Using *Mycobacterium kansasii* as a proxy to study the pathoevolution of *M. tuberculosis*

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Marwan Ghanem wrote the introduction and literature review. Marcel A. Behr edited the draft and authorized the final version of the chapter.

Chapter II. *Heterologous complementation with Rv3377c and Rv3378c elicits the production of 1-tuberculosinyladenosine in Mycobacterium kansasii* (manuscript).

Marcel A. Behr conceived the study. Marwan Ghanem, Joyce Wang and Marcel A. Behr set up the experimental design for the manuscript. Joyce Wang performed all the cloning, electroporation and confirmation of the mutant identity. Marwan Ghanem and Joyce Wang performed lipid extractions for further analysis of lipid production. Pilar Domenech and Michael B. Reed designed the radiolabelled-adenosine TLC assay, which was performed by Joyce Wang. Francis Mann performed and analyzed the GC/MS to detect isotuberculosinol. David Young and Branch D. Moody performed and analyzed the LC/MS to detect 1-tuberculosinyladenosine. Marwan Ghanem performed the *in vitro* growth assessments of the mutant. Marwan Ghanem performed the macrophage infections and all subsequent readouts. Marwan Ghanem, Fiona McIntosh and Daniel Houle performed the murine lung infections. Generated experimental data was analyzed by Marwan Ghanem and Marcel Behr.

Chapter III. Reflections and future directions.

Marwan Ghanem wrote the reflections and future directions. Marcel A. Behr edited the draft and authorized the final version of the chapter.

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"Where is Marwan? Who is Marwan? What is a Marwan?"

LIST OF ABBREVIATIONS

1-TbAd – 1-tuberculosinyladenosine

AIDS – Acquired immunodeficiency syndrome

AM – Alveolar macrophage

ATCC – American type culture collection

ATP – Adenosine triphosphate

BCG – Bacillus Calmette –Guérin

BLAST – Basic local alignment search tool

BMDM – Bone marrow-derived macrophages

cDNA – Coding DNA

CF – Cystic fibrosis

CFU – Colony-forming unit

CID – Collision-induced dissociation

CSF – Cerebrospinal fluid

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

ELISA – Enzyme-linked immunosorbent assay

ESAT-6 – 6 kDa early secretory antigenic target

ESI EIC – Electron spray ionization / extracted-ion chromatography

ESX-1 – ESAT-6 secretion system 1

EV – Empty vector

FPP – Farnesyl pyrophosphate

GC/MS – Gas chromatography-mass spectroscopy

GGPP – Geranylgeranyl pyrophosphate

HAD – Haloacid dehalogenase

HGT – Horizontal gene transfer

HIV – Human immunodeficiency virus

HPLC/MS – Liquid chromatography-mass spectroscopy

IFN-γ – Interferon gamma

IsoTB – Isotuberculosinol

ITS – Internal transcribed spacer

LOS – Lipooligosaccharide

LPS – Lipopolysaccharide

MAC – *Mycobacterium avium* complex

M-CSF – Macrophage-colony stimulating factor

MDR – Multi-drug resistant

Mg^{2+} - Magnesium ion

miscRNA – miscellaneous RNA

MOI – Multiplicity of infection

MTBC – *Mycobacterium tuberculosis* complex

MTMKCA – *M. tuberculosis-M. kansasii* common ancestor

ncRNA – Non-coding RNA

NTM – Non-tuberculous mycobacteria

PCR – Polymerase chain reaction

PMA – Phosphomolybdic acid reagent

PTFE – Polytetrafluoroethylene

qRT-PCR – quantitative reverse transcriptase-PCR

RFLP – Restriction fragment length polymorphism

RNA – Ribonucleic acid

rRNA – Ribosomal RNA

SNP – Single nucleotide polymorphism

TB – Tuberculosis

TLC – Thin layer chromatography

TNF-α – Tumor necrosis factor alpha

tRNA – Transfer RNA

UV – Ultraviolet

WT – Wildtype

XDR – Extensively drug resistant

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ABSTRACT

On a yearly basis, 1.8 million people lose their lives due to tuberculosis, making it the single deadliest infectious disease in the world. *M. tuberculosis* has co-habited with human being for a long time and its evolution was once thought to be purely lateral and degenerative. However, more recent evidence has proven that HGT has played a role in the emergence of the human-tropic professional pathogen from its initially harmless environmental ancestor.

Our understanding of TB pathogenesis is limited by the fact that most virulence factors characterized in *M. tuberculosis* up to date are found in the closely-related environmental NTM species, *M. kansasii.* Our lab has previously identified 55 MTBC-specific genes that are not present in NTMs and exhibit signs of horizontal inheritance. To study the potential of an *M. tuberculosis*-specific HGT-driven pathoevolution, we hypothesized that *M. kansasii* can be used as a proxy for the latest NTM-MTBC common ancestor.

To assess the validity of using *M. kansasii* as our model organism, we complemented it with the *Rv3377-8c* gene pair required to make the *M. tuberculosis*-specific lipid, 1-TbAd. The pathway leading to 1-TbAd production has been previously implicated in the inhibition of phagosomal maturation in macrophages. In this proof-of-concept work, we showed that the generated gain-of-function mutant *M. kansasii*::Rv3377-8c successfully produced 1-TbAd, and that its' production had no consequences on the *in vitro* fitness of the mutant. We also showed that 1-TbAd production led to a higher resistance to acidic environments, with the threshold of growth dropping from a minimum of pH 5.4 to 4.9. Despite the increased *in vitro* acid resistance, 1-TbAd production did not confer an increased fitness inside naïve or activated murine BMDM infection models. The mutant did, however, induce an modest increase in pro-inflammatory cytokine production. Finally, 1-TbAd production did not have an effect on *in vivo* low- and high-dose infection models in mouse lungs.

Put together, our results have successfully validated the use of *M. kansasii* as a model organism to study the effect of HGT on the pathoevolution of *M. tuberculosis*. Although 1-TbAd did not confer an *in vivo* phenotype by itself, we proved it plays a role in acid resistance. Our end goal is to build a library of gain–of-function mutants in order to characterize the role of each of the 55 aforementioned genes, most of which have unknown functions.

RÉSUMÉ

Chaque année, 1,8 million de personnes perdent leure vie à cause de la tuberculose, ce qui en fait la maladie infectieuse la plus meurtrière au monde. *M. tuberculosis* cohabite depuis longtemps avec l'être humain et son évolution était autrefois considérée comme purement latérale et dégénérative. Cependant, des preuves plus récentes ont montré que TGH a joué un rôle dans l'émergence du pathogène humain professionnel de son ancêtre environnemental.

Notre compréhension de la pathogenèse de la tuberculose est limitée par le fait que la plupart des facteurs de virulence caractérisés chez *M. tuberculosis* à ce jour se retrouvent chez l'espèce MNT environnementale et moins virulente, *M. kansasii*. Notre laboratoire a déjà identifié 55 gènes spécifiques au CMTB qui ne se trouvent pas dans les MNT et présentent des signes d'hérédité horizontale. Pour étudier le potentiel d'une pathoévolution dirigée par la TGH spécifique à *M. tuberculosis*, nous avons émis l'hypothèse que *M. kansasii* peut être utilisé comme un proxy pour le dernier ancêtre commun MNT-CMTB.

Pour évaluer la validité de l'utilisation de *M. kansasii* en tant qu'organisme modèle, nous y avons introduit la paire de gènes *Rv3377-8c* nécessaire pour fabriquer le lipide spécifique de *M. tuberculosis*, 1-TbAd. Le mécanisme menant à la production de 1-TbAd a été précédemment impliquée dans l'arrêt de la maturation phagosomale dans les macrophages. Dans ce travail de preuve de concept, nous avons montré que *M. kansasii*::Rv3377-8c, le mutant de gain de fonction généré a produit avec succès 1-TbAd, et que sa production n'avait aucune conséquence sur le comportement général *in vitro* du mutant. Nous avons également montré que la production de 1-TbAd a amélioré la résistance du mutant aux environnements acides, le seuil de croissance passant d'un minimum de 5,4 à 4,9. Malgré l'augmentation de la résistance aux acides *in vitro*, la production de 1-TbAd n'a pas conféré un avantage dans les modèles d'infection MDMO murine naïves ou activés. Le mutant a cependant induit une augmentation modeste de la production de cytokines pro-inflammatoires dans ce modèle. Enfin, la production de 1-TbAd n'a eu aucun effet sur les modèles *in vivo* d'infection pulmonaire à dose faible et élevée dans des souris.

Nos résultats ont collectivement validé l'utilisation de *M. kansasii* en tant qu'organisme modèle pour étudier l'effet de TGH sur la pathoévolution de *M. tuberculosis.* Bien que 1-TbAd

n'ait pas conféré de phénotype *in vivo*, nous avons démontré qu'il joue un rôle dans la résistance à l'acide. Notre objectif final est de construire une collection de mutants de gain de fonction afin de caractériser le rôle de chacun des 55 gènes susmentionnés, dont la plupart ont des fonctions inconnues.

CHAPTER I

Introduction and literature review

1. Mycobacteria

1.1. The *Actinobacteria* phylum

The *Actinobacteria* phylum is a complex bacterial group comprised of a heterogeneous assortment of Gram-positive organisms clustered together due to the high GC content within their genomes, with %GC ranging from 51 to 70 depending on the genus [1].

1.2. The *Mycobacterium* genus

The *Mycobacterium* genus is part of the *Actinobacteria* phylum and has its own family *Mycobacteriaceae*. Mycobacteria are generally aerobic non-motile bacilli that can be 1.0-10 µm in length. They have a notably hydrophobic, waxy and complex mycolic acid-rich cell wall that has an outer membrane; a characteristic that is unusual for Gram-positive bacterial genera [2]. They are readily identifiable by the Ziehl-Neelsen stain due to their acid-fastness (resistance to alcohol-induced decolourization). Colony morphology varies drastically and is species-dependent, ranging from smooth to rough and raised to flat, with some altering between different morphotypes [3-5].

1.3. Mycobacterial phylogeny

There are over 150 recognized species within the *Mycobacterium* genus and they can be sub-classified into larger complexes such as the *Mycobacterium tuberculosis* Complex (MTBC) and the *Mycobacterium avium* Complex (MAC) or smaller clades such as the *M. kansasii* Clade containing the closely related *M. kansasii* and *M. gastri*. The optimal growth temperature ranges from as low as 25 to higher than 50 degrees Celsius, based on the species of interest. They can be classified based on chromogenicity (the ability to produce pigmentation) using the Runyon classification, with photochromogens changing colour after exposure to light, scotochormogens changing colour in the dark and non-chromogens remaining pale yellow or tan at all times [6]. However, mycobacteria are most effectively classified into two main groups that fall in line with phylogenetic analyses using different molecular techniques of classification

based on genomics: (1) rapid-growing mycobacteria and (2) slow-growing mycobacteria [7, 8]. Membership to these groups depends on the average doubling time for each species, with the cut-off being the ability to form mature colonies on solid media within 7 days of plating:

(1) Rapid-growing mycobacteria are usually saprophytic environmental microorganisms. The representative laboratory organism in this group is the non-pathogenic *M. smegmatis*. Some rapid-growing species may cause opportunistic diseases [9]. These include accidental infections due to contaminated medical supplies such as *M. fortuitum* and pulmonary *M. abscessus* infections in CF patients [10].

(2) Slow-growing mycobacteria include the most established mycobacterial pathogens such as different members of the MTBC and MAC, as well as *M. kansasii* and *M. marinum*. Although some are environmental and only rarely cause disease, others have no environmental niche, and their expansion is restricted by the availability of their specific host [11].

There have been many accounts attempting to produce the most predictive phylogenetic tree to establish inter-relationships between mycobacterial species. The analysis that this project bases itself on compares 20 highly conserved housekeeping genes across 16 mycobacterial species with two other *Actinobacteria* members acting as outgroups: *Nocardia farcinica* and *Rhodococcus jostii* [12]. The resulting phylogenetic tree successfully classifies mycobacteria into the two aforementioned groups, with fast-growing mycobacteria emerging at an earlier point compared to slow-growing mycobacteria. This observation is in line with the current dogma that slow-growers are a more recent step in mycobacterial evolution [13].

2. *Mycobacterium tuberculosis*

2.1. History and epidemiology

Although a hotly debated topic, the current consensus is that modern-day *M. tuberculosis* has been cohabitating within humans upwards of at least 10,000 years. Throughout history, TB disease has earned many names including *consumption*, *phthisis* and *White Plague*. It finally got its contemporary nomenclature, tuberculosis (TB) when it became associated with tubercle (or granuloma) formation in the lungs, with the infectious agent

identified by Robert Koch as *Mycobacterium tuberculosis* or Koch's Bacillus after a series of experiments in rabbits [14]. These discoveries also led to the development of the now widely used Koch's postulates for infectious diseases [15]. Today, *M. tuberculosis* is believed to reside quiescently in one-third of the global population, based on indirect measurement of infection (tuberculin skin test reactors) and only progresses to TB disease in a subset (5-10%) of said population [16]. According to the World Health Organization's Global Tuberculosis Report 2017, although treatment is readily available and effective for drug-susceptible forms of the bacteria, there were ~1.3 million TB deaths worldwide in 2016 alone among HIV-negative individuals, making it the deadliest infectious disease today [17]. These deaths are mainly associated with drug-resistant genotypes such as MDR- and XDR-TB and and extra 374,000 deaths are attributable to TB-HIV co-infections [18]. In HIV-positive individuals, the progression to TB disease is accelerated to 5-10% per year.

2.2. Method of infection

Only individuals with pulmonary TB disease can transmit the bacteria to other individuals [16]. *M. tuberculosis* spreads through aerosol generation during expulsive efforts from the lungs, such as coughing and sneezing. The microscopic aerosols may remain in the air for hours and infect individuals through inhalation. However, although TB mainly manifests as a pulmonary infection, the bacteria can also infect other parts of the body, including the bones, meninges and lymphatics or disseminate systemically [19]. Extra-pulmonary forms of TB are non-infectious.

2.3. Pathophysiology

The first line of defense against invading bacilli is at the level of the upper airways, wherein bacteria get stuck onto mucus and expelled by upward ciliary movement [20]. However, bacilli that manage to bypass this mechanism end up in the lower airway within the alveoli, where they are actively phagocytosed by resident alveolar macrophages (AMs) [21, 22].

Upon activation, AMs release pro-inflammatory cytokines and chemokines that attracts other immune cells, such as neutrophils and homing macrophages in a third line of defense to kill still-replicating bacteria [23, 24]. During that time, dendritic cells traffic the intracellular

bacteria to draining lymph nodes, where they present antigens to T cells, bridging the gap between innate and adaptive immune events [25]. The recruitment of innate immune cells as well as T and B cells to the affected area, which in most cases is the lungs, leads to eventual granuloma formation [26]. Granulomas are aggregates of immune cells that surround the infected macrophages and may eventually lead to nutrient deprivation of the bacterial cells. At this point, bacteria are eliminated or chronic infection occurs [27]. Progression to TB disease may happen at any time during the lifetime of the individual, although most cases occur within 18-24 months of infection. This progression from TB infection to TB disease is associated with granuloma resolution and bacteria expansion [28].

2.4. The *M. tuberculosis* genome

According to the Tuberculist knowledge base, the genome of the *M. tuberculosis* H37Rv lab strain is made of a singular circular chromosome [29]. The genome is 4,411,532-bp long, 91.2% of which correspond the 4,018 protein-coding genes. Other features of the genome include 13 pseudogenes, 45 tRNA, 3 rRNA, 30 ncRNA, and 2 miscRNA genes. The GC content is at an average of 65.9% along the whole chromosome.

3. *Mycobacterium kansasii*

3.1. History and epidemiology

First described in 1953 by Buhler and Pollack, *M. kansasii* is characterized as a photochromogenic mycobacterium that produces a characteristic yellow pigmentation (carotenoids) when exposed to light sources for long durations of time [30-32]. It is one of the most common non-tuberculous mycobacteria (NTM) infections worldwide [33-35]. When isolated from humans, it is considered to be the cause of a clinical infection, as opposed to a contaminant or a harmless colonizer. Although it has been reported in immunocompetent individuals and was mainly associated with mine workers in the pre-acquired immunodeficiency syndrome (AIDS) era, *M. kansasii* prevalence increased following the onset of the human immunodeficiency virus (HIV) epidemic [34]. *M. kansasii* infection is also associated with underlying obstructive pulmonary disease and cystic fibrosis. Despite their epidemiological differences, *M. kansasii* shares many microbiologic similarities with *M.*

tuberculosis, including urease production and other characteristics previously used to specifically identify *M. tuberculosis* [36].

To date, there are seven identifiable subtypes of *M. kansasii* based on Restriction Fragment Length Polymorphism (RFLP) analysis, of which only two have major clinical relevance, with the others almost exclusively isolated from environmental samples [37]. More specifically, subtype I is the most prevalent clinical isolate but is rarely grown from environmental sources, while subtype II is less frequent in healthy individuals but more often isolated from immunocompromised people [38-40]. Counterintuitively, Subtypes III-VII are more prominent in nature, but their abundance does not translate into a higher infection rate when compared to the more pathogenic subtypes I and II [41].

3.2. Method of infection

Unlike *M. tuberculosis*, there are no reports of human-to-human transmission with *M. kansasii* [42]. Therefore, human infection leads to a dead end for *M. kansasii*. The environmental mycobacterium is most commonly isolated from artificial water supplies and infection occurs as a consequence of direct inoculation/ingestion into the airway [43, 44].

3.3. Pathophysiology

Both *M. marinum* and *M. kansasii* are closely related to the MTBC. However, *M. marinum* predominately causes subcutaneous infections in humans, known as Fish-tank granulomas, and localized lymphangitis [45, 46]. *M. kansasii* on the other hand causes a more relevant human disease since it infects at the level of the lungs, much like the most common form of *M. tuberculosis* infection [47, 48]. In fact, pulmonary *M. kansasii* disease is similar to and often indistinguishable from tuberculosis [49]. However, the symptoms are more chronic and less severe than is typically seen in *M. tuberculosis*-infected individuals. Once inside the airway, *M. kansasii* has to face the same obstacles previously described for *M. tuberculosis*.

3.4. The *M. kansasii* genome

The genome of the *M. kansasii* ATCC 12478 lab strain is made of a circular chromosome [50]. It is 6,432,277-bp long. The single chromosome encodes 5,712 protein-coding genes. Other features of the genome include 10 pseudogenes, 46 tRNA, 3 rRNA and 2 ncRNA genes. The GC content is at an average of 66.2% along the whole chromosome. Additionally, a small 144,951-bp plasmid (pMK12478) also makes part of the *M. kansasii* ATCC 12478 genome with an average GC content at 65.8% and encodes 154 protein-coding genes. However, this plasmid is not found in all *M. kansasii* isolates and has been deemed dispensable for clinical disease.

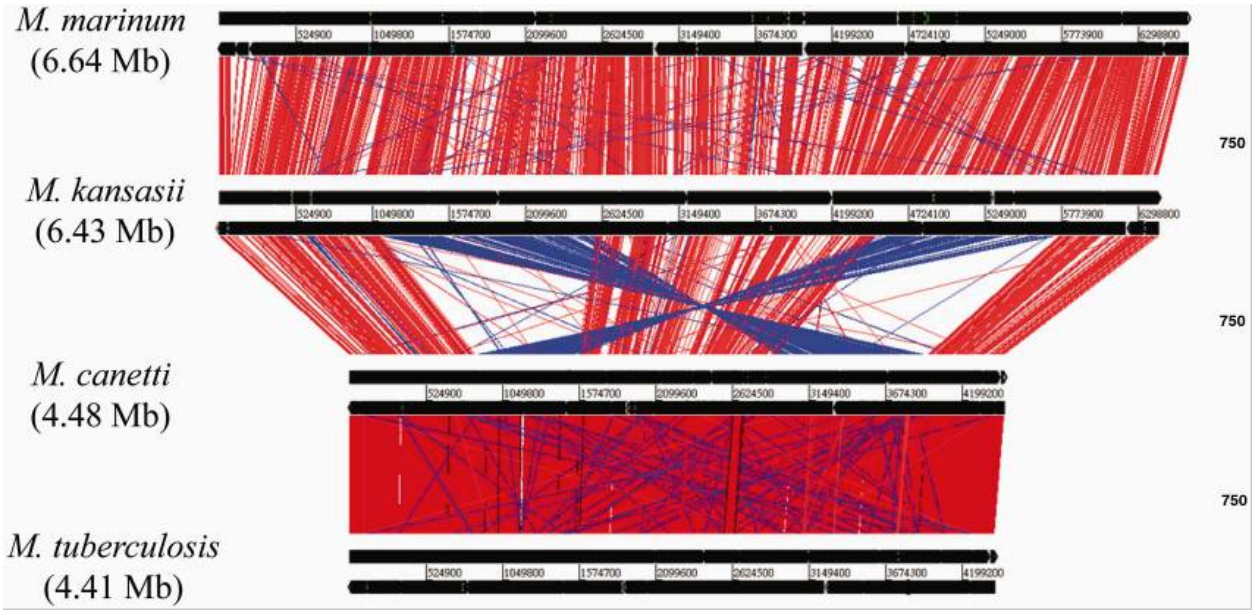

Figure IA. Genomic comparison of *M. marinum*, *M. kansasii*, *M. canetti* and *M. tuberculosis* – adapted from Wang (2015). The red lines represent DNA segments with positions that have been conserved between the two genomes being directly compared to one another. The purple lines represent DNA segments that have had their positions rearranged between the two genomes being directly compared to one another.

In Wang et al (2015), detailed genomic comparisons were performed to assess the similarities between *M. tuberculosis* and *M. kansasii*, shedding some light on the emergence of *M. tuberculosis* as a professional human pathogen [50]. Compared to *M. canetti*, an earlier emerging species within the MTBC, and *M. tuberculosis*, the genomes of *M. marinum* and *M. kansasii* are 2 Mbp larger and exhibit two 1-Mbp genomic inversions. When compared to *M. tuberculosis* more closely at the level of gene composition and function, *M. kansasii* has less

toxin-antitoxin systems and overall genes devoted to regulation and cell signaling. *M. kansasii* also shares 346 genes with other NTMs associated with lipid metabolism, energy manipulation and biosynthesis that have been deleted in the *M. tuberculosis* genome and at least partly explain the greater adaptation to diverse environments seen in NTMs.

Interestingly, the results revealed that most genes that have been attributed virulence functions within *M. tuberculosis* are present and conserved in the *M. kansasii* genome. Notably, the PhoPR 2-component transduction system and DosR/S/T regulon, as well as the ESX-1 secretion system and 65% of all antigens encoded by *M. tuberculosis* are detected in the *M. kansasii* genome. These observations, together with phenotypic characterizations and experimental models, are summarized in the table below and beg the question: *If they have so much in common, what specifically makes M. tuberculosis the better pathogen?*

	M. tuberculosis	***M. kansasii***
Disease	Host-adapted	Opportunistic
Transmission	Human–human	Water–human
Reservoir	Human	Environment
Genome size	4.4 Mb	6.4 Mb
Protein genes	4,018	5,866
% GC content	66	66
Presence of PhoPR 2-component system	Yes (*Rv0757/8*)	Yes (*Mkan_11335/30*)
Presence of DosR/S/T regulon	Yes (*Rv3133c/3132c/2027c*)	Yes (*Mkan_22405/22410/22410*)
# PE/PPE proteins	153	213
# *mce*-associated genes	36	36
Proportion of metabolism genes	0.002316423	0.0084918
Proportion of toxin/antitoxin genes	0.013899	0.001019
Presence of ESX-1	Yes	Yes
T-cell antigens	77	53
Replication in mouse lungs	4.5 log	0.5 log

Table IA. Highlighted similarities and differences between *M. tuberculosis* and *M. kansasii* – adapted from Wang (2015).

4. Mycobacteria and the macrophage

Alveolar macrophages are the first immune cell type encountered by pulmonary pathogens [21, 22]. They are phagocytic in nature and do not induce massive inflammation on their own. When faced with a threat, these cells play an important role in regulating an evenly balanced pro- and anti-inflammatory response in order to (1) eradicate the intruders and (2) protect the airways from excessive inflammation [51, 52]. Once phagocytosis occurs, foreign bodies are engulfed into a vacuole known as a phagosome, which quickly acidifies and fuses with lysosomes to create a structure known as a phagolysosome. Most internalized pathogens succumb to the harsh environment of the phagolysosome including the intense acidification through the action of vesicular proton ATPases and nutrient deprivation as well as pH-dependent lysosomal hydrolases and the production of reactive oxygen and nitrogen species that actively break down the cells [53]. However, a few intracellular pathogens, such as *M. tuberculosis* have developed sophisticated mechanisms to counter the phagosomal attack and abrogate the line of defense [54-57].

4.1. Mycobacteria and phagosomal pH

pH measurement has been linked with antimicrobial phagosomal activity since Elie Metchnikoff used indicator dyes to prove that acidification occurs after exposure to bacteria in 1905 [58]. The pH of the phagosome drops from physiological to ~5.0 within the first 15-60min after the onset of phagocytosis [59]. Among other bacterial genera, mycobacterial species including *M. avium*, *M. kansasii*, *M. bovis* and *M. leprae* and *M. tuberculosis* inhibit phagosomal maturation in initially naïve macrophages [50, 60, 61]. *M. tuberculosis* maintains the intraphagosomal environment at an only mildly acidic pH of 6.2, keeping it within the pathogen's optimal growth range. However, seeing that it shares this ability with other mycobacterial species, even ones that are distantly related, it has been inferred that this capacity to block phagosomal maturation is not specific to *M. tuberculosis* among members of the genus.

Paradoxically, once cell recruitment occurs and innate immunity is at its peak, the alveoli become a milieu of immune activation where IFN-γ is readily detectable. In these settings, the interruption of phagosomal maturation exerted by *M. tuberculosis* is alleviated, at which point

the pH drops down to 4.5-5 [61]. This environment becomes much less hospitable for the bacteria, leading to a growth hindrance and higher susceptibility to killing. In addition, newly recruited immune cells overcome the inhibition of phagosomal maturation without delay, making this characteristic of mycobacteria less relevant as infection progresses. Therefore, *M. tuberculosis* has to have acquired mechanisms to survive within low pH environments that are distinct from those required during the inhibition of phagosomal maturation.

Whether or not acidification by itself is bactericidal or bacteriostatic within the phagosome is yet to be determined. However, it is well established that the pH drop at least indirectly leads to bacterial killing through the activity of pH-dependent enzymes and factors [53]. What *in vitro* studies have been able to demonstrate is the variability of survivability results depending on the composition of the media used in the assay and the initial bacterial density inoculated into the media. In addition, certain saprophytic mycobacteria can grow at even more extreme pH levels than do host-restricted pathogenic mycobacteria [62]. However, that does not nullify the possibility that *M. tuberculosis* may have acquired factors to help it survive more acidic conditions. This is especially relevant given that, unlike most other mycobacteria, *M. tuberculosis* thrives within the acidified phagosome.

5. Mycobacterial speciation and pathoevolution

5.1. Genetics of bacterial evolution

The concept of evolution is defined as the attempt of a species to survive within an equilibrium-altering environment and out-compete others for specific sources of nutrients [63]. Failed evolution is when a genetic event leads to a new bacterial genotype that is less equipped to thrive in its environment and becomes overshadowed by and diluted out of the pre-existing population. Successful evolution leads to the production of offspring that are better equipped than their ancestor and overall population to handle the challenges within their environment and expand through positive selection, or offspring that non-selectively expand through genetic drift.

5.2. Horizontal gene transfer (HGT) in prokaryotes

Evolution occurs as a consequence of a series of changes at the genetic level, characterized by the loss or modification of pre-existing genetic content through vertical inheritance and/or the acquisition of new genetic material from external sources through horizontal or lateral inheritance. If only vertical inheritance occurs, it eventually leads to a stripping down of the genome into the strictly essential genetic content, a phenomenon known as reductive evolution [64, 65]. However, the pathoevolution of originally non-pathogenic environmental microorganisms is more likely to be caused by an initial uptake of new genetic material that aids in disease causation or survival within a specific niche of organisms [66, 67].

5.2.1. Effects of horizontal gene transfer on bacterial fitness

In the cases where it is successfully completed and the DNA is stable within the recipient bacterial cell, the acquisition of new genetic material can have neutral, destructive or constructive effects based on its content and quality [68, 69]. For example, a redundant gene with no additive potential can cause a duplication within the genome that does not provide an instantaneous advantage and may or may not survive the next rounds of replication, depending on random genetic drift. However, a toxic or incomplete gene product may cause harm to the bacterial cell, leading to a reduced capacity to replicate or withstand a given stressor and the instability of the newly acquired DNA within the genetic pool. Finally, a newly acquired gene may be advantageous for bacterial survival within a specific environment, allowing the new genotype to thrive through selective pressure exherted by, for example, acidic stress or antibiotic treatment. The best-studied fitness-increasing traits that are disseminated through HGT are antibiotic resistance, metabolism-related functions and virulence features .

5.2.2. Mechanisms of horizontal gene transfer

The acquisition of new genetic material occurs when a heterogeneous population of organisms co-habit within a defined space, wherein mixing of the exponentially growing genetic pool occurs abundantly and acquisition of new DNA becomes more possible with time

[70]. Although transfer among closely-related organisms is more likely to successfully occur from start to finish, new genetic content can be acquired across phyla and some new evidence has even suggested that this phenomenon may also occur across kingdoms [71].

The main mechanisms of horizontal gene transfer are (1) natural transformation, (2) conjugation, (3) transduction, (4) integrons and (5) nanotubes:

(1) Natural transformation requires that bacteria become temporarily competent in response to a specific stressor within their environment, including but not limited to temperature shock, increased cell density and starvation [72, 73]. Competent bacterial cells pick up cell-free DNA that has been released by nearby live or dead organisms. This free-floating genetic content

(2) can be used as a source of nutrients or may get integrated into their genome and becomes functional DNA.

(3) Conjugation is an active process in which two bacterial cells form junctions or pores in which DNA, for example in the form of a plasmid coding for a sex pilus (type IV secretion system machinery), can freely pass from a donor cell to a recipient cell [74, 75].

(4) Transduction is the process in which genetic information from a donor cell is packaged within a bacteriophage particle during phage assembly, then released following the lytic cycle when virus particles are produced en masse. Subsequently, a newly infected recipient cell can acquire the pre-packaged DNA in the form of a prophage, which gets integrated into the bacterial genome [76, 77]. This allows the bacteriophage genome to replicate with the host chromosome along with the carried DNA fragments.

(5) Integrons are naturally occurring DNA elements found in bacterial chromosomes that can integrate and express multiple unrelated genes that do not otherwise have their own promoter [78].

(6) Nanotubes are non-sex pilus structures that allow inter-cell transfer of DNA and proteins [79]. These tubes are more promiscuous in their transfer than the conjugative pili and depends on the state of nutrition of the cells.

5.3. *M. tuberculosis* evolution

The currently accepted model for the emergence of *M. tuberculosis* is a path starting from an ancient aquatic predecessor (the ancestor of all modern-day mycobacteria) that led to the emergence of a smooth tubercle bacillus, like modern-day *M. canetti* isolates, resulting in an eventual shift to the successful human-adapted *M. tuberculosis* . There are 7 *M. tuberculosis* lineages circulating within the global human population and their abundance varies with geography, disproving the hypothesis that *M. tuberculosis* is globally homogeneous [80, 81].

5.3.1. Evidence of HGT in the making of *M. tuberculosis*

When the genomes of different members of the MTBC were compared to one another, the relative lack of genetic diversity among the group brought forward the hypothesis that *M. tuberculosis* and other pathogenic mycobacteria evolved strictly through modifications and deletions of genetic content [82]. Such was also thought to be the case of *M. leprae*, the uncultivable cause of leprosy in humans, which is known for its degenerative evolution. Consequently, it has the largest number of pseudogenes and smallest genome within the *Mycobacterium* genus [65, 83]. The model at the time suggested that the ancestor of modern-day *M. tuberculosis* lost "anti-virulence" genes in order to become completely adapted to its host, which is not entirely farfetched since anti-virulence-related genes have been characterized in other bacterial genomes, including some mycobacterial species [84-86]. However, more recent advances in molecular epidemiology, including the now wide availability and reduction in costs of whole genome sequencing, led to a drastic change in the accepted model of TB evolution [87]. This shift came to light through independent lines of investigation: 1) compositional analyses, looking at nucleic acid composition within the *M. tuberculosis* genome [88] and 2) comparative analyses, building upon the ongoing characterization of the genomic sequences of NTMs that shed light about the role of HGT in the creation of the ancestral human-adapted species [12].

In addition, the hypothesized ancestor had to overcome many obstacles in order to become what is now known as the MTBC. Such obstacles include, for example, the many stresses of living inside a macrophage phagosome and facing the subsequent components of mammalian immunity, which needed to be overcome for an effective establishment of a host-

tropic/restricted bacterium. Since *M. tuberculosis* is only found within human beings, it had to extensively adapt in order to co-evolve with the human population in order to become a successful human pathogen [89].

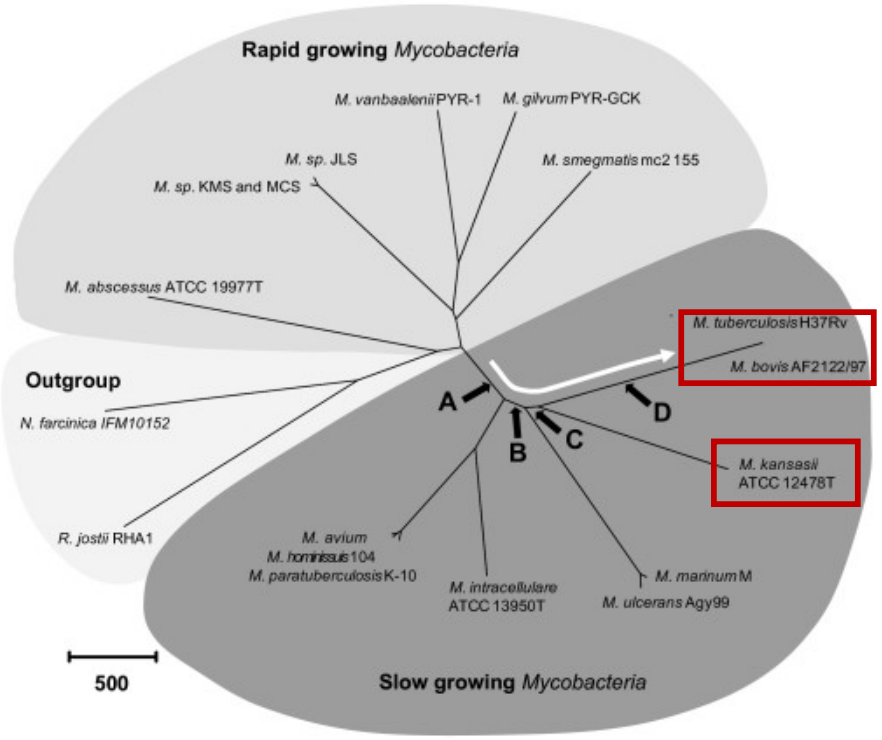

Figure IB. Phylogenetic tree of mycobacteria – adapted from Veyrier (2009). The phylogenetic tree of mycobacterial relationships was constructed using 20 housekeeping genes across 18 bacterial species. The tree is divided into three lineages: the outgroup (light) includes *N. farcinica* and *R. jostii*, two other actinobacteria, the rapid growing mycobacteria (darker) includes 7 mycobacterial species and the slow growing mycobacteria (darkest) includes 9 mycobacterial species. Letters and arrows delineate the common ancestor of the four groups leading to the step-wise emergence of *M. tuberculosis* starting from the common ancestor of all slow-growing mycobacteria. The two red rectangles highlight the MTBC and its most closely related NTM species, *M. kansasii*. The scale indicates the number of different amino acids.

In *Veyrier et al* (2009), the sequences of 16 mycobacterial species from across the board were compared in order to identify HGT events that took place in the evolution of the common ancestor within four different groups within the *Mycobacterium* genus: Group A containing all slow growing mycobacteria, Group B containing *M. marinum*, *M. kansasii* and the MTBC, Group

C containing *M. kansasii* and the MTBC and Group D containing the MTBC members [12]. This phylogenetic analysis established the stepwise divergence of the different slow-growing mycobacteria from the common ancestor and allowed the grouping of more closely-related species together for subsequent analysis.

Using an in-lab-generated software called MycoHIT, sequences specific to each group were identified through a BLAST utility by comparing the group's core genome with all the sequences found in all other mycobacteria and the outgroup members, *R. jostii* and *N. farcinica*. Based on the generated sequences, HGT events were characterized as sequences that have diverging GC content and have evidence of horizontal acquisition, such as close-by insertion elements that identifies plausible HGT events based on %GC (GC deviation) and the presence of an HGT vehicle, for example a predicted transposable element. Collectively, from the time of emergence of all slow-growing mycobacteria, *M. tuberculosis* has acquired 137 genes through horizontal inheritance. Out of these hits, 55 were only present in the MTBC, with no identifiable orthologs in other mycobacteria or the outgroups. Some of these genes were clustered into small genomic islands that were likely acquired in conjunction with one another. The fact that they were not present in the *Actinobacteria* phylum led to conclusion that they were acquired from external sources.

5.4. The *Rv3376-8c* genomic island

Rv3376-8c is a 3.1-kb genomic island that represents 3 of the 55 horizontally-acquired genes in the making of *M. tuberculosis* from its common ancestor with *M. kansasii* [12, 88]. In accordance with features that explain a likely HGT event, this island is found in close proximity to transposases and is composed of 54.7% GC compared to the average of 65.9% GC of the *M. tuberculosis* genome.

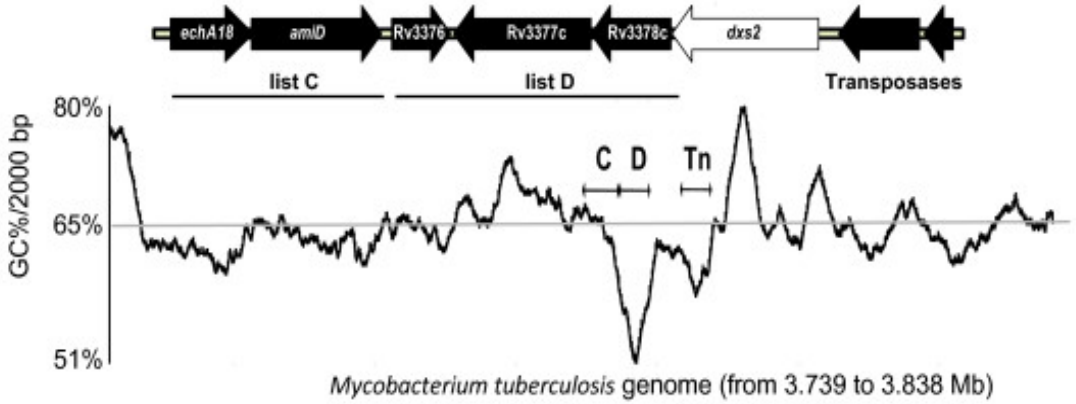

Figure IC. The Rv3376-8c genomic island – adapted from Veyrier (2009). A line-and-arrow representation of the *Rv3375* cluster spanning from Rv3374 to Rv3381c. List C includes genes acquired through HGT by the common ancestor of *M. tuberculosis* and *M. kansasii* while List D includes genes acquired from the ancestor of *M. tuberculosis*. Genes in black are HGT-associated (rightmost are HGT vehicles – transposases). The bottom graph shows the GC% of a 100-kb DNA region surrounding the *Rv3375* gene cluster to emphasize the drop in GC% within the *Rv3376-8c* genomic island and its neighbouring transposase elements as an irregular event within its surroundings.

5.4.1. *Rv3376*

Rv3376 is a 654-bp gene transcribed on the positive strand of the *M. tuberculosis* genome [29]. It encodes a 217-aa hypothetical protein that shares a similar amino acid sequence to several other bacterial proteins, most notably hydrolases/HAD-family phosphatase. It is highly conserved among the MTBC members (100% identity) and *M. canettii* (99% identity) but there are no orthologs found in any other mycobacteria, including closely-related NTMs. Based on transposon mutagenesis studies, it is a non-essential gene for *in vitro* growth but required for survival in primary murine macrophages [90, 91].

5.4.2. *Rv3377c*

Rv3377c is a 1506-bp gene transcribed on the negative strand of the *M. tuberculosis* genome [29]. It encodes a 501-aa halimadienyl diphosphate synthase/class-II terpene cyclase. Although it has orthologs in some members of the MTBC, the WT sequence and functional enzyme are only found in *M. tuberculosis* strains. There are no orthologs found in other mycobacteria, including closely-related NTMs. It is non-essential for *in vitro* growth in one

transposon mutagenesis study and essential in another, and it is required for the arrest of phagosomal maturation/acidification and survival in macrophages [90, 92, 93].

5.4.3. *Rv3378c*

Rv3378c is an 862-bp gene transcribed on the negative strand of the *M. tuberculosis* genome [29]. It encodes a 296-aa diterpene synthase. It is fully conserved in some members of the MTBC, with others having non-functional mutations. It is required for both *in vitro* growth and required for the arrest of phagosomal maturation/acidification and survival in macrophages [90, 93]. Together with *Rv3377c*, the two genes have been shown to be necessary and sufficient for the production of 1-tuberculosinyladenosine (1-TbAd), an adenosine-linked diterpene moiety, in bacteria given the presence of its precursor, geranylgeranyl pyrophosphate (GGPP) [94, 95].

5.5. Geranylgeranyl pyrophosphate (GGPP)

GGPP ($C_{20}H_{36}O_7P_2$) is required for the biosynthesis of many terpenes as well as geranylgeranylated proteins in higher-order eukaryotes. In plants, it acts as a precursor for the production of carotenoids and chlorophylls, among other pigment-related compounds [96]. The biosynthesis of GGPP requires the activity of a GGPP-synthase, an enzyme that is found ubiquitously among bacteria [97]. More specifically, within the *Actinobacteria* phylum, GGPP-synthases *Rv0989c*, *Rv3383c* and *Rv0562* are conserved among most bacteria (including the outgroup) found in the aforementioned phylogenetic tree [98].

GGPP synthesis is outlined in the figure below and is conserved among different taxonomic domains. Whereas the pathway requires two enzymes (FPP-synthase and GGPP-synthase) in yeasts and mammals, it only requires GGPP-synthase in plants and bacteria, but the precursors and end product are the same for both cases.

When strictly comparing its differential uses, GGPP is used as an intermediate in the biosynthesis of 1-TbAd by *M. tuberculosis* [95, 99] while in *M. kansasii*, it is part of the biosynthetic pathway for the production of the environmental bacterium's different carotenoid pigments [31].

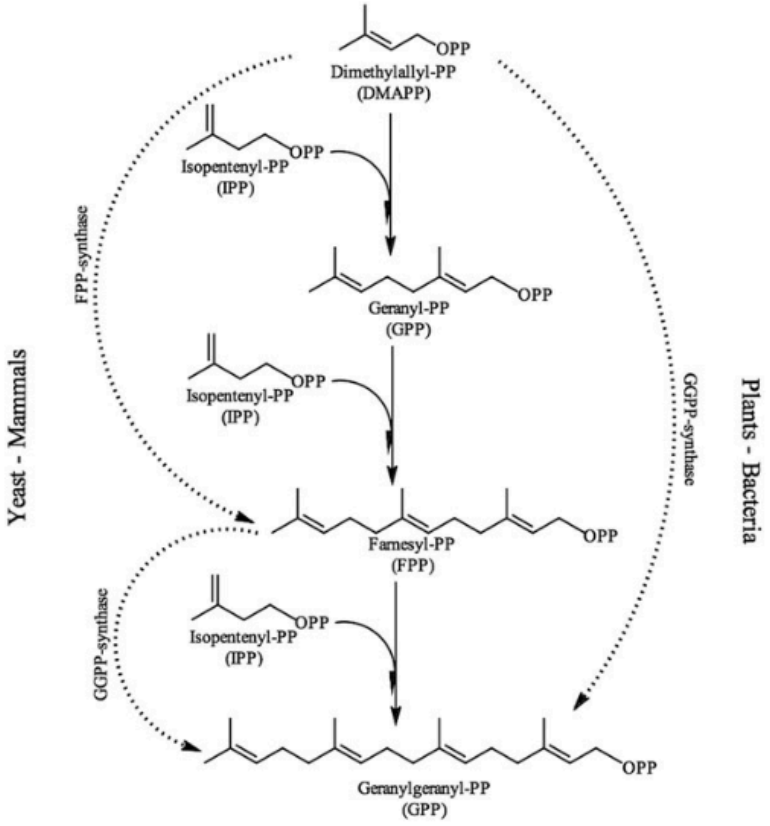

Figure ID. The conserved biosynthesis pathway of GGPP – adapted from Stange (2016).

5.6. 1-Tuberculosinyladenosine (1-TbAd)

Isotuberculosinol (isoTB)($C_{20}H_{34}O$) was believed to be the end product of the biosynthesis cascade of the then-called "isoTB operon", which includes *Rv3377-8c* [99, 100]. It was also implicated in the arrest of phagosomal maturation and acidification [98, 101]. Only recently discovered in 2014, 1-TbAd ($C_{30}H_{45}N_5O_4$) , the final product of the enzymatic activity of *Rv3377c* and *Rv3378c* on GGPP, represents 1% of all lipids produced by *M. tuberculosis* [94, 95]. The biosynthetic pathway of 1-TbAd is shown in the figure below.

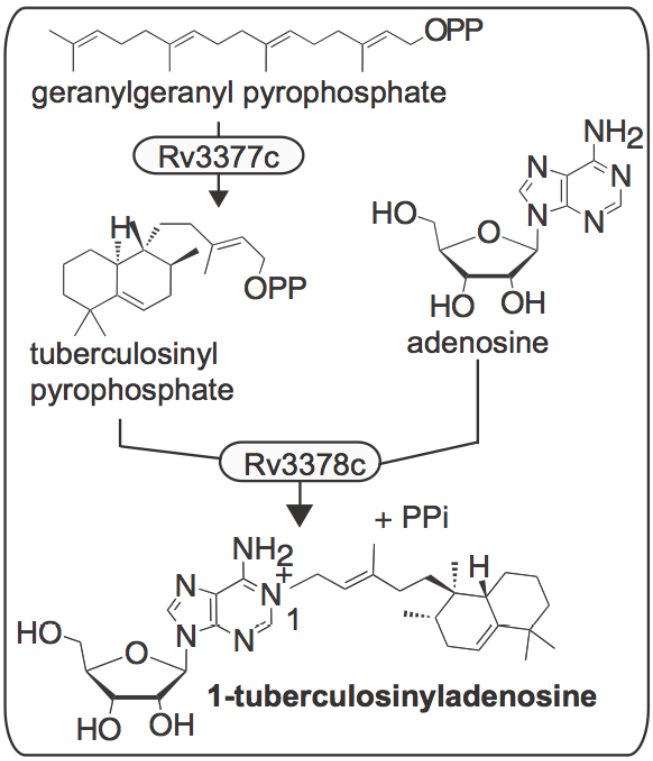

Figure IE. The biosynthetic pathway of 1-TbAd from GGPP – adapted from Layre (2011).

1-TbAd is an adenosine-linked diterpene lipid molecule that is uniquely synthesized by *M. tuberculosis*. The functions 1-TbAd serves for *M. tuberculosis* are yet to be determined. However, (1) *Rv3377c* and *Rv3378c* mutants are defective in phagosomal maturation and unfit to survive in macrophages , (2) the activity of the Rv3377c and Rv3378c enzymes increases in Mg^{2+}-deficient environments and (3) isoTB, now identified as a transient precursor for 1-TbAd, has been implicated in mediating pH within the phagosome. 1-TbAd is found both as a cell-associated lipid and in culture filtrates and is readily detectable in infected mouse lungs as well as a portion of sputum, serum and CSF samples of infected mice and individuals [95, 102, 103]. The *Rv3377-8c* gene pair has side-to-side homologs recently discovered in *Herpetosiphon aurantiacus* that are implicated in diterpene production from GGPP [104]. However, the function of diterpenes in this predatory bacterium remains unknown.

Rationale and research objectives

Unlike *M. tuberculosis,* most species within the genus *Mycobacterium* are environmental microorganisms that are generally harmless to people. Due to its smaller genome, it has been inferred that *M. tuberculosis* emerged as a pathogenic organism through genome reduction. However, more recent evidence has uncovered the presence of horizontally-acquired genes in the *M. tuberculosis* genome that lack homologues in environmental mycobacteria. Given that there is a current lack in species-specific virulence factors for *M. tuberculosis*, we set out to test whether genes acquired in this manner might have contributed to the pathoevolution of the professional human pathogen.

Our overarching hypothesis is that HGT has contributed to the emergence of virulent *M. tuberculosis*, and that *M. kansasii*, an opportunistic lung pathogen associated with artificial water supplies, can be used as a proxy for the ancestor of *M. tuberculosis,* in order to study the effect of HGT on the potential for pathogenesis. More specifically, we hypothesize that *M. kansasii* can be used to recreate the steps during the emergence of *M. tuberculosis* via heterologous complementation.

To test this hypothesis, our experimental aims for this project were:

1. Complement the *M. tuberculosis*-specific *Rv3377-8c* gene pair into *M. kansasii* and ensure the successful expression of the gene products as well as the production of the lipid 1-TbAd.
2. Determine whether *Rv3377-8c* complementation and 1-TbAd production alter the *in vitro* behaviour of the newly generated *M. kansasii* mutant.
3. Test whether *Rv3377-8c* complementation and 1-TbAd production modify the course of experimental *ex vivo* and *in vivo* infection.

CHAPTER II

Heterologous complementation with *Rv3377c* and *Rv3378c* elicits the production of 1-tuberculosinyladenosine in *Mycobacterium kansasii*

This section is adapted from an article in preparation for publication.

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Manuscript in preparation.

1. Abstract

Mycobacterium kansasii is an environmental mycobacterium that causes tuberculosis-like lung disease, but does not use humans for its dissemination. To understand the emergence of *M. tuberculosis* as a host-adapted pathogen, we have used *M. kansasii* as a proxy for their common ancestor, identifying both commonalities – including known *M. tuberculosis* virulence factors – and differences, such as genomic regions unique to *M. tuberculosis* that were presumably acquired via horizontal gene transfer (HGT).

In the current study, we induced the heterologous expression of the *M. tuberculosis*-specific low GC-content gene pair *Rv3377-8c* in *M. kansasii*. The gain-of-function mutant so engineered was shown to produce additional lipid species by adenosine-labeled thin layer chromatography (TLC). Liquid chromatography-mass spectrometry (LC/MS) confirmed that the product of the *M. kansasii* mutant had the same collision-induced dissociation spectra as *M. tuberculosis*-produced 1-tuberculosinyladenosine (1-TbAd). Further investigations revealed that 1-TbAd production did not affect *M. kansasii* growth and morphology in standard growth media, but did confer a growth advantage when the media was acidified to pH < 5.4. Moreover, the pH of the culture media was alkalized by the *M. kansasii* producing 1-TbAd. Production of 1-TbAd did not affect survival and persistence of *M. kansasii* in murine bone marrow-derived macrophages within 2 days post infection, and production of pro-inflammatory cytokines by these cells was minimally altered. Finally, following low- and high-dose infections of C57Bl/6 mice, production of 1-TbAd did not alter *M. kansasii* burdens at 24 hours, 1, 3, 4, 6 and/or 8 weeks post infection.

Our results indicate that *M. kansasii* can serve as a model organism to study the step-wise evolution of *M. tuberculosis* by introducing HGT genes, and that the production of 1-TbAd increased the pH of acidic media and enhanced bacterial growth at low pH, but did not confer a virulence phenotype in isolation.

2. Introduction

M. tuberculosis virulence factors are most classically established by knock-out and complementation strategies within the pathogen, supported by evidence of an attenuation of virulence in *ex vivo* or *in vivo* experimental infections [105-108]. However, while numerous virulence-associated loci have been identified with this approach, the majority of these are intact in the genome of the non-transmissible environmental *M. kansasii*. Consequently, there is currently an incomplete understanding of how *M. tuberculosis* emerged as a human-associated professional pathogen.

The main drivers of bacterial evolution are (1) the modification and/or loss of pre-existing genetic components through vertical inheritance or (2) the acquisition of new genetic information through HGT. While it was previously thought that the latter does not occur in mycobacteria, evidence obtained from our lab and others have shown that HGT events are not uncommon in mycobacterial speciation, and are associated with the step-wise emergence of different mycobacterial species [87, 88, 109-114]. For example, in Veyrier et al., a software utility called mycoHIT was used to predict the presence of 137 HGT-acquired genes throughout four broad stages within the evolution of *M. tuberculosis*, with 55 of these genes belonging exclusively to members of the MTB complex [12].

M. kansasii is readily isolated in clinical settings from pulmonary infections and we have previously shown that it can be studied in an experimental lung model [50]. Since the *M. tuberculosis-M. kansasii* common ancestor (MTMKCA), *M. tuberculosis* has acquired a number of genes through HGT [12]. Although many of these have no postulated function, the *Rv3376-8c* genomic island is known to encode a class II terpene cyclase (Rv3377c) and a tuberculosinyl transferase (Rv3378c), which together are responsible for the production of 1-TbAd [95]. 1-TbAd is exclusively synthesized by *M. tuberculosis* in nature and makes up 1-1.5% of all lipids produced by the pathogen [94]. In this study, we set out to determine whether we could use *M. kansasii* as a proxy of the MTMKCA, by asking whether we could engineer a mutant to produce 1-TbAd as a proof of concept for investigating the step-wise emergence of *M. tuberculosis* from an environmental mycobacterium.

3. Methods and materials

Bacterial strains and culture conditions

M. kansasii ATCC 12478 and *M. tuberculosis* H37Rv were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) containing 0.2% glycerol, 0.1% Tween-80 and 10% albumin-dextrose-catalase (BD Difco) in a rotating incubator set at 37°C or on Middlebrook 7H10 solid medium supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (BD Difco). Chemically competent DH5α (New England Biolabs) were grown in Luria-Bertani (LB) broth (BD Difco) in a shaking incubator at 37°C. When needed, apramycin at 60 µg/ml for DH5α (Wisent), hygromycin at 100 µg/ml for DH5α and *M. kansasii* (Wisent), kanamycin at 50 µg/ml for DH5α and *M. tuberculosis* (Wisent), BBL MGIT PANTA (polymyxin B at 12U/ml, amphotericin B at 1.2 µg/ml, nalidixic acid at 4.8 µg/ml, trimethoprim at 1.2 µg/ml and azlocillin at 1.2 µg/ml) (BD Diagnostic Systems) and/or Congo Red stain at 100 µg/ml (Sigma-Aldrich) were added to the media. Murine bone marrow was isolated from tibiae and femora and cultured in RPMI 1640 media supplemented with 1% non-essential amino acids, 1% HEPES, 10% FBS ± 1% Pen/Strep (Wisent). Bone marrow-derived macrophage (BMDMs) were differentiated through two additions of recombinant M-CSF at 100 U/ml (Peprotech) for a period of 7 days. To activate BMDMs, IFN-γ at 100 U/ml (Peprotech) and LPS at 10 ng/ml (Peprotech) were added on day 5 and 6, respectively. Cell counting was done using a haemocytometer and a 1:1 dilution of stripped cells with 0.4% Trypan Blue (Sigma Aldrich).

Cloning strategy of *Rv3377-8c*

A 2.4-kb PCR fragment of *Rv3377-8c* was generated using primers BamHI-*Rv3377-8c*-F and HindIII-*Rv3377-8c*-R (Sup. Table 1) using high-fidelity Phusion DNA polymerase (New England Biolabs). The fragment was subsequently digested with 1:2 BamHI:HindIII in NEBuffer 3.1 (New England Biolabs) overnight and the product was purified using PCR Purification Kit (Qiagen). The episomal plasmid pMV261 was digested with 1:2 BamHI:HindIII in NEBuffer 3.1 (New England Biolabs) overnight and the digested product was isolated using Gel Extraction Kit (Qiagen). The fragment was subsequently ligated into the vector using T4 DNA ligase (Fermentas) in frame with the constitutive mycobacterial *hsp65* promoter and a selective apramycin resistance marker. The ligation was transformed into chemically-competent DH5α

through heat-shock (30min/ice ➔ 45sec/42°C ➔ 5min/ice ➔ +SOC media ➔ 1hr/37°C shaking) and plasmid-containing colonies were selected for on apramycin-containing LB agar plates. Individual colonies were subsequently picked into LB containing apramycin, grown overnight for plasmid propagation, and the plasmid was subsequently isolated using Plasmid Miniprep Kit (Qiagen). The *hsp65-Rv3377-8c* fragment was isolated using 1:1 XbaI:HindIII in NEBuffer 2.1 (New England Biolabs) overnight followed by gel extraction and shuttled into the pre-digested and gel-extracted integrative vector pMV306 containing a hygromycin resistance cassette using T4 DNA Ligase (Fermentas). All ligations were done using T4 DNA ligase (Fermentas). The resulting plasmid pMV306::*Hsp65-Rv3377-8c* was transformed into chemically-competent DH5α through heat-shock (30min/ice ➔ 45sec/42°C ➔ 5min/ice ➔ +SOC media ➔ 1hr/37°C shaking) and plasmid-containing colonies were selected for on hygromycin-containing LB agar plates. Individual colonies were subsequently picked into LB containing hygromycin, grown overnight for plasmid propagation, and the plasmid was subsequently isolated using Plasmid Miniprep Kit (Qiagen). Plasmids from individual clones were verified by Sanger sequencing to ensure the absence of frameshift or point mutations during the cloning process. The sequences/cloning strategy used are shown in the Appendix.

Electroporation of mycobacterial species

M. kansasii culture was grown in a 100-ml 7H9 suspension to late-log phase (OD_{600} 0.6-0.8) at which point it was incubated with 3ml of 2M Glycine for 16 hours. The *M. kansasii* culture was split in half into two 50-ml conical tubes and centrifuged at 2,000 x g/15min/10°C. The resulting bacterial pellets were resuspended in 1 ml 10% glycerol and washed in 50 ml 10% glycerol at room temperature by pipetting up and down. The washing step was repeated twice in decreasing amounts of 10% glycerol (25 ml and 10 ml) at room temperature and resuspended in a final volume of 1 ml 10% glycerol. A 200-µl volume of concentrated culture was added to a 2-mm electroporation cuvette (Thermo Scientific) containing 0.5 µg of plasmid and electroporation proceeded using GenePulser Xcell (Bio-rad) with the following program: voltage 2500V, capacitance: 25 µF, resistance: 1000 Ω and time constant: 25 ms. 800 µl of pre-warmed 7H9 were added, mixed by inversion, and the cultures were transferred to round-bottom tubes and left to recover at 37°C overnight. Cultures were subsequently plated on

antibiotic-containing 7H10 plates and individual colonies were isolated 2-3 weeks later, grown in 7H9 and 20% glycerol stocks were prepared for subsequent use. Boiled lysates for picked colonies were prepared by resuspending bacterial pellets in 100 µl dH_2O and incubating at 95°C/20min. Boiled lysates were used to confirm the presence of the genes of interest through insert-specific PCR and gel electrophoresis on a 1% agarose gel containing ethidium bromide. Insert-containing colonies were subjected to high-quality genomic DNA isolation, insert-specific PCR and Sanger sequencing with *Rv3377-8c*Seq-F1-4 + *Rv3377-8c*Seq-R1-4 (Table IIIA).

Primer name	***Primer sequence***	***Use***
BamHI-Rv3377-8c-*F*	CGGGATCCATGAACTTGGTTAGCGAAAAAG	Cloning
HindIII-Rv3377-8c-*R*	CCAAGCTTTCATTGGTTACTCTCATCGACC	Cloning
MKAN-ITS-check-F	GGGACGAAGTCGTAACAAGG	Genotyping
MKAN-ITS-check-R	TGCGCCCTTAGACACTTACA	Genotyping
EV-check-F	CCGCGGTACCAGATCTTT	Genotyping
EV-check-R	CTTTCGATGCTGACAAACGA	Genotyping
gDNA-Rv3377c-check-F	TCGAGCACAGCCTATGACAC	Genotyping
gDNA-Rv3377c-check-R	ACCGATCCATTTGTCTCCTG	Genotyping
gDNA-Rv3378c-check-F	CACGAGGTCCACGTTCTTTT	Genotyping
gDNA-Rv3378c-check-R	CAACCCACACCGAAAACTCT	Genotyping
Rv3377-8c*Seq-F1*	GCCTTTGAGTGAGCTGATACC	Sanger sequencing
Rv3377-8c*Seq-F2*	GCTGGTTTCACCTCGAATG	Sanger sequencing
Rv3377-8c*Seq-F3*	GTCATTTCCGGCCCAAAC	Sanger sequencing
Rv3377-8c*Seq-F4*	CTTGGCGTACATCTCATCGA	Sanger sequencing
Rv3377-8c*Seq-R1*	GATAATCTCTCTCCGCGTG	Sanger sequencing
Rv3377-8c*Seq-R2*	CTTATTCGACGTGAGGCTG	Sanger sequencing
Rv3377-8c*Seq-R3*	CATCGTAGGCCACACTTGTG	Sanger sequencing
Rv3377-8c*Seq-R4*	CTGTCGTTCACGGCTCTAG	Sanger sequencing
*qMKAN*SigA-*F*	CGGAGAAGGTGCTCGAAATC	qRT-PCR
*qMKAN*SigA-*R*	TGGTCTGGTCCAGCGAGATC	qRT-PCR
*q*Rv3377c-*F*	CAAGCTCTGGCGCATTGG	qRT-PCR
*q*Rv3377*c*-*R*	GATCTGCGCCGACAAGGA	qRT-PCR
*q*Rv3378c-*F*	CAACGATGCGGCTGAGTCT	qRT-PCR
*q*Rv3378c-*R*	TTACCATGCGTTTCGTTCCA	qRT-PCR

Table IIIA. Primers used for cloning, genotyping, Sanger sequencing and qRT-PCR in this project to identify and quantify Rv3377-8c expression.

RNA isolation and qRT-PCR

Cultures were grown in a 30-ml 7H9 suspension until early log phase (OD_{600} 0.2) and resuspended in 1ml TRIzol Reagent (Ambion). Acid-washed glass beads (Sigma) were added and the mixture was homogenized thrice at maximum speed for 30 seconds using Fastprep-24 (MP Biomedicals). The TRIzol layer was then harvested (12,000 x g/1min) into 200 µl chloroform:isoamyl 24:1 (Sigma), shaken vigorously and separated at 12,000 x g/15min. The aqueous phase was precipitated using 500 µl of isopropanol (Sigma), washed with 70% EtOH (Sigma), air-dried and re-dissolved in 90µl RNAse-free water with 2.5 µl RiboLock (Fermentas) and dissolved for 30min at 4°C. Contaminating DNA was removed with two consecutive Turbo DNAse treatments followed by DNAse Inactivation Reagent (Thermo Fisher Scientific) and RNA was purified using the Qiagen RNease kit (Qiagen). 300ng RNA were subjected to first-strand cDNA synthesis using RevertAid M-MuLV Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer recipe with random primers. qPCR was performed using Maxima SYBR/Fluorescein qPCR Master Mix (Fermentas) following the manufacturer recipe in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems) and run on a 7300 Real-Time PCR System (Applied Biosystems). Gene expression was qualitatively assessed relative to *SigA* expression by qRT-PCR using qMKAN*SigA*-F/R, q*Rv3377c*-F/R and q*Rv3378c*-F/R (Table IIIA).

Detection of isoTB production

M. tuberculosis, *M. kansasii*::EV and *M. kansasii*::Rv3377-8c were grown in modified 7H9 media with decreased Mg^{2+} content at 0.1 instead of 0.43 mM. 10^{11} bacteria were grown in 80 ml of Mg^{2+}-deficient media for 5 days and metabolites were extracted by adding hexane to the culture media and left at rest for 4 days, at which point the hexane layer was isolated and further partitioned with an overnight addition of 5M NaCl at room temperature. The resulting extracts were dried using a light stream of air and subjected to GC/MS as described in Mann et al [99].

Detection of cell filtrate adenosine-linked lipids

M. kansasii::EV (empty vector control), *M. kansasii*::Rv3377-8c (sequence-confirmed clone) and *M. tuberculosis* were grown to mid-log phase and subsequently incubated with radiolabeled [8-14C] adenosine for 14 days. Polar lipid fractions were extracted using different ratios of

$CHCl_3$:CH_3OH:0.3%NaCl (v/v/v) [115]. Extracted lipids were spotted on a TLC Silica Gel 60 (Millipore Sigma) with $CHCl_3$:CH_3OH:H_2O 10:5:1 (v/v/v) used as the mobile phase solvent. The radiolabeled signature was developed using Storm 840 PhosphorImager (GE Healthcare) to visualize adenosine-linked lipids in each lane. [8-14C] adenosine was used as a no-lipid staining control. The plate was stained with 5% phosphomolybdic acid reagent (PMA) (Sigma) and heated briefly using an industrial blow-dryer to visualize the amount of lipids in each lane.

Identification of 1-TbAd in *M. kansasii*::Rv3377-8c

Two biological replicates of *M. kansasii*::EV and four of *M. kansasii*::Rv3377-8c were grown for 14 days in 45ml of 7H9 without Tween-80. Cultures were centrifuged and washed twice with Optima MS-grade water (Thermo Scientific). Whole lipids were extracted in glass heavy-duty round-bottom Pyrex 8422 tubes (Sigma-Aldrich) with PTFE-lined caps (Corning) using $CHCl_3$:CH_3OH at 1:2, 1:1 and 2:1 (v/v) [116]. The supernatants were pooled for each sample, dried under a light stream of air, weighed and deposited at -20°C. Dried lipids were subjected to HPLC/MS as described in Layre et al [116] and Young et al [94].

Congo Red uptake assay

Bacterial cultures were grown on Congo Red-containing 7H10 plates for 2 weeks, scraped into a 15-ml conical tube, washed with water until the supernatant became clear and incubated with 2ml DMSO for 2 hours. Congo Red was measured in the resultant supernatant at A_{488}. The values were normalized to the dry weight of the pellet to define the Congo Red binding index.

***In vitro* growth assessment**

M. kansasii, *M. kansasii::EV* and *M. kansasii::Rv3377-8c* were maintained at mid-log phase (OD_{600} 0.2-0.5), passed through 22-G and 25-G needles and centrifuged at 450 x g/5min to remove clumps and resuspended at a theoretical OD_{600} 0.01. Cultures were grown for 7 days in biological triplicates. At each time point, a volume of 100 µl was taken in duplicate from each culture to measure the OD_{600} on a 96-well tissue-culture plate (Falcon) using the Infinite M200 Pro NanoQuant spectrophotometer (Tecan).

Assessment of pH-dependent *in vitro* growth

M. kansasii::EV, *M. kansasii*::Rv3377-8c and *M. tuberculosis* H37Rv were maintained at mid-log phase (OD_{600} 0.2-0.5), passed through 22-G and 25-G needles and centrifuged at 450 x g/5min to remove clumps, and resuspended in 20ml media with altered pH (prepared using 2M HCl) at a theoretical OD_{600} 0.01. Cultures were grown for 14 days in biological triplicates. At each time point, a volume of 100 µl was taken in duplicate from each culture to measure the OD_{600} on a 96-well tissue-culture plate (Falcon) using the Infinite M200 Pro NanoQuant spectrophotometer (Tecan). At the end of the 14 days, cultures were transferred to a 15-ml conical tube (Falcon) and centrifuged and supernatants were passed through a 0.22 µm filter for subsequent pH measurement.

Murine BMDM infection

On day 7 post M-CSF-induced differentiation, BMDMs were stripped and placed in 24- or 96-well plates (500,000 or 100,000 cells/well in 500 µl complete RPMI media without antibiotics). Cells were infected with an actively growing single-cell culture (5x 22G/25G needle passage and spinning at 450 x g/5min) at an OD_{600} 0.2-0.5 and adjusted in complete RPMI media (without antibiotics) to an OD_{600} 0.05 (MOI 5 assuming OD 1.0 = 10^8 mycobacterial) unless stated otherwise. After 4 hours of infection, the wells were gently washed three times with PBS to eliminate all remaining extracellular bacteria and 1ml of fresh complete RPMI media (without antibiotics) was added to each well. At indicated time points, the plates were centrifuged at 2,000 x g/15min, supernatants were collected for further analysis and stored at -20°C. Each well was subjected to 1 ml (24-well) or 200 µl (96-well) of 1% Triton-X for 10 minutes at room temperature to induce BMDM lysis. CFUs were counted on 7H10 plates 2 weeks post-plating to determine bacterial burden.

Murine pulmonary infection

C57Bl/6 mice were infected through aerosolization of bacterial cultures at OD_{600} 0.4 for 15 minutes using ONARES (CH Technologies, USA) or Lovelace (Intox Products inc). Alternatively, C57Bl/6 mice were intubated using 50 µl of a mixed bacterial suspension containing both *M. kansasii* and *M. kansasii*::Rv3377-8c at OD_{600} 0.2. This model was adapted from the non-invasive

Pseudomonas aeruginosa translaryngeal intubation model [117]. Mouse lungs were harvested at 4 or 24 hours (early time-points to measure survival) and 21, 28, 42 or 56 days post infection (later time-points to measure persistence) into 1ml of 7h9 and homogenized using an Omni Tissue Homogenizer TH (Omni International) at high speed for 45 seconds. Serial dilutions were made in 7H9 liquid media from lung homogenates were plated on 7H10 plates containing PANTA ± hygromycin at 50 µg/ml. CFUs were counted 2 weeks post-plating to determine bacterial burden.

Enzyme-linked immunosorbent assay

Supernatants from BMDM were collected as previously mentioned and stored at -20°C. Different dilutions of samples were subjected to mouse-specific Ready-SET-Go™ ELISA kits (Invitrogen) following the manufacturer's protocol. Alternatively, a 12-plex ELISA (Quansys) was used to assess a panel of pro- and anti-inflammatory cytokines.

4. Results

4.1. *M. kansasii*::Rv3377-8c expresses *Rv3377c* and *Rv3378c*

We searched the *M. kansasii* genome for amino-acid and nucleotide sequences that exhibit homology to *Rv3377c* and *Rv3378c* and found none. After electroporation and colony picking, the *M. kansasii*::Rv3377-8c clone used in this manuscript was first verified by PCR/gel electrophoresis followed by Sanger sequencing to ensure 100% identity of the inserted fragment of interest. Four fragments were amplified during the PCR verification process (Figure IIIA-a): (1) *M. kansasii* internal transcribed spacer (ITS) region joining the 16S and 23S ribosomal RNA genes to ensure the presence of *M. kansasii* DNA in each sample, (2) EV-check to identify the empty-vector (*M. kansasii*::EV) control, with a small portion of the pMV306 plasmid incorporated into its genome at the same insertion site as the gene pair in the mutant, (3) Rv3377-check and (4) Rv3378-check to ensure the presence of the two genes of interest in the mutant (*M. kansasii*::Rv3377-8c) and their absence in *M. kansasii* and *M. kansasii*::EV. When a mutant was confirmed to have the two genes present in its genome, the inserted region was amplified and sent for sequencing using the eight sequencing primers listed in *Rv3377-8cSeq-F1-F4/R1-R4*. The sequencing results were then combine together to recreate the fragment of

interest and compared to the wild-type sequence of the two genes for SNP discovery. When a mutant was found to have 100% sequence identity to *Rv3377-8c*, it was expanded and a large glycerol stock was made for further investigation. Finally, RNA was extracted from *M. kansasii*::EV and *M. kansasii*::Rv3377-8c, cDNA libraries were synthesized using random oligos and targeted qRT-PCR was performed to ensure the expression of the *Rv3377-8c* gene pair using the qRT-PCR primers listed in the table. Gene expression was assessed relative to *SigA* that is constitutively expressed in healthy mycobacterial cultures. Unlike *M. kansasii*::EV, *M. kansasii*::Rv3377-8c expressed *Rv3377c* and *Rv3378c* (data not shown).

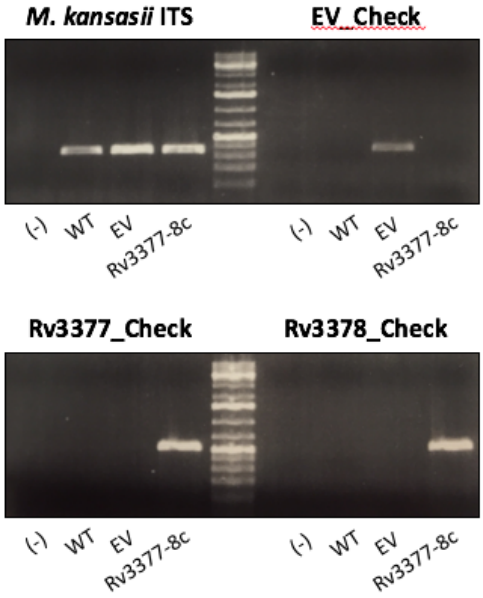

Figure IIIA. *M. kansasii*::Rv3377-8c expresses *Rv3377c* and *Rv3378c.* Boiled lysates from *M. kansasii* (WT), *M. kansasii*::EV (EV) and *M. kansasii*::Rv3377-8c (*Rv3377-8c*) were subjected to insert-specific PCR using primer sets from Table IIIA and gel electrophoresis on a 1% agarose gel. The ladder used was Genruler™ 1kb Plus DNA LadderSD, with the bottommost bright band at 500 bp.

Expected size: ITS 352 bp, EV 352 bp, Rv3377c 535 bp and Rv3378c 492 bp.

4.2. *M. kansasii*::Rv3377-8c produces 1-TbAd

Whole lipids from *M. kansasii*::EV and *M. kansasii*::Rv3377-8c cultured in low-Mg^{2+} media were subjected to GC/MS to detect the presence of isoTB, a transient intermediate in the production of 1-TbAd (Figure IIIB). Compared to *M. kansasii*::EV, *M. kansasii*::Rv3377-8c had a discrete but identifiable signal for isotuberculosinol production.

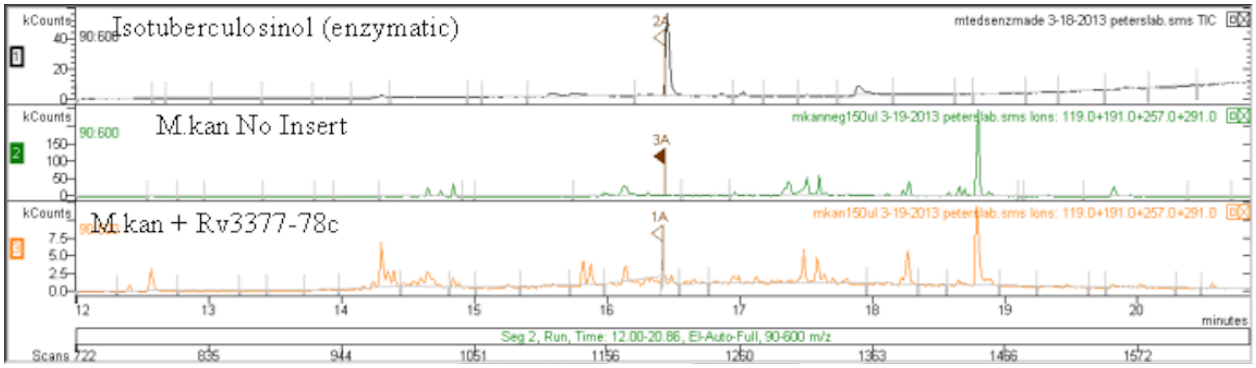

Figure IIIB. *M. kansasii*::Rv3377-8c produces isoTB. IsoTB was detected at 16.64 min in *M. tuberculosis* and *M. kansasii*::Rv3377-8c as indicated by the white flags. The same retention time was indicated in *M. kansasii*::EV with a brown flag.

However, when isoTB was identified as a transient intermediate in 1-TbAd production, we decided that it would be necessary to assess 1-TbAd production. To detect the production of adenosine-linked lipids in *M. kansasii*::EV, *M. kansasii*::Rv3377-8c and *M. tuberculosis*, actively growing cultures were fed radiolabelled adenosine for 14 days, at the end of which the cell membrane-associated lipids were extracted and separated based on polarity using thin layer chromatography. The TLC plate was then developed to visualize the radiolabelled signature of each culture and subsequently stained with PMA to verify the total amount of lipids per sample (Figure IIIC-a). The PMA stain shows that all samples were loaded in similar quantities. *Unlike M. tuberculosis*, *kansasii*::EV did not produce identifiable amounts of adenosine labelling. However, *M. kansasii*::Rv3377-8c showed a nearly identical pattern of radiolabelling signal to that seen in *M. tuberculosis*, suggesting the production of adenosine-linked lipids by the transformant.

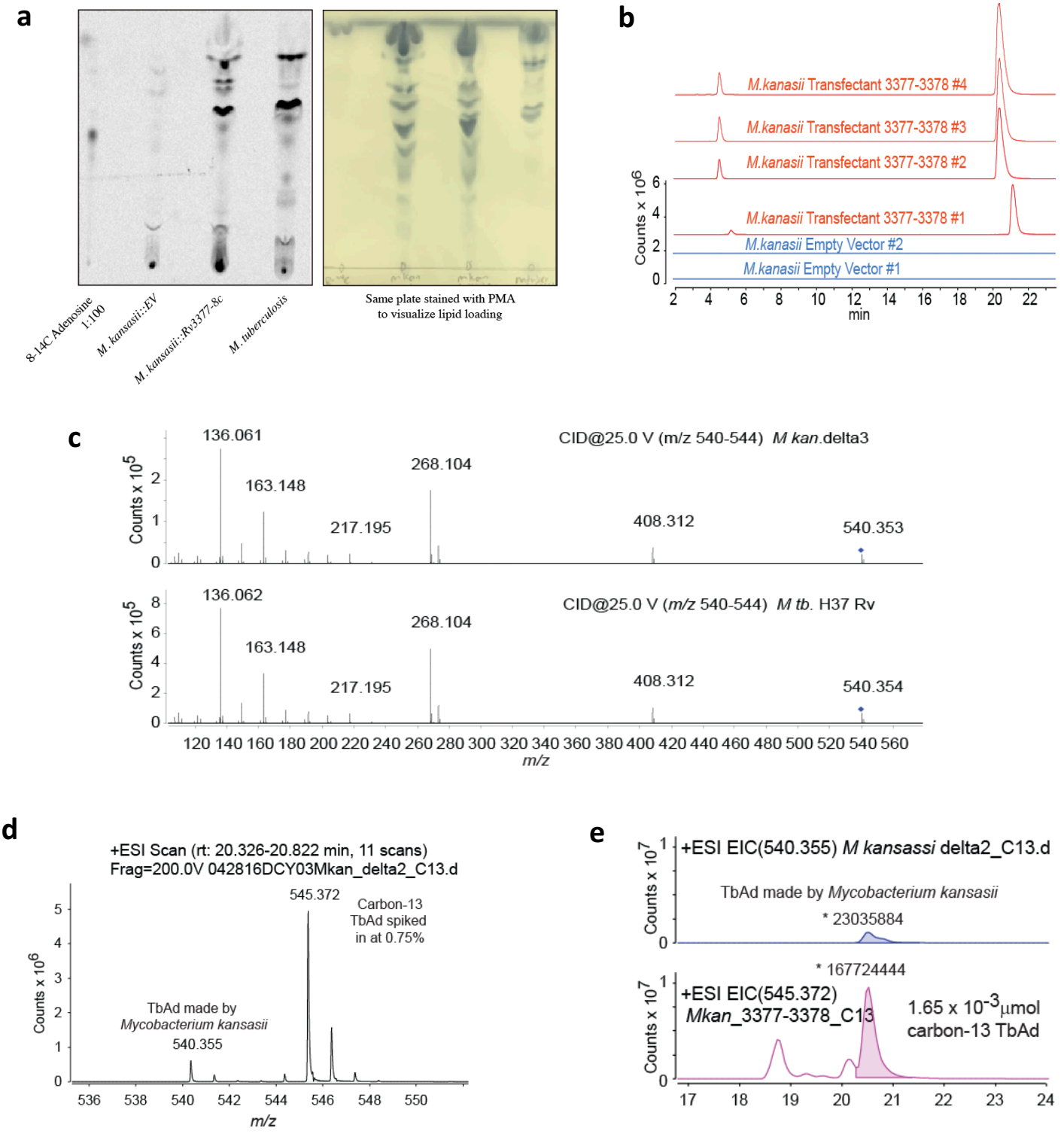

Figure IIIC. *M. kansasii*::Rv3377-8c produces 1-TbAd. (a) Detection of adenosine-linked lipids extracted from *M. kansasii*::EV, *M. kansasii*::Rv3377-8c and *M. tuberculosis* through radiolabelling and separation using normal-phase silica thin-layer chromatography. (b-e) HPLC/MS analysis of extracted lipids from *M. kansasii*::EV and *M. kansasii*::Rv3377-8c. (b) Superimposed ion chromatograms of four different cultures of *M. kansasii*::Rv3377-8c and two cultures of *M. kansasii*::EV to identify retention time of unknown compound in lipid extracts – separation based on *m/z* (540.3545). (c) CID-MS of compound produced by *M. kansasii*::Rv3377-8c (top) compared to 1-TbAd produced by *M. tuberculosis* H37Rv (bottom). (d-e) ESI Scan/ESI-EIC analysis using spiked C^{13}-1-TbAd at 0.75% to identify the (d) percentage and (e) absolute numbers of native 1-TbAd.

To further confirm the production of 1-TbAd, whole lipids were extracted from *M. kansasii*::EV and *M. kansasii*::Rv3377-8c cultures using different ratios of $CHCl_3$:CH_3OH and subjected to LC/MS. First, we identified that the compound produced by *M. kansasii*::Rv3377-8c was present at the correct *m/z* and elution times for 1-TbAd, as seen in Layre et al [15] (Figure IIIC-b). Of the two *M. kansasii*::EV clones, no such products were extracted. Furthermore, collision-induced dissociation-mass spectrometry (CID-MS) analysis produced indistinguishable pattern of dissociation spectra when comparing the *M. kansasii*::Rv3377-8c molecule to 1-TbAd synthesized by *M. tuberculosis* H37Rv, confirming the identity of the compound being produced (Figure IIIC-c). Finally, C^{13}-1-TbAd was spiked into the lipid samples at 0.75% to extrapolate the percentage of the native compound in relation to whole lipids being produced in each sample (Figure IIIC-d). 1-TbAd was produced at 0.125% ± 0.04% in the *M. kansasii*::Rv3377-8c clones, compared to the 0.76% produced by *M. tuberculosis H37Rv*. Absolute amounts of 1-TbAd produced were quantified using UV spectroscopy (Figure IIIC-e).

4.3. *M. kansasii*::Rv3377-8c exhibits unaltered *in vitro* characteristics when compared to *M. kansasii* and *M. kansasii*::EV

To establish the effect of 1-TbAd production on the fitness and behaviour of *M. kansasii*, we first assessed its influence on the *in vitro* characteristics of the bacterial culture. To identify any discrepancies in the growth kinetics of the mutant, we grew *M. kansasii*, *M. kansasii*::EV and *M. kansasii*::Rv3377-8c in liquid 7H9 media and monitored growth through optical density readings at OD_{600}. The daily measurements revealed no changes in the doubling time of *M. kansasii*::Rv3377-8c when compared to the two other strains (Figure IIID-a). When spread on 7H10 plates, the mutant took the typical 2 weeks to grow into mature colonies of 2mm diameter. The colonies on the plates exhibited the typical *M. kansasii* morphology (Figure IIID-b). Since GGPP, the precursor for 1-TbAd production, is also used for carotenoid production in *M. kansasii*, the photochromogenicity of the mutant colonies was tested by prolonged exposure to light. The mutant also retained its capacity as a photochromogen and turned yellow when exposed to light (Figure IIID-c).

A recent publication by Jankute et al described the importance of cell surface composition and relative hydrophobicity in mycobacterial virulence [118]. This study stemmed from previous work with *M. avium paratuberculosis* by Cangelosi et al [119]. Since 1-TbAd is found on the cell surface and has an amphipathic character, we tested the effect of its production on the hydrophobicity of the cell surface of *M. kansasii*::Rv3377-8c. Congo Red is a hydrophobic dye that binds to lipoproteins. When grown on Congo Red-containing 7H10 plates, colonies with a more hydrophobic character in their cell wall retain larger amounts of the dye, which can later-on be extracted using DMSO and spectrophotometrically quantified at A_{488}. Furthermore, Jankute describes the Congo Red retention technique as a general indicator of intra-colony bacterial interaction rather than a hydrophobicity stain [118]. Initial qualitative analysis of the *M. kansasii*::Rv3377-8c mutant colonies grown on Congo Red-containing 7H10 plates showed no sign of increased retention of the lipophilic dye, remaining white similarly to the *M. kansasii*::EV control (Figure IIID-d). Absorbance at 488nm showed no quantitative change between *M. kansasii*::EV and *M. kansasii*::Rv3377-8c when the numbers were normalized to the weight of their respective dry culture pellets (Figure IIID-e).

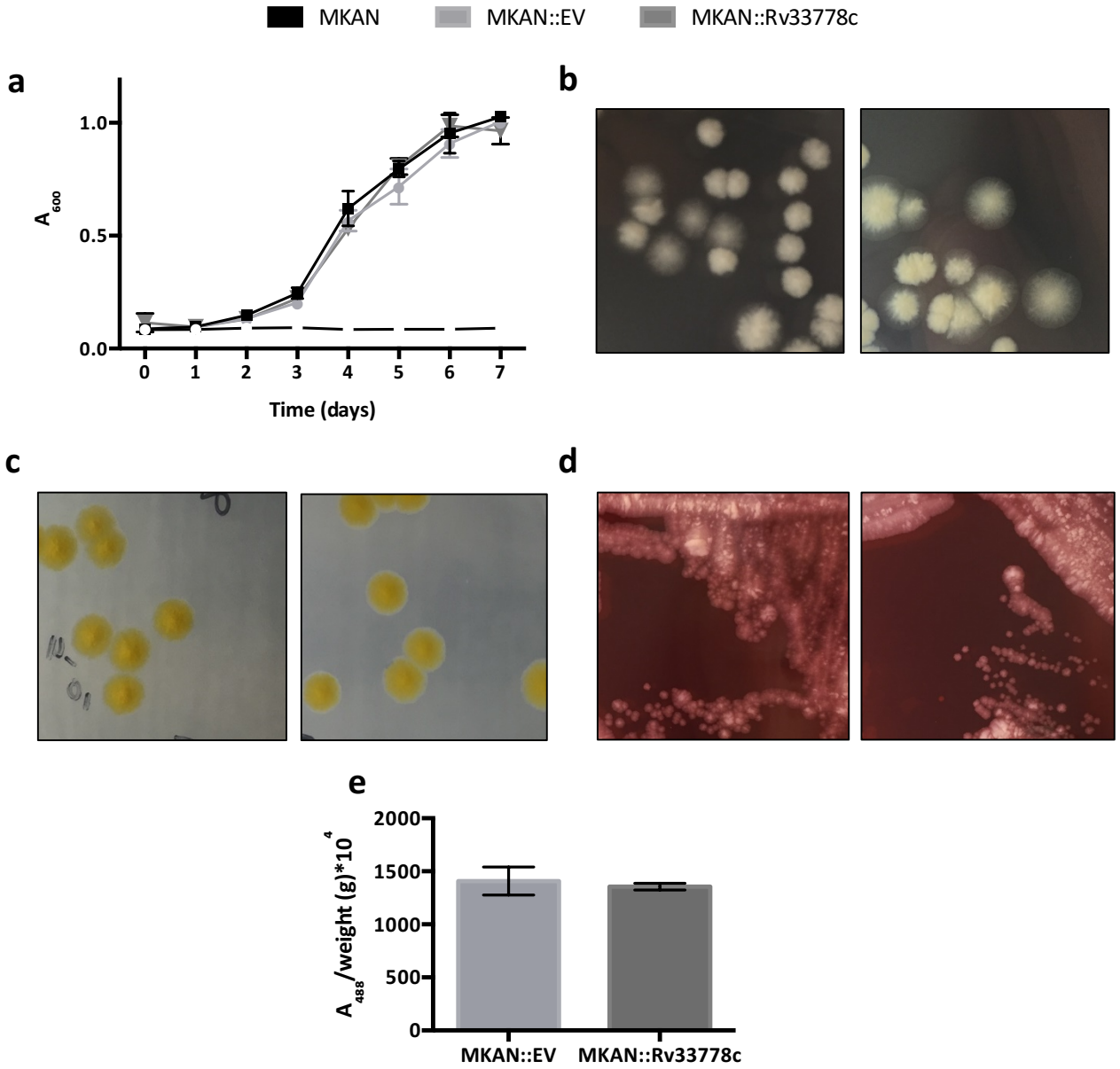

Figure IIID. 1-TbAd production does not influence the *in vitro* growth characteristics and behaviours of *M. kansasii*. (a) Comparative growth kinetics of wild-type *M. kansasii* (MKAN), *M. kansasii*::EV *(*MKAN::EV) and *M. kansasii*::Rv3377-8c (MKAN::33778c) at 37°C in 7H9 broth ± hygromycin at 100 µg/ml. The data are presented as the mean of technical triplicates ± SD. The data are representative of three independent experiments. (b) Colony morphology of *M. kansasii*::EV (left) and *M. kansasii*::Rv3377-8c (right) on 7H10 plates. (c) Colony morphology of *M. kansasii*::EV (left) and *M. kansasii*::Rv3377-8c (right) on 7H10 plates supplemented with Congo Red. (d) Quantitative analysis of Congo Red dye retention by *M. kansasii*::EV (MKAN::EV) and *M. kansasii*::Rv3377-8c (MKAN::Rv33778c). Absorbance at 488nm was divided by the weight of the dry culture pellet after washing (in grams) and multiplied by 10^4 for easier visualization on the graph. The data are plotted as the mean of technical triplicates ± SD. The data are representative of two independent experiments.

4.4. 1-TbAd production elicits a growth advantage for *M. kansasii* at pH ~5.2

Once phagocytosed, mycobacteria have to overcome the sudden pH drop to 5-4.5 by either stopping phagosomal maturation in initially naïve macrophages (alveolar macrophages) or resisting the extreme acidity in pre-activated macrophages (homing BMDMs) within IFN-γ-rich milieu such as sites of active infection. The *Rv3377-8c* gene pair and isoTB have been implicated in changes in acidification levels and maturation of the phagolysosome within macrophages [93]. The expression of the *Rv3377-8c* gene pair is constitutive and unaltered within the macrophage [120-122]. However, their activity is increased in diminished levels of Mg^{2+}, as is the case within the macrophage phagosome [101]. In addition, although the avirulent *M. tuberculosis* strain H37Ra produces 1-TbAd, *M. tuberculosis* mutants with disruptions in either *Rv3377c* or *Rv3378c* become defective in intracellular survival in bone-marrow derived murine macrophages [93-95, 99, 101]. These results suggest a potential role in early stages of infection. To assess the effect of 1-TbAd production on survival within the macrophage phagosome, we first looked at the ability of *M. kansasii*::Rv3377-8c to overcome acidic environments *in vitro*.

To assess the effect of 1-TbAd production on acid resistance, we made modified 7H9 media with pH ranging from 6.6 to 3. The pH was calibrated using 2M HCl solution. H37Rv, *M. kansasii*::EV and *M. kansasii*::Rv3377-8c were grown in the different media for 14 days and their growth levels were monitored at OD_{600} (Figure IIIE-a). *M. tuberculosis* H37Rv grew optimally at pH 6.6, 6, 5.8 and 5.6, its growth was severely attenuated at pH 5.4 and under. Both *M. kansasii*::EV and *M. kansasii*::Rv3377-8c grew readily at pH 5.4 but the growth of *M. kansasii*::EV was attenuated at lower pH, with no growth detected at pH 5.2. While there was an initial delay in growth at pH 5.2, *M. kansasii*::Rv3377-8c otherwise grew at that pH and its growth was attenuated but readily visible at as low as pH 4.9 (Figure IIIE-a-b).

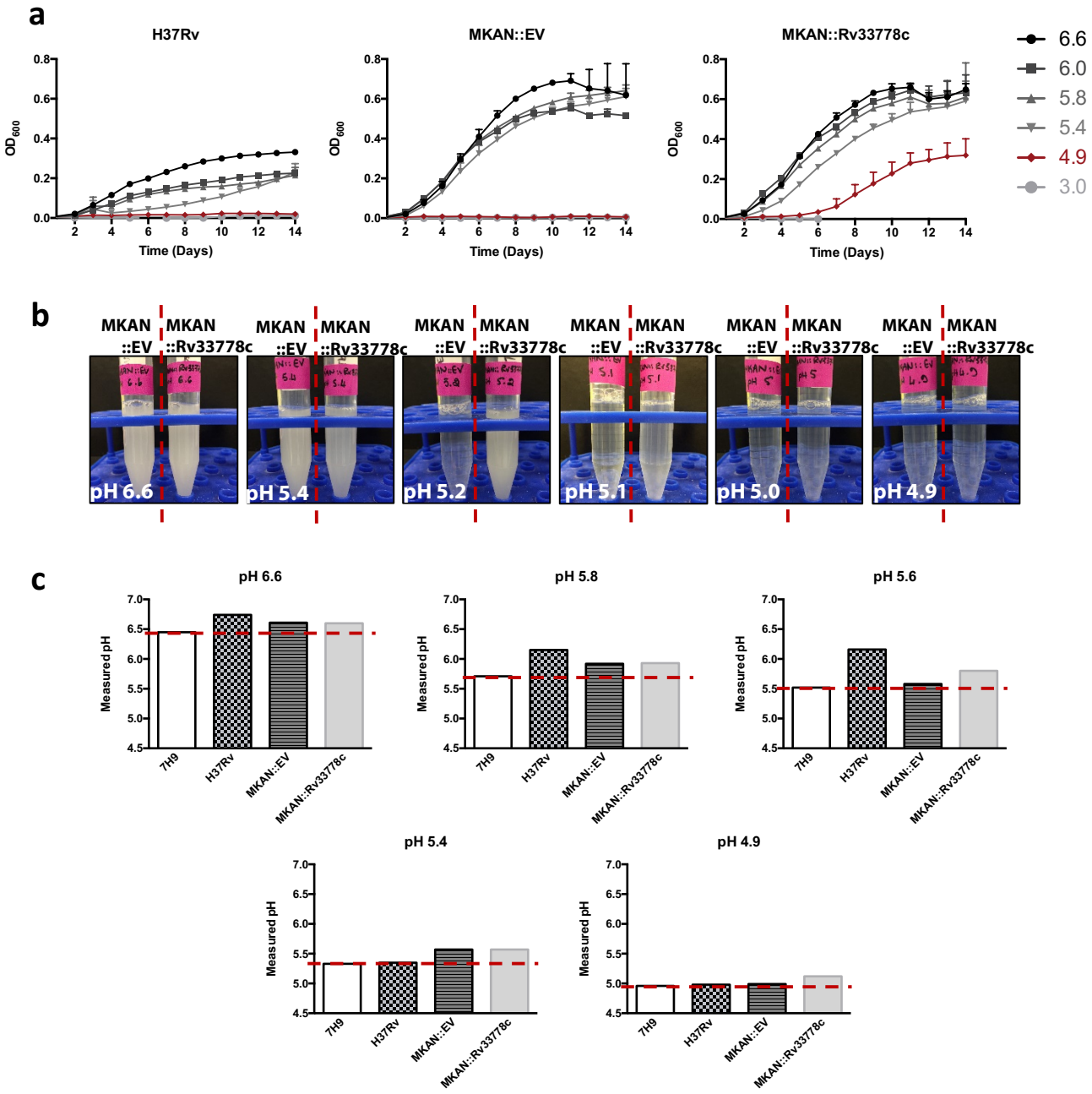

Figure IIIE. 1-TbAd production protects *M. kansasii*::Rv3377-8c from highly acidic environments (pH5.2) reminiscent of the phagosome inside an activated macrophage. *M. tuberculosis* H37Rv (H37Rv), *M. kansasii*::EV (MKAN::EV) and *M. kansasii*::Rv3377-8c (MKAN::Rv33778c) were grown in 7H9 with altered pH (using 2M HCl) for 14 days. (a) Comparative growth kinetics of MTB, MKAN::EV and MKAN::Rv33778c at 37°C in 7H9 broth with pH ranging from 6.6 to 3. The data are presented as the mean of technical triplicates ± SD. The data are representative of at least three independent experiments. (b) Pictures of MKAN::EV and MKAN::Rv33778c cultures after growth for 14 days in the modified 7H9 media for side-to-side comparison. (c) pH values of supernatants from H37Rv, MKAN::EV and MKAN::Rv33778c cultures collected after 14 days of growth within the modified 7H9 media. The data are representative of at least three independent experiments.

Next, the supernatants from cultures grown for 2 weeks were passed through 0.2-µm filters and their pH was measured to check the alkalization potential of each of the three bacteria (Figure IIIE-c). At any point where all three cultures were actively growing, *M. tuberculosis* alkalized the media more than *M. kansasii*. In addition, *M. kansasii*::EV and *M. kansasii*::Rv3377-8c alkalized the media to a similar extent. However, at pH 5.2 and lower, only *M. kansasii*::Rv3377-8c successfully alkalized the media. At the lowest pH points of 4.5 and 3, none of the cultures successfully changed the pH of the media.

4.5. *M. kansasii*::Rv3377-8c exhibits unchanged fitness in naïve or activated BMDM infection models

In order to assess whether the production of 1-TbAd is sufficient for this early successful expansion within macrophages, we infected murine BMDMs with *M. kansasii*, *M. kansasii*::EV and *M. kansasii*::Rv3377-8c for a period of 48 hours. Cells were lysed at 4, 24 and 48 hours post infection. Based on CFU counts, *M. kansasii* (MKAN), *M. kansasii*::EV (MKAN::EV) and *M. kansasii*::Rv3377-8c (MKAN::Rv33778c) were retained in similar proportions at 4-hr post infection, and exhibited a similar fitness throughout the 48 hours of infection, with no significant difference between cultures (Figure IIIF-a).

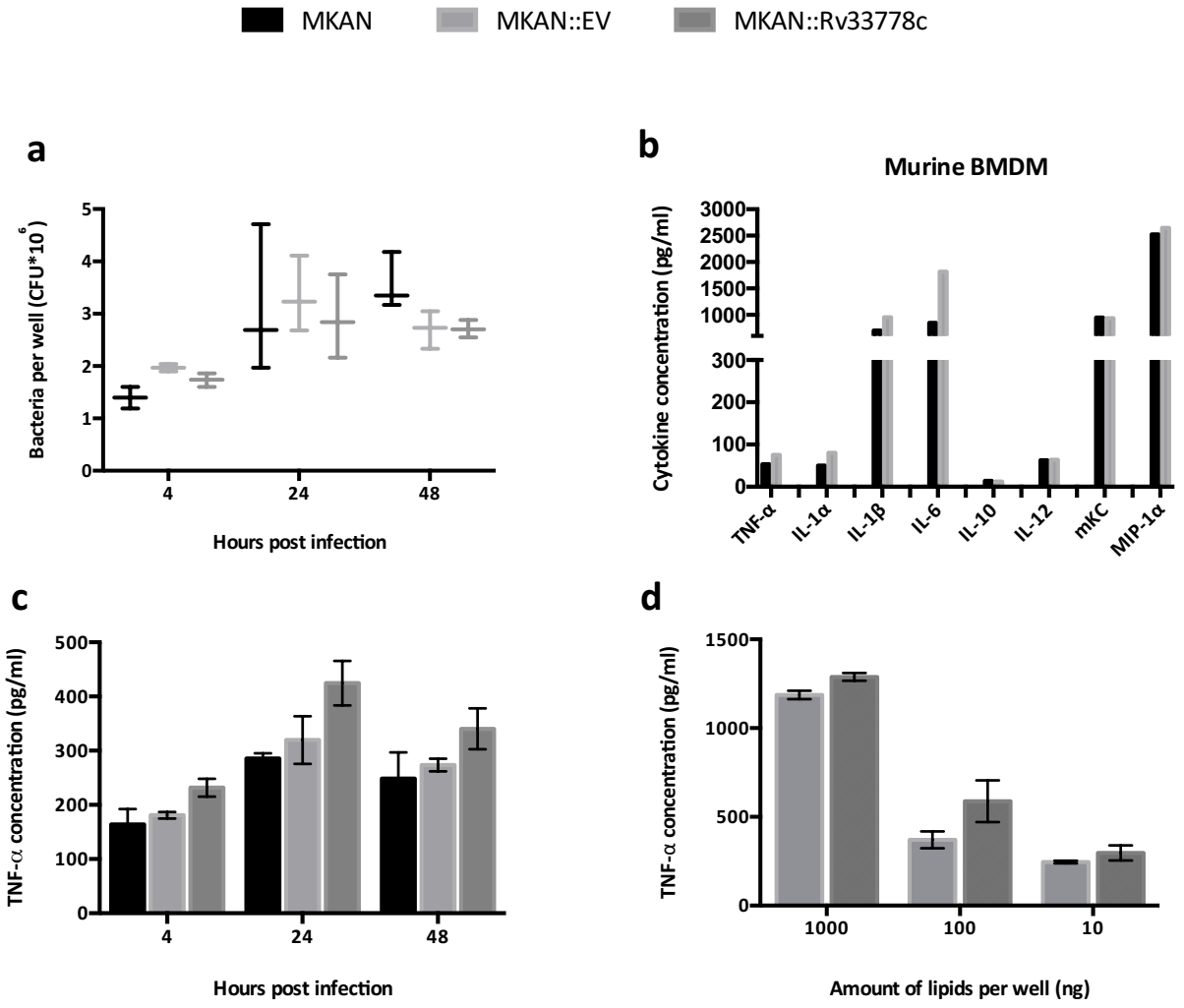

Figure IIIF. 1-TbAd production does not increase mycobacterial fitness in murine BMDMs within the first 48 hours of infection but increases the production of pro-inflammatory cytokines. (a-c) Infection of murine BMDMs using wild-type *M. kansasii* (MKAN), *M. kansasii*::EV (MKAN::EV) or *M. kansasii*::Rv3377-8c (MKAN::Rv33778c) with an MOI=5. (a) Bacterial burden was calculated by counting colonies on 7H10 plates containing 10^1 and 10^3 CFUs. The data are plotted as the mean of technical triplicates ± SD. The data are representative of three independent experiments. (b) A multiplex ELISA was performed to assess the production of pro- and anti-inflammatory cytokines using culture media from one *M. kansasii*::EV-infected and one *M. kansasii*::Rv3377-8c-infected macrophage culture at 24 hours post infection. (c) TNF-α concentrations were calculated using a standard curve generated from each ELISA plate. The data are plotted as the mean of technical triplicates ± SD. The data are representative of three independent experiments. (d) Lipids extracted from MKAN::EV and MKAN::Rv33778c were resuspended in C_6H_{14}:C_2H_5OH 1:1 (v/v) to reach a final concentration of 1000, 100 or 10 ng/well and left to dry out in 96-well plates. TNF-α concentrations were calculated using a standard curve generated from each ELISA plate. The data are plotted as a mean of technical triplicates (three wells per condition).

Although intraspecies studies have shown that the more virulent strains of *M. tuberculosis* (Beijing, K) are the ones that exhibit a diminished TNF-α response [123-125], other studies have shown that virulent *M. tuberculosis* leads to increased TNF-α production, which may be helpful to bacterial growth [126]. Pro-inflammatory cytokines have also been implicated in granuloma stability and latency within the host [127-130]. Although *M. kansasii*::Rv3377-8c did not exhibit differential fitness in macrophages within the first 48 hours of infection based on CFU counts, we tested the possibility of 1-TbAd playing a role as an immunogenic molecule, conditioned upon a similar bacterial burden. A first multiplex ELISA with two samples revealed a potential increase in inflammatory cytokines in supernatants for BMDMs infected with *M. kansasii*::Rv3377-8c compared to *M. kansasii*::EV (Figure IIIF-b). To confirm the preliminary multiplex finding, we collected supernatants from BMDM infections at 4, 24 and 48 hours and assessed the production of pro-inflammatory cytokine TNF-α (Figure IIIF-c). 1-TbAd production leads to the production of discretely larger amounts of TNF-α when compared to non-1-TbAd producing cultures.

In an effort to pinpoint the effect of the lipid on cytokine production, dried lipids from each culture were resuspended in C_6H_{14}:C_2H_5OH 1:1 (v/v) and layered onto 96-well plates. When the solvent was completely dried, BMDMs were added to each well. Exposure to whole lipid extracts led to the same increase in TNF-α production. However, this phenotype was only successfully achieved once (Figure IIIF-d).

Next, we infected naïve (-/-), IFN-γ-stimulated (+/-) and IFN-γ- and LPS-stimulated (+/+) BMDMs with *M. kansasii*::EV or *M. kansasii*::Rv3377-8c and lysed the cells at 4, 24 and 48 hours to quantify the bacterial load (Figure IIIF-e). Based on CFU counts, *M. kansasii*::EV and *M. kansasii*:Rv3377-8c were retained within activated macrophages in a similar fashion. Although the initial infectious dose was not equal for both infections, the trend remains the same with or without 1-TbAd production, with an expected growth hindrance seen in the doubly-stimulated macrophages.

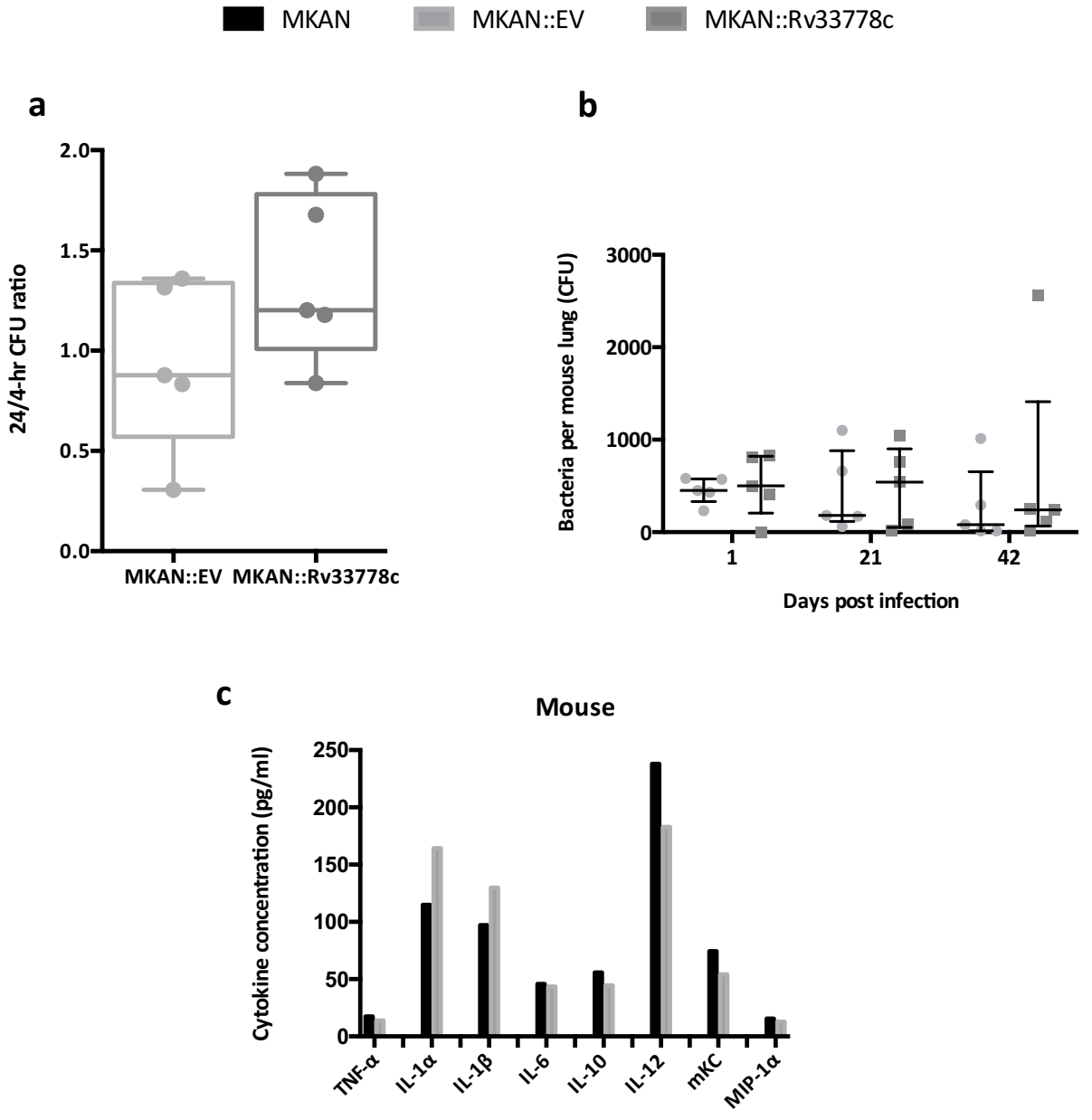

Figure IIIG. 1-TbAd production does not increase mycobacterial fitness in murine pulmonary infections but alters the production of pro- and anti-inflammatory cytokines within the first 24 hours of infection. (a) CFUs were counted from C57Bl/6 mouse lungs isolated at 4 hours vs. 24 hours post aerosol infection. The ratio was generated by dividing the 24 hr/4-hr CFU counts (n=5 lungs/condition/time point). (b) CFUs were counted from C57Bl/6 mouse lungs isolated at 1, 21 and 42 days post aerosol infection (n=5 lungs/condition/time point). The data are plotted as the median ± IQ range. (c) A multiplex ELISA was performed to assess the production of pro- and anti-inflammatory cytokines using lung homogenate from one *M. kansasii*::EV-infected and one from *M. kansasii*::Rv3377-8c-infected mouse at 24 hours post infection.

4.6. *M. kansasii*::Rv3377-8c exhibits unchanged fitness in murine infection models

In C57Bl/6 mice, *M. tuberculosis* expands in a logarithmic scale within the course of infection. In sharp contrast, *M. kansasii* remains at its initial levels of infection, making it a good model for acquisition-of-virulence studies within the mouse [50]. 1-TbAd has been isolated from different tissues in mice and humans infected with *M. tuberculosis* and its production pathway is implicated in the capacity of the organism to survive early host defence mechanisms [93, 103].

To identify the effect of 1-TbAd production on establishing infection within the first hours, mice were aerosolized with *M. kansasii*::EV or *M. kansasii*::Rv3377-8c and their lungs were collected and homogenized at 4 or 24 hours (Figure IIIG-a). The ability to establish infection early after initial exposure to mycobacteria was expressed as the ratio of CFUs at 24hrs/4hrs. *M. kansasii*::Rv3377-8c exhibited a ratio of 1.36 ± 0.42 compared to *M. kansasii*::EV at 0.94 ± 0.43. Therefore, 1-TbAd does not significantly change the ability of *M. kansasii* to establish infection. Next, to elucidate the persistence of *M. kansasii*::Rv3377-8c in a long-term mouse infection, we aerosolized *M. kansasii*::EV and *M. kansasii*::Rv3377-8c into mice and collected theirs lungs at 1, 21 and 42 d.p.i. (Figure IIIG-b). Both *M. kansasii*::EV and *M. kansasii*::Rv3377-8c remained within the same log of initial infection up to day 42.

Next, to identify the effect of 1-TbAd on cytokine production in mouse lungs, homogenates from 24-hr low-dose infection were passed through a 0.22-µm filter and subjected to a 12-plex ELISA. As seen in macrophages, pro-inflammatory cytokines were increased and anti-inflammatory cytokines were decreased inside the lung homogenate.

We then tested the effect of 1-TbAd in a high-dose competitive infection starting with a mixed culture of 1:1 *M. kansasii-M. kansasii*::Rv3377-8c. The method of infection for this experiment was altered to ensure the appropriate proportions of bacteria were introduced into the lungs. Instead of performing an aerosolization, we performed a trans-laryngeal intubation by introducing bacteria to each lung in a 50-µl liquid suspension. First, to optimize this experiment, we infected mice with two infectious doses of *M. kansasii*, with 10^6 CFUs introduced to one group of mice and 10^5 to another group. Mouse lungs were collected at 1 and 42 d.p.i. for assessment of bacterial loads through CFU plating (Figure IIIH-a). The mice were weighed and their behaviours were monitored for 42 days to assess levels of sickness (Figure

IIIH-b). The initial infectious dose was fully attained in its entirety and the bacteria persisted but decreased in overall numbers during the 42 d.p.i.

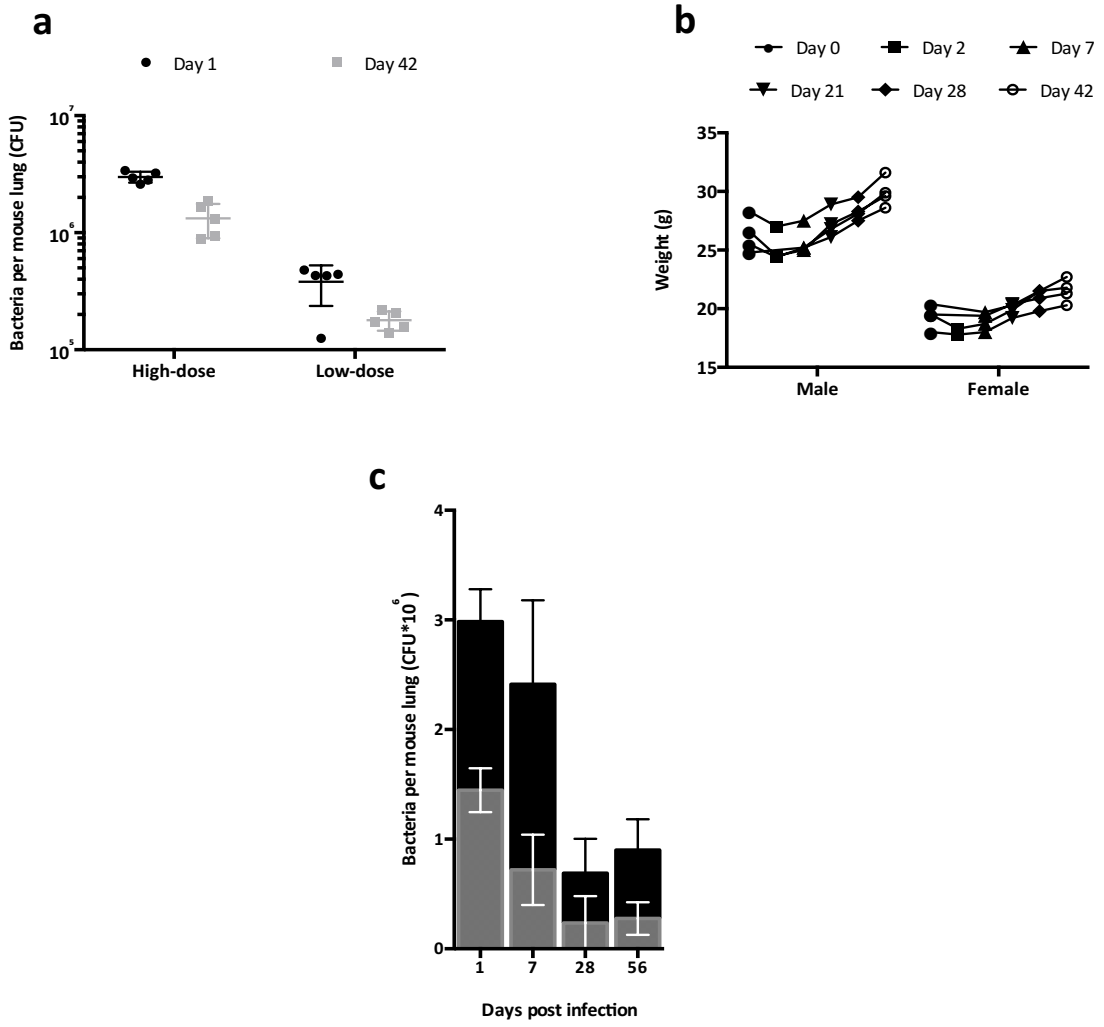

Figure IIIH. 1-TbAd production does not provide protection for an overall high-dose *M. kansasii* infection, and does not provide a selective advantage for *M. kansasii*::Rv3377-8c. (a-b) *M. kansasii* was used to infect C57Bl/6 mice with 10^6 and 10^5 CFUs. (a) Mice were sacrificed at 1 and 42 d.p.i to establish initial and persistent infectious dose, respectively. (b) Mice were weighed over the 42-day period to establish their level of sickness. (c) Mice were infected with 1:1 *M. kansasii-M. kansasii*::Rv3377-8c at 10^6 CFUs. CFUs were counted on 7H10 plates + PANTA ± hyg50 from C57bl/6 mouse lungs isolated at 1, 7, 28 and 56 days post high-dose trans-laryngeal intubation (n=10 lungs/condition/time point). The data are plotted as the mean ± SD.

Finally, mice were infected with a 1:1 mixture of *M. kansasii-M. kansasii*:Rv3377-8c for 52 days. To identify the *M. kansasii*::Rv3377-8c proportion of the CFUs, lung homogenates were collected at 1, 7, 28 and 56 d.p.i. and plated on 7H10 plates containing PANTA ± hygromycin at

50 µg/ml (Figure IIIH-c). These proportions did not change significantly, with *M. kansasii*::Rv3377-8c remaining between 30-50% at all the time points. Moreover, the infectious dose exhibited a similar overall reduction to that seen in the optimization experiment.

5. Discussion

In summary, our results indicate the feasibility of using *M. kansasii* to query the function of the 55 genes found exclusively in *M. tuberculosis*. The less virulent NTM species is capable of expressing *M. tuberculosis*-specific products and can readily be used in cell culture and murine lung infections. It can also be used in a level-2 setting, making it easier to manipulate than *M. tuberculosis.* As previously shown in *M. smegmatis* by Layre et al [95], the addition of *Rv3377-8c* alone was sufficient to produce 1-TbAd, shown here in the TLC and LC-MS data (Figure IIIB). An initial low-dose infection suggested that the mutant so generated was clearly of less virulence than the attenuated vaccine strain *M. bovis* BCG (data not shown), enabling further investigations to be performed on the strain in a containment level 2 laboratory setting.

Previous studies have suggested that 1-TbAd acts as a pH mediator that hinders the acidification of the phagolysosome within macrophages. Our results have validated the hypothesis that 1-TbAd plays a role in pH-dependent growth using *M. kansasii* as a surrogate for 1-TbAd production. We showed that 1-TbAd production leads to a resistance to and eventual unhindered growth within an initially acidic environment. We also showed that while both bacterial species alkalize the media, *M. tuberculosis* does so to a further extent, which might be partially due to the fact that it produces more 1-TbAd than our mutant. However, we contradictorily showed that when both *M. kansasii*::EV and *M. kansasii*::Rv3377-8c actively grow within their optimal pH levels at 5.4 and higher, they alkalize the media in the same manner, suggesting that the role of 1-TbAd is more likely to confer an initial small shift in pH or an acid resistance shell that leads to growth, which independently leads to alkalization and ease of growth (Figure IIII).

We might have predicted increased survival and/or increased persistence in a macrophage infection model, translating into greater *ex vivo* and *in vivo* fitness. However, we did not see an *ex vivo* or *in vivo* effect on the overall fitness of *M. kansasii.* Through the competition infections, we established that the production of 1-TbAd does not introduce an

advantage either in a cis or trans fashion to *M. kansasii*. Its presence on the cell surface did not afford a selective pressure for the bacteria and its secretion and presence within the mouse lung did not lead to better survival of the lung-residing bacteria in general.

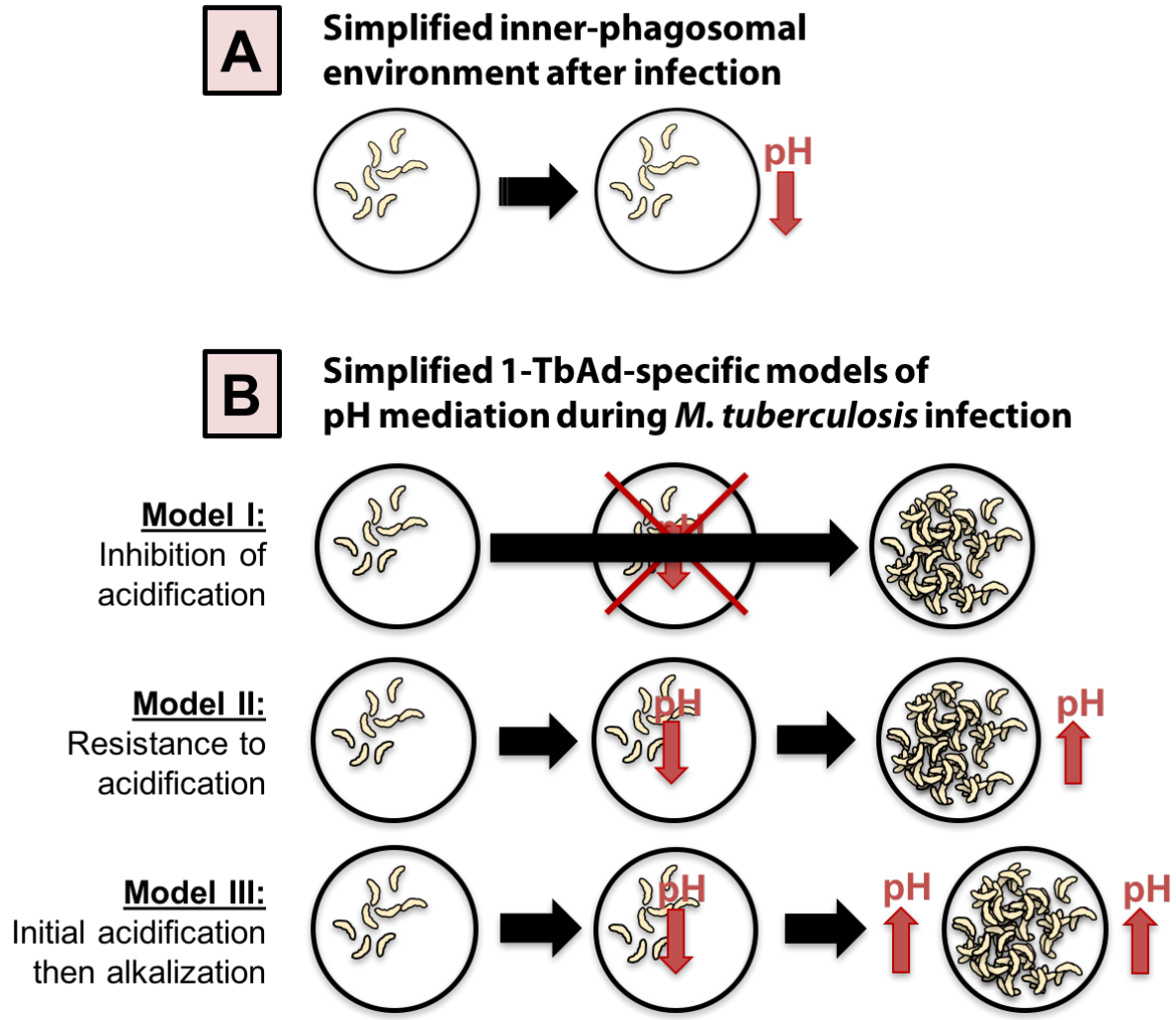

Figure IIII. Suggested models of 1-TbAd-specific intraphagosomal pH mediation during *M. tuberculosis* infection of macrophages and other immune cell types.

A potential reason why we failed to see a marked phenotype in the *ex vivo* and *in vivo* infections is that the generated mutant *M.* kansasii::Rv3377-8c produced significantly less 1-TbAd (0.15%) than *M. tuberculosis* (1-1.5%), thus possibly diluting the full effect of 1-TbAd in infection settings. This reduction might be due to (1) gene complementation strategy and (2) the utilization of GGPP by *M. kansasii* for carotenoid production. Another reason for the lack of

a prominent effect is that 1-TbAd may not act in an independent manner, requiring the assistance of other *M. tuberculosis*-specific products to perform a collective of activities required as a whole to make a more fit pathogen. Another potential reason is that some lipid products in *M. tuberculosis* are only recognized by human receptors that do not have homologs in murine cells such as cD1b [42, 43]. If that is the case, then 1-TbAd production could have a human-specific phenotype, in which case further investigations need to be made. However, this explanation is less likely to be true seeing that 1-TbAd production did lead to an increase in inflammatory cytokine production. In addition to that, further studies contrasting our *M. kansasii* knock-in mutants and the pre-existing *M. tuberculosis* knock-out strains may reveal the reasons for these discrepancies.

In conclusion, our data show that *M. kansasii* can be used as a proxy for its common ancestor with *M. tuberculosis* to study TB-specific HGT-acquired genes through complementation. It provides an opportunity to study the function of each of the 55 genes present exclusively within the MTBC among all mycobacterial species.

6. Author Contributions and Funding

M. A. B. conceived the study. M. G., J. W. and M. B. set up the experimental plan. J. W. performed the cloning and electroporation. M. G. and J. W. extracted the lipids for further analysis and use. J. W., P. D. and M. B. R. performed the adenosine-radiolabelled TLC. F. Mann, D. Y. and B. D. M. performed the GC/MS and LC/MS analyses. M. G. performed the *in vitro* mutant characterization and *ex vivo* macrophage infections. M. G., F. McIntosh and D. H. performed the murine infections.

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CHAPTER III

Reflections and future directions

To date, very few virulence attributes have been identified as being specific to *M. tuberculosis*. Most established virulence factors of *M. tuberculosis* are encoded in the genome of *M. kansasii*. Seeing that the natural habitat of *M. kansasii* is environmental, these previously established virulence factors common to both species, including the ESX-1 secretion system, the PhoPR 2-component system and the DosR/S/T regulon, may play a role in a more broad-spectrum survival strategy in mycobacteria [131-133]. Some findings have identified proof of *M. kansasii* living within free-living phagocytic amoeba [134-136]. This means that the ESX-1 secretion system, for example, which has been shown to have pore-forming activity against the macrophage phagosomal membrane for the release of secreted mycobacterial products into the cytosol, could play a role against phagocytic cells in many different environments, and not just inside the human lung.

Our team has previously made use of bioinformatics tools to identify genes that have potentially been acquired through HGT during the divergent speciation of *M. tuberculosis* from its common ancestor with *M. kansasii* [12]. We have hypothesized that these genes have played an important role in shaping *M. tuberculosis* into the professional human pathogen it is today. Our goal in this thesis project was to use the gene pair *Rv3377-8c* as a proof of concept to establish *M. kansasii* as an experimental model for its common ancestor with *M. tuberculosis*, wherein we can introduce *M. tuberculosis*-specific HGT-acquired genes to study their effects on the environmental mycobacterium in infection models. We have proven, in our findings, that *M. kansasii* can be engineered to produce the *M. tuberculosis*-specific 1-TbAd. We have subsequently shown that 1-TbAd production led to an *M. kansasii* mutant better fit to withstand acidic stress in an *in vitro* experimental study.

To continue studying this phenotype, we will re-perform the pH-dependent growth curves in Mg^{2+}-low media reminiscent of the environment seen within a phagosome, since the activity of *Rv3377-8c* has been shown to increase in Mg^{2+}-deficient environments [101]. We will also repeat the pH-dependent growth curve using Tyloxapol as a substitute for Tween-80, since it has been shown to be less toxic to mycobacteria in acidic media [137]. This will provide us

with proof that 1-TbAd is not acting on Tween-80 toxicity but specifically on the acidity of the media. Next, to directly attribute this alkalization effect to 1-TbAd, we will extract whole lipids were extracted from *M. kansasii*::EV and *M. kansasii*::Rv3377-8c and induce the formation of bilayer lipid membranes that will be mixed into 7H9 at different pH levels. The pH will be measured at 0, 4, 24 and 48 hours as well as 7 and 14 days. We will then grow *M. kansasii* in 7H9 at different pH levels for 14 days in the presence of lipids extracted from *M. kansasii*::EV and *M. kansasii*::Rv3377-8c to further validate and determine the transferability of the resistance phenotype in trans.

Furthermore, the Hsp65 promoter is a heat shock protein-family member that has been shown to be upregulated under stress [138], which in our model may lead to an increased production of 1-TbAd, given non-limiting amounts of precursor molecule, GGPP. In the future, we want to assess the possibility of increased expression of *Rv3377c* and *Rv3378c* through the increased activity of Hsp65 in *M. kansasii*::Rv3377-8c under acidic stress. This will be tested through qRT-PCR at different timepoints after culturing the mutant in media with different pH levels. If that is the case, then we will, pre-condition *M. kansasii*::Rv3377-8c to produce high levels of 1-TbAd before subjecting it to another macrophage infection, to see whether or not increased production would lead to a bacterium better fit to thrive within infection settings.

Although further studies have proven that this effect did not translate into better fitness in for the gain-of-function mutant on its own in *ex vivo* infection models used in this project, murine BMDMs were only fully viable until 48 hours post infection, at which point they started losing confluency even in the absence of bacteria. Therefore, we are in the process of repeating these macrophage infections using murine macrophage RAW264.7 cell lines to study the effect on 1-TbAd production in a long-term *ex vivo* infection model. However, its function within the host may be synergic with other factors found across mycobacteria, such as the pore-forming and secretory ESAT-6, as well as *M. tuberculosis*-specific virulence enablers and effectors. The latter group includes the immunomodulatory sulfoglycolipids as well as the recently characterized HGT-acquired moaA1-D1required for survival in hypoxic conditions, and whose function was partly identified using an *M. kansasii* gain-of-function mutant [139, 140].

Beyond 1-TbAd, our experiments have proven the usefulness of *M. kansasii* as a model organism for further experimentation with the 55 other HGT-acquired genes that have been

found back in 2009 [12]. Our overarching goal is to identify the functions of individually acquired attribute in order to get closer to solving the puzzle of the evolution of *M. tuberculosis* from its distant environmental ancestor. Therefore, our next step will be to create a gain-of-function mutant library with all the remaining *M. tuberculosis*-specific HGT genes to understand their effect on *M. kansasii* behaviour at the different levels that were assessed in this project.

So far, we have discussed HGT in *M. tuberculosis* as one of the main pillars of our molecular approach into understanding mycobacterial pathogenesis. However, we find it essential to fully characterize the model organism that we are using in our studies. In parallel to the complementation project that was the topic of this thesis, we are currently performing an *in vivo* transposon sequencing experiment to identify virulence- and anti-virulence-related genes within the *M. kansasii* genome. This direction will allow us to ascertain (1) the level of conservation of the conditional *in vivo* essentiality of pre-established virulence-related genes shared between *M. kansasii* and *M. tuberculosis*, (2) virulence-related genes that are unique to and HGT-acquired by *M. kansasii* and (3) anti-virulence-related genes that are unique to *M. kansasii* and may have been lost from *M. tuberculosis*. Whereas virulence genes are commonly studied, anti-virulence genes are mostly overlooked in the conversation about pathoevolution. However, the concept of anti-virulence emerged in 2008 in a comparative genomics study that looked at *M. ulcerans* haplotypes and found a number of genes that led to enhanced host adaptation when inactivated/pseudogenized [141]. More recently, this concept has been established in *Mycobacterium canettii*, wherein the loss-if-function mutation in the *pks5* gene leads to a more virulent mutant in cell and murine infection models [142]. The function of *pks5* and LOS biosynthesis are conserved in *M. marinum*, *M. kansasii* and *M. canettii* but the gene has undergone extensive recombination and complete loss of function in *M. tuberculosis* [143]. The identification of such genes in our experimental model would allow us to assess their origins and presence in other mycobacteria, to allow us to ascertain whether or not they were specifically acquired by *M. kansasii* or lost by *M. tuberculosis*.

Finally, we are also interested in studying the previously-identified *M. tuberculosis* virulence attributes that are conserved in *M. kansasii*. We have initiated a series of experiments to understand the function of the ESX-1 secretion system and the ESX-1-dependent secretome of *M. kansasii* and contrast it to its *M. tuberculosis* equivalent, to understand any differences in

secreted effectors between the two organisms. This will be done through genetic recombineering and knockout of the *esxA* gene in *M. kansasii* ATCC 12478 and performing mass spectrometry studies on the supernatant of liquid culture to compare it to the already accessible *M. tuberculosis*::Δ*esxA* we have in our lab.

It is important to mention that we do not think that *M. tuberculosis* became a professional pathogen through the addition of single gene-pair to its genome, but it is the stepwise addition/deletion/modification of genetic content that allowed it to be what it is today. Although modelling this exact evolution may be impossible with genetic engineering tools that are used today, we intend to use bioinformatics to infer the genomic structure and composition of the MKMKCA in order to then attempt to elucidate the stepwise acquisition, loss and modification of genetic content based on molecular evidence that may be left behind within each of the two genomes. This process will allow us to decipher key events that occurred in the differential speciation of *M. tuberculosis* as a human pathogen and *M. kansasii* as an environmental mycobacterium. Ultimately, we hope that through understanding what enables *M. tuberculosis* to be so successful as a human-adapted pathogen, and so destructive to the patients suffering from *M. tuberculosis*-induced pathology, we can devise better tools towards the detection, management and/or prevention of TB.

REFERENCES

1. Ventura, M., et al., *Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum.* Microbiol Mol Biol Rev, 2007. **71**(3): p. 495-548.
2. Niederweis, M., et al., *Mycobacterial outer membranes: in search of proteins.* Trends Microbiol, 2010. **18**(3): p. 109-16.
3. Wright, E.L., et al., *Monoclonal infection involving Mycobacterium avium presenting with three distinct colony morphotypes.* J Clin Microbiol, 1996. **34**(10): p. 2475-8.
4. Mukherjee, S., et al., *The white morphotype of Mycobacterium avium-intracellulare is common in infected humans and virulent in infection models.* The Journal of infectious diseases, 2001. **184**(11): p. 1480-4.
5. R¸ger, K., et al., *Characterization of rough and smooth morphotypes of Mycobacterium abscessus isolates from clinical specimens.* Journal of clinical microbiology, 2014. **52**(1): p. 244-50.
6. Rogall, T., et al., *Towards a phylogeny and definition of species at the molecular level within the genus Mycobacterium.* International journal of systematic bacteriology, 1990. **40**(4): p. 323-30.
7. Rastogi, N., E. Legrand, and C. Sola, *The mycobacteria: an introduction to nomenclature and pathogenesis.* Rev Sci Tech, 2001. **20**(1): p. 21-54.
8. Stahl, D.A. and J.W. Urbance, *The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria.* Journal of bacteriology, 1990. **172**(1): p. 116-24.
9. Pitulle, C., et al., *Phylogeny of rapidly growing members of the genus Mycobacterium.* International journal of systematic bacteriology, 1992. **42**(3): p. 337-43.
10. Bryant, J.M., et al., *Whole-genome sequencing to identify transmission of Mycobacterium abscessus between patients with cystic fibrosis: a retrospective cohort study.* Lancet (London, England), 2013. **381**(9877): p. 1551-60.
11. Kazda, J. and I. Pavlik, *Obligate Pathogenic Mycobacteria*, in *The Ecology of Mycobacteria: Impact on Animal's and Human's Health*. 2009, Springer Netherlands: Dordrecht. p. 13-19.
12. Veyrier, F., et al., *Phylogenetic detection of horizontal gene transfer during the step-wise genesis of Mycobacterium tuberculosis.* BMC Evol Biol, 2009. **9**: p. 196.
13. Devulder, G., M. Perouse de Montclos, and J.P. Flandrois, *A multigene approach to phylogenetic analysis using the genus Mycobacterium as a model.* Int J Syst Evol Microbiol, 2005. **55**(Pt 1): p. 293-302.
14. Sakula, A., *Robert Koch: The story of his discoveries in tuberculosis.* Irish Journal of Medical Science, 1985. **154**(1): p. 3-9.
15. Blevins, S.M. and M.S. Bronze, *Robert Koch and the 'golden age' of bacteriology.* Int J Infect Dis, 2010. **14**(9): p. e744-51.
16. Lin, P.L. and J.L. Flynn, *Understanding latent tuberculosis: a moving target.* Journal of immunology (Baltimore, Md. : 1950), 2010. **185**(1): p. 15-22.
17. *Global Tuberculosis Report.* World Health Organization, 2017.
18. Calver, A.D., et al., *Emergence of increased resistance and extensively drug-resistant tuberculosis despite treatment adherence, South Africa.* Emerg Infect Dis, 2010. **16**(2): p. 264-71.
19. Lee, J.Y., *Diagnosis and Treatment of Extrapulmonary Tuberculosis.* Kyorhaek mit hohupki chirhwan. Tuberculosis and respiratory diseases., 2015. **78**(2): p. 47-55.
20. Fokkens, W.J. and R.A. Scheeren, *Upper airway defence mechanisms.* Paediatr Respir Rev, 2000. **1**(4): p. 336-41.
21. Johnson, J.D., et al., *Activation of alveolar macrophages after lower respiratory tract infection.* J Immunol, 1975. **115**(1): p. 80-4.

22. Gordon, S.B. and R.C. Read, *Macrophage defences against respiratory tract infections.* Br Med Bull, 2002. **61**: p. 45-61.
23. Kim, S.J., et al., *Production of tumor necrosis factor-alpha by alveolar macrophages from patients with pulmonary tuberculosis.* Journal of Korean medical science, 1991. **6**(1): p. 45-53.
24. Flynn, J.L., J. Chan, and P.L. Lin, *Macrophages and control of granulomatous inflammation in tuberculosis.* Mucosal immunology, 2011. **4**(3): p. 271-8.
25. Wolf, A.J., et al., *Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo.* Journal of immunology (Baltimore, Md. : 1950), 2007. **179**(4): p. 2509-19.
26. *Modeling the Mycobacterium tuberculosis granuloma - The critical battlefield in host immunity and disease.* frontiers in immunology, 2013. **4**.
27. Gengenbacher, M. and S.H.E. Kaufmann, *Mycobacterium tuberculosis: success through dormancy.* FMR FEMS Microbiology Reviews, 2012. **36**(3): p. 514-532.
28. Cadena, A.M., S.M. Fortune, and J.L. Flynn, *Heterogeneity in tuberculosis.* Nature reviews. Immunology, 2017. **17**(11): p. 691-702.
29. Lew, J.M., et al., *TubercuList--10 years after.* Tuberculosis (Edinb), 2011. **91**(1): p. 1-7.
30. Buhler, V.B. and A. Pollak, *Human infection with atypical acid-fast organisms; report of two cases with pathologic findings.* Am J Clin Pathol, 1953. **23**(4): p. 363-74.
31. David, H.L., *Carotenoid pigments of Mycobacterium kansasii.* Appl Microbiol, 1974. **28**(4): p. 696-9.
32. Canueto-Quintero, J., et al., *Epidemiological, clinical, and prognostic differences between the diseases caused by Mycobacterium kansasii and Mycobacterium tuberculosis in patients infected with human immunodeficiency virus: a multicenter study.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2003. **37**(4): p. 584-90.
33. Sheu, L.C., et al., *Non-tuberculous mycobacterial infections at San Francisco General Hospital.* CRJ The Clinical Respiratory Journal, 2015. **9**(4): p. 436-442.
34. Bloch, K.C., et al., *Incidence and clinical implications of isolation of Mycobacterium kansasii: results of a 5-year, population-based study.* Annals of internal medicine, 1998. **129**(9): p. 698-704.
35. Johnson, M.M. and J.A. Odell, *Nontuberculous mycobacterial pulmonary infections.* Journal of thoracic disease, 2014. **6**(3): p. 210-20.
36. Roberts, G.D., *Mycobacteria and Nocardia*, in *Laboratory Procedures in Clinical Microbiology*, J.A. Washington, Editor. 1985, Springer New York: New York, NY. p. 379-418.
37. Bakula, Z., et al., *Short communication: subtyping of Mycobacterium kansasii by PCR-restriction enzyme analysis of the hsp65 gene.* BioMed research international, 2013. **2013**.
38. Taillard, C., et al., *Clinical implications of Mycobacterium kansasii species heterogeneity: Swiss National Survey.* J Clin Microbiol, 2003. **41**(3): p. 1240-4.
39. Santin, M., et al., *Incidence and molecular typing of Mycobacterium kansasii in a defined geographical area in Catalonia, Spain.* Epidemiology and infection, 2004. **132**(3): p. 425-32.
40. Chimara, E., et al., *Molecular characterization of Mycobacterium kansasii isolates in the State of S„o Paulo between 1995-1998.* mioc MemÛrias do Instituto Oswaldo Cruz, 2004. **99**(7): p. 739-743.
41. Thomson, R., et al., *Strain variation amongst clinical and potable water isolates of M. kansasii using automated repetitive unit PCR.* IJMM International Journal of Medical Microbiology, 2014. **304**(3-4): p. 484-489.
42. Ricketts, W.M., T.C. O'Shaughnessy, and J. van Ingen, *Human-to-human transmission of Mycobacterium kansasii or victims of a shared source?* The European respiratory journal, 2014. **44**(4): p. 1085-7.

43. Ulmann, V., A. Kracalikova, and R. Dziedzinska, *Mycobacteria in water used for personal hygiene in heavy industry and collieries: a potential risk for employees.* International journal of environmental research and public health, 2015. **12**(3): p. 2870-7.
44. Thomson, R., et al., *Isolation of nontuberculous mycobacteria (NTM) from household water and shower aerosols in patients with pulmonary disease caused by NTM.* J Clin Microbiol, 2013. **51**(9): p. 3006-11.
45. Saleh, M. and M. Saleh, *Nodular lymphangitis caused by Mycobacterium marinum.* PID Pediatric Infectious Disease, 2016. **8**(3): p. 88-90.
46. Aubry, A., et al., *Sixty-three Cases of Mycobacterium marinum Infection.* Arch Intern Med Archives of Internal Medicine, 2002. **162**(15): p. 1746.
47. Lillo, M., et al., *Pulmonary and disseminated infection due to Mycobacterium kansasii: a decade of experience.* Reviews of infectious diseases, 1990. **12**(5).
48. Sato, R., et al., *Interferon-gamma release assays in patients with Mycobacterium kansasii pulmonary infection: A retrospective survey.* The Journal of infection, 2016. **72**(6): p. 706-712.
49. Griffith, D.E., et al., *An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases.* American journal of respiratory and critical care medicine, 2007. **175**(4): p. 367-416.
50. Wang, J., et al., *Insights on the emergence of Mycobacterium tuberculosis from the analysis of Mycobacterium kansasii.* Genome biology and evolution, 2015. **7**(3): p. 856-70.
51. Kopf, M., C. Schneider, and S.P. Nobs, *The development and function of lung-resident macrophages and dendritic cells.* Nature immunology, 2015. **16**(1): p. 36-44.
52. Kaur, M., et al., *Macrophage adaptation in airway inflammatory resolution.* European respiratory review : an official journal of the European Respiratory Society, 2015. **24**(137): p. 510-5.
53. Weiss, G.n. and U.E. Schaible, *Macrophage defense mechanisms against intracellular bacteria.* IMR Immunological Reviews, 2015. **264**(1): p. 182-203.
54. Welin, A., et al., *Incorporation of Mycobacterium tuberculosis lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block.* Infection and immunity, 2008. **76**(7): p. 2882-7.
55. Molmeret, M., et al., *Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the Legionella-containing phagosome.* Cellular microbiology, 2004. **6**(1): p. 33-48.
56. Dermine, J.-F., et al., *Leishmania promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis.* CMI Cellular Microbiology, 2000. **2**(2): p. 115-126.
57. Eissenberg, L.G., W.E. Goldman, and P.H. Schlesinger, *Histoplasma capsulatum modulates the acidification of phagolysosomes.* The Journal of experimental medicine, 1993. **177**(6): p. 1605-11.
58. Metchnikoff, E. and F.G. Binnie, *Immunity in Infective Diseases*. 1905: University Press.
59. Yates, R.M., et al., *Macrophage Activation Downregulates the Degradative Capacity of the Phagosome.* Traffic, 2007. **8**(3): p. 241-250.
60. Nalpas, N.C., et al., *RNA sequencing provides exquisite insight into the manipulation of the alveolar macrophage by tubercle bacilli.* Scientific reports, 2015. **5**.
61. Vandal, O.H., C.F. Nathan, and S. Ehrt, *Acid resistance in Mycobacterium tuberculosis.* Journal of bacteriology, 2009. **191**(15): p. 4714-21.
62. Portaels, F. and S.R. Pattyn, *Growth of mycobacteria in relation to the pH of the medium.* Ann Microbiol (Paris), 1982. **133**(2): p. 213-21.
63. Eyre-Walker, A. and P.D. Keightley, *The distribution of fitness effects of new mutations.* Nature reviews. Genetics, 2007. **8**(8): p. 610-8.

64. MendonÁa, A.G., R.J. Alves, and J.B. Pereira-Leal, *Loss of genetic redundancy in reductive genome evolution.* PLoS computational biology, 2011. **7**(2).
65. *Reductive Evolution in Bacteria: Buchnera sp., Rickettsia prowazekii and Mycobacterium leprae.* comparative and functional genomics, 2001. **2**(1): p. 44-48.
66. Arber, W., *Horizontal Gene Transfer among Bacteria and its Role in Biological Evolution.* Life Life, 2014. **4**(2): p. 217-224.
67. Kelly, B.G., A. Vespermann, and D.J. Bolton, *The role of horizontal gene transfer in the evolution of selected foodborne bacterial pathogens.* Food and Chemical Toxicology, 2009. **47**(5): p. 951-968.
68. Francino, M.P., *Horizontal gene transfer in microorganisms.* 2012.
69. Dong, H., L. Nilsson, and C.G. Kurland, *Gratuitous overexpression of genes in Escherichia coli leads to growth inhibition and ribosome destruction.* Journal of bacteriology, 1995. **177**(6): p. 1497-504.
70. Soucy, S.M., J. Huang, and J.P. Gogarten, *Horizontal gene transfer: building the web of life.* Nature reviews. Genetics, 2015. **16**(8): p. 472-82.
71. Dunning Hotopp, J.C., *Horizontal gene transfer between bacteria and animals.* Trends in genetics : TIG, 2011. **27**(4): p. 157-63.
72. Johnston, C., et al., *Bacterial transformation: distribution, shared mechanisms and divergent control.* Nature reviews. Microbiology, 2014. **12**(3): p. 181-96.
73. Solomon, J.M. and A.D. Grossman, *Who's competent and when: regulation of natural genetic competence in bacteria.* Trends in genetics : TIG, 1996. **12**(4): p. 150-5.
74. Grohmann, E., G. Muth, and M. Espinosa, *Conjugative plasmid transfer in gram-positive bacteria.* Microbiology and molecular biology reviews : MMBR, 2003. **67**(2): p. 277-301.
75. Porter, C.J., et al., *The conjugation protein TcpC from Clostridium perfringens is structurally related to the type IV secretion system protein VirB8 from Gram-negative bacteria.* Molecular microbiology, 2012. **83**(2): p. 275-88.
76. Eggers, C.H., et al., *Phage-mediated horizontal gene transfer of both prophage and heterologous DNA by varphiBB-1, a bacteriophage of Borrelia burgdorferi.* Pathog Dis, 2016. **74**(9).
77. Balcazar, J.L., *How do bacteriophages promote antibiotic resistance in the environment?* Clin Microbiol Infect, 2017.
78. Engelst‰dter, J., K. Harms, and P.J. Johnsen, *The evolutionary dynamics of integrons in changing environments.* The ISME journal, 2016. **10**(6): p. 1296-307.
79. Dubey, G.P. and S. Ben-Yehuda, *Intercellular nanotubes mediate bacterial communication.* Cell, 2011. **144**(4): p. 590-600.
80. Hershberg, R., et al., *High functional diversity in Mycobacterium tuberculosis driven by genetic drift and human demography.* PLoS Biol, 2008. **6**(12): p. e311.
81. Comas, I., et al., *Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans.* Nat Genet, 2013. **45**(10): p. 1176-82.
82. Brosch, R., et al., *The evolution of mycobacterial pathogenicity: clues from comparative genomics.* TIMI</cja:jid> Trends in Microbiology, 2001. **9**(9): p. 452-458.
83. Singh, P. and S.T. Cole, *Mycobacterium leprae: genes, pseudogenes and genetic diversity.* Future Microbiol, 2011. **6**(1): p. 57-71.
84. Maurelli, A.T., et al., *"Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of Shigella spp. and enteroinvasive Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(7): p. 3943-8.
85. Kaser, M. and G. Pluschke, *Differential gene repertoire in Mycobacterium ulcerans identifies candidate genes for patho-adaptation.* PLoS Negl Trop Dis, 2008. **2**(12): p. e353.
86. Boritsch, E.C., et al., *pks5-recombination-mediated surface remodelling in Mycobacterium tuberculosis emergence.* Nat Microbiol, 2016. **1**: p. 15019.

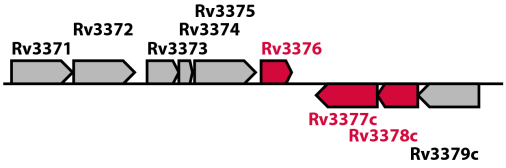
87. Veyrier, F.J., A. Dufort, and M.A. Behr, *The rise and fall of the Mycobacterium tuberculosis genome.* Trends Microbiol, 2011. **19**(4): p. 156-61.
88. Becq, J., et al., *Contribution of horizontally acquired genomic islands to the evolution of the tubercle bacilli.* Mol Biol Evol, 2007. **24**(8): p. 1861-71.
89. Mostowy, S. and M.A. Behr, *The origin and evolution of Mycobacterium tuberculosis.* Clin Chest Med, 2005. **26**(2): p. 207-16, v-vi.
90. Griffin, J.E., et al., *High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism.* PLoS Pathog, 2011. **7**(9): p. e1002251.
91. Rengarajan, J., B.R. Bloom, and E.J. Rubin, *Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages.* Proc Natl Acad Sci U S A, 2005. **102**(23): p. 8327-32.
92. Sassetti, C.M., D.H. Boyd, and E.J. Rubin, *Genes required for mycobacterial growth defined by high density mutagenesis.* Mol Microbiol, 2003. **48**(1): p. 77-84.
93. Pethe, K., et al., *Isolation of Mycobacterium tuberculosis mutants defective in the arrest of phagosome maturation.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13642-7.
94. Young, D.C., et al., *In vivo biosynthesis of terpene nucleosides provides unique chemical markers of Mycobacterium tuberculosis infection.* Chem Biol, 2015. **22**(4): p. 516-526.
95. Layre, E., et al., *Molecular profiling of Mycobacterium tuberculosis identifies tuberculosinyl nucleoside products of the virulence-associated enzyme Rv3378c.* Proc Natl Acad Sci U S A, 2014. **111**(8): p. 2978-83.
96. Rosas-Saavedra, C. and C. Stange, *Biosynthesis of Carotenoids in Plants: Enzymes and Color.* Subcell Biochem, 2016. **79**: p. 35-69.
97. Stange, C., *Carotenoids in Nature : Biosynthesis, Regulation and Function.* 2016.
98. Mann, F.M., et al., *Functional characterization and evolution of the isotuberculosinol operon in Mycobacterium tuberculosis and related Mycobacteria.* Front Microbiol, 2012. **3**: p. 368.
99. Mann, F.M., et al., *Edaxadiene: a new bioactive diterpene from Mycobacterium tuberculosis.* J Am Chem Soc, 2009. **131**(48): p. 17526-7.
100. Maugel, N., et al., *Synthesis of (+/-)-nosyberkol (isotuberculosinol, revised structure of edaxadiene) and (+/-)-tuberculosinol.* Org Lett, 2010. **12**(11): p. 2626-9.
101. Mann, F.M., B.C. VanderVen, and R.J. Peters, *Magnesium depletion triggers production of an immune modulating diterpenoid in Mycobacterium tuberculosis.* Mol Microbiol, 2011. **79**(6): p. 1594-601.
102. Lau, S.K., et al., *Identification of specific metabolites in culture supernatant of Mycobacterium tuberculosis using metabolomics: exploration of potential biomarkers.* Emerg Microbes Infect, 2015. **4**(1): p. e6.
103. Pan, S.J., et al., *Biomarkers for Tuberculosis Based on Secreted, Species-Specific, Bacterial Small Molecules.* J Infect Dis, 2015. **212**(11): p. 1827-34.
104. Kiss, H., et al., *Complete genome sequence of the filamentous gliding predatory bacterium Herpetosiphon aurantiacus type strain (114-95(T)).* Stand Genomic Sci, 2011. **5**(3): p. 356-70.
105. Dunphy, K.Y., et al., *Attenuation of Mycobacterium tuberculosis functionally disrupted in a fatty acyl-coenzyme A synthetase gene fadD5*. The Journal of infectious diseases, 2010. **201**(8): p. 1232-9.
106. Sander, P., et al., *Lipoprotein processing is required for virulence of Mycobacterium tuberculosis.* Molecular microbiology, 2004. **52**(6): p. 1543-52.
107. Peirs, P., et al., *Mycobacterium tuberculosis with disruption in genes encoding the phosphate binding proteins PstS1 and PstS2 is deficient in phosphate uptake and demonstrates reduced in vivo virulence.* Infection and immunity, 2005. **73**(3): p. 1898-902.

108. Kalscheuer, R., et al., *Trehalose-recycling ABC transporter LpqY-SugA-SugB-SugC is essential for virulence of Mycobacterium tuberculosis.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(50): p. 21761-6.
109. Brosch, R., et al., *A new evolutionary scenario for the Mycobacterium tuberculosis complex.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(6): p. 3684-9.
110. Stinear, T.P., et al., *Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis.* Genome Res, 2008. **18**(5): p. 729-41.
111. Smith, S.E., et al., *Comparative genomic and phylogenetic approaches to characterize the role of genetic recombination in mycobacterial evolution.* PLoS One, 2012. **7**(11): p. e50070.
112. Reva, O., I. Korotetskiy, and A. Ilin, *Role of the horizontal gene exchange in evolution of pathogenic Mycobacteria.* BMC Evol Biol, 2015. **15 Suppl 1**: p. S2.
113. Ummels, R., et al., *Identification of a novel conjugative plasmid in mycobacteria that requires both type IV and type VII secretion.* MBio, 2014. **5**(5): p. e01744-14.
114. Boritsch, E.C., et al., *Key experimental evidence of chromosomal DNA transfer among selected tuberculosis-causing mycobacteria.* Proceedings of the National Academy of Sciences of the United States of America, 2016. **113**(35): p. 9876-81.
115. Slayden, R.A. and C.E. Barry, 3rd, *Analysis of the Lipids of Mycobacterium tuberculosis.* Methods Mol Med, 2001. **54**: p. 229-45.
116. Layre, E., et al., *A comparative lipidomics platform for chemotaxonomic analysis of Mycobacterium tuberculosis.* Chem Biol, 2011. **18**(12): p. 1537-49.
117. Guilbault, C., et al., *Cystic fibrosis lung disease following infection with Pseudomonas aeruginosa in Cftr knockout mice using novel non-invasive direct pulmonary infection technique.* Lab Anim, 2005. **39**(3): p. 336-52.
118. Jankute, M., et al., *The role of hydrophobicity in tuberculosis evolution and pathogenicity.* Sci Rep, 2017. **7**(1): p. 1315.
119. Cangelosi, G.A., et al., *Colony morphotypes on Congo red agar segregate along species and drug susceptibility lines in the Mycobacterium avium-intracellulare complex.* Microbiology, 1999. **145 (Pt 6)**: p. 1317-24.
120. Stewart, G.R., et al., *Mycobacterial mutants with defective control of phagosomal acidification.* PLoS Pathog, 2005. **1**(3): p. 269-78.
121. Rohde, K.H., R.B. Abramovitch, and D.G. Russell, *Mycobacterium tuberculosis invasion of macrophages: linking bacterial gene expression to environmental cues.* Cell Host Microbe, 2007. **2**(5): p. 352-64.
122. Waddell, S.J. and P.D. Butcher, *Microarray analysis of whole genome expression of intracellular Mycobacterium tuberculosis.* Curr Mol Med, 2007. **7**(3): p. 287-96.
123. Sohn, H., et al., *In vitro and ex vivo activity of new derivatives of acetohydroxyacid synthase inhibitors against Mycobacterium tuberculosis and non-tuberculous mycobacteria.* Int J Antimicrob Agents, 2008. **31**(6): p. 567-71.
124. Wang, C., et al., *Innate immune response to Mycobacterium tuberculosis Beijing and other genotypes.* PLoS One, 2010. **5**(10): p. e13594.
125. Olsen, A., et al., *Targeting Mycobacterium tuberculosis Tumor Necrosis Factor Alpha-Downregulating Genes for the Development of Antituberculous Vaccines.* MBio, 2016. **7**(3).
126. Engele, M., et al., *Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent Mycobacterium tuberculosis.* J Immunol, 2002. **168**(3): p. 1328-37.
127. Yu, K., et al., *Toxicity of nitrogen oxides and related oxidants on mycobacteria: M. tuberculosis is resistant to peroxynitrite anion.* Tuber Lung Dis, 1999. **79**(4): p. 191-8.
128. Mohan, V.P., et al., *Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology.* Infect Immun, 2001. **69**(3): p. 1847-55.

129. Clay, H., H.E. Volkman, and L. Ramakrishnan, *Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death*. Immunity, 2008. **29**(2): p. 283-94.
130. Watkins, B.Y., et al., *Mycobacterium marinum SecA2 promotes stable granulomas and induces tumor necrosis factor alpha in vivo*. Infect Immun, 2012. **80**(10): p. 3512-20.
131. Fortune, S.M., et al., *Mutually dependent secretion of proteins required for mycobacterial virulence*. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(30): p. 10676-81.
132. Boon, C. and T. Dick, *Mycobacterium bovis BCG response regulator essential for hypoxic dormancy*. Journal of bacteriology, 2002. **184**(24): p. 6760-7.
133. Walters, S.B., et al., *The Mycobacterium tuberculosis PhoPR two-component system regulates genes essential for virulence and complex lipid biosynthesis*. Molecular microbiology, 2006. **60**(2): p. 312-30.
134. Thomas, V., et al., *Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality*. FMR FEMS Microbiology Reviews, 2010. **34**(3): p. 231-259.
135. Salah, I.B., E. Ghigo, and M. Drancourt, *Free-living amoebae, a training field for macrophage resistance of mycobacteria*. CLM Clinical Microbiology and Infection, 2009. **15**(10): p. 894-905.
136. Goy, G., et al., *The Neff strain of Acanthamoeba castellanii, a tool for testing the virulence of Mycobacterium kansasii*. RESMIC Research in Microbiology, 2007. **158**(4): p. 393-397.
137. Darby, C.M., et al., *Whole cell screen for inhibitors of pH homeostasis in Mycobacterium tuberculosis*. PLoS ONE, 2013. **8**(7).
138. Choudhary, E., et al., *Expression of a Subset of Heat Stress Induced Genes of Mycobacterium tuberculosis Is Regulated by 3',5'-Cyclic AMP*. PLoS ONE PLoS ONE, 2014. **9**(2): p. e89759.
139. Levillain, F., et al., *Horizontal acquisition of a hypoxia-responsive molybdenum cofactor biosynthesis pathway contributed to Mycobacterium tuberculosis pathoadaptation*. PLoS Pathog, 2017. **13**(11): p. e1006752.
140. Blanc, L., et al., *Mycobacterium tuberculosis inhibits human innate immune responses via the production of TLR2 antagonist glycolipids*. Proc Natl Acad Sci U S A, 2017. **114**(42): p. 11205-11210.
141. K‰ser, M. and G. Pluschke, *Differential gene repertoire in Mycobacterium ulcerans identifies candidate genes for patho-adaptation*. PLoS neglected tropical diseases, 2008. **2**(12).
142. Boritsch, E.C., et al., *pks5-recombination-mediated surface remodelling in Mycobacterium tuberculosis emergence*. Nature microbiology, 2016. **1**.
143. Nataraj, V., et al., *MKAN27435 is required for the biosynthesis of higher subclasses of lipooligosaccharides in Mycobacterium kansasii*. PloS one, 2015. **10**(3).

APPENDIX

Gene sequences and cloning strategy information



Sequences

Rv3377c

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Rv3378c

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Rv3377-8c fragment generated with BamHI, intergenic and HindIII sites

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pMV261 unique restriction sites for XbaI, BamHI and HindIII and the **HSP65 promoter**

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GTGGTGGCATCCGTGGCGCGGCCGCGGTACCAGATCTTTAAATCTAGACC**GGTGACCACAACGACGCGCCCGCTTTGA**
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pMV306 unique restriction sites for XbaI and HindIII

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