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Research paper

Mutations of *Mycobacterium tuberculosis* induced by anti-tuberculosis treatment result in metabolism changes and elevation of ethambutol resistance

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ABSTRACT

Selective pressure from antibiotic use is one of the most important risk factors associated with the development of drug resistance in *Mycobacterium tuberculosis* (MTB). However, the mechanisms underlying drug resistance at the molecular level remain partly unclear. Therefore, the purpose of this study was to investigate the potential functional effect of novel mutations arising from anti-tuberculosis treatment. We analyzed two multidrug-resistant TB (MDR-TB) isolates from the same patient; one collected before and one almost a year after commencing MDR-TB treatment. The post-treatment isolate exhibited elevated ethambutol resistance. We sequenced the whole genomes of the two clinical isolates and detected six novel polymorphisms affecting the genes *Rv1026*, *nc0021*, *Rv2155c*, *Rv2437*, and *Rv3696c*, and the intergenic region between *Rv2764c* and *Rv2765*. Metabolomics approach was used to reveal the effect of the found variation on the metabolic pathways of MTB. Partial least squares–discriminant analysis showed a clear differentiation between the two isolates, involving a total of 175 metabolites. Pathway analysis showed that these metabolites are mainly involved in amino sugar and nucleotide sugar metabolism, β-alanine metabolism, sulfur metabolism, and galactose metabolism. The increased ethambutol resistance exhibited by the post-treatment MDR-TB strain could speculatively be linked to the identified genetic variations, which affected the synthesis of a number of metabolites associated with sources of carbon and energy. This may have been the main factor underlying the increased ethambutol resistance of this isolate.

1. Introduction

The latest World Health Organization (WHO) report stated that tuberculosis (TB) has been the single infectious disease with the most impact, with a global estimate of 10.4 million new cases and 1.4 million deaths in 2016 (World Health Organization, 2017). As first-line drugs,

Abbreviations: WGS, whole-genome sequencing; MABA, Microplate Alamar Blue Assay; PCA, Principal Component Analysis; PLS-DA, Partial least squares–discriminant analysis

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isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) have been used for decades to treat active TB. However, the emerging crisis of drug-resistant TB has become a serious threat to global TB treatment and control.

Besides the transmission of drug-resistant TB strains between individuals, the most important causative factors contributing to the development of drug-resistant strains are mismanagement of patients and inadequate administration of antimicrobial therapy. The main molecular mechanism underlying drug resistance in MTB is the occurrence of chromosomal mutations, which alter the drug target or other genes governing treatment susceptibility. Therefore, identification of the mutations induced by anti-tuberculosis treatment is critical for not only the detection of drug resistance but also for investigating the mechanisms involved.

Previous studies identified specific mutations strongly associated with TB drug resistance. For example, the *katG* (S315T) mutation is found in up to 94% of INH-resistant *Mycobacterium tuberculosis* (MTB) clinical isolates (Vilchèze and Jacobs Jr, 2014). Mutations in the well-defined 81-bp region of the *rpoB* gene have been detected in approximately 97% of all RIF-resistant MTB strains (Telenti et al., 1993). Acquired mutations in *embCAB* operon have been suggested associated with EMB-resistance (Mikusova et al., 1995; Telenti et al., 1997) although these mutations were also found in EMB-susceptible isolates (Mokrousov et al., 2002; Hazbón et al., 2005). Increasing evidence has confirmed that ethambutol resistance, and the *embB* codon 306 mutation in particular, is an important risk factor in the development of RIF resistance and multidrug-resistant tuberculosis (MDR-TB) (Cuevas-Córdoba et al., 2015; Perdigao et al., 2009).

Genomic techniques such as the polymerase chain reaction (PCR) and DNA microarrays have been widely used to explore the genetic mechanisms of anti-tuberculosis drug resistance, but these techniques are limited to detecting known drug-resistance mutations. The mutations uncovered to date cannot however explain all the genetic mechanisms of drug resistance, evolution, and transmission. Whole-genome sequencing (WGS) is therefore increasingly being used as it can identify novel functional mutations responsible for the observed phenotypical changes (Nimmo et al., 2017).

Investigations of the potential functional effect of novel mutations on drug resistance are critical for active TB treatment and control. The majority of current research focuses on the functional effect of mutations at the genome- and protein-level, and studies investigating the drug-resistant phenotype from a metabolomics perspective are lacking. Metabolomics is an emerging research approach that enables insights into the underlying mechanisms of interactions between pathogen and host. Metabolomics applications in the field of TB research include investigations of antimicrobial therapy-induced drug resistance, monitoring of treatment outcomes, and screening of diagnostic biomarkers for the differentiation of *Mycobacterium* species (Olivier and Loots, 2011; Lau et al., 2015). Metabolomics studies can also provide new insights into the altered metabolic pathways that result in drug-resistant phenotypes.

In this study, we found that two MTB clinical isolates from the same patient (pre- and post-treatment) differed in their EMB-resistant phenotype. After one year of anti-tuberculosis therapy, the patient's EMB resistance level had increased significantly. To investigate the potential mechanisms involved in elevated EMB resistance, we applied whole genome sequencing to screen the underlying drug-resistance mutations, and metabolomics methods to identify changes in the relevant metabolic pathways.

2. Materials and methods

2.1. Bacterial strains

Two MTB isolates 10522 and 12543 were collected from the same patient at Beijing Chest Hospital. The patient was diagnosed with pulmonary tuberculosis and hospitalized for anti-tuberculosis treatment. The first isolate 10522 (pre-treatment strain) was collected before treatment in July 2012. The pre-treatment strain was later confirmed to be MDR-TB by drug susceptibility tests. The second isolate 12543 (post-treatment strain) was collected in June 2013, nearly 12 months after commencement of anti-tuberculosis therapy which including levofloxacin, amikacin, prothionamide, ethambutol and pyrazinamide. MTB laboratory strain H37Rv (GenBank accession no. NC_000962.3) from the strain library established at Beijing Chest Hospital was used as the reference strain.

2.2. Drug susceptibility testing

The isolates were cultured on Lowenstein–Jensen medium for 4 weeks at 37 °C. Susceptibility towards four first-line anti-TB drugs was tested using the absolute concentration method. The concentrations of the tested drugs in the medium were as follows: INH, 0.2 μg/ml; RIF, 50 μg/ml; streptomycin, 10 μg/ml; EMB, 5 μg/ml.

Susceptibility testing was also performed to determine the EMB minimum inhibitory concentration (MIC) of the isolates. The Microplate Alamar Blue Assay (MABA) was performed using Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD, USA) including 10% oleic acid-albumin-dextrose-catalase supplement and EMB at concentrations of 0, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml (Franzblau et al., 1998).

2.3. DNA extraction, genotyping and sanger sequencing

DNA was extracted from *M. tuberculosis* cultures using the recommended method (van Embden et al., 1993). The genomic DNA was purified and then checked with the Qubit dsDNA BR Assay (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the purified DNA was confirmed with agarose gel electrophoresis.

Multilocus variable-number tandem repeat analysis (MLVA) was performed for 24 loci as described previously (Supply et al., 2006). The H37Rv strain was used as the quality control and a 100-bp ladder (Beijing SBS Genetech; Beijing, China) was used as the molecular weight marker. 24-MIRU profiles were compared to MIRU-VNTRplus (http://www.miru-vntrplus.org).

Sanger sequencing of resistance-related mutations was performed according to previously published methods. The sequenced genes were those associated with EMB resistance (*embA*, *embB*, *embC*, *ubiA*, and *aftA*) and resistance towards other first-line drugs (*rpoB*, *katG*, and *rpsL*).

2.4. Whole-genome sequencing

Whole-genome sequencing was performed on the Illumina HiSeq PE150 platform at the Beijing Novogene Technology Co., Ltd. The DNA was “A-tailed”, ligated to paired-end adapters, and PCR-amplified to generate 350-bp inserts used for library construction. Short reads were assembled with SOAP denovo (http://soap.genomics.org.cn), a genome assembler developed specifically for next-generation short-read sequences. SOAP GapCloser was used to close gaps where possible after assembly.

Data for the *M. tuberculosis* sequenced genomes were deposited in the National Center for Biotechnology Information Sequence Read Archive (project number PRJNA 490398).

The short sequencing reads (fastq files) were submitted to TGS-TB online tool at https://gph.niid.go.jp/tgs-tb/ for genome-wide SNP based comparison of the isolates. The sequencing reads were mapped to the genome of reference strain H37Rv (NC_00962.3) using Geneious 9.0 package (Biomatters Ltd).

2.5. Bacterial cultures and sample extraction for metabolomics

The strains were incubated in Middlebrook 7H9 broth supplemented with 0.2% (*v*/v) glycerol and 10% oleic acid-albumin-dextrose-catalase. The two strains had the same number of 4 passages, culture conditions and bacteria was collected at the same concentration. The inocula were incubated at 37 °C until an OD600 of 1.5 was obtained, to achieve comparable bacterial concentrations at the early stationary phase for MTB strains. OD600 1.5 was chosen for harvest because it represents early stationary phase in MTB strains as shown in previous studies (Tufariello et al., 2004). Seven or eight biological replicates of each culture were used, and 200 µl of each sample was mixed with three equivalent volumes of 100% acetonitrile and vortexed for 1–3 min. After 10 min incubation at 4 °C, the samples were centrifuged at 13,000 rpm for 10 min to obtain the supernatant. The supernatants were then lyophilized with the vacuum method.

2.6. LC-MS/MS analysis

All LC-MS/MS analyses were performed with the Acquity Nexera X2 system (Shimadzu; Kyoto, Japan) coupled to the TripleTOF 5600+ system (AB Sciex, Framingham, MA, USA). Chromatographic separation of the sample was performed with a ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 3.5 µm; Agilent; Santa Clara, CA, USA). Mobile phase A was 0.1% formic acid, phase B was acetonitrile in 0.1% formic acid, and the linear gradient program was as follows: phase B was maintained at 2% for 1 min, linearly increased to 90% for 15 min, then reduced to 2% for 4 min. The flow rate was 0.50 ml/min and the sample injection volume was 2 µl. The column was equilibrated for 5 min before each sample introduction and the operating temperature was set to 40 °C. The following conditions were applied on the mass spectrometer: capillary temperature, 300 °C; source voltage, 3.0 kV; capillary voltage, 35 V for ESI+ analysis, source temperature, 120 °C. The mass scan range was set between *m/z* 50–1500.

2.7. Data processing and statistical data analysis

Potential discriminatory variables were determined after the raw data were processed for peak discovery and peak alignment and filtered. Principal component analysis (PCA) and partial least squares–discriminant analysis (PLS-DA) which were commonly used for metabolic studies were performed by SIMCA-P (Version 11.0, Umetrics, Umea, Sweden) software. PCA and hierarchical clustering were performed for unsupervised multivariate statistical analysis. PCA is a statistic method which enables to identify the principal components (*e.g.* individual amino acids or patterns of amino acids) out of a complex data set to explain biological differences between strains. The resulting PCA score plot visualizes the metabolic distance, which is defined as distance between metabolites on the real metabolic map. Partial least squares–discriminant analysis (PLS-DA) was performed as a supervised method to identify important variables with discriminatory power. PLS-DA models were validated based on their multiple correlation coefficient (R^2) and cross-validated R^2 (Q^2) values from the cross-validation and permutation test by applying 200 iterations (FDR < 0.05). R^2 (*cum*) and Q^2 (*cum*) parameters usually indicate the fitness and prediction of the model. The model is stable and reasonable when R^2 and Q^2 are < 1 and the two parameters are close to 1. Biomarkers with variable importance in projection (VIP) > 1 were recognized as potential markers contributing to group discrimination. MS/MS fragmentation was performed on the potential biomarkers, and hits were identified with a literature and database search using the exact molecular weights or MS/MS fragmentation pattern data. These databases were the METLIN database (http://metlin.scripps.edu/), Human Metabolome Database (http://www.hmdb.ca/), *E. coli* Metabolome Database (http://www.ecmdb.ca/), MassBank (http://www.massbank.jp/), Lipid Maps (http://www.lipidmaps.org/), and KEGG database (http://www.genome.jp/kegg).

3. Results

3.1. Drug susceptibility testing of the pre- and post-treatment strains

M. tuberculosis isolates were collected from the same patient before and one year after commencing anti-TB therapy. Susceptibility of the two *M. tuberculosis* isolates towards four first-line anti-TB drugs (EMB, RIF, INH, and streptomycin) was tested. The MIC of EMB was 4 µg/ml for the pre-treatment isolate, while the post-treatment isolate showed 4-fold elevation of MIC to 16 µg/ml. The MIC of the other three first-line drugs were maintained at their initial level of and exhibited to be resistant following treatment.

3.2. Genotyping and genomics analysis

The strains were genotyped with the standard 24-locus variable-number tandem-repeat (VNTR) method and were confirmed to share the same 24-locus VNTR genotype pattern, implying that the two MDR-TB strains were clonally related. This profile was assigned Mlva15-9 code 9380-32 according to MIRU-VNTRplus resource.

Based on WGS/NGS data analysis, the isolates from both patients belonged to L2.2.1 according to the SNP-barcode scheme (Coll et al., 2014). They belong to early ancient sublineage of the Beijing genotype with mutant alleles of *mutT4*-48 and *mutT2*-58 and deleted *RD181* region.

Previously reported resistance-related mutations were analyzed with direct Sanger sequencing. A total of eight resistance-related genes were examined. The two *M. tuberculosis* isolates shared the same mutations of these genes, included *embA* C76C– TGT, *embB* M306L-CTG, *embC* R927R-CGT, *rpoB* S450L-TTG, *katG* S315 T-ACC, *rpsL* K43R-AGG. No mutations were detected in gene *ubiA*, and *aftA*.

After whole-genome sequencing, six variant SNPs were identified between the two isolates (Table 1).

3.3. Metabolomics changes between the two strains

To analyze the metabolic changes in relation to identified inter-strain gene variation, LC-MS/MS analysis was used to obtain the metabolomics profile of the two culture supernatants. Totally, 2121 and 2182 metabolites were identified for pre-treatment and post-treatment strains respectively. The LC-MS/MS spectra of culture supernatants from the two strains are shown in Fig. 1, with major metabolites labeled in the spectra. Several differences in the spectra were observed between the two isolates.

A score plot of the PCA model for sample data collected from the two isolates is shown in Fig. 2A. To improve the separation, the PLS-DA model was applied to more easily visualize the metabolic differences between the two isolates. A distinct separation was obtained according

Table 1
Six mutations detected by WGS between two isolates using MTB H37Rv as reference.

Position	Ref.	Pre-treatment strain	Post-treatment strain	Type	Gene
1147754	C	A (Thr > Pro)	C (Thr > Thr)	missense	*Rv1026*
1471656	G	G (NA)	C	*	*nc0021*
2415166	G	G (Pro > Pro)	C (Pro > Arg)	missense	*Rv2155c (murD)*
2734453	G	C (Pro > Pro)	G (Pro > Pro)	synonym.	*Rv2437*
3074495	G	G (NA)	A	*	*Rv2764c (thyA)-Rv2765* intergenic region
4138983	A	C (Gly > Ala)	A (Gly > Gly)	missense	*Rv3696c*

*non-coding region.

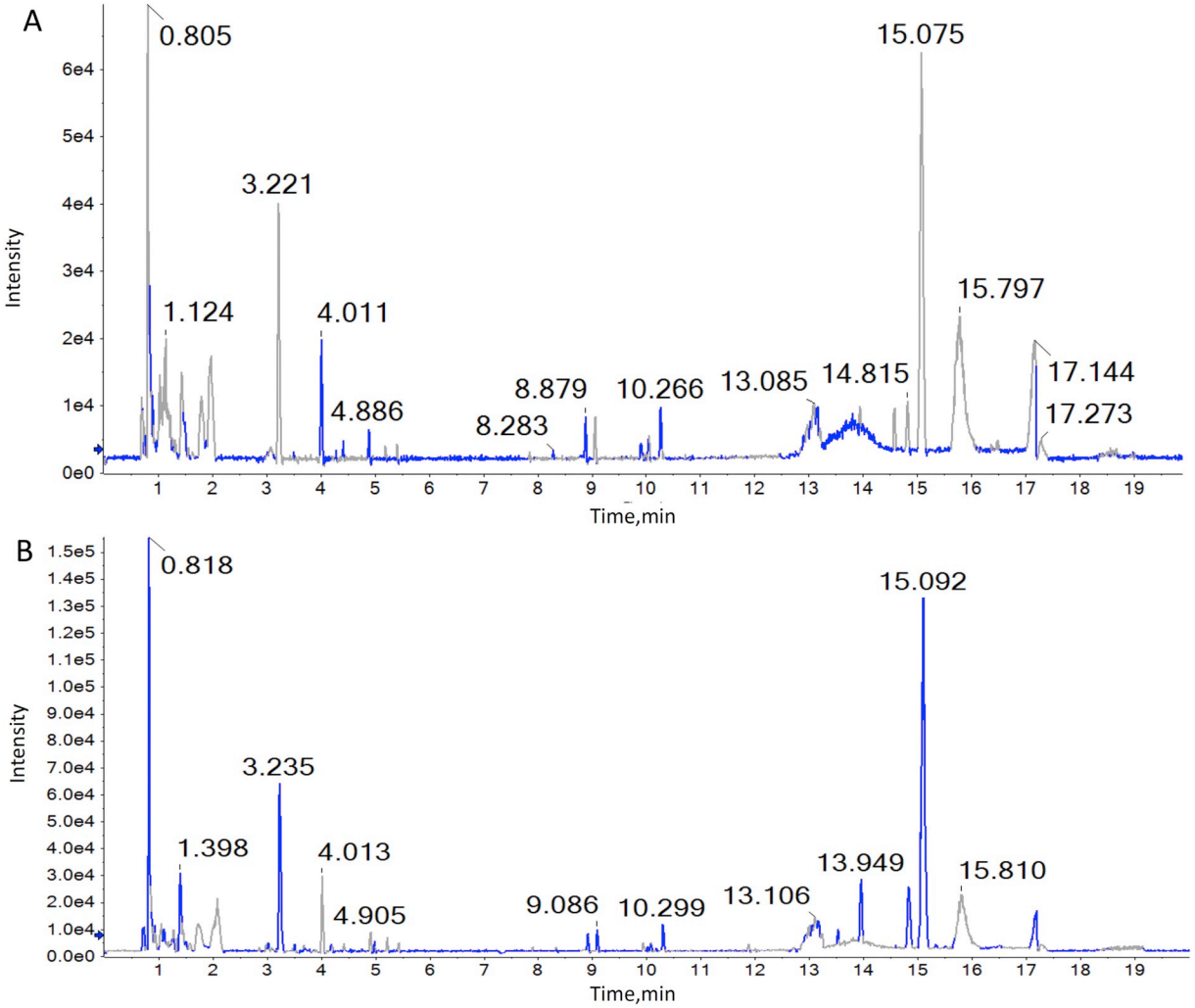

Fig. 1. Metabolomics profiling of the culture supernatants from pre-treatment isolate (A) and post-treatment isolate (B).

to the score plots of the model, indicating that these isolates had significantly different metabolic characteristics (Fig. 2B). The parameters for assessing quality of PLS-DA model (R^2X = 21.3%, R^2Y = 93.2%, Q^2 = 84.4%) showed that the model is stable and reasonable.

Subsequently, the compounds contributing the most to the variation observed between the two isolates were identified based on their MS data. We then analyzed their potential as markers for explaining the variation between the highly EMB-resistant strain and the pre-treatment EMB-resistant strain for the purpose of elucidating the mechanisms related to increased EMB resistance. A total of 590 compound peaks were identified that could discriminate between the two isolates (VIP > 1 and p < 0.05).

3.4. Pathway analysis

Among the 590 compound peaks identified, 175 compounds were annotated by the KEGG PATHWAY Database. Using a p value cut-off of < 0.05 and an impact factor threshold of > 0, the metabolites most affected were those involved in amino sugar and nucleotide sugar metabolism, β-alanine metabolism, sulfur metabolism, and galactose metabolism, with impact factors of 0.38, 0.69, 0.10, and 0.50, respectively (Fig. 3). These altered compounds play important roles in their respective pathways, as exemplified by the pathway analysis of the metabolites participating in amino sugar and nucleotide sugar metabolism (Fig. 4).

4. Discussion

Because of the intense use and misuse of antibiotics, drug resistance is now a critical public health issue facing today's society. TB is ranked as one of the most difficult infections to treat. In 2016 there was an estimated 490,000 new cases of MDR-TB globally (World Health Organization, 2017). The wide spread of MTB is mainly attributable to its ability to persist in the face of the host immune system, and its capacity to develop resistance towards most available antimicrobial agents (Nguyen and Pieters, 2009). Therefore, besides efforts to develop new antibiotics, further studies are urgently needed to elucidate key factors involved in the novel resistance mechanisms of MTB.

Most of the acquired resistance of TB is mediated through chromosomal mutations arising from the selective pressure of antibiotic use. Accumulation of these genetic alternations results in the emergence of MTB strains that are antibiotic resistant. A series of genes involved in the acquired resistance of MTB to first-line and second-line anti-tuberculosis drugs have been reported; these include (but are not limited to) the drug target genes *inhA*, *rpoB*, *embCAB*, and *rpsL*, and genes associated with prodrug activity or activity modulation such as *katG*, *pncA*, and *ethA* (Smith et al., 2013). However, these mutations cannot explain the full repertoire of drug resistance observed. The current list of chromosomal mutations conferring drug resistance is far from comprehensive although recent multicenter genomics studies have significantly advanced knowledge in this direction (Farhat et al., 2016; Miotto et al., 2017; Zignol et al., 2018).

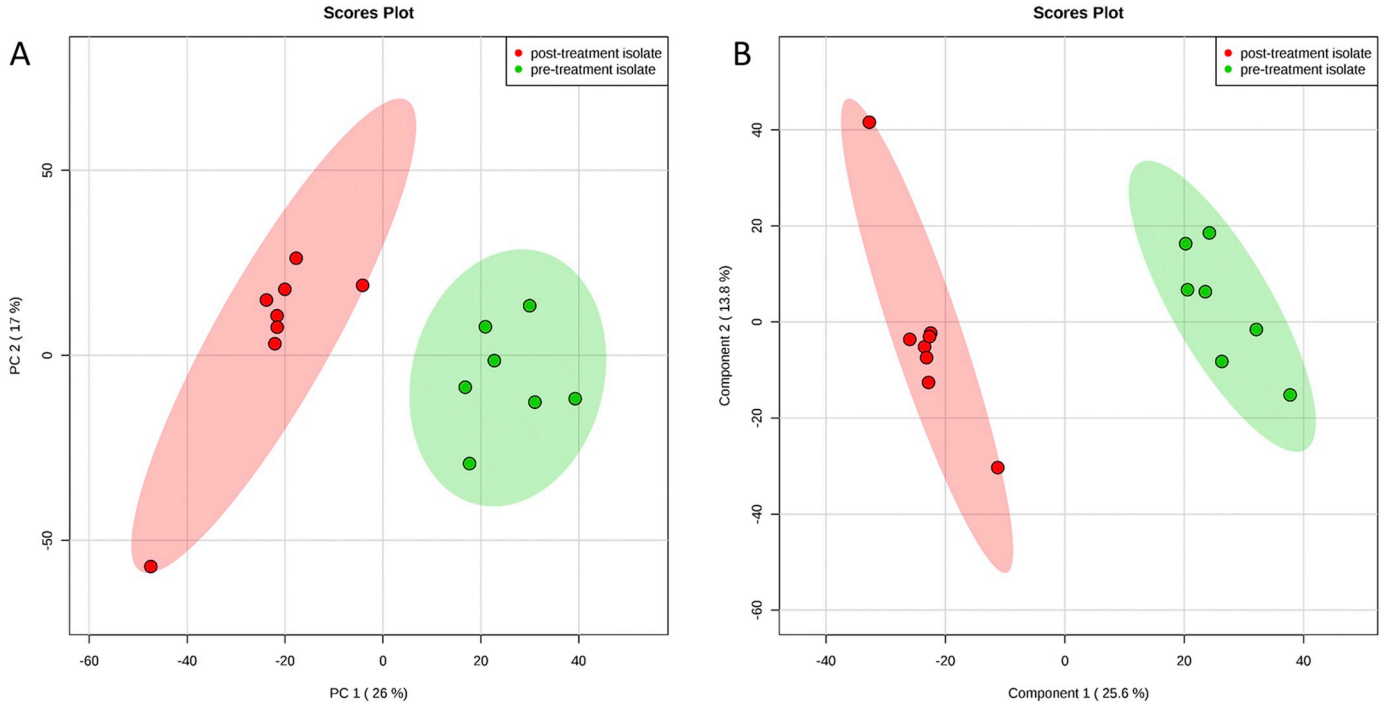

Fig. 2. Results of multivariate statistical analysis for the two MTB isolates. (A) PCA score plots; (B) PLS-DA score plots. The analysis was limited to the first two principal components (PC). The vertical axis shows PC1 and the horizontal axis shows PC2. The numbers in parentheses on the axis labels indicate the contribution rate. The number of plots represent the biological replicates of each culture.

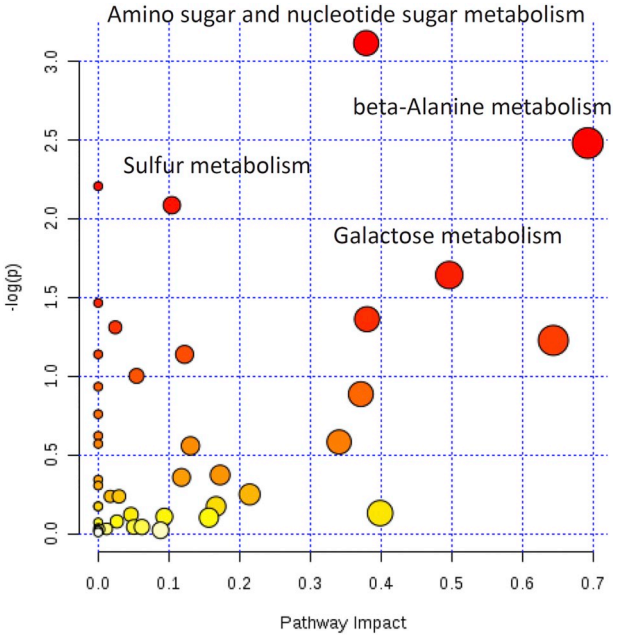

Fig. 3. Impact factors of pathways calculated by Metabolite set enrichment analysis. Powerful pathway enrichment was performed in R package on log-transformed data. The major metabolic pathways in which the altered metabolites between pre-treat and post-treat strains enriched were shown based on the *p* value and impact factor.

While most of the recent studies focused on the comparative genomics of drug-sensitive and drug-resistant strains, only few studies have paid close attention to the evolution of drug resistance within the context of underlying mechanisms following an *in vivo* challenge with anti-TB drugs. In the present study, MDR isolates were collected before treatment and after 12 months of treatment from the same patient, and the post-treatment strain was shown to exhibit an elevated level of EMB resistance. Sanger sequencing found that the previously reported polymorphisms associated with drug resistance were the same for both isolates. WGS was therefore used to uncover any novel genetic alterations related to the evolution of antibiotic resistance, and a metabolomics approach was used to identify the corresponding changes in the metabolic pathways involved.

WGS is now used not only for understanding the transmission dynamics of an epidemic, but also to identify molecular evidence for strain-specific phenotypic variability such as the acquisition of anti-mycobacterial drug resistance. A series of studies using WGS have been performed to map genetic diversity among MTB isolates and identify the genomic signatures associated with drug resistance (Sharma et al., 2017; Ali et al., 2015). The evolution of antibiotic resistance is critical for the bacterium's survival, and it has been reported that micro-evolution events can lead to a higher than expected accumulation of variability (Pérez-Lago et al., 2014). However, the underlying mechanisms affected by these mutations still require in-depth investigation.

In the present study, we found 6 variant positions in the genes *Rv1026*, *Rv2155c*, *Rv2437*, *Rv3696c*, *nc0021*, and in the intergenic region between *Rv2764c* and *Rv2765* in pre- and post-treatment isolates. To investigate the potential genetic mechanisms involved in elevated EMB resistance, metabolomics methods were used to identify changes in the relevant metabolic pathways.

Inorganic polyphosphate (polyP) plays an essential role in microbial stress adaptation, virulence, and drug tolerance. Polyphosphatases encoded by *Rv0496* and *Rv1026* are critical in the maintenance of intracellular polyP levels (Singh et al., 2016). Chuang et al. (2015) identified an MTB *Rv1026* knockdown strain that exhibited reduced susceptibility towards isoniazid and increased bacterial survival within macrophages. Metabolic downshifts were also observed in the *Rv1026* knockdown strain, including reductions in the level of glycerol 3-phosphate (G3P; involved in phospholipid biosynthesis (Kanehisa et al., 2006)), glucose phosphorylation (important during MTB chronic infection in mice (Marrero et al., 2013)), glucose metabolism, fatty acid synthesis, and nucleotide biogenesis.

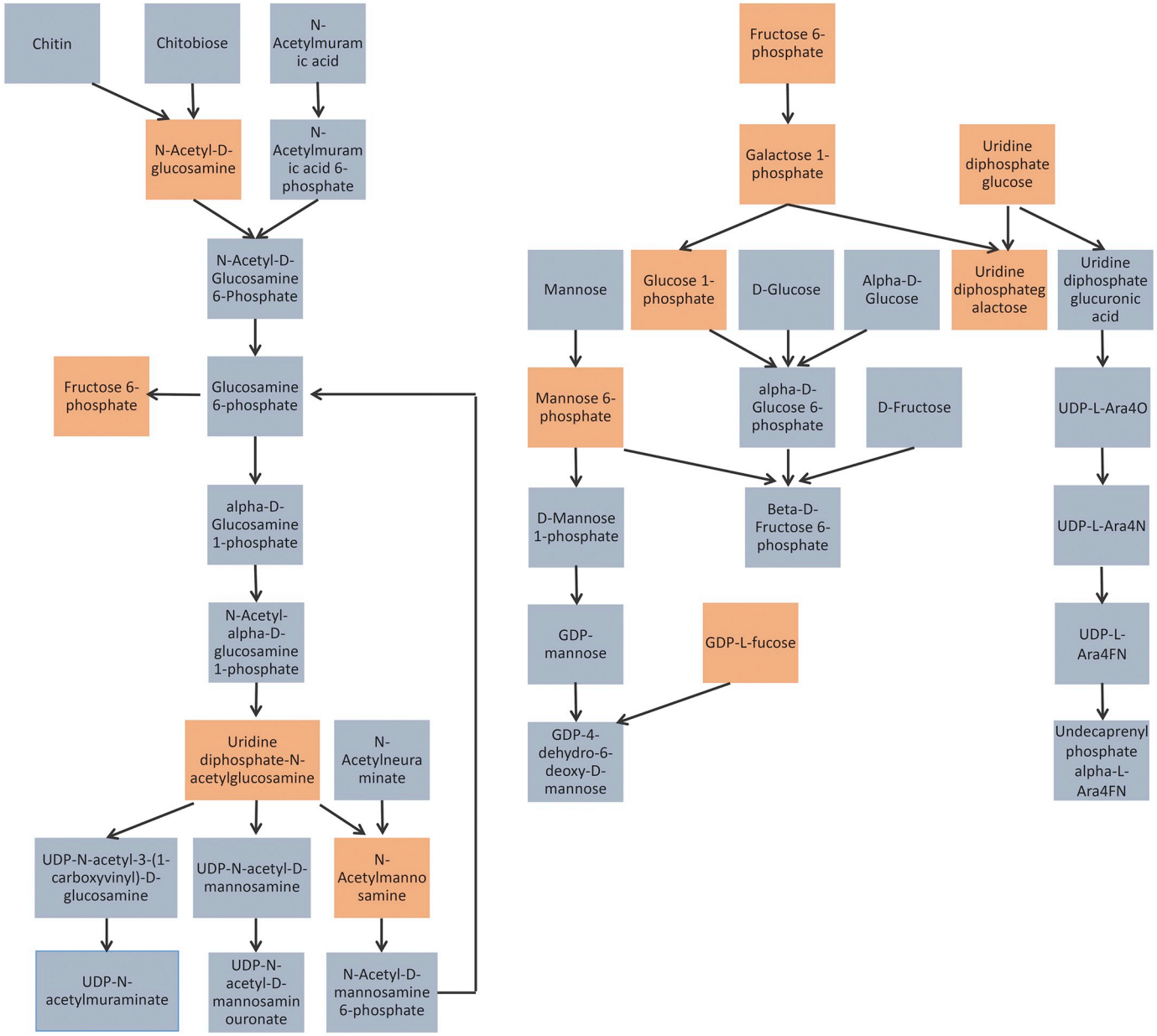

Fig. 4. The amino sugar and nucleotide sugar metabolism pathway of bacterium. The metabolites marked with red color were altered ones detected between the two isolates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Another two missense mutations were located in *Rv2155c* and *Rv3696c* respectively. MTB *murD* (*Rv2155c*) is located in a cluster near the cell division genes such as *ftsW*, *murX*. *murD* encodes UDP-*N*-acetylmuramoyl-L-alanine:Dglutamate-ligase, a ligase involved in the process of peptidoglycan biosynthesis (Thakur and Chakraborti, 2008). Through a metabolic pathway analysis, MurD ligase was considered as potential drug targets (Anishetty et al., 2005). *Rv3696c* of MTB encodes glycerol kinase, one of the important enzyme for the intake and metabolism of glycerin. Because of the degeneracy of the triplet codon, the substitution of G with C in *Rv2437* did not resulted in amino acid changes. *Nc0021* recodes Rvnc0021 which is considered as putative small regulatory RNA. This kind of RNAs have been recognized as a major class of gene regulators in bacteria. The studies that focused on the function of *nc0021* are few.

In this study, we detected one variation in the intergenic region between *thyA* (*Rv2764c*) and *Rv2765*, and it is predicted to lie within the promoter of *thyA*. Based on previous studies, *thyA* may be important gene associated with bacterial resistance. Mutations in this exon of the gene, which encodes a thymidylate synthase, accounts for 36% of para-aminosalicylic acid (PAS)-resistant MTB strains (Zhang et al., 2007). The *thyA* mutations are useful markers for predicting PAS treatment outcome (Leung et al., 2010). In the present study, the patient received treatment including PAS-INH for one month until the drug susceptibility testing results were received. Consequently, PAS was withdrawn and the conventional treatment was used. Whether the mutation was induced by PAS needs further analysis. Altogether whether this promoter variant position is associated with EMB resistance is not clear and further mechanism studies are needed.

Alterations in the MTB metabolome induced by genetic mutations has previously been demonstrated. For example, *katG* codons 315 and 321 mutations affect the intake and utilization of alkanes and fatty acids and the metabolites synthesized which are directly involved in reducing oxidative stress (Loots, 2014), while *rpoB* mutations may trigger the phthiocerol dimycocerosate biosynthetic pathway (Bisson et al., 2012). To our knowledge, this is the first study to explore the EMB resistance mechanism using metabolic method. In the present

study, the metabolites most affected were those involved in amino sugar and nucleotide sugar metabolism, β-alanine metabolism, sulfur metabolism, and galactose metabolism. According to the previous reported studies (Loots, 2014; Bisson et al., 2012), the changes of metabolites induced by the mutations of resistance gene were different and may differ in case of different anti-TB drugs.

Considering the metabolomics data we collected in the present study, it is evident that the elevated resistance towards EMB during *in vivo* challenge with anti-TB drugs is associated with the metabolic pathway of amino sugar and nucleotide sugar metabolism, β-alanine metabolism, sulfur metabolism, and galactose metabolism. However, the specific functional significance of the genetic variation detected in this study remains unclear based on the present data. Previous studies have found some evidence of gene functions in bacterial drug resistance and metabolic changes. One study focusing on the mechanisms of resistance and virulence of *Staphylococcus aureus* revealed that changes in amino sugar and nucleotide sugar metabolism present one of the putative modes of increased virulence (Subramanian and Natarajan, 2015). The enzymes involved in these processes are also often used as precursor targets for designing novel drugs against bacteria such as *Legionella pneumophila* (Hasan et al., 2014). However, how these metabolic changes are triggered by treatment with anti-TB drugs is unknown, and the underlying mechanisms remain to be further explored; such studies will be critical in the improved diagnosis and treatment of this globally important disease.

In conclusion, a combination of WGS and metabolomics was applied to study two sequential isolates from the some patient with EMB resistance level increased during *in vivo* challenge (treatment with anti-TB drugs). As a result, variant alleles were identified in the genes *Rv1026*, *Rv2155c*, *Rv2437*, *Rv3696c*, and *nc0021*, and *Rv2764c-Rv2765* intergenic region and distinct metabolite profiles for found for the two isolates. The increased EMB resistance exhibited by the post-treatment MDR-TB isolate can speculatively be linked to these variations, which affected the synthesis of a number of metabolites associated with sources of carbon and energy. This may have been the main factor underlying the increased resistance of this isolate towards ethambutol. Metabolomics investigations are only the preliminary step in the elucidation of the functional effect of these novel mutations and further studies are required.

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Competing interests

None declared.

Ethical approval

This study was approved by the Health Research Ethics Committee of Beijing Chest Hospital, Capital Medical University [decision # 2014-36-1].

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References

Ali, A., Hasan, Z., McNerney, R., Mallard, K., Hill-Cawthorne, G., Coll, F., Nair, M., Pain, A., Clark, T.G., Hasan, R., 2015. Whole genome sequencing based characterization of extensively drug-resistant *Mycobacterium tuberculosis* isolates from Pakistan. PLoS One 10, e0117771.

Anishetty, S., Pulimi, M., Pennathur, G., 2005. Potential drug targets in *Mycobacterium tuberculosis* through metabolic pathway analysis. Comput. Biol. Chem. 29, 368–378.

Bisson, G.P., Mehaffy, C., Broeckling, C., Prenni, J., Rifat, D., Lun, D.S., Burgos, M., Weissman, D., Karakousis, P.C., Dobos, K., 2012. Upregulation of the phthiocerol dimycocerosate biosynthetic pathway by rifampin-resistant, rpoB mutant *Mycobacterium tuberculosis*. J. Bacteriol. 194, 6441–6452.

Chuang, Y.M., Bandyopadhyay, N., Rifat, D., Rubin, H., Bader, J.S., Karakousis, P.C., 2015. Deficiency of the novel exopolyphosphatase Rv1026/PPX2 leads to metabolic downshift and altered cell wall permeability in *Mycobacterium tuberculosis*. MBio 6, e02428.

Coll, F., McNerney, R., Guerra-Assunção, J.A., Glynn, J.R., Perdigão, J., Viveiros, M., Portugal, I., Pain, A., Martin, N., Clark, T.G., 2014. A robust SNP barcode for typing *Mycobacterium tuberculosis* complex strains. Nat. Commun. 5, 4812.

Cuevas-Córdoba, B., Juárez-Eusebio, D.M., Almaraz-Velasco, R., Muñiz-Salazar, R., Laniado-Laborin, R., Zenteno-Cuevas, R., 2015. Mutation at embB codon 306, a potential marker for the identification of multidrug resistance associated with ethambutol in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 59, 5455–5462.

van Embden, J.D., Cave, M.D., Crawford, J.T., Dale, J.W., Eisenach, K.D., Gicquel, B., Hermans, P., Martin, C., McAdam, R., Shinnick, T.M., Small, P., 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. 31 (2), 406–409 (Feb.).

Farhat, M.R., Sultana, R., Iartchouk, O., Bozeman, S., Galagan, J., Sisk, P., Stolte, C., Nebenzahl-Guimaraes, H., Jacobson, K., Sloutsky, A., Kaur, D., Posey, J., Kreiswirth, B.N., Kurepina, N., Rigouts, L., Streicher, E.M., Victor, T.C., Warren, R.M., van Soolingen, D., Murray, M., 2016. Genetic determinants of drug resistance in *Mycobacterium tuberculosis* and their diagnostic value. Am. J. Respir. Crit. Care Med. 194 (5), 621–630.

Franzblau, S.G., Witzig, R.S., McLaughlin, J.C., Torres, P., Madico, G., Hernandez, A., Degnan, M.T., Cook, M.B., Quenzer, V.K., Ferguson, R.M., Gilman, R.H., 1998. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J. Clin. Microbiol. 36, 362–366.

Hasan, A., Mazumder, H.H., Khan, A., Hossain, M.U., Chowdhury, H.K., 2014. Molecular characterization of legionellosis drug target candidate enzyme phosphoglucosamine mutase from legionella pneumophila (strain Paris): an in silico approach. Genomics Inform. 12, 268–275.

Hazbón, M.H., Bobadilla Del Valle, M., Guerrero, M.I., Varma-Basil, M., Filliol, I., Cavatore, M., Colangeli, R., Safi, H., Billman-Jacobe, H., Lavender, C., Fyfe, J., García-García, L., Davidow, A., Brimacombe, M., León, C.I., Porras, T., Bose, M., Chaves, F., Eisenach, K.D., Sifuentes-Osornio, J., Ponce De León, A., Cave, M.D., Alland, D., 2005. Role of embB codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. Antimicrob. Agents Chemother. 49 (9), 3794–3802.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K.F., Itoh, M., Kawashima, S., Katayama, T., Araki, M., Hirakawa, M., 2006. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. 34, D354–D357.

Lau, S.K., Lam, C.W., Curreem, S.O., Lee, K.C., Lau, C.C., Chow, W.N., Ngan, A.H., To, K.K., Chan, J.F., Hung, I.F., Yam, W.C., Yuen, K.Y., Woo, P.C., 2015. Identification of specific metabolites in culture supernatant of *Mycobacterium tuberculosis* using metabolomics: exploration of potential biomarkers. Emerg. Microbes Infect. 4, e6.

Leung, K.L., Yip, C.W., Yeung, Y.L., Wong, K.L., Chan, W.Y., Chan, M.Y., Kam, K.M., 2010. Usefulness of resistant gene markers for predicting treatment outcome on second-line anti-tuberculosis drugs. J. Appl. Microbiol. 109, 2087–2094.

Loots, du T., 2014. An altered *Mycobacterium tuberculosis* metabolome induced by katG mutations resulting in isoniazid resistance. Antimicrob. Agents Chemother. 58, 2144–2149.

Marrero, J., Trujillo, C., Rhee, K.Y., Ehrt, S., 2013. Glucose phosphorylation is required for *Mycobacterium tuberculosis* persistence in mice. PLoS Pathog. 9, e1003116.

Mikusova, K., Slayden, R.A., Besra, G.S., Brennan, P.J., 1995. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob. Agents Chemother. 39, 2484–2489.

Miotto, P., Tessema, B., Tagliani, E., Chindelevitch, L., Starks, A.M., Emerson, C., Hanna, D., Kim, P.S., Liwski, R., Zignol, M., Gilpin, C., Niemann, S., Denkinger, C.M., Fleming, J., Warren, R.M., Crook, D., Posey, J., Gagneux, S., Hoffner, S., Rodrigues, C., Comas, I., Engelthaler, D.M., Murray, M., Alland, D., Rigouts, L., Lange, C., Dheda, K., Hasan, R., Ranganathan, U.D.K., McNerney, R., Ezewudo, M., Cirillo, D.M., Schito, M., Köser, C.U., Rodwell, T.C., 2017. A standardised method for interpreting the association between mutations and phenotypic drug resistance in *Mycobacterium tuberculosis*. Eur. Respir. J. 50 (6) (pii: 1701354).

Mokrousov, I., Otten, T., Vyshnevskiy, B., Narvskaya, O., 2002. Detection of embB306 mutations in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotypic resistance testing. J. Clin. Microbiol. 40 (10), 3810–3813 (Oct.).

Nguyen, L., Pieters, J., 2009. Mycobacterial subversion of chemotherapeutic reagents and host defense tactics: challenges in tuberculosis drug development. Annu. Rev. Phamacol. Toxicol. 49, 427–453.

Nimmo, C., Doyle, R., Burgess, C., Williams, R., Gorton, R., McHugh, T.D., Brown, M., Morris-Jones, S., Booth, H., Breuer, J., 2017. Rapid identification of a *Mycobacterium tuberculosis* full genetic drug resistance profile through wholegenome sequencing directly from sputum. Int. J. Infect. Dis. 62, 44–46.

Olivier, I., Loots, D.T., 2011. An overview of tuberculosis treatments and diagnostics. What role could metabolomics play? J. Cell. Tissue. Res. 11, 2655–2671.

Perdigao, J., Macedo, R., Ribeiro, A., Brum, L., Portugal, I., 2009. Genetic characterisation of the ethambutol resistance-determining region in *Mycobacterium tuberculosis*:

prevalence and significance of embB306 mutations. Int. J. Antimicrob. Agents 33, 334–338.
Pérez-Lago, L., Comas, I., Navarro, Y., González-Candelas, F., Herranz, M., Bouza, E., García-De-Viedma, D., 2014. Whole genome sequencing analysis of intrapatient microevolution in *Mycobacterium tuberculosis*: potential impact on the inference of tuberculosis transmission. J. Infect. Dis. 209, 98–108.
Sharma, K., Verma, R., Advani, J., Chatterjee, O., Solanki, H.S., Sharma, A., Varma, S., Modi, M., Ray, P., Mukherjee, K.K., Sharma, M., Dhillion, M.S., Suar, M., Chatterjee, A., Pandey, A., Prasad, T.S.K., Gowda, H., 2017. Whole genome sequencing of *Mycobacterium tuberculosis* isolates from extrapulmonary sites. OMICS 21, 413–425.
Singh, M., Tiwari, P., Arora, G., Agarwal, S., Kidwai, S., Singh, R., 2016. Establishing virulence associated polyphosphate kinase 2 as a drug target for *Mycobacterium tuberculosis*. Sci. Rep. 6, 26900.
Smith, T., Wolff, K.A., Nguyen, L., 2013. Molecular biology of drug resistance in *Mycobacterium tuberculosis*. Curr. Top. Microbiol. Immunol. 374, 53–80.
Subramanian, D., Natarajan, J., 2015. Network analysis of S. aureus response to ramoplanin reveals modules for virulence factors and resistance mechanisms and characteristic novel genes. Gene 574, 149–162.
Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rüsch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C., van Soolingen, D., 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 44, 4498–4510.
Telenti, A., Imboden, P., Marchesi, F., Lowrie, D., Cole, S., Colston, M.J., Matter, L., Schopfer, K., Bodmer, T., 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet 341, 647–650.
Telenti, A., Philipp, W.J., Sreevatsan, S., Bernasconi, C., Stockbauer, K.E., Wieles, B., Musser, J.M., Jacobs Jr., W.R., 1997. The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nat. Med. 3, 567–570.
Thakur, M., Chakraborti, P.K., 2008. Ability of PknA, a mycobacterial eukaryotic-type serine/threonine kinase, to transphosphorylate MurD, a ligase involved in the process of peptidoglycan biosynthesis. Biochem. J. 415, 27–33.
Tufariello, J.M., Jacobs Jr., W.R., Chan, J., 2004. Individual *Mycobacterium tuberculosis* resuscitation-promoting factor homologues are dispensable for growth in vitro and in vivo. Infect. Immun. 72, 515–526.
Vilchèze, C., Jacobs Jr., W.R., 2014. Resistance to isoniazid and ethionamide in *Mycobacterium tuberculosis*: genes, mutations, and causalities. Microbiol. Spectr. 2 (MGM2-0014-2013).
World Health Organization, 2017. Global Tuberculosis Control. WHO Report, Geneva, Switzerland.
Zhang, Z.D., Zhao, Y.L., Li, Z.H., Jia, H.Y., Liu, Y.H., Chen, X., Liu, Z.Q., Du, B.P., Xing, A.Y., Ma, Y., 2007. Mutations in the thymidylate synthase gene is a major mechanism in the para-aminosalicylic acid resistance of *M. tuberculosis*. Zhonghua Jie He He Hu Xi Za Zhi. 30, 683–685.
Zignol, M., Cabibbe, A.M., Dean, A.S., Glaziou, P., Alikhanova, N., Ama, C., Andres, S., Barbova, A., Borbe-Reyes, A., Chin, D.P., Cirillo, D.M., Colvin, C., Dadu, A., Dreyer, A., Driesen, M., Gilpin, C., Hasan, R., Hasan, Z., Hoffner, S., Hussain, A., Ismail, N., Kamal, S.M.M., Khanzada, F.M., Kimerling, M., Kohl, T.A., Mansjö, M., Miotto, P., Mukadi, Y.D., Mvusi, L., Niemann, S., Omar, S.V., Rigouts, L., Schito, M., Sela, I., Seyfaddinova, M., G5, Skenders, A, Skrahina, Tahseen, S., Wells, W.A., Zhurilo, A., Weyer, K., Floyd, K., Raviglione, M.C., 2018. Genetic sequencing for surveillance of drug resistance in tuberculosis in highly endemic countries: a multi-country population-based surveillance study. Lancet Infect. Dis. 18 (6), 675–683.