Probing differences in gene essentiality between the human and animal adapted lineages of the *Mycobacterium tuberculosis* complex using TnSeq

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Abstract

Members of the Mycobacterium tuberculosis complex (MTBC) show distinct host adaptations, preferences and phenotypes despite being >99% identical at the nucleic acid level. Previous studies have explored gene expression changes between the members, however few studies have probed differences in gene essentiality. To better understand the functional impacts of the nucleic acid differences between *Mycobacterium bovis* and *Mycobacterium tuberculosis* we used the Mycomar T7 phagemid delivery system to generate whole genome transposon libraries in laboratory strains of both species and compared the essentiality status of genes during growth under identical *in vitro* conditions. Libraries contained insertions in 54% of possible TA sites in *M. bovis* and 40% of those present in *M. tuberculosis*, achieving similar saturation levels to those previously reported for the MTBC. The distributions of essentiality across the functional categories were similar in both species. 527 genes were found to be essential in *M. bovis* whereas 477 genes were essential in *M. tuberculosis* and 370 essential genes were common in both species. CRISPRi was successfully utilised in both species to determine the impacts of silencing genes including *wag31*, a gene involved in peptidoglycan synthesis and *Rv2182c*/*Mb2204c,* a gene involved in glycerophospholipid metabolism. We observed species specific differences in the response to gene silencing, with the inhibition of expression of *Mb2204c* in *M. bovis* showing significantly less growth impact than silencing its ortholog (*Rv2182c*) in *M. tuberculosis.* Given that glycerophospholipid metabolism is a validated pathway for antimicrobials, our observations suggest that target vulnerability in the animal adapted lineages cannot be assumed to be the same as the human counterpart. This is of relevance for zoonotic tuberculosis as it implies that the development of antimicrobials targeting the human adapted lineage might not necessarily be effective against the animal adapted lineage. The generation of a transposon library and the first reported utilisation of CRISPRi in *M. bovis* will enable the use of these tools to further probe the genetic basis of survival under disease relevant conditions.

Introduction

Mycobacterium bovis and *Mycobacterium tuberculosis* are closely related members of the Mycobacterium tuberculosis complex (MTBC). Although both species are >99% identical at the nucleotide level each species shows distinct host tropisms. *M. bovis*, the animal adapted species, is the main causative agent of bovine tuberculosis in cattle (1) while *M. tuberculosis* is the main cause of human tuberculosis (TB) and is responsible for ~1.5 million deaths annually (1,2). *M. bovis* exhibits a broader host range than *M. tuberculosis* and is also able to cause TB in humans through zoonotic transfer, representing a serious public health risk in countries without a control programme in domestic livestock (2,3). The WHO recognises that zoonotic transfer of tuberculosis threatens the delivery of the end TB strategy, highlighting the importance of understanding the differences between the two species (3).

Many studies have explored the genotypic and phenotypic differences between *M. tuberculosis* and *M. bovis* in order to better understand host preference. Genome sequencing of the reference strains (H37Rv and AF2122/97) showed that the main genetic differences between these pathogens were several large-scale deletions, or regions of difference (RD), and over 2,000 single-nucleotide polymorphisms (SNPs) (4–7). More recently, studies that include clinically circulating strains have confirmed that all animal adapted lineages share deletions RD7, 8, 9, and 10 (8). Transcriptomic studies which have measured significant changes in gene expression between H37Rv and AF2122/97 have provided a functional insight into the impacts of some of these polymorphisms (9–11). For instance, a SNP in *rskA* (*Mb0452c*) an anti-sigma factor in *M. bovis,* prevents repression of *sigK* activity, leading to constitutively high levels of expression of *mpb70* and *mpb83,* genes that encode key immunogenic antigens; MPB70 and MPB83 (12,13). Recent studies have shown that MPB70 mediates multi-nucleated giant cell formation in *M. bovis* infected bovine macrophages, but not in *M. bovis* (or *M. tuberculosis*) infected human macrophages, providing insight into bacterial effectors of the species-specific response (14). Transcriptomic studies have also indicated a differential response to *in vitro* mimics of host stresses such as acid shock and highlight the impact of SNPs in the signalling and response regulons in two-component systems such as PhoPR and DosSRT (15–18).

Genome-wide transposon mutagenesis coupled with next-generation sequencing (TnSeq) has allowed genome wide predictions of gene essentiality in *M. tuberculosis* (19–24). These studies have provided information on the genetic requirements for *in vitro* growth under a number of conditions and also for growth in disease relevant models such as macrophages (20). Most of these studies performed in the MTBC have used strain H37Rv. More recently Tnseq of different clinical strains of *M. tuberculosis* has shown that there are strain specific differences in fitness associated with Tn insertions in certain genes. The implication of this observation is that different strains can show different antibiotic sensitivities as a result (25). To date, there has been a single reported Tnseq study performed in *M. bovis* (AF2122/97) which focused on intra-cellular genetic requirements (26)

A direct comparison of gene essentiality in *M. bovis* and *M. tuberculosis* has not been reported. Therefore, we created dense transposon libraries in both *M. bovis* (AF2122/97) and *M. tuberculosis* (H37Rv) generated on the same medium to enable direct comparisons between the two related species. We identified that there are key differences in essentiality in *M. bovis* compared to *M. tuberculosis*. We used CRISPRi to directly demonstrate that silencing the expression of a gene annotated to be involved in glycerophospholipid metabolism has different impacts on growth in the two species. This has implications for target discovery programmes as it implies that inhibition of therapeutically relevant pathways may have different impacts in the different species. This is important in the context of zoonotic tuberculosis.

Materials and Methods

Bacterial Strains and Culture Methods

M. bovis AF2122/97 was maintained on modified Middlebrook 7H11 solid medium containing 0.5% lysed defibrinated sheep blood, 10% heat inactivated foetal bovine serum and 10% oleic acid-albumin-dextrose-catalase (OADC) (27). Liquid cultures of *M. bovis* were grown in Middlebrook 7H9 medium containing 75 mM sodium pyruvate, 0.05% Tween®80 and 10% albumin-dextrose-catalase (ADC). *M. tuberculosis* H37Rv and *Mycobacterium smegmatis* mc^2155 were maintained on Middlebrook 7H11 solid medium supplemented with 0.5% glycerol and 10% OADC. Liquid cultures were grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween®80 and 10% ADC unless stated otherwise. MycomarT7 Phagemid was propagated on *M. smegmatis* mc^2155 lawns grown on Middlebrook 7H10 solid medium supplemented with 0.5% glycerol and 10% OADC in a 0.6% agar overlay. The strains and plasmids used or made in this study are given in Table 1.

Generation of Transposon Libraries

Transposon libraries in *M. bovis* (AF2122/97) and *M. tuberculosis* (H37Rv) were generated using the previously described MycomarT7 phagemid system as per Majumdar *et al* with modifications (28). Briefly, 50 ml cultures of *M. bovis* and *M. tuberculosis* at $OD_{600}\cong1$ were washed twice with MP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgSO_4$ and 2 mM $CaCl_2$) at 37 °C, and then incubated with ~ 10^{11} pfu of φMycoMarT7 phage for 16-18 h at 37 °C without rolling. Transduced bacteria were washed in pre-warmed PBS + 0.05% Tween®80 to remove extra-cellular phage and plated on Middlebrook 7H11 solid medium containing 0.5% lysed defibrinated sheep blood, 10% heat inactivated foetal bovine serum, 10% OADC, 25 µg/ml kanamycin and 0.05% Tween®80. Cultures were allowed to grow for 5-6 weeks. Concurrent CFU plating was performed to estimate transduction efficiency. Approximately 15-20 colonies from each library were used for validation of random insertion using a nested PCR strategy followed by Sanger sequencing, method and data are shown in Supplementary File S1. Libraries were scraped from the plates and incubated in liquid medium at 37°C with hourly vortexing for 3 h to homogenise. Homogenised mutants were distributed to cryovials and stored at -80°C for further selection or gDNA extraction.

DNA Extraction

Unless stated otherwise, reagents were acquired from Sigma Aldrich. Genomic DNA from harvested libraries was isolated by a bead beating procedure (mechanical lysis) or using de-lipidation followed by enzymatic lysis as previously described by Long *et al* 2015 (29) and Belisle *et al* 2009 (30). Briefly, for mechanical lysis, library aliquots were disrupted using 0.1 mm glass beads and bead-beating by 3 X 15 sec bursts (5000 rpm) interspersed with 2 min on ice using a beat-beater (Biospec). For enzymatic lysis, libraries were de-lipidated with equal volumes chloroform-methanol (2:1) for 1 h with agitation every 15 min, suspension was centrifuged at 3,488 x g for 10 min the bacterial pellet allowed to dry for 2 h after removal of both solvent layers. De-lipidated bacteria were suspended in TE buffer and incubated with 100 µg/ml lysozyme in the presence of 100 mM TrisBase (pH 9.0) at 37°C for 12-16 h. Bacterial lysis was completed by incubating for 3 h at 55°C in the presence of 1% SDS and 100 µg/ml proteinase K (NEB). Lysates from both methods were extracted twice with equal volumes of phenol–chloroform–isoamyl alcohol (25:24:1). The aqueous layer was harvested by centrifugation at 12,000 x g for 30 min and DNA was precipitated with 0.1 volumes of 3M sodium acetate (pH 5.2) and one volume of ice-cold isopropanol overnight at -20°C. DNA pellets were washed several times in ethanol. DNA was re-suspended in water and quantity and quality were determined using a DeNovix Spectrophotometer (DeNovix Inc, USA), agarose gel electrophoresis and fluorometry using Qubit4 (Invitrogen).

Library Preparation for Transposon Directed Inserted Sequencing

2 µg of extracted DNA libraries were resuspended in purified water and sheared to approximately 550 bp fragments using a S220 focussed-ultrasonicator (Covaris), according to the manufacturer's protocol. Sheared DNA was repaired using NEBNext blunt-end repair kit (New England Biolabs) and purified using Monarch PCR clean-up kit (New England Biolabs). Blunted DNA was A-tailed using NEBNext dA-tailing kit and column-purified. Custom transposon sequencing adaptors, or "TraDIS tags", (Table 2) were generated by heating an equimolar mix of adaptor standard primer and adaptor P7+index to 95°C for 7 min and then allowed to cool to room temperature. Adaptors were ligated to A-tailed library fragments using NEBNext quick ligase kit. Transposon-containing fragments were enriched by PCR using ComP7 primers ComP5 using Phusion

DNA polymerase (New England Biolabs) in a 20-cycle reaction. Library fragments were subsequently cleaned up with AMPureXP purification beads (Beckman).

Data Analysis

Indexed libraries were combined, spiked with 20% PhiX, and sequenced on the Illumina HiSeq 3000 platform, using v2 chemistry, generating single-end reads of 150 bp. Raw FASTQ sequence files were pre-processed using the TPP utility of TRANSIT python package (DeJesus *et al*., 2015), including removing TRADIS tags and adapter sequences and mapping using BWA-MEM algorithm [32], to generate insertion files in .wig format. Custom annotations, 'prot tables', were created from the *M. bovis* strain AF2122/97 annotation file (NCBI Accession Number LT708304, version LT708304.1) and for the *M. tuberculosis* strain, H37Rv (NCBI Accession Number AL123456, version AL123456.3, assembly build GCA_000195955.2 (ENA)). TRANSIT was run on both *M.bovis* and *M. tuberculosis* files using the default normalisation (TTR) and the TRANSIT HMM algorithm (31) to make calls of essentiality for each TA insertion site, and for each gene based on annotated gene boundaries. Data files (fastq) are deposited in SRA (PRJNA754037)

CRISPRi mediated gene silencing

We utilised dCas9 from *Streptococcus pyogenes* ($dCas9_{Spy}$) for silencing as previously described (32). sgRNA targeting $wag31_{Mtb/Mb}$ and *Rv2182c*/*Mb2204c* were designed according to the parameters derived from Larson *et al* 2013 (33). Protospacer adjacent motif (PAM) sites, "NGG," were chosen and putative sgRNAs 20 bp downstream of the PAM were selected. All sgRNAs designed targeted the coding non-template strand. The probability of complementarity to any other region of the genome and predicted secondary structure of the sgRNA transcript was analyzed using a basic local alignment search tool (BLAST) and M-fold, respectively (34,35). Complementary forward and reverse primers using the sequence (without the PAM) with appropriate ends for ligation to the pRH2521 vector were designed (Table 2). Oligos were annealed and cloned into pRH2521 using BbsI as previous (32,36). One microgram of pRH2502 was electroporated at 25 kV, 25 µF with 1000 Ω resistance into electrocompetent *M. bovis* and *M. tuberculosis* to generate strains expressing $dCas9_{Spy}$ ($Mtb_{\_dCas9}$/ $Mb_{\_dCas9}$). These strains were grown and

197 further electroporated with 1 µg of pRH2521 expressing sgRNAs targeting *wag31*$_{Mtb/Mb}$
198 and *Rv2182c*/*Mb2204c* or pRH2521, the sgRNA -ve plasmid.

199 **RNA Extraction and RT-qPCR**

200 Cultures were grown to OD_{600} ≅ 0.1–0.2 and the CRISPRi machinery induced with
201 200 ng/ml of aTc for 1 h. Total RNA was extracted as previously described (37). Briefly,
202 cultures were centrifuged at 3,488 x g at 4°C for 10 min. Pellets were resuspended in 1
203 ml of TRIzol containing 0.1 mm glass beads and were disrupted by three cycles of 30 sec
204 pulses at 6000 rpm using a Precellys homogenizer. RNA was purified using a Qiagen
205 RNeasy kit combined with on-column DNase digestion according to the manufacturer's
206 instructions. Quantity and quality were determined using a DeNovix Spectrophotometer
207 (DeNovix Inc, USA) and agarose gel electrophoresis.

208 To remove traces of contaminating DNA, RNA samples were treated with RNase-
209 free DNase I (Invitrogen) according to the manufacturer's instructions. cDNA was
210 synthesized from 100 ng of RNA using Superscript III Reverse transcriptase according to
211 manufacturer instructions. qPCRs were performed using PowerUp SYBR Green Master
212 Mix with 1 µl of cDNA and 0.3 µM of either *sigA* primers or gene specific primers (Table
213 2) in a final volume of 20 µl. Samples were run on a BioRad CFX96 analyser at 50 °C for 2
214 min, 95 °C for 2 min, followed by 40 cycles of 50 °C for 2 min, 95 °C for 2 min, followed by
215 40 cycles of 95 °C for 15 sec, 72 °C for 1 min and 85 °C for 5 sec at which point fluorescence
216 was captured. A melt curve analysis was also carried out for each run at 65 °C – 95 °C in
217 increments of 0.5 °C. Gene expression data was analysed using the 2-ΔΔCT method (38).
218 Reverse transcriptase -ve samples were used as a control to ensure removal of gDNA. All
219 results were normalised against the house keeping gene *sigA.* Two or three biological
220 replicates were run, with each measured in duplicate, unless otherwise stated.
221

Results

High-density transposon libraries in *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv were generated

The Mycomar transposon inserts randomly into TA sites in bacterial genomes. There are 73,536 and 74,604 TA sites present in the *M. bovis* (AF2122/97) and *M. tuberculosis* (H37Rv) genomes, respectively. The smaller number of TA sites in *M. bovis* is likely to be reflective of a smaller genome. We successfully generated transposon libraries in *M. bovis* and *M. tuberculosis* containing 39,987 (*M. bovis*) and 29,919 (*M. tuberculosis*) unique mutants, representing 54 % (*M. bovis*) and 40 % (*M. tuberculosis*) saturation. The distribution of transposon insertions in the two species is shown in figure 1.

Himar1 transposase has been previously suggested to exhibit local sequence preferences rendering ~9% of possible TA sites non-permissive to insertion (23) and others have also observed TA insertion cold spots within the *M. tuberculosis* genome. Using the non-permissive sequence pattern, 'SGNTANCS' (where S is either G/C), we identified 6657 sites in both *M. bovis* and *M. tuberculosis* genomes (data not shown). Taking a similar approach to Carey *et al*, we found that removing these sites prior to determining gene essentiality as described below did not affect the gene calls (25).

Comparisons of essentiality between *M. bovis* and *M. tuberculosis*

We examined *in vitro* gene essentiality in *M. bovis* and *M. tuberculosis* using the TRANSIT HMM method (31). This approach classifies genes into four categories; those that are essential for growth and cannot sustain a transposon insertion (ES), those where the transposon insertion results in a growth defect (GD) and those where the transposon insertion results in a growth advantage (GA). Those that show no impact as a result of the transposon insertion are considered non-essential (NE). From this analysis, 527 genes were classified as ES (15.3%), 176 genes were classified as GD (5.1%) and 131 as GA (3.8%) in the *M. bovis* genome. In *M. tuberculosis* 477 genes were classified as ES (13.7%), 179 genes were classified as GD (5.1%) and 1 gene as GA (0.03%). A complete list of calls

for the genes that are conserved between both species is given in supplementary table S1. The status of the genes that are *M. bovis* specific are also included in the table.

Early sequencing and functional annotation of the genome of *M. tuberculosis* categorised genes into several different functional classes with an uneven distribution of genes across the classes (4,5). We examined the distribution of the genes classified as ES in *M. tuberculosis* (477) and *M. bovis* (527) across the functional classes to determine if (i) ES genes are over-represented in any particular functional class when compared to the genome as a whole (ii) there are differences between the two species. The results are shown in figure 2 and table 4. Chi squared testing showed that the distribution of ES genes across the functional classes was significantly different to the distribution of all orthologs ($p=<0.01$). ES genes in both species are over-represented in "information pathways" and "intermediary metabolism and respiration" and under-represented in "conserved hypotheticals" and "PE/PPE" functional classes. Our data are in line with previous reports; Griffin *et al* noted that the distribution of ES genes across the different functional classes were different compared to the genome as a whole (22). DeJesus *et al* also noted that insertions in PE/PPE genes were under-represented likely due to GC rich sequences and an increased proportion of non-permissive TA sites in the PE/PPE genes (23). There were no major differences in distribution of ES genes across the functional classes when *M. tuberculosis* and *M. bovis* were compared with each other except for "insertion sequences and phages" which did not contain any genes classified as ES in the *M. bovis* genome.

Genes categorised as ES in this study were compared between the two species and also compared to previously reported studies (21–23,26,39) (supplementary file, table S1). We found that the *M. bovis* dataset generated in our study shared 370 (70%) of genes classified as ES with *M. tuberculosis in vitro* (this study; figure 3A) and up to 86% overlap with three key published *M. tuberculosis* data sets: DeJesus *et al* 2017 (71%), Griffin *et al* 2011 (86%) and Minato *et al* 2019 (79%) indicating good correlation with previous reports (figure 3D). Similarly, the *M. tuberculosis* dataset generated in our study shared good overlap with other published datasets (figure 3C). When comparing *M. bovis* genes classified as ES with those reported by Butler *et al* 2020 (40) we found that 220 (42%) genes were shared between these data sets (figure 3B). Butler *et al* reported a total of 318 genes to be essential in *M. bovis in vitro* prior to selection in *Dictyostelium discoideum*

compared to 527 reported in this study. Both libraries showed similar saturation levels (58% vs. 54% in this study) therefore differences might be due to the conditions under which the libraries were generated (although both studies used Middlebrook 7H11 solid medium supplemented with lysed sheep blood, heat inactivated foetal bovine serum and OADC) or between laboratory variation as might be expected for whole genome techniques such as Tnseq. It should also be noted that the similarities between the studies increases when GD genes are considered, for instance of the 307 genes that appear to be uniquely ES in our study, 212 of these are classified as GD in the study by Butler *et al.,* indicating a debilitating impact of the transposon insertion.

Differences in gene essentiality between *M. bovis* and *M. tuberculosis*

Genes uniquely classified as ES in either species are of interest to determine potential genetic insights for phenotypic differences between these closely related mycobacterial species. In this study 157 genes were uniquely ES in *M. bovis* when compared to the *M. tuberculosis* (figure 3A), however, of these 157, 61 were classified as GD in *M. tuberculosis*. The remaining 96 were classified as NE in *M. tuberculosis* (supplementary file, table S2). The existence of multiple datasets allows for a robust meta-analysis and so we compared across datasets and found that there were 42 genes that were either ES or GD in this study and the study by Butler *et al*., and were classified as NE in *M. tuberculosis* in this study and the study by DeJesus et al. (supplementary file, table S3). Included in this subset of genes is *Rv3543c* (*fadE19*), *Rv3541c* and *Rv3540c* (*lpt2*), genes which are encoded on the same operon (*Rv3545c-Rv3540c* - based on intergenic gaps) regulated by *kstR* and involved in cholesterol catabolism. This study and the Butler *et al.,* study indicates that insertional mutagenesis of this operon has a debilitating impact in *M. bovis* but not in *M. tuberculosis.*

Data for the entire *kstR* regulon is given in supplementary file, table S4. Interestingly, the media used in this study and the study by Butler *et al*., contains traces of cholesterol due to the presence of lysed sheep blood, although there is no evidence that cholesterol presented a selective pressure (for *M. tuberculosis*) in this study as there is little overlap of the *M. tuberculosis* dataset with the study by Griffin *et al*., In addition to the *Rv3545c-Rv3540c* operon considered above, several orthologs in the *kstR* regulon

were classified as ES in *M. bovis*; *Mb3538* (*Rv3508*), *Mb3568* (*Rv3538*) and *Mb3581* (*Rv3551*), and *Mb3595* (*Rv3565*). Others such as *Mb3541* (*Rv3511*) and *Mb3574c* (*Rv3544c*) were classified as GD. Interestingly insertions in the genes belonging to the *mce4* operon and required for growth on cholesterol mostly confer a GA for *M. bovis.* These observations might reflect a difference in the requirement for cholesterol catabolism *in vitro* in a complex carbon mixture compared to *M. tuberculosis.*

One of the key metabolic differences between *M. bovis* and *M. tuberculosis* is the inability of *M. bovis* to utilise carbohydrates. Genes in the glycolytic pathway (supplementary file, table S5) such as, enolase (*eno*), pyruvate kinase (*pykA)* and pyruvate carboxylase (*pca*) might be expected to be NE in *M. bovis* as *pykA* is non-functional in *M. bovis* (41). The datasets show that *eno* is ES in *M. bovis* as well as *M. tuberculosis* perhaps indicating that its essentiality is linked to a role other than glycolysis. Similarly, the suggestion that a transposon insertion in *pykA* confers a GA (this study only) is counter-intuitive and might suggest a non-glycolytic role for this enzyme. Only our dataset suggests that a transposon insertion in *icl1,* an enzyme required for growth on fatty acids, confers a growth advantage in *M. bovis.*

The two-component system PhoPR has been shown to regulate *de novo* PDIM synthesis and also co-ordinate the acid-stress response (16,42). It is of particular interest because a non-synonymous SNP in the sensor histidine kinase *phoR* in *M. bovis* renders signalling through the system defective, however, the existence of compensatory mechanisms that restore PDIM synthesis obscures the role of the regulon in *M. bovis.* Of the genes in the PhoPR regulon (supplementary file, table S6) only *Rv3778c* seems to be consistently required across species and studies. Genes in the redox sensing WhiB family are included in the operon (*whiB1, whiB3* and *whiB6*) but only *whiB1* is ES in *M. bovis* in our study.

Finally, as the electron transport chain and ATP synthesis is a relatively new therapeutic pathway we chose to examine ES more closely in these pathways (supplementary table, S7). These pathways are targets of recently introduced drugs such as bedaquiline (ATP synthase) and those in development e.g. Q203 which targets the terminal cytochrome bc_1-aa_3 oxidase (43). Unsurprisingly, the genes encoding the ATP synthase are largely ES in both species in all studies (*Rv1304-Rv1311*) with the exception of *Rv1304* (*atpB*). The genes that encode a sub-unit of the terminal cytochrome bc1-aa3

oxidase complex (*qcrCAB*) the target of Q203 are classified as either ES or GD. One interesting observation is that both our study and the study by Butler *et al*., is that a GD occurs as a result of an insertion in *nuoG* but this is not observed in any of the *M. tuberculosis* studies. *nuoG* forms part of the multi-subunit NADH reductase-I complex in the respiratory chain and transfers electrons to the menaquinone pool while simultaneously contributing to the proton gradient through its proton pumping function

Establishment of CRISPRi in *Mycobacterium bovis* using *wag31*

Wag31 is required for peptidoglycan synthesis and previously published datasets have classified *wag31* in *M. tuberculosis* as ES *in vitro* (21–23). This study classified *wag31* in *M. bovis* as ES but NE in *M. tuberculosis*. The study by Butler *et al*., assigned *wag31* as NE in *M. bovis*. In order probe this discrepancy and to establish CRISPRi silencing in *M. bovis* this gene was chosen for silencing. Early CRISPRi studies in *M. tuberculosis* performed by Singh *et al*. successfully utilised two plasmids encoding sgRNAs guides targeting +26 bp to +45 bp and +144 bp to +163 bp downstream of the annotated start codon of $wag31_{\text{Mtb}}$ (table 1 and figure 4A). We utilised these plasmids to investigate the impact of silencing $wag31_{\text{Mb}}$. *M. bovis* AF2122/97 was transformed with pRH2502 to create a strain expressing $dcas9_{Spy}$ (Mb_{dCas9}). Mb_{dCas9} was then transformed with plasmids expressing the sgRNA guides. Strains were cultured to exponential phase and serial dilutions were spotted onto agar containing 200 ng/ml aTc. Controls (without aTc, without sgRNA) were also included. The results, presented in figure 4B, show that silencing $wag31_{Mb}$ in *M. bovis* results in a severe growth defect, visible at 10^{-1} dilution with complete cessation of growth at 10^{-2} dilution. This growth defect is identical to that seen in *M. tuberculosis* and supports the ES classification.

Silencing *Rv2182c* and its ortholog *Mb2204c* shows a species-specific growth impact

Rv2182c/*Mb2204c* is annotated as a 1-acylglycerol-3-phosphate O-acyltransferase (agpat) and involved in glycerophospholipid metabolism. It is thought to synthesize diacylglycerol-3P through the addition of acyl chains to monoacylglycerol-3P. It is classified as ES in *M. tuberculosis* in this study and by others (22,23,44). It is classified

as ES in *M. bovis* in this study but NE in the study by Butler *et al*.,. Strains of *M. tuberculosis* and *M. bovis* were constructed expressing sgRNAs targeting +2 bp to +21 bp and +40 bp to +59 bp downstream of the annotated start codon of *Rv2182c*/*Mb2204c* (table 2 and figure 5A). The impact of inducing the system on expression of *Rv2182c/Mb2204c* was measured using RT-qPCR. The results, which are shown in figure 5B show that $dCas9_{Spy}$ is similarly induced in both *M. tuberculosis* and *M. bovis* with 150 to 350-fold induction of expression in the presence of aTc. Additionally, the results show that, in the presence of the sgRNA, there is a clear reduction in expression of *Rv2182c/Mb2204c* in both species. These data demonstrate effective gene silencing of *Rv2182c/Mb2204c* in both *M. tuberculosis* and *M. bovis*, respectively.

To determine the impact of induction of the guides, strains were cultured to exponential phase and serial dilutions were spotted onto agar containing 200 ng/ml aTc. The results (figure 5C) show that silencing *Rv2182c* in *M. tuberculosis* results in a severe growth defect, with almost complete cessation of growth at 10^{-1} dilution. However, the consequence of silencing *Mb2204c* on the growth of *M. bovis* is far less impactful with a small reduction visible at the lowest dilution 10^{-4}. This demonstrates that, unlike *wag31*, silencing of *Rv2182c* and its ortholog *Mb2204c* in *M. tuberculosis* and *M. bovis* respectively, has a differential impact on growth, with *M. tuberculosis* being more vulnerable and showing a greater growth defect. These results do not support the classification of *Mb2204c* as an ES gene in *M. bovis* but they clearly highlight that there are different phenotypic consequences as a result of silencing the ortholog in both species.

Discussion

The aim of this work was to directly compare gene essentiality in the human and animal adapted members of the MTBC. In order to do this we generated transposon libraries in *M. bovis* AF2122/97and *M. tuberculosis* H37Rv using a rich medium that supported the growth of both species. We assessed gene essentiality using the TRANSIT HMM method to define 527 and 477 genes as ES for *M. bovis* and *M. tuberculosis*, respectively. Datasets from each species were compared with each other and with previously published datasets. Genes classified as ES were congruent between the species and also with existing studies of gene essentiality in *M. tuberculosis* (21–23,39).

Comparing this study with a previously published *M. bovis* dataset revealed a 42% overlap which increased when genes predicted to show a GD as a result of the transposon insertion were taken into account (26). There were some indications of differences between the species, and a meta-analysis of the data indicated that 42 genes were differentially essential between the species. A recent study using whole genome CRISPRi screens showed that a similar number (80 genes) were differentially essential in two different strains of *M. tuberculosis* (H37Rv vs HN878) (45). Genes that appear to show differential essentiality between the two species include those involved in cholesterol catabolism.

Whole-genome TnSeq provides a high-throughput assessment of fitness costs and has allowed the classification of genes based on essentiality but does not provide information on target vulnerability. More recent studies highlight the limitations of the (near) binary classification of genes into and ES/NE and utilise CRISPRi to assess vulnerability (45,46). Additionally, datasets are prone to false calls of ES due to non-saturating mutagenesis. In this study CRISPRi was utilised to show that there are different impacts on bacterial growth as a result of silencing *Rv2182c*/*Mb2204c* in their respective species, despite achieving similar levels of gene silencing. Significant growth inhibition was seen as a result of silencing in *Rv2182c* in *M. tuberculosis* while only marginal impacts on growth were observed on silencing the ortholog *Mb2204c* in *M. bovis.* *Rv2182c*/*Mb2204c* is annotated as a 1-acylglycerol-3-phosphate O-acyltransferase and involved in glycerophospholipid metabolism. It is thought to synthesize diacylglycerol-3P through the addition of acyl chains to monoacylglycerol-3P. This pathway may be involved in detoxification and further work is required to fully understand the differential impact of silencing this gene in the two species. Given that *Rv2182c* was a predicted target in a recent compound screen (47), differential essentiality estimates in *M. bovis* and *M. tuberculosis* are important to predict if zoonotic TB caused by *M. bovis* can also be suitably treated with drugs designed to be effective against *M. tuberculosis.*

We have provided a comparative analysis of the genetic requirements for growth of two key MTBC members: *M. bovis* and *M. tuberculosis*. Genes which are uniquely ES for either *M. bovis* or *M. tuberculosis* have the potential to provide insights into niche specific aspects e.g., host tropism, survival in the environment, phenotype, and anti-tubercular drugs. Host tropism is of particular interest when considering the zoonotic nature of *M.*

bovis and the involvement of wildlife hosts as reservoirs of infection for bovine TB. Use of *M. bovis* libraries in the context of the host i.e., through experimental infection of bovine TB will enable the study of the genetic requirements for survival *in vivo*. Further investigations exploring the role and function of ES genes between *M. bovis* and *M. tuberculosis* is necessary to better understand the physiological differences in these key MTBC species.

References

1. Malone KM, Gordon S V. "Mycobacterium tuberculosis complex members adapted to wild and domestic animals," in *Advances in Experimental Medicine and Biology* (Springer New York LLC), 135–154. doi:10.1007/978-3-319-64371-7_7
2. WHO. WHO | Global tuberculosis report 2019. *World Heal Organ* (2020) doi:.1037//0033-2909.I26.1.78
3. Fao. Roadmap for zoonotic tuberculosis. *WHO* (2017) Available at: www.who.int [Accessed May 11, 2020]
4. Camus JC, Pryor MJ, Médigue C, Cole ST. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. *Microbiology* (2002) **148**:2967–2973. doi:10.1099/00221287-148-10-2967
5. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon S V., Eiglmeier K, Gas S, Barry CE, et al. Deciphering the biology of mycobacterium tuberculosis from the complete genome sequence. *Nature* (1998) **393**:537–544. doi:10.1038/31159
6. Malone KM, Farrell D, Stuber TP, Schubert OT, Aebersold R, Robbe-Austerman S, Gordon S V. Updated reference genome sequence and annotation of mycobacterium bovis AF2122/97. *Genome Announc* (2017) **5**: doi:10.1128/genomeA.00157-17
7. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C, Monsempe C, et al. The complete genome sequence of Mycobacterium bovis. *Proc Natl Acad Sci U S A* (2003) **100**:7877–7882. doi:10.1073/pnas.1130426100
8. Brites D, Loiseau C, Menardo F, Borrell S, Boniotti MB, Warren R, Dippenaar A, Parsons SDC, Beisel C, Behr MA, et al. A new phylogenetic framework for the animal-adapted mycobacterium tuberculosis complex. *Front Microbiol* (2018) **9**:2820. doi:10.3389/fmicb.2018.02820
9. Golby P, Hatch KA, Bacon J, Cooney R, Riley P, Allnutt J, Hinds J, Nunez J, Marsh PD, Hewinson RG, et al. Comparative transcriptomics reveals key gene expression differences between the human and bovine pathogens of the Mycobacterium tuberculosis complex. *Microbiology* (2007) **153**:3323–3336. doi:10.1099/mic.0.2007/009894-0
10. Rehren G, Walters S, Fontan P, Smith I, Zárraga AM. Differential gene expression between Mycobacterium bovis and Mycobacterium tuberculosis. *Tuberculosis* (2007) **87**:347–359. doi:10.1016/j.tube.2007.02.004
11. Malone KM, Rue-Albrecht K, Magee DA, Conlon K, Schubert OT, Nalpas NC, Browne JA, Smyth A, Gormley E, Aebersold R, et al. Comparative 'omics analyses differentiate mycobacterium tuberculosis and mycobacterium bovis and reveal distinct macrophage responses to infection with the human and bovine tubercle bacilli. *Microb Genomics* (2018) **4**: doi:10.1099/mgen.0.000163
12. Saïd-Salim B, Mostowy S, Kristof AS, Behr MA. Mutations in Mycobacterium tuberculosis Rv0444c, the gene encoding anti-SigK, explain high level expression of MPB70 and MPB83 in Mycobacterium bovis. *Mol Microbiol* (2006) **62**:1251–1263. doi:10.1111/j.1365-2958.2006.05455.x
13. Wiker HG. MPB70 and MPB83 - Major antigens of mycobacterium bovis. *Scand J Immunol* (2009) **69**:492–499. doi:10.1111/j.1365-3083.2009.02256.x
14. Queval CJ, Fearns A, Botella L, Smyth A, Schnettger L, Mitermite M, Wooff E, Villarreal-Ramos B, Garcia-Jimenezi W, Heunis T, et al. Macrophage-specific

responses to humanand animal-adapted tubercle bacilli reveal pathogen and host factors driving multinucleated cell formation. *PLoS Pathog* (2021) **17**:e1009410. doi:10.1371/journal.ppat.1009410

15. Bigi MM, Blanco FC, Araújo FR, Thacker TC, Zumárraga MJ, Cataldi AA, Soria MA, Bigi F. Polymorphisms of 20 regulatory proteins between Mycobacterium tuberculosis and Mycobacterium bovis. *Microbiol Immunol* (2016) **60**:552–560. doi:10.1111/1348-0421.12402
16. García EA, Blanco FC, Klepp LI, Pazos A, McNeil MR, Jackson M, Bigi F. Role of PhoPR in the response to stress of Mycobacterium bovis. *Comp Immunol Microbiol Infect Dis* (2021) **74**:101593. doi:10.1016/j.cimid.2020.101593
17. Broset E, Martín C, Gonzalo-Asensio J. Evolutionary landscape of the mycobacterium tuberculosis complex from the viewpoint of phoPR: Implications for virulence regulation and application to vaccine development. *MBio* (2015) **6**: doi:10.1128/mBio.01289-15
18. Sohaskey CD, Modesti L. Differences in nitrate reduction between mycobacterium tuberculosis and mycobacterium bovis are due to differential expression of both narGHJI and narK2. *FEMS Microbiol Lett* (2009) **290**:129–134. doi:10.1111/j.1574-6968.2008.01424.x
19. Sassetti CM, Boyd DH, Rubin EJ. Comprehensive identification of conditionally essential genes in mycobacteria. *Proc Natl Acad Sci U S A* (2001) **98**:12712–12717. doi:10.1073/pnas.231275498
20. Rengarajan J, Bloom BR, Rubin EJ. Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. *Proc Natl Acad Sci U S A* (2005) **102**:8327–8332. doi:10.1073/pnas.0503272102
21. Minato Y, Gohl DM, Thiede JM, Chacón JM, Harcombe WR, Maruyama F, Baughn AD. Genomewide Assessment of Mycobacterium tuberculosis Conditionally Essential Metabolic Pathways. *mSystems* (2019) **4**: doi:10.1128/MSYSTEMS.00070-19
22. Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS Pathog* (2011) **7**:e1002251. doi:10.1371/journal.ppat.1002251
23. Dejesus MA, Gerrick ER, Xu W, Park SW, Long JE, Boutte CC, Rubin EJ, Schnappinger D, Ehrt S, Fortune SM, et al. Comprehensive essentiality analysis of the Mycobacterium tuberculosis genome via saturating transposon mutagenesis. *MBio* (2017) **8**: doi:10.1128/mBio.02133-16
24. Zhang YJ, Ioerger TR, Huttenhower C, Long JE, Sassetti CM, Sacchettini JC, Rubin EJ. Global assessment of genomic regions required for growth in Mycobacterium tuberculosis. *PLoS Pathog* (2012) **8**:e1002946. doi:10.1371/journal.ppat.1002946
25. Carey AF, Rock JM, Krieger I V., Chase MR, Fernandez-Suarez M, Gagneux S, Sacchettini JC, Ioerger TR, Fortune SM. TnSeq of Mycobacterium tuberculosis clinical isolates reveals strain-specific antibiotic liabilities. *PLOS Pathog* (2018) **14**:e1006939. doi:10.1371/journal.ppat.1006939
26. Butler RE, Smith AA, Mendum TA, Chandran A, Wu H, Lefrançois L, Chambers M, Soldati T, Stewart GR. Mycobacterium bovis uses the ESX-1 Type VII secretion system to escape predation by the soil-dwelling amoeba Dictyostelium discoideum. (2020) **14**:919–930. doi:10.1038/s41396-019-0572-z
27. Gallagher J, Horwill DM. A selective oleic acid albumin agar medium for the

549 cultivation of Mycobacterium bovis. *J Hyg (Lond)* (1977) **79**:155–60.
550 doi:10.1017/s0022172400052943
551 28. Majumdar G, Mbau R, Singh V, Warner DF, Dragset MS, Mukherjee R. "Genome-
552 Wide Transposon Mutagenesis in Mycobacterium tuberculosis and
553 Mycobacterium smegmatis," in *Methods in molecular biology (Clifton, N.J.)*, 321–
554 335. doi:10.1007/978-1-4939-6472-7_21
555 29. Long JE, DeJesus M, Ward D, Baker RE, Ioerger T, Sassetti CM. "Identifying
556 Essential Genes in Mycobacterium tuberculosis by Global Phenotypic Profiling," in
557 (Humana Press, New York, NY), 79–95. doi:10.1007/978-1-4939-2398-4_6
558 30. Belisle JT, Sonnenberg MG. Isolation of genomic DNA from mycobacteria. *Methods*
559 *Mol Biol* (1998) **101**:31–44. doi:10.1385/0-89603-471-2:31
560 31. DeJesus MA, Ioerger TR. A Hidden Markov Model for identifying essential and
561 growth-defect regions in bacterial genomes from transposon insertion
562 sequencing data. *BMC Bioinformatics* (2013) **14**: doi:10.1186/1471-2105-14-303
563 32. Singh AK, Carette X, Potluri LP, Sharp JD, Xu R, Prisic S, Husson RN. Investigating
564 essential gene function in Mycobacterium tuberculosis using an efficient CRISPR
565 interference system. *Nucleic Acids Res* (2016) **44**:e143–e143.
566 doi:10.1093/nar/gkw625
567 33. Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS. CRISPR interference
568 (CRISPRi) for sequence-specific control of gene expression. *Nat Protoc* (2013)
569 **8**:2180–2196. doi:10.1038/nprot.2013.132
570 34. M Z. Mfold web server for nucleic acid folding and hybridization prediction.
571 *Nucleic Acids Res* (2003) **31**:3406–3415. doi:10.1093/NAR/GKG595
572 35. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
573 tool. *J Mol Biol* (1990) **215**:403–410. doi:10.1016/S0022-2836(05)80360-2
574 36. Choudhary E, Thakur P, Pareek M, Agarwal N. Gene silencing by CRISPR
575 interference in mycobacteria. *Nat Commun* (2015) **6**: doi:10.1038/ncomms7267
576 37. Rustad TR, Roberts DM, Liao RP, Sherman DR. Isolation of mycobacterial RNA.
577 *Methods Mol Biol* (2008) **465**:13–22. doi:10.1007/978-1-59745-207-6_2
578 38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
579 quantitative PCR and the 2-ΔΔCT method. *Methods* (2001) **25**:402–408.
580 doi:10.1006/meth.2001.1262
581 39. Serafini A, Tan L, Horswell S, Howell S, Greenwood DJ, Hunt DM, Phan M,
582 Schembri M, Monteleone M, Montague CR, et al. *Mycobacterium tuberculosis*
583 requires glyoxylate shunt and reverse methylcitrate cycle for lactate and pyruvate
584 metabolism. *Mol Microbiol* (2019) **112**:1284–1307. doi:10.1111/mmi.14362
585 40. Butler RE, Smith AA, Mendum TA, Chandran A, Wu H, Lefrançois L, Chambers M,
586 Soldati T, Stewart GR. Mycobacterium bovis uses the ESX-1 Type VII secretion
587 system to escape predation by the soil-dwelling amoeba Dictyostelium
588 discoideum. *ISME J 2020 144* (2020) **14**:919–930. doi:10.1038/s41396-019-
589 0572-z
590 41. Keating LA, Wheeler PR, Mansoor H, Inwald JK, Dale J, Hewinson RG, Gordon S V.
591 The pyruvate requirement of some members of the Mycobacterium tuberculosis
592 complex is due to an inactive pyruvate kinase: implications for in vivo growth.
593 *Mol Microbiol* (2005) **56**:163–174. doi:10.1111/J.1365-2958.2005.04524.X
594 42. Gonzalo-Asensio J, Malaga W, Pawlik A, Astarie-Dequeker C, Passemar C, Moreau
595 F, Laval F, Daffé M, Martin C, Brosch R, et al. Evolutionary history of tuberculosis
596 shaped by conserved mutations in the PhoPR virulence regulator. *Proc Natl Acad*
597 *Sci U S A* (2014) **111**:11491–11496. doi:10.1073/pnas.1406693111

43. Pethe K, Bifani P, Jang J, Kang S, Park S, Ahn S, Jiricek J, Jung J, Jeon HK, Cechetto J, et al. Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat Med* (2013) **19**:1157–1160. doi:10.1038/nm.3262
44. Minato Y, Gohl DM, Thiede JM, Chacón JM, Harcombe WR, Maruyama F, Baughn AD. Genome-wide assessment of Mycobacterium tuberculosis conditionally essential metabolic pathways. *bioRxiv* (2019)534289. doi:10.1101/534289
45. Bosch B, DeJesus MA, Poulton NC, Zhang W, Engelhart CA, Zaveri A, Lavalette S, Ruecker N, Trujillo C, Wallach JB, et al. Genome-wide gene expression tuning reveals diverse vulnerabilities of M. tuberculosis. *Cell* (2021) doi:10.1016/j.cell.2021.06.033
46. McNeil MB, Keighley LM, Cook JR, Cheung C, Cook GM. CRISPR interference identifies vulnerable cellular pathways with bactericidal phenotypes in Mycobacterium tuberculosis. *Mol Microbiol* (2021)mmi.14790. doi:10.1111/mmi.14790
47. Martínez-Jiménez F, Papadatos G, Yang L, Wallace IM, Kumar V, Pieper U, Sali A, Brown JR, Overington JP, Marti-Renom MA. Target Prediction for an Open Access Set of Compounds Active against Mycobacterium tuberculosis. *PLoS Comput Biol* (2013) **9**:1003253. doi:10.1371/journal.pcbi.1003253
48. Gu Z, Gu L, Eils R, Schlesner M, Brors B. circlize implements and enhances circular visualization in R. *Bioinformatics* (2014) **30**:2811–2812. doi:10.1093/BIOINFORMATICS/BTU393

Author Contributions

AJG, SW, IP and SLK designed the study. AJG, IP, VF, carried out the experimental work. Data analysis was done by IN, JS and DX. SLK, DW, BWW and BVR did funding acquisition. AJG and SLK wrote the first draft of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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634 **Table 1. Strains and plasmids used in this study**

Strain/plasmid	Genotype/Description	Source
Strains		
E. coli DH5α	*SupE44 ΔlacU169 (lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
M. bovis AF2122/97	*M. bovis* reference strain	
M. tuberculosis H37Rv	*M. tuberculosis* reference strain	
$Mtb_{\_dCas9}$/ $Mb_{\_dCas9}$	*M. tuberculosis* or *M. bovis* with integrative plasmid containing $dCas9_{Spy}$ (pRH2502), kan^R	This study
Mtb_{dCas9}_control/ Mb_{dCas9}_control	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ with sgRNA –ve control plasmid (pRH2521), kan^R, hyg^R	This study
Mtb_{dCas9}_wag1/ Mb_{dCas9}_wag1	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ expressing sgRNA targeting +26 bp to +45 bp downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon. kan^R hyg^R	This study
Mtb_{dCas9}_wag2/ Mb_{dCas9}_wag2	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ expressing sgRNA targeting +144 bp to +163 bp downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon kan^R, hyg^R	This study
Mtb_{dCas9}_ agpat1/ Mb_{dCas9}_agpat1	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ expressing sgRNA targeting +2 bp to +21 bp downstream of the *Rv2182c/Mb2204c* annotated start codon. kan^R hyg^R	This study
Mtb_{dCas9}_ agpat2/ Mb_{dCas9}_agpat2	$Mtb_{\_dCas9}$/ $Mb_{\_dCas9}$ expressing sgRNA targeting +40 bp to +59 bp downstream of the *Rv2182c/Mb2204c* annotated start codon. kan^R hyg^R	This study
Plasmids		
pRH2502	Integrative plasmid derived from pTC-0X-1L, expressing $dCas9_{Spy}$ from an inducible tetRO promoter (uv15tetO). kan^R	(32)
pRH2521	Non-integrative plasmid derived from pTE-10M-0X, expressing sgRNA from an inducible tetRO promoter (Pmyc1tetO). hyg^R	(32)
pRH2521_wag1	pRH2521 with an sgRNA targeting +26 bp to +45 downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon. hyg^R	(32)
pRH2521_wag2	pRH2521 with an sgRNA targeting +144 bp to +163 downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon. hyg^R	(32)
pRH2521_agpat1	pRH2521 with an sgRNA targeting +2 bp to +21 bp over and downstream of the *Rv2182c/Mb2204c* annotated start codon. hyg^R	This study
pRH2521_agpat2	pRH2521 with an sgRNA targeting +40 bp to +59 bp downstream of the *Rv2182c/Mb2204c* annotated start codon. hyg^R	This study

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Table 2. Oligonucleotides used in this study.

Primer	Sequence
Primers used for Tn library	
Adaptor standard	GATCGGAAGAGCACAC
Adaptor P7+index[a]	CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ComP7 primer	CAAGCAGAAGACGGCATACG
ComP5 primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGGGACTTATCAGCCAACCTG
Oligonucleotides used for nested PCR verification	
HiMar_Right_1	CCTCGTGCTTTACGGTATCG
Arb_primer_1c*	GCCAGCGAGCTAACGAGACNNNNN
HiMar_Tn_Jnct_PCR	ACTATAGGGGTCTAGAGACCGGG
Arb_primer_1*	GCCAGCGAGCTAACGAGAC
Oligonucleotides used for CRISPRi silencing sgRNA[b]	
agpat1_F	AAACTGTGGTACTACCTGTTCAAG
agpat1_R	GGGACTTGAACAGGTAGTACCACA
agpat2_F	AAACCTCTTTACGTTGCTTGGTCG
agpat2_R	GGGACGACCAAGCAACGTAAAGAG
Oligonucleotides used for RT-qPCR	
sigA_Fq	CCTACGCTACGTGGTGGATT
sigA_Rq	TGGATTTCCAGCACCTTCTC
agpat1_Fq	CTTTACGTTGCTTGGTCGCC
agpat1_Rq	AGAACCAGCGGTTGATCCAG
dCas9$_{Spy}$_Fq	AAGAAGTACAGCATCGGCCTGG
dCas9$_{Spy}$_Rq	TTCTTGCGCCGCGTGTATCG

[a] **XXXXXXX** in AdaptorP7+index primer denotes sequence of variable indices used.

[b] The four bases underlined at the start of each oligo were used for cloning into pRH2521.

640 **Table 3. Summary statistics of the Tn libraries created in this study**

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	***M. bovis* AF2122/97**	***M. tuberculosis* H37Rv**
Unique Mutants	39,987 (of 73,536)	29,919 (of 74,604)
Saturation	54%	40%
Essential Genes	528	477

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643 **Table 4. Distribution of genes classified as ES across functional class**

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Functional class	% ES Mb	%ES Mtb	% all
cell wall and cell processes	19.7	18.66	18.71
conserved hypotheticals	10.23	10.90	28.11
information pathways	17.61	18.87	5.96
insertion seqs and phages	0	0.63	2.54
intermediary metabolism and respiration	42.23	38.57	23.1
lipid metabolism	4.73	5.03	6.5
PE/PPE	0.95	1.68	4.44
regulatory proteins	2.27	3.35	4.93
virulence, detoxification and adaptation	2.27	2.31	5.71

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Figure Legends

Figure 1: Distribution of Tn insertions in both *M. tuberculosis* and *M. bovis.* Transposon libraries were created in *M. tuberculosis* and *M. bovis* using the *Himar1* system and sequenced on a HiSeq NGS platform (Illumina, UK) as described in the materials and methods. Insertion locations of *Himar1* across the *M. tuberculosis* genome (green) and *M. bovis* genome (blue) were visualised using Circlize (48).

Figure 2: Functional category distribution. Gene essentiality was determined for *M. bovis* and *M. tuberculosis* using custom HMM analyses with TRANSIT software. Functional categories were assigned to orthologous genes and compared for *Himar1* insertion distribution between *M. bovis* (left) and *M. tuberculosis* (right). Transposon insertions were found to be similar across functional categories. Data were analysed using pivot tables in Excel.

Figure 3: Essential Gene Comparisons. Gene essentiality was determined for *M. bovis* and *M. tuberculosis* using custom HMM analyses with TRANSIT software and compared to previously published datasets. **(A)** *M. bovis* and *M. tb* (both this study), **(B)** *M. bovis* (this study) and *M. bovis* (Butler *et al* 2020) **(C)** *M. tb* (this study) and *M. tb* DJ (DeJesus *et al* 2017), *M. tb* G (Griffin *et al* 2011) and *M. tb* M (Minato *et al* 2019) and **(D)** *M. bovis* (this study) and *M. tb* DJ (DeJesus *et al* 2017), *M. tb* G (Griffin *et al* 2011) and *M. tb* M (Minato *et al* 2019).

Figure 4. Using CRISPRi/dCas9 to inhibit *wag31* expression in *M. bovis* and *M. tuberculosis* **(A)** Schematic showing the target regions of two sgRNAs designed to target and inhibit *wag31* expression. The numbers show the nucleotide position of the sgRNA relative to the annotated start site **(B)** CRISPRi strains were cultured in 10 ml of supplemented 7H9 medium to exponential phase and diluted to 2 x 10^7 CFU/ml. A 10-fold serial dilution to 10^{-4} was performed and 20 µl of each dilution was spotted onto 7H11 agar without aTc and with 200 ng / ml aTc to induce CRISPRi/ dCas9 and the sgRNA in those strains that carried the guide. Two biological replicates were carried out.

Figure 5. Using CRISPRi/dCas9 to inhibit *Rv2182c/Mb2204c* expression in *M. bovis* and *M. tuberculosis.* **(A)** Schematic showing the target regions of two sgRNAs designed to target and inhibit *Rv2182c/Mb2204c* expression. The numbers show the nucleotide position of the sgRNA relative to the annotated start site **(B)** *dCas9* expression and

678 *Rv2182c/Mb2204c* expression were measured by RT-qPCR as described in the methods
679 section. Gene expression was analysed using the $2^{-\Delta\Delta CT}$ method, normalised against *sigA.*
680 Results represent two biological repeats with two technical repeats each. *P* = * <0.05, **
681 <0.01, *** <0.001, **** <0.0001 or not significant (ns), analysed using a 2-way ANOVA
682 test **(C)** CRISPRi strains were cultured in 10 ml of supplemented 7H9 medium to
683 exponential phase and diluted to 2 x 10^7 CFU/ ml. A 10-fold serial dilution to 10^{-4} was
684 performed and 20 µl of each dilution was spotted onto 7H11 agar without aTc and with
685 200 ng/ml aTc to induce CRISPRi/ dCas9 and the sgRNA in those strains that carried the
686 guide. Two biological replicates were carried out.

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References

1. Malone KM, Gordon S V. "Mycobacterium tuberculosis complex members adapted to wild and domestic animals," in *Advances in Experimental Medicine and Biology* (Springer New York LLC), 135–154. doi:10.1007/978-3-319-64371-7_7

2. WHO. WHO | Global tuberculosis report 2019. *World Heal Organ* (2020) doi:.1037//0033-2909.I26.1.78

3. Fao. Roadmap for zoonotic tuberculosis. *WHO* (2017) Available at: www.who.int [Accessed May 11, 2020]

4. Camus JC, Pryor MJ, Médigue C, Cole ST. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. *Microbiology* (2002) **148**:2967–2973. doi:10.1099/00221287-148-10-2967

5. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon S V., Eiglmeier K, Gas S, Barry CE, et al. Deciphering the biology of mycobacterium tuberculosis from the complete genome sequence. *Nature* (1998) **393**:537–544. doi:10.1038/31159

6. Malone KM, Farrell D, Stuber TP, Schubert OT, Aebersold R, Robbe-Austerman S, Gordon S V. Updated reference genome sequence and annotation of mycobacterium bovis AF2122/97. *Genome Announc* (2017) **5**: doi:10.1128/genomeA.00157-17

7. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C, Monsempe C, et al. The complete genome sequence of Mycobacterium bovis. *Proc Natl Acad Sci U S A* (2003) **100**:7877–7882. doi:10.1073/pnas.1130426100

8. Brites D, Loiseau C, Menardo F, Borrell S, Boniotti MB, Warren R, Dippenaar A, Parsons SDC, Beisel C, Behr MA, et al. A new phylogenetic framework for the animal-adapted mycobacterium tuberculosis complex. *Front Microbiol* (2018) **9**:2820. doi:10.3389/fmicb.2018.02820

9. Golby P, Hatch KA, Bacon J, Cooney R, Riley P, Allnutt J, Hinds J, Nunez J, Marsh PD, Hewinson RG, et al. Comparative transcriptomics reveals key gene expression differences between the human and bovine pathogens of the Mycobacterium tuberculosis complex. *Microbiology* (2007) **153**:3323–3336. doi:10.1099/mic.0.2007/009894-0

10. Rehren G, Walters S, Fontan P, Smith I, Zárraga AM. Differential gene expression between Mycobacterium bovis and Mycobacterium tuberculosis. *Tuberculosis* (2007) **87**:347–359. doi:10.1016/j.tube.2007.02.004

11. Malone KM, Rue-Albrecht K, Magee DA, Conlon K, Schubert OT, Nalpas NC, Browne JA, Smyth A, Gormley E, Aebersold R, et al. Comparative 'omics analyses differentiate mycobacterium tuberculosis and mycobacterium bovis and reveal distinct macrophage responses to infection with the human and bovine tubercle bacilli. *Microb Genomics* (2018) **4**: doi:10.1099/mgen.0.000163

12. Saïd-Salim B, Mostowy S, Kristof AS, Behr MA. Mutations in Mycobacterium tuberculosis Rv0444c, the gene encoding anti-SigK, explain high level expression of MPB70 and MPB83 in Mycobacterium bovis. *Mol Microbiol* (2006) **62**:1251–1263. doi:10.1111/j.1365-2958.2006.05455.x

13. Wiker HG. MPB70 and MPB83 - Major antigens of mycobacterium bovis. *Scand J Immunol* (2009) **69**:492–499. doi:10.1111/j.1365-3083.2009.02256.x

14. Queval CJ, Fearns A, Botella L, Smyth A, Schnettger L, Mitermite M, Wooff E, Villarreal-Ramos B, Garcia-Jimenezi W, Heunis T, et al. Macrophage-specific responses to humanand animal-adapted tubercle bacilli reveal pathogen and host factors driving multinucleated cell formation. *PLoS Pathog* (2021) **17**:e1009410. doi:10.1371/journal.ppat.1009410

15. Bigi MM, Blanco FC, Araújo FR, Thacker TC, Zumárraga MJ, Cataldi AA, Soria MA, Bigi F. Polymorphisms of 20 regulatory proteins between Mycobacterium tuberculosis and Mycobacterium bovis. *Microbiol Immunol* (2016) **60**:552–560. doi:10.1111/1348-0421.12402

16. García EA, Blanco FC, Klepp LI, Pazos A, McNeil MR, Jackson M, Bigi F. Role of PhoPR in the response to stress of Mycobacterium bovis. *Comp Immunol Microbiol Infect Dis* (2021) **74**:101593. doi:10.1016/j.cimid.2020.101593

17. Broset E, Martín C, Gonzalo-Asensio J. Evolutionary landscape of the mycobacterium tuberculosis complex from the viewpoint of phoPR: Implications for virulence regulation and application to vaccine development. *MBio* (2015) **6**: doi:10.1128/mBio.01289-15

18. Sohaskey CD, Modesti L. Differences in nitrate reduction between mycobacterium tuberculosis and mycobacterium bovis are due to differential expression of both narGHJI and narK2. *FEMS Microbiol Lett* (2009) **290**:129–134. doi:10.1111/j.1574-6968.2008.01424.x

19. Sassetti CM, Boyd DH, Rubin EJ. Comprehensive identification of conditionally essential genes in mycobacteria. *Proc Natl Acad Sci U S A* (2001) **98**:12712–12717. doi:10.1073/pnas.231275498

20. Rengarajan J, Bloom BR, Rubin EJ. Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. *Proc Natl Acad Sci U S A* (2005) **102**:8327–8332. doi:10.1073/pnas.0503272102

21. Minato Y, Gohl DM, Thiede JM, Chacón JM, Harcombe WR, Maruyama F, Baughn AD. Genomewide Assessment of Mycobacterium tuberculosis Conditionally Essential Metabolic Pathways. *mSystems* (2019) **4**: doi:10.1128/MSYSTEMS.00070-19

22. Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS Pathog* (2011) **7**:e1002251. doi:10.1371/journal.ppat.1002251

23. Dejesus MA, Gerrick ER, Xu W, Park SW, Long JE, Boutte CC, Rubin EJ, Schnappinger D, Ehrt S, Fortune SM, et al. Comprehensive essentiality analysis of the Mycobacterium tuberculosis genome via saturating transposon mutagenesis. *MBio* (2017) **8**: doi:10.1128/mBio.02133-16

24. Zhang YJ, Ioerger TR, Huttenhower C, Long JE, Sassetti CM, Sacchettini JC, Rubin EJ. Global assessment of genomic regions required for growth in Mycobacterium tuberculosis. *PLoS Pathog* (2012) **8**:e1002946. doi:10.1371/journal.ppat.1002946

25. Carey AF, Rock JM, Krieger I V., Chase MR, Fernandez-Suarez M, Gagneux S, Sacchettini JC, Ioerger TR, Fortune SM. TnSeq of Mycobacterium tuberculosis clinical isolates reveals strain-specific antibiotic liabilities. *PLOS Pathog* (2018) **14**:e1006939. doi:10.1371/journal.ppat.1006939

26. Butler RE, Smith AA, Mendum TA, Chandran A, Wu H, Lefrançois L, Chambers M, Soldati T, Stewart GR. Mycobacterium bovis uses the ESX-1 Type VII secretion system to escape predation by the soil-dwelling amoeba Dictyostelium discoideum. (2020) **14**:919–930. doi:10.1038/s41396-019-0572-z

27. Gallagher J, Horwill DM. A selective oleic acid albumin agar medium for the cultivation of Mycobacterium bovis. *J Hyg (Lond)* (1977) **79**:155–60. doi:10.1017/s0022172400052943

28. Majumdar G, Mbau R, Singh V, Warner DF, Dragset MS, Mukherjee R. "Genome-Wide Transposon Mutagenesis in Mycobacterium tuberculosis and Mycobacterium smegmatis," in *Methods in molecular biology (Clifton, N.J.)*, 321–335. doi:10.1007/978-1-4939-6472-7_21

29. Long JE, DeJesus M, Ward D, Baker RE, Ioerger T, Sassetti CM. "Identifying Essential

Genes in Mycobacterium tuberculosis by Global Phenotypic Profiling," in (Humana Press, New York, NY), 79–95. doi:10.1007/978-1-4939-2398-4_6

30. Belisle JT, Sonnenberg MG. Isolation of genomic DNA from mycobacteria. *Methods Mol Biol* (1998) **101**:31–44. doi:10.1385/0-89603-471-2:31

31. DeJesus MA, Ioerger TR. A Hidden Markov Model for identifying essential and growth-defect regions in bacterial genomes from transposon insertion sequencing data. *BMC Bioinformatics* (2013) **14**: doi:10.1186/1471-2105-14-303

32. Singh AK, Carette X, Potluri LP, Sharp JD, Xu R, Prisic S, Husson RN. Investigating essential gene function in Mycobacterium tuberculosis using an efficient CRISPR interference system. *Nucleic Acids Res* (2016) **44**:e143–e143. doi:10.1093/nar/gkw625

33. Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat Protoc* (2013) **8**:2180–2196. doi:10.1038/nprot.2013.132

34. M Z. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* (2003) **31**:3406–3415. doi:10.1093/NAR/GKG595

35. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* (1990) **215**:403–410. doi:10.1016/S0022-2836(05)80360-2

36. Choudhary E, Thakur P, Pareek M, Agarwal N. Gene silencing by CRISPR interference in mycobacteria. *Nat Commun* (2015) **6**: doi:10.1038/ncomms7267

37. Rustad TR, Roberts DM, Liao RP, Sherman DR. Isolation of mycobacterial RNA. *Methods Mol Biol* (2008) **465**:13–22. doi:10.1007/978-1-59745-207-6_2

38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* (2001) **25**:402–408. doi:10.1006/meth.2001.1262

39. Serafini A, Tan L, Horswell S, Howell S, Greenwood DJ, Hunt DM, Phan M, Schembri M, Monteleone M, Montague CR, et al. *Mycobacterium tuberculosis* requires glyoxylate shunt and reverse methylcitrate cycle for lactate and pyruvate metabolism. *Mol Microbiol* (2019) **112**:1284–1307. doi:10.1111/mmi.14362

40. Butler RE, Smith AA, Mendum TA, Chandran A, Wu H, Lefrançois L, Chambers M, Soldati T, Stewart GR. Mycobacterium bovis uses the ESX-1 Type VII secretion system to escape predation by the soil-dwelling amoeba Dictyostelium discoideum. *ISME J 2020 144*

(2020) **14**:919–930. doi:10.1038/s41396-019-0572-z

41. Keating LA, Wheeler PR, Mansoor H, Inwald JK, Dale J, Hewinson RG, Gordon S V. The pyruvate requirement of some members of the Mycobacterium tuberculosis complex is due to an inactive pyruvate kinase: implications for in vivo growth. *Mol Microbiol* (2005) **56**:163–174. doi:10.1111/J.1365-2958.2005.04524.X

42. Gonzalo-Asensio J, Malaga W, Pawlik A, Astarie-Dequeker C, Passemar C, Moreau F, Laval F, Daffé M, Martin C, Brosch R, et al. Evolutionary history of tuberculosis shaped by conserved mutations in the PhoPR virulence regulator. *Proc Natl Acad Sci U S A* (2014) **111**:11491–11496. doi:10.1073/pnas.1406693111

43. Pethe K, Bifani P, Jang J, Kang S, Park S, Ahn S, Jiricek J, Jung J, Jeon HK, Cechetto J, et al. Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat Med* (2013) **19**:1157–1160. doi:10.1038/nm.3262

44. Minato Y, Gohl DM, Thiede JM, Chacón JM, Harcombe WR, Maruyama F, Baughn AD. Genome-wide assessment of Mycobacterium tuberculosis conditionally essential metabolic pathways. *bioRxiv* (2019)534289. doi:10.1101/534289

45. Bosch B, DeJesus MA, Poulton NC, Zhang W, Engelhart CA, Zaveri A, Lavalette S, Ruecker N, Trujillo C, Wallach JB, et al. Genome-wide gene expression tuning reveals diverse vulnerabilities of M. tuberculosis. *Cell* (2021) doi:10.1016/j.cell.2021.06.033

46. McNeil MB, Keighley LM, Cook JR, Cheung C, Cook GM. CRISPR interference identifies vulnerable cellular pathways with bactericidal phenotypes in Mycobacterium tuberculosis. *Mol Microbiol* (2021)mmi.14790. doi:10.1111/mmi.14790

47. Martínez-Jiménez F, Papadatos G, Yang L, Wallace IM, Kumar V, Pieper U, Sali A, Brown JR, Overington JP, Marti-Renom MA. Target Prediction for an Open Access Set of Compounds Active against Mycobacterium tuberculosis. *PLoS Comput Biol* (2013) **9**:1003253. doi:10.1371/journal.pcbi.1003253

48. Gu Z, Gu L, Eils R, Schlesner M, Brors B. circlize implements and enhances circular visualization in R. *Bioinformatics* (2014) **30**:2811–2812. doi:10.1093/BIOINFORMATICS/BTU393

Figure 1

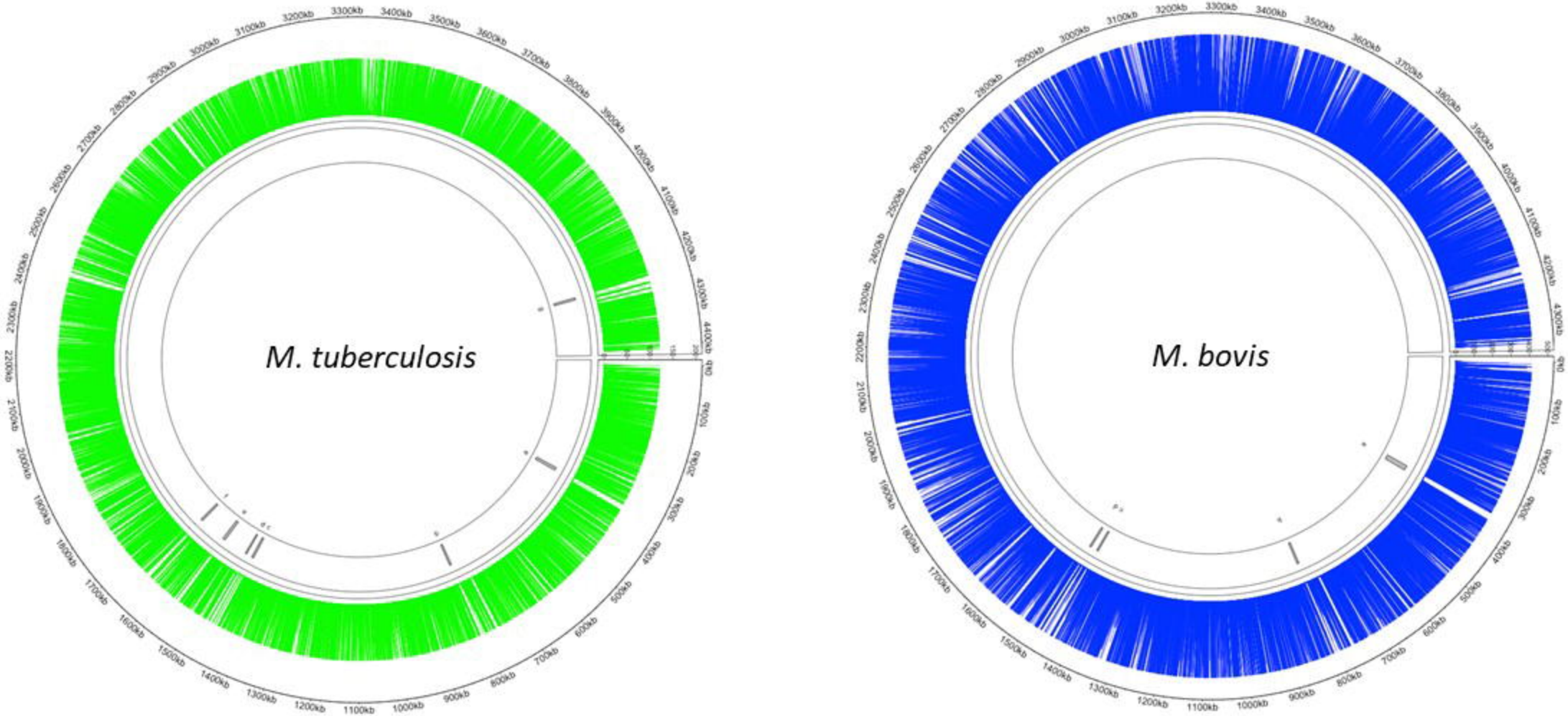
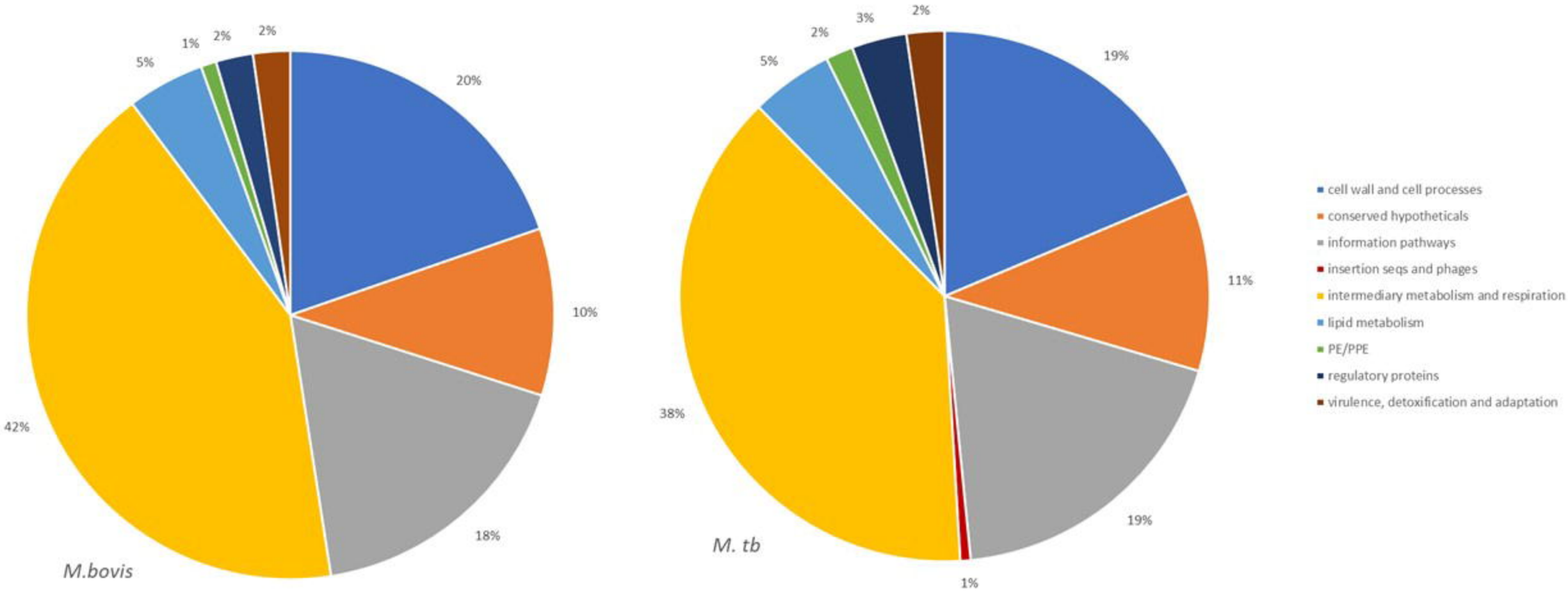


Figure 2


20%
10%
18%
42%
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M.bovis
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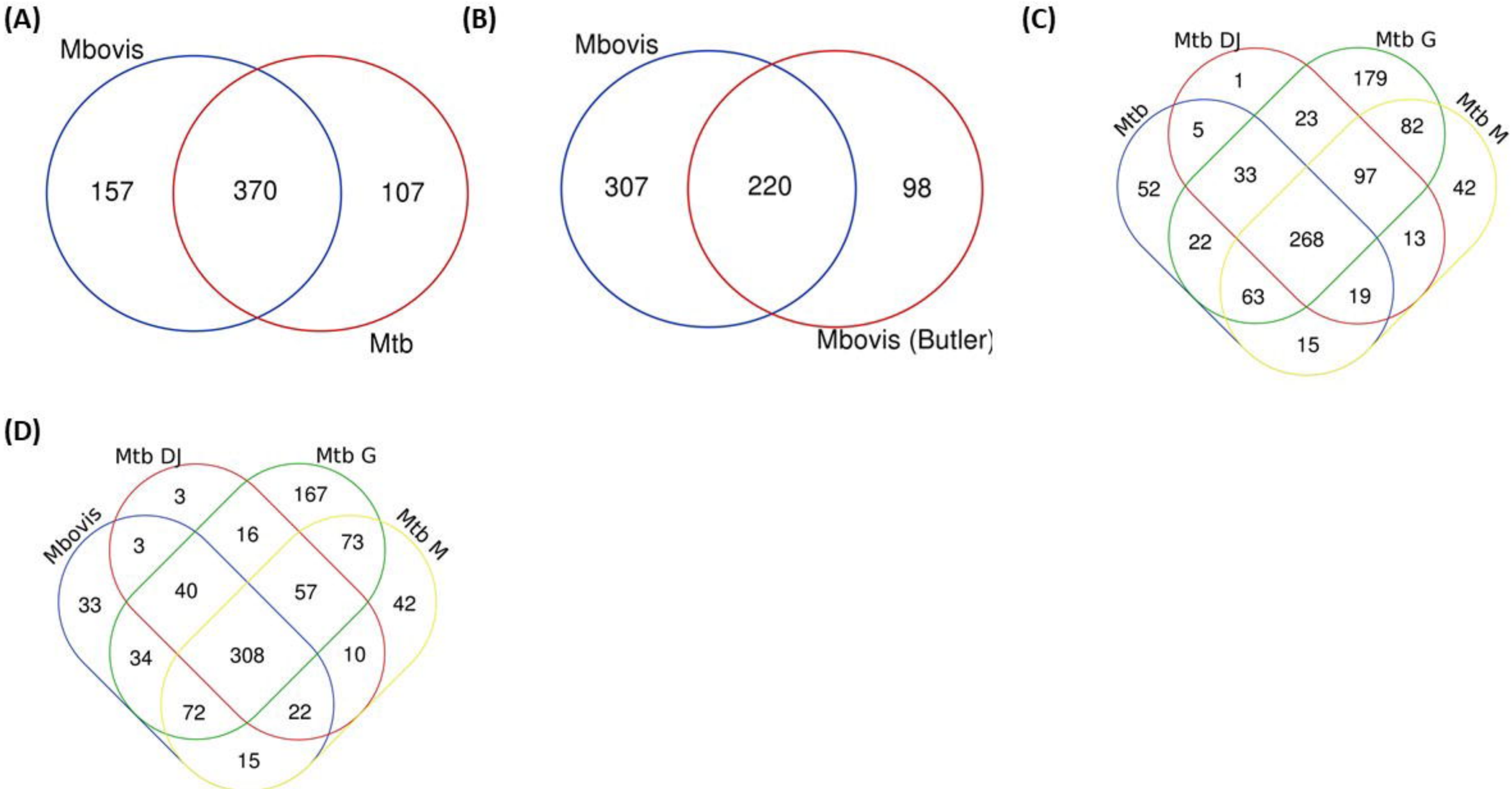
M. tb
cell wall and cell processes
conserved hypotheticals
information pathways
insertion seqs and phages
intermediary metabolism and respiration
lipid metabolism
PE/PPE
regulatory proteins
virulence, detoxification and adaptation

Figure 3


(A)
Mbovis
157
370
107
Mtb
(B)
Mbovis
307
220
98
Mbovis (Butler)
(C)
Mtb DJ
Mtb G
Mtb
Mtb M
1
179
5
23
82
33
52
97
42
22
268
13
63
19
15
(D)
Mtb DJ
Mtb G
Mbovis
Mtb M
3
167
3
16
73
40
57
33
42
34
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10
72
22
15

Figure 4

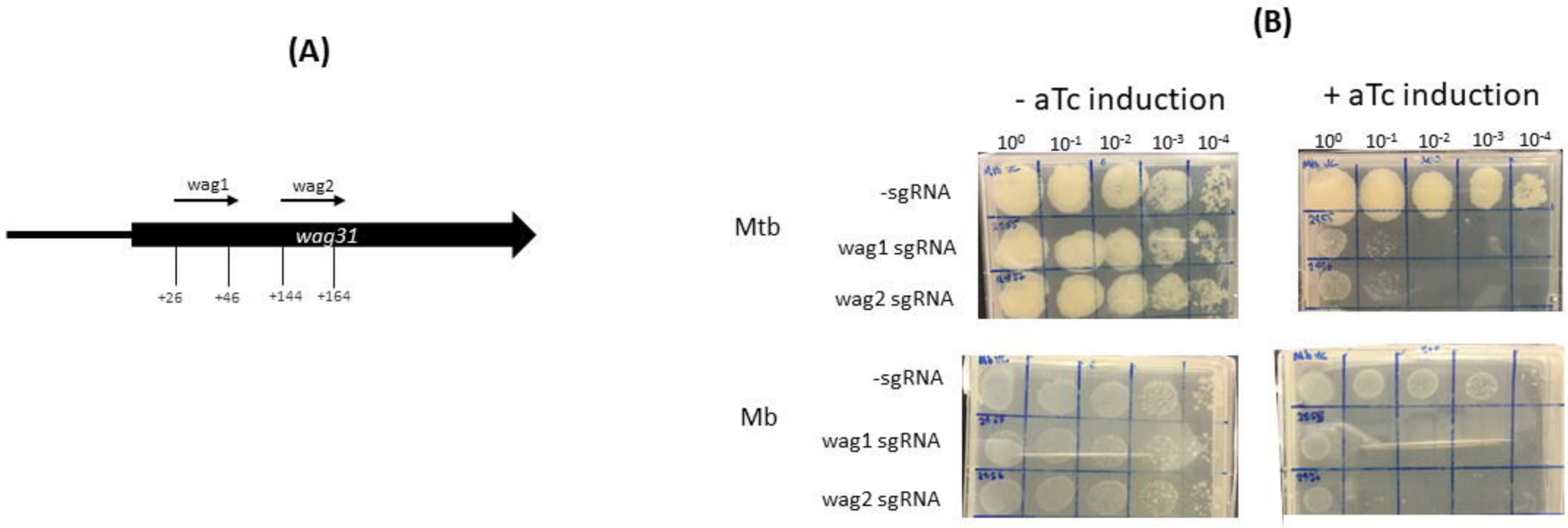
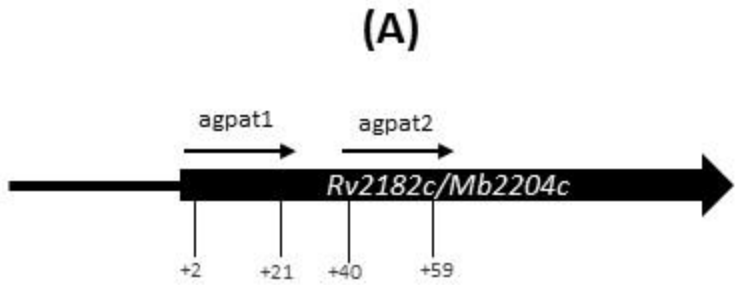
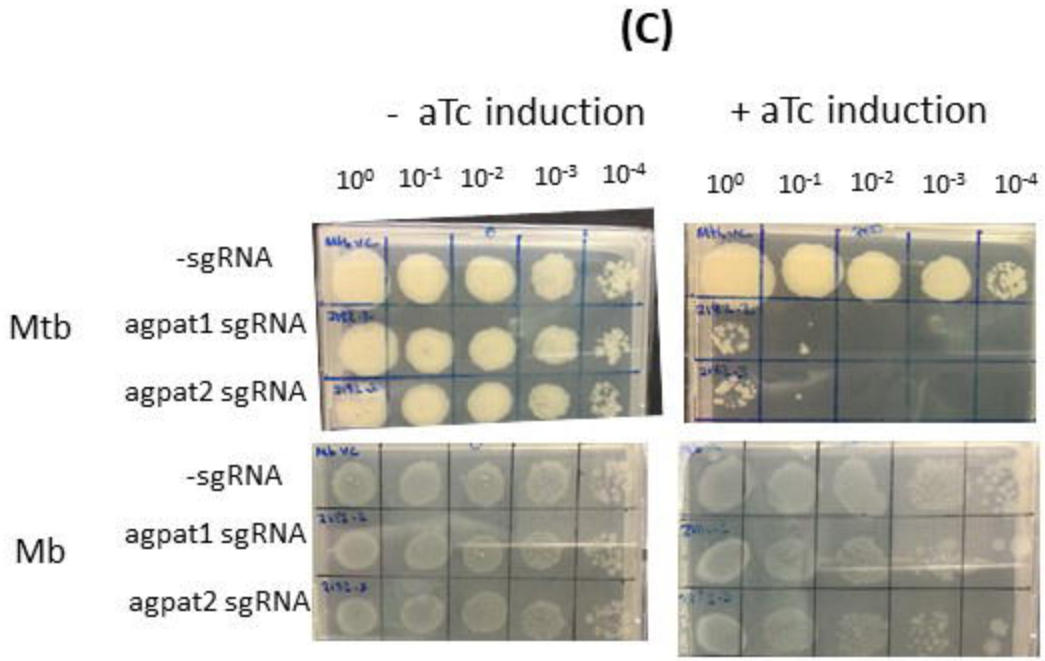
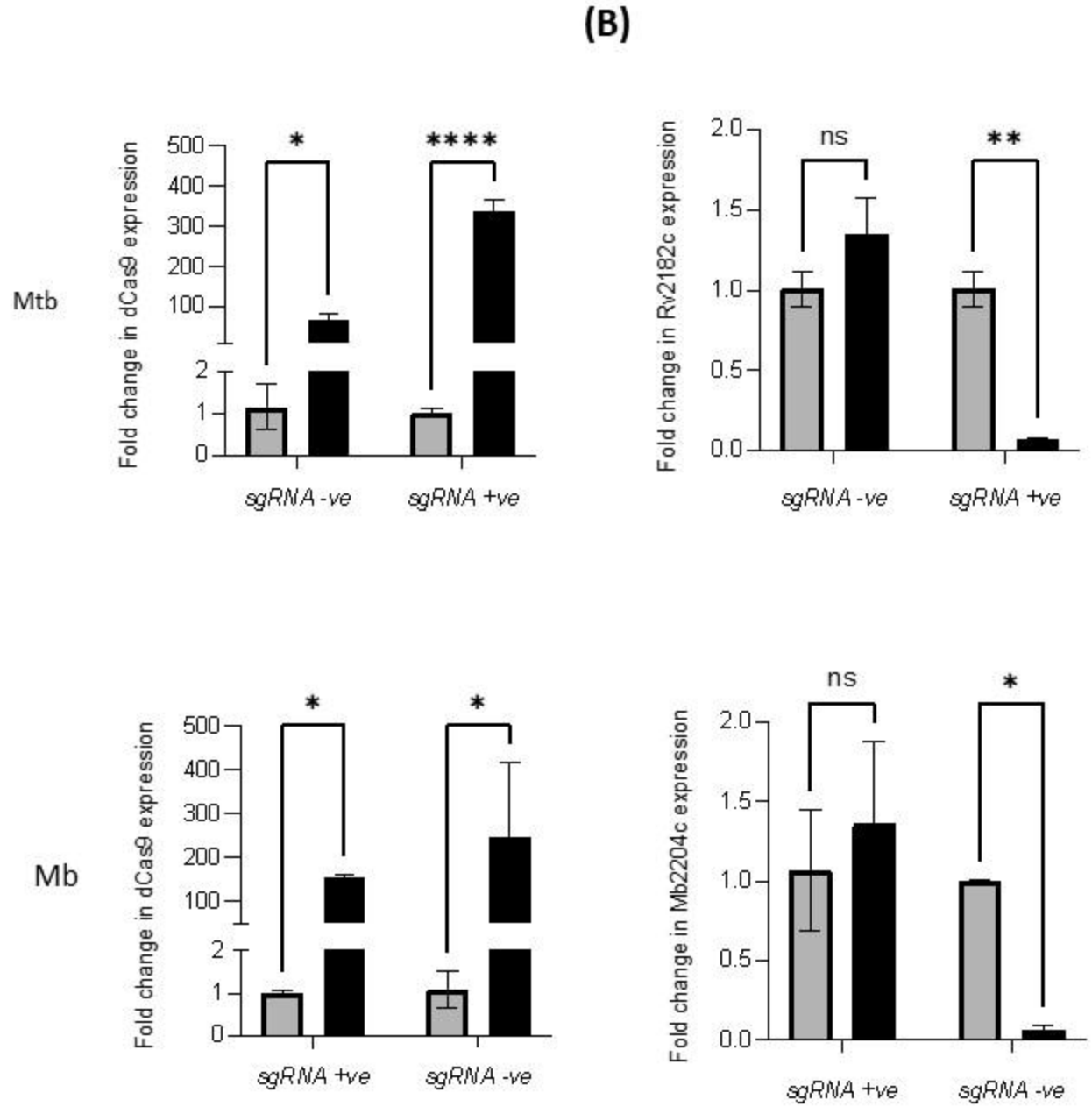

(A)
wag1
wag2
wag31
+26
+46
+144
+164
(B)
- aTc induction
+ aTc induction
10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4}
Mtb
-sgRNA
wag1 sgRNA
wag2 sgRNA
Mb
-sgRNA
wag1 sgRNA
wag2 sgRNA

Figure 5


(A)
agpat1
agpat2
Rv2182c/Mb2204c
+2
+21
+40
+59


(C)
- aTc induction
+ aTc induction
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
Mtb
-sgRNA
agpat1 sgRNA
agpat2 sgRNA
Mb
-sgRNA
agpat1 sgRNA
agpat2 sgRNA


(B)
Mtb
Fold change in dCas9 expression
500
400
300
200
100
2
1
0
*
****
sgRNA -ve
sgRNA +ve
Fold change in Rv2182c expression
2.0
1.5
1.0
0.5
0.0
ns
**
sgRNA -ve
sgRNA +ve
Mb
Fold change in dCas9 expression
500
400
300
200
100
2
1
0
*
*
sgRNA +ve
sgRNA -ve
Fold change in Mb2204c expression
2.0
1.5
1.0
0.5
0.0
ns
*
sgRNA +ve
sgRNA -ve