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Parallel reaction monitoring of clinical *Mycobacterium tuberculosis* lineages reveals pre-existent markers of rifampicin tolerance in the emerging Beijing lineage

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ABSTRACT

The spread of multidrug resistant *Mycobacterium tuberculosis* is one of the major challenges in tuberculosis control. In Eurasia, the spread of multidrug resistant tuberculosis is driven by the *M. tuberculosis* Beijing genotype. In this study, we examined whether selective advantages are present in the proteome of Beijing isolates that contribute to the emergence of this genotype. To this end, we compared the proteome of *M. tuberculosis* Beijing to that of *M. tuberculosis* H37Rv, both in the presence and absence of the first-line antibiotic rifampicin. During rifampicin exposure, both *M. tuberculosis* genotypes express proteins belonging to the DosR dormancy regulon, which induces a metabolically hypoactive-, drug tolerant phenotype. However, these markers of rifampicin tolerance were already more abundant in the *M. tuberculosis* Beijing isolate prior to drug exposure. To determine whether the *a priori* high abundance of specific proteins contribute to the formation of antibiotic resistance in *M. tuberculosis* Beijing, we quantified the abundance of 33 selected proteins in 27 clinical isolates from the five most common *M. tuberculosis* lineages using parallel reaction monitoring. The observed pre-existing high abundance of dormancy proteins in Beijing strains provides an evolutionary advantage that allows these strains to persist for prolonged periods during rifampicin treatment.

Significance: M. tuberculosis is the leading cause of death by a bacterial infection worldwide. Treatment-regimen to eradicate this pathogen make use of the first-line antibiotic rifampicin, which is considered to be the cornerstone of modern day *anti*-tuberculosis treatment. Despite the potency of rifampicin, there is an increasing occurrence of rifampicin resistant mutants in a specific cluster of *M. tuberculosis*, the Beijing genotype. Using both a data dependent acquisition and a targeted proteomic approach we identified markers of rifampicin tolerance to be high abundant in members of the *M. tuberculosis* Beijing genotype, already prior drug exposure. The identification of this *M. tuberculosis* Beijing specific trait will contribute to improved diagnostics and treatment of *M. tuberculosis*.

1. Introduction

Mycobacterium tuberculosis has recently been declared the most important worldwide cause of death by a single infectious disease [1]. In 2014 tuberculosis (TB) caused the death of 890,000 men, 480,000 women and 140,000 children [1]. Of major concern is the spread of multidrug resistant tuberculosis (MDR-TB), a form of TB that is resistant to at least rifampicin and isoniazid, the cornerstones in *anti*-TB treatment. In Eurasia, the dissemination of MDR-TB is largely attributable to the *M. tuberculosis* Beijing genotype [2,3].

Strains of the *M. tuberculosis* Beijing lineage seem to possess selective advantages to acquire rifampicin resistance [4–7], to circumvent BCG-vaccine induced immunity [8–10] and hyper-transmissibility compared to other *M. tuberculosis* lineages [11–14]. Not surprisingly, the

Abbreviations: CAS, Central Asian strains; DDA, data dependent acquisition; EAI, East-African Indian; FDR, false discovery rate; FASP, filter aided sample preparation; MIC, minimal inhibitory concentration; MDR-TB, multidrug resistant tuberculosis; MGIT, mycobacterial growth indicator tube; MIRU, mycobacterial interspersed repeat units; nsSNP, non synonymous single nucleotide polymorphisms; PRM, parallel reaction monitoring; RRDR, rifampicin resistance determining region; SIL, stable isotopically labelled; SCX, strong cation exchange; TTD, time-till detection; TB, tuberculosis; VNTR, variable number of tandem repeats.

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M. tuberculosis Beijing genotype is correlated with (multi)drug resistance, hyper-virulence and relapses after curative treatment in many geographical areas [15–20].

Although it is well accepted that *M. tuberculosis* Beijing manifests itself differently from other *M. tuberculosis* lineages regarding transmission, drug resistance and virulence, there is some discrepancy between the various studies published. This heterogeneity is partly attributable to differences between *M. tuberculosis* sub-lineages in the Beijing genotype family [21]. Phylogenetically, the Beijing genotype can be delineated into the more "ancient" or atypical and the more "modern" or typical Beijing sub-lineage. However, both sub-lineages are geneticallly highly similar [22,23]. In fact, only 31 non synonymous single nucleotide polymorphisms (nsSNP) have been identified that discriminate both sub-lineages [24]. Although genetically similar, the degree of genetic conservation is much higher within the typical Beijing sublineage, which suggests that these strains acquired selective advantages over atypical Beijing strains in relatively recent years [24]. This hypothesis is further strengthened by the fact that it is mainly the modern typical Beijing sub-lineage that is associated with (multi)drug resistance and the ability to spread and cause disease [25]. Currently, there is no unequivocal model that can clarify the evolutionary success of *M. tuberculosis* (typical) Beijing strains [26]. However, the key to the evolutionary success of *M. tuberculosis* (typical) Beijing might be traced within the proteomic composition of strains in this lineage.

Over a decade ago, a two-dimensional gel electrophoresis approach was used to identify proteins that are differentially abundant in *M. tuberculosis* Beijing, a second "non-Beijing" clinical isolate, and *M. tuberculosis* H37Rv, a frequently studied mycobacterial laboratory strain [27]. One protein was identified to be more abundant in *M. tuberculosis* Beijing (Rv2031c/hspX), whereas three other proteins were identified to be less abundant (Rv0440/GroEL2, Rv0934/PstS1 and Rv1860/47 kDa). In a recent study, we quantitatively compared the proteomes of typical with that of atypical Beijing strains, in which we found that Beijing sub-lineages are extremely conserved in terms of protein abundance [28]. In fact, the two proteomes were so similar that we could only identify two proteins to be differentially regulated between both sub-lineages; Rv0450c/mmpL4 and Rv3283/sseA. Subsequently, when we examined the initial response of a typical Beijing isolate to rifampicin, we found the so-called DosR dormancy regulon to be induced within 24 h after treatment [29]. The DosR dormancy regulon induces a non-replicative, metabolically inactive and drug tolerant mycobacterial phenotype which may severely compromise treatment efficacy [30]. We demonstrated that dormancy can be actively induced by the pathogen to protect itself from toxic compounds, whereas it was previously only known that environmental factors can induce dormancy [31,32]. If *M. tuberculosis* Beijing strains possess an increased ability to induce dormancy upon exposure to rifampicin, this could possibly reflect the mechanism through which Beijing genotype bacteria developed a higher tolerance to antibiotics within patients, which consequently leads to a longer persistence, development of resistance and higher relapse rates even after curative treatment. However, the induction of the dormancy regulon upon rifampicin exposure has so far only been demonstrated in *M. tuberculosis* Beijing.

Therefore, we here address the question of what proteins typify the proteome of *M. tuberculosis* Beijing and what proteins contribute to the emergence of drug-resistant *M. tuberculosis* Beijing. To this end, we used a data-dependent acquisition (DDA) approach to compare the proteome of the *M. tuberculosis* Beijing B0/W-148, which resembles the "successful" Russian typical Beijing strain [33], with the proteome of the *M. tuberculosis* laboratory-strain H37Rv, both with and without exposure to rifampicin. Based on the proteins quantified by DDA proteomics, we made a selection of 33 proteins and examined their abundance using parallel reaction monitoring (PRM) in multiple lineages of well characterized clinical *M. tuberculosis* isolates; typical Beijing, atypical Beijing, East-African Indian (EAI), Haarlem and Central Asian Strain (CAS) lineages.

Following this approach, we provide a thorough inter-lineage comparison of protein abundance in well characterized *M. tuberculosis* genotypes. The results presented in this manuscript show that proteins required to circumvent rifampicin-induced killing are already highly abundant in strains of the *M. tuberculosis* Beijing genotype, prior to drug exposure.

2. Materials & methods

2.1. Culture conditions

Mycobacterial cells were re-cultured from frozen stocks in 5 ml Tween-Albumin liquid culture broth (Tritium Microbiologie, the Netherlands) at 36 °C to an O.D. of 0.4 AU at 600 nm, as described previously [28]. One ml of this pre-culture was transferred to a 250 ml Erlenmeyer flask containing 100 ml Tween-Albumin broth and incubated under constant rotation at 36 °C to provide aeration. Once the cultures reached an O.D. of 0.6 AU at 600 nm, representing the mid-log phase, the cells were exposed to 1 μg/ml rifampicin (Sigma Aldrich, the Netherlands) or DMSO as a control. Cells were harvested after 24 h of incubation and washed three times with ice-cold PBS (Braun, Germany), the resulting pellet was suspended in 5 ml Lysis-buffer (4% SDS, 100 mM Tris-HCl, pH 7.6) and heat-killed at 95 °C for 10 min. Lysates were stored at −20 °C until further usage. Biological duplicates were grown, processed and analysed in parallel.

2.2. Rifampicin susceptibility determination

To exclude the selection of rifampicin resistant mycobacteria we analysed the rifampicin resistance determining region (RRDR) of *rpoB*, the hotspot for drug resistance, for presence of mutations using the MTBDR*plus* assay according to the manufacturers' recommendations, as described previously [34]. Phenotypic rifampicin susceptibility, as determined by the minimal inhibitory concentration (MIC), was determined according to Clinical and Laboratory Standards Institute guidelines [35], using the BACTEC MGIT-960 system (Becton, Dickinson and Co., Franklin Lakes, NJ).

2.3. Sample preparation

Protein digests were prepared as described previously [28]. In brief, mycobacterial cell lysates were mechanically disrupted by bead-beating in a mini bead-beater 16 (BioSpec, USA) after which the lysates were transferred to a fresh tube. Proteins in the lysate were digested using the filter aided sample preparation (FASP) method, including reduction, removal of SDS by urea and carbamidomethylation of cysteines [36]. The tryptic digest was desalted on C18 SepPak columns (Waters Corporation, Massachusetts, USA) and on column labelled by dimethylation [37]. Samples analysed by data dependent acquisition (DDA) were fractionated into 15 fractions by strong cation exchange (SCX) on a Agilent 1100 system equipped with an in-house packed SCX-column (320 μm ID, 15 cm, polysulfoethyl A 3 μm, Poly LC, Columbia, USA), run at 4 μl/min. The SCX gradient started for 10 min at 100% solvent A (water/acetonitrile/formic acid; 70/30/0.1), after which a linear gradient reached 100% solvent B (250 mM KCl/acetonitrile/formic acid 70/30/0.1) in 15 min, followed by 100% solvent C (500 mM KCl/acetonitrile/formic acid 70/30/0.1) for 15 min. To clean the column, the gradient was held at 100% solvent C for 5 min. Next, the column was washed with 100% solvent A. Fifteen fractions were collected in 1 min intervals, lyophilized and reconstituted in 30 μl (water/acetonitrile/formic acid 95/3/0.1) for nanoLC-MS/MS.

2.4. Data acquisition

Samples prepared for DDA analysis were analysed on a nanoLC-MS/MS system consisting of an Easy nLC 1000 gradient HPLC system