The assessment of changes to the nontuberculous mycobacterial metabolome in response to anti-TB drugs.

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Abbreviations: AG, arabinogalactan; cfu, colony forming units; DPA, decaprenylphosphate-arabinose; EMB, ethambutol; FAS, fatty acid synthase; GC/MS, gas chromatography mass spectrometry; HPLC, high performance liquid chromatography; INH, isoniazid; LAM, lipoarabinomannan; LM, lipomannan; MK9, menaquinone with nine isoprenoid side chain residues; NRP, non-replicating persistence; NTM, nontuberculous mycobacteria; OD, optical density; TAG, triacylglycerides; TB, tuberculosis; TCA, tricarboxylic acid.
Abstract

*Mycobacterium* species can cause a range of nontuberculous infections of healthy and immunocompromised people as well as infect people during and after surgical procedures. The similarity of nontuberculous mycobacteria (NTM) to the tuberculosis bacilli (TB) could ultimately enable the use of anti-TB drugs for the genus. Hence, three NTM (*M. smegmatis, M. phlei* and *M. avium*) were cultured under different lab conditions, causing two mycobacterial phenotypes (active and dormant), and treated with isoniazid (INH) and ethambutol (EMB) independently or in combination. Metabolite profiling was applied to facilitate the investigation and characterisation of intracellular targets affected by the antibiotics. Aliquots of the cell culture were taken over the treatment period and the metabolite profile of the cells analysed by GC/MS. Comparative analysis of the metabolite levels to untreated mycobacteria confirmed the successful action of the antibiotics on the metabolism of all three species. Furthermore, single metabolites and metabolite pathways affected by the antibiotics could be identified and included, besides the known target sites for INH and EMB on mycobacterial cells, changes in e.g. nucleotide and saccharide levels. The combined treatment highlighted the property of EMB to enhance the effects of INH even under hypoxic culture conditions.
Introduction

The genus *Mycobacterium* includes the causative agent of tuberculosis (TB) as well as nontuberculous mycobacteria (NTM) which can cause lung disease and a range of nosocomial infections (Phillips and von Reyn 2001, Wolinsky 1979). NTM can be found in soil, dust, shallow waters and water systems; generally have a low, non-lethal infection rate and are classified in four categories: photochromogens, scotochromogens, non-photochromogens and rapid growers (Abdallah, et al. 2012, Karnam, et al. 2011, Timpe and Runyon 1954). A recent rise of infections with NTM, predominantly with the *M. avium* complex causing lung disease, was reported in Japan and stated effective long-term treatment with anti-mycobacterial drugs (Ide, et al. 2015). Due to the rarity of NTM infections, no guidelines for effective treatment are available (Karnam, et al. 2011) and bear the question whether the standard TB multi-drug regime can be applied for other mycobacterial infections?

*Mycobacterium* species are well known for their unique cell wall composition with high lipid levels and a triple layer structure bearing a considerable constraint for the transport of hydrophilic compounds into the cell under aerobic conditions (Niederweis 2008). Under oxygen limiting conditions, the structural and functional changes of the cell wall increase the natural resistance to many antibiotics even further, preventing the interaction of the antibiotics with their intracellular targets (Chao and Rubin 2010, Karakousis, et al. 2008). Hence, the present study focused on two TB drugs with the specific ability to weaken the cell wall structure and facilitate the uptake of additional anti-mycobacterials (WHO 2010). The intracellular targets of ethambutol (EMB) and isoniazid (INH) are the saccharide backbone and long chain fatty acids of the cell envelope, respectively. Specifically, EMB inhibits the transfer of arabinose to the arabinogalactan (AG) and lipoarabinomannan (LAM) and INH, after intracellular activation, inhibits the assimilation of shorter fatty acids to mycolic acids by the fatty acid synthase (FAS)-II (Da Silva and Palomino 2011, Ma, et al. 2007, Takayama, et al. 1972). Both inhibitory reactions can be easily measured by subsequent changes of metabolites involved.

Metabolic studies present a comprehensive analysis of metabolites – the end products of cellular processes (Harrigan and Goodacre 2003). For antibiotics without growth inhibiting effects, metabolite profiling can elucidate the primary mode of action, “off” targets and their metabolic plasticity leading to resistance (Hoerr, et al. 2016). Additionally, monitoring metabolic changes of multi-drug treatments will establish synergistic and antagonistic effects. The *in vitro* model was chosen in the present study
to ensure a regulated environment in which metabolic processes to an inhibitor can be monitored without additional variables. Gas chromatography mass spectrometry (GC/MS) was chosen as the analysis platform due to its ability to provide informative, relative quantification of a complementary range of metabolites (up to 150 metabolites). Additionally, the GC/MS workflow includes a robust validation of metabolites with customised libraries and can be used in its own right as a routine, accessible, generic profiling methodology; or as part of a larger workflow with complementary platforms. Examples for these platforms are the more focused approaches such as flux analysis and lipidomics (de Carvalho, et al. 2010, Layre, et al. 2011). These systems do not display a diverse coverage of the overall metabolism and are less cost-effective compared to GC/MS.

In the present study the anti-TB drugs EMB and INH were used as inhibitors to 1) establish whether EMB and INH affect NTM metabolism in a holistic manner beyond their primary targets and 2) elucidate the inhibitory and accompanying effects of the antibiotics under single and combined application in more detail, which could contribute to treatment of mycobacterial infections in the long term. Three NTM were chosen to represent several NTM categories and included *M. avium* (slow-growing, non-photochromogenic pathogen), *M. smegmatis* (rapid-growing scotochromogen) and *M. phlei* (chromogen with a medium growth rate). The antibiotics EMB and INH were applied under aerated and hypoxic culture conditions facilitating the occurrence of actively growing and dormant mycobacteria (Chao and Rubin 2010, Russell, et al. 2010). The concentration of both drugs was based on the current treatment for mycobacterial diseases (Ryu, et al. 2016, WHO 1991, WHO 2010), taking into account reported minimal inhibitory concentrations under *in vitro* conditions and serum concentrations from patients to cause a perturbation in the metabolism (Chaturvedi, et al. 2007, Kaur and Khuller 2001, Park, et al. 2016, Schönfeld, et al. 2012, Udekwu, et al. 2009). The metabolism of the treated *Mycobacterium* species was captured with a comprehensive profiling method previously adapted for the elucidation of common metabolic traits (Drapal, et al. 2014, Drapal, et al. 2016). The effect of all treatments, except for INH under hypoxic conditions, could be observed through several specific changes in the metabolite profiles. Using this approach we have been able to assess the effect not just on the primary target sites but secondary effects either from (i) direct interaction of the antibiotic with its target or (ii) changes resulting from metabolite perturbation initiated from the primary target.
Materials and Methods

Bacterial strains

*M. smegmatis* (National Collection of Type Cultures (NCTC) 8159, Public Health England,), *M. phlei* (NCTC 8151), and *M. avium* (serotype 8, private collection) were plated from five different glycerol stocks representing biological replicates. For the following culture conditions the five isolates of each species were grown in parallel. The starting cultures were prepared and cultivated as published previously (Drapal, et al. 2014).

Growth and antibiotic treatment conditions

For both the aerobic and hypoxic culture conditions, the five starting cultures of each species were used to inoculate separate flasks of fresh Middlebrook 7H9 medium supplemented with 10% OADC (oxalic acid-albumin-dextrose-catalase) and 0.4% Tween 80 until an initial optical density of 0.1 was reached. These cultures were then incubated shaking (180 rpm, 37°C) under aerated conditions in vent-cap flasks. After 16h (*M. smegmatis*), 1d (*M. phlei*) or 7d (*M. avium*), the culture in each flask was divided and used for the aerated and hypoxic antibiotic treatments. Before the addition of antibiotics to the hypoxic cultures, the cultures were aliquoted (8ml) and cultivated without stirring at 37°C for two days to transfer the cells into a hypoxic environment. The depletion of oxygen was visualised as described previously (Tan, et al. 2010). At the initiation of the antibiotic treatment (0d), isoniazid (INH, 5µg/ml cell culture), ethambutol (EMB, 10µg/ml cell culture) or a combination of INH and EMB (5µg and 10µg/ml cell culture, respectively) was added to the cultures and a control without antibiotics was cultivated in parallel. Aliquots (8ml) of the treated and non-treated cultures were sampled at 0d, 1d and 7d after treatment initiation.

Metabolite profiling of bacterial cultures

The collected aliquots were immediately quenched and the bacterial cells extracted and analysed by GC/MS and high performance liquid chromatography (HPLC) as previously published (Drapal, et al. 2014). A customized library was used to identify the metabolites in the samples (Drapal, et al. 2014). The GC/MS data was normalised to the weight of the dried sample and the internal standard. The identified metabolites within each sampling point of the inhibitor treatments were compared to the corresponding time point of the non-treated culture. Differences between the treated and the non-
treated bacteria were tested for statistical significance \((P<0.05)\) by one-way ANOVA with Dunnett’s post-test (Supplementary File).

**Results**

**Effects of antibiotics under aerated and hypoxic cultivation**

The growth of *M. smegmatis*, *M. phlei* and *M. avium* was monitored throughout the treatment to visualise that the mycobacteria maintained a stationary phase-like growth (Fig. 1A and B). This guaranteed monitoring the active metabolism of viable cells and not apoptotic processes. The most obvious bacteriostatic effect was monitored for *M. avium* under aerobic conditions, as the treated cultures maintained the same cfu values, whereas the corresponding non-treated culture showed a ~10-fold increase. The two fast-growers showed an increase of cfu values throughout the aerobic culture conditions.

The metabolite profiles of all treatments were analysed to establish the effects of the antibiotic treatments on the metabolism of each *Mycobacterium* species. The comparison of the treated cultures with an untreated control culture enabled the detection of changes for each metabolite monitored (Supplementary File). The overall changes of metabolites are displayed with principal component analysis (PCA) and as a summary of metabolite classes for each species separately (Fig. 2, 3, 4).

**M. smegmatis** under antibiotic treatment

Overall, the single EMB and combined EMB-INH treatment of *M. smegmatis* (Fig. 2) showed a greater number of changes in steady state metabolite levels under both oxygen and hypoxic conditions than the single INH treatment and clustered away from the non-treated culture in the PCA score plot. The metabolites affected over the single and combined EMB treatments included cell wall related saccharides (e.g. galactose and trehalose), nucleotides at 1d, amino acids at 7d, fatty acids, glycerol-phosphates and –esters and menaquinone (MK9). All of these metabolites were decreased in the treated cultures with the exception of MK9 and glycerol-esters. MK9, an essential part of the electron transport chain in *Mycobacterium* species (Mathew, et al. 2010), did not show a definite increase compared to the non-treated culture but rather an increasing trend from decreased levels at 0d to similar levels/a slight increase (~1.8 times) at 7d. The differences of fatty acid levels detected under
aerobic EMB treatments included about half of the measured odd, even, antiso- and iso- fatty acids. The aerobic INH treatment showed decreased nucleotide levels similar to the EMB treatments and various changes, including in- and decreases, of amino acids. Very little change was detected in fatty acid levels, which comprised a lack of fatty acid C20:0 and glycerol-esters with C18:0 and C14:0 fatty acids and increased levels of glycerol-phosphates at 7d. Both single treatments showed an increase of homocysteine, the key intermediate of the sulphur metabolism, at 7d, contrary to the combined treatment which showed no change over the whole treatment period.

Under hypoxic conditions, the single and combined EMB treatment caused an increase in lipid precursors (almost all fatty acids and glycerol compounds) at 7d (up to 6.7-fold) which was already seen at 1d (up to 3-fold) for the combined treatment and can be observed in the PCA score plot (Fig. 2). Contrary to the aerated EMB treatments, almost no change of cell wall related saccharides was detected under the hypoxic conditions. The differences detected were a lack of inositol at 7d and an 80% decrease of mannose-phosphate. Another distinct feature of the aerated treatments were amino acid and nucleotide levels. Under hypoxic conditions, those metabolites were mostly unchanged in their relative levels. The exception were fatty acid precursors, isoleucine and leucine, which were decreased at 0d followed by no difference to the non-treated mycobacteria from 1d onwards. For the hypoxic INH treatment, even less change of metabolite levels was detected compared to the aerated conditions. Identified differences were similar to the other treatments and included steadily increasing levels of MK9 over the treatment period and increased levels (from 30 up to 100-fold) of the fatty acid C18:0.

**M. phlei under antibiotic treatment**

All three aerobic antibiotic treatments showed a large number of changes of metabolite levels over the whole treatment period and show a distinct cluster away from the non-treated mycobacteria (Fig. 3). Similar changes of metabolite groups were detected for the single and combined treatments and included nucleotides, several amino acids (e.g. pyroglutamic acid), fatty acids, glycerol-esters and – phosphates and carotenoids. The majority of changes of nucleotides and amino acids were a decrease compared to the non-treated condition. For an interpretation of fatty acid and glycerol-1-ester levels, the trend of increased levels at the induction time point towards no change at 7d for all three aerobic treatments has to be taken into consideration and indicated a decrease of fatty acid...
levels over the treatment period. The glycerol-phosphates showed a clear decrease at 7d and for the EMB treatments already at 1d. Contrary, glycerol-esters with fatty acid C16:0 and C18:0 esterified at the second OH-position were significantly increased at 7d in the single aerobic treatments and from 1d onwards in the combined aerobic treatment. Carotenoid levels were increased over the treatment period and trehalose levels were decreased at 7d for all aerobic treatments. Additionally, the EMB single and combined treatment showed a decrease of mannose and –phosphate at 1d.

The hypoxic antibiotic treatments showed less metabolite changes than the aerobic treatments and a separation of the treatment cluster from the non-treated mycobacteria can only be observed at 7d in the score plot (Fig. 3). Contrary to the results of *M. smegmatis*, those changes were similar between conditions with and without oxygen. The exception were nucleotide levels which overall showed no change. Both single treatments had increased levels of some metabolites groups (amino acids, organic acids, saccharides and lipid components) at the induction time point, which were significantly decreased or without change at 7d. The majority of significant changes were detected for the hypoxic EMB treatments. This included decreases of cell wall and plasma membrane components/precursors comprised of saccharides; glucose, mannose, their –phosphates, galactose, and trehalose; as well as fatty acids and glycerol–phosphates. Glycerol-esters were an exception as they were increased in the single hypoxic EMB treatment but decreased in the combined hypoxic treatment. Another difference between those two treatments was the increase of MK9 at 1d and 7d of the combined treatment. The hypoxic INH treatment, showing barely any change of lipid components/precursors, had increased levels of glycerol-phosphates at 7d, contrary to the EMB treatments.

*M. avium* under antibiotic treatment

The slow-growing *M. avium* showed different effects to the antibiotic treatments under aerobic conditions compared to the two rapid growers. Even though all antibiotic treatments had the highest inhibition of growth on *M. avium* under aerobic conditions, the metabolite profile of *M. avium* showed the least changes (Fig. 1, Fig. 4). The nucleotides were increased or without change for all three antibiotic treatments and the INH seemed to have more influence on the changes in the combined treatment. The saccharides glucose, fructose, their- phosphates and inositol were increased and lipid components/precursors showed no change at 7d in both the aerobic single INH and combined treatments, whereas no change for the mentioned saccharides and increased levels of fatty acids and
glycerol compounds was detected in the single EMB treatment. Additionally, the single EMB treatment had increased levels of MK9 at 7d. All three treatments had decreased levels of mannose at 7d and the combined aerobic INH-EMB treatment showed an increase of arabinose at 7d (Supplementary File).

Under hypoxic treatment conditions, *M. avium* showed no changes of lipid components/precursors (fatty acids and glycerol compounds). Most other metabolites followed this trend with the exception of nucleotides. The latter metabolite group was mainly increased at 1d for the single INH treatment and at 7d for the combined EMB-INH treatment. Furthermore, inositol phosphate was decreased at 7d for all hypoxic antibiotic treatments, mannose levels were increased under the single EMB treatment and the combined treatment showed a decrease of fructose and glucose at 7d. These changes at 7d can be observed in the score plot, as only at this time point did the treatments separate from the non-treated mycobacteria (Fig. 4). Pyruvic acid, the metabolite linking glycolysis and the TCA cycle, was increased at the induction time point and remained increased throughout the treatment period, with the exception of the combined treatment which showed no change of pyruvic acid levels at 1d.

**Discussion**

The two anti-mycobacterials INH and EMB, chosen for the present study for their inhibition of cell wall components, are primarily effective against replicating bacteria (Da Silva and Palomino 2011, Ma, et al. 2007). Nevertheless, metabolite changes could be detected under both oxygen conditions (Supplementary File 1). The combination of cfu values and metabolite changes showed an indirect correlation and indicates that the physiological effect of the drugs is dependent on the specificity rather than the quantity of metabolic changes and on how the cell adapts metabolically in response to the inhibited pathways.

**Antibiotics under aerobic conditions**

A common feature for both single treatments were changes in nucleotide levels which were decreased for the rapid growers and increased for the slow-grower. Previous publications report an inhibitory effect of nucleotides through INH as well as inhibition of RNA, DNA and subsequent protein synthesis through EMB (Forbes, et al. 1965, Gangadharam, et al. 1963, Takayama, et al. 1972). Even though in later reports DNA replication was excluded as a target of EMB (Deng, et al. 1995), the
metabolite data of the present study indicates a significant effect on nucleotides as well as amino acids. Both compound classes were decreased simultaneously in *M. smegmatis* and *M. phlei*. This could indicate the utilisation of available resources (nucleotides and amino acids) for increased RNA replication and protein synthesis as part of a defence response e.g. transporters for active removal of the antibiotics or an inhibition of the biosynthesis pathways as speculated previously (Beggs 1979, Hemmerlin, et al. 2014). A different effect was observed for *M. avium* which included increased nucleotide levels and hardly any change involved in amino acids of the nitrogen metabolism. A more detailed radiolabelled study would be needed to determine an exact mode of action of the antibiotics on nucleotides and amino acids (e.g. Forbes, et al. 1965, Gangadharam, et al. 1963).

For the INH treatment only subtle changes of fatty acid levels were detected for all three mycobacteria. These levels included increases of long chain fatty acids C\(_{24}\) and C\(_{26}\), especially at 1d, followed by no change at 7d for *M. phlei* and *M. avium* and decreases of C\(_{20}\) and C\(_{18}\)-OH at 7d for *M. smegmatis*. The detected changes correspond to the known INH-inhibition of FAS-II and the resulting lack of incorporation of long chain fatty acids for mycolic acids biosynthesis (Crellin, et al. 2013). The expected accumulation of short chain fatty acids (<C\(_{26}\)) from FAS-I could not be observed at 7d as acyl-CoA hydrogenases are known to recycle excess fatty acids (Wilson, et al. 1999). The effects of FAS-II inhibition were more pronounced under the combined treatment and a synergistic effect of both antibiotics could be seen for the lipid precursors of the two rapid growers. For the combined treatment of *M. avium*, the effects of INH (increase followed by decrease) and EMB (no change followed by increase) seemed to have cancelled each other out as no change could be detected.

The primary target of EMB is the transfer of arabinose to LAM or AG, which should result in increased levels of arabinose (Belanger, et al. 1996). No such change was detected in any of the species studied with the exception of the combined treatment of *M. avium*. A previous study reported a small remaining LAM production under EMB treatment as well as the inhibition of glucose conversion to arabinose and galactose by EMB (Deng, et al. 1995). This observation would explain the lack of arabinose in the present study, as no arabinose could be produced from glucose and the residual arabinose used for the remaining LAM production. The decrease of other saccharides linked to the cell envelope could be related to a compensation mechanism for the lack of arabinose. Previous studies with EMB reported a higher content of galactose in AG for *C. glutamicum* (Radmacher, et al. 2005) and an overproduction of mycolic acid moieties with trehalose and glucose as less mycolic
acids can be bound to the arabinose in LAM and AG (Radmacher, et al. 2005). This metabolic adaptation involving saccharides was most pronounced in \textit{M. smegmatis} which is known to be susceptible to EMB (Belanger, et al. 1996). \textit{M. avium}, a known EMB-resistant phenotype (Belanger, et al. 1996), showed none of these changes. The incorporation of other saccharides instead of arabinose suggested the saccharide transporters and isoprenoid derived compounds as a new antibiotic target.

Besides affecting the saccharide content, the EMB treatment also impacted levels of fatty acids and glycerol-esters - precursors of mycolic acids and phospholipids (Crellin, et al. 2013) - in both the single and the combined treatments. Both rapid growing mycobacteria had decreasing trends of fatty acids and increased levels of glycerol-esters which correlates with the increase of LM units with a glycerol-ester anchor and a higher incorporation of fatty acids into components of the plasma membrane and cell wall (Radmacher, et al. 2005). The lack of change for intermediates of the energy cycles (TCA cycle and glyoxylate shunt), providing precursors for even-chain fatty acid synthesis, contradicts the proposed effect of EMB on the mycolic acid synthesis via the central metabolism (Silve, et al. 1993). Interestingly, in both rapid growers the glycerol-2-fatty acids seemed to be the preferred glycerol-esters, which is consistent with previous studies showing a higher activity of the glycerol-2-fatty acid acyltransferase compared to the glycerol-1-fatty acid acyltransferase (Okuyama, et al. 1977). Under stress conditions, the cell wall synthesis, including e.g. phospholipid biosynthetic processes, is activated (Cossu, et al. 2012) and combined with the higher activity of the former acyltransferase the interrupted membrane bound electron transport system could be stabilised quicker (White and Tucker 1969). This finding suggests a new target for a combination drug treatment, as mammalians can synthesise phosphatidic acid for phospholipids from acyl-3-glycerol via two pathways (Athenstaedt and Daum 1999).

The results of the combined treatments are adding further support to the beneficial properties of EMB for drug synergy (Deng, et al. 1995, Hoffner, et al. 1994).

**Antibiotics under hypoxic conditions**

All antibiotic treatments were least potent under hypoxic conditions. The weaker effect of antibiotics on NRP cells has been described before (e.g. Esmail, et al. 2012), as a result of cell wall alterations (Cunningham and Spreadbury 1998) and less metabolic activity (Karakousis, et al. 2008).
As expected, the single treatment of INH showed the least change of metabolites. INH resistance of mycobacteria during NRP is mainly related to the reduced activity of the catalase peroxidase (KatG) required to convert the prodrug INH into its active form (Karakousis, et al. 2008). Nevertheless, the combined treatment showed an enhanced effect on metabolite changes compared to the single EMB treatment. This would indicate that the presence of EMB either enables the activation of INH or a small amount of converted INH is sufficient in a combined treatment to add to the overall inhibitory effect. Another hypothesis is that the stress of EMB induced the metabolic activity of the bacterial cells, including the KatG activity, and rendered the cells more prone to INH (Bhargava and Collins 2015). Even though EMB has a low activity against non-replicating organisms, all treated hypoxic cultures showed a change of saccharides and/or the fatty acid metabolism (Ma, et al. 2007).

In all three mycobacteria changes in saccharide levels were mainly decreases, which coincides with the compensation mechanism for arabinose observed under aerobic conditions (Radmacher, et al. 2005). In addition to this approach, further experimentation could potentially be performed by calculating the in vitro MIC for all strains and then perform metabolomic analyses at these concentrations.

In summary, the present study highlighted the successful application of anti-TB drugs to three NTM under lab conditions. The profiling method applied showed robust results and can be transferred to an in vivo study observing drug action under “real” infection conditions. The metabolic responses detected in the three Mycobacterium species highlight the difficulty to draw overall conclusions about the antibiotic effects from one species to the genus, as previously observed for the general metabolism (Drapal, et al. 2016). Nevertheless, the metabolite profiling of the treated mycobacteria elucidated primarily changes related to precursors of the cell wall (e.g. saccharides under EMB treatment) as well as secondary and/or "off" targets (e.g. nucleotides under INH and EMB treatment) (Fig. 5).
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Fig. 1. Growth curves of M. smegmatis, M. phlei and M. avium under antibiotic treatments with aerated (A) and hypoxic (B) culture conditions. All graphs show the growth of the cultures from the initiation of the antibiotic treatments, at which point the cultures were at the end of log phase for the aerated condition and in a non-replicating state under hypoxic conditions. Cfu/ml values displaying the number of viable cells throughout the treatments.

Figure 2. Score plot of all treatments and time points under aerobic (A) and hypoxic (B) conditions of M. smegmatis. The PCA analysis included the averaged metabolite levels after normalisation. The legend for treatments (shapes) and time points (white to black) are labelled to the left of the PCAs. The heat map (C) displays the metabolite changes as higher (blue), lower (red) than or equal to the non-treated control culture as displayed in the legend to the left. The time points 1d (left) and 7d (right) are shown next to each other under the label of each antibiotic treatment and metabolites are summarised according to chemical class.
Figure 3. Score plot of all treatments and time points under aerobic (A) and hypoxic (B) conditions of *M. phlei*. The PCA analysis included the averaged metabolite levels after normalisation. The legend for treatments (shapes) and time points (white to black) are labelled to the left of the PCAs. The heat map (C) displays the metabolite changes as higher (blue), lower (red) than or equal to the non-treated control culture as displayed in the legend to the left. The time points 1d (left) and 7d (right) are shown next to each other under the label of each antibiotic treatment and metabolites are summarised according to chemical class.

Figure 4. Score plot of all treatments and time points under aerobic (A) and hypoxic (B) conditions of *M. avium*. The PCA analysis included the averaged metabolite levels after normalisation. The legend for treatments (shapes) and time points (white to black) are labelled to the left of the PCAs. The heat map (C) displays the metabolite changes as higher (blue), lower (red) than or equal to the non-treated control culture as displayed in the legend to the left. The time points 1d (left) and 7d (right) are shown next to each other under the label of each antibiotic treatment and metabolites are summarised according to chemical class.
Fig. 5. Summarising schematic representation of metabolic processes detected in mycobacteria under antibiotic treatments. Metabolic pathways (black arrows) and incorporation of compounds into the cell wall (grey arrows, grey circle) are displayed. Compound classes highlighted (bold and grey background) were affected by presence of antibiotics in all three Mycobacterium species analysed.