A proteomic portrait of Mycobacterium tuberculosis

Keijzer, J. de

7

General discussion

General Discusion

The ongoing spread of (multi-)drug resistant *Mycobacterium tuberculosis* presents a major burden on the management of tuberculosis (TB).[1] Early detection of drug resistance or drug tolerance can be essential to minimize the spread of resistant strains. Furthermore, a better knowledge of interstrain variation, mechanisms of action of anti-TB drugs and mycobacterial drug tolerance will facilitate the development of improved diagnostic assays, new drug targets and novel drug treatment strategies. In this thesis, we made use of mass spectrometry-based proteomics as an unbiased hypothesis generating tool to study protein regulation in *M. tuberculosis* in relation to the development and transmission of drug resistance. The (major) novel findings described in this thesis are summarized and discussed in this Chapter; Box 1.

BOX 1: Summary of novel findings described in this thesis.

In this thesis, we examined to what extent regulation of the M. tuberculosis proteome contributes to drug tolerance, a (sub-)lineage specific phenotype and the anti-TB mechanism of antibiotics. The main findings are listed below:

- Chapter 2 & 4 a gap is bridged between the M. tuberculosis Beijing sub-lineages genotype and phenotype by identifying the differential abundance of Rv0450c/MmpL4 and Rv3283/sseA in modern Beijing strains relative to other clinically relevant M. tuberculosis lineages.
- In Chapter 3 it is demonstrated that the DosR dormancy regulon is actively induced by M. tuberculosis Beijing B0/W148 upon rifampicin treatment. Furthermore, it is demonstrated that the M. tuberculosis Beijing B0/W148 strain displays a pre-dormant phenotype prior to rifampicin treatment.
- In Chapter 4 it is demonstrated that next to M. tuberculosis Beijing B0/W148, also M. tuberculosis H37Rv induces the dormancy regulon upon rifampicin treatment. Additionally, it is shown that the proteinaceous regulators of dormancy are a priori more abundant in M. tuberculosis Beijing strains compared to other clinically relevant M. tuberculosis strains.
- In Chapter 5 a novel mechanism of action for the potential new antibiotic thioridazine is described.
- In Chapter 6 we demonstrate that phylogenetic relationships between closely related mycobacteria and M. tuberculosis strains can be reconstructed based on their phenotype using tandem mass spectrometry.

Proteomic analysis of *M. tuberculosis*

In order to establish a comprehensive, representable proteomic view of *M. tuberculosis* using mass spectrometry, mycobacterial proteins need to be extracted from the cell. Reproducible, unbiased extraction of an entire mycobacterial proteome is hampered by the thick, lipid-rich cell wall surrounding mycobacteria.[2, 3] To obtain a representative extract of the mycobacterial proteome, we made use of a chemical disruption, using sodium dodecyl sulfate, combined with heat treatment and mechanical disruption by means of bead-beating. Based on the identified recovered proteins, we demonstrate that this procedure yields a reproducible extraction of the mycobacterial proteome without a bias towards a protein's physiochemical properties and cellular localization (**Chapter 2**).

The proteomic coverage in a proteomics experiment is dependent on several variables, including pre-fractionation of the proteome and the type of mass spectrometer used. Within this thesis, we made use of a Thermo Scientific LTQ-FT Ultra hybrid mass spectrometer and a Thermo Scientific Q-Exactive quadrupole orbitrap mass spectrometer. As expected, the Q-Exactive outperformed the LTQ-FT Ultra in total number of identified proteins; Table 1. The Q-Exactive is a newer generation of mass spectrometer, that can generate more high resolution mass spectra per second, which resulted in more peptide identifications and ultimately more protein identifications.

In the proteomic dataset generated with the Q-Exactive mass spectrometer, we reproducibly quantified a total of 2,429 proteins with ≥2 peptides per protein and a 1% false discovery rate (**Chapter 4**). Thereby, this proteomic dataset covers ±60% of all proteins theoretically present in *M. tuberculosis* and ±80% of the expressed proteome reported to date.[4-6]. Considering that we analyzed a homogenous culture of *M. tuberculosis* cells that was in mid-logarithmic growth phase, we assume that for example stationary growth phase specific proteins were likely to be absent, or in quantities too low to be detected.[7]

The comprehensive, unbiased proteome-wide quantifications performed in this thesis were obtained after pre-fractionation of the digested proteome to decrease complexity and thereby increase proteomic coverage. However, with the availability of new bioinformatic tools and even faster scanning mass spectrometers, pre-fractionation of mycobacterial protein digests can possibly be omitted. One novel powerful approach to study unfractionated proteomes is the data-independent acquisition (DIA) method SWATH (Sequential Window Acquisition of all THeoretical Mass Spectra).[8]

Analysis of the *M. tuberculosis* proteome using SWATH allowed for the quantification of up to

2,458 proteins in a single LC-MS/MS run on a Sciex TripleTOF 5600 mass spectrometer.[6] Using SWATH, *M. tuberculosis* proteomes can be quantitatively studied with a high-throughput, but SWATH also has several disadvantages. A major drawback of SWATH assays is that the method is not compatible with conventional proteomic database search alghorithms. The generation of a SWATH assay specific proteomics library requires a major investment. For the generation of the *M. tuberculosis* SWATH library, a total of 17,463 peptides were selected, synthesized and analyzed by data-dependent acquisition (DDA) on a TripleTOF 5600 mass spectrometer.[5, 6] The resulting tandem mass spectra were compiled into a SWATH assay library. Albeit very powerful, this SWATH library resource is only of use for researchers that have acces to a TripleTOF mass analyzer or other mass spectrometers that produce peptide fragmentation patterns similar to the curated tandem mass spectra stored in the SWATH library.

Due to the limitations of DIA/SWATH analysis, DDA remains to be an attractive alternative. Even more than DIA, DDA analysis of complex samples relies on the acquisition speed of the mass analyzer. The ongoing development of mass spectrometers continues to lead to new mass spectrometers with increased resolution and acquisition speed. For example, with the introduction of the Thermo Scientific Orbitrap Fusion mass analyser, comprehensive analysis of the *Saccharomyces cerevisiae* proteome could be achieved using a single 95 minute analysis.[9] Considering the fact that the *S. cerevisiae* proteome consists of 6,100 theoretical proteins, compared to *M. tuberculosis* producing 4,000 proteins, and the fact that the dynamic range of the proteome in *S. cerevisiae* is an order of magnitude larger than that of *M. tuberculosis*, we can conclude that the observable proteome of *S. cerevisiae* is more complex than that of *M. tuberculosis*.[5, 10, 11] Therefore, we would anticipate that comprehensive DDA proteomic analysis of *M. tuberculosis* can be achieved within 95 minutes using the latest generation of mass spectrometers.

Despite the ongoing development of mass spectrometers, the current, state-of-the-art, equipment is sufficiently powerful to comprehensively study the proteome of *M. tuberculosis* and address scientific questions. The development and implementation of new mass spectrometers with increased resolution and acquisition speed will be of limited added value to the *M. tuberculosis* proteomics research field that performs bottom-up proteomic analysis, i.e. faster mass spectrometer will mainly allow for a higher sample throughput. However, the development of novel fragmentation techniques, that are orthoganol to the commonly used fragmentation method collision induced dissociation, will be of more interest as the characterization of (semi-) intact proteins and post-translational modifications become increasingly important.[12-14]

In the cell, proteins can engage into highly specific interactions that affect the cellular phenotype. With the hardware available to identify and quantify these proteins within a reasonable

timeframe, the generated mass spectrometry data should be analyzed to add biological meaning to data and generate new hypotheses. Proteomic analysis is heavily dependent on downstream software solutions, both for the identification & quantification of proteins and interpretation of the large datasets generated. The functional interpretation of quantitative proteomic datasets can be performed using pathway and protein-protein interaction analysis. Pathway analysis refers to a type of data analysis that aims to identify (de)activated biologicals pathways typically including; signalling pathways, gene regulatory pathways and metabolic pathways.[15] The interpretation of proteomic datasets by either network or pathway analysis requires prior information of the proteins of interest, such as protein-protein interactions, a proteins cellular localization and its molecular function. For well-studied organisms, this type of data can be derived from well curated scientific publications.

Regarding the functional assignment of the proteins present in *M. tuberculosis*, relatively little pre-existing functional biological information is present. This is well illustrated by the functional categorization of the *M. tuberculosis* proteome as performed by Tuberculist, a regularly updated database of *M. tuberculosis* proteins, where a quarter of the proteome is categorized as hypothetical.[16] Despite the, mainly bioinformatic, attemps to assign these hypothetical proteins to a molecular function, specific functional information is limited for most of the *M. tuberculosis* proteome.([17] More information on this aspect will improve the functional biological interpretation of new and previously published proteomic datasets, and thereby further advance our understanding of cellular processes in *M. tuberculosis*. Now that the analytical hardware reached a level of maturity that is sufficient for the rapid, comprehensive study of *M. tuberculosis* proteomes, it is highly conceivable that the availibity of protein functionality data will become the next bottleneck at the frontier of *M. tuberculosis* proteomics.

TABLE 1: Overview of the proteomics analyses performed and proteins identified in this thesis. Proteins identified contain ≥2 peptides/ protein and 1% False Discovere Rate (FDR).

Chapter	*M. tuberculosis* Strains	Multiplex	SCX fractions	Bio./Tech. duplicate	Total no. of fractions analysed	Mass analyzer	Total no. of proteins identified
2	Atypical and Typical Beijing	2	3 x 15	No/ Yes	90	LTQ-FTICR Ultra	2392
3	Typical Beijing	2	5 x 15	Yes/ Yes	300	LTQ-FTICR Ultra	2534
4	Typical Beijing & H37Rv	3	2 x 15	Yes/ Yes	60	Q-Exactive	2903
5	H37Rv	2	1 x 15	Yes/ Yes	30	LTQ-FTICR Ultra	2479

Metabolic reprogramming of *M. tuberculosis* in response to rifampicin treatment

M. tuberculosis has a wide variety of molecular tools that can increase the cells' tolerance to drugs and thereby prevent rapid drug induced killing of the pathogen (**Chapter 1**). In this thesis, we set out to identify the mechanisms that come to play in *M. tuberculosis* during the initial 24 hrs of rifampicin exposure (**Chapter 3** & **Chapter 4**). As described in **Chapter 3**, we reasoned that short-term exposure of the pathogen to rifampicin will yield a more accurate representation of the *in vivo* situation than long-term rifampicin exposure due to the short half-life time of rifampicin *in vivo*.[18]

Although we aimed to identify the molecular tools that contribute to the development of rifampicin resistance, we did not study rifampicin resistant strains. Strains that have acquired a rifampicin resistance conferring mutation will no longer be susceptible to the drug. As a consequence, it is not to be expected that a rifampicin resistant mutant will regulate stress induced proteins or any of the molecular tools that provide drug tolerance when the cells are exposed to rifampicin. Therefore, we reasoned that the physiology and proteome of a drug resistant strain will not provide information on the proteins required to develop an enduring drug tolerant phenotype.

Rifampicin targets *rpoB*, an enzyme whose RNA polymerase activity is required for the synthesis of proteins.[19, 20] However, in this thesis we demonstrated that despite the presence of high levels of rifampicin, *M. tuberculosis* still manages to regulate the abundance of proteins in the initial 24 hrs after the start of rifampicin exposure. Strikingly, when we exposed *M. tuberculosis* Beijing B0/W148 to 16 µg/ml of rifampicin for 24 hrs, we did observe the increased abundance of DosR dormancy proteins (**Chapter 3**). Using DosR proteins, *M. tuberculosis* can enter a metabolicly hypoactive latent state where the pathogen is less susceptible to antibiotics which act as inhibitors of active cellular or metabolic processes.[21] In this thesis, we did not only describe the proteomic changes induced by rifampicin exposure, but using cellular assays, we demonstrated that, following the induction of DosR dormancy proteins, rifampicin exposed cells develop a more dormant phenotype (**Chapter 3**).

To determine whether this reponse is shared by other *M. tuberculosis* strains, we also exposed *M. tuberculosis* H37Rv, a laboratory *M. tuberculosis* strain, to rifampicin (**Chapter 4**). Similar to the results obtained after exposure of *M. tuberculosis* Beijing B0/W148 to rifampicin, we found that *M. tuberculosis* H37Rv also induces dormancy proteins as a response to rifampicin treatment. Thereby, the outcomes of this thesis suggest that transition to dormancy, as a response to rifampicin induced stress, is conserved throughout various *M. tuberculosis*

genotypes, although this may be more pronounced in genotypes such as the Beijing family, that is often associated with the spread of drug resistant TB.[22-28]

If rifampicin treatment induces the transition into a dormant phenotype, it will eventually make the pathogen less susceptible to a variaty of antibiotics, since the mechanism of action of most drugs share the requirement for growth or active metabolism.[21] The increased tolerance of the pathogen during dormancy will lead to extended survival times of the cells during treatment, but the pathogen can still be killed by antibiotics. However, dormant *M. tuberculosis* cells maintain the same mutation rate as actively growing cells.[29] Thereby, dormancy increases the window of opportunity of the pathogen to establish a drug resistant genotype, which can ultimately result in relapses of drug resistant *M. tuberculosis*.

The dramatic effects of drug induced dormancy are not limited to the *in vivo* situation. *In vitro*, drug susceptibility testing aims to determine the growth inhibitory concentrations of antibiotics, known as the minimal inhibitory concentration (MIC). Since mycobacterial growth in a known concentration of a given antibiotic is the only measure of these assays, it cannot diagnose a situation in which growth is actively inhibited by the pathogen to prevent killing. As a consequence, the read-out of the assay does provide the tester with a growth limiting concentration of the antibiotic examined, but it does not provide a measure of antibiotic tolerance. Therefore, the currently available drug susceptibility testing is not capable of diagnosing bacteria more prone to escape treatment and develop drug resistance.

As an alternative to MIC testing, it has been suggested to supplement current microbial drug susceptibility testing with minimum duration for killing (MDK) testing.[30] The combination of MIC and MDK values can be very powerful to determine mycobacterial tolerance to specific drugs and combinatorial therapy. However, the time required to perform both MIC and MDK testing for slow growing *M. tuberculosis* cells would significantly increase diagnostic turnaround times. Ideally, in the future, the abundance of phenotypic markers of drug tolerance, as the DosR proteins identified in this thesis, can be rapidly monitored to provide a quantitative measure of drug tolerance.

Finally, it is known that a wide variety of stimuli can induce dormancy, including nitric oxide, acidity, nutrient deprivation and hypoxia.[31-34] Therefore, we hypothesized that the observed DosR regulated metabolic reprogramming can be part of a general stress response for *M. tuberculosis* (**Chapter 3** & **Chapter 4**). In support of this hypothesis, it was recently reported that the DosR protein Rv0081 is involved in the mycobacterial response to isoniazid, rifampicin, moxifloxacin, mefloquine and bedquiline.[35] Thereby, it is conceivable that not only rifampicin, but also other antibiotics can induce a more dormant phenotype in *M. tuberculosis*, indicating

that the impact of the findings presented in this thesis can have a much broader impact than we can foresee with our current knowledge.

The regulators of dormancy are more abundant in the emerging *M. tuberculosis* Beijing genotype

In **Chapter 3**, we demonstrated that *M. tuberculosis* Beijing B0/W148 is actively inducing a more dormant phenotype as a mechanism to circumvent killing by rifampicin. Furthermore, in **Chapter 4** we observed a similar response to rifampicin in *M. tuberculosis* H37Rv. In addition, we also observed that out of the 23 identified DosR regulon proteins, ten were significantly more abundant prior to drug exposure in *M. tuberculosis* Beijing B0/W148 than *M. tuberculosis* H37Rv (**Chapter 4**). Finally, we found that the *M. tuberculosis* Beijing B0/W148 cultures posses a highly homogenous, close to dormant, phenotype (**Chapter 3**). To determine whether the *a priori* high abundance of DosR proteins is a Beijing specific trait, we examined the abundance of eight DosR proteins in five of the globally most prevalent *M. tuberculosis* (sub-)lineages; atypical Beijing, typical Beijing, East-African Indian, Haarlem and Central Asian strains (**Chapter 4**). Strikingly, we observed that the proteinaceous regulators of dormancy, Rv3132c/devS and Rv3133c/devR, are more abundant in the *M.tuberculosis* Beijing genotype than in any of the other clinically relevant *M. tuberculosis* lineages examined.

In **Chapter 4**, we demonstrated that *M. tuberculosis* Beijing B0/W148 induces three-fold more copies of the DosR protein Rv2031c/hspX within the initial 24 hrs of rifampicin exposure than H37Rv. This observation is an indication that *M. tuberculosis* Beijing B0/W148 transitions faster to dormancy than *M. tuberculosis* H37Rv. It is tempting to assume that the quantity of DosR proteins is a direct reflection of a pathogen's potential to switch to a fully dormant state. However, this conclusion can only be drawn based on cellular assays, where the induction of dormancy, and phenotypic markers of dormancy, correlate with the quantity of DosR proteins present. Nevertheless, in **Chapter 3** we did demonstrate that *M. tuberculosis* Beijing strains posses a pre-existent dormant phenotype during logarhitmic growth, indicating that the cells are always prepared to enter a fully dormant state.

The *a priori* high abundance of DosR proteins that are required for the response to rifampicin and pre-existing dormant phenotype in *M. tuberculosis* Beijing, provide a potential advantage over other strains to establish an enduring drug tolerant phenotype. Therefore, we reasoned that part of the success of *M. tuberculosis* Beijing strains might be attributable to the constant high abundance of dormancy proteins. As the abundance of DosR proteins is conserved in the *M. tuberculosis* Beijing lineage, it is plausible that phenotype associated drug tolerance is

conserved throughout entire *M. tuberculosis* genotypes. In the case of *M. tuberculosis* Beijing it has been demonstrated that this specific genotype is more tolerant to rifampicin, which correlates with the specific pre-dormant phenotype we described in this thesis.[22, 23]

It should be noted that the results in this thesis are obtained in an *in vitro* model of *M. tuberculosis*. Although the here described results impact *in vitro M. tuberculosis* drug susceptiblity testing, the *in vivo* impact and validaty of these findings remain to be examined. However, the dormancy regulon has been pinpointed in this thesis as a key factor in drug tolerance and hence most likely in resistance development. Using targeted proteomic approaches, these proteins can potentially be quantified in patient sputum or in an *in vivo M. tuberculosis* infection model.

Finally, in view of the importance of DosR for mycobacterial persistence and the findings presented in this thesis, it can be concluded that DosR proteins are promising drug targets.[36, 37] It could be speculated that if novel drugs designed to target the DosR dormancy regulon and prevent the pathogen from entering dormancy, TB treatment times can potentially be reduced due to synergism with other antibiotics and even MIC-testing can improve with regard to forecasting drug tolerance.[35]

M. tuberculosis Beijing sublineages specific traits

In **Chapter 2** & **Chapter 4**, we demonstrated that the abundance of DosR proteins is conserved between the more ancient "atypical" Beijing and more modern "typical" Beijing strains. Nevertheless, both sublineages differ in their capacity to cause and spread active disease, leading to global prevalence of the typical Beijing strain, except for Japan.[38-40] Therefore, we hypothesized that there are selective advantages present in the more modern typical Beijing strain, which resulted in the dominance of this sublineage over other *M. tuberculosis* strains (**Chapter 2**).

Genetically, the atypical and typical Beijing strains are highly related, with a mere total of 31 non-synonymous single nucleotide polymorphisms (nsSNPs) that separate the two sublineages.[41] Since the genome of *M. tuberculosis* can be seen as the blueprint for the proteome of the organism, it could be that the polymorphisms present in the genome can be displayed in the proteome.

By systematically comparing the proteomes of well characterized *M. tuberculosis* Beijing sublineages, we identified four proteins to be differentially abundant between typical and atypical Beijing strains: Rv0450c/MmpL4, Rv1269c, Rv3137, and Rv3283/sseA. By analysing enzymatic activity and the expression of mRNA of these genes in a large cohort of 29 clinically

derived Beijing strains, we could confirm the differential regulation of Rv0450c/MmpL4 and Rv3283/sseA in the *M. tuberculosis* Beijng sublineages (**Chapter 2**). In **Chapter 4** we further compared the abundance of Rv0450c/MmpL4 and Rv3283/sseA in five clinically prevalent *M. tuberculosis* genotypes and found that Rv0450c/MmpL4 is always highly abundant in *M. tuberculosis* typical Beijing, whereas Rv3283/sseA is always less abundant in typical Beijing strains, relative to all other *M. tuberculosis* (sub-)lineages examined.

For Rv3283/sseA, we were able to bridge the observed typical Beijing specific proteomic phenotype with the typical Beijing specific genotype. It is known that typical Beijing strains posses a typical Beijing specific nsSNP in Rv3283/sseA.[41] In **Chapter 2**, we described how this nsSNP impacts protein stability, and as result, we demonstrate in **Chapter 3** that once protein production is inhibited by the presence of rifampicin, the abundance of Rv3283/sseA rapidly declines due to the instability of the protein.

The relatively low abundance of Rv3283/sseA in the typical Beijing strains could provide the sub-lineage with an advantage over other genotypes, as a Rv3283/sseA deficiency was reported to promote enhanced growth of *M. tuberculosis* in macrophages relative to wild-type *M. tuberculosis*.[42] Thereby, the inheritable differential abundance of Rv3283/sseA in typical Beijing strains presents a potential factor that provides the sublineage with a selective advantage. Regarding Rv0450c/MmpL4, there is no typical Beijing specific nsSNP described in the gene of this protein. However, Rv0450c/MmpL4, together with Rv0451c/MmpS4 or Rv0677c/MmpS5 and Rv0676c/MmpL5, are required for the secretion of iron-scavenging siderophores in *M. tuberculosis*.[43] The typical Beijing specific nsSNP present in Rv0676c/MmpL5 does, using the bioinformatic algorhitims used in this thesis, not impact the functionality or half-life time of the protein (**Chapter 2**). Nevertheless, considering the shared functionality of Rv0450c/MmpL4 and Rv0676c/MmpL5, it is plausible that increased levels of Rv0450c/MmpL4 are present to compensate for the mutated Rv0676c/MmpL5 in typical Beijing strains. Since both Rv0450c/MmpL4 and Rv0676c/MmpL5 play an important role in mycobacterial iron metabolism, our data suggest a unique role for iron metabolism in typical Beijing strains.

The importance of iron metabolism in *M. tuberculosis* Beijing strains has been stressed before. From the host's perspective, it has been demonstrated that patients with a mutation in SLC11A1/NRAMP1, a divalent transition metal transporter involved in human iron metabolism, have been associated with increased odds of contracting *M. tuberculosis* Beijing.[44] Adaption of the mycobacterial iron metabolism to the altered situation in the host is evidence for the co-evolution of *M. tuberculosis* Beijing and humans in the region.

In **Chapter 3**, we found more evidence linking iron metabolism to the success of *M. tuberculosis*

typical Beijing strains. Rv2986c/HupB, also known as iron-regulated protein (Irep-28), was observed to be phosphorylated during rifampicin treatment. Rv2986c/HupB can positively regulate the biosynthesis of siderophores.[45, 46] Next to Rv2986c/HupB, we also found Rv3458c/rpsD to be increasingly phosphorylated during initial exposure to rifampicin (**Chapter 3**). Similar to Rv2986c/HupB, Rv3458c/rpsD plays a role in the pathogens iron metabolism.[47] Thereby, we were not only the first to link the regulation of post-translational modifications to drug treatment, but also support the correlation between iron metabolism and drug tolerance.[48]

Finally, others recently reported that Rv1346/MbtN, an acyl-CoA dehydrogenase that is involved in the production siderophores, was significantly more abundant in the *M. tuberculosis* Beijing strains, compared to the other *M. tuberculosis* lineages examined.[49] Eventually, iron metabolism and dormancy are related. *M. tuberculosis* cells that enter dormancy, or a metabolic hypoactive state, attempt to increase their iron storages.[50, 51] The link of iron metabolism with dormancy fits our overall, re-occurring, picture that iron metabolism is an important feature of *M. tuberculosis* Beijing strains, especially typical Beijing strains, which requires further investigation.

Dormancy and iron metabolism are potentially related within *M. tuberculosis* Beijing, with even a genomic link between the typical Beijing specific nsSNP in Rv0676c/MmpL5 and iron metabolism (**Chapter 2**). In relation to the typical Beijing genome and dormancy, there is a typical Beijing specific mutation present in the DosR gene Rv2027c/DosT.(52) It has been reported that the presence of this mutation correlates with an increased abundance of Rv3133c/devR mRNA, where typical Beijing strains express on average 12-fold more Rv3133c/devR transcripts than atypical Beijing strains. However, both in our comparative proteomic (**Chapter 2**) and parallel reaction monitoring (**Chapter 4**) studies, we did not find this relation on the protein level. Nevertheless, the presence of a typical Beijing specific mutation in a DosR regulon protein strengthens the hypothesis that there is a unique relation between the genotype of typical Beijing strains in relation to dormancy and iron metabolism.

Now that we are bridging the gap between the typical Beijing specific genotype and phenotype, we can supplement molecular differentiation between Beijing sub-lineages using genotypic markers of which we know they more closely reflect a sub-lineage specific phenotype; Rv0676c/MmpL5, Rv2027c/DosT and Rv3283/sseA.

Thioridazine, the magic bullet for dormant tuberculosis?

Antibiotics are essential for curative treatment of TB patients and prevention of dissemination of the disease. Despite the presence of effective antibiotics, *M. tuberculosis* is increasingly found to be resistant to one or several antibiotics.[53] In this thesis, we reasoned that antibiotic tolerance, and thereby resistance, is at least partially caused by dormancy (**Chapter 3** & **Chapter 4**). As we described in **Chapter 4**, dormancy is related to the *M. tuberculosis* Beijing genotype and can be induced by anti-TB treatment regimens. Also other factors can induce dormancy, including nitric oxide, acidity, nutrient deprivation and hypoxia.[31-34] As a result, *in vivo M. tuberculosis* often transitions itself in its dormant state. Since most antibiotics target replicating metabolically active cells, they loose activity once the cellular metabolism is down regulated. The ideal novel antibiotic would not only target metabollicaly active cells, but also the dormant forms of *M. tuberculosis*.[54-58]

Several phenothiazine neuroleptics, such as thioridazine, exhibit *in vitro* and *in vivo* activity against *M. tuberculosis*.[59] Thioridazine has already been successfully used, off-label, in combination with third-line antibiotics for compassionate therapy of patients presenting with extensively drug resistant infections of TB in Buenos Aires.[60] In this and other studies, thioridazine has proven to work synergistically with other antibiotics.[61-63]

The synergy between thioridazine and other antibiotics has been attributed to the potential inhibition of drug secreting efflux pumps by thioridazine. The inhibition of efflux pumps was thought to increase the accumulation of antibiotics, which could explain the synergy between thioridazine and other drugs.[64] However, the inhibition of drug efflux pumps solely does not fully explain the reported bactericidal effects of thioridazine, leaving the mode of action of thioridazine unknown. Using an unbiased proteomic approach, we set out to unravel the molecular mechanism of this potential new anti-TB component by examining the impact of continuous thioridazine exposure on the proteome of *M. tuberculosis* (**Chapter 5**).

Long-term exposure of *M. tuberculosis* to thioridazine requires the drug to be thermostable over a culture period of approximately two weeks. To determine the thermostability of thioridazine, we designed a straightforward, easy to implement, bioassay that demonstrated the stability of thioridazines' anti-TB activity over a period of 21 days (**Chapter 5**).

By analyzing the proteomic composition of thioridazine treated and untreated cells, we discovered that under the influence of thioridazine several proteins, including two large clusters of proteins involved in the maintenance of the cell wall permeability barrier, are differentially regulated. Our study data did not provide evidence for the differential regulation of specific

mycobacterial efflux pumps that are potentially inhibited by thioridazine.

Following our proteomic data that suggested a difference in cell envelope permeability, we assessed the accumulation of fluorescent dyes in *M. tuberculosis* over time. Our findings show that long-term thioridazine exposure of *M. tuberculosis* increased the accumulation of both hydrophilic and hydrophobic fluorescent compounds. Furthermore, using gas chromatography, we demonstrated that treatment of *M. tuberculosis* with thioridazine altered the composition of the mycobacterial plasma membrane, similarly to other cell envelope permeabilizing drugs.[65] The observed increase of cell envelope permeability upon thioridazine treatment explains the reported synergistic effects and increased accumulation of other antibiotics when thioridazine was included in multidrug treatment regimens, i.e. drugs can more easily cross the mycobacterial cell envelope after thioridazine treatment. Although the hypothesis that thioridazine treatment leads to higher intercellular drug concentrations has not changed in this study, the more exact knowledge of its mode of action is a step forward and could facilitate further development of this class of drugs for therapy of multidrug resistant pulmonary infections of TB. In fact, due to the increased permeability of the cell envelope after thioridazine exposure, old drugs can be accumulated to an effective therapeutic level, and also the dose levels of new, but toxic, anti-TB compounds may be significantly reduced.

The mechanism of action described for thioridazine in **Chapter 5** does not only explain why thioridazine acts synergestically with other antibiotics, but also serves as a plausible explanation for the effectivity of thioridazine to both metabolically active and hypoactive cells, as thioridazine seems to directly target the cell envelope.[54, 66] Despite reported adverse events associated with the usage of thioridazine, the need for novel antibiotics could justify the risk associated with usage of this repurposed drug for the treatment of drug resistant TB.[67] However, the development of less toxic, more potent thioridazine analogues, will bring the routine usage of thiordazine closer to the clinic, which could ultimately result in more effective treatment of drug tolerant, dormant and to dormant transitioning *M. tuberculosis*.[68]

Differentiation of mycobacterial species using tandem mass spectrometry

The mycobacterium genus, including *M. tuberculosis*, consists out of more than 140 species.[69] The various mycobacterial species cause various diseases, some leading to morbidity and mortality, but not all mycobacteria are of clinical significance, and not all mycobacteria are susceptible to the same antibiotics.[69, 70] In order to properly diagnose and treat patients infected with a *Mycobacterium*, the pathogen needs to be correctly identified.[71] However, the

occurrence of >140 *Mycobacterium* species, of which some species are genetically very closely related, hampers the routine identification with a single test.

In **Chapter 6**, we demonstrated that using a rapid proteomics-based method, phylogenetic relationships between closely related mycobacterial species can be established by directly comparing tandem mass spectra derived from a proteolytic digest of the mycobacterial proteome. The method brings the following advantages compared to other currently employed mycobacterial typing approaches; prior knowledge of the species to be identified is not required when performing this untargeted assay (e.g. specific primers), the workflow can be applied to virtually all (micro-)organisms, all acquired tandem mass spectra can be used for clustering of species, the acquisition of tandem mass spectra can be performed on both high and low resolution mass spectrometers, and the technique can easily be implemented in a proteomic laboratory. Ideally, the method would not only be capable of species identification as demonstrated in **Chapter 6**, but it would also be able to discriminate between antibiotic susceptible, tolerant and resistant strains. However, since drug resistant bacteria could differ from drug susceptible bacteria by as little as a single mutation, it would be difficult to predict drug resistance using an untargeted method as described in this thesis. Theoretically, a single mutation could alter the physiology of a cell and thereby the proteome, making identification of drug resistant strains a possibility. However, as such data is not available in literature, other approaches should be explored.

As discussed above, current i*n vitro* drug susceptibility testing methods often determine the growth inhibitory concentrations of antibiotics, the MIC. Since the outgrowth of the pathogen in a pre-determined drug concentration is the only measure of these assays, it is not possible to identify drug tolerant bacteria which inhibit their own growth to prevent killing as we presented in **Chapter 3** & **Chapter 4**. As a consequence, based on the outcomes of these types of drug susceptibility assays, one can solely conclude whether a strain is drug resistant, i.e. drug resistant strains will grow in high concentrations of antibiotics, whereas drug susceptible strains will not be able to grow in the presence of antibiotics. Theoretically, the in **Chapter 6** described mycobacterial typing method can be applied to discriminate between bacterial drug responses, as discussed below.

It is conceivable that rifampicin treatment will not inhibit the production of new proteins in rifampicin resistant strains. Therefore, it is to be expected that the proteome of a rifampicin resistant strain will not change dramatically during rifampicin treatment. In contrast, rifampicin treatment of fully drug susceptible cells will lead to cell death and might eventually even lead to cell lysis, which will result in a proteomic signature that differs from that of drug resistant, viable, cells. Finally, drug tolerant cells that will persist during drug treatment, but

stop growing, will need to adjust their metabolism and thereby their proteome, described in **Chapter 3** & **Chapter 4**. As a result, drug susceptible, tolerant and resistant cells are likely to present a different proteome after drug exposure. Therefore, it would be of interest to perform a traditional phenotypic drug susceptibility assay of which the cultures are also analysed using the described compareMS2 method.

If the pathogen is able to grow in the presence of an antibiotic, we can state that the pathogen is drug resistant, as can be determined using current drug susceptibility testing. If there is no growth, the pathogen can be either drug susceptible or drug tolerant. In this situation, the mass spectrometer can be used to determine whether the cells are drug tolerant, based on their proteomic signature. As a control, the bacteria could be transferred to fresh cultured broths without antibiotics, so non-killed, drug tolerant cells can be re-cultured, as also described in **Chapter 3**. However, the re-culture of cells would take significantly longer than the identification of drug tolerant cells using tandem mass spectra. Thereby, phenotypic identification methods, as compareMS2, could shorten the diagnostic turn-around times significantly.

Although the hypothetical situation outlined above could lead to the discrimination of drug tolerant and drug susceptible strains, it is to be expected that in a cultured population both drug tolerant and drug susceptible strains are present due to the heterogeneity of cell cultures, even with highly defined culture conditions.[72] However, as recently reported for meat products, the described compareMS2 method is capable to determine the relative composition of a sample containing meat derived from two or more species.[73] Thereby, the method has, theoretically, the potential to not only discriminate between closely-related mycobacterial species, but also determine the relative composition of drug susceptible, tolerant or resistant cells present within a single culture.

The limitations and possibilities of the method should be further examined before it can reach its full potential and being considered for routine use in the clinic. However, based on the data presented in **Chapter 6**, we can conclude that the approach has the potential to advance our understanding of phenotypic relationships between mycobacterial species. Furthermore, the principles of this method can provide accurate species identification and could be of help in directing rapid, accurate and effective patient treatment in the future.

Concluding remarks

The research presented in this thesis has increased our understanding of the mechanisms that provide rifampicin tolerance, the limitations of drug susceptibility testing, inter- and intra-strain

variation in *M. tuberculosis*, thioridazine's mechanism of action and potential new diagnostic methods. The hypotheses generated, tested and confirmed in this thesis were the result of unbiased proteomic approaches that allowed for novel insights into the regulation of the *M. tuberculosis* proteome in well-defined experimental settings. Although we found that the phenotype *M. tuberculosis* Beijing lineage is more equipped to withstand antibiotic treatment, the outcomes of this thesis indicate that if we advance our understanding of the etiology of drug resistance in *M. tuberculosis*, improved treatment strategies and diagnostic methods will be on the horizon.

References

1. Donald, P. R.; van Helden, P. D., The global burden of tuberculosis--combating drug resistance in difficult times. *N Engl J Med* **2009,** 360, (23), 2393-5.
2. Lanigan, M. D.; Vaughan, J. A.; Shiell, B. J.; Beddome, G. J.; Michalski, W. P., Mycobacterial proteome extraction: comparison of disruption methods. *Proteomics* **2004,** 4, (4), 1094-100.
3. Rabodoarivelo, M. S.; Aerts, M.; Vandamme, P.; Palomino, J. C.; Rasolofo, V.; Martin, A., Optimizing of a protein extraction method for Mycobacterium tuberculosis proteome analysis using mass spectrometry. *J Microbiol Methods* **2016,** 131, 144-147.
4. Kelkar, D. S.; Kumar, D.; Kumar, P.; Balakrishnan, L.; Muthusamy, B.; Yadav, A. K.; Shrivastava, P.; Marimuthu, A.; Anand, S.; Sundaram, H.; Kingsbury, R.; Harsha, H. C.; Nair, B.; Prasad, T. S.; Chauhan, D. S.; Katoch, K.; Katoch, V. M.; Kumar, P.; Chaerkady, R.; Ramachandran, S.; Dash, D.; Pandey, A., Proteogenomic analysis of Mycobacterium tuberculosis by high resolution mass spectrometry. *Mol Cell Proteomics* **2011,** 10, (12), M111 011627.
5. Schubert, O. T.; Mouritsen, J.; Ludwig, C.; Rost, H. L.; Rosenberger, G.; Arthur, P. K.; Claassen, M.; Campbell, D. S.; Sun, Z.; Farrah, T.; Gengenbacher, M.; Maiolica, A.; Kaufmann, S. H.; Moritz, R. L.; Aebersold, R., The Mtb proteome library: a resource of assays to quantify the complete proteome of Mycobacterium tuberculosis. *Cell Host Microbe* **2013,** 13, (5), 602-12.
6. Schubert, O. T.; Ludwig, C.; Kogadeeva, M.; Zimmermann, M.; Rosenberger, G.; Gengenbacher, M.; Gillet, L. C.; Collins, B. C.; Rost, H. L.; Kaufmann, S. H.; Sauer, U.; Aebersold, R., Absolute Proteome Composition and Dynamics during Dormancy and Resuscitation of Mycobacterium tuberculosis. *Cell Host Microbe* **2015,** 18, (1), 96-108.
7. Pheiffer, C.; Betts, J.; Lukey, P.; van Helden, P., Protein expression in Mycobacterium tuberculosis differs with growth stage and strain type. *Clin Chem Lab Med* **2002,** 40, (9), 869-75.
8. Gillet, L. C.; Navarro, P.; Tate, S.; Rost, H.; Selevsek, N.; Reiter, L.; Bonner, R.; Aebersold, R., Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics* **2012,** 11, (6), O111 016717.
9. Hebert, A. S.; Richards, A. L.; Bailey, D. J.; Ulbrich, A.; Coughlin, E. E.; Westphall, M. S.; Coon, J. J., The one hour yeast proteome. *Mol Cell Proteomics* **2014,** 13, (1), 339-47.
10. Kumar, A.; Agarwal, S.; Heyman, J. A.; Matson, S.; Heidtman, M.; Piccirillo, S.; Umansky, L.; Drawid, A.; Jansen, R.; Liu, Y.; Cheung, K. H.; Miller, P.; Gerstein, M.; Roeder, G. S.; Snyder, M., Subcellular localization of the yeast proteome. *Genes Dev* **2002,** 16, (6), 707-19.
11. Picotti, P.; Bodenmiller, B.; Mueller, L. N.; Domon, B.; Aebersold, R., Full dynamic range proteome analysis of S. cerevisiae by targeted proteomics. *Cell* **2009,** 138, (4), 795-806.
12. Olsen, J. V.; Mann, M., Status of large-scale analysis of post-translational modifications by mass spectrometry. *Mol Cell Proteomics* **2013,** 12, (12), 3444-52.
13. Brodbelt, J. S., Ion Activation Methods for Peptides and Proteins. *Anal Chem* **2016,** 88, (1), 30-51.
14. Cannon, J. R.; Kluwe, C.; Ellington, A.; Brodbelt, J. S., Characterization of green fluorescent proteins by

193 nm ultraviolet photodissociation mass spectrometry. *Proteomics* **2014,** 14, (10), 1165-73.

15. Wu, X.; Hasan, M. A.; Chen, J. Y., Pathway and network analysis in proteomics. *J Theor Biol* **2014,** 362, 44-52.
16. Lew, J. M.; Kapopoulou, A.; Jones, L. M.; Cole, S. T., TubercuList--10 years after. *Tuberculosis (Edinb)* **2011,** 91, (1), 1-7.
17. Doerks, T.; van Noort, V.; Minguez, P.; Bork, P., Annotation of the M. tuberculosis hypothetical orfeome: adding functional information to more than half of the uncharacterized proteins. *PLoS One* **2012,** 7, (4), e34302.
18. Acocella, G., Clinical pharmacokinetics of rifampicin. *Clin Pharmacokinet* **1978,** 3, (2), 108-27.
19. Taniguchi, H.; Aramaki, H.; Nikaido, Y.; Mizuguchi, Y.; Nakamura, M.; Koga, T.; Yoshida, S., Rifampicin resistance and mutation of the rpoB gene in Mycobacterium tuberculosis. *FEMS Microbiol Lett* **1996,** 144, (1), 103-8.
20. Floss, H. G.; Yu, T. W., Rifamycin-mode of action, resistance, and biosynthesis. *Chem Rev* **2005,** 105, (2), 621-32.
21. Connolly, L. E.; Edelstein, P. H.; Ramakrishnan, L., Why is long-term therapy required to cure tuberculosis? *PLoS Med* **2007,** 4, (3), e120.
22. den Hertog, A. L.; Menting, S.; van Soolingen, D.; Anthony, R. M., Mycobacterium tuberculosis Beijing genotype resistance to transient rifampin exposure. *Emerg Infect Dis* **2014,** 20, (11), 1932-3.
23. de Steenwinkel, J. E.; ten Kate, M. T.; de Knegt, G. J.; Kremer, K.; Aarnoutse, R. E.; Boeree, M. J.; Verbrugh, H. A.; van Soolingen, D.; Bakker-Woudenberg, I. A., Drug susceptibility of Mycobacterium tuberculosis Beijing genotype and association with MDR TB. *Emerg Infect Dis* **2012,** 18, (4), 660-3.
24. Devaux, I.; Kremer, K.; Heersma, H.; Van Soolingen, D., Clusters of multidrug-resistant Mycobacterium tuberculosis cases, Europe. *Emerg Infect Dis* **2009,** 15, (7), 1052-60.
25. Buu, T. N.; Huyen, M. N.; Lan, N. T.; Quy, H. T.; Hen, N. V.; Zignol, M.; Borgdorff, M. W.; Cobelens, F. G.; van Soolingen, D., The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. *Int J Tuberc Lung Dis* **2009,** 13, (7), 900-6.
26. Buu, T. N.; van Soolingen, D.; Huyen, M. N.; Lan, N. T.; Quy, H. T.; Tiemersma, E. W.; Kremer, K.; Borgdorff, M. W.; Cobelens, F. G., Increased transmission of Mycobacterium tuberculosis Beijing genotype strains associated with resistance to streptomycin: a population-based study. *PLoS One* **2012,** 7, (8), e42323.
27. Glynn, J. R.; Whiteley, J.; Bifani, P. J.; Kremer, K.; van Soolingen, D., Worldwide occurrence of Beijing/W strains of Mycobacterium tuberculosis: a systematic review. *Emerg Infect Dis* **2002,** 8, (8), 843-9.
28. Parwati, I.; Alisjahbana, B.; Apriani, L.; Soetikno, R. D.; Ottenhoff, T. H.; van der Zanden, A. G.; van der Meer, J.; van Soolingen, D.; van Crevel, R., Mycobacterium tuberculosis Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. *J Infect Dis* **2010,** 201, (4), 553-7.
29. Ford, C. B.; Lin, P. L.; Chase, M. R.; Shah, R. R.; Iartchouk, O.; Galagan, J.; Mohaideen, N.; Ioerger, T. R.; Sacchettini, J. C.; Lipsitch, M.; Flynn, J. L.; Fortune, S. M., Use of whole genome sequencing to estimate the mutation rate of Mycobacterium tuberculosis during latent infection. *Nat Genet* **2011,** 43, (5), 482-6.

30. Brauner, A.; Fridman, O.; Gefen, O.; Balaban, N. Q., Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol* **2016,** 14, (5), 320-30.
31. Wayne, L. G.; Sohaskey, C. D., Nonreplicating persistence of mycobacterium tuberculosis. *Annu Rev Microbiol* **2001,** 55, 139-63.
32. Voskuil, M. I.; Schnappinger, D.; Visconti, K. C.; Harrell, M. I.; Dolganov, G. M.; Sherman, D. R.; Schoolnik, G. K., Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J Exp Med* **2003,** 198, (5), 705-13.
33. Schnappinger, D.; Ehrt, S.; Voskuil, M. I.; Liu, Y.; Mangan, J. A.; Monahan, I. M.; Dolganov, G.; Efron, B.; Butcher, P. D.; Nathan, C.; Schoolnik, G. K., Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J Exp Med* **2003,** 198, (5), 693-704.
34. Betts, J. C.; Lukey, P. T.; Robb, L. C.; McAdam, R. A.; Duncan, K., Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol Microbiol* **2002,** 43, (3), 717-31.
35. Danelishvili, L.; Shulzhenko, N.; Chinison, J. J. J.; Babrak, L.; Hu, J.; Morgun, A.; Burrows, G.; Bermudez, L. E., Mycobacterium tuberculosis Proteome Response to Antituberculosis Compounds Reveals Metabolic "Escape" Pathways That Prolong Bacterial Survival. *Antimicrob Agents Chemother* **2017,** 61, (7).
36. Sharma, S.; Tyagi, J. S., Mycobacterium tuberculosis DevR/DosR Dormancy Regulator Activation Mechanism: Dispensability of Phosphorylation, Cooperativity and Essentiality of alpha10 Helix. *PLoS One* **2016,** 11, (8), e0160723.
37. Hu, Y.; Liu, A.; Menendez, M. C.; Garcia, M. J.; Oravcova, K.; Gillespie, S. H.; Davies, G. R.; Mitchison, D. A.; Coates, A. R., HspX knock-out in Mycobacterium tuberculosis leads to shorter antibiotic treatment and lower relapse rate in a mouse model--a potential novel therapeutic target. *Tuberculosis (Edinb)* **2015,** 95, (1), 31-6.
38. Yang, C.; Luo, T.; Sun, G.; Qiao, K.; Sun, G.; DeRiemer, K.; Mei, J.; Gao, Q., Mycobacterium tuberculosis Beijing strains favor transmission but not drug resistance in China. *Clin Infect Dis* **2012,** 55, (9), 1179-87.
39. Mokrousov, I.; Ly, H. M.; Otten, T.; Lan, N. N.; Vyshnevskyi, B.; Hoffner, S.; Narvskaya, O., Origin and primary dispersal of the Mycobacterium tuberculosis Beijing genotype: clues from human phylogeography. *Genome Res* **2005,** 15, (10), 1357-64.
40. Bifani, P. J.; Mathema, B.; Kurepina, N. E.; Kreiswirth, B. N., Global dissemination of the Mycobacterium tuberculosis W-Beijing family strains. *Trends Microbiol* **2002,** 10, (1), 45-52.
41. Schurch, A. C.; Kremer, K.; Warren, R. M.; Hung, N. V.; Zhao, Y.; Wan, K.; Boeree, M. J.; Siezen, R. J.; Smith, N. H.; van Soolingen, D., Mutations in the regulatory network underlie the recent clonal expansion of a dominant subclone of the Mycobacterium tuberculosis Beijing genotype. *Infect Genet Evol* **2011,** 11, (3), 587-97.
42. Rengarajan, J.; Bloom, B. R.; Rubin, E. J., Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. *Proc Natl Acad Sci U S A* **2005,** 102, (23), 8327-32.
43. Wells, R. M.; Jones, C. M.; Xi, Z.; Speer, A.; Danilchanka, O.; Doornbos, K. S.; Sun, P.; Wu, F.; Tian, C.; Niederweis, M., Discovery of a siderophore export system essential for virulence of Mycobacterium

tuberculosis. *PLoS Pathog* **2013,** 9, (1), e1003120.

44. van Crevel, R.; Parwati, I.; Sahiratmadja, E.; Marzuki, S.; Ottenhoff, T. H.; Netea, M. G.; van der Ven, A.; Nelwan, R. H.; van der Meer, J. W.; Alisjahbana, B.; van de Vosse, E., Infection with Mycobacterium tuberculosis Beijing genotype strains is associated with polymorphisms in SLC11A1/NRAMP1 in Indonesian patients with tuberculosis. *J Infect Dis* **2009,** 200, (11), 1671-4.
45. Pandey, S. D.; Choudhury, M.; Yousuf, S.; Wheeler, P. R.; Gordon, S. V.; Ranjan, A.; Sritharan, M., Iron-regulated protein HupB of Mycobacterium tuberculosis positively regulates siderophore biosynthesis and is essential for growth in macrophages. *J Bacteriol* **2014,** 196, (10), 1853-65.
46. Yeruva, V. C.; Duggirala, S.; Lakshmi, V.; Kolarich, D.; Altmann, F.; Sritharan, M., Identification and characterization of a major cell wall-associated iron-regulated envelope protein (Irep-28) in Mycobacterium tuberculosis. *Clin Vaccine Immunol* **2006,** 13, (10), 1137-42.
47. Rao, P. K.; Rodriguez, G. M.; Smith, I.; Li, Q., Protein dynamics in iron-starved Mycobacterium tuberculosis revealed by turnover and abundance measurement using hybrid-linear ion trap-Fourier transform mass spectrometry. *Anal Chem* **2008,** 80, (18), 6860-9.
48. Pandey, R.; Rodriguez, G. M., A ferritin mutant of Mycobacterium tuberculosis is highly susceptible to killing by antibiotics and is unable to establish a chronic infection in mice. *Infect Immun* **2012,** 80, (10), 3650-9.
49. Peters, J. S.; Calder, B.; Gonnelli, G.; Degroeve, S.; Rajaonarifara, E.; Mulder, N.; Soares, N. C.; Martens, L.; Blackburn, J. M., Identification of Quantitative Proteomic Differences between Mycobacterium tuberculosis Lineages with Altered Virulence. *Front Microbiol* **2016,** 7, 813.
50. Voskuil, M. I.; Visconti, K. C.; Schoolnik, G. K., Mycobacterium tuberculosis gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis (Edinb)* **2004,** 84, (3-4), 218-27.
51. Hampshire, T.; Soneji, S.; Bacon, J.; James, B. W.; Hinds, J.; Laing, K.; Stabler, R. A.; Marsh, P. D.; Butcher, P. D., Stationary phase gene expression of Mycobacterium tuberculosis following a progressive nutrient depletion: a model for persistent organisms? *Tuberculosis (Edinb)* **2004,** 84, (3-4), 228-38.
52. Fallow, A.; Domenech, P.; Reed, M. B., Strains of the East Asian (W/Beijing) lineage of Mycobacterium tuberculosis are DosS/DosT-DosR two-component regulatory system natural mutants. *J Bacteriol* **2010,** 192, (8), 2228-38.
53. WHO, Multidrug-resistant tuberculosis (MDR-TB) 2013 Update. Factsheet. *Available at:* http://www.who.int/tb/challenges/mdr/MDR_TB_FactSheet.pdf **2013,** (Accessed: 16 January 2015).
54. Xie, Z.; Siddiqi, N.; Rubin, E. J., Differential antibiotic susceptibilities of starved Mycobacterium tuberculosis isolates. *Antimicrob Agents Chemother* **2005,** 49, (11), 4778-80.
55. de Steenwinkel, J. E.; de Knegt, G. J.; ten Kate, M. T.; van Belkum, A.; Verbrugh, H. A.; Kremer, K.; van Soolingen, D.; Bakker-Woudenberg, I. A., Time-kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of Mycobacterium tuberculosis. *J Antimicrob Chemother* **2010,** 65, (12), 2582-9.
56. Deb, C.; Lee, C. M.; Dubey, V. S.; Daniel, J.; Abomoelak, B.; Sirakova, T. D.; Pawar, S.; Rogers, L.; Kolattukudy, P. E., A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis

generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS One* **2009,** 4, (6), e6077.

57. Zahrt, T. C., Molecular mechanisms regulating persistent Mycobacterium tuberculosis infection. *Microbes Infect* **2003,** 5, (2), 159-67.
58. Kapoor, N.; Pawar, S.; Sirakova, T. D.; Deb, C.; Warren, W. L.; Kolattukudy, P. E., Human granuloma in vitro model, for TB dormancy and resuscitation. *PLoS One* **2013,** 8, (1), e53657.
59. Kristiansen, J. E.; Dastidar, S. G.; Palchoudhuri, S.; Roy, D. S.; Das, S.; Hendricks, O.; Christensen, J. B., Phenothiazines as a solution for multidrug resistant tuberculosis: From the origin to present. *Int Microbiol* **2015,** 18, (1), 1-12.
60. Amaral, L.; Boeree, M. J.; Gillespie, S. H.; Udwadia, Z. F.; van Soolingen, D., Thioridazine cures extensively drug-resistant tuberculosis (XDR-TB) and the need for global trials is now! *Int J Antimicrob Agents* **2010,** 35, (6), 524-6.
61. Dutta, N. K.; Pinn, M. L.; Karakousis, P. C., Reduced emergence of isoniazid resistance with concurrent use of thioridazine against acute murine tuberculosis. *Antimicrob Agents Chemother* **2014,** 58, (7), 4048-53.
62. Machado, D.; Couto, I.; Perdigao, J.; Rodrigues, L.; Portugal, I.; Baptista, P.; Veigas, B.; Amaral, L.; Viveiros, M., Contribution of efflux to the emergence of isoniazid and multidrug resistance in Mycobacterium tuberculosis. *PLoS One* **2012,** 7, (4), e34538.
63. Viveiros, M.; Amaral, L., Enhancement of antibiotic activity against poly-drug resistant Mycobacterium tuberculosis by phenothiazines. *Int J Antimicrob Agents* **2001,** 17, (3), 225-8.
64. Amaral, L.; Viveiros, M., Why thioridazine in combination with antibiotics cures extensively drug-resistant Mycobacterium tuberculosis infections. *Int J Antimicrob Agents* **2012,** 39, (5), 376-80.
65. Sareen, M.; Khuller, G. K., Effect of ethambutol on the phospholipids of ethambutol susceptible and resistant strains of Mycobacterium smegmatis ATCC 607. *Indian J Biochem Biophys* **1990,** 27, (1), 39-42.
66. Kopec, W.; Khandelia, H., Reinforcing the membrane-mediated mechanism of action of the anti-tuberculosis candidate drug thioridazine with molecular simulations. *J Comput Aided Mol Des* **2014,** 28, (2), 123-34.
67. Amaral, L.; Viveiros, M., Thioridazine: A Non-Antibiotic Drug Highly Effective, in Combination with First Line Anti-Tuberculosis Drugs, against Any Form of Antibiotic Resistance of Mycobacterium tuberculosis Due to Its Multi-Mechanisms of Action. *Antibiotics (Basel)* **2017,** 6, (1).
68. Pieroni, M.; Machado, D.; Azzali, E.; Santos Costa, S.; Couto, I.; Costantino, G.; Viveiros, M., Rational Design and Synthesis of Thioridazine Analogues as Enhancers of the Antituberculosis Therapy. *J Med Chem* **2015,** 58, (15), 5842-53.
69. van Ingen, J., Diagnosis of nontuberculous mycobacterial infections. *Semin Respir Crit Care Med* **2013,** 34, (1), 103-9.
70. Johnson, M. M.; Odell, J. A., Nontuberculous mycobacterial pulmonary infections. *J Thorac Dis* **2014,** 6, (3), 210-20.
71. Li, W.; Raoult, D.; Fournier, P. E., Bacterial strain typing in the genomic era. *FEMS Microbiol Rev* **2009,** 33, (5), 892-916.

72. Zhang, Y., Persisters, persistent infections and the Yin-Yang model. *Emerg Microbes Infect* **2014,** 3, (1), e3.
73. Ohana, D.; Dalebout, H.; Marissen, R. J.; Wulff, T.; Bergquist, J.; Deelder, A. M.; Palmblad, M., Identification of meat products by shotgun spectral matching. *Food Chem* **2016,** 203, 28-34.