with the lone published study dating back to 1964 [151]. Recently, the *M. smegmatis* PNPase was characterized and found to possess DNA polymerization and phosphorolysis activities besides its active role in RNA metabolism [152]. The DNA modifying activities of *M. smegmatis* PNPase coupled with an earlier report by Juan Alonso and colleagues [153] suggested that mycobacterial PNPases could possibly be involved in DNA repair and mutagenesis *in vivo*. Juan and colleagues provided genetic evidence that *B. subtilis* PNPase participates in the homologous recombination (HR) and non-homologous end joining (NHEJ) pathways of double-strand break (DSB) repair in response to damage by hydrogen peroxide [153]. Considering that mycobacteria possess

three distinct pathways for the repair of DNA double-strand breaks [HR, NHEJ, and single-strand annealing (SSA)], we hypothesized a similar participation by *M. tuberculosis* Rv2783 (PNPase). While the mechanisms by which PNPase affects DNA repair are not yet clear, Alonso and colleagues suggest that PNPase reacts with broken DNA ends, either converting them from non-ligatable "dirty" breaks to clean ends that can be sealed by DNA ligase or by adding non-templated single-stranded 3′ tails that can then influence repair pathway choice [153, 154].

RNA degradosome, a multi-enzyme complex comprised of proteins such as RNAse E and PNPase, is involved in RNA metabolism and post-transcriptional control of gene expression in *E. coli* and many other bacteria [155-157]. Although the mechanism of action of RNA degradosome in *E. coli* is relatively well understood, very little is known about the mechanisms involved in the RNA metabolism in other species [158, 159], including mycobacteria. *M. tuberculosis* is a very successful pathogen which is able to persist under stressful conditions for a long time in a non-replicating state inside the host [2]. Given the central role of RNA degradosome in RNA metabolism in *E. coli*, it is conceivable that the RNA degradosome-dependent regulation of RNA stability in *M. tuberculosis* might also be a very important mechanism, adjusting the cellular metabolism to environmental stress. It has been reported during dormancy, the bacilli exhibit a decline in sensitivity to antibiotics and an increase in RNase E levels [160], which is a component of the RNA degradosome. Likewise, we conjecture a similar involvement of *M. tuberculosis* PNPase in the regulation of RNA metabolism of the non-replicating persister bacilli during dormancy. Taken together, *M. tuberculosis Rv2783c* protein merited our consideration as essential gene for the bacilli survival growth under conditions that impact PZA susceptibility.

1.6.4. Mechanisms of PZA resistance in *M. tuberculosis*

Despite the association of some gene mutations to drug resistance in *M. tuberculosis,* some phenotypic anti-TB drug resistances are not associated with any known mutation or resistance mechanism. For successful treatment and control of TB, the availability of diagnostic tools that are user friendly, inexpensive and can provide rapid results of drug sensitivity profile of a *M. tuberculosis* strain is of great importance. However, regarding the epidemiology of TB, and also in view of the need to develop new anti-TB drugs, it is paramount to further our knowledge on the molecular basis of drug resistance to major anti-

TB drugs and all its complexity. For example, it is necessary to show the contribution of specific mutations and the emergence of MDR-TB or the complex interaction between drug resistance and fitness. This would allow better evaluation of the emergence and spread of resistant strains and more accurate prediction of a future trajectory of drug-resistant TB. Eventually, such knowledge will contribute to the development of new diagnostic tools and drugs; something that is urgently needed, when taking into account the increasing rates of drug-resistant variants globally. It is on this account that this article presents an updated review of resistance mechanisms of *M. tuberculosis* against PZA. Unlike common antibiotics that act on growing bacteria, PZA is dissimilar in that it has no activity against growing *M. tuberculosis* bacteria in normal pH. It is this unique synergistic activity that makes PZA indispensable in TB therapy.

Considering that PZA is such an important drug in the current and future regimens for drug-susceptible, drug-resistant TB and latent TB, a better understanding of its molecular mechanisms of action and resistance is requisite in the exploration of novel drug targets and resistance mechanisms in *M. tuberculosis*. While the availability of new genetic information derived from the complete genome sequence of *M. tuberculosis* and major advances in molecular biology has increased knowledge of the mechanisms of drug action and resistance to the main anti-TB drugs, this has not been much the case for this indispensable first-line drug. Although PZA resistance in *M. tuberculosis* was shown by McDermott's group to be related to loss of nicotinamidase and pyrazinamidase in 1967 [161], the mechanism of PZA resistance was not known until 1996 when mutation in the *pncA* gene encoding nicotinamidase and pyrazinamidase was demonstrated to cause PZA resistance [99].

1.6.4.1. Role of *pncA* mutations in PZA resistance

In 1996, the *pncA* gene was confirmed to be strongly associated with PZA resistance in *M. tuberculosis* [99], and in the following year, it was found that *pncA* mutations constituted the principal mechanism (70-97%) of PZA resistance [103]. However, some (3–30%) PZA-resistant strains without *pncA* mutations have been reported [107, 136]. This indicates that other genes and mechanisms are involved in PZA resistance. Studies have showed that mutations in recombinant *pncA* decreased the enzymatic activity to about 10-fold, depending on the localization and type of mutation [162, 163]. The important residues associated with *pncA* function are those of the active site (Asp8, IIe133, Ala134 and

Cys138); and mutations at these residues highly affect the PZase function [162-165] (**Figure 5**).

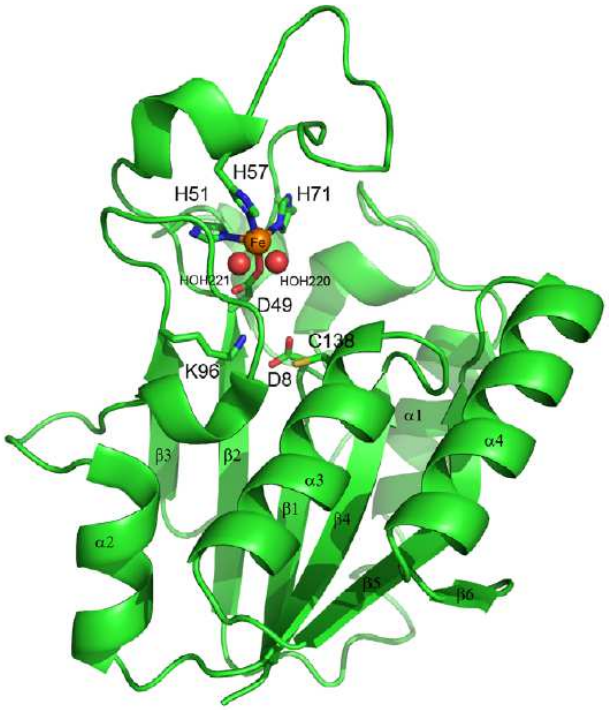

Figure 5: Ribbon representation of the structure of the *M. tuberculosis* pyrazinamidase.

The secondary structures, the iron binding site (Asp49, His51, His57, and His71) and the catalytic triad (Cys138, Asp8, Lys96) are annotated. The iron ion is represented by the orange sphere, the two water molecules by red spheres [165]. Illustration adopted from a previous report [165].

Most of the reported alterations in *pncA* occur in the 561 bp region of the open reading frame or in an 82 bp region of its flanking region [99]. However, the mutations in *pncA* are highly diverse within the *M. tuberculosis* complex in different geographical regions [166, 167]. Clinical isolates with new *pncA* mutations are encountered frequently, which hampers the development and application of diagnostic tools based on the molecular characterization of gene mutations. To link these new mutations to PZA phenotypic resistance usually requires going back to the conventional DST [168]. However, the issues of reliability of conventional DST are also of concern, as previously alluded.

In this regard, a very recent study by Tan et al. [169] reported the regional disparities in *pncA* mutations and the highly diverse patterns in *pncA* mutations. In this study, the *pncA* mutations were randomly dispersed along the entire gene and observed at a high ratio in

PZA-resistant clinical isolates in southern China. Of the clinical isolates with *pncA*-plus-FR (flanking region) mutations, 28.9% (13 of 45) were new, which displayed the high diversity of mutations in the *pncA* gene in different regions. In addition, some PZA-resistant strains associated with *pncA* mutations do not show mutations in *pncA* or its regulatory region. In this case, it has been postulated that resistance to PZA could be due to mutations occurring in an unknown *pncA* regulatory gene. An alternative explanation could be the difficulty in performing drug susceptibility testing for PZA and that these strains are falsely resistant by the phenotypic test. Furthermore, a small proportion of PZA-resistant strains that have low-level resistance and retain PZase activity are considered to have other alternative mechanism(s) of resistance [170]. The highly specific activity of PZA for *M. tuberculosis*, with little or no activity against other mycobacteria, can be explained by the fact that *pncA* is altered in many species of mycobacteria. For example, in *Mycobacterium bovis* subsp. *bovis*, the natural substitution H57A produces a non-effective PZase resulting into intrinsic resistance to PZA, while *M. canettii* is intrinsically resistant to PZA despite lacking non-synonymous mutations in *pncA* [131].

Correlation of drug resistance with a defect in a specific “drug-resistant” gene has been observed for several antitubercular drugs. Resistance against INH, RIF, EMB and OFX show variable correlation between the drug resistance phenotype and mutations in the drug resistance genes *katG* (31–97 %)/*inhA* (8–43 %), *rpoB* (90–95%), *embBC* (47–65%) and *gyrA/gyrB* (75–94%), respectively [171, 172]. However, PZA appear to have multiple cellular targets resulting in a variable correlation between PZA resistance and the presence of mutations in the *pncA* gene. A majority (70-97%) of PZA-resistant isolates of *M. tuberculosis* harbor mutations in their *pncA* gene or putative regulatory region. However, some other clinical PZA-resistant isolates with PZase activity and wild type *pncA* gene have also been reported in several studies, indicating the existence of alternate resistance mechanism(s) or target(s) of PZA [112, 130, 173]. In several studies of PZA resistance, low to moderate correlation (41–80%) between PZA resistance and *pncA* mutations has been reported in places such as in Taiwan (41%) [174], Rio de Janeiro, Brazil (45.7%) [175], South Africa (67%) [176], Japan (77%) [177], Brazil (72%) [178], Hong Kong, China (80%) [179], New Zealand (42%) [180] and Thailand (75%) [181]. Similarly, a higher correlation (>90%) such as in South Africa (91-92%) [182, 183], China (91%) [184], Japan (97%) [103] and S. Korea (97%) [185] has been reported. Such a disparity could be attributed to some

extent to the limitation of phenotypic susceptibility testing. False resistance is the most common cause of error in the results of susceptibility testing to PZA. Recently Simons and colleagues [186] reported that this could be influenced by the inoculum size. The ammonia produced by bacterial metabolism can increase the pH of the culture medium therefore inactivating the drug [113]. However, limitations in susceptibility testing are not the sole reason for the varied correlation between PZA resistance and *pncA* mutation. In some studies, susceptibility testing was repeated carefully, including the use of the Bactec MGIT for the same discordant strains. Therefore, identification and understanding of other novel mutations conferring resistance to PZA is highly desirable as this would shed light to new resistance mechanism(s) or target(s) of PZA and hence more effective TB control and treatment.

More intriguing is the lack of correlation between the observed *pncA* mutation genotypes and the resultant PZA resistance phenotypes. The study by Klemens et al. [132] showed the lack of consistent correlation between the absolute MICs and the reductions in organ viable cell counts seen within isolates for which the MICs were ≥256 µg/mL. In their study, PZA at 150 mg/kg had activity in the murine test system against *M. tuberculosis* isolates for which the MICs were ≤256 µg/mL by the broth dilution method. However, treatment with PZA did not reduce organ viable cell counts against isolates for which the MICs were above 256 µg/mL. Furthermore, Aono and colleagues [187] recently reported some novel findings in which three *M. tuberculosis* strains failed to yield *pncA* Polymerase chain reaction *(*PCR) amplicons. Each of the three strains carried a complete deletion of *pncA*, and had MICs exceeding 1600 µg/mL. The first strain contained a 4,475-bp deletion from *Rv2041* to *Rv2046*; the second one contained a 1,565-bp deletion from *Rv2042* to *Rv2044*; while the third strain contained a 6,258-bp deletion from *Rv2037* to *Rv2045*. These results suggest that the entire *pncA* was deleted and that the *M. tuberculosis* strains lacking *pncA* were PZA-resistant. These findings point out to the need of a better understanding of PZA resistance with respect to the correlations between PZA resistance, PZase activity, and *pncA* mutations. In regard to the above, Yoon and colleagues [188] recently reported on the effect of *pncA* mutations on protein function, where they analyzed expression and PZase activity of 8 *pncA* point mutants identified in 19 PZA-resistant *M. tuberculosis* clinical isolates. Among them, 2 mutants (Y99D and T135P) showed high expression level and solubility

comparable to those of the wild-type PZase protein, 2 (K48E and G97D) displayed low expression level and solubility, and four (C14R, H51P, W68S, and A146V) were insoluble.

Interestingly, when possible structural effects of these mutations were predicted by the Cologne University Protein Stability Analysis Tool (CUPSAT) program based on the proposed three-dimensional structure of *M. tuberculosis* PZase, only the 2 highly soluble mutant proteins (Y99D and T135P) were predicted to be stabilizing and had favorable torsion angles. However, the others exhibiting either low solubility or precipitation were foreseen to be destabilizing and/or had unfavorable torsion angles, suggesting that the alterations could have interfered with proper protein folding, thereby decreasing or depleting protein solubility. A PZase activity assay demonstrated virtually no activity for two mutants (G97D and T135P), while other two mutants (K48E and Y99D) exhibited wild-type activity, indicating that the PZase residues (Cys14, His51, Trp68, Gly97, Thr135, and Ala146) may be important for PZase activity and/or proper protein folding. In addition, other reports have also suggested the existence of alternative mechanisms of PZA resistance that do not affect the PZase structure and activity or POA target.

1.6.4.2. Role of *rpsA* mutations in PZA resistance

Recently, Shi et al. [101] demonstrated that the binding of POA to the 30S ribosomal protein S1 inhibits the trans-translation activity required for efficient protein synthesis. However, mutations in *rpsA (Rv1630,* 1446 nucleotides*)*, which encodes the S1 protein, result in altered POA binding and can thus mediate PZA resistance in strains wild type for the *pncA* gene ($pncA^{WT}$). Previously, out of 38 PZA-resistant clinical isolates, this group [131] had identified a low level PZA-resistant *M. tuberculosis* clinical isolate DHM444 (MIC=200–300 μg/mL PZA) that lacked *pncA* mutations. Later, Shi et al. [101] sequenced the *rpsA* gene from the DHM444 strain and found that it contained a ΔA438 deletion at the C-terminus of RpsA protein. They further overexpressed and purified the mutant RpsAΔA438 protein, and found that unlike the wild type H37RV RpsA protein, it did not bind to POA using isothermal titration calorimetry. Shi and colleagues aligned the protein sequence of RpsA of different mycobacterial species and found out that the C-terminal region, where the ΔA438 deletion occurred in the PZA-resistant *M. tuberculosis* strain DHM444, is also the region that varies most between PZA-sensitive and -resistant mycobacterial species indicating that changes in this region may alter PZA susceptibility [101]. Trans-translation

is essential for rescuing stalled ribosomes during translation in non-replicating organisms, and its inhibition may explain the ability of PZA to eradicate persisting organisms. PZA inhibition of the trans-translation process may therefore interfere with survival under stressful, non-replicating conditions in *M. tuberculosis* [101]. This finding that POA binds to RpsA and inhibits the trans-translation process helps to explain how diverse stress conditions, such as starvation, acid pH, hypoxia, energy inhibitors and other drugs could all potentiate PZA activity [106].

In another recent study, Tan et al. [169] also supported the inclusion of the C-terminal of the RpsA protein as a new target of POA. In their study, 3 of 7 phenotypically PZA-resistant but genotypically $pncA^{WT}$ + FR^{WT} isolates had *rpsA* mutations (E433, A438, and R474) which were all clustered in the C-terminus of RpsA protein and highly conserved among all the mycobacteria spp. One PZA-susceptible clinical isolate also contained one Q162R single mutation which was away from the C-terminal, as were all other *rpsA* mutations in the PZA-sensitive strains. An alignment of C-terminal amino acid sequences of RpsA from 33 strains of 28 mycobacterial species was highly variable. This observation was also concordant with an earlier study by Ilina et al. [189] who reported a PZA-sensitive strain with a mutated *rpsA* (M432T) at its C-terminal. The frequency of *rpsA* mutations in the study by Tan and colleagues [54] showed no significant difference (P=0.099) between PZA-resistant and PZA-susceptible isolates, which they attributed to the small sample size of PZA-resistant isolates. The low frequency of *rpsA* mutations in this study again indicated this was not the main mechanism of PZA resistance in *M. tuberculosis*, although it could affect PZA susceptibility [101]. From these reports, there is need for further verification of the influence of these *rpsA* mutations on PZA susceptibility, because several studies did not find any meaningful *rpsA* mutations in their assays.

In Canada, Alexander and colleagues [136] reported no *rpsA* mutations in 11 PZA-resistant clinical isolates that were genotypically wild type for the *pncA* gene. However, one *rpsA* mutation (A364G), located at the C-terminal of the RpsA protein was found in one PZA-sensitive strain. In their conclusion, Alexander et al. noted that even though the RpsA protein may contribute to PZA resistance, *rpsA* sequencing required analysis of five overlapping PCR products, and no phenotypically informative mutations were identified. This again indicates that DNA sequencing of *pncA* but not *rpsA* would be a robust tool for routine and rapid verification of PZA susceptibility in *M. tuberculosis*. Simons and

colleagues [135] further supported this suggestion when they reported that only 1 of 5 PZA-resistant isolates with *pncA*^{WT} had an RpsA (V260I) mutation. However, the reported mutation by Simons et al. was even questionable based on the disparity of the reported mutated codon and the amino acid that it encoded. In a more recent study, Akhmetova and colleagues [190] also reported that gene sequencing revealed that mutations in *pncA* and its FR, but not in *rpsA*, occurred in PZA-resistant *M. tuberculosis* isolates circulating in Kazakhstan. Gene sequencing showed that only synonymous substitutions occurred in the *rpsA* gene of 3 out of 5 phenotypically PZA-resistant clinical isolates with wild type *pncA* gene [190]. In light of the above, the impact of *rpsA* mutations in PZA resistance seem to be innocuous and thus more studies that correlate PZA MICs with *rpsA* mutations would be necessary to define specific *rpsA* mutations that confer PZA resistance. The PZA-resistant determinant region may be used as a second-step molecular marker as indicated by Tan et al [55].

1.6.4.3. Role of *panD* mutations in PZA resistance

Furthermore, some other PZA-resistant strains lack mutations in both *pncA* or *rpsA* genes and their flanking regions. Identifying new mechanisms of PZA resistance has been challenging due to the diverse genetic background of the clinical strains that differ from each other and from the sequenced type strains [107]. To identify potential new mechanisms of PZA resistance, Zhang et al. [107] isolated 174 *in vitro* generated isogenic mutants of *M. tuberculosis* H37Rv that were PZA-resistant and characterized them for novel mutations in their genomes by whole genome sequencing. Sequence analyses identified 5 low level PZA-resistant isolates without *pncA* or *rpsA* mutations which had mutations in the *panD* gene encoding aspartate alpha-decarboxylase, which is involved in synthesis of β-alanine that is a precursor for pantothenate and co-enzyme A biosynthesis. In addition, Zhang et al. [107] also found a T400C nucleotide change causing amino acid substitution of P134S in an MDR *M. tuberculosis* clinical isolate, and in the naturally PZA-resistant *M. canettii* (M117T). In a follow-up study by the same group [134], they isolated 30 POA-resistant mutants lacking mutations in *pncA* and *rpsA* from *M. tuberculosis in vitro*, and whole-genome sequencing of 3 mutants identified various mutations in the *panD* gene. Further sequencing analysis revealed that the remaining 27 POA-resistant mutants all harbored *panD* mutations affecting the C-terminus of the PanD protein, with PanD M117I mutant being the most frequent mutation (24/30, 80%) [134]. Inducible PanD overexpression caused significant resistance

to POA and PZA in *M. tuberculosis*. In addition, they found out that the activity of the *M. tuberculosis* PanD enzyme was inhibited by POA at therapeutically relevant concentrations in a concentration-dependent manner but was not inhibited by the prodrug PZA or the control compound nicotinamide. These finding suggests that *panD* mutations are closely associated with POA resistance and that the C-terminus of the PanD protein may be involved in POA binding, and in particular to residue M117, which was most altered in the POA-resistant mutants, resulting to inhibition of PanD enzymatic activity.

In another most recent study [108], evaluation of PZA activity against various laboratory strains of *M. tuberculosis* revealed that supplementation of cultures with β-alanine, pantothenate, or pantetheine could antagonize the action of PZA and some of its structural analogs. These findings were consistent with the previous [107, 134] indication of a link between pantothenate synthesis and PZA action. However, when a pantothenate-auxotrophic strain was cultivated using a sub-antagonistic concentration of pantetheine in lieu of pantothenate, susceptibility to PZA and POA was restored [108]. In this study, β-alanine was also found not to antagonize PZA and POA activity against the pantothenate-auxotrophic strain, indicating that the antagonism was specific to pantothenate. In addition, Dillon and colleagues [108] found that an *M. tuberculosis* strain with *panC* (encoding pantoate β-alanine) and *panD* deleted could be cultivated with pantetheine at a concentration that did not antagonize PZA activity, indicating that *panD* is unlikely to be the principal target for PZA. This finding uncouples pantothenate synthesis and PZA action, indicating that pantothenate-mediated antagonism occurs via a novel mechanism that is independent of *panD*. Taken together, these data demonstrated that while pantothenate can interfere with the action of PZA, pantothenate synthesis is not directly targeted by PZA. These findings suggest that targeting of pantothenate/ Coenzyme A synthesis has the potential to enhance PZA efficacy and possibly to restore PZA susceptibility in isolates with *panD*-linked resistance.

Earlier on, *panD* mutations in *M. tuberculosis* had been shown to cause higher attenuation of virulence in mice than BCG vaccine [191], further indicating the critical importance of the gene for survival and persistence of the bacilli *in vivo*. The possibility then that PZA may inhibit pantothenate and CoA synthesis thereby interfering with diverse metabolic functions such as energy production and fatty acid metabolism in *M. tuberculosis* needs to be further verified. Although Zhang et al. [107] identified a few other mutations such as

mutations in *hadC* (β-hydroxyacyl-acyl carrier protein dehydratase) involved in cell wall mycolic acid elongation in the PZA-resistant mutants without *pncA* or *rpsA* mutations, they are less likely causal in PZA-resistance. This is because mycolic acid synthesis occurs in actively growing TB bacilli, and inhibition of HadC by PZA while cannot be excluded, is inconsistent with the specific sterilizing activity of PZA for semi-dormant persister bacilli. Nonetheless, further studies are required to verify the actual role of *hadC* mutations in *M. tuberculosis* PZA resistance.

1.6.5. PZA susceptibility testing and diagnostic implications

PZA DST is crucial for successful management of patients with susceptible and drug-resistant TB, especially with MDR/XDR/TDR–TB. Furthermore, as mentioned earlier, future shorter regimens for both DS- and DR-TB will include PZA as a key drug for achieving both sterilization and prevention of the development of drug resistance to new drugs. Thus, reliable PZA resistance data for clinical isolates are crucial for guiding the clinical management of patients. The Clinical and Laboratory Standards Institute (CLSI) - recommended method for PZA testing [192] is the Bactec 460TB radiometric system (Becton Dickinson, Sparks, MD). Most laboratories have however replaced the 460TB system with the non-radiometric Bactec MGIT 960 (BT960) system (Becton Dickinson, Sparks, MD). Both methods utilize an acidified Middlebrook broth and an MIC cut-off for resistance at 100 µg/mL. However, the BT960 system has generated inconsistent test outcomes when assessing PZase activity and the presence of *pncA* mutations [182, 193, 194]. The BT960 system does not give a 100% agreement when compared with the 460TB reference method, and most cite problems with false resistance. The effects of inoculum concentration, volume, and homogeneity contribute to the lack of reproducibility in BT960 PZA tests [195, 196]. Several differences exist in the inocula used for testing PZA in the two systems. Firstly, in the 460TB, the ratio of inoculum to medium is 1:42, whereas in the BT960 system, the ratio is 1:16.6. Thus, the concentration of inoculum in the test medium in the BT960 is more than 2.5 times greater than that used in the 460TB. The volume of inoculum used in the BT960 is 0.5 ml, while in the 460TB is 0.1 ml. The higher concentration and volume of inoculum used in the BT960 could possibly contain PZA-resistant bacilli, as the probability of finding cells resistant to any drug in *M. tuberculosis* is between 1 in 10^7 and 1 in 10^{10} cells [196]. Secondly, there is variation in the concentration of the inoculum used in the BT960 test according to the day of test setup. Usually, there is

no dilution of the MGIT seed vial for the first and second day after the culture flags positive, but from the third to the fifth day, the inoculum is diluted 1:5. This may lead to considerable variation in the amount of the bacilli in the inoculum and could cause the lack of reproducibility found during repeat testing. Thirdly, the inoculation method differs between the two systems. A fine-needle tuberculin syringe is used in the 460TB, while the inoculum is dispensed with a disposable pipette tip in the BT960, which may result in uneven distribution of bacilli due to "clumping." Following the differences aforementioned between the BT960 and 469TB, susceptibility testing using the BT960 alone could lead to a significant number of false resistant and erroneous results for PZA [197], as well as therapeutic issues in patient management.

The ambiguous results obtained with culture-based DST of PZA are attributed to poor buffering of test media, the use of acidic medium pH that inhibits bacilli growth, and excessively large inocula that reduce the activity of PZA, thereby leading to false resistance [198]. As mentioned, the antimicrobial activity of PZA is highly sensitive to pH. In liquid media, there is a narrow pH range bounded on the alkaline side by no activity, and on the acid side by complete inhibition of growth in drug-free medium [102]. As growth in this pH range is usually very slow even in the absence of PZA, the drug often appears to exert only slow or no bactericidal activity. Using the BACTEC 7H12B medium (pH 5.6), Heifets and Lindholm-Levy [199] found that PZA killed 0, 33, 60, 68, 57, and 72% of the bacterial population at 31, 62, 125, 250, 500 and 1000 µg/ml during a 2-week drug exposure period. The minimum bactericidal concentration (MBC, defined as killing 99% bacterial population) could not be determined since even at the highest concentration used (1000 µg/mL) PZA killed no more than 72% of the bacterial population. This concentration is at least 20 times greater than the MIC (50 µg/ml) and the peak concentration attainable in humans (30–60 µg/mL) [200].

The Wayne PZase test [201] is oftenly used as an alternative, although it is usually difficult to interpret the results. In brief, a heavy inoculum of fresh mycobacterial culture is inoculated into 16 x 125 mm glass tubes containing 5 mL Dubois broth medium supplemented with 100 µg/mL PZA and 2 mg/mL sodium pyruvate. After incubation at 37°C for 4 or 7 days, 1 mL of freshly prepared 1% ferrous ammonium sulphate is added to each tube, and the cultures are placed in the refrigerator for 4 hours. Then the tubes are examined for a pink band in the agar. Appearance of a pink band on diffusion of the ferrous

salt is indication of hydrolysis of PZA to free POA. Gonzalo and colleagues [202] recently proposed a further alternative of testing PZA drug susceptibility using a biphasic media assay (BMA). The BMA might serve as a reliable low-cost DST alternative for PZA, particularly in laboratories using locally made solid media. However, its major drawback is the time to result.

Host cell-based methods using macrophages of either human or animal origin are also documented [115, 118, 203, 204]. However, these methods are limited by the heterogeneity of different batches of animal cells and subsequent problems of reproducibility. In addition, it takes more than 2 weeks to obtain results by the conventional method, because the number of live bacilli has to be counted after growth on agar plates [118]. Nevertheless, data on the actual activity of PZA against intracellular *M. tuberculosis* are controversial, with some authors reporting both inhibitory and bactericidal effects [205], inhibitory activity alone [115], or no activity at all [206, 207].

Considering the aforementioned challenges with existing phenotypic test methods, the design of molecular tests for predicting PZA resistance is a priority especially in settings where drug-resistant TB is increasing. These techniques would help overcome some of the limitations of the classical techniques of phenotypic DST, which may require standard mycobacterial culture for up to several months [71]. Hence, developing improved methods of molecular PZA DST in TB is critical. However, a significant proportion (3-30%) [107, 136] of PZA resistance-conferring mutations has not been systematically investigated [172, 208] and remains unknown. Hence, culture-based PZA DST remains indispensable. Therefore more efforts are needed to unravel novel PZA targets and resistance determinants mechanisms in *M. tuberculosis*, which could then be used to develop more sensitive diagnostic tools for PZA-resistant TB. Once more sensitive molecular diagnostics become available, PZA-resistance profiles could be determined at treatment onset, and drug regimens could be tailored to the individual patient needs especially those infected with MDR/XDR/TDR-TB. Ultimately, this would help curb the transmission of drug-resistant strains. Therefore, it is on this account of these gaps that this study was undertaken.

1.6.6. PZA efficacy in persistent *M. tuberculosis* bacilli

The unusual efficacy of PZA on persisters was demonstrated using Hu/Coates models of dormant and rifampicin tolerant *M. tuberculosis* [209]. The results obtained with them are

therefore crucial to the overall thesis concerning the bacillary populations against which it is most effective. In model 1, cultures of *M. tuberculosis* incubated without shaking for up to 100 days were sampled, PZA added and bactericidal activity measured. As the duration of incubation and starvation increased from 4 days (log phase) to 30 days and then 100 days, the bactericidal activity of PZA increased. In model 2, the cultures were sampled immediately after selection of the RIF-tolerant population when re-growth was occurring upon subculture into fresh RIF-free medium and when bacterial metabolism was high, and PZA had little bactericidal activity against these actively growing bacilli. In model 3, the cultures were sampled at 3 days after inoculation in RIF-containing liquid medium when growth and metabolism of the sub-population would be expected to be minimal, and PZA was highly bactericidal against this population. The action of PZA in these three models demonstrates clearly that PZA is most bactericidal when cultures of *M. tuberculosis* are the most static. Similarly, starvation was found to decrease the membrane potential in old bacilli and enhanced PZA activity [210]. Starved *M. tuberculosis* had increased expression of *pncA* [211], which could increase pyrazinamidase enzyme levels needed for enhanced conversion of PZA to active form POA, and may thus contribute to increased killing of tubercle bacilli by PZA under starvation conditions.

Following the above, PZA validates a principle that drugs active against non-replicating persisters are important for improved treatment of persistent infections. PZA thus serves as a prototype model persister drug that plays an indispensable role in any new drug combination. Indeed, in a recent study on the sterilizing activities of new drug regimens in the mouse model, all regimens contained PZA [212]. Improved understanding of PZA targets and mechanism(s) of action is thus important for design of new drugs that further shorten TB therapy. From this prototype persister drug, one envisions a future of potent antibiotic for killing the MTB persisters more effectively and thus shorten the duration of chemotherapy.

1.7. Clinical implications of PZA resistance in TB therapy

Several studies such as in Kazakhstan [190], China [169], South Africa [183] and Thailand [103] have indicated that MDR *M. tuberculosis* strains are more likely to harbor PZA resistance, as compared to non-MDR, especially drug-susceptible *M. tuberculosis* strains. For example, the recent study by Tan et al. [169] indicated that of all the MDR strains,

68.4% (39/57) were PZA-resistant and only 12.5% (13/104) of non-MDR strains harbored PZA resistance. From another territory-wide registry of MDR-TB cases diagnosed between 1995 and 2009, Chang et al. [213] assembled a cohort of 194 patients with MDR-TB given fluoroquinolone-containing regimens. Stratified by PZA use and susceptibility, this study reported that PZA use with susceptibility among PZA users considerably increased the incidence proportion of early sputum culture conversion and that of treatment success by a best estimate of 38% for both. As this magnitude of change exceeds the 15 to 20% increase in the 2-month culture conversion rate of drug-susceptible TB that results from adding PZA to INH and RIF [214-216], this study suggested that PZA has an important role in fluoroquinolone-based treatment of MDR-TB. Taken together, these findings emphasize the importance of PZA in treating MDR-TB, and this had earlier on been demonstrated in a murine model in which all PZA-containing regimens tested showed better activities than the corresponding regimens without PZA when combined with second-line drugs [217].

Patients with DR-TB currently require a minimum of 18 to 24 months of treatment. This more extensive therapy requires more than 14,000 pills and daily injections for at least 6 months. The long duration of MDR-TB treatment, combined with the pain and side effects that treatment causes, are the major obstacles to treatment compliance. On the basis of these, on March 18, 2015, TB Alliance and its partners launched a global phase 3 clinical trial named STAND (Shortening Treatment by Advancing Novel Drugs) in both DS- and MDR-TB patients [218]. The STAND trial will test PaMZ, a three-drug regimen comprised of two candidate drugs that are not yet licensed for use against TB: pretomanid (PA-824) and moxifloxacin, and PZA [218]. STAND researchers expect to enroll 1,500 patients in 15 countries in Africa, Asia, Caribbean, Eastern Europe, and Latin America in this study. PaMZ will be tested in STAND as a 4- and 6-month treatment for drug-sensitive TB, a 6-month treatment for patients with MDR-TB susceptible to both PZA and fluoroquinolones, and also enroll those co-infected with HIV. If successful in this Phase 3 trial, the PaMZ regimen would eliminate the need for injectable drugs and reduce the cost of MDR-TB therapy by more than 90% in those patients infected with *M. tuberculosis* isolates susceptible to the three drugs. It also promises to be compatible with commonly used HIV drugs, helping the millions of people co-infected with TB/HIV. Again, these developments signify the importance of rapid detection of PZA resistance in controlling both DS- as well as DR-TB.

A number of molecular methods have been developed for the detection of other TB drug resistance-associated mutations including the line probe assays [219-221]. However, *pncA* mutations associated with majority (70-97%) of PZA resistance are dispersed throughout the entire gene plus it's FR, which makes it difficult to develop probe-based methods that cover all the mutations. So far, the most accurate identification of *pncA* mutations is the polymerase chain reaction (PCR) sequencing. PCR single-stranded conformational polymorphism (PCR-SSCP) is however not sensitive enough to detect all *pncA* mutations, and cannot be used as a screening method for detecting PZA resistance. In addition, the occurrence of about 3-30% of PZA resistance not associated with *pncA* mutations, and the rare association of other genes such as *rpsA* and *panD*, further complicates the development of rapid and reliable molecular detection tools. Therefore, reliable and accurate molecular approaches to detect PZA resistance in clinical isolates need to cover at least a significant number of possible *pncA* mutant variants, plus the *rpsA* and *panD* genes, to reach a high sensitivity. This could be achieved through direct sequencing of the gene amplicons using approaches based on classical Sanger sequencing or next-generation genome sequencing. These techniques must be combined with an appropriate interpretation algorithm and database that distinguishes Single nucleotide polymorphisms (SNPs) clearly associated with PZA resistance from those for which their impact for developing resistance is undefined. Accordingly, knowledge of the variants found in PZA resistant strains combined with evidence-based correlation with resistance phenotypes are needed to develop large-scale databases ensuring valid data interpretation. For example, Miotto and colleagues [222] performed a large multicenter study assessing *pncA* genetic variants based on their predictive value for PZA resistance. The results of p*ncA* sequencing were correlated with phenotype, enzymatic activity, structural and phylogenetic data. They identified 280 genetic variants which were divided into four classes: (i) very high confidence resistance mutations that were found only in PZA-resistant strains (85%), (ii) high-confidence resistance mutations found in more than 70% of PZA-resistant strains, (iii) mutations with an unclear role found in less than 70% of PZA-resistant strains, and (iv) mutations not associated with phenotypic resistance (10%) [222]. The sharing of such elaborate databases of mutations involved in PZA-resistance could contribute to a better understanding of molecular mechanisms of resistance, improved molecular diagnostics, new diagnostic algorithms, and better public health control of DS- and DR-TB.

2. Aim

The aim of this study is to explore molecular targets and mechanisms of action of pyrazinamide in *M. tuberculosis.* This includes analyzing the actual roles of Rv2783, a probable bifunctional enzyme [polyribonucleotide nucleotidyltransferase (PNPase) and guanosine pentaphosphate synthetase (GpsI)]; the reported ribosomal protein S1 (*rpsA*) mutations and the A-11G pyrazinamidase (*pncA*) mutation in *M. tuberculosis* pyrazinamide resistance. A better understanding of this unique sterilizing drug is important for the design of new drugs targeting *M. tuberculosis* persisters for improved treatment; and would also facilitate development and application of rapid and reliable molecular detection tools thereby enhancing the reliability of testing resistance to this increasingly important anti-TB drug.

3. Materials and Methods

3.1. Media and Buffer preparation

Table 3 Luria-Bertani (LB) broth and agar for *E. coli*

Reagent	Quantity	
NaCl（Mw=58.44）	10 g	
Yeast extract	5 g	Autoclave
Tryptone	10 g	
ddH_2O	1L	

For LB agar, the agar was added to a final concentration of 1.5%.

Table 4: NaOH, Oleic acid and Oleate-albumin-dextrose-catalase (OADC) solutions

Reagent	Quantity
• **6M NaOH solution**	
ddH_20	10 mL
NaOH (MW=40)	2.4 g
• **Oleic acid solution**	
ddH_20	120 mL
6M NaOH	2.4 mL
Oleic acid	2.4 ml
• **OADC solution**	
ddH_20	460 mL
NaCl（Mw=58.44）	4.05 g
Bovine serum albumin (BSA)	25 g
Catalase	20 mg
Glucose	10 g
Oleic acid solution	15 mL

Table 5: Middlebrook 7H11 agar for *M. tuberculosis*

Reagent	Quantity	
ddH_20	900 mL	
7H11 powder	19 g	Autoclave
Glycerol	5 mL	

When cool to 65°C, 100 mL of Middlebrook OADC was added.

Table 6: Middlebrook 7H9 broth for *M. tuberculosis*

Reagent	Quantity	
ddH_20	900 mL	
7H9 powder	4.7 g	Autoclave
Glycerol	2 mL	

When cool to 65°C, 100 mL of Middlebrook OADC was added.

Table 7: Super Optimal Broth (SOB) for *E. coli* DH5α competent cells

Reagent	Quantity	Final Concentration	
Yeast extract	5 g	0.5 %	
Tryptone	20 g	2 %	Autoclave
NaCl	584.4 m g	10 mM	
KCL	186.4 mg	2.5 mM	
ddH_2O	Volume to1L	Adjust pH to 6.7-7.0	

When cool to 65°C, sterile $MgCl_2$ solution was added to a final concentration of 10 mM.

Table 8: *E. coli* Transformation Buffer (TB)

Reagent	Quantity	Final Concentration	
$MnCl_2.4H_2O$	10.88 g	55 mM	
$CaCl_2$	1.66 g	15mM	Autoclave
KCl	18.65 g	250 mM	
PIPES (0.5 M, pH 6.7)	20mL	10mM	
ddH_2O	Volume to1L	Adjust pH to 6.7-7.0	

The *E. coli* TB solution was filter sterilized with 0.45μm filter and stored at 4°C.

3.2. Identification of new targets of PZA in *M. tuberculosis*

3.2.1. Isolation of genomic DNA from *M. tuberculosis*

As *M. tuberculosis* is a hazard group 3 pathogen, all stages of the DNA extraction, up to and including the addition of chloroform, were performed in a class 2 biological safety cabinet in a containment level 3 laboratory. DNA extraction of wild type *M. tuberculosis* H37Rv and the 4 previously reported [169] PZA-resistant clinical strains that lacked mutations in *pncA*, *rpsA, panD* and *hadC* and their flanking regions were extracted using the Cetyltrimethyl ammonium bromide (CTAB) method as previously described [223]. Briefly, a loopful of colonies from Middlebrook 7H11agar was added to 400 L 1x TE buffer and incubated at 80 °C for 20 minutes. The tubes were cooled to room temperature and 50 mL 10 mg/mL lysozyme (Sigma, UK) was added. The tubes were incubated at 37 °C overnight. The following morning, 70 mL 10% sodium dodecyl sulfate (SDS) and 5 mL 10 mg/ml proteinase K (Sigma, UK) were added before incubation at 65 °C for 10 minutes. Then, 100 mL of 5M NaCl and CTAB/NaCl (pre-warmed to 65 °C) were added and incubated at 65 °C for 10 minutes. 750 mL chloroform/isoamylalcohol (24:1) was added and the tubes were mixed by inversion. The tubes were centrifuged at 10,000 g for 5 minutes and the upper aqueous phase containing the DNA was removed and added to 450 mL of ice-cold isopropanol. These tubes were mixed gently before chilling at –20 °C for at least 30 minutes. The tubes were centrifuged at 10,000 g for 15 minutes at room temperature, the supernatant was removed and the DNA pellet was washed with 1mL ice-cold 70% ethanol. The tubes were centrifuged at 10,000 g for 5 minutes at room temperature, the supernatant was removed and the pellet was allowed to air-dry. The pellets were rehydrated using TE buffer overnight at 4 °C. The concentration of DNA obtained was estimated by comparing with serial dilutions of lambda DNA using agarose gel electrophoresis. The genomic DNA was used as template for the subsequent PCR reactions.

3.2.2. PCR and DNA sequencing of new genes associated with PZA resistance

To identify possible new genes associated with PZA resistance in 4 previously reported [169] PZA-resistant clinical strains lacking *pncA* and *rpsA* mutations, we amplified 3 genes plus their FRs, (*Rv2731, Rv2783c* and *Rv3169*), which encode proteins previously reported to bind with a POA derivative, 5-hydroxyl-2- pyrazinecarboxylic acid **(Figure 6)** [101], using primer pairs: Rv2731F and Rv2731R; Rv2783cF and Rv2783cR; Rv3169F and Rv3169R, respectively **(Table 9)**. We also amplified the *panD* (*Rv3601c*) and *hadC* (*Rv0637*) genes plus their FRs, also implicated in PZA resistance [101, 134] using primer pairs; panDF and panDR; and hadCF and hadCR **(Table 9**), respectively. We also amplified *pncA* plus its FR using primers pncAF and pncAR (**Table 9**) to verify the absence of any mutation. The PCR master mix was prepared in 25 µL reaction mixture with final reagent concentrations as follows; 1x KCl buffer, which contains $MgCl_2$ at a final concentration of 1.5 mM; 0.2 mM dNTPs, 0.5 µM of each primer and 0.1 µM of *pfu* DNA polymerase (TransGen Biotech, China). Dimethyl sulfoxide (DMSO) (5%) was added to the reaction mixture due to the high G+C content of the mycobacterial genomes. Amplification conditions comprised of an initial denaturation at 95 ℃ for 5 min; followed by 30 cycles of denaturation at 94 ℃ for 30 sec, annealing step for 30 sec (temperature dependent on the specific gene), an extension at 72 ℃ (time duration dependent on the size of the specific gene); then a final extension at 72 ℃ for 10 min. The PCR-amplified DNA products were analyzed by electrophoresis in agarose gels and purified using PCR purification kits (Qiagen, Hilden, Germany). Purified PCR products were sequenced at BGI, Shenzhen, China. The gene sequences from the 4 PZA-resistant strains were compared against the respective wild type gene sequences of *M. tuberculosis* H37Rv to identify potential mutations.

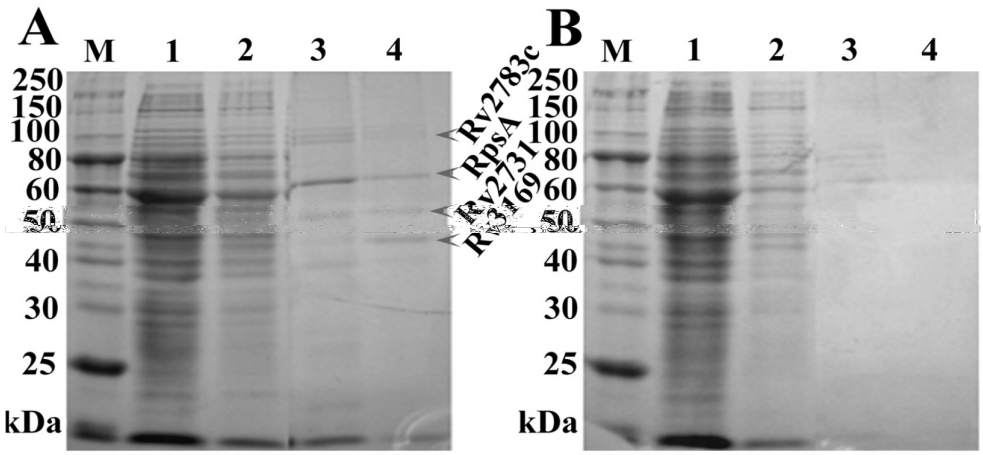

Figure 6: Protein binding study using POA-linked columns.

M. tuberculosis whole cell lysates were loaded onto the POA-linked and control columns and the proteins that bound to POA **(A)** and the control column **(B)** were analyzed by SDS-PAGE. Lane M, protein ladder; Lane 1, whole cell lysate; 2, flow-through fraction; 3, wash fraction; 4, elution fraction. The bands indicated by arrows are Rv2783c, RpsA, Rv2731 and Rv3169, respectively. Figure adopted from a previous report [101].

Table 9: DNA primer used in this study

Primer pairs	Nucleotide sequences (5'-3'), restriction enzyme sites underlined.	Restriction enzymes (F/R)
Rv2731F/ Rv2731R	ATCGGCAAATATCGCG/ GCACCATCGTGCAGCT	
Rv2783cF/ Rv2783cR	GGGAATTCCATATGTCTGCCGCTGAAAT/ CCCAAGCTTATTCGGTGACCACTCG	*Nde*I/ *Hin*dIII
Rv3169F/ Rv3169R	AACTTGGTGACCTGCA/ CCGCATTTCGGCGGTT	
panDF/ panDR	TCGACTACCTGGAGCT/ TGACTTCGGATTCGGT	
hadCF/ hadCR	GGGTGAAATCGGTTGA/ TGAACTCACGGAAAGC	
pncAF/ pncAR	ATTTGTCGCTCACTAC/ ATGCCCCACCTGCGGCT	
Ms2656F/ Ms2656R1	GGGAATTCCATATGTCTGTAGTCGAACT/ AAAAGTACTCGGACCTGCGACGTTTC	*Nde*I/ *Sca*I
HygF/ HygR	AGAGCACCAACCCCGTACTG/ GTGAAGTCGACGATCCCGGT	
Ms2656F/ Ms2656R2	GGGAATTCCATATGTCTGTAGTCGAACT/ ATAAGAATGCGGCCGCCGGACCTGCGACGTTTC	*Nde*I/ *Not*I
RelF/ RelR	GGGAATTCCATATGGTGGCCGAGGACCAG/ CCCAAGCTTAAATCAGCCCGCCCAAT	*Nde*I/ *Hin*dIII
rpsAF/ rpsAR	TCGTCTAGAATTTCCGCCCTGAGTTCAC CCGAAGCTTACGATTCCGCCGCATTG	*Xba*I/ *Hin*dIII
pncAF1	GGAATTCCATATGCGGGCGTTGATCATC	*Nde*I
pncAF2	GGAATTCCATATGATTGGTGTTCCGGGC	*Nde*I
pncAF3	GGAATTCCATATGACACCTCTGTCAC	*Nde*I
pncAR1	CCCAAGCTTTCAGGAGCTGCAAACC	*Hin*dIII

3.2.2.1. Pyrazinamidase assay of the 4 PZA-resistant clinical *M. tuberculosis* strains

PZase activity of the of 4 PZA-resistant clinical strains lacking *pncA* and *rpsA* mutations was evaluated as described by Wayne [201] with some modifications [224]. Briefly, 2 to 3 discrete colonies from the same batch were picked and transferred into 200 µl Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (ADC) medium containing PZA (100 µg/mL and 200 µg/mL) in a 1.5 mL Eppendorf tube. The cells were incubated overnight at 37 °C while shaking. 15 µL of 2% Fe^{2+} and was added to the cells and incubated at 4 °C for 2 hours for color reaction. Wild type H37Rv, was used as a positive controls (PZA-sensitive), while *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG Tice) and a clinical PZA^R strain with a *pncA* mutation were used as a negative controls (PZA-resistant). PZA in the presence of positive PZase enzyme from the *M. tuberculosis* would be converted to POA, which then reacts with ferrous ion to produce a brown colored compound, which can be detected as an indication of positive PZase activity.

3.2.3. Role of *M. tuberculosis Rv2783c* gene in PZA resistance.

A series of experimental manipulations were undertaken to evaluate the actual role and association of *M. tuberculosis Rv2783*c gene in PZA resistance (**Figure 7**).

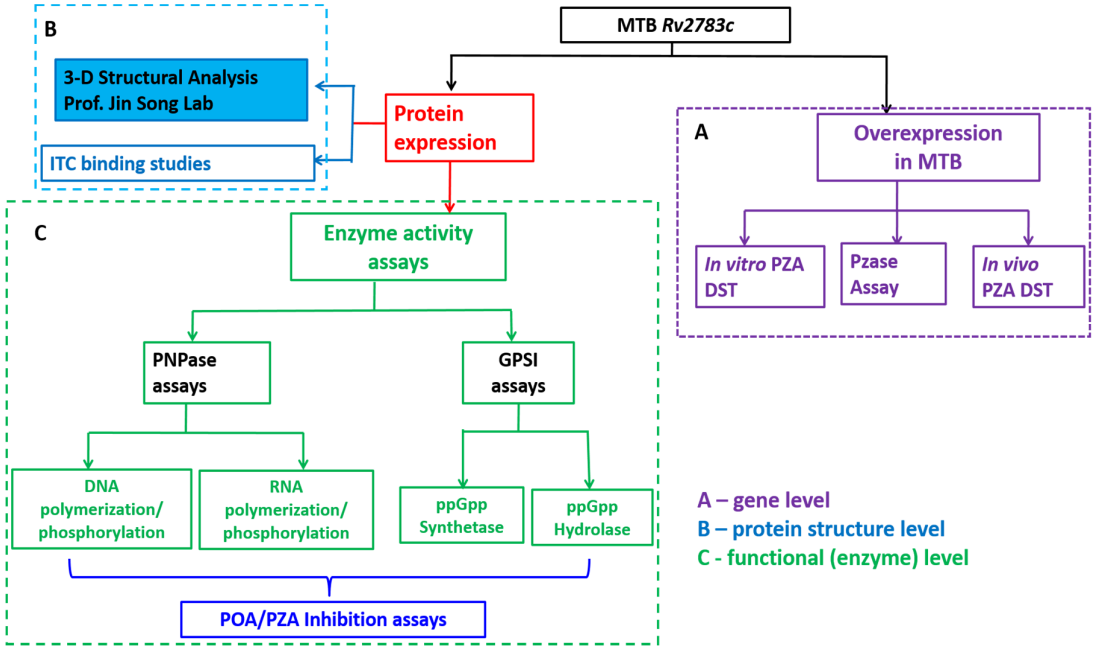

Figure 7: Technique route for evaluating the role of *M. tuberculosis Rv2783c* in PZA resistance.

3.2.3.1. Cloning of *M. tuberculosis Rv2783c* gene

The sequential steps, DNA templates and vectors used to clone and overexpress wild type *Rv2783c* and $Rv2783c_{G199A}$ mutant in wild type *M. tuberculosis* H37Rv plus PZA susceptibility testing of the transformants illustrated in **Figure 8.**

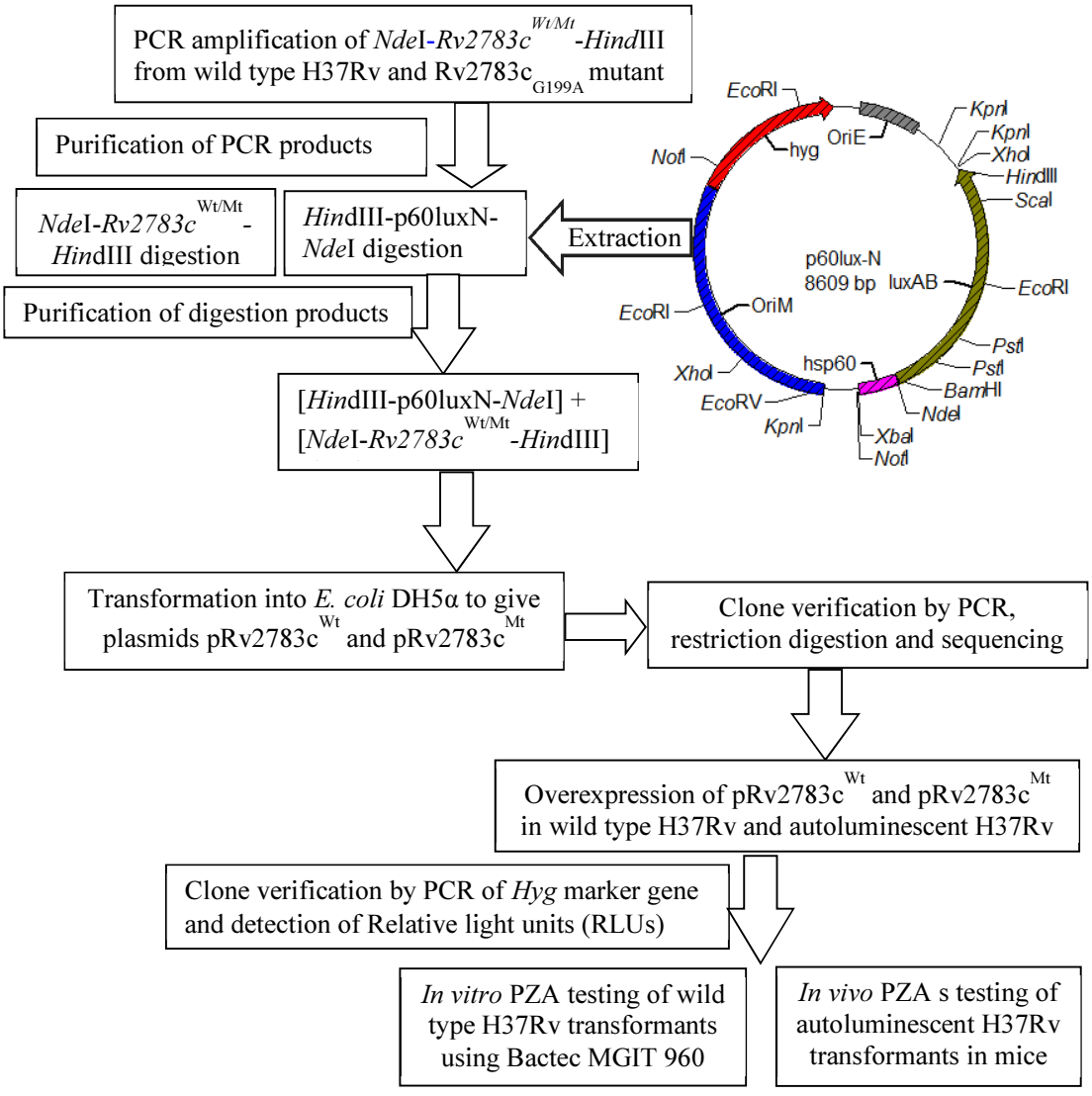

Figure 8: Technique route for cloning, overexpression and PZA susceptibility testing of *M. tuberculosis Rv2783c* transformants.

3.2.3.1.1. PCR Amplification

Genomic DNA from wild type *M. tuberculosis* H37Rv and $Rv2783c_{G199A}$ mutants were used as templates for PCR amplification of the *Rv2783c* gene (2259), using primer pair Rv2783cF

and Rv273cR **(Table 9)**. The PCR master mix was prepared as described in section 4.2.2 above. Amplification conditions comprised of an initial denaturation at 95 ℃ for 5 min; followed by 30 cycles of denaturation at 94 ℃ for 30 sec, annealing at 56.4 ℃ for 30 sec and extension at 72 ℃ for 2.5 min; then a final extension at 72 ℃ for 10 min. The PCR-amplified DNA products were analyzed, purified and sequenced as described in section 4.2.2.

3.2.3.1.2. Restriction enzyme digestion

The p60luxN [225] extrachromosomal plasmid vector carrying the strong *hsp60* promoter was extracted from *E. coli* strain DH5α host cells using a plasmid extraction kit (Magen, China). The purified PCR products plus the extracted p60luxN plasmid vector were digested with the same restriction enzymes as indicated in **Table 10**.

Table 10: Restriction enzyme digestion system

p60luxN / *Rv2783c*$^{Wt/Mt}$	15 µL	
1×K buffer	2 µL	Incubated at 37°C
*Nde*I	1.5 µL	for 8 hours
*Hin*dIII	1.5 µL	
Reaction mixture	**20 µl**	

The digested PCR products were purified using a DNA purification kit (Magen, China) and analyzed by electrophoresis in agarose gels. The digested plasmid vector was first analyzed by electrophoresis in agarose gels and recovered using a plasmid gel purification kit (Magen, China).

3.2.3.1.3. Ligation

The digested plasmid vector, *Hin*dIII-p60luxN-*Nde*I, and the digested PCR fragments, *Nde*I-*Rv2783c*$^{Wt/Mt}$-*Hin*d III were ligated together as in the ligation system in **Table 11**. The ligation mixture was first heated (without enzyme) for 3 minutes at 72℃; chilled on ice immediately (about 5 minutes) and mixed by pipetting several times. One microliter (1µl) of T_4 ligase buffer and T_4 DNA ligase were added to the ligation mixture while on ice, followed by incubation of the mixture for 3 hours at room temperature.

Table 11: Ligation system

Insert DNA [*Nde*I-*Rv2783c*$^{Wt/Mt}$-*Hin*d III]	5 µL	Incubated at room temperature for 3 hours
Promoter + vector [*Hin*dIII-p60luxNew-*Nde*I]	3 µL	
1X T_4 Ligation Buffer	1 µL	
T_4 DNA ligase	1 µL	
Reaction mixture	**10** µL	

3.2.3.1.4. Preparation of competent *E. coli* DH5α cells

E. coli DH5α competent cells were prepared as described by Inoue and colleagues [226] with slight modifications. Briefly, *E. coli* DH5α cells were cultured on LB agar and incubated at 37° C overnight. A loopful of the bacterial cells on LB agar were inoculated into 250 mL super optimal broth (SOB) in a 1 L flask and incubated at 18-20 °C with vigorous shaking to OD_{600} ~ 0.4-0.6. The bacterial culture in flask was placed on ice for 10 min. The cells were centrifuged at 4000 rpm for 10 min at 4 ºC. The pellets were gently resuspended in 80 mL ice-cold TB and chilled on ice for 10 min. The cells were centrifuged again at 4000 rpm for 10 min at 4 °C. The pelleted cells were again gently resuspended in 20 mL ice-cold TB and 1.4 mL DMSO, and chilled on ice for 10 min. The competent cells were then aliquoted (50 µL) while on ice, frozen under liquid Nitrogen and stored at -80 °C.

3.2.3.1.5. Transformation of competent *E. coli* DH5α cells

Ten microliters (10 µL) of the transforming DNA (insert DNA+promoter+vector) was added to 50 µL of *E. coli* DH5α competent cells in eppendorf tubes and mixed gently by pipetting several times. An empty p60luxN vector was included as a control. The reaction mixture was placed on ice for 30 minutes and then placed in a pre-heated water bath at 42 ºC for 90 seconds. The reaction mixture was then rapidly transferred to an ice bath and chilled for 2 minutes. LB broth (1000 µL) was added to the reaction mixture aseptically and transferred to a shaking incubator set at 37 ºC for 1 hour. The transforming mixture from each tube was plated on LB agar medium supplemented with 200 µg/mL hygromycin (Hyg) antibiotic for selection and incubated overnight at 37 ºC. The transforming mixture was also plated on LB agar medium without Hyg as a control. Single transformant colonies were picked, verified by PCR amplification of the respective genes, restriction digested with same cloning enzymes and sequenced at BGI, Shenzhen, China.

3.2.3.2. Overexpression of *Rv2783c* in wild type *M. tuberculosis* H37Rv

The verified mycobacterial recombinant plasmids, p60Rv2783c^{Wt}, p60Rv2783c^{Mt} and p60luxN^{C} (control), were transformed into competent cells of *M. tuberculosis* H37Rv through an electroporation method as previously described [227]. Briefly, 40 mL of wild type *M. tuberculosis* H37Rv in Middlebrook 7H9 broth were transferred into 50 mL centrifuge tube and centrifuged at 5000 rpm for 5 minutes, and supernatant discarded 40 mL of sterile 10% glycerol was added to the bacteria sediment and centrifuged at 5000 rpm for 5 minutes, and supernatant discarded. This was repeated once again and 1 mL of the supernatant was retained. Plasmid DNA (10 µL) and 200 µL of competent *M. tuberculosis* H37Rv cells were added into 0.2 cm electroporation tubes, mixed gently by pipetting and incubated at 37 °C for 10 minutes. Electroporation was then done using a Bio-Rad Gene Pulser electroporation device (2.5 KV voltage, 1000 Ω resistance). The bacteria mixture was then washed off the electroporation cuvettes 2 times using Middlebrook 7H9: OADC media (mixed in the ratio of 9:1 respectively) and transferred into 50 mL centrifuge tubes. The bacteria were incubated at 37 °C for 20 hours. A 500 µL transformation mixture was cultured on Middlebrook 7H11 agar plates supplemented with Hyg (40 µg/mL), wrapped with polythene bags and incubated at 37 °C for 30 days. Colony growth and contamination with other bacteria was monitored on a weekly basis.

3.2.3.2.1. Verification of true *M. tuberculosis* transformants

Single *M. tuberculosis Rv2783c* transformant colonies were subcultured in Middlebrook 7H9 broth containing Hyg (10 µg/mL). After 4 weeks, portions of the single colony subcultures were serial diluted [10^{0} - 10^{-4}], plated on both 7H11 agar with Hyg (40 µg/mL) and without Hyg. The remaining single colony broth cultures were temporarily stored in -80 °C until further use. After 5 weeks, the single colony cultures on 7H11 Hyg plates were verified by amplification of the 529 bp *Hyg* marker gene open reading frame using the primer pair: HygF and HygR **(Table 9).** For the wild type H37Rv transformants, if positive for the *Hyg* gene, the culture 7H9 plates were used for *in vitro* PZA susceptibility testing using the Bactec MGIT 960 system (BD, Franklin Lakes, NJ, USA) at Guangzhou Chest Hospital.

3.2.3.2.2. *In vitro* PZA susceptibility testing of *M. tuberculosis Rv2783c* recombinant strains

PZA susceptibility tests were carried out in MGIT PZA medium (BD, Franklin Lakes, NJ, USA) according to manufacturer's protocol. Briefly, a 0.5 McFarland suspension was diluted into 1:5 and 1:50 in sterile distilled water. Dilutions of 1:50 were inoculated into MGIT PZA medium without drug, while 1:5 dilutions were inoculated into MGIT PZA medium supplemented with 25 - 500 µg/mL PZA drug, and incubated in the MGIT instrument at 37 °C. Results were read automatically within7, 14 and 21 days after inoculation of media. *M. tuberculosis* H37Rv parental strain, susceptible to PZA, was used as a positive control, while *M. bovis* Bacillus Calmette-Guérin (BCG Tice), naturally resistant to PZA, was used as a negative control. At the same time, the *M. tuberculosis Rv2783c* recombinant strains were evaluated for PZase activity as described in section **3.2.2.1** above.

3.2.3.3. Protein expression and purification

3.2.3.3.1. Construction of mycobacterial protein expression plasmids.

The pET-28a-c(+) protein expression vector plasmid **(Figure 9)** containing an N-terminal His-tag was used for expressing the *M. tuberculosis* Rv2783, *M. smegmatis* M.smeg_2656 (positive control for the PNPase assays) and *M. tuberculosis* Rv2583c (Rel_{Mtb}–positive control for the ppGpp synthetase and hydrolase assays) proteins.

The wild type *M. tuberculosis Rv2783c* and *M. tuberculosis* $Rv2783c_{G199A}$ mutant DNA fragments were PCR-amplified from the genomic DNA of *M. tuberculosis* H37Rv and the PZA-resistant $Rv2783c_{G199A}$ mutant respectively, using primer pair Rv2783cF and Rv2783cR (**Table 9**). The *M. smegmatis* PNPase protein, encoded by *M.smeg_2656* gene (2292 bp) was expressed to serve as positive control for the PNPase assays of the *M. tuberculosis* Rv2783 protein. Genomic DNA from wild type *M. smegmatis* was used to amplify the *M.smeg_2656* gene using primer pair Ms2656F and Ms2656R2 **(Table 9)**. The *M. tuberculosis* Rel_{Mtb} protein, encoded by *Rv2583c* gene (2373 bp) was also expressed to serve as positive control for the ppGpp synthetase and hydrolysis assays of the *M. tuberculosis* Rv2783 protein.

Genomic DNA from wild type *M. tuberculosis* H37Rv was used to amplify the *Rv2583c* gene using primer pair RelF and RelR Rv2583cF **(Table 9)**. The PCR-amplified DNA fragments were digested with the appropriate cloning enzymes, ligated to pET-28a(+) plasmid vector digested with the same cloning enzymes and cloned into *E. coli* strain DH5α to yield recombinant plasmids. The PCR master mix was prepared as described in section **3.2.2.** Amplification conditions comprised of an initial denaturation at 95 ℃ for 5 min; followed by 30 cycles of denaturation at 94 ℃ for 30 sec, annealing at 56.4 ℃ (for *Rv2783c*), 53.2 ℃ (for *M.smeg_2656*) and 55.5 ℃ (for Rel_{Mtb}) for 30 sec, an extension at 72 ℃ for 2.5 min; and then a final extension at 72 ℃ for 10 min. The PCR-amplified DNA products were analyzed, purified and sequenced as described in section **3.2.2.** The purified PCR products plus the pET-28a (+) protein expression vector were digested and ligated as described in sections **3.2.3.1.2** and **3.2.3.1.2** respectively**.**

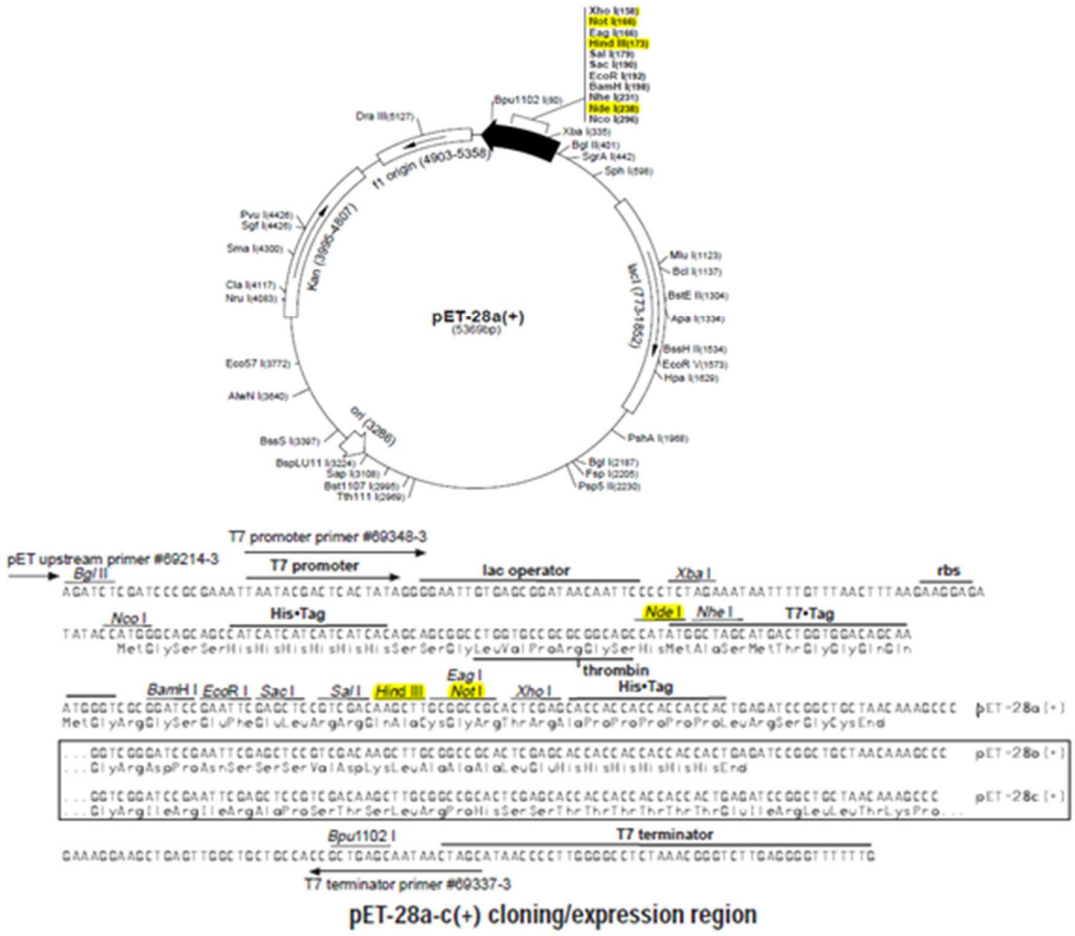

Figure 9: pET-28a-c(+) plasmid vector indicating the restriction enzymes and expression region.

Ligation products were then first cloned into *E. coli* DH5α and verified by restriction digestion and sequencing as described in the preceding sections**.** The plasmids obtained, pETTv2783c^{Wt}, pETTv2783c^{Mt}, pETMs2656 and pETRv2583c, were then transformed into *E. coli* strain BL21 (DE3) expression host and selected on Kan (50 µg/mL).

3.2.3.3.2. Optimization of protein expression

This was done following the pET system manual (Novagen) with some modifications. Briefly, 20 Ml fresh LB medium supplemented with 50 µg/mL KAN was inoculated with 200 µL of the mycobacterial recombinant plasmids – pETRv2783c^{Wt}, pETRv2783c^{Mt}, pETMs2656 and pETRv2583c – contained in *E. coli* strain BL21 (DE3) expression host bacteria cultures. The cultures were incubated at 37 °C with shaking at 220 rpm and the OD_{600} monitored until it reached 0.6. Over-expression of the recombinant proteins was then induced by adding Isopropyl β-D-1-thiogalactopyranoside **(**IPTG) to final concentrations of 0.1-1.0 mM, and incubated at 16°C, 30°C and 37°C for 3 h/overnight to determine the optimum temperature. Un-induced samples were also included as negative controls.

3.2.3.3.3. Monitoring of protein expression by SDS PAGE

Each sample was centrifuged at 13,000 rpm for 10 min at 4°C and the supernatants discarded. Each pellet was resuspended in 40 mL extraction buffer [50mM Tris-HCl, 200mM NaCl, pH 8.0] and sonicated to break the cells. The cells were centrifuged at 13,000 rpm for 10 minute at 4 °C and the topmost supernatants carefully aspirated without taking any cell debris.

To 20 µL protein samples, 5 µL of 5X loading buffer (0.5 M Tris-HCl, pH=6.8, Glycerol, 10% SDS, 2.9 mM β-mercaptoethanol and 0.5% bromophenol blue) was added. The samples were heated for 10 min at 100°C to denature the proteins. Each protein sample was centrifuged at 12,000 rpm for 2 minute. About 6-10 µL of each protein sample [both supernatant and resuspended precipitate] were loaded into the wells carefully ensuring no overflow. The protein marker was loaded into the first well. The top of the electrophoresis tank was covered on the top and connected to electricity. The samples were run in 1X running buffer, at 90Vin the stacking gel and 120V in the separating gel until the bromophenol blue reached the bottom.

The gel was stained with Coomassie Brilliant Blue, warmed for one minute in microwave oven and slowly shaken on a horizontal rotator for about 10-20 min to visualize the induced protein. The gel was immersed in a destaining solution, warmed for one minute in microwave oven and put on the same shaker for about 10-20 min. The destaining solution was changed for 3-5 times until clear bands with almost no blue background was observed.

3.2.3.3.3.1. Preparation of the SDS-PAGE gels

Table 12: Separating SDS-PAGE preparation [20 mL, 12%]

Reagents	Quantity
30% Acrylamide/Bis	8.0 mL
1.5 M Tris-HCl pH=8.8	5.0 mL
10% SDS	200 µL
Distilled water	6.6 mL
10% Ammonium Persulfate	200 µL
Tetramethylethylenediamine (TEMED)	8 µL

The casting frames were set by clamping two glass plates in the casting frames on the casting stands. The separating gel solution was swirled gently but thoroughly and an appropriate amount was pipetted into the gap between the glass plates (1.5 mm thick). To level off the separating gel, water (or isopropanol) was added to fill up gap between the glass plates until overflow. The gel was let to stand for 20-30 min to gelate. Water was discarded off the casting frames leaving only the separating gel.

Table 13: Stacking SDS-PAGE preparation [10 mL, 5%]

Reagents	Quantity
30% Acrylamide/Bis	1.7 mL
0.5 M Tris/HCl pH=6.8	2.6 mL
10% SDS	100 µL
Distilled water	5.5 mL
10% Ammonium Persulfate	100 µL
TEMED	10 µL

The stacking gel solution was pipetted on top of the separating gel until overflow. The well-forming comb was inserted without trapping air under the teeth, and left to stand for 20-30min to gelate. After complete gelation of the stacking gel, the comb was removed carefully not to damage the wells. The glass plates were removed from the casting frame

and set into the cell buffer dam. The running electrophoresis buffer was poured into the inner chamber until the overflow reached the required level in the outer chamber.

3.2.3.3.4. Scaled up protein expression and purification

Having optimized the expression conditions, the recombinant proteins were expressed in large scale in *E. coli* BL21 (D13) after induction with IPTG (0.5 mM) at 16 °C overnight as His-tagged derivatives and purified from soluble bacterial extracts by sequential nickel-affinity, anion-exchange, and gel-filtration chromatography steps.

Briefly, the cells were harvested by centrifugation at 4°C, and all subsequent procedures were performed at 4 °C. The bacteria pellets were resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl) and then sonicated for 5 min to achieve lysis. Insoluble material was removed by centrifugation at 16000 rpm in a Sorval SS34 rotor. The supernatants were applied to 10-mL columns of Ni^{2+}-NTA (Qiagen, Hilden, Germany) that had been equilibrated with buffer A (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM Imidazole). The columns were washed with buffer A and then eluted with buffer B (50mM Tris-HCl (pH 8.0), 500 mM NaCl, 300 mM Imidazole). The protein compositions of the fractions were monitored by SDS-PAGE. The appropriate protein fractions were pooled and loaded onto a Q-Sepharose column (180 mL) previously equilibrated with buffer C (20 mM Tris-HCl (pH 80), 5 mM NaCl). The unbound proteins were washed off the column using buffer C and eluted with buffer D (20 mM Tris-HCl (pH 8.0), 1 M NaCl) with a linear gradient (0-100%). The protein fractions were again monitored by SDS-PAGE. The right protein fractions were pooled together and gel-filtered through a column of Superdex-200 equilibrated with buffer E (20 mM Tris-HCl (pH 8.0), 150 mM NaCl). The peak fractions were pooled, concentrated by centrifugal ultrafiltration. The protein concentrations were determined using the Bio-Rad dye reagent with 5 BSA standards (0.1-0.5 mg/mL). The Micro Win Software was used to measure the protein concentration (at 595 nm absorption wavelength) and used to generate a standard curve using the BSA standards. The standard curve equation was used to calculate the protein sample concentrations by multiplying with the dilution factor. The proteins were frozen under liquid nitrogen and stored at -80 °C until further use.

3.2.3.4. Isothermal titration calorimetry binding studies.

Titration of the mycobacterial recombinant proteins with POA/PZA was performed in a VP-ITC 200 microcalorimeter (MicroCal, LLC, USA) according to the manufacturer's instructions and as described by Duff and colleagues [228]. The VP-ITC system was used to determine whether any interaction existed between wild type Rv2783, $Rv2783_{D67N}$, and *M. smegmatis* PNPase and POA/PZA. Initially, the recombinant proteins in 10 mM phosphate buffer (pH 7.5) were titrated with 100 µM POA or PZA (molar concentration ratio of 1:10). Blank titration of drug solution in the same buffer in the absence of the protein was performed. Further, the proteins were titrated with saturated POA/PZA (up to a 1:200 molar concentration ratio) at pH 5.5 and pH 6.5. The binding constants were estimated from the obtained isotherms using the calorimetric analysis origin software. The titrations were run at room temperature and repeated three times to get reproducible data. A control where the drug was titrated into buffer in the sample cell was run to determine the heat of dilution for the drugs. Typically, 20 injections of 2 µL per injection were made at 300s intervals, and reaction temperature was at 25 °C. The heat of reaction per injection (microcalories per second) was determined by integration of the peak areas.

3.2.3.5. ss-DNA and RNA catalytic activities of *M. tuberculosis* Rv2783

Polyribonucleotide nucleotidyltransferases (PNPases) plays synthetic and degradative roles in bacterial RNA metabolism, it has also been suggested to participate in bacterial DNA transactions [153, 154, 229-231]. In this study, we characterized and compared the RNA and DNA modifying activities of *M. tuberculosis* Rv2783 according to recently published work on *M. smegmatis* PNPase [152], which served as a positive control, with some modifications.

3.2.3.5.1. ss-DNA polymerization assay

Reaction mixtures (10 µL) containing 20 mM Tris-HCl (pH 7.5), 5 or 10 mM $MnCl_2$, 2 mM dADP, 1 pmol (0.1 µM) 36-mer ssDNA substrate (5'-FAM-GCCCTGCTGCCGACCAACGAAGGTAAAAAAAAAAAA-3'), and different concentrations of $Rv2783^{Wt/Mt}$ and M.smeg_2656 proteins were incubated for 30 min at 37°C. The reactions were quenched by adding 10 µL of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100°C and then analyzed by capillary electrophoresis at BGI, Wuhan, China.

3.2.3.5.2. ss-DNA 3′-phosphorylase assay

Reaction mixtures (10 µL) containing 20 mM Tris-HCl (pH 7.5), 5 mM $MnCl_2$, 30 µM $(NH_4)_3PO_4$, 0.1 µM (1 pmol) of 36-mer ssDNA substrate (5'-FAM-GCCCTGCTGCCGACCAACGAAGGTAAAAAAAAAAAA-3'), and different concentrations of $Rv2783^{Wt/Mt}$ and M.smeg_2656 proteins were be incubated for 60 min at 37°C. The reactions were quenched by adding 10 µL of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100 °C and then analyzed by capillary electrophoresis at BGI, Wuhan, China.

3.2.3.5.3. RNA polymerization assay

Reaction mixtures (10 µL) containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ADP, 1 pmol (0.1 µM) 24-mer ssRNA substrate (5'- FAM-GGGUCGCAAUUGAUUCCGAUAGUG-3'), and different concentrations of $Rv2783^{Wt/Mt}$ and M.smeg 2656 proteins were incubated at 37 °C for 15 min. The reactions were quenched by adding 10 µL of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100°C and then analyzed by capillary electrophoresis at Sangon Biotech, Shanghai, China.

3.2.3.5.4. RNA 3′-phosphorylase assay

RNA phosphorylase reaction mixtures (10 µL) containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $(NH_4)_3PO_4$, 1 pmol (0.1 µM) of 24-mer ssRNA substrate (5'- FAM-GGGUCGCAAUUGAUUCCGAUAGUG-3') and different concentrations of $Rv2783^{Wt/Mt}$ and M.smeg 2656 proteins were incubated for 15 min at 37 °C. The reactions were quenched by adding 10 µL of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100°C and then analyzed by capillary electrophoresis at Sangon Biotech, Shanghai, China.

3.2.3.5.5. Inhibition of Rv2783 ss-DNA and RNA catalytic activities by POA/PZA

Both DNA and RNA polymerization and phosphorylation assays were repeated as described above but in the presence of POA/PZA.

3.2.3.6. ppGpp synthetase and hydrolysis assays of *M. tuberculosis* Rv2783 protein

These were done as described by Avarbock and colleagues on the ribosome-independent (p)ppGpp synthetase and hydrolase activities of Rel_{Mtb} from *M. tuberculosis* [232] with some modifications.

3.2.3.6.1. ppGpp synthetase assays

Briefly, reaction mixtures (25 µL) contained 50 mM HEPES (pH 5.5- 8.0), 150 mM NaCl, 1 mM DTT, 2 mM GDP (Sigma Aldrich), 2 mM ATP (Sigma Aldrich), 4 mM $MgCl_2$, and 5 µM *M. tuberculosis* $Rv2783^{Wt/Mut}$ proteins were incubated at 30 °C for 1 hour. The *M. tuberculosis* Rel_{Mtb} protein was used as the positive control at similar concentrations. Three negative control reactions were also run which included no $Rv2783^{Wt/Mut}$ protein, no GDP and no ATP. The reactions were quenched by adding 1µL of formic acid. Additionally, the reactions were repeated in the presence of different concentrations of POA/PZA at different pH to evaluate the inhibitory effects of POA/PZA. After 5 min at room temperature, reaction tubes were centrifuged in an eppendorf centrifuge, filtered to remove precipitated proteins and analyzed by High-Pressure liquid chromatographic (HPLC) using ppGpp, ATP, GTP, GDP and AMP as standards.

3.2.3.6.2. ppGpp hydrolysis assays

Briefly, hydrolysis reaction mixtures containing 50 mM HEPES (pH 5.5-8.0), 150 mM NaCl, 1 mM DTT, 0.1 µM pppGpp (Trilink Biotechnologies), 20 mM $MnCl_2$, and 5 µM *M. tuberculosis* $Rv2783^{Wt/Mut}$ proteins were incubated at 30 °C for 20 minutes. The *M. tuberculosis* Rel_{Mtb} protein was used as the positive control. The reactions were quenched by adding 1µL of formic acid. Additionally, the reactions were repeated in the presence of different concentrations of POA/PZA at different pH to evaluate the inhibitory effects of POA/PZA. After 5 min at room temperature, reaction tubes were centrifuged in an eppendorf centrifuge, filtered to remove precipitated proteins and analyzed by HPLC.

3.2.3.6.3. HPLC analysis of the ppGpp synthetase and hydrolysis products

The chromatographic system consisted of a Waters in-line degasser, a Waters-600 controller connected to a Waters-600 pump and a Waters 486 tunable absorbance detector and a Waters Millennium workstation (Version 3.05) chromatography manager. The nucleotides were separated by reversed-phase chromatography using a Waters Symmetry C_{18} 3.5 µm

(150 by 4.6 mm) column, equipped with a NovaPak C_{18} Sentry guard column (Waters) with UV detection at 254 nm, adapting a prior method used for the separation of nucleotides [112]. Separation was done using a method that started with a 70:30 concentration (A:B) at a flow rate of 0.8 ml/min, which was changed in a linear gradient to 40:60 (A:B) after 30 min. From 30 to 60 min the gradient was changed linearly from 40:60 (A:B) to 0:70:30 (A:B:C). The injection volume was 50 µL, and detection was by UV at 254 nm. Buffer A consisted of 5 mM *t*-butyl ammonium phosphate (PicA Reagent), 10 mM KH_2PO_4, and 0.25% methanol adjusted to pH 6.9. Buffer B consisted of 5 mM *t*-butyl ammonium phosphate, 50 mM KH_2PO_4, and 30% methanol (pH 7.0). Buffer C was acetonitrile. Buffer A and B were degassed, passed through a 0.45 µm filter to avoid the risk of microbial contamination, and were prepared afresh and stored in the dark at 4 °C prior to use. The millennium work station (version 3.05) chromatographic manager was used to pilot the HPLC instrument and to process the data throughout the method validation and sample analysis.

3.2.4. Role of reported *rpsA* (*Rv1630*) mutations in PZA resistance

From our previous report [169], we indicated that the influence of *rpsA* mutations on PZA susceptibility needs to be further evaluated. Additionally, as aforementioned in the introduction section, the impact of *rpsA* mutations on PZA resistance seem to be innocuous and thus more studies that correlate PZA susceptibility with specific *rpsA* mutations is necessary to define their association and role in PZA resistance. Therefore, to query the actual role of particular missense mutations in the *M. tuberculosis rpsA* in PZA resistance, a number of previously reported *rpsA* mutations **(Table 14**) located at both the N- and C termini were overexpressed in wild type *M. tuberculosis* H37Rv, and tested for PZA susceptibility *in vitro* .

The primer pair rps*A*F and rpsAR **(Table 9)** was used for amplification of the 1.4-kb wild type *M. tuberculosis rpsA* gene. The PCR master mix was prepared as described in the preceding sections and amplification conditions comprised of an initial denaturation at 95 ℃ for 5 min; followed by 30 cycles of denaturation at 94 ℃ for 30 sec, annealing at 55 ℃ for 30 sec, extension at 72 ℃ for 1.5 min; and then a final extension at 72 ℃ for 10 min. The purified 1.4-kb PCR-amplified wild type *rpsA* DNA fragment was then ligated to p60luxNvector, cloned into *E. coli* DH5α and sequence verified as described in the

preceding sections. The verified plasmid, p60luxNrpsA^{Wt}, was then used to generate different point mutations in the *rpsA* gene (**Table 14**).

Table 14: Plasmids overexpressing reported mutated *rpsA* gene

Plasmid No.	Plasmid overexpressing reported mutated *rpsA* genes	Locus terminal of mutation	References
1	Thr5Ser [C14G], Asp123Ala [A368C]	N terminal	[101]
2	Ala440Thr [G1318A]	C terminal	[136]
3	Arg474Trp [C1420T]	C terminal	[169]
4	Thr5ser [C14G]	N terminal	[101]
5	Gln162Arg [A484G]	N terminal	[169]
6	Arg474Leu [G1421T]	C terminal	[169]
7	Thr5ala [A13G] and Thr210Ala [A628G]	N terminal	[136]
8	Lys122Glu [A364G]	N terminal	[136]
9	Ala438 deletion [1312GCC1314]	C terminal	[101]
10	Glu433Asp [G1299C)]	C terminal	[169]
11	Wild type *rpsA*		**Control**

The mycobacterial *rpsA* recombinant plasmids were then overexpressed in wild type *M. tuberculosis* H37Rv and autoluminescent H37Rv and verified as described in the preceding section. The autoluminiscent H37Rv *rpsA* transformant strains were stored at -80 °C for *in vivo* PZA susceptibility testing in mice, while the wild type H37Rv *rpsA* transformants were used for *in vitro* PZA susceptibility testing using the Bactec MGIT 960 system (BD, Franklin Lakes, NJ, USA) as described in section **3.2.3.2.2** at Guangzhou Chest Hospital.

3.2.5. Role of the reported A-11G *pncA* mutation in PZA resistance.

There exists no typical promoter upstream of the *pncA* gene from nucleotide -1 to nucleotide -358 including its upstream *Rv2044c* gene. From our previous report [169], we identified 2 *M. tuberculosis* PZA-resistant clinical strains (P160 and P78) that contained an A-11G mutation at the putative upstream regulatory region of the *pncA* gene, and no mutation within its 561 bp coding region. Reverse transcription of fragments spanning *Rv2045c-Rv2044c-pncA-Rv2042c* genes indicated that the *pncA* gene is co-transcribed with its surrounding gene [169]. We therefore set out to evaluate if the A-11G *pncA* mutation would affect translation of the *pncA* gene.

Genomic DNA from wild type *M. tuberculosis* H37Rv and the A-11G PZA-resistant clinical mutant strains was used as templates for PCR amplifying different fragments spanning *Rv2045c-Rv2044c-pncA* genes (**Figure 10**). The primer pairs: pncAF2 and pncAR1; pncAF3 and pncAR1 were used for amplifying fragments B (713 bp) and C (1185 bp) (Figure 9) spanning part of *Rv2045c-Rv2044c-pncA* and part of *Rv2044c-pncA* respectively. Primer pair pncAF1 and pncAR1 was used to amplify fragment A (516 bp), comprising only the *pncA* gene as a positive control. The PCR master mix was prepared as described in the preceding sections. Amplification conditions comprised of an initial denaturation at 95 ℃ for 5 min; followed by 30 cycles of denaturation at 94 ℃ for 30 sec, annealing at 56 ℃ for 30 sec, extension at 72 ℃ for 1 min (for fragments A and B) and 1.5 min (for fragment C); then followed by a final extension at 72 ℃ for 10 min.

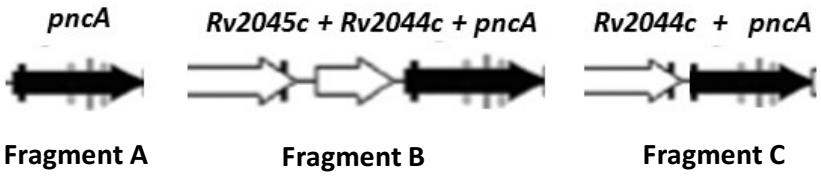

Figure 10: Primer designs for amplifying fragments A, B and C spanning genes surrounding *pncA* gene.

The PCR-amplified DNA fragments were then ligated to p60luxN vector, cloned into *E. coli* DH5α, and verified by restriction digestion and sequencing**.** The verified *pncA* recombinant plasmids were overexpressed in wild type *M. tuberculosis* H37Rv, and the P160 A-11G PZA-resistant clinical mutant strains, and verified as earlier described in section **3.2.3.2.1.** The *M. tuberculosis pncA* transformants were then tested for PZA susceptibility *in vitro* using the Bactec MGIT 960 system (BD, Franklin Lakes, NJ, USA) at Guangzhou Chest Hospital. At the same time, all the *M. tuberculosis pncA* transformants were tested for pyrazinamidase enzyme expression as described in section **3.2.2.1.**

4. Results

4.1. PZA-resistant *M. tuberculosis* clinical strains had no mutations in *panD* gene

To identify possible new targets of POA in PZA-resistant mutants that do not have *pncA* and *rpsA* mutations which would indicate possible new mechanisms of PZA resistance, we isolated genomic DNA from the 4 mutants and performed PCR to amplify the *panD* (*Rv3601c)* and *hadC* (*Rv0637*) gene plus their FR. Sequencing analysis of *panD*, another gene reported as a target of PZA [134, 224], did not reveal any *panD* mutations in the 4 mutants. Similarly, no mutations were identified in the *hadC* gene of the 4 mutants. Mutations in the *hadC* gene had previously been reported in 3 of 5 PZA-resistant isogenic mutants [224] without any *pncA* and *rpsA* mutations. PZase assay showed that the 4 mutants were positive for the enzyme activity, which was consistent with the *pncA* sequence analysis previously reported [169] ruling out a mutation at the putative upstream regulatory region of the *pncA* gene that could result in lack of PZase enzyme activity as a possible cause of the PZA resistance in the 4 mutants. The above findings suggest that the 4 PZA-resistant mutants harbor possible new mechanisms of PZA resistance independent of *pncA, rpsA, panD* or *hadC* mutations.

4.2. Sequence analysis identified a new gene, *Rv2783c*, associated with PZA resistance

We hypothesized that an uncharacterized mechanism and target(s) existed in the four previously reported PZA-resistant *M. tuberculosis* clinical strains [169] that lacked mutations in *pncA,* r*psA*, *panD* and *hadC*. To test this hypothesis, we amplified, sequenced and analyzed other potential genes, *Rv273*1, *Rv2783c* and *Rv3169* from them. *Rv2731, Rv2783c* and *Rv3169* encode proteins previously reported to bind with a POA derivative 5-hydroxyl-2-pyrazinecarboxylic acid [101]. Analysis of sequences of these genes identified two strains (P71 and P136) that harbored the same C948A synonymous and G199A nonsynonymous mutations in the *Rv2783c*. (**Table 15**).

Table 15: Sequence analysis of 2 PZA^{R} clinical strains containing *Rv2783c* mutations

Strain Number	P71 and P136 Mutants
Rv2783c（2259 bp）	C948A synonymous; G199A (Asp67Asn) nonsynonymous mutations

4.3. Overexpression of *Rv2783c* in *M. tuberculosis*

4.3.1. Cloning of *M. tuberculosis Rv2783c* gene

We successfully cloned the *M. tuberculosis* wild type *Rv2783c* and $Rv2783c_{G199A}$ mutant genes into *E. coli* DH5α using the p60luxN extrachromosomal plasmid vector carrying the strong *hsp60* promoter to produce, p60Rv2783c^{Wt}, p60Rv2783c^{Mt} and p60luxN^{C} (control) recombinant plasmids (**Figure 11**). The recombinant plasmid sequences were also verified by sequencing at BGI, Shenzhen, China.

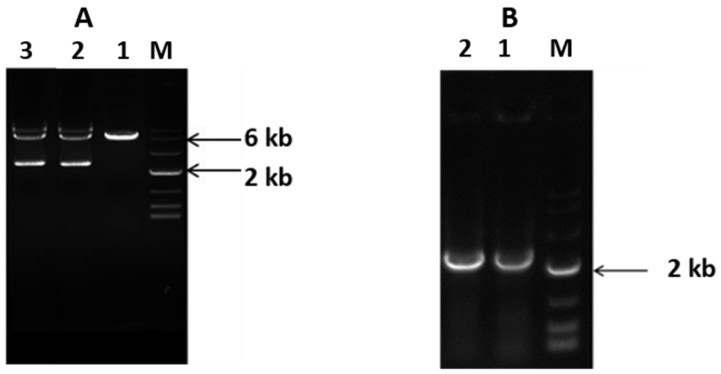

Figure 11: Clone verification of *M. tuberculosis Rv2783c* recombinant overexpression plasmids.

(A) Clone verification by restriction digestion with *Nde*I and *Hin*dIII: Lane M, 10-kb DNA marker; Lane 1, p60luxN^{C} empty vector control plasmid; Lane 2, p60Rv2783c^{Wt} plasmid restriction digestion; Lane 3, p60Rv2783c^{Mt} plasmid restriction digestion **(B)** Clone verification by PCR of *Rv2783c* gene: Lane M, 5-kb DNA marker; Lane 1, *Rv2783c* in plasmid p60Rv2783c^{Wt}; Lane 2, Rv2783c in plasmid p60Rv2783c^{Mt} (expected fragment sizes; 2259 bp for *RV2783c* gene and 6.2 Kb for the p60luxNew vector).

4.3.2. Overexpression of $Rv2783_{G199A}$ in *M. tuberculosis* H37Rv causes PZA resistance

To query the involvement of *Rv2783c* in PZA resistance, we successfully overexpressed the p60Rv2783c^{Wt}, p60Rv2783c^{Mt} and p60luxN^{C} (control) recombinant plasmids into wild type *M. tuberculosis* H37Rv and tested their PZA susceptibility *in vitro*. Overexpression of the mutant $Rv2783c_{G199A}$ in *M. tuberculosis* H37Rv using the p60luxN vector containing the strong *hsp60* promoter caused a 5-fold increase in the minimum inhibitory concentration (MIC) of PZA (MIC=500 µg/ml) compared with the *hsp60* vector control and the parental *M. tuberculosis* H37Rv strain (MIC=100 µg/ml) at pH 5.5. On the other hand, the MIC of the wild-type Rv2783 overexpressing *M. tuberculosis* strain was 150 µg/ml. (**Table 16**).

Table 16: PZA susceptibility testing of *M. tuberculosis Rv2783c* recombinant strains

Sample / Conc. (µg/ml)	Mutant Rv2783c$_{D67N}$			Wild-type Rv2783c			Hsp60Rv^{C} (Vector control)			Parental *M. tuberculosis*		
	Day			Day			Day			Day		
	7	14	21	7	14	21	7	14	21	7	14	21
25	R	R	R	R	R	R	S/R	R	R	S/R	R	R
50	R	R	R	S/R	R	R	S	S/R	R	S	S/R	R
100	S/R	R	R	S	S/R	S/R	S	S	S	S	S	S
200	S	S/R	R	S	S	S	S	S	S	S	S	S
300	S	S/R	R	S	S	S	S	S	S	S	S	S
400	S	S/R	S/R	S	S	S	S	S	S	S	S	S
500	S	S	S	S	S	S	S	S	S	S	S	S

It is also interesting to note that all the recombinant strains, overexpressing both wild type and mutant *Rv2783c*, were positive for the PZase enzyme activity assay (**Figure 12**), which was again consistent with the earlier *pncA* sequence analysis previously reported [169].

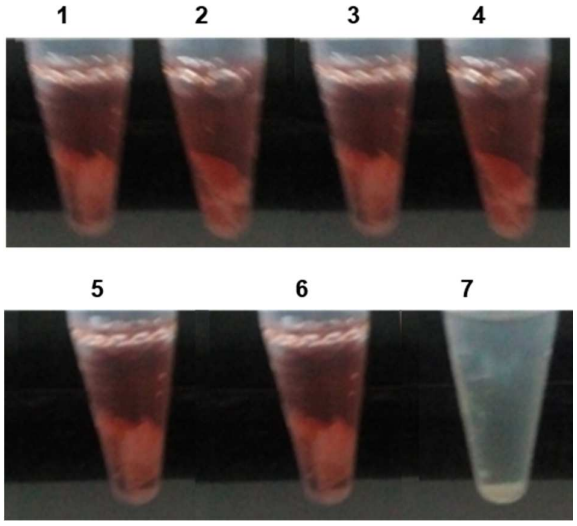

Figure 12: PZase enzyme assay of PZA^{R} clinical and *M. tuberculosis Rv2783c* recombinant strains.

M. tuberculosis recombinant strains (1-3)**: (1)** Wild-type Rv2783; **(2)** Rv2783$_{D67N}$ mutant; **(3)** Hsp60 vector control; **(4)** *M. tuberculosis* H37Rv parental strain; **(5-6)** PZA-resistant clinical strains harboring the G199A mutation in *Rv2783c*; **(7)** *M. bovis* Bacillus Calmette-Guérin (BCG Tice).

4.4. Protein expression and purification

M. tuberculosis PNPase is a 756-aa polypeptide encoded by the *Rv2783c* gene, whose primary structure is 87% identical to that of the 763-aa *M. smegmatis* PNPase encoded by the *Msmeg_2656* gene, and 73% identical to that of *S. antibioticus* PNPase (757-aa) encoded by *SCO5737* gene, the first PNPase for which a crystal structure [233] was solved.

We successfully cloned the *M. tuberculosis* wild type *Rv2783c*, $Rv2783c_{G199A}$ mutant, *M. smegmatis Msmeg_2656* (positive control for the PNPase assays) and *M. tuberculosis Rv2583c* (positive control for the ppGpp synthetase/hydrolysis assays) genes into *E. coli* strain DH5α using the pET-28a(+) expression vector to create pETRv2783c^{Wt}, pETRV2783c^{Mt}, pETMs2656 and pETRv2583c recombinant expression plasmids respectively (**Figure 13A**). The sequences of the recombinant expression plasmids were also verified by sequencing at BGI, Shenzhen, China.

To query the enzymatic and biochemistry of *M. tuberculosis* Rv2783, we used the recombinant expression plasmids to produce full length mycobacterial proteins (**Figure 13 B**) through IPTG induction (0.5 mM final concentration) at 16°C overnight as His-tagged derivatives. The proteins were purified from soluble bacterial extracts by sequential nickel-affinity, anion-exchange, and gel-filtration chromatography steps.

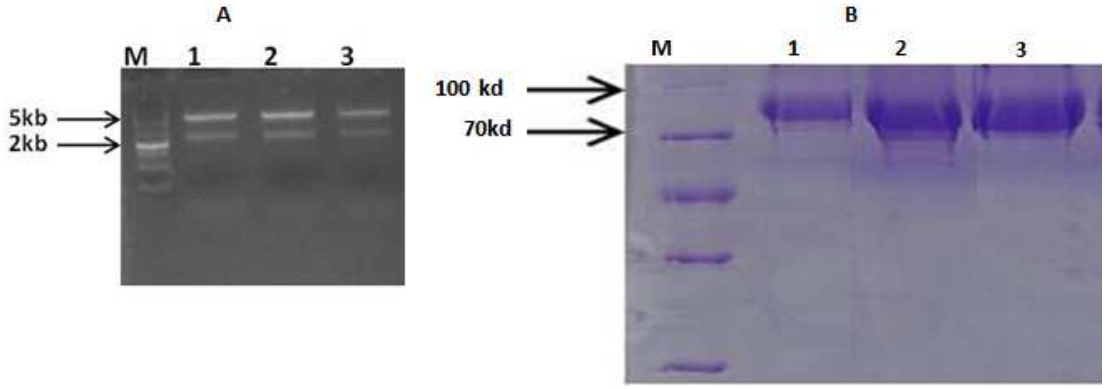

Figure 13: Clone verification and SDS-PAGE of mycobacterial proteins

A) Clone verification of mycobacterial protein expression recombinant plasmids by restriction digestion with *Nde*I and *Hin*dIII**:** Lane M, 5-kb DNA marker; Lane 1, pETRv2783c^{Wt}; Lane 2, pETRV2783c^{Mt}; Lane 3, pETMs2656 (expected fragment sizes; 5.6 kb for pET28a(+) vector, 2259 bp for $Rv2783c^{Wt/Mt}$ and 2292 bp for *Msmeg_2656*). **(B)** SDS-PAGE of the purified mycobacterial proteins expressed through IPTG induction (0.5 mM final concentration) at 16°C overnight. Lane M, 100 kd protein molecular weight marker; Lane 1, *M. smegmatis* PNPase [81.02 kd] encoded by *Msmeg_2656* gene*;* Lane 2, mutant *M. tuberculosis* PNPase [79.7 kd] encoded by $Rv2783c_{G199A}$ mutant; Lane 3, wild type *M. tuberculosis* PNPase [79.7 kd] encoded by wild type *Rv2783c* gene**.**

4.5. Wild type *M. tuberculosis* Rv2783 binds to POA and not to PZA

To determine if POA binds to Rv2783 and whether the G199A mutation affects binding to POA, we overexpressed and purified the wild-type Rv2783, the mutant $Rv2783_{D67N}$ and the *M. smegmatis* PNPase proteins, and used isothermal titration calorimetry (ITC) to measure binding interactions with POA or PZA (**Figure 14**).

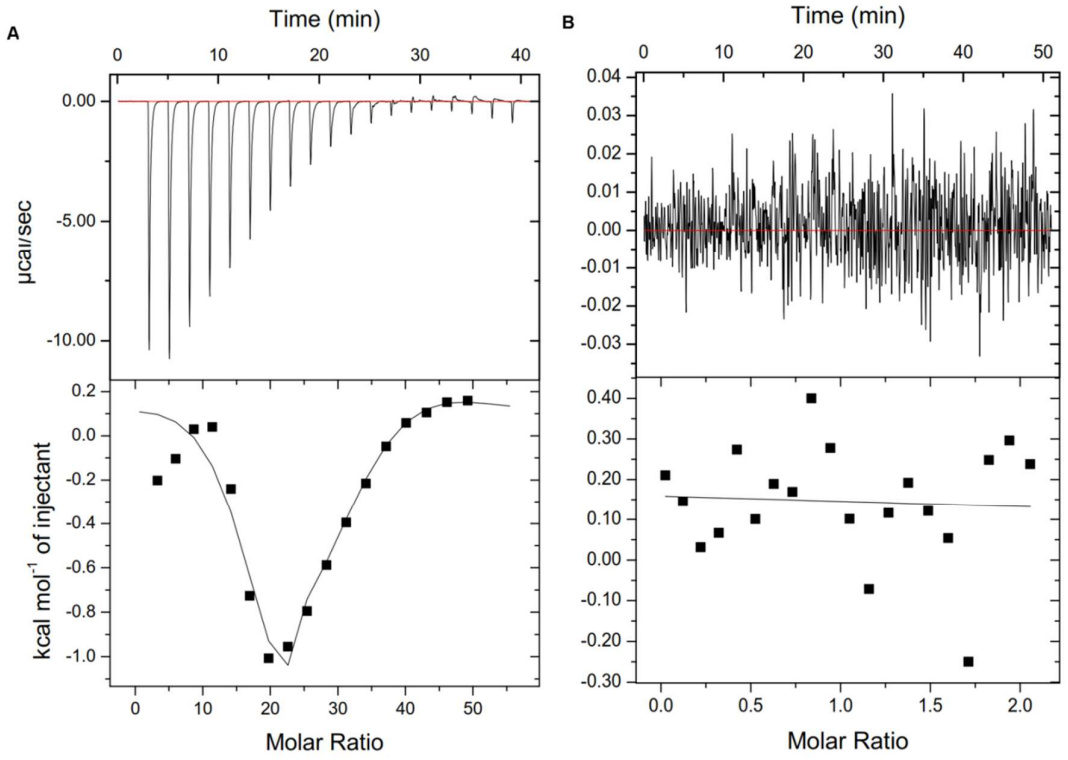

Figure 14: ITC titration of POA/PZA binding to *M. tuberculosis* Rv2783.

ITC binding studies indicate that the wild type *M. tuberculosis* H37Rv Rv2783 bound to POA **(A)** but not to PZA **(B)**. The upper panel shows raw data, and the Y axis indicates the heat released per second during Rv2783 and POA or PZA binding. The lower panel shows integrated heat in each injection of POA or PZA together with a fit, and Y axis is expressed by heat release per mole in each injection. The association constants were obtained from fits of wild type Rv2783 binding with POA and not with PZA.

The wild type *M. tuberculosis* Rv2783 was found to bind to POA via a sequential binding sites model (N=2) with dissociation constant K_{D1}=1.05 mM and K_{D2}=3.17 mM **(Figure 14A);** and not to the prodrug PZA even in higher concentrations **(Figure 14B**). However, we observed

that POA bound to the wild-type *M. tuberculosis* Rv2783 at relatively higher concentration, and only weakly at lower concentrations.

On the contrary, the *M. tuberculosis* Rv2783$_{D67N}$ mutant and PNPase from naturally PZA-resistant *M. smegmatis* failed to bind to POA or PZA even at higher concentrations **(Figure 15).**

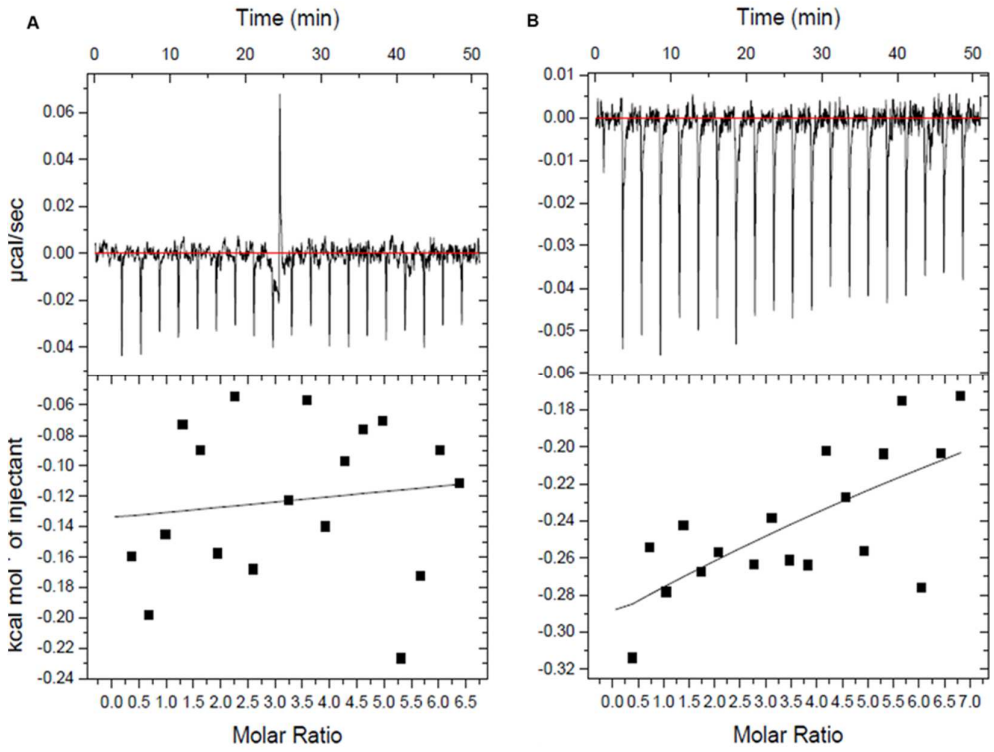

Figure 15: ITC titration of POA binding to mutant *M. tuberculosis* Rv2783$_{D67N}$ and *M. smegmatis* PNPase.

ITC binding studies indicate POA did not bind to the mutant *M. tuberculosis* Rv2783$_{D67N}$ **(A)** and to the *M. smegmatis* PNPase **(B)** proteins**.** The upper panel shows raw data, and the Y axis indicates that there was no heat released during the interaction between POA and the 2 proteins. The lower panel shows there was no integrated heat following each injection of POA, and thus there was no curve of fit.

4.6. PNPase activities of *M. tuberculosis* Rv2783c

Although PNPases from various bacteria are generally regarded as RNA modifying enzymes, herein we characterized and compared the RNA and ss-DNA catalytic activities of *M. tuberculosis* Rv2783 according to recently published work on *M. smegmatis* PNPase [152], which served as a positive control, with some modifications.

4.6.1. ss-DNA catalytic activities of *M. smegmatis* PNPase

As had been previously demonstrated by Unciuleac and Shuman [152], the *M. smegmatis* PNPase catalyzed non-templated ss-DNA synthesis, as gauged by its ability, in the presence of 5 mM Mn^{2+} and 2 mM dADP, to extend a 5′ FAM-labeled 24-mer ssDNA template strand when incubated for 60 min at 37°C. On the other hand, the substitution dADP with of phosphate diminished the polymerization activity and elicited the appearance of fluorescently FAM-labeled decay products shorter than the input 36-mer ss-DNA when incubated for the same period and at same temperature **(Figure 16).**

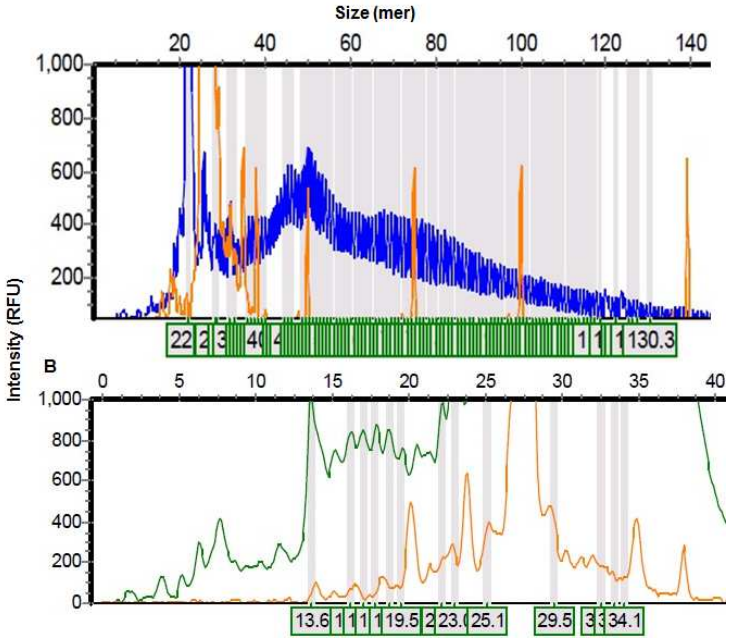

Figure 16: ss-DNA catalytic activities of *M. smegmatis* PNPase.

(A) ss-DNA polymerization activity of *M. smegmatis* PNPase (control) analysis by capillary electrophoresis. Blue peaks indicates the elongation of the fluorescently FAM-labeled 24-mer ssDNA template, Orange peaks indicates the internal size standard. **(B) ss-DNA phosphorolysis** activity of *M. smegmatis* PNPase analysis by capillary electrophoresis. Green color indicates the degradation of the fluorescently FAM-labeled 36-mer ssDNA template, Orange color indicates the internal size standard.

4.6.1.2. ss-DNA polymerization activity of *M. tuberculosis* Rv2783

Like the *M. smegmatis* PNPase, wild-type *M. tuberculosis* Rv2783 protein (at 2 µM concentration) also catalyzed non-templated ss-DNA elongation, as gauged by its ability, in the presence of 5 mM Mn^{2+} and 2 mM dADP, to extend a 5′ FAM-labeled 36-mer ss-DNA template when incubated for 30 min at 37°C**.** The ss-DNA polymerization activity of wild-type *M. tuberculosis* Rv2783 protein yielded a bimodal distribution of fluorescently labeled end

products ranging between 45 mer to 93 mer in size . On evaluating the inhibitory effects of PZA/POA, it was observed that POA and not the pro-drug PZA significantly inhibited the ss-DNA polymerization activity of the *M. tuberculosis* Rv2783 protein (**Figure 17**).

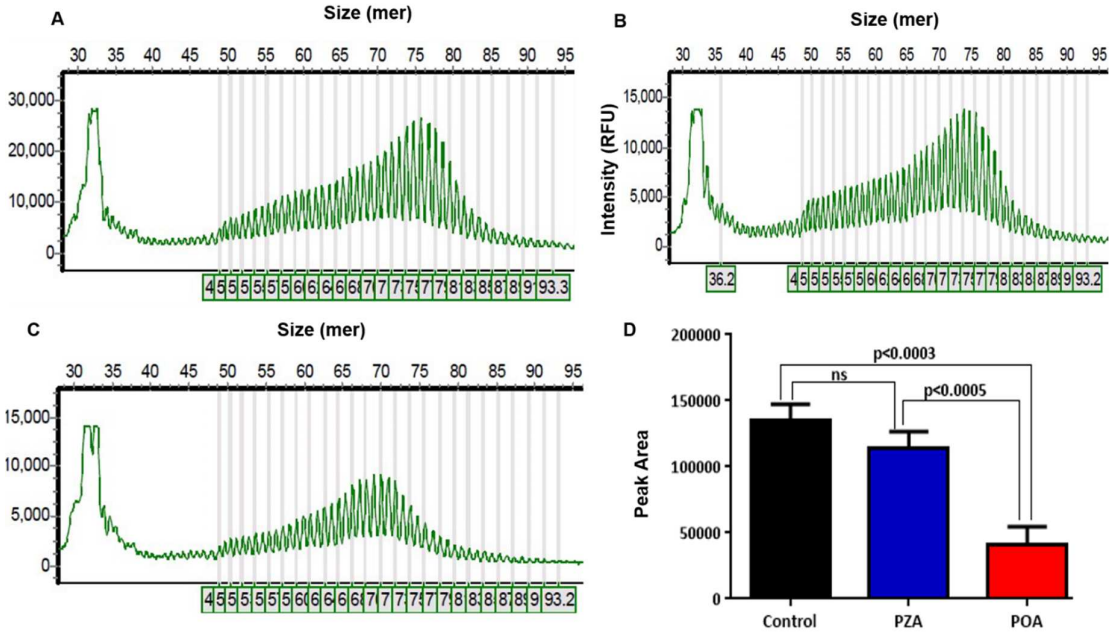

Figure 17: ss-DNA polymerization activity of wild type *M. tuberculosis* Rv2783 protein analysis by capillary electrophoresis.

(A) Uninhibited ss-DNA polymerization of wild type *M. tuberculosis* Rv2783. **(B)** Inhibition of ss-DNA polymerization activity of wild type *M. tuberculosis* Rv2783c protein by PZA and **(C)** by POA. **(D)** Comparison of the effect of POA and PZA on the polymerization activity of *M. tuberculosis* Rv2783 protein. Data is presented as mean ± SEM; t-test was used in the statistical analysis.

SS-DNA polymerization activity was also observed for the *M. tuberculosis* Rv2783$_{D67N}$ mutant protein under similar conditions, also yielding a bimodal distribution of elongated ss-DNA end products. However, unlike for the wild type *M. tuberculosis* Rv2783 protein, no significant inhibition of the DNA polymerization activity of the *M. tuberculosis* Rv2783c$_{G199A}$ protein by either POA was observed **(Figure 18).** This implies that the *M. tuberculosis* Rv2783$_{G199A}$ mutant and not the wild type *M. tuberculosis* Rv2783 protein can withstand the inhibitory effects of POA.

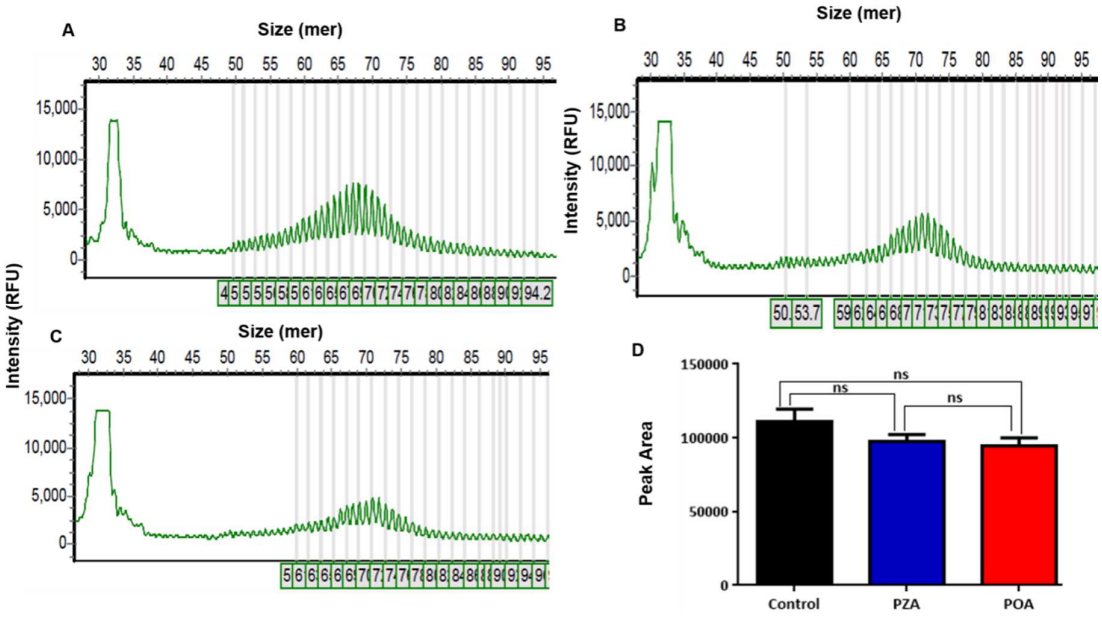

Figure 18: ss-DNA polymerization activity of *M. tuberculosis* Rv2783$_{D67N}$ mutant protein analysis by capillary electrophoresis.

(A) Uninhibited DNA polymerization of mutant Rv2783$_{D67N}$. **(B)** Inhibition of DNA polymerization activity of mutant Rv2783$_{D67N}$ by PZA and **(C)** by POA. **(D)** Comparison of the effect of POA and PZA on the DNA polymerization activity of mutant Rv2783$_{D67N}$ protein. Data is presented as mean ± SEM; t-test was used in the statistical analysis.

4.6.1.3. SS-DNA phosphorolysis activity of *M. tuberculosis* Rv2783

On the other hand, we tested the wild type *M. tuberculosis* RV2783 protein (2 µM concentration) for its ability to resect the 3′ end of a 5′ FAM-labeled 36-mer ss-DNA substrate. Like the *M. smegmatis* PNPase, we observed that, the substitution of dADP with inorganic phosphate abolished the polymerization activity and elicited a phosphorolytic activity resulting to the appearance of fluorescently FAM-labeled decay products shorter than the input 36-mer ss-DNA when incubated for 60 min at 37°C. Again, we observed that POA and not PZA significantly inhibited the ss-DNA phosphorolytic activity of the *M. tuberculosis* Rv2783 protein (**Figure 19**).

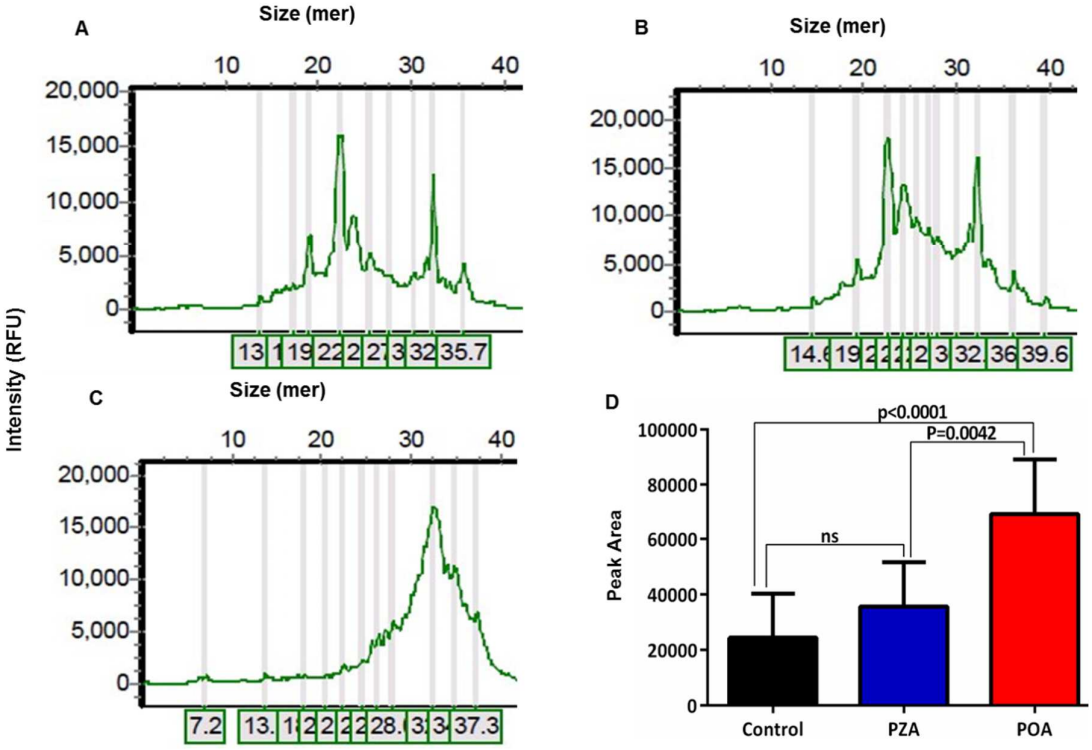

Figure 19: ss-DNA phosphorolytic activity of wild type *M. tuberculosis* Rv2783 protein analysis by capillary electrophoresis.

(A) Uninhibited ss-DNA phosphorolysis activity of wild type Rv2783. **(B)** Inhibition of ss-DNA phosphorolysis activity of wild type Rv2783 by PZA and **(C)** by POA. **(D)** Comparison of the effect of POA and PZA on the ss-DNA phosphorolysis activity of wild type *M. tuberculosis* Rv2783 protein. Data is presented as mean ± SEM; t-test was used for the statistical analysis.

Single stranded-DNA phosphorolytic activity was also observed for the *M. tuberculosis* $Rv2783_{G199A}$ mutant protein under similar conditions **(Figure 20).** However, unlike for the wild type *M. tuberculosis* Rv2783 where the inhibitory effects of POA on ss-DNA phosphorolysis was significantly high ($P<0.0001$), the inhibitory effect on DNA phosphorolytic activity of *M. tuberculosis* $Rv2783_{D67N}$ by POA was significantly less ($P= 0.0399$).

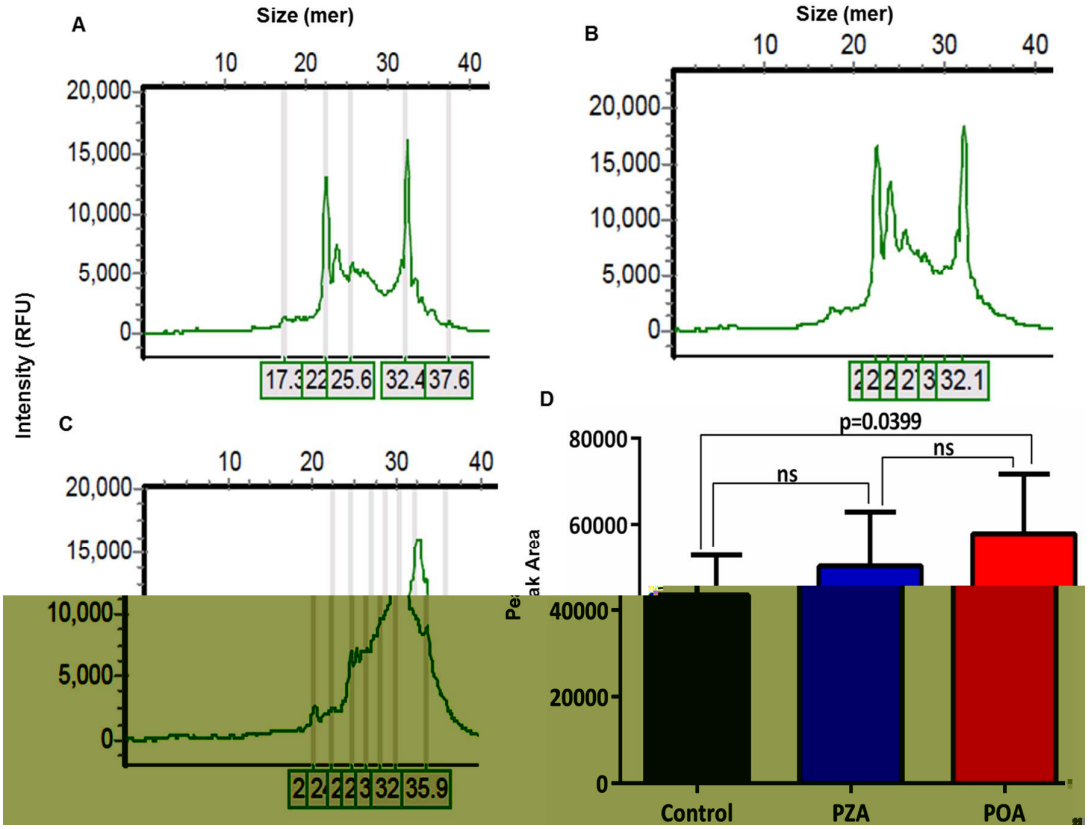

Figure 20: ss-DNA phosphorolytic activity of *M. tuberculosis* Rv2783$_{D67N}$ mutant protein analysis by capillary electrophoresis.

(A) Uninhibited ss-DNA phosphorolysis of mutant Rv2783$_{D67N}$. **(B)** Inhibition of ss-DNA phosphorolysis activity of mutant Rv2783$_{D67N}$ by PZA and **(C)** by POA. **(D)** Comparison of the effect of POA and PZA on the ss-DNA phosphorolysis activity of mutant Rv2783$_{D67N}$ protein. Data is presented as mean ± SEM; t-test was used for the statistical analysis.

On comparing the POA/PZA-free ss-DNA modifying activities of wild type *M. tuberculosis* Rv2783 and *M. tuberculosis* Rv2783$_{D67N}$ proteins, we observed that there was no significant difference in the POA/PZA-free ss-DNA polymerization activities of wild-type and mutant proteins. However, a significant difference was observed between the POA/PZA-free ss-DNA phosphorolysis activities of the wild type and mutant *M. tuberculosis* Rv2783 proteins **(Figure 21).**

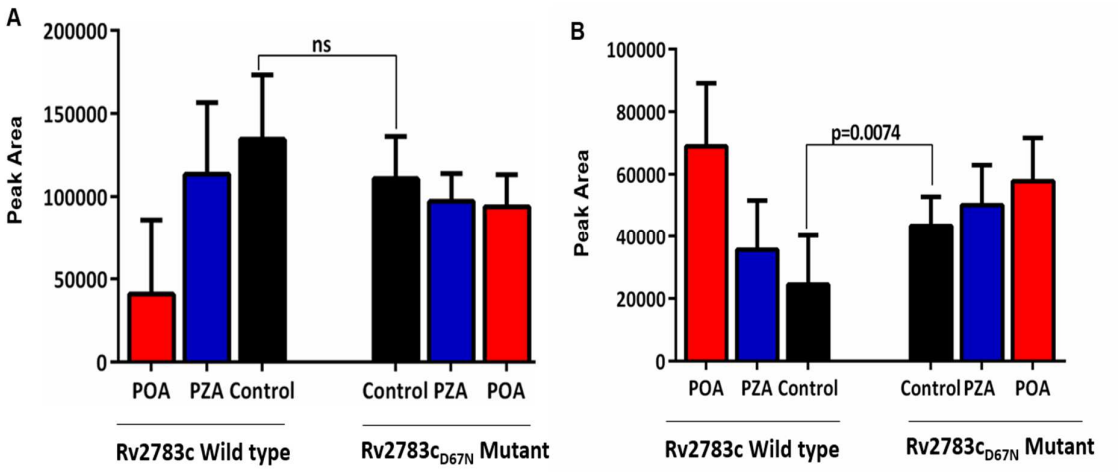

Figure 21: Graphical presentation comparing the ss-DNA catalytic activities of *M. tuberculosis* Rv2783 proteins.

(A) Comparison of the ss-DNA polymerization and **(B)** ss-DNA phosphorolysis activities of wild type Rv2783 and mutant Rv2783$_{D67N}$ proteins**.** Data is presented as mean ± SEM; t-test was used for the statistical analysis.

4.6.2. RNA modifying activities of *M. tuberculosis* Rv2783

4.6.2.1. RNA polymerization activity of *M. tuberculosis* Rv2783

To test wild-type *M. tuberculosis* Rv2783 protein for RNA polymerase activity, the protein, at a final concentration of 2 µM, was reacted with a 5′ FAM-labeled 24-mer RNA (0.1 µM) in the presence of 5 mM Mg^{2+} (in lieu of Mn^{2+}) and 2 mM ADP for 15 min. It was observed that 2 µM of the wild type *M. tuberculosis* Rv2783 protein sufficed to extend the input fluorescently 5' FAM-labeled 24-mer ssRNA into a polynucleotide tail**.** In addition, it was observed that POA and not the pro-drug PZA significantly inhibited the polymerization activity of the wild type *M. tuberculosis* Rv2783 protein (**Figure 22**).

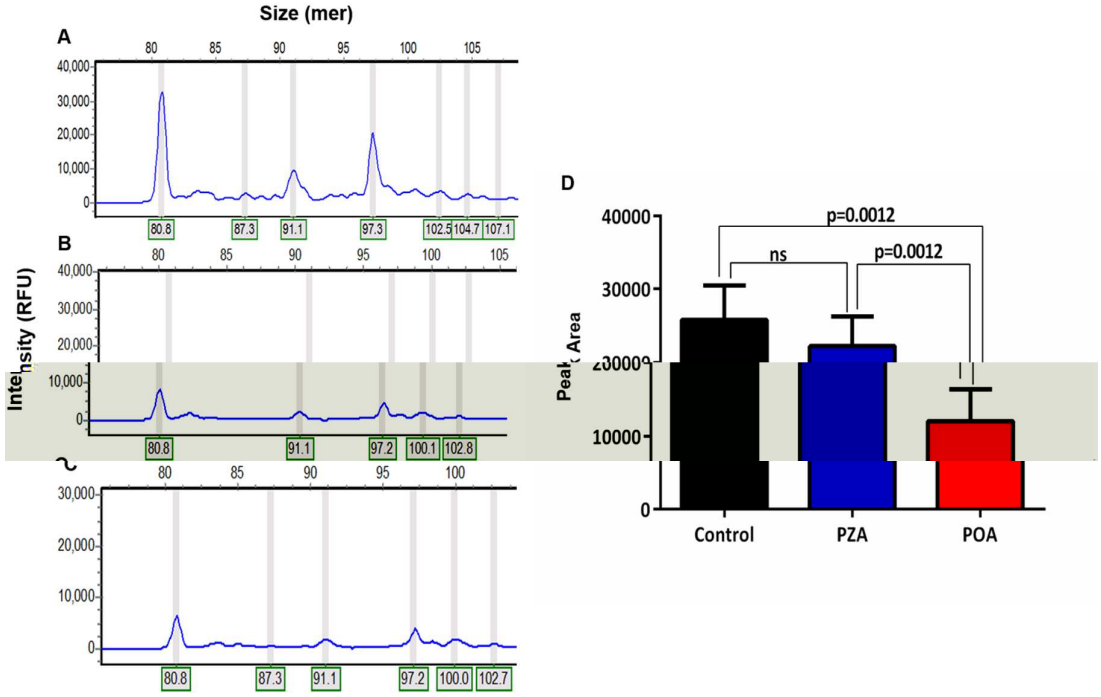

Figure 22: RNA polymerization activity of wild type *M. tuberculosis* Rv2783 protein analysis by capillary electrophoresis.

(A) Uninhibited RNA polymerization activity of wild type Rv2783. **(B)** Inhibition of RNA polymerization activity of wild type Rv2783 by PZA and **(C)** by POA. **(D)** Comparison of the effect of POA and PZA on RNA polymerization activity of wild type *M. tuberculosis* Rv2783 protein. Data is presented as mean ± SEM; t-test was used for the statistical analysis.

RNA polymerization activity was also observed for the *M. tuberculosis* $Rv2783_{D67N}$ mutant protein under similar conditions as of the wild type *M. tuberculosis* Rv2783 protein. However, the inhibitory effect of POA on the RNA polymerization activity of *M. tuberculosis* $Rv2783_{D67N}$ mutant was significantly less than for the wild type *M. tuberculosis* Rv2783 protein **(Figure 23).**

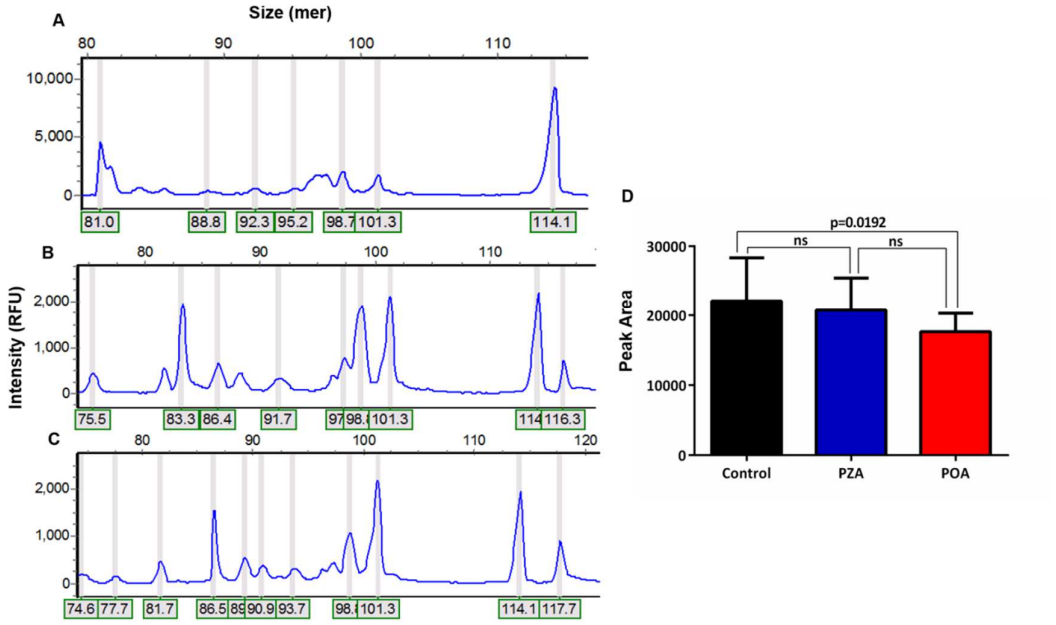

Figure 23: RNA polymerization activity of *M. tuberculosis* Rv2783$_{D67N}$ mutant protein analysis by capillary electrophoresis.

(A) Uninhibited RNA polymerization activity of Rv2783$_{D67N}$ mutant protein. **(B)** Inhibition of RNA polymerization activity of Rv2783$_{D67N}$ mutant by PZA and **(C)** by POA. **(D)** Graphical presentation and data analysis of the effect of POA and PZA on RNA polymerization activity of Rv2783$_{D67N}$ mutant protein. Data is presented as mean ± SEM; t-test was used for the statistical analysis.

This observation also implied that the mutant *M. tuberculosis* Rv2783$_{D67N}$ protein withstood the inhibitory effects of POA, as was also observed in the DNA polymerization activity of the mutant protein.

On comparing the POA/PZA-free RNA polymerization activities of the wild type *M. tuberculosis* Rv2783 and *M. tuberculosis* Rv2783$_{D67N}$ proteins, just like for the POA/PZA-free DNA polymerization, we observed that there was no significant difference in the POA/PZA-free RNA polymerization activities of wild-type and mutant proteins. (**Figure 24)**.

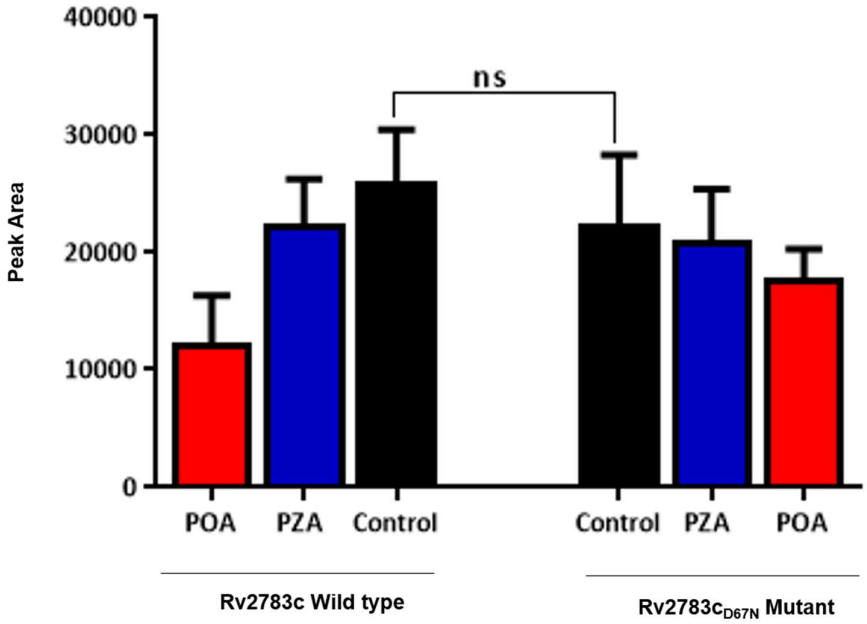

Figure 24: Comparison of the RNA polymerization activities of *M. tuberculosis* Rv2783 proteins.

RNA polymerization activities of wild type Rv2783 and $Rv2783_{D67N}$ mutant proteins. Data is presented as mean ± SEM; t-test was used for the statistical analysis.

4.6.2.2. RNA phosphorolytic activity of *M. tuberculosis* Rv2783

On the other hand, when the 5′ FAM-labeled 24-mer RNA was reacted 2 µM of both the wild-type and mutant *M. tuberculosis* Rv2783 proteins in the presence of 5 mM Mg^{2+} and 0.5 mM phosphate (in lieu of ADP) for 15 min at 37°C, the proteins switched to a phosphorolysis mode, such that most of the input fluorescently 5' FAM-labeled 24-mer ssRNA template was degraded into shorter nucleotide products than the input 24-mer ssRNA. Similarly, it was also observed that POA and not PZA significantly inhibited the RNA phosphorolytic activity of the wild-type *M. tuberculosis* Rv2783 and not the $Rv2783_{D67N}$ mutant protein (**Figures 25).**

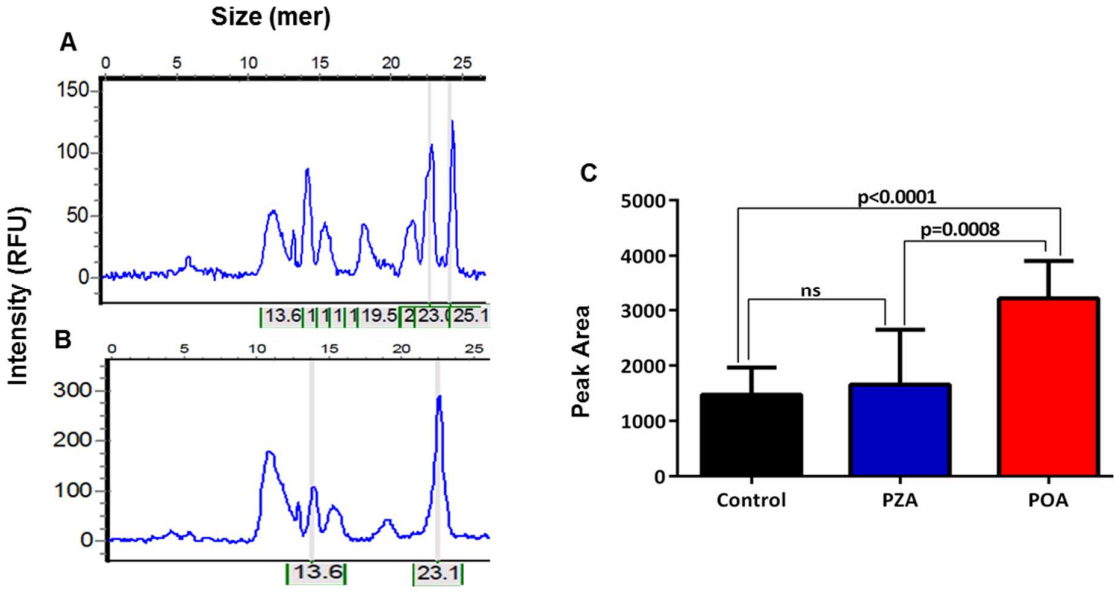

Figure 25: RNA phosphorolysis activity of wild type *M. tuberculosis* Rv2783 proteins analysis by capillary electrophoresis.

(A) Uninhibited RNA phosphorolysis activity of wild type *M. tuberculosis* Rv2783. (B) Inhibition of RNA phosphorolysis activity of wild type *M. tuberculosis* Rv2783 by POA (C) Comparison of the effect of POA and PZA on the RNA phosphorolysis activity of wild type *M. tuberculosis* Rv2783 protein. Data is presented as mean ± SEM; t-test was used for the statistical analysis.

4.7. ppGpp synthetase and hydrolysis assays

M. tuberculosis Rv2783 have been designated as a probable guanosine pentaphosphate synthetase (GpsI) enzyme, which could be involved in the synthesis and hydrolysis of ppGpp implicated in the stringent response. Therefore, it was of considerable interest to determine whether the protein possessed ppGpp synthesis and hydrolysis activities, as have been previously demonstrated by the *M. tuberculosis* Rel_{Mtb} protein. When reacted with 2 mM ATP and 2 mM GTP in the presence of varying concentrations of Mg^{2+} and Na^{+} at varying pH values (5.5-8.0), for 1 hour at 30°C, both wild type Rv2783 and $Rv2783_{D67N}$ mutant proteins demonstrated only weak ppGpp synthesis activities (**Figure 26 A)**, while the *M. tuberculosis* Rel^{Mtb} control protein demonstrated strong ppGpp synthesis activity under similar reaction conditions (**Figure 26 B**).

On the other hand, when the same concentrations of wild-type Rv2783 protein was reacted with 0.1 mM ppGpp in the presence of varying concentrations of Mn^{2+} and Na^{+}, the ppGpp was strongly hydrolyzed into GDP and ATP (**Figure 27A**). However, the strong ppGpp hydrolysis activity of wild-type Rv2783 protein was significantly inhibited by POA (**Figure 27B**).

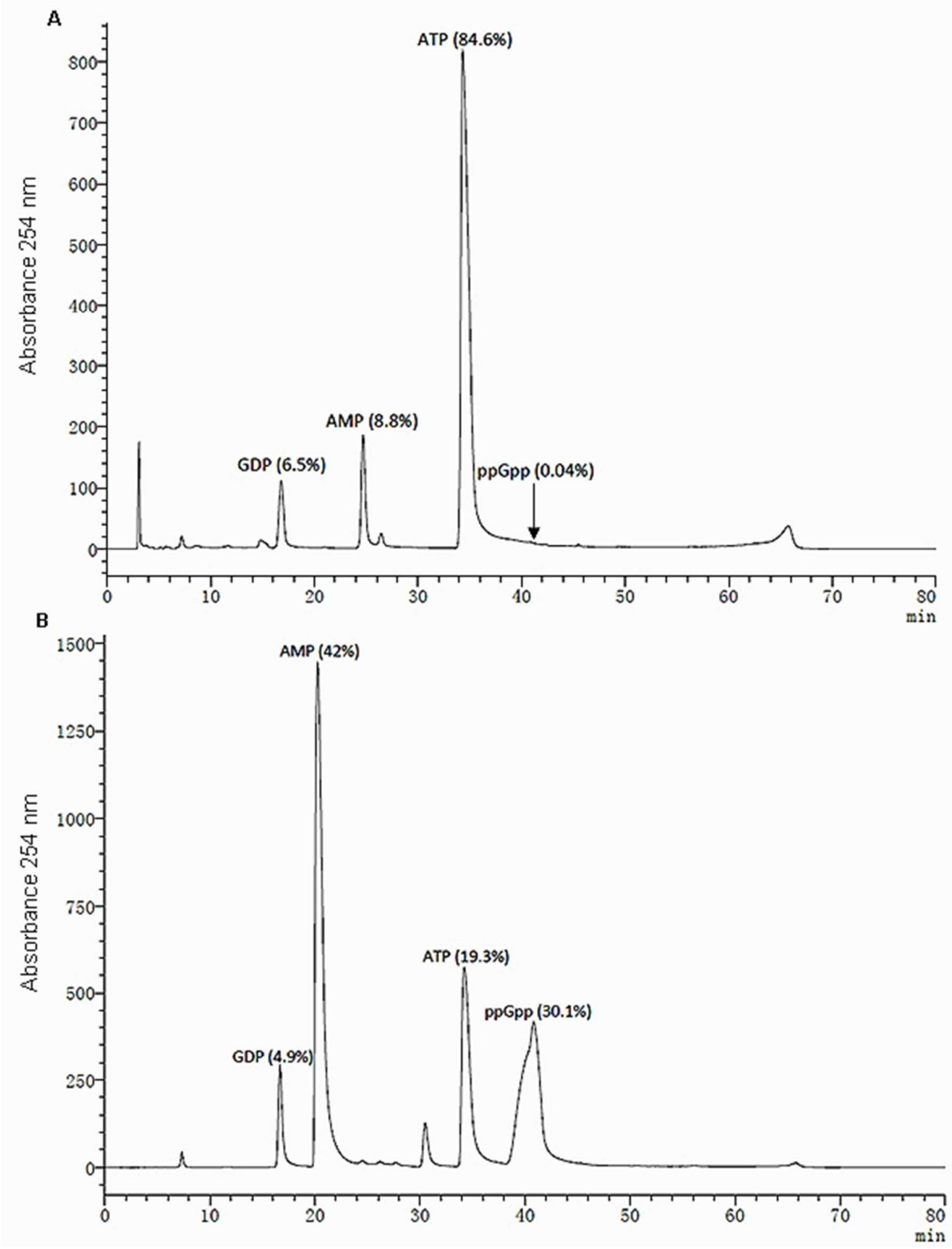

Figure 26: HPLC separation of ppGpp synthesis reaction products.

Reactions catalyzed by **(A)** wild type *M. tuberculosis* Rv2783 protein and (**B**) Wild type *M. tuberculosis* Rel_{Mtb} protein (control).

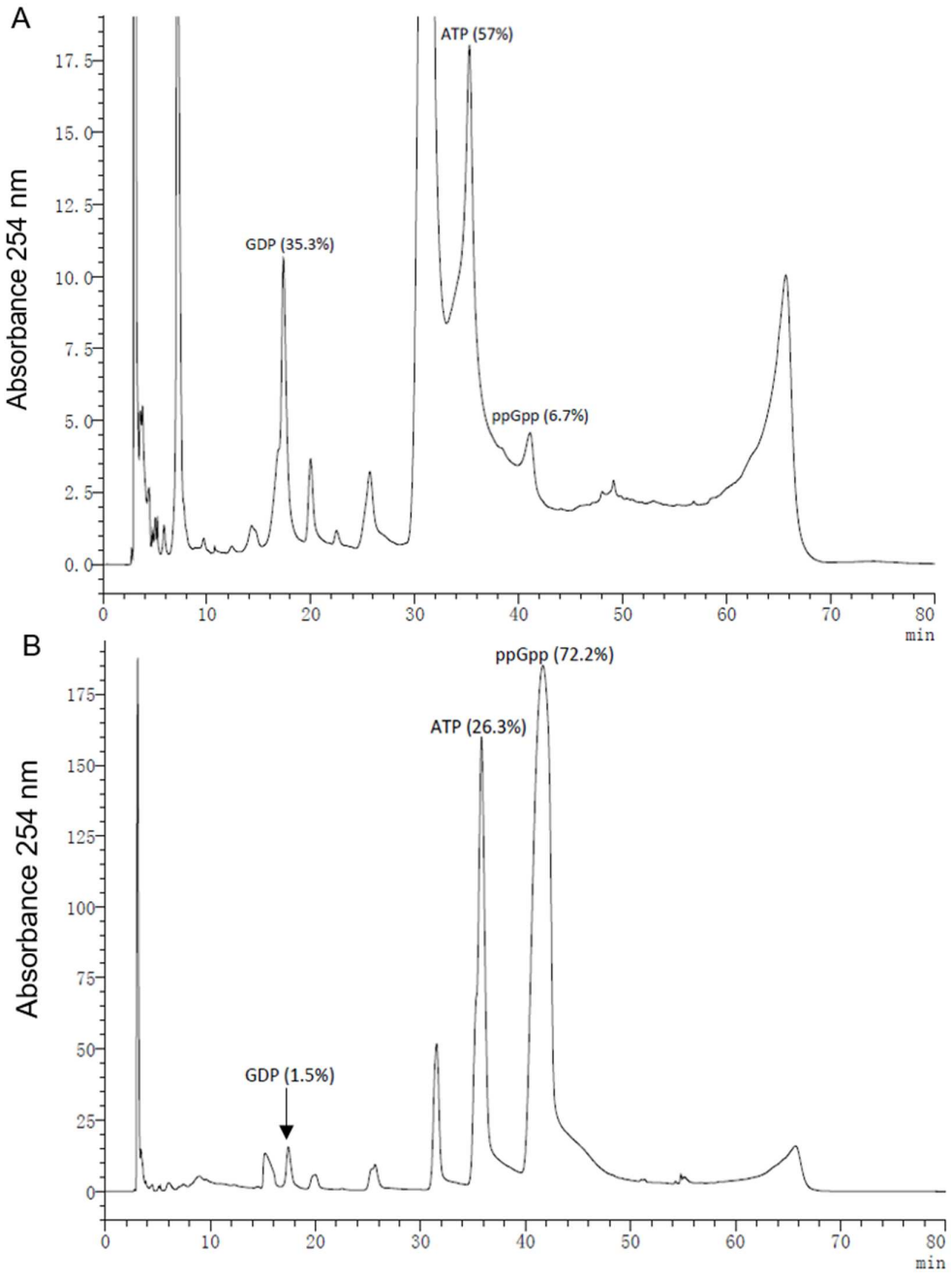

Figure 27: HPLC separation of wild type Rv2783 ppGpp hydrolysis reaction products.

Reactions catalyzed by wild type *M. tuberculosis* Rv2783 protein in the absence (**A**) and presence (**B**) of POA.

Like the wild type Rv2783 protein, the $Rv2783_{D67N}$ mutant protein also demonstrated ppGpp hydrolysis activity, which was comparatively less than for the wild type Rv278 protein under similar reaction conditions **(Figure 28A)**.

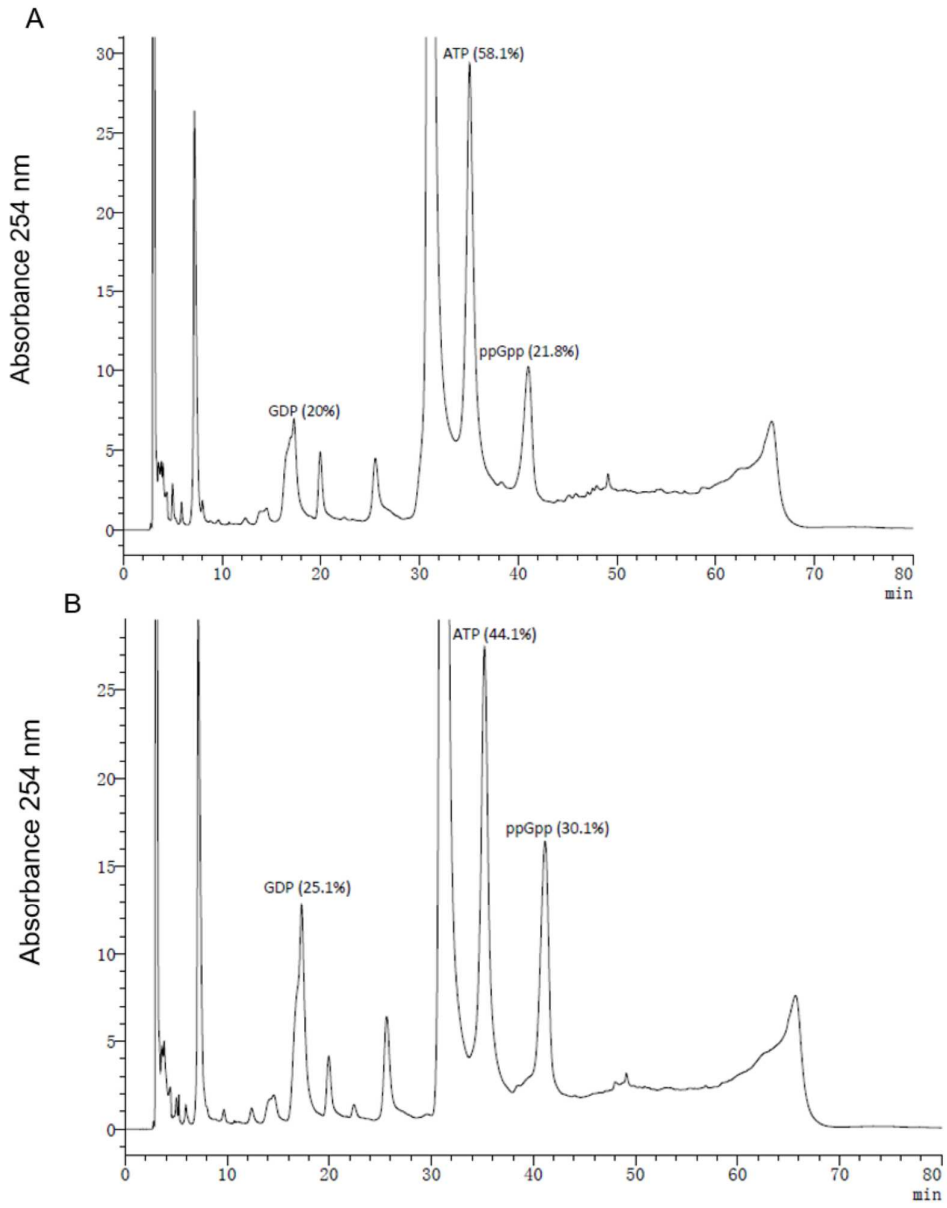

Figure 28: HPLC separation of Rv2783$_{D67N}$ mutant ppGpp hydrolysis reaction products.
Reactions catalyzed by *M. tuberculosis* Rv2783$_{D67N}$ protein in the absence (**A**) and presence (**B**) of POA.

However, unlike for the wild type Rv2783 protein, the ppGpp hydrolytic activity of the Rv2783$_{D67N}$ mutant protein was not significantly inhibited by POA under similar reaction conditions (**Figure 28B**).

4.8. Role of reported *rpsA* mutations in PZA resistance

A total of 9 non-synonymous substitutions and 1 codon deletion were created in the wild type *M. tuberculosis rpsA* gene through point mutagenesis (**Table 17**).

Table 17: Plasmids overexpressing mutated *rpsA* gene in wild type *M. tuberculosis* H37Rv

Plasmid No.	Plasmid overexpressing reported mutated *rpsA* genes	*In vitro* Bactec MGIT 960 Growth Unit	Locus terminal of mutation	PZA susceptibility from References	
		P^{100}			
1	Thr5Ser [C14G], Asp123Ala [A368C]	S	N terminal	R	[101]
2	Ala440Thr [G1318A]	R	C terminal	R	[136]
3	Arg474Trp [C1420T]	R	C terminal	R	[169]
4	Thr5ser [C14G]	S	N terminal	R	[101]
5	Gln162Arg [A484G]	S	N terminal	S	[169]
6	Arg474Leu [G1421T]	R	C terminal	R	[169]
7	Thr5ala [A13G] and Thr210Ala [A628G]	R	N terminal	R	[136]
8	Lys122Glu [A364G]	S	N terminal	S	[136]
9	Ala438 deletion [1312GCC1314]	R	C terminal	R	[101]
10	Glu433Asp [G1299C)]	S	C terminal	R	[169]
11	Wild type *rpsA* (Control)	S		R	

The mutated *rpsA* genes were overexpressed in wild type *M. tuberculosis* H37Rv using the extrachromosomal p60luxN vector containing the strong *hsp60* mycobacterial promoter. The wild type *M. tuberculosis* H37Rv *rpsA* gene was also overexpressed as a control. The *M. tuberculosis rpsA* recombinant strains were verified as earlier described and tested for PZA susceptibility *in vitro* using Bactec MGIT 960 system (MIC cut-off for resistance at 100 µg/mL) as earlier described. Out of the 10 *M. tuberculosis* H37Rv recombinant strains overexpressing mutated *rpsA* genes, 3 of them which included the Thr5Ser and Asp123Ala double mutant, Thr5Ser and Glu433Asp single mutants, were susceptible to PZA (MIC cut-off for resistance at 100 µg/mL), contrary to having been previously reported as resistant. The remaining 7 *rpsA* were concordant with the previous findings (**Table 17**).

4.9. Role of the reported A-11G *pncA* mutation in PZA resistance.

4.9.1. Cloning of *M. tuberculosis Rv2783c* gene

We successfully cloned both wild type A-11 pncA and mutated A-11G *pncA* in fragments B and C spanning *Rv2045c-Rv2044c-pncA* and *Rv2044c-pncA,* and named them as p60luxNpncA$^{BWt/Mt}$ and p60luxnpncA$^{CWt/Mt}$ respectively. We also cloned fragment A comprising only of the *pncA* gene and named it p60luxNpncA^{A}. We verified the sequences of the plasmids as earlier described **(Figure 29).**

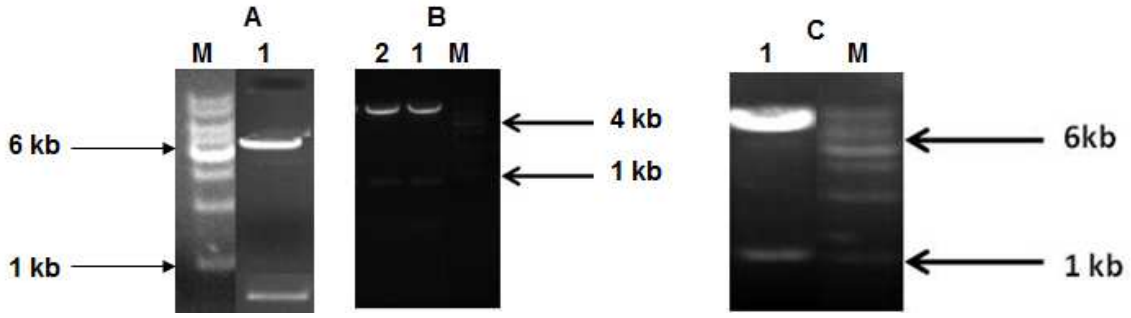

Figure 29: Clone verification of *M. tuberculosis* A-11G *pncA* recombinant overexpression plasmids by restriction digestion with *Nde*I and *Hin*dIII.

(A) Plasmid p60luxnpncAA: Lane M, 10-kb DNA marker; Lane 1, restriction digestion of p60luxnpncAA control plasmid. **(B)** Plasmid p60luxNpncAB: Lane M, 10-kb DNA marker; Lanes 1 and 2, restriction digestion of wild type and mutant p60luxNpncAB respectively. **(C)** Plasmid p60luxNpncAC: Lane M, 10-kb DNA marker; Lanes 1, restriction digestion of wild type p60luxNpncAC (expected fragment sizes; 6.2 kb for p60luxN vector, 516 bp for fragment A, 713 bp for fragment B and 1185 bp for fragment C).

4.9.2 Overexpression of A-11G *pncA* in wild type *M. tuberculosis* H37Rv

To query whether the A-11G *pncA* would affect the translation of the *pncA* gene, we successfully overexpressed the wild type A-11 *pncA* recombinant plasmids containing fragments A, B and C into the P160 *M. tuberculosis* PZA-resistant clinical strain (A-11G *pncA* mutant) to form *M. tuberculosis* recombinant strains p160luxNpncA^{AWt} (control), p160luxNpncA^{BWt} and p160luxNpncA^{CWt}. We also successfully overexpressed the mutated A-11G *pncA* recombinant plasmids containing fragments A, B and C into wild type *M. tuberculosis* H37Rv to form H37RvluxNpncA^{AMt} (control), H37RvluxNpncA^{BMt} and H37RvluxNpncA^{CMt} recombinant strains. Verification of successful overexpression was done as previously described.

We tested the PZA susceptibility *in vitro* using the Bactec MGIT 960 system (Becton Dickinson, Sparks, MD) and PZase activity of all the *M. tuberculosis pncA* recombinant strains

as earlier described. We found that overexpression of the wild type A-11 *pncA* contained in fragments B and C in the P160 A-11G *pncA* PZA-resistant strain did not alter its PZA susceptibility and neither did it restore its PZase activity. Moreover, we also observed that overexpression of the mutated A-11G *pncA* also contained in fragments B and C in wild type *M. tuberculosis* H37Rv did not cause PZA resistance (MIC cut-off for resistance at 100 µg/mL) and neither did it abolish the PZase activity of *M. tuberculosis* H37Rv **(Figure 30)**.

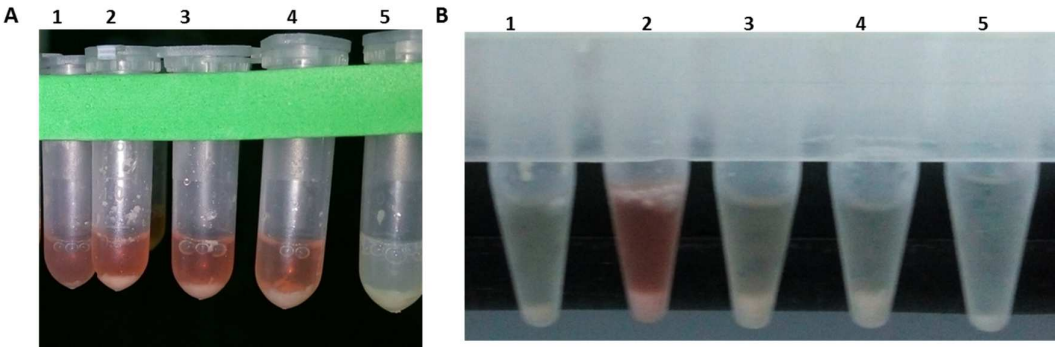

Figure 30: PZase enzyme assay of *M. tuberculosis* A-11/ A-11G *pncA* recombinant strains.

(A) PZase assay of wild type *M. tuberculosis* H37Rv overexpressing mutated A-11G *pncA* mutation: Tube 1, *M. tuberculosis* H37Rv parental strain; Tube 2, Fragment A (control), Tube 3-4, Fragments B and C mutants; Tube 5, BCG (Tice). **(B) PZase assay of clinical PZA^{R} strain overexpressing wild type A-11 *pncA*:** Tube1, Parental A-11G *pncA* mutant; Tube 2, wild type *M. tuberculosis* H37Rv; Tube 3, Fragments A (control); Tube 4-5, Fragments B and C wild type.

5. Discussion

New anti-TB regimens are urgently needed to further shorten TB treatment and manage patients with drug-resistant TB. Due to the role of PZA in shortening TB treatment duration, identification of its precise cellular targets in *M. tuberculosis* is a vital consideration for understanding its mode of action and thus further optimizing and shortening TB short-course therapy. While the mechanism of PZA activation into its active moiety POA by the PZase enzyme has been characterized, many questions remain in refining our understanding of the precise cellular targets and mode of action of POA in *M. tuberculosis.* In this study, we identified a new gene, *M. tuberculosis Rv2783c,* which was associated with PZA resistance and a probable new target of POA in *M. tuberculosis. M. tuberculosis Rv2783c* encode a probable bifunctional enzyme: polyribonucleotide nucleotidyltransferase (PNPase), a major component of the RNA degradosome involved in RNA metabolism [137], and guanosine pentaphosphate synthetase (GpsI), involved in the synthesis of the alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) [138] implicated in the stringent response [139] in *E. coli* and other bacteria .

For two decades, alterations in *pncA* gene [99], encoding the PZase required for conversion of PZA prodrug to POA, have been implicated as the major cause of PZA resistance in *M. tuberculosis*. Recently, mutations in the targets of POA/PZA, ribosomal protein S1 (RpsA) [101] and the aspartate decarboxylase (panD) [134, 224], involved in trans-translation and the synthesis of the essential metabolic cofactors pantothenate and coenzyme A respectively, were also implicated with PZA resistance. However, PZA-resistant clinical isolates that do not have mutations in *pncA, rpsA* or *panD* and their flanking regions have been reported [169, 234]. In this study, we shed light on a possible new target of PZA/POA and report that mutations in *M. tuberculosis Rv2783c* are closely associated with PZA resistance. By using whole genome sequencing, we were able to identify G199A (Asp67Asn) missense mutation in the *Rv2783c* gene of 2 clinical *M. tuberculosis* PZA-resistant strains, suggesting a possible new target of PZA/POA. The 2 PZA-resistant strains were positive for the PZase enzyme activity assay, which was consistent with the earlier *pncA* sequence analysis previously reported [169].

The involvement of *M. tuberculosis Rv2783c* in PZA resistance was demonstrated by the overexpression of the $Rv2783c_{G199A}$ (Asp67Asn) mutant in wild type *M. tuberculosis*

H37Rv. Increasing transcription of the target gene, leading to its overexpression is a well-known drug resistance mechanism in bacteria. Our results showed that overexpression of the *M. tuberculosis* $Rv2783c_{G199A}$ mutant in wild-type *M. tuberculosis* H37Rv caused PZA resistance *in vitro* (MIC=500 µg/mL). In contrast, strains overexpressing the wild-type *Rv2783c* and the empty p60luxN vector were still susceptible to PZA *in vitro* (MIC=100 µg/mL), indicating that the induced $Rv2783c_{G199A}$ expression was responsible for the PZA resistance. It is also noteworthy that all the transformant strains were positive for the PZase enzyme activity assay, which again ruled out a mutation at the putative upstream regulatory region of the *pncA* gene as possible cause of the PZA resistance in the transformant strain overexpressing $Rv2783c_{G199A}$. These findings suggest that POA may possibly bind to Rv2783, and in particular to residue D67 as it was the altered site in the 2 PZA-resistant mutants, thereby influencing PZA susceptibility of the *M. tuberculosis* bacilli.

We further performed an ITC experiment to determine the thermodynamic parameters (affinity, enthalpy and stoichiometry) of the binding interaction between POA/PZA and *M. tuberculosis* Rv2783 protein. We found that the wild type *M. tuberculosis* Rv2783 was able to bind to the active entity, POA, and not to the inactive pro-drug, PZA. This finding suggests that *M. tuberculosis* Rv2783 is a probable target of POA, and a confirmation that indeed at it is POA that has the anti-tuberculosis activity and not the pro-drug PZA. Most interestingly, the finding that the *M. tuberculosis* $Rv2783_{D67N}$ mutant protein failed to bind to POA or PZA further suggest that *M. tuberculosis* Rv2783 is a highly probable target of POA and that the Asp67Asn mutation may affect the binding of POA. This suggestion was further supported by the observation that the PNPase from naturally PZA-resistant *M. smegmatis* (87% homology) failed to bind either POA or PZA. We however found that POA bound to the wild type *M. tuberculosis* Rv2783 at relatively higher concentration, and only weakly at lower concentrations. This finding was concordant with an earlier report by Shi and colleagues [101], in which, *M. tuberculosis* Rv2783 was also shown to only bind weakly to a POA derivative, 5-hydroxyl-2- pyrazinecarboxylic acid. This could probably explain why PZA is usually clinically administered in relatively higher dosage (up to 50 mg/kg) as compared with other anti-TB drugs such as INH (5 mg/kg) and RIF (10 mg/kg).

M. tuberculosis Rv2783 is a 756-aa polypeptide whose primary structure is 87% identical to that of the 763-aa *M. smegmatis* PNPase encoded by the Msmeg_2656 gene, and 73%

identical to that of *S. antibioticus* PNPase (757-aa), the first PNPase for which a crystal structure [233] was solved. Our results on the ss-DNA polymerase and phosphorylase reactions of *M. tuberculosis* Rv2783 (here in functioning as a PNPase) contribute to an emerging picture of mycobacterial PNPases as catalysts of DNA metabolism in addition to their synthetic and degradative roles in RNA metabolism [152]. This suggests that *M. tuberculosis* Rv2783 could possibly be involved in conserving DNA integrity through DNA repair and mutagenesis *in vivo*. Juan Alonso and colleagues demonstrated the direct involvement of *Bacillus subtilis* PNPase in DNA repair [153]. By studying the effects of a *ΔpnpA* null allele on clastogen sensitivity and its epistasis relationships to null alleles of other DNA repair factors, they provide genetic evidence that PNPase participates in the homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways of *B. subtilis* double-strand break (DSB) repair in response to damage by hydrogen peroxide [153]. Considering that mycobacteria possess three distinct pathways for the repair of DNA double-strand breaks (HR, NHEJ, and single-strand annealing), a similar participation by PNPase is highly possible. While the mechanisms by which PNPase affects DNA repair are not yet clear, Alonso and colleagues suggest that PNPase reacts with broken DNA ends, either converting them from non-ligatable "dirty" breaks to clean ends that can be sealed by DNA ligase or by adding non-templated single-stranded 3′ tails that can then influence repair pathway choice [153, 154].

The finding that POA inhibited the ss-DNA polymerization activity of the wild type *M. tuberculosis* Rv2783 and not the $Rv2783_{D67N}$ mutant protein also explains the difference in the PZA susceptibilities of the respective transformants. Comparatively, the ss-DNA phosphorolytic activity of the wild type Rv2783 was highly inhibited by POA ($p \leq 0.0001$) as opposed to that of the $Rv2783_{D67N}$ mutant protein ($p=0.0399$). Taken together, it thus appears that the D67N substitution in the *M. tuberculosis* PNPase altered the binding of POA conferring to the bacilli the ability to evade its sterilizing effects, thereby causing resistance. In that case, the *M. tuberculosis* $Rv2783_{D67N}$ mutant protein would therefore proceed on catalyzing both ss-DNA polymerization and phosphorolytic reactions needful during DNA repair and mutagenesis during dormancy without being inhibited by POA. The ability of such non-replicating persister bacilli to carry out DNA repair and mutagenesis would highly impact on its survival during such stressful environmental conditions of limited nutrients, antibiotic treatment among others.

Next, we found out that *M. tuberculosis* Rv2783 catalyzed both RNA polymerization and RNA phosphorolysis, confirming its important role in RNA metabolism. Comparatively, as observed in the DNA transactions, the RNA polymerization and phosphorolysis activities of the wild type Rv2783 and not the mutant $Rv2783_{D67N}$ protein were significantly inhibited by POA. In addition, there was no significant difference in the POA-free RNA modifying activities between the wild type Rv2783 and the mutant $Rv2783_{D67N}$ proteins. Taken together, these findings suggest an important role of Rv2783 protein in RNA metabolism. The RNA degradosome, a multi-enzyme complex comprised of proteins such as RNAse E and PNPase, is involved in RNA metabolism and post-transcriptional control of gene expression in *E. coli* and many other bacteria [155-157]. Although the mechanism of action of RNA degradosome in *E. coli* is relatively well understood, very little is known about the mechanisms involved in the RNA metabolism in other bacterial species [158, 159], including mycobacteria. *M. tuberculosis* is a very successful pathogen which is able to persist under stressful conditions for a long time in a non-replicating state inside the host [2]. Given the central role of RNA degradosome in RNA metabolism in *E. coli*, it is conceivable that the RNA degradosome-dependent regulation of RNA stability in *M. tuberculosis* might also be a very important mechanism, adjusting the cellular metabolism to environmental stress during dormancy.

It has been reported that during dormancy, the bacilli exhibit a decline in sensitivity to antibiotics and an increase in RNase E levels [160], which is a component of the RNA degradosome. Likewise, having demonstrated the involvement of *M. tuberculosis* PNPase in RNA metabolism, we infer its involvement in influencing PZA susceptibility during dormancy. This is especially because the mutant $Rv2783_{D67N}$ protein was able to catalyze RNA metabolism even in the presence of POA, and could in addition contribute towards sustaining RNA stability during dormancy. The finding that the RNA catalytic activities of the wild type Rv2783 and not the mutant $Rv2783_{D67N}$ protein were significantly inhibited by POA again suggest that the D67N mutation may affect the binding of POA without necessarily eliminating the protein's RNA modifying enzymatic activities. Similarly, the *M. tuberculosis* $Rv2783_{D67N}$ mutant protein would therefore catalyze RNA metabolism uninhibited by POA, and especially soo during dormancy where RNA stability is at stake. Moreover, it is likely that the D67 residue on the N-terminal region of *M. tuberculosis* Rv2783 is flexible and dispensable for enzymatic activity but is required for POA binding.

However, further studies on the structural organization and functional analysis of some specific amino acid residues of the protein are needed to confirm this hypothesis and delineate their roles. On the other hand, the ability of the Rv2783 to catalyze RNA polymerization in a template-independent manner would possibly result in producing inaccurate mRNAs, rRNAs and tRNAs, and further mistranslation. New protein variants with an adaptive function could be synthesized, producing new bacillary phenotypes that can tolerate or resist POA sterilizing effect. This only happens to a small population which explains why most of the bacilli are killed by antibiotics for lack of adaptive "protein variants". Tolerance to antibiotics, like other stresses encountered by *M. tuberculosis* bacilli during persistence, is an important part of their survival, and would give rise to clones that are not killed and can even grow in the presence of antibiotics [235]. Altering translational fidelity was reported to represent a unique form of environmental adaptation under stressful conditions through increasing proteome diversity [236]. In their study, Javid and colleagues [236] provided evidence that specific mistranslation of the mycobacterial RNA polymerase, the target of RIF, is necessary and sufficient for RIF phenotypic resistance. In addition, we speculate that the potential diverse small RNAs synthesized by Rv2783 may regulate the gene expression of *M. tuberculosis* bacilli in different ways, enabling some of them to survive during persistence.

We further investigated whether or not *M. tuberculosis* Rv2783 is involved in ppGpp synthesis and ppGpp hydrolysis reactions. Our finding that both wild type *M. tuberculosis* Rv2783 and *M. tuberculosis* $Rv2783_{D67N}$ mutant proteins demonstrated only weak ppGpp synthesis activities but strong hydrolytic activities suggest the involvement of *M. tuberculosis* Rv2783 in the regulation of intracellular concentrations of ppGpp in the bacilli under conditions that impart PZA susceptibility. The catalytic activities were similar to those of *E. coli* SpoT protein, which is a bi-functional enzyme with strong (p)ppGpp hydrolase and weak synthetase activities activated during glucose, fatty acid and possibly other starved conditions [237]. Herein, we used GDP as the substrate leading to the formation of ppGpp, while the reverse hydrolysis reaction yielded GDP and ATP as the building blocks. In many bacteria, (p)ppGpp acts as a signaling molecule to control gene expression in several metabolic pathways involved in long-term survival under stressful conditions. In *M. tuberculosis*, the long-term survival of non-replicating bacilli has hitherto been associated with the Rel_{Mtb} – mediated (p)ppGpp synthesis, which triggers the stringent

response [238]. PZA exhibits a preferential sterilizing activity against non-replicating persister bacilli during dormancy [239]. Therefore, our finding that overexpression of *Rv2783c*$_{G199A}$ causes PZA resistance and that the Rv2783$_{D67N}$ mutant protein retained the strong ppGpp hydrolysis activity even in the presence of POA, strongly suggest the protein's involvement in stringent response. Weiss and Stallings predicted that the sole expression of (p)ppGpp without its hydrolysis may directly impact cellular pathways in an uncontrolled manner that is detrimental for the bacteria [240]. They demonstrated that expression of an allele encoding the hydrolase-dead Rel$_{Mtb}$ mutant incapable of hydrolyzing (p)ppGpp but still able to synthesize (p)ppGpp decreased the growth rate of *M. tuberculosis* and changed the colony morphology of the bacteria. Weiss and Stallings further demonstrated that the Rel$_{Mtb}$ mutant expression during acute or chronic *M. tuberculosis* infection in mice was lethal to the infecting bacteria. Taken together, our findings further highlight the distinct importance of (p)ppGpp hydrolysis that is essential for *M. tuberculosis* pathogenesis. We propose that *M. tuberculosis* Rv2783 plays an important role in the general homeostasis of (p)ppGpp during dormancy, and while this function is inhibited by POA in the wild type, the D67N substitution mutation helps circumvent POA effects. Again, the finding that *M. tuberculosis* Rv2783$_{D67N}$ protein retains the strong ppGpp hydrolase and weak synthetase activities further suggest that the mutation may affect the binding of POA without diminishing the protein's catalytic activities.

The *M. tuberculosis Rv2783c* gene is located within a cluster of co-oriented open reading frames (**Figure 31**) in the order: *rpsO* (encoding ribosomal protein S15), *lppU* (a liporotein), *Rv2783c* (PNPase/GpsI), *pepR* (a zinc protease), and *Rv2781c* (a putative oxidoreductase). The upstream *lppU* gene and the downstream *pepR* and *Rv2781c* genes are deemed non-essential for *M. tuberculosis* growth, as was illustrated by the recovery of viable bacteria with transposon insertions within their respective open reading frames [140].

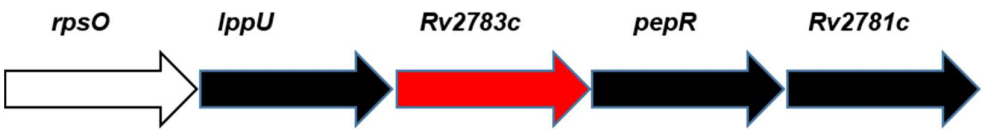

Figure 31: The *M. tuberculosis Rv2783c* gene locus and its surrounding genes.

In contrast, no transposon insertion mutant within the *Rv2783c* gene was recovered [140], suggesting that *Rv2783c* might be essential for growth of *M. tuberculosis*. Therefore, it is likely that the D67 residue on the N-terminal region of *M. tuberculosis* Rv2783 is flexible and dispensable for enzymatic activity but is required for POA binding. However, further studies on the structural organization and functional analysis of some specific amino acid residues of the protein are needed to confirm this hypothesis and delineate their roles.

In summary, we have demonstrated *M. tuberculosis* Rv2783 as a probable target of POA, and illustrated that mutations in *Rv2783c* confer resistance to POA. For the first time, we have also demonstrated that Rv2783 is a bifunctional enzyme; it is a polynucleotide phosphorylase (PNPase) and guanosine pentaphosphate synthetase (GpsI)), and its functions can be inhibited by POA. Taken together, we have provided a better understanding of this unique sterilizing drug; important for the design of new drugs targeting *M. tuberculosis* persisters for improved treatment of TB. Following the above, we thus propose a model of the association and peculiar roles of Rv2783 in PZA resistance (**Figure 32**).

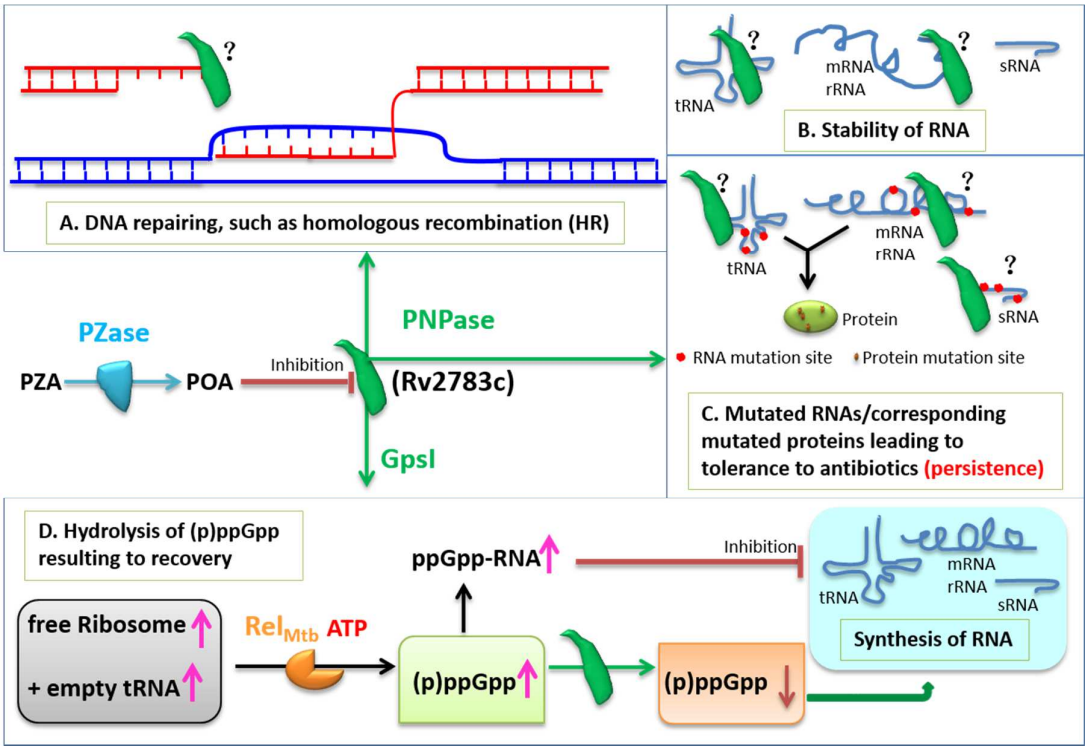

Figure 32: Role of Rv2783 in PZA resistance.

POA kills *M. tuberculosis* by inhibiting the dual catalytic activities of Rv2783 under stressful conditions.

During persistence, the conditions which impact PZA susceptibility, Rv2783 catalyzes both PNPase and ppGpp hydrolysis activities. As a PNPase, Rv2783 catalyzes DNA metabolism resulting to conservation of DNA integrity through DNA repair and mutagenesis. On RNA metabolism, Rv2783 catalyzes the increased production of RNA molecules resulting to RNA stability. On the other hand, Rv2783 catalyzes mistranslation producing variant proteins that enable the bacilli to tolerate or resist the sterilizing effects of POA. As a GpsI, Rv2783 catalyzes the hydrolysis of ppGpp when it reaches a critical concentration following its production by Rel_{Mtb}. By regulating the general homeostasis of ppGpp, Rv2783 facilitates the synthesis of mRNA in the ribosomes, subsequent cytoplasmic RNA molecules (such as tRNAs and sRNAs) and the eventual translation of the RNA transcripts.

While it is often rather difficult to demonstrate the activity of PZA especially in mouse models of short duration owing to its short half-life in mice, animal studies should be carried out to further confirm the functionality of *M. tuberculosis* Rv2783 so as to demonstrate its *in vivo* relevance for drug discovery and development. Detailed studies on structural organization and functional analysis of the specific catalytic domains of *M. tuberculosis* Rv2783 protein to delineate their roles would be needful for drug discovery and development. In addition, gene knockdown of *M. tuberculosis Rv2783c* would be necessary to allow study what the effects of POA would be on the growth of the bacilli upon suppressing the expression of *Rv2783c*.

Following the questionable impact of *rpsA* mutations in PZA resistance [133], we set out to verify the effect of some reported rpsA mutations through overexpression in wild type *M. tuberculosis* H37Rv. In this study, 6 out of the 10 overexpressed *rpsA* mutations were concordant with the previous reports, while the remaining 4 were inconsistent with previous findings. Shi and colleagues [101] had previously reported 2 PZA-resistant strains wild type for the *pncA* gene but bearing dual RpsA Thr5Ser and Asp123Ala mutations. This double mutant was however not observed in another study by Alexander and colleagues [136], but who instead reported an almost similar mutation, *rpsA* A364G (Lys122Glu), in one PZA-sensitive strain. The phenotypic findings of Alexander and colleagues suggested that RpsA Lys122Glu had no impact on PZA resistance, and thus, Asp123Ala could also be innocuous. The later suggestion is concordant with our findings as overexpression of the dual Thr5Ser and Asp123Ala mutations, and the singular Thr5Ser mutation in wild type *M. tuberculosis*

H37Rv did not cause PZA resistance. The Glu433Asp mutation had previously been reported by Tan and colleagues [169] in a PZA-resistant clinical strain wild type for the *pncA* gene. The Glu433 amino acid is located on the C-terminal of the RpsA, a region highly conserved among all the *Mycobacterium* species, and thus Tan and colleagues [169] had suggested the involvement of this region in PZA resistance. However, this was contrary to our findings as overexpression of the Glu433Asp mutation in wild type *M. tuberculosis* H37Rv did not cause PZA resistance. Taken together, further work on the protein analysis are required to determine if any Thr5Ser, Asp123Ala, Lys122Glu and Glu433Asp mutations impact POA/PZA binding or simply represent regions of RpsA that tolerate amino acid substitutions.

While high numbers of PZA-resistant cases are attributed to inactivation of the PZase, numerous genetic variants are highly diverse and scattered over the full length of the 561 bp of the *pncA* gene [172, 241]. About 50 cases of missense mutations at the -11 upstream putative regulatory region of *pncA* has been reported [222]. We therefore set to explore whether this mutation would affect gene expression of *pncA* at the translation level. Our finding that overexpression of the wild type A-11 *pncA,* contained in the fragments spanning the upstream *Rv2044c* and part of *Rv2045c* genes, in the P160 A-11G mutant a did not make it susceptible to PZA or restore its PZase expression was intriguing. Similarly, the finding that overexpression of the A-11G *pncA* mutant, contained in the same fragments, in wild type *M. tuberculosis* did not cause PZA resistance or abolish its PZase activity was more intriguing. The A-11G *pncA* mutation had previously been reported not to affect the transcription of *pncA* gene, as it was co-transcribed together with its surrounding genes in a polycistron [154]. However, from our results, it appears that there could be some other regulatory factors that recognize the A-11G *pncA* mutation thus affecting the expression of *pncA* at the translation level.

6. Publications

1. **Moses Njire**, Na Wang, Bangxing Wang, Yaoju Tan, Xingshan Cai, Yanwen Liu, Julius Mugweru, Jintao Guo, Shouyong Tan, Wing Wai Yew, Eric Nuermberger, Jinsong Liu, Tianyu Zhang. "Pyrazinoic acid Inhibits the Polynucleotide Phosphorylase and Guanosine Pentaphosphate Synthetase Bifunctional Enzyme in *Mycobacterium tuberculosis*". ***Manuscript submitted.***
2. Gaëlle Guiewi Makafe*, Yuanyuan Cao*, Yaoju Tan*, Mugweru Julius, Zhiyong Liu, Changwei Wang, **Moses M Njire**, Xingshan Cai, Tianzhou Liu, Bangxing Wang, Wei Pang, Shouyong Tan, Buchang Zhang, Wing Wai Yew, Gyanu Lamichhane, Jintao Guo, Tianyu Zhang. "Role of Cys154Arg substitution in the ribosomal protein L3 in Oxazolidinone resistance in *Mycobacterium tuberculosis*". *Antimicrobial Agents and Chemotherapy* 2016; 60(5): 3202-3206. **[IF 4.415].**
3. **Njire Moses*,** Yaoju Tan*, Julius Mugweru, Changwei Wang, Jintao Guo, WingWai Yew, Shouyong Tan, and Tianyu Zhang. "Pyrazinamide resistance in *Mycobacterium tuberculosis*: Review and update." *Advances in Medical Sciences* 2016; 61(1):63-71. [**IF 1.211;** Co-First author with equal contribution with Yaoju Tan].
4. Tang Jian, Wang Bangxing, Wu Tian, Wan Junting, TuZheng-Chao, **Njire Moses**, Wan Baojie, Franzblau Scott, Zhang Tianyu, Ding Ke, Lu Xiaoyun. "Design, Synthesis, and Biological Evaluation of Pyrazolo [1, 5-a] pyridine-3-carboxamides as Novel Antitubercular Agents." *ACS Medicinal Chemistry Letters* 2015; 6(7): 814-818. **[IF 3.355].**
5. Tianzhou Liu, BangxingWang, Jintao Guo, Yang Zhou, MugweruJulius, **Moses Njire,** YuanyuanCao, Tian Wu, ZhiyongLiu, ChangweiWang, Yong Xu, Tianyu Zhang. "Role of *folP1* and *folP2* genes in the action of sulfamethoxazole and trimethoprim against mycobacteria". *Journal of Microbiology and Biotechnology* 2015; 25(9):1559-1567. **[IF 1.685]**
6. Yang, Feng*, **Moses M. Njire***, Jia Liu, Tian Wu, Bangxing Wang, Tianzhou Liu, Yuanyuan Cao, Zhiyong Liu, Junting Wan, ZhengchaoTu, Yaoju Tan, Shouyong Tan, Tianyu Zhang. "Engineering more stable, selectable marker-free autoluminescent mycobacteria by one step." *PLoS One* 2015; 10(3): e0119341. **[IF 3.057;** Co-First author with equal contribution with Yang Feng**].**
7. Tan Shouyong, Danxiong Sun, Tiantuo Zhang, Yanqiong Li, **Moses M. Njire,** Yuanyuan Cao, Changwei Wang and Tianyu Zhang. "Risk Factors for Hemoptysis in Pulmonary Tuberculosis Patients from Southern China: A Retrospective Study." *Journal of Tuberculosis Research* 2014; 2(4): 173-180. **[Google-based IF 0.63]**

Patent:

张天宇，**Moses M Njire**，王邦兴，刘志永，韩轶，刘燕，刘洋。一种检测结核分枝杆菌耐药性的 DNA 标记物及其应用 (2016). 201610564946.X.

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Finally and not the least, To God be the Glory!

8. Abbreviations

TB	Tuberculosis
PZA	Pyrazinamide
DS-TB	Drug-sensitive tuberculosis
DR-TB	Drug-resistant tuberculosis
PZase	Pyrazinamidase
POA	Pyrazinoic acid
PNPase	Polyribonucleotide nucleotidyltransferase
GPSI	Guanosine pentaphosphate synthetase
RNA	Ribonucleic acid
mRNA	Messenger Ribonucleic acid
ssRNA	Single-strand Ribonucleic acid
ppGpp	Guanosine tetraphosphate
pppGpp	Guanosine pentaphosphate
ITC	Isothermal titration calorimetry
DNA	Deoxyribonucleic acid
ssDNA	Single-strand Deoxyribonucleic acid
G+C	Guanine + Cytosine
TST	Tuberculin skin test
PPD	Purified protein derivative
BCG	Bacillus Calmette–Guérin
HIV	Human immunodeficiency virus
EEA1	Early endosomal antigen 1
LAMP1	Lysosomes-associated membrane glycoprotein 1
STR	Streptomycin
PAS	Para-aminosalicylic acid
AIDS	Acquired Immune Deficiency Syndrome
MDR-TB	Multi-drug resistant tuberculosis
XDR-TB	Extremely-drug resistant tuberculosis
TDR-TB	Totally-drug resistant tuberculosis
WHO	World Health Organization
RIF	Rifampicin
INH	Isoniazid
NE	New cases
PT	Previously treated cases
DST	Drug susceptibility testing
EMB	Ethambutol

KAN	Kanamycin
AMK	Amikacin
CAP	Capreomycin
ACP	Acyl-carrier-protein
CoA	Coenzyme A
BMRC	British Medical Research Council
MICs	Minimum inhibitory concentrations
MtQAPRTase	*M. tuberculosis* quinolinic acid phosphoribosyltransferase
NAD	Nicotinamide adenine dinucleotide
FAS-I	Fatty acid synthetase
5-Cl-PZA	5-chloro-pyrazinamide
RpsA	Ribosomal protein S1
Rel_{Mtb}	*M. tuberculosis* Rel protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
ATP	Adenosine triphosphate
NHEJ	Non-homologous end joining
DSB	Double-strand break
SSA	Single-strand annealing
FR	Flanking region
PCR	Polymerase chain reaction
CUPSAT	Cologne University Protein Stability Analysis Tool
CLSI	Clinical and Laboratory Standards Institute
MBC	Minimum bactericidal concentration
BMA	Biphasic media assay
STAND	Shortening Treatment by Advancing Novel Drugs
PaMZ	Pretomanid, moxifloxacin and pyrazinamide
SNPs	Single nucleotide polymorphisms
LB	Luria-Bertani
NaCl	Sodium chloride
NaOH	Sodium hydroxide
KCl	Potassium hydrochloride
$CaCl_2$	Calcium chloride
$MgSO_4$	Magnesium sulphate
OADC	Oleate-albumin-dextrose-catalase
ADC	Albumin-dextrose-catalase
BSA	Bovine serum albumin

PIPES	Piperazine-N,N'-bis(ethanesulfonic acid) buffer
CTAB	Cetyltrimethyl ammonium bromide
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
dNTPs	Deoxyribonucleotide triphosphates
DMSO	Dimethyl sulfoxide
F/R	Forward/Reverse
SOB	Super optimal broth
OD_{600}	Optical density at 600 nanometer
rpm	Revolutions per minute
Hyg	Hygromycin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Tris-HCl	Tris-Hydrocloride buffer
TEMED	Tetramethylethylenediamine
Ni^{2+}-NTA	Nickel-Nitroacetic acid
EDTA	Ethylenediaminetetraacetic acid
dADP	Deoxyadenosine diphosphate
ADP	Adenosine diphosphate
$(NH_4)_3PO_4$	Ammonium phosphate
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer
DTT	Dithiothreitol buffer
HPLC	High-Pressure liquid chromatography
AMP	Adenosine monophosphate
$MnCl_2$	Manganese chloride
UV	Ultra violet light
KH_2PO_4	Potassium dihydrogen phosphate
Kb	Kilo base

9. List of Figures

10. List of Tables

11. References

1. Koch R. The etiology of tuberculosis. Berl Klin Wochenschr. 1882:15: 221–30.
2. Russell DG. *Mycobacterium tuberculosis*: here today, and here tomorrow. Nature Reviews Molecular Cell Biology. 2001;2(8):569-86.
3. Jones GH. Purification and properties of ATP: GTP 3'-pyrophosphotransferase (guanosine pentaphosphate synthetase) from *Streptomyces antibioticus*. Journal of Bacteriology. 1994;176(5):1475-81.
4. World Health Organization. Global tuberculosis report 2015. 2015.
5. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. JAMA. 1999;282(7):677-86.
6. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. Annual review of immunology. 2013;31:475-527.
7. Wheeler PR, Ratledge C. In Tuberculosis: Pathogenesis, protection, and control (ed. Bloom, B. R.) 353–385 American Society for Microbiology press, Washington DC; 1994.
8. Brennan PJ, Draper P. In Tuberculosis: pathogenesis, protection, and control (ed. Bloom, B. R.) 271–284 American Society for Microbiology press, Washington DC.; 1994.
9. Kolattukudy P, Fernandes ND, Azad A, Fitzmaurice AM, Sirakova TD. Biochemistry and molecular genetics of cell-wall lipid biosynthesis in mycobacteria. Molecular Microbiology. 1997;24(2):263-70.
10. Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 1998;393(6685):537-44.
11. Harisinghani MG, McLoud TC, Shepard J-AO, Ko JP, Shroff MM, Mueller PR. Tuberculosis from Head to Toe 1: (CME available in print version and on RSNA Link). Radiographics. 2000;20(2):449-70.
12. Vynnycky E, Fine PE. Lifetime risks, incubation period, and serial interval of tuberculosis. American Journal of Epidemiology. 2000;152(3):247-63.
13. Pirquet V. Frequency of tuberculosis in childhood. JAMA. 1907:52:675–78.
14. Mantoux MC. La voie intradermique en tubercalinothérapie. Presse Med. 1912;20:146-8.
15. Reichman LB. Tuberculin skin testing. The state of the art. Chest. 1979;76(6 Suppl):764-70.
16. Holden M, Dubin MR, Diamond PH. Frequency of negative intermediate-strength tuberculin sensitivity in patients with active tuberculosis. New England Journal of Medicine. 1971;285(27):1506-9.
17. Richeldi L. An update on the diagnosis of tuberculosis infection. American Journal of Respiratory and Critical Care Medicine. 2006;174(7):736-42.

18. Horsburgh Jr CR. Priorities for the treatment of latent tuberculosis infection in the United States. New England Journal of Medicine. 2004;350(20):2060-7.

19. Van Rhijn I, Nguyen TKA, Michel A, Cooper D, Govaerts M, Cheng TY, et al. Low cross-reactivity of T-cell responses against lipids from *Mycobacterium bovis* and *Mycobacterium avium* paratuberculosis during natural infection. European Journal of Immunology. 2009;39(11):3031-41.

20. Young DB, Perkins MD, Duncan K, Barry III CE. Confronting the scientific obstacles to global control of tuberculosis. The Journal of Clinical Investigation. 2008;118(4):1255.

21. Zimmerli S, Edwards S, Ernst JD. Selective receptor blockade during phagocytosis does not alter the survival and growth of *Mycobacterium tuberculosis* in human macrophages. American Journal of Respiratory Cell and Molecular Biology. 1996;15(6):760-70.

22. Duclos S, Diez R, Garin J, Papadopoulou B, Descoteaux A, Stenmark H, et al. Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages. Journal of Cell Science. 2000;113(19):3531-41.

23. Sturgill-Koszycki S, Schaible U, Russell D. Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. The EMBO journal. 1996;15(24):6960.

24. Hao M, Maxfield FR. Characterization of rapid membrane internalization and recycling. Journal of Biological Chemistry. 2000;275(20):15279-86.

25. Rohrer J, Schweizer A, Russell D, Kornfeld S. The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. The Journal of Cell Biology. 1996;132(4):565-76.

26. Schweizer A, Kornfeld S, Rohrer J. Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. The Journal of Cell Biology. 1996;132(4):577-84.

27. Waksman SA, Reilly HC, Schatz A. Strain specificity and production of antibiotic substances V. Strain resistance of bacteria to antibiotic substances, especially to streptomycin. Proceedings of the National Academy of Sciences. 1945;31(6):157-64.

28. Crofton J, Mitchison D. Streptomycin resistance in pulmonary tuberculosis. British Medical Journal. 1948;2(4588):1009.

29. British Medical Research Council ". Streptomycin treatment of pulmonary tuberculosis. British Medical Journal 1948; 2(4582): 769-782. 1948.

30. Lehmann J. On the effect of isomers of PAS (para-aminosalicylic acid) and related substances on the tuberculostatic effect of PAS. Cellular and Molecular Life Sciences. 1949;5(9):365-7.

31. Smith P, Moss A. Epidemiology of tuberculosis. Tuberculosis: Pathogenesis, protection, and control. 1994;47.

32. McShane H, Jacobs WR, Fine PE, Reed SG, McMurray DN, Behr M, et al. BCG: myths, realities, and the need for alternative vaccine strategies. Tuberculosis. 2012;92(3):283-8.

33. World Health Organization. The stop TB strategy. World Health Organization, Geneva, Switzerland. 2006.

34. World Health Organization. Global Tuberculosis Report 2014: World Health Organization; 2014.

35. Loewenberg S. India reports cases of totally drug-resistant tuberculosis. The Lancet. 2012;379(9812):205.

36. Klopper M, Warren RM, Hayes C, van Pittius NCG, Streicher EM, Müller B, et al. Emergence and spread of extensively and totally drug-resistant tuberculosis, South Africa. Emerging Infectious Diseases. 2013;19(3):449.

37. Udwadia Z, Vendoti D. Totally drug-resistant tuberculosis (TDR-TB) in India: every dark cloud has a silver lining. Journal of Epidemiology and Community Health. 2013;67(6):471-2.

38. Migliori G, De Iaco G, Besozzi G, Centis R, Cirillo D. First tuberculosis cases in Italy resistant to all tested drugs. Euro surveillance. 2007;12(5):E070517.

39. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, ZiaZarifi AH, et al. Emergence of new forms of totally drug-resistant tuberculosis bacilli: Super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. Chest Journal. 2009;136(2):420-5.

40. Alanis AJ. Resistance to antibiotics: are we in the post-antibiotic era? Archives of Medical Research. 2005;36(6):697-705.

41. Pooran A, Pieterson E, Davids M, Theron G, Dheda K. What is the cost of diagnosis and management of drug resistant tuberculosis in South Africa. PLoS One. 2013;8(1):e54587.

42. Fischbach MA, Walsh CT. Antibiotics for emerging pathogens. Science. 2009;325(5944):1089-93.

43. World Health Organization. Global tuberculosis control: surveillance, planning, financing: World Health Organization Report 2012.

44. Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, Van Soolingen D, et al. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. The Lancet. 2010;375(9728):1830-43.

45. McKinney JD, zu Bentrup KH, Muñoz-Elías EJ, Miczak A, Chen B, Chan W-T, et al. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature. 2000;406(6797):735-8.

46. Wayne L. Dormancy of *Mycobacterium tuberculosis* and latency of disease. European Journal of Clinical Microbiology and Infectious Diseases. 1994;13(11):908-14.

47. Elliott A, Beming S, Iseman M, Peloquin C. Failure of drug penetration and acquisition of drug resistance in chronic tuberculous empyema. Tubercle and Lung Disease. 1995;76(5):463-7.

48. Iseman M, Madsen L. Chronic tuberculous empyema with bronchopleural fistula resulting in treatment failure and progressive drug resistance. Chest Journal. 1991;100(1):124-7.

49. Jindani A, Aber V, Edwards E, Mitchison D. The early bactericidal activity of drugs in patients with pulmonary tuberculosis 1, 2. American Review of Respiratory Disease. 1980;121(6):939-49.

50. Mitchison D. The action of antituberculosis drugs in short-course chemotherapy. Tubercle. 1985;66(3):219-25.

51. Mohapatra BN, Mohanty C. Treatment of Tuberculosis WHO Guidelines 2010.

52. East African/ British Medical Research Councils. Controlled trial of five short course regimens of chemotherapy regimens for pulmonary tuberculosis. Am Rev Respir Dis. 1981:123: 65–70.

53. Cole S, Telenti A. Drug resistance in *Mycobacterium tuberculosis*. The European Respiratory Journal Supplement. 1995;20:701s-13s.

54. Nguyen L, Pieters J. Mycobacterial subversion of chemotherapeutic reagents and host defense tactics: challenges in tuberculosis drug development. Annual Review of Pharmacology and oxicology. 2009;49:427-53.

55. Dale J. Mobile genetic elements in mycobacteria. The European Respiratory Journal Supplement. 1995;20:633s-48s.

56. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tubercle and Lung disease. 1998;79(1):3-29.

57. Pasipanodya JG, Gumbo T. A new evolutionary and pharmacokinetic–pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs. Current Opinion in Pharmacology. 2011;11(5):457-63.

58. Perlman DC, Segal Y, Rosenkranz S, Rainey PM, Remmel RP, Salomon N, et al. The clinical pharmacokinetics of rifampin and ethambutol in HIV-infected persons with tuberculosis. Clinical Infectious Diseases. 2005;41(11):1638-47.

59. Müller B, Streicher E, Hoek K, Tait M, Trollip A, Bosman M, et al. inhA promoter mutations: a gateway to extensively drug-resistant tuberculosis in South Africa? The International Journal of Tuberculosis and Lung Disease. 2011;15(3):344-51.

60. Louw GE, Warren RM, Gey van Pittius NC, Leon R, Jimenez A, Hernandez-Pando R, et al. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. American Journal of Respiratory and Critical Care Medicine. 2011;184(2):269-76.

61. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Medicine. 2009;6(2):e2.

62. David HL. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. Applied microbiology. 1970;20(5):810-4.

63. Gillespie SH. Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. Antimicrobial Agents and Chemotherapy. 2002;46(2):267-74.

64. Ford CB, Lin PL, Chase MR, Shah RR, Iartchouk O, Galagan J, et al. Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. Nature Genetics. 2011;43(5):482-6.

65. Jolivet-Gougeon A, Kovacs B, Le Gall-David S, Le Bars H, Bousarghin L, Bonnaure-Mallet M, et al. Bacterial hypermutation: clinical implications. Journal of Medical Microbiology. 2011;60(5):563-73.

66. Zhang Y, Yew WW, Barer MR. Targeting persisters for tuberculosis control. Antimicrobial Agents and Chemotherapy. 2012;56(5):2223-30.

67. Zhang Y. Persisters, persistent infections and the Yin–Yang model. Emerging Microbes and Infections. 2014;3(1):e3.

68. Balaban N. Persistence: mechanisms for triggering and enhancing phenotypic variability. Current Opinion in Genetics and Development. 2011;21(6):768-75.

69. Dhar N, McKinney JD. *Mycobacterium tuberculosis* persistence mutants identified by screening in isoniazid-treated mice. Proceedings of the National Academy of Sciences. 2010;107(27):12275-80.

70. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M. Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. Infection, Genetics and Evolution. 2012;12(4):695-700.

71. Van Deun A, Martin A, Palomino J. Diagnosis of drug-resistant tuberculosis: reliability and rapidity of detection [State of the art series. Drug-resistant tuberculosis. Edited by CY. Chiang. Number 3 in the series]. The International Journal of Tuberculosis and Lung Disease. 2010;14(2):131-40.

72. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. New England Journal of Medicine. 2010;363(11):1005-15.

73. Dalmer O, Walter E, Firma E. Merck in Darmstadt. Verfahren zur Herstellung von Abkömmlingen der Pyrazinmonocarbonsäure Patentiert im Deutschen Reiche vom 8 Juli 1934 ab Germany patent 632 257 Klasse 12 p Gruppe 6 M 127990 IV a/12 p 1936.

74. Yeager R, Munroe W, Dessau FI. Pyrazinamide (aldinamide) in the treatment of pulmonary tuberculosis. American Review of Tuberculosis and Pulmonary Diseases. 1952;65(5):523-46.

75. Chorine V, editor Action de l'amide nicotinique sur les bacilles du genre Mycobacterium. CR Acad Sci (Paris) 1945; 220:150–151.

76. Malone L, Schurr A, Lindh H, McKenzie D, Kiser J, Williams J. The Effect of pyrazinamide (Aldinamide) on experimental tuberculosis in mice. American Review of Tuberculosis and Pulmonary Diseases. 1952;65(5):511-18.

77. Solotorovsky M, Gregory F, Ironson E, Bugie E, O'Neill R, Pfister K. Pyrazinoic acid amide-an agent active against experimental murine tuberculosis. Experimental Biology and Medicine. 1952;79(4):563-5.

78. McCune RM, Tompsett R. Fate of *Mycobacterium tuberculos*is in mouse tissues as determined by the microbial enumeration technique I. The persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. The Journal of Experimental Medicine. 1956;104(5):737-62.

79. Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council Tuberculosis Units, 1946–1986, with relevant subsequent publications. The International Journal of Tuberculosis and Lung Disease. 1999;3(10s2):S231-S79.

80. World Health Organization. Guidelines for the programmatic management of drug-resistant tuberculosis. World Health Organization; Geneva: 2011. 2011.

81. Tasneen R, Li S-Y, Peloquin CA, Taylor D, Williams KN, Andries K, et al. Sterilizing activity of novel TMC207-and PA-824-containing regimens in a murine model of tuberculosis. Antimicrobial Agents and Chemotherapy. 2011;55(12):5485-92.

82. Deye GA, Gettayacamin M, Hansukjariya P, Im-erbsin R, Sattabongkot J, Rothstein Y, et al. Use of a rhesus Plasmodium cynomolgi model to screen for anti-hypnozoite activity of pharmaceutical substances. The American Journal of Tropical Medicine and Hygiene. 2012;86(6):931-5.

83. Wade MM, Zhang Y. Effects of weak acids, UV and proton motive force inhibitors on pyrazinamide activity against *Mycobacterium tuberculosis in vitro*. Journal of Antimicrobial Chemotherapy. 2006;58(5):936-41.

84. Lewis K. Persister cells. Annual Review of Microbiology. 2010;64:357-72.

85. Coates AR, Hu Y. Targeting non-multiplying organisms as a way to develop novel antimicrobials. Trends in Pharmacological Sciences. 2008;29(3):143-50.

86. Nathan C. Fresh approaches to anti-infective therapies. Science Translational Medicine. 2012;4(140):140sr2-sr2.

87. Gopal P, Dick T. Reactive dirty fragments: implications for tuberculosis drug discovery. Current Opinion in Microbiology. 2014;21:7-12.

88. Speirs R, Welch J, Cynamon M. Activity of n-propyl pyrazinoate against pyrazinamide-resistant *Mycobacterium tuberculosis*: investigations into mechanism of action of and mechanism of resistance to pyrazinamide. Antimicrobial Agents and Chemotherapy. 1995;39(6):1269-71.

89. Salfinger M, Heifets LB. Determination of pyrazinamide MICs for *Mycobacterium tuberculosis* at different pHs by the radiometric method. Antimicrobial Agents and Chemotherapy. 1988;32(7):1002-4.

90. Gumbo T, Chigutsa E, Pasipanodya J, Visser M, van Helden PD, Sirgel FA, et al. The pyrazinamide susceptibility breakpoint above which combination therapy fails. Journal of Antimicrobial Chemotherapy. 2014:dku136.

91. Alsultan A, Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis: an update. Drugs. 2014;74(8):839-54.

92. British Thoracic Tuberculosis Association. Short course chemotherapy in pulmonary tuberculosis. Lancet. 1976; ii:1102–1104.

93. British Thoracic Association. A controlled trial of six months chemotherapy in pulmonary tuberculosis: Second Report: Results during the 24 months after the end of chemotherapy. The American Review of Respiratory Disease 1982; 126:460–462. 1981.

94. McCune RM, Tompsett R, McDermott W. The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique II. The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. The Journal of Experimental Micine. 1956;104(5):763-802.

95. Nuermberger E, Tyagi S, Tasneen R, Williams KN, Almeida D, Rosenthal I, et al. Powerful bactericidal and sterilizing activity of a regimen containing PA-824, moxifloxacin, and pyrazinamide in a murine model of tuberculosis. Antimicrobial Agents and Chemotherapy. 2008;52(4):1522-4.

96. Rosenthal IM, Zhang M, Williams KN, Peloquin CA, Tyagi S, Vernon AA, et al. Daily dosing of rifapentine cures tuberculosis in three months or less in the murine model. PLoS Medicine. 2007;4(12):e344.

97. Veziris N, Ibrahim M, Lounis N, Chauffour A, Truffot-Pernot C, Andries K, et al. A once-weekly R207910-containing regimen exceeds activity of the standard daily regimen in murine tuberculosis. American Journal of Respiratory and Critical Care Medicine. 2009;179(1):75-9.

98. Konno K, Feldmann F, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. The American Review of Respiratory Disease. 1967;95:461-9.

99. Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nature Medicine. 1996;2(6):662-7.

100. Heifets L, Lindholm-Levy P. Comparison of bactericidal activities of streptomycin, amikacin, kanamycin, and capreomycin against Mycobacterium avium and *M. tuberculosis.* Antimicrobial Agents and Chemotherapy. 1989;33(8):1298-301.

101. Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry CE, et al. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. Science. 2011;333(6049):1630-2.

102. McDermott W, Tompsett R. Activation of pyrazinamide and nicotinamide in acidic environments *in vitro*. American Review of Tuberculosis. 1954;70(4):748.

103. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tubercle and Lung Disease. 1998;78(2):117-22.

104. Budavari S, O'Neil MJ, Smith A, Heckelman PE. The Merck Index, Merck & Co. Inc, Rahway, NJ. 1989;1481.

105. Zimhony O, Cox JS, Welch JT, Vilchèze C, Jacobs WR. Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*. Nature medicine. 2000;6(9):1043-7.

106. Zhang Y, Wade MM, Scorpio A, Zhang H, Sun Z. Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid. Journal of Antimicrobial Chemotherapy. 2003;52(5):790-5.

107. Zhang Y, Shi W, Zhang W, Mitchison D. Mechanisms of pyrazinamide action and resistance. Microbiology Spectrum. 2013;2(4):1.

108. Dillon NA, Peterson ND, Rosen BC, Baughn AD. Pantothenate and pantetheine antagonize the antitubercular activity of pyrazinamide. Antimicrobial Agents and Chemotherapy. 2014;58(12):7258-63.

109. Manca C, Koo M-S, Peixoto B, Fallows D, Kaplan G, Subbian S. Host targeted activity of pyrazinamide in *Mycobacterium tuberculosis* infection. PloS One. 2013;8(8):e74082.

110. Mendez S, Traslavina R, Hinchman M, Huang L, Green P, Cynamon MH, et al. The antituberculosis drug pyrazinamide affects the course of cutaneous leishmaniasis *in vivo* and increases activation of macrophages and dendritic cells. Antimicrobial Agents and Chemotherapy. 2009;53(12):5114-21.

111. Zhang Y, Scorpio A, Nikaido H, Sun Z. Role of acid pH and deficient efflux of pyrazinoic acid in unique susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. Journal of Bacteriology. 1999;181(7):2044-9.

112. Huang D, Zhang Y, Chen X. Analysis of intracellular nucleoside triphosphate levels in normal and tumor cell lines by high-performance liquid chromatography. Journal of Chromatography B. 2003;784(1):101-9.

113. Zhang Y, Permar S, Sun Z. Conditions that may affect the results of susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide. Journal of Medical Microbiology. 2002;51(1):42-9.

114. Zimhony O, Vilchèze C, Arai M, Welch JT, Jacobs WR. Pyrazinoic acid and its n-propyl ester inhibit fatty acid synthase type I in replicating tubercle bacilli. Antimicrobial Agents and Chemotherapy. 2007;51(2):752-4.

115. Salfinger M, Crowle AJ, Reller LB. Pyrazinamide and pyrazinoic acid activity against tubercle bacilli in cultured human macrophages and in the BACTEC system. Journal of Infectious Diseases. 1990;162(1):201-7.

116. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, et al. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994;263(5147):678-81.

117. Heifets L, Higgins M, Simon B. Pyrazinamide is not active against *Mycobacterium tuberculosis* residing in cultured human monocyte-derived macrophages. Unresolved issues. The International Journal of Tuberculosis and Lung Disease. 2000;4(6):491-5.

118. Vandal OH, Pierini LM, Schnappinger D, Nathan CF, Ehrt S. A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. Nature Medicine. 2008;14(8):849-54.

119. Zhang T, Li S-Y, Nuermberger EL. Autoluminescent *Mycobacterium tuberculosis* for rapid, real-time, non-invasive assessment of drug and vaccine efficacy. PloS One. 2012;7(1):e29774.

120. Boshoff HI, Mizrahi V, Barry CE. Effects of pyrazinamide on fatty acid synthesis by whole mycobacterial cells and purified fatty acid synthase I. Journal of Bacteriology. 2002;184(8):2167-72.

121. Kim H, Shibayama K, Rimbara E, Mori S. Biochemical characterization of quinolinic acid phosphoribosyltransferase from *Mycobacterium tuberculosis* H37Rv and inhibition of its activity by pyrazinamide. PloS One. 2014;9(6):e100062.

122. Via LE, Savic R, Weiner DM, Zimmerman MD, Prideaux B, Irwin SM, et al. Host-mediated bioactivation of pyrazinamide: Implications for efficacy, resistance, and therapeutic alternatives. ACS Infectious Diseases. 2015; 1(5):203-14

123. Kamal N, Mukerji P, Kishore K, Kumar S, Khanna B. Serum levels of pyrazinamide and pyrazinoic acid following long term administration of pyrazinamide orally. Indian Journal of Tuberculosis. 1976;23(1):19.

124. Lacroix C, Hoang TP, Nouveau J, Guyonnaud C, Laine G, Duwoos H, et al. Pharmacokinetics of pyrazinamide and its metabolites in healthy subjects. European Journal of Clinical Pharmacology. 1989;36(4):395-400.

125. World Health Organization. Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. Report No HO/HTM/TB/20103. 2010.

126. Fernandes ND, Kolattukudy PE. Cloning, sequencing and characterization of a fatty acid synthase-encoding gene from *Mycobacterium tuberculosis var. bovis* BCG. Gene. 1996;170(1):95-9.

127. Brindley D, Matsumura S, Bloch K. Mycobacterium phlei fatty acid synthetase-a bacterial multienzyme complex. Nature. 1969;224:666-9.

128. Cynamon MH, Speirs RJ, Welch JT. In vitro antimycobacterial activity of 5-chloropyrazinamide. Antimicrobial Agents and Chemotherapy. 1998;42(2):462-3.

129. Ahmad Z, Tyagi S, Minkowski A, Almeida D, Nuermberger EL, Peck KM, et al. Activity of 5-chloro-pyrazinamide in mice infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis.* The Indian Journal of Medical Research. 2012;136(5):808.

130. Ngo SC, Zimhony O, Chung WJ, Sayahi H, Jacobs WR, Welch JT. Inhibition of isolated *Mycobacterium tuberculosis* fatty acid synthase I by pyrazinamide analogs. Antimicrobial Agents and Chemotherapy. 2007;51(7):2430-5.

131. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, et al. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 1997;41(3):540-3.

132. Klemens S, Sharpe C, Cynamon M. Activity of pyrazinamide in a murine model against *Mycobacterium tuberculosis* isolates with various levels of *in vitro* susceptibility. Antimicrobial Agents and Chemotherapy. 1996;40(1):14-6.

133. Personne Y, Parish T. *Mycobacterium tuberculosis* possesses an unusual tmRNA rescue system. Tuberculosis. 2014;94(1):34-42.

134. Shi W, Chen J, Feng J, Cui P, Zhang S, Weng X, et al. Aspartate decarboxylase (PanD) as a new target of pyrazinamide in *Mycobacterium tuberculosis*. Emerging Microbes and Infections. 2014;3(8):e58.

135. Simons SO, Mulder A, van Ingen J, Boeree MJ, van Soolingen D. Role of *rpsA* gene sequencing in diagnosis of pyrazinamide resistance. Journal of Clinical Microbiology. 2013;51(1):382-.

136. Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: a role for *pncA* but not *rpsA*. Journal of Clinical Microbiology. 2012;50(11):3726-8.

137. Mohanty BK, Kushner SR. Polynucleotide phosphorylase functions both as a 3′→ 5′ exonuclease and a poly (A) polymerase in *Escherichia coli.* Proceedings of the National Academy of Sciences. 2000;97(22):11966-71.

138. Jones GH, Bibb MJ. Guanosine pentaphosphate synthetase from *Streptomyces antibioticus* is also a polynucleotide phosphorylase. Journal of Bacteriology. 1996;178(14):4281-8.

139. Cashel M, Gentry D, Hernandez V. Vinella (1996) The stringent response. *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology American Society for Microbiology, Washington, District of Columbia.1458-96.

140. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. Molecular Microbiology. 2003;48(1):77-84.

141. Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. Recent functional insights into the role of (p)ppGpp in bacterial physiology. Nature Reviews Microbiology. 2015;13(5):298-309.

142. Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS. ppGpp conjures bacterial virulence. Microbiology and Molecular Biology Reviews. 2010;74(2):171-99.

143. Geiger T, Francois P, Liebeke M, Fraunholz M, Goerke C, Krismer B, et al. The stringent response of *Staphylococcus aureus* and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. PLoS Pathology. 2012;8(11):e1003016..

144. Poole K. Bacterial stress responses as determinants of antimicrobial resistance. Journal of Antimicrobial Chemotherapy. 2012:dks196.

145. Maisonneuve E, Gerdes K. Molecular mechanisms underlying bacterial persisters. Cell. 2014;157(3):539-48.

146. Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, Barry CE. The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. Journal of Bacteriology. 2000;182(17):4889-98.

147. Dahl JL, Kraus CN, Boshoff HI, Doan B, Foley K, Avarbock D, et al. The role of Rel_{Mtb}-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. Proceedings of the National Academy of Sciences. 2003;100(17):10026-31.

148. Avarbock A, Avarbock D, Teh J-S, Buckstein M, Wang Z-m, Rubin H. Functional regulation of the opposing (p)ppGpp synthetase/hydrolase activities of Rel_{Mtb} from *Mycobacterium tuberculosis*. Biochemistry. 2005;44(29):9913-23.

149. Atkinson GC, Tenson T, Hauryliuk V. The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. PloS One. 2011;6(8):e23479.

150. Bralley P, Jones GH. Organization and expression of the polynucleotide phosphorylase gene (pnp) of Streptomyces: processing of pnp transcripts in *Streptomyces antibioticus.* Journal of Bacteriology. 2004;186(10):3160-72.

151. Malathi V, Sirsi M, Ramakrishnan T, Maller R. Polynucleotide phosphorylase of *Mycobacterium tuberculosis* H37Rv. Indian Journal of Biochemistry. 1964;1(2):71-6.

152. Unciuleac M-C, Shuman S. Distinctive effects of domain deletions on the manganese-dependent DNA polymerase and DNA phosphorylase activities of *Mycobacterium smegmatis* polynucleotide phosphorylase. Biochemistry. 2013;52(17):2967-81.

153. Cardenas PP, Carrasco B, Sanchez H, Deikus G, Bechhofer DH, Alonso JC. *Bacillus subtilis* polynucleotide phosphorylase 3′-to-5′ DNase activity is involved in DNA repair. Nucleic Acids Research. 2009;37(12):4157-69.

154. Cardenas PP, Carzaniga T, Zangrossi S, Briani F, Garcia-Tirado E, Dehò G, et al. Polynucleotide phosphorylase exonuclease and polymerase activities on single-stranded DNA ends are modulated by RecN, SsbA and RecA proteins. Nucleic Acids Research. 2011;39(21):9250-61.

155. Carpousis AJ, Van Houwe G, Ehretsmann C, Krisch HM. Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. Cell. 1994;76(5):889-900.

156. Miczak A, Kaberdin VR, Wei C-L, Lin-Chao S. Proteins associated with RNase E in a multicomponent ribonucleolytic complex. Proceedings of the National Academy of Sciences. 1996;93(9):3865-9.

157. Py B, Higgins CF, Krisch HM, Carpousis AJ. A DEAD-box RNA helicase in the E*scherichia coli* RNA degradosome. 1996.

158. Condon C. RNA processing and degradation in *Bacillus subtilis*. Microbiology and Molecular Biology Reviews. 2003;67(2):157-74.

159. Condon C, Putzer H. The phylogenetic distribution of bacterial ribonucleases. Nucleic Acids Research. 2002;30(24):5339-46.

160. Archuleta RJ, Hoppes PY, Primm TP. *Mycobacterium avium* enters a state of metabolic dormancy in response to starvation. Tuberculosis. 2005;85(3):147-58.

161. Konno K, Feldmann FM, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli 1, 2. American Review of Respiratory Disease. 1967;95(3):461-9.

162. Lemaitre N, Callebaut I, Frenois F, Jarlier V, Sougakoff W. Study of the structure–activity relationships for the pyrazinamidase (PncA) from *Mycobacterium tuberculosis.* Biochemistry. 2001;353:453-8.

163. Sheen P, Ferrer P, Gilman RH, López-Llano J, Fuentes P, Valencia E, et al. Effect of pyrazinamidase activity on pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuberculosis. 2009;89(2):109-13.

164. Du X, Wang W, Kim R, Yakota H, Nguyen H, Kim S-H. Crystal structure and mechanism of catalysis of a pyrazinamidase from *Pyrococcus horikoshii*. Biochemistry. 2001;40(47):14166-72.

165. Petrella S, Gelus-Ziental N, Maudry A, Laurans C, Boudjelloul R, Sougakoff W. Crystal structure of the pyrazinamidase of *Mycobacterium tuberculosis*: insights into natural and acquired resistance to pyrazinamide. PLoS One. 2011;6(1):e15785.

166. Juréen P, Werngren J, Toro J-C, Hoffner S. Pyrazinamide resistance and *pncA* gene mutations in *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 2008;52(5):1852-4.

167. Köser CU CI, Feuerriegel S, Niemann S, Gagneux S, Peacock SJ. Genetic diversity within *Mycobacterium tuberculosis* complex impacts on the accuracy of genotypic pyrazinamide drug-susceptibility assay. Tuberculosis. 2014;94:451-3.

168. Zhou M, Geng X, Chen J, Wang X, Wang D, Deng J, et al. Rapid colorimetric testing for pyrazinamide susceptibility of *Mycobacterium tuberculosis* by a PCR-based *in-vitro* synthesized pyrazinamidase method. PloS one. 2011;6(11):e27654

169. Tan Y, Hu Z, Zhang T, Cai X, Kuang H, Liu Y, et al. Role of *pncA* and *rpsA* gene sequencing in detection of pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from southern China. Journal of Clinical Microbiology. 2014;52(1):291-7.

170. Sreevatsan S, Pan X, Zhang Y, Kreiswirth BN, Musser JM. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. Antimicrobial Agents and Chemotherapy. 1997;41(3):636-40.

171. Kalokhe AS, Shafiq M, Lee JC, Ray SM, Wang YF, Metchock B, et al. Multidrug-resistant tuberculosis drug susceptibility and molecular diagnostic testing: a review of the literature. The American Journal of the Medical Sciences. 2013;345(2):143.

172. Zhang Y, Yew W. Mechanisms of drug resistance in *Mycobacterium tuberculosis* [State of the art series. Drug-resistant tuberculosis. Edited by CY. Chiang. Number 1 in the series]. The International Journal of Tuberculosis and Lung Disease. 2009;13(11):1320-30.

173. Raynaud C, Lanéelle M-A, Senaratne RH, Draper P, Lanéelle G, Daffé M. Mechanisms of pyrazinamide resistance in mycobacteria: importance of lack of uptake in addition to lack of pyrazinamidase activity. Microbiology. 1999;145(6):1359-67.

174. Huang T-S, Lee SS-J, Tu H-Z, Huang W-K, Chen Y-S, Huang C-K, et al. Correlation between pyrazinamide activity and *pncA* mutations in *Mycobacterium tuberculosis* isolates in Taiwan. Antimicrobial Agents and Chemotherapy. 2003;47(11):3672-3.

175. Bhuju S, de Souza Fonseca L, Marsico AG, de Oliveira Vieira GB, Sobral LF, Stehr M, et al. *Mycobacterium tuberculosis* isolates from Rio de Janeiro reveal unusually low correlation between pyrazinamide resistance and mutations in the *pncA* gene. Infection, Genetics and Evolution. 2013;19:1-6.

176. Bishop K, Blumberg L, Trollip A, Smith A, Roux L, York D, et al. Characterisation of the *pncA* gene in *Mycobacterium tuberculosis* isolates from Gauteng, South Africa. The International Journal of Tuberculosis and Lung Disease. 2001;5(10):952-7.

177. Miyagi C, Yamane N, Yogesh B, Ano H, Takashima T. Genetic and phenotypic characterization of pyrazinamide-resistant *Mycobacterium tuberculosis* complex isolates in Japan. Diagnostic Microbiology and Infectious Disease. 2004;48(2):111-6.

178. Rodrigues VdFS, Telles MA, Ribeiro MO, Cafrune PI, Rossetti MLR, Zaha A. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* in Brazil. Antimicrobial Agents and Chemotherapy. 2005;49(1):444-6.

179. Chan RC, Hui M, Chan EW, Au T, Chin ML, Yip CK, et al. Genetic and phenotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates in Hong Kong. Journal of Antimicrobial Chemotherapy. 2007;59(5):866-73.

180. Pandey S, Newton S, Upton A, Roberts S, Drinković D. Characterisation of *pncA* mutations in clinical *Mycobacterium tuberculosis* isolates in New Zealand. Pathology. 2009;41(6):582-4.

181. Jonmalung J, Prammananan T, Leechawengwongs M, Chaiprasert A. Surveillance of pyrazinamide susceptibility among multidrug-resistant *Mycobacterium tuberculosis* isolates from Siriraj Hospital, Thailand. BMC Microbiology. 2010;10(1):223.

182. Louw G, Warren R, Donald P, Murray M, Bosman M, Van Helden P, et al. Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. The International Journal of Tuberculosis and Lung Disease. 2006;10(7):802-7.

183. Mphahlele M, Syre H, Valvatne H, Stavrum R, Mannsåker T, Muthivhi T, et al. Pyrazinamide resistance among South African multidrug-resistant *Mycobacterium tuberculosis* isolates. Journal of Clinical Microbiology. 2008;46(10):3459-64.

184. Hou L, Osei-Hyiaman D, Zhang Z, Wang B, Yang A, Kano K. Molecular characterization of *pncA* gene mutations in *Mycobacterium tuberculosis* clinical isolates from China. Epidemiology and Infection. 2000;124(02):227-32.

185. Lee KW, Lee JM, Jung KS. Characterization of *pncA* mutations of pyrazinamide-resistant *Mycobacterium tuberculosis* in Korea. Journal of Korean Medical Science. 2001;16(5):537.

186. Simons SO, van Ingen J, van der Laan T, Mulder A, Dekhuijzen PR, Boeree MJ, et al. Validation of *pncA* gene sequencing in combination with the mycobacterial growth indicator tube method to test susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. Journal of Clinical Microbiology. 2012;50(2):428-34.

187. Aono A, Chikamatsu K, Yamada H, Kato T, Mitarai S. The association between *pncA* gene mutations, pyrazinamidase activity, and pyrazinamide susceptibility testing in *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 2014;58(8):4928-30..

188. Yoon J-H, Nam J-S, Kim K-J, Ro Y-T. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* isolates from Korea and analysis of the correlation between the mutations and pyrazinamidase activity. World Journal of Microbiology and Biotechnology. 2014:1-8.

189. Ilina EN, Shitikov EA, Ikryannikova LN, Alekseev DG, Kamashev DE, Malakhova MV, et al. Comparative genomic analysis of *Mycobacterium tuberculosis* drug resistant strains from Russia. PloS One. 2013;8(2):e56577.

190. Akhmetova A, Kozhamkulov U, Bismilda V, Chingissova L, Abildaev T, Dymova M, et al. Mutations in the *pncA* and *rpsA* genes among 77 *Mycobacterium tuberculosis* isolates in Kazakhstan. The International Journal of Tuberculosis and Lung Disease. 2015;19(2):179-84.

191. Sambandamurthy VK, Wang X, Chen B, Russell RG, Derrick S, Collins FM, et al. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. Nature medicine. 2002;8(10):1171-4.

192. CLSI N. Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes, vol. 23, no. 18. Approved standard M24-A. National Committee for Clinical Laboratory Standards, Wayne, PA. 2003.

193. Marttila HJ, Marjamäki M, Vyshnevskaya E, Vyshnevskiy BI, Otten TF, Vasilyef AV, et al. *pncA* Mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* isolates from Northwestern Russia. Antimicrobial Agents and Chemotherapy. 1999;43(7):1764-6.

194. Werngren J, Sturegård E, Juréen P, Ängeby K, Hoffner S, Schön T. Reevaluation of the critical concentration for drug susceptibility testing of *Mycobacterium tuberculosis* against pyrazinamide using wild-type MIC distributions and *pncA* gene sequencing. Antimicrobial Agents and Chemotherapy. 2012;56(3):1253-7.

195. Kontos F, Nicolaou S, Kostopoulos C, Gitti Z, Petinaki E, Maniati M, et al. Multicenter evaluation of the fully automated Bactec MGIT 960 system for susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide: comparison with the radiometric Bactec 460TB system. Journal of Microbiological Methods. 2003;55(1):331-3.

196. Parsons LM, Somoskovi A, Urbanczik R, Salfinger M. Laboratory diagnostic aspects of drug resistant tuberculosis. Frontiers in Bioscience. 2004;9:2086-105.

197. Clark R, Lewinski M, Loeffelholz M, Tibbetts R. Cumitech 31A, Verification and validation of procedures in the clinical microbiology laboratory. SE Sharp (Coordinating ed ASM Press Washington DC. 2009:2-5.

198. Tummon R. Growth inhibition of *Mycobacterium tuberculosis* by oleate in acidified medium. Medical Laboratory Technology. 1975;32(3):229-32.

199. Heifets LBb, Lindholm-Levy J. Is pyrazinamide bactericidal against *Mycobacterium tuberculosis*? 1-3. The American review of respiratory disease. 1990;141:250-2.

200. Ellard G. Absorption, metabolism and excretion of pyrazinamide in man. Tubercle. 1969;50(2):144-58.

201. Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. American Review of Respiratory Disease. 1974;109(1):147-51.

202. Gonzalo X, Drobniewski F, Hoffner S, Werngren J. Evaluation of a biphasic media assay for pyrazinamide drug susceptibility testing of *Mycobacterium tuberculosis*. Journal of Antimicrobial Chemotherapy. 2014:dku230.

203. Carlone N, Acocella G, Cuffini A, Forno-Pizzoglio M. Killing of macrophage-ingested mycobacteria by rifampicin, pyrazinamide, and pyrazinoic acid alone and in combination. The American Review of Respiratory Disease. 1985;132(6):1274-7.

204. Crowle A, Sbarbaro J, May M. Inhibition by pyrazinamide of tubercle bacilli within cultured human macrophages. The American Review of Respiratory Disease. 1986;134(5):1052-5.

205. Crowle AJ, Dahl R, Ross E, May MH. Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. Infection and Immunity. 1991;59(5):1823-31.

206. Rastogi N, Potar M, David H. Pyrazinamide is not effective against intracellularly growing *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 1988;32(2):287.

207. Heifets L, Higgins M, Simon B. Pyrazinamide is not active against *Mycobacterium tuberculosis* residing in cultured human monocyte-derived macrophages [Unresolved

issues]. The International Journal of Tuberculosis and Lung Disease. 2000;4(6):491-5.

208. Singh P, Mishra A, Malonia S, Chauhan D, Sharma V, Venkatesan K, et al. The paradox of pyrazinamide: an update on the molecular mechanisms of pyrazinamide resistance in Mycobacteria. Journal of Communicable Diseases. 2006;38(3):288.

209. Hu Y, Coates A, Mitchison D. Sterilising action of pyrazinamide in models of dormant and rifampicin-tolerant *Mycobacterium tuberculosis*. The International Journal of Tuberculosis and Lung Disease. 2006;10(3):317-22.

210. Huang Q, Chen Z-F, Li Y-Y, Zhang Y, Ren Y, Fu Z, et al. Nutrient-starved incubation conditions enhance pyrazinamide activity against *Mycobacterium tuberculosis*. Chemotherapy. 2007;53(5):338-43.

211. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Molecular Microbiology. 2002;43(3):717-31.

212. Andries K, Gevers T, Lounis N. Bactericidal potencies of new regimens are not predictive of their sterilizing potencies in a murine model of tuberculosis. Antimicrobial Agents and Chemotherapy. 2010;54(11):4540-4.

213. Chang K-C, Leung C-C, Yew W-W, Leung EC-C, Leung W-M, Tam C-M, et al. Pyrazinamide may improve fluoroquinolone-based treatment of multidrug-resistant tuberculosis. Antimicrobial Agents and Chemotherapy. 2012:AAC. 01300-12.

214. British Medical Research Councils. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis: third report. The Lancet. 1974;304(7875):237-40.

215. East African/ British Medical Research Councils . Controlled clinical trial of four 6-month regimens of chemotherapy for pulmonary tuberculosis. Second report. American Review of Respiratory Disease. 1976;114:471.

216. The East African/ British Medical Research Councils Study. Controlled clinical trial of four short-course regimens of chemotherapy for two durations in the treatment of pulmonary tuberculosis Second report. Tubercle. 1980;61(2):59-69.

217. Ahmad Z, Tyagi S, Minkowski A, Peloquin CA, Grosset JH, Nuermberger EL. Contribution of moxifloxacin or levofloxacin in second-line regimens with or without continuation of pyrazinamide in murine tuberculosis. American Journal of respiratory and Critical Care Medicine. 2013;188(1):97-102.

218. Dawson R, Diacon AH, Everitt D, van Niekerk C, Donald PR, Burger DA, et al. Efficiency and safety of the combination of moxifloxacin, pretomanid (PA-824), and pyrazinamide during the first 8 weeks of antituberculosis treatment: a phase 2b, open-label, partly randomised trial in patients with drug-susceptible or drug-resistant pulmonary tuberculosis. The Lancet. 2015;385(9979):1738-47.

219. Brossier F, Veziris N, Jarlier V, Sougakoff W. Performance of MTB-DR plus for detecting high/low levels of *Mycobacterium tuberculosis* resistance to isoniazid. The International Journal of Tuberculosis and Lung Disease. 2009;13(2):260-5.

220. Ajbani K, Nikam C, Kazi M, Gray C, Boehme C, Balan K, et al. Evaluation of genotype MTBDRsl assay to detect drug resistance associated with fluoroquinolones, aminoglycosides and ethambutol on clinical sediments. 2012.

221. Juréen P, Werngren J, Hoffner SE. Evaluation of the line probe assay (LiPA) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. Tuberculosis. 2004;84(5):311-6.

222. Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniewski F, Rodionova Y, et al. *Mycobacterium tuberculosis* pyrazinamide resistance determinants: a multicenter study. American Society for Microbiology. 2014;5(5):e01819-14.

223. van Helden PD, Victor TC, Warren RM, van Helden EG. Isolation of DNA from *Mycobacterium tubercolosis*. *Mycobacterium tuberculosis* protocols. 2001:19-30.

224. Zhang S, Chen J, Shi W, Liu W, Zhang W, Zhang Y. Mutations in *panD* encoding aspartate decarboxylase are associated with pyrazinamide resistance in *Mycobacterium tuberculosis*. Emerging Microbes and Infections. 2013;2(6):e34.

225. Yang F, Njire MM, Liu J, Wu T, Wang B, Liu T, et al. Engineering more stable, selectable marker-free autoluminescent mycobacteria by one step. PloS One. 2015;10(3):e0119341.

226. Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. Gene. 1990;96(1):23-8.

227. Saviola B, Bishai WR. Method to integrate multiple plasmids into the mycobacterial chromosome. Nucleic Acids Research. 2004;32(1):e11-e.

228. Duff Jr MR, Grubbs J, Howell EE. Isothermal titration calorimetry for measuring macromolecule-ligand affinity. Journal of visualized experiments. 2011;55:e2796.

229. Chou JY, Singer MF. Deoxyadenosine diphosphate as a substrate and inhibitor of polynucleotide phosphorylase of *Micrococcus luteus* I. Deoxyadenosine diphosphate as a substrate for polymerization and the exchange reaction with inorganic 32P. Journal of Biological Chemistry. 1971;246(24):7486-96.

230. Kaufmann G, Littauer U. Deoxyadenosine diphosphate as substrate for polynucleotide phosphorylase from *Escherichia coli*. FEBS letters. 1969;4(2):79-83.

231. Beljanski M. *De novo* synthesis of DNA-like molecules by polynucleotide phosphorylase in vitro. Journal of Molecular Evolution. 1996;42(5):493-9.

232. Avarbock D, Avarbock A, Rubin H. Differential regulation of opposing Rel_{Mtb} activities by the aminoacylation state of a tRNA•Ribosome•mRNA•RelMtb complex. Biochemistry. 2000;39(38):11640-8.

233. Symmons MF, Jones GH, Luisi BF. A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation. Structure. 2000;8(11):1215-26.

234. Cheng S-J, Thibert L, Sanchez T, Heifets L, Zhang Y. pncA mutations as a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*: spread of a monoresistant strain in Quebec, Canada. Antimicrobial Agents and Chemotherapy. 2000;44(3):528-32.

235. Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S, et al. Dynamic persistence of antibiotic-stressed mycobacteria. Science. 2013;339(6115):91-5.

236. Javid B, Sorrentino F, Toosky M, Zheng W, Pinkham JT, Jain N, et al. Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. Proceedings of the National Academy of Sciences. 2014;111(3):1132-7.

237. Das B, Pal RR, Bag S, Bhadra RK. Stringent response in *Vibrio cholerae*: genetic analysis of spoT gene function and identification of a novel (p)ppGpp synthetase gene. Molecular Microbiology. 2009;72(2):380-98.

238. Avarbock D, Salem J, Li L-s, Wang Z-m, Rubin H. Cloning and characterization of a bifunctional RelA/SpoT homologue from *Mycobacterium tuberculosis*. Gene. 1999;233(1):261-9.

239. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity *in vitro* against semidormant *Mycobacterium tuberculosis* bacterial populations. The American Review of Respiratory Disease. 1992;145(5):1223-5.

240. Weiss LA, Stallings CL. Essential roles for *Mycobacterium tuberculosis* Rel beyond the production of (p) ppGpp. Journal of Bacteriology. 2013;195(24):5629-38.

241. Njire M, Tan Y, Mugweru J, Wang C, Guo J, Yew W, et al. Pyrazinamide resistance in *Mycobacterium tuberculosis*: Review and update. Advances in Medical Sciences. 2016;61(1):63-71.

12. Curriculum vitae

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