

CHAPTER 4

The effect of rifampicin-resistance conferring mutations on the fatty acid metabolome of *M. tuberculosis*

A part of this chapter has been submitted for publication to *OMICS: A journal of integrative biology*

OLIVIER, I., LOOTS, D.T. Altered fatty acid metabolism due to rifampicin-resistance conferring mutations in the *rpoB* gene of *M. tuberculosis*. Manuscript nr. OMI-2012-0028.

1. INTRODUCTION

More than 9 million new TB cases are recorded annually, of which 0.4 - 0.5 million are multi-drug resistant (MDR) (WHO, 2010). MDR-TB is defined as resistance to at least rifampicin and isoniazid, the two most important drugs currently used to treat *M. tuberculosis* infection. As almost 90% of rifampicin-resistant strains are also resistant to isoniazid, resistance to rifampicin is used as a surrogate marker for detecting MDR-TB (Aziz *et al.*, 2006).

Rifampicin functions by inhibiting RNA-polymerase, an enzyme essential for the transcription of bacterial DNA to RNA (Wehrli, 1983). Resistance to rifampicin is primarily caused by an alteration in the β -subunit of RNA-polymerase, owing to mutations in the coding *rpoB* gene. Various specific resistance-conferring mutations (accounting for over 95% of the rifampicin-resistant strains), occur in an 81-bp region of *rpoB*, known as the rifampicin-resistance determining region (RRDR) (Ahmad *et al.*, 2000; Anthony *et al.*, 2005). The majority of these are point mutations, resulting in a replacement of aromatic with non-aromatic amino acids. These replacements lead to drug resistance by interrupting the forces that bind rifampicin to RNA polymerase, but do, however, also initially impair the fitness of these bacteria, which may be restored by secondary mutations (Gagneux *et al.*, 2006; Mariam *et al.*, 2004). Since the *rpoB* gene codes for the β -subunit of RNA-polymerase, mutations in this gene are thought to lead in disruptions to many parts of the bacterial metabolism (Bergval *et al.*, 2007), which to date have not yet been elucidated.

Genomic, transcriptomic, and proteomic changes eventually result in changes in the metabolites (small-molecular weight compounds), and consequently, the study of these metabolites may provide information on a higher level of integration (Van Ravenzwaay *et al.*, 2007). As metabolomics reflects changes in gene expression (due to, for example, mutations), it is considered an important functional genomics tool, which, when interpreted with information generated from the other “omics” techniques, gives a more holistic description of the expression profile. Fiehn *et al.* (2000) successfully used metabolomics as a tool for functional genomics by comparing four *Arabidopsis* genotypes, including two homozygous ecotypes and a mutant of each ecotype. A clustering of the individual samples into each of the respective genotype groups was observed when applying PCA. This grouping was ascribed to the distinct variations in the metabolic profiles of the individual samples in the respective groups. Since then, other research groups have also identified variances in the metabolite profiles of a variety of bacterial species, due to genetic perturbations (Baran *et al.*, 2009). Proving the capacity of metabolomics as a tool to investigate previously unknown mechanisms of the mycobacterial metabolome, in a lipidomics study, Jain *et al.* (2007), indicated that *M. tuberculosis* undergoes a metabolism shift

to preferentially use host lipids as a carbon source during infection, consequently increasing their virulence associated lipid anabolism. Similarly, using an untargeted metabolic profiling approach, de Carvalho *et al.* (2010) successfully proved that *M. tuberculosis* can simultaneously catabolise multiple carbon sources.

Considering this, we applied the metabolomics approach developed in Chapter 3, in order to differentiate and potentially better characterise two rifampicin-resistant mutants, by comparing their fatty acid metabolite profiles to that of a fully drug-sensitive *M. tuberculosis* parent strain. This was done for the purpose of identifying new metabolite markers, in order to potentially better explain the altered metabolism induced by these rifampicin-resistance conferring mutations. The two rifampicin-resistant strains selected are the previously described *rpoB* S531L mutant, which is present in well over 50% of all clinical isolates, and the *rpoB* S522L mutant, which is a well known laboratory isolate (Gagneux *et al.*, 2006).

Aim: To apply the GC-MS fatty acid metabolomics approach developed in Chapter 3 to metabolite marker identification for better characterising two rifampicin-resistance conferring *rpoB* mutations in *M. tuberculosis*.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

KOH, nonadecanoic acid (C19:0) and glacial acetic acid were obtained from Merck. Chloroform, methanol and hexane were ultra-purity Burdick & Jackson brands (Honeywell International Inc.).

2.2 Bacterial cultures

All the organisms used in this study were obtained from the Royal Tropical Institute (KIT), Amsterdam, The Netherlands. The rifampicin-resistant *M. tuberculosis* strains used are spontaneous mutants derived from the wild-type parent strain (MTB72, ATCC35801), and were picked after one round of selection with rifampicin. For this selection, bacteria were cultured in Middlebrook 7H9 (Difco) broth for 14 days in a shaking incubator at 37°C. Then, 0.5 mL of broth was plated out onto Middlebrook 7H11 (Difco) media containing rifampicin (8 µg mL⁻¹), and selected colonies were once again streaked on rifampicin containing media (8 µg mL⁻¹) to confirm the resistant phenotype (Anthony *et al.*, 2005). Mutations in the *rpoB* gene were identified by sequencing a 271-bp region containing the 81-bp RRDR hotspot and if no mutation was detected in this region, an additional 365-bp region at the N-terminus was sequenced

(Anthony *et al.*, 2005). The mutations in the two rifampicin-resistant strains used in this study were identified as S522L and S531L. Twelve repeats of the two rifampicin resistant and wild-type parent strains were then separately cultured in Middlebrook 7H9 supplemented with oleic acid-albumin-dextrose-catalase, in a shaking incubator at 37°C. For each of these samples, a liquid starting culture was made by inoculating pure colonies from slopes into 10 mL culture medium until these cultures reached the late logarithmic growth phase. Prior to extraction, bacteria were isolated from each separate culture for each of the 3 strains, and re-suspended in ddH₂O at a concentration of 1×10^8 bacteria mL⁻¹.

2.3 Extraction procedure

After the addition of 100 µL of C19:0 (14 µg mL⁻¹) as internal standard to 1 mL of the above mentioned separately cultured and isolated *M. tuberculosis* cell suspensions (n = 12) for each of the three strains, samples were extraction using the modified Bligh-Dyer fatty acid extraction procedure described in Chapter 3 section 2.3.

2.4 GC-MS analysis and data processing

Chromatographic analyses, peak deconvolution and alignment, was done as described in Chapter 3 sections 2.4 and 2.5. Briefly, the raw GC-MS data were deconvoluted and analysed using AMDIS software (V2.65). Alignment of the detected compounds across the samples analysed was achieved by creating a new reference library in AMDIS, which contained the mass spectra of all the compounds detected above a threshold of 0.01% of the total signal, for all the samples analysed. Each analysed sample was subsequently processed using the aforementioned reference library, and the resulting output of each sample was combined into a data matrix containing the relative concentrations (normalised with the internal standard) for all compounds present or absent in each sample.

2.5 Statistical data analysis

The statistical packages, “R” (version 2.13.0) and Statistica (version 10) were used for all the statistical data analyses. Data were normalised relative to the internal standard in order to compensate for any potential sample loss during the extraction or chromatographic injection. To prevent variables with high concentrations from dominating the multivariate statistical analyses, data pre-treatment using a non-parametric transformation function (Koekemoer & Swanepoel, 2008) was used to scale the data prior to statistical data analyses, after which mean centering was applied.

As described by Madsen *et al.*, (2010), PCA was applied in order to determine whether or not a natural grouping (differentiation) exists between the investigated sample groups on the basis of the extracted metabolite profiles. PLS-DA was used to identify those metabolites which best characterise the differentiated sample groups by ranking the metabolites according to the VIP parameter, as described in Chapter 3, section 2.6. Using the mass fragmentation patterns generated by the MS, together with their respective GC retention times, the identities of these metabolite markers were determined using libraries generated from previously injected standards.

3. RESULTS

3.1 Differentiation between wild-type and rifampicin-resistant strains

Prior to multivariate statistical data analyses, a 50% filter was applied to the data, excluding those compounds which do not appear in at least 50% of the samples, in one or more of the sample groups. This filter, together with the removal of compounds with no detected variation between the groups, led to total number of 87 compounds. Using these 87 variables, a clear differentiation of all 3 sample groups was achieved when using the first three PCs of the PCA (Figure 4.1). The total amount of variance explained by the first three PCs (R^2X cum) was 65.37%, of which PC 1 explained 29.66%, PC 2 explained 16.38%, and PC 3 explained 10.33%.

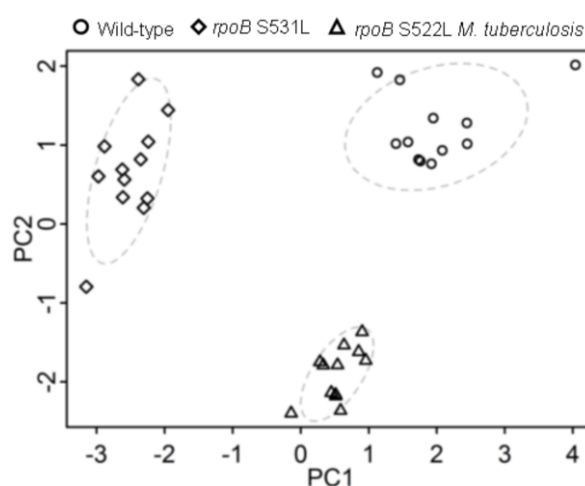


Figure 4.1: PCA scores plot showing PC 1 vs. PC 2 of the *M. tuberculosis* wild-type parent strain and the two rifampicin-resistant conferring *rpoB* mutants (twelve individually cultured sample isolates for each strain), subsequent to fatty acid extraction and GC-MS analyses, indicating a differentiation of the three *M. tuberculosis* strains.

3.2 Identification of potential biomarkers characteristic of specific rifampicin-resistant conferring *rpoB* mutations

The PLS-DA model used two components, with the modelling parameter R^2Y (cum) being 47.6%, indicative of the total explained variation of the response Y . Q^2 (cum), the cross-validated variation explained by the response Y , was 92.1%.

The relative concentrations of the metabolites (normalised with the internal standard) with the highest modelling powers, i.e., those best explaining the variation in the data (as identified by PCA), in addition to those metabolites identified with the highest VIP values, i.e., those which vary the most between the three sample groups (as identified by PLS-DA), are listed in Table 4.1.

Table 4.1: Mean relative concentrations ($\mu\text{g mg}^{-1}$ sample) and rankings of metabolite markers, as identified by PCA and PLS-DA. Standard deviations are given in parenthesis.

PCA ranking	PLS-DA ranking	Compound	Wild-type <i>M. tuberculosis</i>	Rifampicin-resistant mutants	
				<i>rpoB</i> S522L	<i>rpoB</i> S531L
			Mean relative concentration (SD)		
1	1	C17:0	60.3 (5.4)	42 (2.7)	T
2	9	10Me-C16:0	6 (0.6)	0	0
3	2	Unknown 422	48.9 (3)	5.1 (0.6)	22.8 (3)
4	8	Unknown 394	6.6 (0.6)	0	5.7 (0.9)
5		10Me-C15:0	1.2 (0.09)	0	0
6	11	Hexacosane	40.8 (59.7)	24.6 (1.8)	19.5 (2.7)
7		C22:0	3.6 (0.27)	3 (0.6)	7.2 (1.2)
8		Tetradecane	11.7 (12.3)	8.4 (12.6)	5.1 (8.7)
9		Heptacosane	33.3 (44.1)	21.6 (1.8)	18.3 (2.7)
10	10	C20:0	28.2 (1.2)	20.7 (0.6)	30.3 (2.4)
11	12	C24:0	9.9 (1.5)	8.4 (1.2)	16.2 (1.8)
12		Tricosane	13.8 (1.8)	6 (0.6)	5.1 (1.2)
13		C18:2n6c	3.3 (0.9)	5.7 (0.6)	6.3 (3.3)
14		C14:0	24.6 (2.7)	23.7 (4.5)	29.4 (3.6)
15		Unknown 459	2.7 (0.3)	3 (0.3)	4.8 (0.9)
	3	C6:0 2-ethyl-, hexadecyl ester	1.8 (4.2)	0.9 (0.6)	18.9 (13.2)
	4	Butyl phthalate	22.5 (37.2)	4.5 (1.5)	28.5 (53.7)
	5	TBSA	201.3 (28.5)	262.5 (26.1)	293.7 (45.6)
	6	C16:0	582 (42)	768 (34.2)	762 (65.7)
	7	C26:0	36.3 (9.6)	27 (2.4)	43.8 (5.1)
	13	Pentacosane	34.5 (55.8)	18.9 (1.2)	15.6 (2.7)
	14	BPA	53.4 (6.3)	46.2 (8.7)	60 (9)
	15	C16:1 ω7c	42.9 (7.8)	52.2 (3)	43.5 (6)

BPA, benzenepropanoic acid. Me, methyl. T, trace amounts.

When considering the results in Table 4.1, it is clear that the differentiation of the wild-type and rifampicin-resistant strains are dependant not only on the occurrence of those metabolite makers which are novel to a particular sample group, but also those considered important due to constant concentration differences between two or more of these sample groups. Two novel branched-chain fatty acids (10-methyl-pentadecanoic acid (10Me-C15:0) and 10-methyl-hexadecanoic acid (10Me-C16:0)) as well as an unknown fatty acid with a relative molecular mass of 503 (Unknown 503), were identified exclusively in the wild-type *M. tuberculosis* strain, and were totally absent in the *rpoB* mutants. Interestingly, another branched-chain fatty acid, the well known *Mycobacterium* biomarker, tuberculostearic acid (TBSA, 10-methyl-octadecanoic acid), was seen to be increased in both resistant strains, comparative to the wild-type strain. According to the metabolite rankings for both the PCA and PLS-DA, as indicated in Table 4.1, the compound considered most important for differentiating the investigated sample groups,

was heptadecanoic acid (C17:0). This compound was detected in reduced amounts in both the *rpoB* mutant *M. tuberculosis* strains (with only trace concentrations detected for the S531L *rpoB* mutant), as compared to the wild-type strain. A second straight-chain fatty acid, hexadecanoic acid (C16:0), on the other hand, was detected in elevated concentrations in both *rpoB* mutants. A general reduction in the mean relative concentrations of a number of alkanes was also observed in these rifampicin-resistant strains, slightly more so in the S531L mutant sample group.

4. DISCUSSION

Using the described metabolomics research approach, we were able to differentiate between two rifampicin-resistant *M. tuberculosis* *rpoB* mutants and the wild-type parent strain, and additionally identified, amongst others, a number of fatty acid metabolite markers characterising these rifampicin-resistant mutants. The fact that these sample groups are closely related and only differ by a single *in vitro* selection step, highlights the capacity of this metabolomics approach to detect minor metabolic changes induced by genetic alterations.

As previously mentioned, because the *rpoB* gene in *M. tuberculosis* encodes for the β -subunit of RNA-polymerase, a mutation in this gene may lead to disruptions in various parts of the bacteria's metabolism (Bergval *et al.*, 2007). In this study, we specifically investigated possible alterations to the fatty acid metabolome of these rifampicin-resistant conferring *rpoB* mutants. It should be noted that all conclusions made are based on hypotheses, which should be further investigated in future, using a number of targeted research approaches.

According to the "Kyoto Encyclopedia of Genes and Genomes database" and shown in the schematic summary of our results (Figure 4.2), the biosynthesis of fatty acids, and in particular mycolic acids in mycobacteria, involves at least two discrete types of enzyme systems, namely fatty acid synthase I and II (FAS I and FAS II) (Takayama *et al.*, 2005).

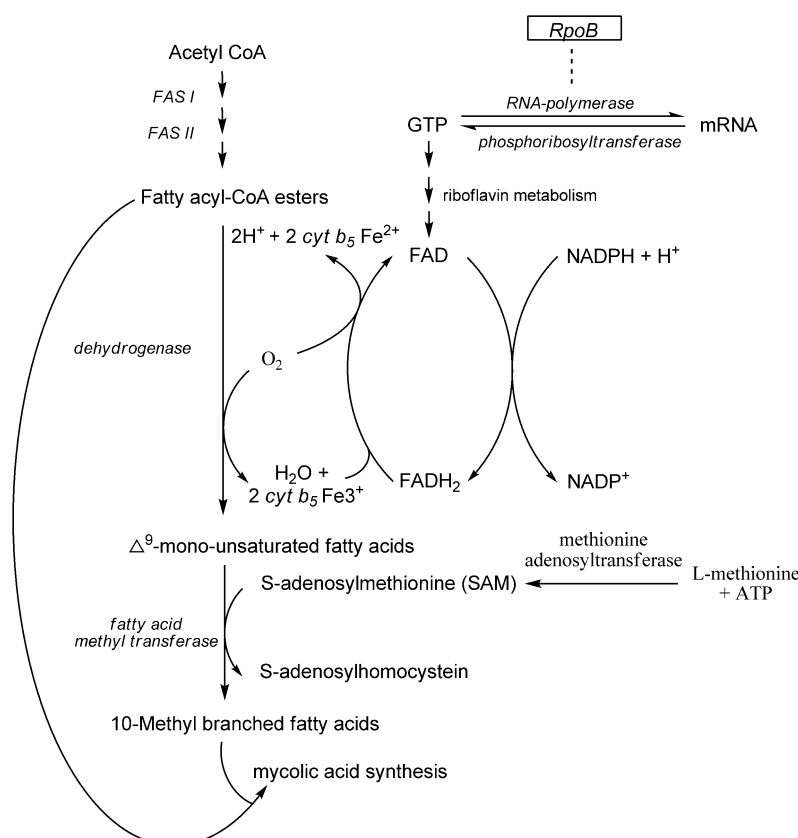


Figure 4.2: The role of the *rpoB* gene in the biosynthesis of 10-methyl-branched fatty acids in *M. tuberculosis*. FAS I and FAS II generate fatty acyl-CoA esters from acetyl-CoA, which are dehydrogenated to their corresponding Δ^9 -mono-unsaturated straight-chain fatty acids, and ultimately methylated to form 10-methyl branched fatty acids, with S-adenosylmethionine acting as the methyl donor. The dehydrogenation step requires Fe^{2+} , GTP derived FMN, NADPH and O_2 . The availability of GTP for this process is dependent on its equilibrium with mRNA, regulated by phosphoribosyltransferase and RNA polymerase (*rpoB* expression).

Mycolic acids are long-chain, high-molecular-weight, α -alkyl, β -hydroxyl fatty acids and are crucial cell wall components of *Mycobacterium* species. FAS I, a single polypeptide with multiple catalytic activities, mediates *de novo* synthesis of the intermediate-length, saturated α -chains of these mycolic acids and generates short chain CoA primers from acetyl-CoA (Takayama *et al.*, 2005). FAS II consists of a host of enzymes that synthesises the full-length meromycolate chain of these mycolic acids, by elongating the FAS I synthesised acyl-CoA substrates (Bhatt *et al.*, 2007; Cole *et al.*, 1998; Takayama *et al.*, 2005). Furthermore, saturated mid-chain, methyl-branched fatty acids are well known to occur in various *Mycobacterium* species, including *M. tuberculosis* (Cohen *et al.*, 2003; Chou *et al.*, 1998). In these species, these saturated mid-chain methyl-branched fatty acids are formed via the methylation of a delta-9-unsaturated fatty acid with S-adenosylmethionine (SAM) acting as the methyl donor. The resulting residue is then reduced to the 10-methyl compound, with NADPH acting as co-substrate for this reaction (Ramakrishnan *et al.*, 1972). The synthesis of these delta-9-unsaturated fatty acids takes place by the dehydrogenation of the corresponding saturated fatty

acid and it has been shown that, unlike other aerobic bacteria, mycobacteria require Fe^{2+} , and a flavin (FAD or FMN), in addition to NADPH and O_2 (Fulco & Bloch, 1974) for this reaction.

Furthermore, in prokaryotes, RNA polymerases use ribonucleoside 5'-triphosphates (NTPs including ATP, GTP, CTP, and UTP) as substrates to synthesise mRNA. The degradation of mRNA leads to a release of free nucleotide bases (adenine, guanine etc.), which may subsequently be used to re-synthesise NTPs via phosphoribosyltransferases (Garrett & Grisham, 2005), ensuring an mRNA - NTP equilibrium. Hence, it is not unlikely that a change in the configuration of RNA polymerase, as is the case in the *rpoB* mutants investigated in this study, may lead to a disturbance in this equilibrium. Furthermore, as GTP is a precursor of riboflavine (FAD and FMN) synthesis (KEGG, 2011), this disturbance may ultimately influence the mid-chain methyl-branched fatty acids (Figure 4.2), explaining the reduced amounts of 10-Me-C15:0 acid and 10-Me-C16:0 detected for the *rpoB* mutants used in this study.

Furthermore, SAM is synthesised by methionine adenosyltransferase, using both L-methionine and ATP as substrates (Gonzales *et al.*, 2003). Hence, a general decrease in NTPs, including ATP, as discussed, will result in a decrease in SAM, and consequently a slight elevation in the immediate upstream delta-9-unsaturated fatty acids, as detected in our study by the somewhat elevated concentrations of oleic acid (C18:1 ω 9c) and palmitoleic acid (C16:1 ω 7c) detected in the *rpoB* mutants. This, in turn, would also be expected to contribute to the previously mentioned reduction of the 10-methyl branched-chain fatty acids in these mutant *M. tuberculosis* strains.

It is also well known, that high amounts of ATP are required for mycolic acid synthesis (Shi *et al.*, 2010). It has furthermore been observed that the synthesis of cyclopropane-containing mycolates in *M. tuberculosis* is catalyzed by SAM-dependant enzymes (Barry *et al.*, 1998). Considering this and the above, the proposed reduction in ATP and resulting reduction in SAM, would also be expected to negatively influence mycolic acid synthesis in these *rpoB* mutants. Additionally, the FAS I and FAS II systems are directly linked by the activity of the enzyme β -ketoacyl-AcpM synthase III (coded by *fabH*), which catalyses the condensation of malonyl-AcpM and the FAS I acyl-CoA synthesised primers (Choi *et al.*, 2000). During conditions of stress, *fabH* is strongly down regulated, uncoupling the FAS I and FAS II systems, resulting in a state where the FAS II system only elongates the meromycolate chain of the existing mycolic acids, instead of using freshly synthesized FAS I primers (Shi *et al.*, 2010). Further confirmation of this was shown by Betts *et al.* (2002), who also indicated a down regulation of several *M. tuberculosis* genes involved in mycolic acid modification and transfer into the cell wall, under nutrient deprived conditions, serving as a stressor. Additionally, it was shown that a range of transcriptional changes can be induced in *M. tuberculosis* as a result of exposure to sub-lethal

antibiotic concentrations, and that these changes are under the control of various stress inducible systems (Morris *et al.*, 2005). Hence, an exposure to rifampicin, or a mutation in the *rpoB* gene, would be considered a stressor, potentially leading to a down regulation of mycolic acid synthesis.

The framework of the mycobacterial cell-wall, mycolylarabinogalactan peptidoglycan, consists of a mycolic acid complex embedded with lipoarabinomannan (LAM) (Chatterjee, 1997). Considering this, we suggest that the proposed down regulation of mycolic acid synthesis would in turn lead to a decrease of the synthesized LAM incorporated into this framework. This is further verified by the detection of elevated levels of C16:0 and TBSA, which are the major fatty acid components of LAM (Nigou *et al.*, 2003), for the *rpoB* mutants investigated in this study. Considering the markers identified in this metabolomics investigation in the light of the above explanations, it may be useful to investigate the influence of these *rpoB* mutations on the mycolic acid content of *M. tuberculosis*, through targeted mycolic acid analysis, at a later stage.

In addition to the down regulation of mycolic acid synthesis, stressors in *M. tuberculosis* are well known to up-regulate the glyoxylate shunt enzyme, isocitrate lyase (Icl), allowing these bacteria to survive on acetate or fatty acids as a primary carbon source (Glickman & Jacobs, 2001; Shi *et al.*, 2010). Furthermore, various stressors may also lead to transcriptional changes in *M. tuberculosis*, which are under the control of the stress inducible systems, resulting in a decreased growth rate (Morris *et al.*, 2005). As these *rpoB* mutations may be considered a stressor, and the fact that these *M. tuberculosis* strains are generally associated with a competitive fitness cost (Gagneux *et al.*, 2006), one would expect these mutants to reroute their carbon source from sugars to fatty acids. This rerouting consequently explains the pronounced reduction in the highest ranked biomarker, C17:0, and also contributes to the reduction in the 10-methyl branched-chain fatty acids detected. Additionally, the oxidation of these odd-chain and methyl-branched fatty acids is well known to produce propionyl-CoA and acetyl-CoA, which in turn are oxidised to pyruvate via the methylcitrate cycle (Muñoz-Elías *et al.*, 2006). This oxidation may explain the elevated concentrations of benzenepropionic acid, a propionic acid derivative, detected in the S531L mutants. Apart from these fatty acids, the use of alkanes as an alternative energy source by a variety of bacterial species is also well-known (Rojo, 2009), although, to our knowledge, this has not yet been proven for *M. tuberculosis*. However, should this be the case, it would explain the overall decrease in alkanes seen in the *M. tuberculosis* mutants in this study.

From Table 4.1 it is also clear that differences in the concentrations of a number of metabolite markers exists, when comparing the two *rpoB* mutant strains to one another. Gagneux *et al.* (2006) compared the competitive fitness of various clinical and laboratory generated rifampicin-

resistant *M. tuberculosis* strains by allowing each of the drug-resistant and the drug-susceptible organisms to compete with each other for limited resources, in identical environments. They determined that all the laboratory mutant strains tested had a significant fitness cost, and that this disadvantage was noticeably less in the S531L mutant strain. When tested on clinically derived mutants, no fitness cost was observed for the S531L strain, as opposed to considerable fitness defects detected for all other strains. According to our results, we hypothesise that the differences seen in the fitness costs of these strains are not necessarily because the specific S531L *rpoB* mutation has a less severe effect on the overall fitness of these bacteria, but due to the fact that this strain has other characteristics, allowing for a better comparative capacity to use alternative energy sources, such as fatty acids and alkanes. This hypothesis is supported by the fact that, although the concentrations of the detected compounds which serve as substrates for alternative energy pathways (specifically C17:0 and the alkanes) were reduced in both the *rpoB* mutants, this reduction was more pronounced in the S531L mutant strain comparative to that of the S522L mutant strain, indicating its capacity to better utilise these for energy. This would also explain the higher concentrations of the propionic acid derivative, benzenepropionic acid, detected in the S531L mutant strain comparatively, as this is a product of the β -oxidation of the odd-chain and methyl-branched fatty energy substrates.

Furthermore, Bergval *et al.* (2007) indicated that the expression of an inducible DNA polymerase (DnaE2) was seen to be increased in both the *rpoB* mutants used in our study. This increase for the S522L mutant was however, determined to be almost double that of the S531L mutant strain. As an increased expression in DnaE2 is related to DNA damage (Boshoff *et al.*, 2003), this perhaps suggests increased DNA damage to those genes required for β -oxidation, the glyoxylate shunt, and rerouting of the carbon source from sugars to fatty acids in the S522L mutants. The sequencing of a larger area of the genome of these rifampicin-resistant *rpoB* mutants, would therefore be another interesting topic to investigate.

5. CONCLUSION

Bergval *et al.* (2007) stated: “Mutations in a gene as critical for bacterial survival as *rpoB* most likely have effects on many areas of cellular function” and “perhaps all strains carrying mutations in *rpoB* have an altered expression of a wide range of genes”. Considering this, this study is the first of its kind to use metabolomics to indicate the effects of two *rpoB* mutations and the role of the β -subunit of RNA-polymerase, on the fatty acid metabolism of rifampicin-resistant *M. tuberculosis*. Furthermore, this study proves the capacity of a metabolomics research approach to identify previously unknown metabolite markers, never before associated with rifampicin-resistance. These markers could ultimately broaden our understanding of rifampicin-resistance and the role of the *rpoB* gene on certain aspects of the bacterial

metabolome, which may, in time, lead to a better characterisation of these *Mycobacterium* mutants, in a quest for improved diagnostics and treatments.

6. REFERENCES

- AHMAD, S., ARAJ, G.F., AKBAR, P.K., FARES, E., CHUGH, T.D., MUSTAFA, A.S. 2000. Characterization of *rpoB* mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from the Middle East. *Diagn. Microbiol. Infect. Dis.* 38:227–232.
- ANTHONY, R.M., SCHUITEMA, A.R.J., BERGVAL, I.L., BROWN, T.J., OSKAM, L., KLATSER, P.R. 2005. Acquisition of rifabutin resistance by a rifampicin resistant mutant of *Mycobacterium tuberculosis* involves an unusual spectrum of mutations and elevated frequency. *Ann. Clin. Microbiol. Antimicrob.* 4:9.
- AZIZ, M. A., WRIGHT, A., LASZLO, A. DE MUYNCK, A., PORTAELS, F., VAN DEUN, A., WELLS, C., NUNN, P., BLANC, L., RAVIGLIONE, M. 2006. Epidemiology of antituberculosis drug resistance (the Global Project on Anti-tuberculosis Drug Resistance Surveillance): an updated analysis. *Lancet.* 368:2142–54.
- BARAN, R., REINDL, W., NORTEN, T.R. 2009. Mass spectrometry based metabolomics and enzymatic assays for functional genomics. *Curr. Opin. Microbiol.* 12:547–552.
- BARRY, C.E., III, LEE, R.E., MDLULI, K., SAMPSON, A.E., SCHROEDER, B.G., SLAYDEN, R.A., YUAN, Y. 1998. Mycolic acids: structure, biosynthesis and physiological functions. *Prog. Lipid Res.* 37(2/3):43-179.
- BERGVAL, I.L., KLATSER P.R., SCHUITEMA, A.R.J., OSKAM, L., ANTHONY, R.M. 2007. Specific mutations in the *Mycobacterium tuberculosis rpoB* gene are associated with increased *dnaE2* expression. *FEMS Microbiol. Lett.* 275:338–343.
- BETTS, J.C., LUKEY, P.T., ROBB, L.C., MCADAM, R.A., DUNCAN, K. 2002. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* 43(3):717–731.
- BHATT, A., MOLLE, V., BESRA, G.S., JACOBS, W.R., JR, KREMER, L. 2007. The *Mycobacterium tuberculosis* FAS-II condensing enzymes: their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. *Mol. Microbiol.* 64:1442–1454.
- BOSHOF, H.I.M., REED, M.B., BARRY, C.E. III, MIZRAHI, V. 2003. DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. *Cell.* 113:183–193.
- CHATTERJEE, D. 1997. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. *Curr. Opin. Chem. Biol.* 1:579-568.
- CHOI, K.H., KREMER, L., BESRA, G.S., ROCK, C.O. 2000. Identification and substrate specificity of beta-ketoacyl (acyl carrier protein) synthase III (mtFabH) from *Mycobacterium tuberculosis*. *J. Biol. Chem.* 275: 28201–28207.

CHOU, S., CHEDORE, P., KASATIYA, S. 1998. Use of gas chromatographic fatty acid and mycolic acid cleavage product determination to differentiate among *Mycobacterium genavense*, *Mycobacterium fortuitum*, *Mycobacterium simiae*, and *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 36(2):577–579.

COHEN, T., SOMMERS, B., MURRAY, M. 2003. The effect of drug resistance on the fitness of *Mycobacterium tuberculosis*. *Lancet Infect. Dis.* 3: 13–21.

COLE, S. T., BROSCHE, R., PARKHILL, J., GARNIER, T. CHURCHER, C., HARRIS, D. GORDON, S. V., EIGLMEIER, K. GAS, S., BARRY III, C. E., TEKAIA, F., BADCOCK, K., BASHAM, D., BROWN, D., CHILLINGWORTH, T., CONNOR, R., DAVIES, R., DEVLIN, K., FELTWELL, T., GENTLES, S., HAMLIN, N., HOLROYD, S., HORNSBY, T., JAGELS, K., KROGH, A., MCLEAN, J., MOULE, S., MURPHY, L., OLIVER, K., OSBORNE, J., QUAIL, M. A., RAJANDREAM, M.-A., ROGERS, J., RUTTER, S., SEEGER, K., SKELTON, J., SQUARES, R., SQUARES, S., SULSTON, J. E., TAYLOR, K., WHITEHEAD S., BARRELL B. G. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature.* 393:537-544.

DE CARVALHO, L.P.S., FISCHER, S.M., MARRERO J., NATHAN, C., EHRT, S., RHEE, L.Y. 2010. Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chem. Biol.* 17(10):1122-1131.

FIEHN, O., KOPKA, J., DÖRMANN, P., ALTMANN, T., TRETHEWEY, R.N., WILLMITZER, L. 2000. Metabolite profiling for plant functional genomics. *Nature Biotechnol.* 18:1157-1161.

FULCO, A.J., BLOCH, K. 1964. Cofactor Requirements for the Formation of Δ^9 -Unsaturated Fatty Acids in *Mycobacterium phlei**. *J. Biol. Chem.* 239(4):993-997.

GAGNEUX, S., LONG, C.D., SMALL, P.M., VAN, T., SCHOOLNIK, G.K., BOHANNAN, B.J.M. 2006. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science.* 312:1944-1946.

GARRETT R.H., GRISHAM C.M. Biochemistry, third ed., Thomas Brooks/Cole. Belmont, CA. 2005. p. 860-862.

GLICKMAN, M.S., JACOBS, W.R. Jr. 2001. Microbial Pathogenesis of *Mycobacterium tuberculosis*: Dawn of a Discipline. *Cell*, 104: 477–485.

GONZÁLEZ, B., PAJARES, M.A., HERMOSO, J.A., GUILLERM, D., GUILLERM, G., SANZ-APARICIO, J. 2003. Crystal structures of methionine adenosyltransferase complexed with substrates and products reveal the methionine-ATP recognition and give insights into the catalytic mechanism. *J. Mol. Biol.* 331:407–416.

JAIN, M., PETZOLD, C.J., SCHELLE, M.W., LEAVELL, M.D., MOUGOUS, J.D., BERTOZZI, C.R., LEARY, J.A., COX, J.S. 2007. Lipidomics reveals control of *Mycobacterium tuberculosis* virulence lipids via metabolic coupling. *PNAS.* 104(12):5133–5138.

KEGG. Kyoto Encyclopedia of Genes and Genomes. 2011. <http://www.genome.jp/kegg/pathway>.

KOEKEMOER G, SWANEPOEL J.W.H. 2008. A semi-parametric method for transforming data to normality. *Stat Comput.* 18:241-257.

- MADSEN, R., LUNDSTEDT, T., TRYGG, J. 2010. Chemometrics in metabolomics - A review in human disease diagnosis. *Anal. Chim. Acta.* 659:23–33.
- MARIAM, D.H., MENGISTU, Y., HOFFNER, S.E., ANDERSSON, D.I. 2004. Effect of *rpoB* mutations Conferring Rifampin Resistance on Fitness of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 48(4):1289–1294.
- MORRIS, R.P., NGUYEN, L., GATFIELD, J., VISCONTI, K., NGUYEN, K., SCHNAPPINGER, D., EHRT, S., LIU, Y., HEIFETS, L., PIETERS, J., SCHOOLNIK, G., THOMPSON, C.J. 2005. Ancestral antibiotic resistance in *Mycobacterium tuberculosis*. *PNAS.* 102(34):12200–12205.
- MUÑOZ-ELÍAS, E.J., UPTON, A.M., CHERIAN, J., MCKINNEY, J.D. 2006. Role of the methylcitrate cycle in *Mycobacterium tuberculosis* metabolism, intracellular growth, and virulence. *Mol. Microbiol.* 60(5):1109–1122.
- NIGOU, J., GILLERON, M., PUZO, G. 2003. Lipoarabinomannans: from structure to biosynthesis. *Biochimie.* 85:153–166.
- RAMAKRISHNAN, T. MURTHY, P.S., GOPINATHAN, K.P. 1972. Intermediary metabolism of mycobacteria. *Bacteriol. Rev.* 36(1):65-108.
- ROJO F. Degradation of alkanes by bacteria. *Environ. Microbiol.* 2009;11:2477-2490.
- SHI, L., SOHASKEY, C.D., PFEIFFER, C., DATTA, P., PARKS, M., MCFADDEN, J., NORTH, R.J., GENNARO, M.L. 2010. Carbon flux rerouting during *Mycobacterium tuberculosis* growth arrest. *Mol. Microbiol.* 78(5):1199–1215.
- TAKAYAMA, K., WANG, C., BESRA, G.S. 2005. Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* 18(1):81–101.
- VAN RAVENZWAAY, B., COELHO-PALERMO CUNHA, G., LEIBOLD, E., LOOSER, R., MELLERT, W., PROKOUDINE, A., WALKB, T., WIEMER, J. 2007. The use of metabolomics for the discovery of new biomarkers of effect. *Toxicol. Lett.* 172:21–28.
- WEHRLI, W. 1983. Rifampin: Mechanisms of Action and Resistance. *Rev. Infect. Dis.* 5(3):S407-S411.
- WHO 2010. Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. Geneva, Switzerland.