Metabolite analysis of *Mycobacterium* species under aerobic and hypoxic conditions reveals common metabolic traits

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Abbreviations: ACAT, acyl-CoA acetyltransferase; BCG, Bacillus Calmette-Guerin; cfu, colony forming units; DGAT, diacylglycerol O-acyltransferase; ESAT, early secretory antigen target; GC, gas chromatography; LC, liquid chromatography; MK9, menaquinone with nine isoprenoid side chain residues; MS, mass spectrometry; Mtb, Mycobacterium tuberculosis; NRP, non-replicating persistence; OD, optical density; TAG, triacylglycerides; TCA, tricarboxylic acid; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

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Abstract (250 words)

A metabolite profiling approach has been implemented to elucidate metabolic adaptation at set culture conditions in five Mycobacterium species with the potential to act as model organisms for Mycobacterium tuberculosis (Mtb). Analysis has been performed over designated growth phases and under representative environments (nutrient and oxygen depletion) experienced by Mtb during infection. The procedure was able to determine a range of metabolites (60 - 120 compounds) covering nucleotides, amino acids, organic acids, saccharides, fatty acids, glycerols, -esters, -phosphates and isoprenoids. Among these classes of compounds key biomarker metabolites were identified that can act as indicators of pathway/process activity. In numerous cases, common metabolite traits were observed for all five species across the experimental conditions. Amino acid content, especially glutamic acid, highlighted the different properties between the fast- and slow-growing mycobacteria studied. The greatest similarities in metabolite composition between fast- and slow-growing mycobacteria were apparent under hypoxic conditions. A comparison to previously reported transcriptomic data revealed a strong correlation between changes in transcription and metabolite content. Collectively, these data validate the changes in the transcription at the metabolite level, suggesting transcription exists as one of the predominant modes of cellular regulation in Mycobacterium. Whilst, sectors with restricted correlation between metabolites and transcription (e.g. hypoxic cultivation) warrant further study to elucidate and exploit post-transcriptional modes of regulation. The strong correlation between the laboratory conditions used and data derived from in vivo conditions, indicates that the approach applied is a valuable addition to our understanding of cell regulation in these Mycobacterium species.

Introduction

The genus *Mycobacterium* contains a variety of species with different phenotypes (e.g. fast- and slow-growers, chromogens, pathogens and saprophytes) and hence, provides a range of suitable model organisms for Mtb. The suitability of *Mycobacterium* species as model organisms has been extensively discussed highlighting differences/similarities in growth rate, cell envelope chemistry, pathogenicity/biosafety and their phylogenetic relationship compared to Mtb (e.g. Barry, 2001). For the purpose of this study, two fast-growing (*M. smegmatis* and *M. phlei*) and three slow-growing (*M. bovis* BCG, *M. avium* and *M. intracellulare*) mycobacteria were chosen to represent a diverse range of *Mycobacterium* species. These mycobacteria were analysed at set growth stages to show adaptation processes upon the induction of abiotic stresses (e.g. nutrient and oxygen limitation through natural depletion of given resources) which are comparable to the microenvironment experienced by Mtb. The metabolite levels monitored elucidated metabolic pathways common to the genus *Mycobacterium* (e.g. mycolic acid synthesis (Gago *et al.*, 2011)) and specific to certain phenotypes mentioned above (e.g. poly-glutamine synthesis in slow-growers (Harth and Horwitz, 1999)).

The culture conditions in the present study were based on published *in vitro* models (Wayne and Hayes, 1996) to represent typical environments experienced by Mtb under physiological conditions. The prevalent stress conditions experienced within the host, including infection stages of active replication in alveolar macrophages and dormancy within granulomas (Chao and Rubin, 2010), involve a gradual depletion of oxygen and nutrients (Russell *et al.*, 2010). To minimise environmental influences on the mycobacterial cells, they were cultivated in a standardised volume of nutrient rich medium with no additionally supplementation over the duration of the experiments creating a natural depletion of nutrients over the period of batch cultivation. The three culture conditions investigated included growth from lag to stationary phase (Phase I) and growth from logarithmic (log) phase for 28d under aerated (Phase II) or hypoxic (Phase III) conditions. To capture the changes in metabolites occurring under these conditions a quantitative metabolite profiling approach was used (Drapal *et al.*, 2014). The resulting changes at the metabolite level were compared with previous published transcriptomic and proteomic data (e.g. (Betts *et al.*, 2002; Hampshire *et al.*, 2004)) for a better understanding of cellular regulation in *Mycobacterium* and its changing chemotype with environmental adaptation. Collectively, these data will enable an assessment of how representative the laboratory conditions used are compared to real life scenarios.

Material and Methods

Bacteria

M. smegmatis (National Collection of Type Cultures (NCTC) 8159, Public Health England,), *M. phlei* (NCTC 8151), *M. bovis* BCG (Pasteur strain, from AHVLA Collection, Weybridge, UK), *M. avium* (serotype 8, private collection) and *M. intracellulare* (serotype 7, private collection) were cultivated and prepared as a starting culture as published previously (Drapal *et al.*, 2014). For the following culture conditions five replicates per species were grown in parallel.

For Phase I cultivation, fresh Middlebrook 7H9 supplemented with 10% OADC (oxalic acid,-albumin-dextrose-catalase) and 0.4% Tween 80 was inoculated with the starting culture until an initial optical density at 600nm (OD) of 0.1 or 0.05, for fast- and slow-growing mycobacteria respectively, was reached. The cultures (1.5 times the total volume sampled) were then incubated shaking (180 rpm, 37°C) under aerated conditions in vent-cap flasks.

For the other two culture conditions, fresh medium (1.5 times the total volume sampled) was inoculated as described for Phase I and incubated shaking (180 rpm, 37°C) under constant aeration in vent-cap flasks. After 16h (*M. smegmatis*), 1d (*M. phlei*) or 7d (*M. bovis* BCG, *M. avium*, *M. intracellulare*) an aliquot (8 ml) of the inoculated medium was sampled representing the induction point of the two different oxygen conditions. The aerated, shaking condition was maintained for Phase II cultivation and samples (8 ml) were taken at 1d, 2d and 28d after induction for all mycobacteria. For the hypoxic cultivation (Phase III), the cultures were aliquoted (8 ml) at the induction point and all aliquots cultivated without stirring at 37°C until sampled for analysis. The depletion of oxygen in the hypoxic culture was visualised by discolouration of methylene blue (1.5 µg/ml cell culture). Aliquots of inoculated medium (8 ml) were sampled following the same time regime as described for Phase II.

Sampling and analysis of polar and non-polar metabolites

Aliquots of the cultures were quenched with isotonic 60% (v/v) methanol, centrifuged and the resulting cell pellet weighed. The cells were the extracted with a methanol/chloroform method followed by analysis with gas chromatography mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) as previously published (Drapal *et al.*, 2014). The metabolites of each sample were identified through a customized library (Drapal *et al.*, 2014). Variation in the number and type of metabolites identified occurred at each sampling point. The GC/MS results were normalised to the internal standard and weight of the cell pellet. The identified compounds within each culture condition were compared to the first time point taken. Statistically significant changes of metabolites were analysed with one-way ANOVA with Dunnett's post-test and were then plotted over a pathway diagram.

Identification of lipids and proteins of the secreted layer

After four days of induction of hypoxia, the secreted layer was carefully removed from the cell pellet and separately analysed for lipid and protein content. For the identification of lipids, the collected secreted layer was dissolved (1:2) in chloroform/methanol/water (10:10:3, v/v/v). The diluted sample was then mixed (1:1) with 2,5-dihydroxybenzoic acid matrix (5 mg/ml in acetonitrile with 0.1% (v/v) trifluoroacetic

acid) and placed on a 600 µm Anchor chip 384 format MALDI target plate (Bruker Daltonics) before analysis with MALDI–TOF/TOF-MS as described previously (Jones *et al.*, 2013).

For the identification of proteins of the secreted layer, a previously published protocol (Robertson *et al.*, 2012; Mora *et al.*, 2013) was followed which included precipitation of proteins and separation by SDS-PAGE followed by in-gel digestion of the four major protein bands and analysis with nano-LC/MS/MS. The proteins were identified through the detected peptides as previously published (Nogueira *et al.*, 2013) with SwissProt protein database for taxonomy *M. tuberculosis* complex.

Results

Growth properties under aerated and hypoxic cultivation

The growth curves for all three culture conditions were measured by monitoring optical density (OD) and colony forming units (cfu) (Supplementary File 1). Both these measurements over Phase I showed the typical sigmoid shape for all five mycobacteria (Fig. 1). The culture condition Phase II was characterised by an almost horizontal linear curve at at least 2d after log phase. Again all five mycobacteria showed similar growth properties (Fig. 1). The OD data was consistent with the cfu measurements and indicated a constant viable count of culturable cells which is associated with data found for non-replicating persistence (NRP) 1 (Chao and Rubin, 2010; Shi *et al.*, 2010; Bacon *et al.*, 2014). The exception was *M. phlei* which showed a decline of growth from 1d to 2d after the log phase followed by constant values until 28d suggesting a death phase before the switch to NRP (Bacon *et al.*, 2014). Interestingly, a resurgent increase of cfu values was detected at 28d (Phase II) only for *M. smegmatis, M. avium* and *M. intracellulare* which indicated a reactivation of cells or a second log phase of the remaining active cells. Phase III cultures, contrary to Phase II, showed an immediate stop of replication at the first time point after induction of hypoxic conditions conferred by the removing of agitation (Fig. 1). This was shown through constant values similar to the induction point (log phase) measured until 28d and would indicate that the cells are capable of rapid detection/adaptation to the change in oxygen concentration. This change in condition was monitored *in situ* by the reduction of methylene blue in the medium. Discolouration arose four hours after induction, which was approximately half the duration required for all cells to settle at the bottom of the culture tube and was the same for all five mycobacteria. All samples retained the discolouration throughout Phase III.

The metabolite profile was measured at each set time point and compared to the first time point of the culture condition for each *Mycobacterium* species separately. The resulting changes of metabolite levels were calculated and their significance evaluated (Supplementary File 2). Key metabolites within the data set were chosen to visualise changes of nucleotides, the nitrogen and sulphur pathways, TCA cycle, glyoxylate shunt, the biosynthesis of fatty acids and complex lipids and thus describe the metabolic state over the growth stages.

Metabolite changes occurring at the logarithmic and stationary growth phases (Phase I)

Overall ~70 compounds were detected throughout Phase I for four of the five mycobacteria tested. *M. phlei* was the exception with 126 metabolites detected. The summarised Phase I data for all five mycobacteria (Fig. 2) highlighted the differences in metabolite levels between the fast-growing and the slow-growing *Mycobacterium* species. Interestingly, the slow-growers had decreased metabolite levels predominantly, when compared to lag phase, which suggested that metabolites associated with primary metabolism were not detectable or rapid utilisation occurred. The data on the fast-growers, *M. phlei* and *M. smegmatis*, showed increased levels of polar metabolites suggesting accumulation of the latter. *M. bovis* BCG, being one of the slow-growing mycobacteria, showed similar metabolite changes to *M. avium* and *M. intracellulare* but differed from the latter two as more metabolites were detected in *M. bovis* BCG including uracil, glycine, fatty acid precursors and intermediates of the TCA cycle.

The common nucleotide detected in *M. smegmatis, M. bovis* BCG and *M. phlei* was uracil and showed increased levels from log phase onwards. *M. phlei* had the most nucleotides detected which showed the same increasing trends as uracil (Supplementary File 2). No nucleotides were detected for *M. avium* and *M. intracellulare* over Phase I which would be compliant with rapid utilisation.

Homocysteine, the key intermediate of the sulphur pathway, can be metabolised via cysteine to mycothiol (Zeng *et al.*, 2013) or converted to methionine (Singhal *et al.*, 2013) leading to reduced levels of the latter as detected throughout Phase I, in four out of five mycobacteria tested. *M. phlei* was the only *Mycobacterium* species in this study for which no homocysteine but increased cysteine levels were detected.

Glutamic acid, the preferred nitrogen assimilation product (Amon *et al.*, 2009), showed higher levels at log compared to the stationary phase in *M. phlei* and *M. smegmatis*. *M. intracellulare* was the only slow-growing species which showed up-regulation of glutamic acid at stationary phase. The other two slow-growing species, *M. bovis* BCG and *M. avium*, had decreased levels of glutamic acid. Additionally, in *M. bovis* BCG levels of proline were increased.

The only intermediate of the TCA cycle which was detected in all five mycobacteria studied throughout Phase I was succinic acid. This intermediary metabolite connects the TCA cycle, glyoxylate shunt and methylcitrate cycle, which are all involved in fatty acid synthesis and catabolism (Eoh and Rhee, 2013). Succinic acid and odd-chain fatty acids were decreased in the three slow-growing mycobacteria studied. In the case of *M. phlei* and *M. smegmatis,* levels of succinic acid showed no change or an increase at log phase, respectively. For glyoxylate, the key intermediate of the glyoxylate shunt, the derivative glycolate was detected by GC/MS, due to chemical structures created during derivatisation process. Glycolate was down-regulated throughout Phase I in all mycobacteria studied, except *M. smegmatis* for which no glyoxylate was detected. Additionally, malonic acid, the dicarboxylic acid of malonyl-CoA, was detected and its levels increased at the stationary phase for *M. bovis* BCG and *M. smegmatis* and at the log phase of *M. phlei.* The even-chain fatty acids, consecutive intermediates of malonyl-CoA and precursors for biosynthesis of mycolic acids (Crellin *et al.*, 2013), were reduced in all five mycobacteria studied throughout Phase I. Similarly, the content of glycerol, utilised for glycero- and phospholipid biosynthesis (Crellin *et al.*, 2013), was decreased or at levels below the limit of detection. Besides malonic acid, fatty acid precursors isoleucine, threonine, leucine and valine were detected with increased levels for *M. bovis* BCG, *M. smegmatis* and *M. phlei*.

Saccharides associated with components of the cell wall (arabinose, galactose, inositol, mannose and trehalose) showed different trends in all mycobacteria tested over Phase I. Arabinose, part of the arabinogalactan, was only detected in *M. smegmatis* at stationary phase. Galactose, also part of the arabinogalactan, was decreased in all mycobacteria except *M. phlei* which showed increased levels at log and no change at the stationary phase. Contrary to this, inositol and mannose-6-phosphate, both components of plasma membrane related lipids, were increased in all mycobacteria except *M. avium,* which showed decreased levels throughout Phase I. Trehalose, the backbone of trehalose mono- and dimycolates (TMM, TDM), showed the most differences in contents between the five mycobacteria tested. It was increased throughout Phase I for *M. smegmatis*, at log phase for *M. phlei,* at stationary

phase for *M. intracellulare* and showed no change and decreased levels throughout in *M. avium* and *M. bovis* BGC, respectively.

An essential metabolite for the electron transport chain is menaquinone (MK) 9, the major lipoquinone in *Mycobacterium* species (Mathew *et al.*, 2010). It was detected with overall decreased contents compared to lag phase in all mycobacteria studied and showed increasing levels between log and stationary phase for all species except for *M. phlei*. The data for *M. phlei* showed further decrease of MK9 from log to stationary phase and increased levels of β-carotene throughout Phase I. *M. intracellulare,* the other chromogenic *Mycobacterium* species studied, produced only phytoene, a colourless carotenoid which represent the first step in the C40 pathway, during Phase I. Metabolite profiling features found over Phase I, such as even-chain fatty acid and glycerol metabolism and MK9 levels, highlighted similarities between the mycobacteria studied.

Metabolic changes under elongated aerated cultivation (Phase II)

In all five mycobacteria, more compounds were detected over Phase II compared to Phase I. *M. phlei* had the most metabolite changes measured over Phase II which were mainly lower levels compared to the initial time point of measurement (Fig. 3).

Nucleotide metabolism appeared to be active in all five mycobacteria as a variety of changes in nucleotide constituents were detected throughout Phase II. Amino acid content was unchanged for *M. avium* and *M. intracellulare* at 1d and 2d, respectively, and decreased for *M. phlei*. Contrary to this, *M. smegmatis* and *M. bovis* BCG showed an increase in number and levels of amino acids especially at 2d of Phase II.

Even though the overall trends of amino acid levels differed between the five mycobacteria studied, the amino acids involved in the sulphur and nitrogen pathways were similar between them. Homocysteine/cysteine content showed no change overall and glutamic acid levels showed a decreasing trend throughout Phase II after an initial increase for *M. smegmatis* and *M. intracellulare.* Additionally, *M. phlei* had decreased levels of biochemically related consecutive intermediates of glutamic acid (ornithine and citrulline), whereas *M. bovis* BCG and *M. smegmatis* showed increased levels at 1d or 2d (proline and ornithine) (Supplementary File 2).

The glyoxylate shunt seemed activated after 1d for *M. smegmatis* and after 2d for BGC and *M. avium* which was indicated by increased levels of glyoxylate and/or glycine*. M. intracellulare* showed decreased levels of glyoxylate at 2d and no change of glycine. Whereas, *M. phlei* had decreased levels of both glyoxylate and glycine throughout Phase II with a further decrease from 1d to 2d.

The metabolite profiling data of Phase II showed that trehalose and glycerol contents were related to even chain fatty acid levels which are all precursors for the biosynthesis of TDM or triacylglyceride (TAG). In *M. phlei,* fatty acids and trehalose were decreased, whereas in the three slow-growing species no change was detected for both trehalose and fatty acids within the first two days of Phase II. Furthermore, glycerol-3-phosphate was increased for *M. bovis* BCG and *M. intracellulare* and together

with glycerol-esters decreased in *M. avium* and *M. phlei.* Only *M. smegmatis* showed no change of fatty acids, an increase in trehalose and a decrease of glycerol-3-phosphate.
The chromogenic mycobacteria (*M. phlei* and *M. intracellulare*) and *M. smegmatis* had increased levels of isoprenoids throughout Phase II and increased levels of MK9 at 1d except for *M. phlei* which showed no change in MK9 levels. *M. avium* and *M. bovis* BCG showed no change of MK9 and an additional MK (MK8) was detected for *M. bovis* BCG.

Cultivation of mycobacteria tested over Phase II highlighted differences between the *Mycobacterium* species, especially for *M. phlei* which showed down-regulated metabolite levels throughout Phase II and even further decreases at 28d whereas the other mycobacteria showed a predominantly positive metabolic switch at the last time point. At 28d, *M. smegmatis* showed increased levels of the glyoxylate shunt, sulphur pathway and fatty acids and *M. bovis* BCG showed increased levels of the glyoxylate shunt, nucleotides and fatty acid precursors indicating activated energy metabolism and induced activity for cell replication. At the same time, *M. avium* and *M. intracellulare* showed a distinct metabolic activation of all pathways with mainly increased levels except for glycerol-esters which were decreased. For *M. smegmatis, M. avium* and *M. intracellulare* these metabolic changes at 28d were also reflected with increased cfu values as described earlier.

Effects of hypoxic culture conditions on metabolite levels (Phase III)

Over Phase III, mycobacterial cells were not agitated and became oxygen-depleted, a well-documented effect (Wayne and Sohaskey (2001). The measured changes of metabolites over Phase III were more similar between the five mycobacteria tested (Fig. 4) compared to the data described for Phase II. In general, fewer metabolites were detected in Phase III (~90) than Phase II (~110).
The number of nucleotides detected followed this trend with a decrease in amount and number or a general lack of nucleotides detected for *M. smegmatis, M. avium* and *M. intracellulare*, respectively. In *M. bovis* BCG uracil showed increased levels after 2d and in *M. phlei* the highest number of nucleotides was detected with increased levels of most nucleotides at 1d (Supplementary File 2).

No change of homocysteine levels was detected for *M. bovis* BCG throughout Phase III, for *M. intracellulare* after 1d and for *M. smegmatis* and *M. avium* until 28d. All three mycobacteria had increased levels of homocysteine at the other time points. As seen in Phase I and II, only cysteine was detected in *M. phlei* with decreased levels throughout Phase III.
A striking observation was the decrease of glutamic acid in all three slow-growers throughout Phase III whereas the fast-growing species showed increased levels. Only two biochemically related consecutive metabolites, proline and ornithine, were detected in *M. smegmatis* and *M. phlei,* respectively, with increased content at 1d.
The succinic acid levels varied for all five mycobacteria tested. *M. smegmatis* and *M. avium* showed decreased levels from 1d and 2d, respectively, in *M. bovis* BCG no change occurred and *M. phlei* and *M. intracellulare* showed increased followed by decreased levels of succinic acid at 1d and 2d, respectively. Similar to succinic acid, precursors for lipid and cell wall synthesis (glycerol, fatty acids

and saccharides) were expressed with different metabolite levels for all mycobacteria. *M. smegmatis* and *M. avium* showed decreased levels of glyercol-3-phosphate whereas *M. phlei* and *M. intracellulare* showed increased levels of glycerol-3-phosphate and -esters, respectively, at 1d followed by no change from 2d onwards. *M. bovis* BCG showed an increase of glycerol, -phosphate and –esters and most fatty acids at 2d with the exception of decreased levels of C18:0 throughout Phase III, a fatty acid favoured for first or second position of TAG (Walker *et al.*, 1970). Arabinose was detected in *M. smegmatis* at 1d and in *M. phlei* and *M. avium* at 28d. Galactose content was mainly unchanged over Phase III apart from decrease throughout in *M. smegmatis* and increase at 1d in *M. phlei*. At 1d *M. phlei* also had an increased content of mannose which was otherwise unchanged for the other four species. Inositol and trehalose were detected with similar trends of levels over Phase III. For *M. smegmatis* they were increased over the first two days and at 2d, respectively. In *M. avium* both saccharides were decreased after 1d and 2d, respectively. Inositol and trehalose were unchanged in *M. bovis* BCG until 28d at which point they were decreased. *M. intracellulare* also showed unchanged levels of inositol and an increase at 28d whereas trehalose was increased until 2d and showed no change at 28d. In *M. phlei*, trehalose and inositol were increased at 1d followed by no change of trehalose and decreased levels of inositol.

MK9 levels showed no change over Phase III for all mycobacteria tested and for *M. intracellulare* after 1d. For *M. bovis* BCG MK8 and MK9 were detected as described for Phase II. A smaller number of carotenoids (less than half) was detected for *M. phlei* and *M. intracellulare* over Phase III compared to Phase II. The carotenoids showed no change except for phytoene which was increased throughout Phase III.

At 28d of Phase III a renewed change of metabolite levels were detected similar to Phase II but without an increase in cfu number. *M. bovis* BCG and *M. smegmatis* showed activation of sulphur and energy metabolism (e.g. glyoxylate shunt, fatty acid metabolism) and an increase of nucleotide levels (Supplementary File 2). *M. smegmatis* and *M. intracellulare* had increased contents of almost all fatty acids detected. Furthermore, *M. avium* and *M. intracellulare* showed activity for all metabolic pathways as described for Phase II with the exception of increased levels of nucleotides for *M. avium*.

The most striking property of the hypoxic cultivation was a secreted lipid layer of only the two fast-growing *Mycobacterium* species (Fig. 5a). Three to four days after induction of hypoxic conditions, a translucent layer became visible right above the cell pellet and showed a clear separation from the medium. The control tube, containing bacterial-free medium, showed no such occurrence, indicating that the layer was a product of the bacteria. In the case of the chromogenic *M. phlei,* the extracellular material was coloured yellow/orange throughout and showed a zone of denser colouration at the border to the cell pellet. Analysis of carotenoids extracted from the secretion did not show any results as the amount of carotenoids was below the level of detection due to the volume of secretion per tube (~50 µl/10 mg cell pellet). Thus, the concentration of carotenoids could be significant but the total amount of extracellular material precludes adequate extractable amounts.

After collection, the secreted layer was analysed for metabolite and protein composition. The analysis with MALDI showed two series of peaks from 525 to 917 m/z and 1005 to 2035 m/z (Fig. 5b). The fingerprint of the molecular ion peaks was typical for MALDI spectra of TAG as seen through

315 comparison with TAG spectra of *M. brumae* (Rafidinarivo *et al.*, 2009). The MALDI data suggested that
316 TAG was the main compound of the secretion as no other spectra were detected in the sample. The
317 two series of peaks in the TAG spectrum of *M. smegmatis* further indicated two different types of TAGs
318 due to a combination of fatty and mycolic acids with different chain length as described for *M. brumae*
319 (Rafidinarivo *et al.*, 2009).
320 For the protein analysis, proteins were extracted from the secreted layer and separated by SDS-PAGE.
321 The same pattern of protein bands was seen for both fast-growing mycobacteria. The two major protein
322 bands of both species were identified as a putative diacylglycerol O-acyltransferase (DGAT, ~63 kDa)
323 from *Mycobacterium* and acyl-CoA acetyltransferase (ACAT, ~40 kDa). Furthermore, a match for the
324 early secreted antigen target (ESAT)-6-like protein EsxB (~11kDa) was found.
325
326

Discussion

The metabolome, as the end product of gene expression and protein regulation (Harrigan and Goodacre, 2003; Boshoff and Lun, 2010), can be used to give a mechanistic insight into adaptation processes of an organism. An example in the present study is increasing levels of uracil detected through all culture conditions as part of DNA repair of G+C rich bacteria in macrophage infections (Venkatesh *et al.*, 2003; Cossu *et al.*, 2012). Difficulties of metabolite profiling methods, including the quick metabolic turn-over and leakage during sampling, were minimised in the approach used (Drapal *et al.*, 2014) and results reveal snapshots of the intracellular metabolism. Furthermore, the comparison of the metabolic data with transcriptional and translational data elucidates the principal mode of cellular regulation during adaptation to a stress condition.

The identified common properties of all mycobacteria specific to a culture condition (Fig. 6) were consistent with transcriptional and translational data published (e.g. during macrophage infection (Sassetti and Rubin, 2003)). For Phase I this included an active nucleotide and amino acid metabolism (Wang *et al.*, 2005) which ceased with cell replication over Phase III as reported under *in vivo* and *in vitro* conditions (Wayne and Sohaskey, 2001; Betts *et al.*, 2002). Additionally, changes in homocysteine levels as precursor for cell wall maintenance (Dhiman *et al.*, 2009) and for protein and nucleotide synthesis (Singhal *et al.*, 2013) were detected. MK9 and isoprenoids, both involved in the electron transport chain, seemed to play an essential interconnected role over all three conditions tested (Lee *et al.*, 2008; Dhiman *et al.*, 2009). Another important and already well known compound class for mycobacteria are lipids which act as a barrier to the environment (e.g. TDM) (Archuleta *et al.*, 2005) and as carbon/energy storage (e.g. TAG) (Crellin *et al.*, 2013). Their precursor metabolites detected included fatty acids, monosaccharides and glycerol derivatives. All of these metabolites displayed an active metabolism during replication cycles for plasma membrane and cell wall (Sassetti *et al.*, 2003; Crellin *et al.*, 2013) as well as part of the adaptation to environmental changes (Archuleta *et al.*, 2005) and the transition to the dormant state in Mtb as reported in mycobacterial infections (Stehr *et al.*, 2013).

One typical mycobacterial feature is the redirection of the carbon flow under reduced oxygen conditions which results in a switch of the TCA cycle to the glyoxylate shunt (Sassetti and Rubin, 2003; Eoh and Rhee, 2013). Succinic acid and glyoxylate, both products of the isocitrate lyase in the glyoxylate shunt (Eoh and Rhee, 2013), were detected in this study. The detection of both of those metabolites over Phase I suggested that the glyoxylate shunt was already activated during active replication contrary to published literature (e.g. Wayne and Sohaskey, 2001; Chao and Rubin, 2010). Levels of glyoxylate and related metabolites indicated a far less active glyoxylate shunt over Phase II than that described for a transcriptomic study following progressive nutrient depletion similar to the conditions in the present study (Hampshire *et al.*, 2004).

The main metabolic changes, which showed distinct differences between fast- and slow-growing mycobacteria, were glutamic acid levels and sequestration of TAG over Phase III. Increased levels of glutamic acid in the fast-growing species over Phase I were coherent with nitrogen assimilation under nitrogen excess in the medium (Amon *et al.*, 2009) and suggests that fast-growing species can sense upcoming limitation in the surrounding medium and adapt accordingly (Smeulders *et al.*, 1999). Contrary to this, glutamic acid levels over Phase I in the slow-growing species were consistent with utilisation of the latter for nucleotide and protein synthesis and transcriptional data for optimal growth

(Sassetti *et al.*, 2003). Over Phase III the lack of nucleotides in combination with decreased levels of glutamic acid in slow-growing species suggested secretion of glutamic acid into the extracellular environment. Pathogenic mycobacteria (e.g. *M. bovis* and *M. tuberculosis*) are known to release glutamine synthetase during infection and synthesise poly-L-glutamate/glutamine as an extracellular carbon and nitrogen storage (Harth and Horwitz, 1999). The intracellular accumulation of glutamic acid for non-pathogenic mycobacteria (e.g. *M. phlei* and *M. smegmatis*) supports the hypothesis that glutamic acid secretion is a trait of pathogenic mycobacteria.

For fast-growing species a release of TAG into the medium was observed a few days after Phase III induction (Fig. 5). Lipid body formation with TAG is a common property of mycobacteria during the hypoxic phase of the infection (e.g. Garton *et al.*, 2002; Low *et al.*, 2009) and *M. smegmatis* is known to constantly releasing fatty acids or TAG into the medium (Selishcheva *et al.*, 2012). The proteins detected in the secreted layer suggest simultaneous synthesis of TAG by DGAT similar to mycobacterial infections (Stehr *et al.*, 2013) and β-oxidation of fatty acids by ACAT for butyryl-CoA and acetyl-CoA synthesis (Kanehisa *et al.*, 2006). DGAT was detected in higher amounts than ACAT, resulting in more TAG synthesis compared to β-oxidation and consequently the visible TAG layer. Saprophytic mycobacteria usually cannot gain access to lipids originated from a host such as pathogenic mycobacteria (Russell *et al.*, 2010). In the present study, oleic acid was available in the medium in its free form and through hydrolysis of Tween 80. Hence, the production and secretion of TAG with oleic acid might be the adaptation process of choice for non-pathogenic mycobacteria to create an environment similar to foamy macrophages during infection with Mtb (Stehr *et al.*, 2013) and secure storage of extracellular signalling metabolites (oleic acid) for resuscitation (Selishcheva *et al.*, 2012). Similar metabolic changes related to TAG synthesis were detected over Phase II and highlight that TAG synthesis and secretion might be a reaction to stressful conditions including nutrients and/or oxygen depletion, leading to a NRP state (Wayne and Sohaskey, 2001). The metabolic changes related to phenotypes suggests a possible genetic variation between pathogenic and non-pathogenic mycobacteria due to necessities presented by their respective ecological niches.

In the present study a renewed increase in metabolic activity and growth rate was detected at 28d of Phase II and III cultivation for all mycobacteria tested. The metabolic activities correlated with gene expression for optimal growth over Phase I (Sassetti *et al.*, 2003), which indicates the presence of active and “dormant” cells in the culture. This supports reports of heterogeneous culture in *in vivo* and *in vitro* studies and suggested that this phenomenon is a mycobacterial trait and might provide new insight into NRP cultures (Smeulders *et al.*, 1999; Chao and Rubin, 2010).

Despite the metabolic differences of non-pathogenic and pathogenic mycobacteria, a comparison of their metabolic features provided valuable insight to *in vivo* studies as metabolic reactions detected were consistent with results described for mycobacteria under macrophage infection. The present study revealed that all *Mycobacterium* species maintain an active turn-over of metabolites involved in transcription, translation and other general cellular processes through all three culture conditions. The comparison with published literature highlighted that metabolic changes over a gradually occurring nutrient depletion (Phase I and II) seemed to be more influenced by transcriptional and translational processes, whereas cells experiencing a sudden oxygen reduction (Phase III) showed a more independent metabolic regulation related to a phenotype. This suggested a more complex and diverse regulation of intracellular processes over Phase III for the different phenotypes, contrary to the orderly

shift-down previously described (Cunningham and Spreadbury, 1998). This observation emphasises the importance of integrated data from several levels of cellular regulation to guarantee sufficient data for a more complex understanding of the biological processes as a whole.

In conclusion, all five *Mycobacterium* species can be considered as model organisms for Mtb in relation to cell wall and MK9 synthesis over the cultures conditions tested. Whereas, most other metabolic changes detected over the three culture conditions (e.g. poly-L-glutamate layer over Phase III) showed a divide between fast- and slow-growing mycobacteria. This suggested that for metabolite profiling study purposes *Mycobacterium* species phenotypically closer related to Mtb (e.g. *M. bovis* BCG and *M. avium*) should be recommended as model organism. Given that a chloroform/methanol/water step, lethal to mycobacteria, is used for the extraction of metabolites, the methodology is applicable to use with ACDP3 pathogens such as virulent Mtb. The strong correlation between responses detected in the present laboratory based study and published data, often acquired under conditions approaching *in vivo* scenarios, adds support to the metabolomic approach as a means of obtaining conclusive datasets for interpretation into exploitable networks. Thus, a metabolite profiling of at least one Mtb strain should be done to further validate the presented conclusions and lead to a more precise consideration for the choice of model species for metabolic modelling.

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549 **SUPPORTING INFORMATION**

550 Supplementary File 1 – OD and cfu values of all growth conditions and mycobacteria

551 Supplementary File 2 - Average ratios and P-values of all growth conditions and mycobacteria

552

553 **FIGURE LEGENDS**

554 **Fig. 1.** Growth curves of *M. smegmatis* (a) and *M. bovis* BCG (b) under aerated and hypoxic cultivation.
555 These are representative of all the mycobacteria studied, though the actual OD values and sampling
556 points (diamonds, crosses and circles) vary for each species tested is (see methods). For Phase I
557 measurements were taken from lag to stationary phase. The induction time of elongated, aerated
558 (Phase II) and hypoxic (Phase III) cultivation is indicated by an arrow.

559 **Fig. 2.** Heat map of metabolite levels measured from lag phase over log and stationary phase. *M.*
560 *smegmatis, M. phlei, M. bovis* BCG, *M. avium* and *M. intracellulare* were grown under aerated
561 conditions and sampled at lag, log and stationary phase. The reference point for metabolite levels was
562 lag phase. Metabolites were grouped in polar (nucleotides, amino acids, organic acids and saccharides)
563 and non-polar (fatty acids, glycerol, -esters, -phosphates and isoprenoids) extracts. Changes are
564 indicated as increase (green), no change (grey), decrease (red) and not detected (white) shown in the
565 legend. Results were means of five replicates.

566 **Fig. 3.** Heat map of metabolite levels measured over Phase II. *M. smegmatis, M. phlei, M. bovis* BCG,
567 *M. avium* and *M. intracellulare* were grown under aerated conditions throughout and sampled from log
568 phase onwards over 28d. The reference point for metabolite levels was the first sampling point at log
569 phase. Metabolites were grouped in polar (nucleotides, amino acids, organic acids and saccharides)
570 and non-polar (fatty acids, glycerol, -esters, -phosphates and isoprenoids) extracts. Changes are
571 indicated as increase (green), no change (grey), decrease (red) and not detected (white) shown in the
572 legend. Results were means of five replicates.

573 **Fig. 4.** Heat map of metabolite levels measured over Phase III. *M. smegmatis, M. phlei, M. bovis* BCG,
574 *M. avium* and *M. intracellulare.* Those mycobacteria were grown under hypoxic conditions from log
575 phase onwards and sampled over this period until 28d. The reference point for metabolite levels was
576 the first sampling point at log phase. Metabolites were grouped in polar (nucleotides, amino acids,
577 organic acids and saccharides) and non-polar (fatty acids, glycerol, -esters, -phosphates and
578 isoprenoids) extracts. Changes are indicated as increase (green), no change (grey), decrease (red) and
579 not detected (white) shown in the legend. Results were means of five replicates.

580 **Fig. 5** Lipid **s**ecretion of *M. smegmatis* under hypoxic cultivation. (a) The secretion layer occurred
581 after three to four days of hypoxic cultivation of *M. smegmatis*. The translucent secretion layer (arrow)
582 was collected and analysed with MALDI for metabolite composition. (b) An average MALDI spectrum is
583 presented.

584 **Fig. 6.** Schematic representation of metabolic processes of mycobacteria over Phase I, II and III.
585 Mycobacterial cells were schematically displayed for Phase I, II and III. Arrows represented the

transition from Phase I to Phase II or III with depletion (cross) of oxygen (O_2) and/or nutrients. Intracellular metabolic processes were indicated with black arrows and utilisation of compound classes for the cell wall (grey circle) with grey arrows. The same changes of compound classes for all mycobacteria tested were highlighted as up- and down- regulated (green and red, respectively).