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].

83   Genome-wide transposon mutagenesis coupled with next-generation sequencing

84   (TnSeq) has allowed genome wide predictions of gene essentiality in *M. tuberculosis* (19–

85   24). These studies have provided information on the genetic requirements for *in vitro*

86   growth under a number of conditions and also for growth in disease relevant models such

87   as macrophages (20). Most of these studies performed in the MTBC have used strain

88   H37Rv. More recently Tnseq of different clinical strains of *M. tuberculosis* has shown that

89   there are strain specific differences in fitness associated with Tn insertions in certain

90   genes. The implication of this observation is that different strains can show different

91   antibiotic sensitivities as a result (25). To date, there has been a single reported Tnseq

92   study performed in *M. bovis* (AF2122/97) which focused on intra-cellular genetic

93   requirements (26)

94   A direct comparison of gene essentiality in *M. bovis* and *M. tuberculosis* has not

95   been reported. Therefore, we created dense transposon libraries in both *M. bovis*

96   (AF2122/97) and *M. tuberculosis* (H37Rv) generated on the same medium to enable

97   direct comparisons between the two related species. We identified that there are key

98   differences in essentiality in *M. bovis* compared to *M. tuberculosis*. We used CRISPRi to

99   directly demonstrate that silencing the expression of a gene annotated to be involved in

100   glycerophospholipid metabolism has different impacts on growth in the two species. This

101   has implications for target discovery programmes as it implies that inhibition of

102   therapeutically relevant pathways may have different impacts in the different species.

103   This is important in the context of zoonotic tuberculosis.

104

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* mc²155 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* mc²155 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 $OD_{600} \cong 1$ 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

167   DNA polymerase (New England Biolabs) in a 20-cycle reaction. Library fragments were

168   subsequently cleaned up with AMPureXP purification beads (Beckman).

Data Analysis

170   Indexed libraries were combined, spiked with 20% PhiX, and sequenced on the

171   Illumina HiSeq 3000 platform, using v2 chemistry, generating single-end reads of 150 bp.

172   Raw FASTQ sequence files were pre-processed using the TPP utility of TRANSIT python

173   package (DeJesus *et al.*, 2015), including removing TRADIS tags and adapter sequences

174   and mapping using BWA-MEM algorithm [32], to generate insertion files in .wig format.

175   Custom annotations, 'prot tables', were created from the *M. bovis* strain AF2122/97

176   annotation file (NCBI Accession Number LT708304, version LT708304.1) and for the

177   *M. tuberculosis* strain, H37Rv (NCBI Accession Number AL123456, version AL123456.3,

178   assembly build GCA_000195955.2 (ENA)). TRANSIT was run on both *M.bovis* and

179   *M. tuberculosis* files using the default normalisation (TTR) and the TRANSIT HMM

180   algorithm (31) to make calls of essentiality for each TA insertion site, and for each gene

181   based on annotated gene boundaries. Data files (fastq) are deposited in SRA

182   (PRJNA754037)

CRISPRi mediated gene silencing

184   We utilised dCas9 from *Streptococcus pyogenes* (dCas9$_{Spy}$) for silencing as

185   previously described (32). sgRNA targeting *wag31*$_{Mtb/Mb}$ and *Rv2182c/Mb2204c* were

186   designed according to the parameters derived from Larson *et al* 2013 (33). Protospacer

187   adjacent motif (PAM) sites, "NGG," were chosen and putative sgRNAs 20 bp downstream

188   of the PAM were selected. All sgRNAs designed targeted the coding non-template strand.

189   The probability of complementarity to any other region of the genome and predicted

190   secondary structure of the sgRNA transcript was analyzed using a basic local alignment

191   search tool (BLAST) and M-fold, respectively (34,35). Complementary forward and

192   reverse primers using the sequence (without the PAM) with appropriate ends for ligation

193   to the pRH2521 vector were designed (Table 2). Oligos were annealed and cloned into

194   pRH2521 using BbsI as previous (32,36). One microgram of pRH2502 was electroporated

195   at 25 kV, 25 µF with 1000 Ω resistance into electrocompetent *M. bovis* and *M. tuberculosis*

196   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.

RNA Extraction and RT-qPCR

200   Cultures were grown to $OD_{600} \cong 0.1$–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

251 for the genes that are conserved between both species is given in supplementary table

252 S1. The status of the genes that are *M. bovis* specific are also included in the table.

253   Early sequencing and functional annotation of the genome of *M. tuberculosis*

254 categorised genes into several different functional classes with an uneven distribution of

255 genes across the classes (4,5). We examined the distribution of the genes classified as ES

256 in *M. tuberculosis* (477) and *M. bovis* (527) across the functional classes to determine if

257 (i) ES genes are over-represented in any particular functional class when compared to

258 the genome as a whole (ii) there are differences between the two species. The results are

259 shown in figure 2 and table 4. Chi squared testing showed that the distribution of ES genes

260 across the functional classes was significantly different to the distribution of all orthologs

261 (p=<0.01). ES genes in both species are over-represented in "information pathways" and

262 "intermediary metabolism and respiration" and under-represented in "conserved

263 hypotheticals" and "PE/PPE" functional classes. Our data are in line with previous

264 reports; Griffin *et al* noted that the distribution of ES genes across the different functional

265 classes were different compared to the genome as a whole (22). DeJesus *et al* also noted

266 that insertions in PE/PPE genes were under-represented likely due to GC rich sequences

267 and an increased proportion of non-permissive TA sites in the PE/PPE genes (23). There

268 were no major differences in distribution of ES genes across the functional classes when

269 *M. tuberculosis* and *M. bovis* were compared with each other except for "insertion

270 sequences and phages" which did not contain any genes classified as ES in the *M. bovis*

271 genome.

272   Genes categorised as ES in this study were compared between the two species and

273 also compared to previously reported studies (21–23,26,39) (supplementary file, table

274 S1)*. We found that the *M. bovis* dataset generated in our study shared 370 (70%) of genes

275 classified as ES with *M. tuberculosis in vitro* (this study; figure 3A) and up to 86% overlap

276 with three key published *M. tuberculosis* data sets: DeJesus *et al* 2017 (71%), Griffin *et al*

277 2011 (86%) and Minato *et al* 2019 (79%) indicating good correlation with previous

278 reports (figure 3D). Similarly, the *M. tuberculosis* dataset generated in our study shared

279 good overlap with other published datasets (figure 3C). When comparing *M. bovis* genes

280 classified as ES with those reported by Butler *et al* 2020 (40) we found that 220 (42%)

281 genes were shared between these data sets (figure 3B). Butler *et al* reported a total of

282 318 genes to be essential in *M. bovis in vitro* prior to selection in *Dictyostelium discoideum*

283   compared to 527 reported in this study. Both libraries showed similar saturation levels

284   (58% vs. 54% in this study) therefore differences might be due to the conditions under

285   which the libraries were generated (although both studies used Middlebrook 7H11 solid

286   medium supplemented with lysed sheep blood, heat inactivated foetal bovine serum and

287   OADC) or between laboratory variation as might be expected for whole genome

288   techniques such as Tnseq. It should also be noted that the similarities between the studies

289   increases when GD genes are considered, for instance of the 307 genes that appear to be

290   uniquely ES in our study, 212 of these are classified as GD in the study by Butler *et al.,*

291   indicating a debilitating impact of the transposon insertion.

292

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

294   Genes uniquely classified as ES in either species are of interest to determine

295   potential genetic insights for phenotypic differences between these closely related

296   mycobacterial species. In this study 157 genes were uniquely ES in *M. bovis* when

297   compared to the *M. tuberculosis* (figure 3A), however, of these 157, 61 were classified as

298   GD in *M. tuberculosis*. The remaining 96 were classified as NE in *M. tuberculosis*

299   (supplementary file, table S2). The existence of multiple datasets allows for a robust

300   meta-analysis and so we compared across datasets and found that there were 42 genes

301   that were either ES or GD in this study and the study by Butler *et al.*, and were classified

302   as NE in *M. tuberculosis* in this study and the study by DeJesus et al. (supplementary file,

303   table S3). Included in this subset of genes is *Rv3543c* (*fadE19*), *Rv3541c* and *Rv3540c*

304   (*lpt2*), genes which are encoded on the same operon (*Rv3545c-Rv3540c* - based on

305   intergenic gaps) regulated by *kstR* and involved in cholesterol catabolism. This study and

306   the Butler *et al.,* study indicates that insertional mutagenesis of this operon has a

307   debilitating impact in *M. bovis* but not in *M. tuberculosis.*

308   Data for the entire *kstR* regulon is given in supplementary file, table S4.

309   Interestingly, the media used in this study and the study by Butler *et al.*, contains traces

310   of cholesterol due to the presence of lysed sheep blood, although there is no evidence that

311   cholesterol presented a selective pressure (for *M. tuberculosis*) in this study as there is

312   little overlap of the *M. tuberculosis* dataset with the study by Griffin *et al.,* In addition to

313   the *Rv3545c-Rv3540c* operon considered above, several orthologs in the *kstR* regulon

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

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

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

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

346   oxidase complex (*qcrCAB*) the target of Q203 are classified as either ES or GD. One

347   interesting observation is that both our study and the study by Butler *et al.*, is that a GD

348   occurs as a result of an insertion in *nuoG* but this is not observed in any of the *M.*

349   *tuberculosis* studies. *nuoG* forms part of the multi-subunit NADH reductase-I complex in

350   the respiratory chain and transfers electrons to the menaquinone pool while

351   simultaneously contributing to the proton gradient through its proton pumping function

352

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

354   Wag31 is required for peptidoglycan synthesis and previously published datasets

355   have classified *wag31* in *M. tuberculosis* as ES *in vitro* (21–23). This study classified *wag31*

356   in *M. bovis* as ES but NE in *M. tuberculosis*. The study by Butler *et al.*, assigned *wag31* as

357   NE in *M. bovis*. In order probe this discrepancy and to establish CRISPRi silencing in

358   *M. bovis* this gene was chosen for silencing. Early CRISPRi studies in *M. tuberculosis*

359   performed by Singh *et al.* successfully utilised two plasmids encoding sgRNAs guides

360   targeting +26 bp to +45 bp and +144 bp to +163 bp downstream of the annotated start

361   codon of *wag31*$_{Mtb}$ (table 1 and figure 4A). We utilised these plasmids to investigate the

362   impact of silencing *wag31*$_{Mb}$. *M. bovis* AF2122/97 was transformed with pRH2502 to

363   create a strain expressing *dcas9*$_{Spy}$ (Mb_dCas9). Mb_dCas9 was then transformed with

364   plasmids expressing the sgRNA guides. Strains were cultured to exponential phase and

365   serial dilutions were spotted onto agar containing 200 ng/ml aTc. Controls (without aTc,

366   without sgRNA) were also included. The results, presented in figure 4B, show that

367   silencing *wag31*$_{Mb}$ in *M. bovis* results in a severe growth defect, visible at 10^{-1} dilution with

368   complete cessation of growth at 10^{-2} dilution. This growth defect is identical to that seen

369   in *M. tuberculosis* and supports the ES classification.

370

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

373   *Rv2182c/Mb2204c* is annotated as a 1-acylglycerol-3-phosphate O-

374   acyltransferase (agpat) and involved in glycerophospholipid metabolism. It is thought to

375   synthesize diacylglycerol-3P through the addition of acyl chains to monoacylglycerol-3P.

376   It is classified as ES in *M. tuberculosis* in this study and by others (22,23,44). It is classified

377   as ES in *M. bovis* in this study but NE in the study by Butler *et al*.,. Strains of *M. tuberculosis*

378   and *M. bovis* were constructed expressing sgRNAs targeting +2 bp to +21 bp and +40 bp

379   to +59 bp downstream of the annotated start codon of *Rv2182c/Mb2204c* (table 2 and

380   figure 5A). The impact of inducing the system on expression of *Rv2182c/Mb2204c* was

381   measured using RT-qPCR. The results, which are shown in figure 5B show that dCas9$_{Spy}$

382   is similarly induced in both *M. tuberculosis* and *M. bovis* with 150 to 350-fold induction of

383   expression in the presence of aTc. Additionally, the results show that, in the presence of

384   the sgRNA, there is a clear reduction in expression of *Rv2182c/Mb2204c* in both species.

385   These data demonstrate effective gene silencing of *Rv2182c/Mb2204c* in both

386   *M. tuberculosis* and *M. bovis*, respectively.

387   To determine the impact of induction of the guides, strains were cultured to

388   exponential phase and serial dilutions were spotted onto agar containing 200 ng/ml aTc.

389   The results (figure 5C) show that silencing *Rv2182c* in *M. tuberculosis* results in a severe

390   growth defect, with almost complete cessation of growth at 10^{-1} dilution. However, the

391   consequence of silencing *Mb2204c* on the growth of *M. bovis* is far less impactful with a

392   small reduction visible at the lowest dilution 10^{-4}. This demonstrates that, unlike *wag31*,

393   silencing of *Rv2182c* and its ortholog *Mb2204c* in *M. tuberculosis* and *M. bovis* respectively,

394   has a differential impact on growth, with *M. tuberculosis* being more vulnerable and

395   showing a greater growth defect. These results do not support the classification of

396   *Mb2204c* as an ES gene in *M. bovis* but they clearly highlight that there are different

397   phenotypic consequences as a result of silencing the ortholog in both species.

398

Discussion

400   The aim of this work was to directly compare gene essentiality in the human and

401   animal adapted members of the MTBC. In order to do this we generated transposon

402   libraries in *M. bovis* AF2122/97and *M. tuberculosis* H37Rv using a rich medium that

403   supported the growth of both species. We assessed gene essentiality using the TRANSIT

404   HMM method to define 527 and 477 genes as ES for *M. bovis* and *M. tuberculosis*,

405   respectively. Datasets from each species were compared with each other and with

406   previously published datasets. Genes classified as ES were congruent between the species

407   and also with existing studies of gene essentiality in *M. tuberculosis* (21–23,39).

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

417 Whole-genome TnSeq provides a high-throughput assessment of fitness costs and
418 has allowed the classification of genes based on essentiality but does not provide
419 information on target vulnerability. More recent studies highlight the limitations of the
420 (near) binary classification of genes into and ES/NE and utilise CRISPRi to assess
421 vulnerability (45,46). Additionally, datasets are prone to false calls of ES due to non-
422 saturating mutagenesis. In this study CRISPRi was utilised to show that there are different
423 impacts on bacterial growth as a result of silencing *Rv2182c/Mb2204c* in their respective
424 species, despite achieving similar levels of gene silencing. Significant growth inhibition
425 was seen as a result of silencing in *Rv2182c* in *M. tuberculosis* while only marginal impacts
426 on growth were observed on silencing the ortholog *Mb2204c* in *M. bovis.*
427 *Rv2182c/Mb2204c* is annotated as a 1-acylglycerol-3-phosphate O-acyltransferase and
428 involved in glycerophospholipid metabolism. It is thought to synthesize diacylglycerol-
429 3P through the addition of acyl chains to monoacylglycerol-3P. This pathway may be
430 involved in detoxification and further work is required to fully understand the differential
431 impact of silencing this gene in the two species. Given that *Rv2182c* was a predicted target
432 in a recent compound screen (47), differential essentiality estimates in *M. bovis* and
433 *M. tuberculosis* are important to predict if zoonotic TB caused by *M. bovis* can also be
434 suitably treated with drugs designed to be effective against *M. tuberculosis.*

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

440 *bovis* and the involvement of wildlife hosts as reservoirs of infection for bovine TB. Use

441 of *M. bovis* libraries in the context of the host i.e., through experimental infection of bovine

442 TB will enable the study of the genetic requirements for survival *in vivo*. Further

443 investigations exploring the role and function of ES genes between *M. bovis* and

444 *M. tuberculosis* is necessary to better understand the physiological differences in these

445 key MTBC species.

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620 **Author Contributions**

621 AJG, SW, IP and SLK designed the study. AJG, IP, VF, carried out the experimental work.

622 Data analysis was done by IN, JS and DX. SLK, DW, BWW and BVR did funding acquisition.

623 AJG and SLK wrote the first draft of the manuscript. All authors contributed to the

624 manuscript revision, read, and approved the submitted version.

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633

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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636

637   **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	<u>AAAC</u>TGTGGTACTACCTGTTCAAG
agpat1_R	<u>GGGA</u>CTTGAACAGGTAGTACCACA
agpat2_F	<u>AAAC</u>CTCTTTACGTTGCTTGGTCG
agpat2_R	<u>GGGA</u>CGACCAAGCAACGTAAAGAG
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

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

639   [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**

641

	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

642

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

644

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 2×10^7 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.

687

688

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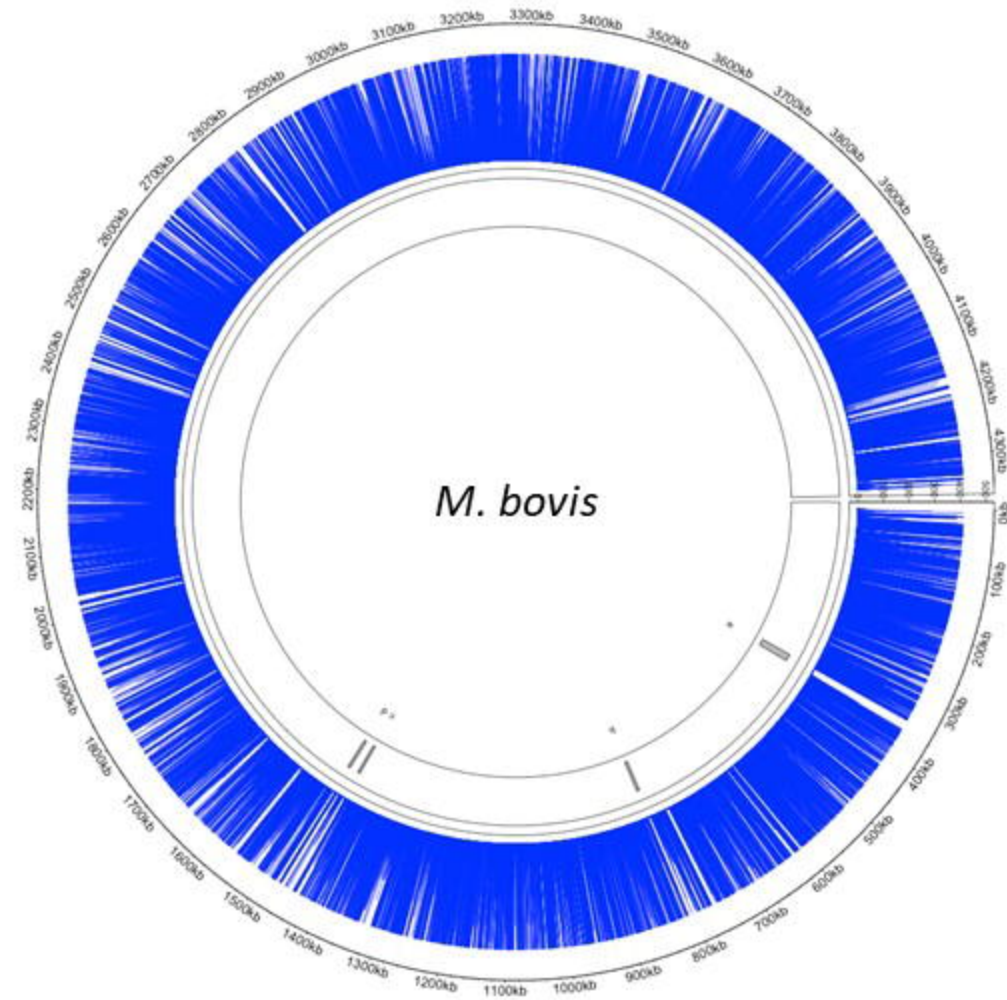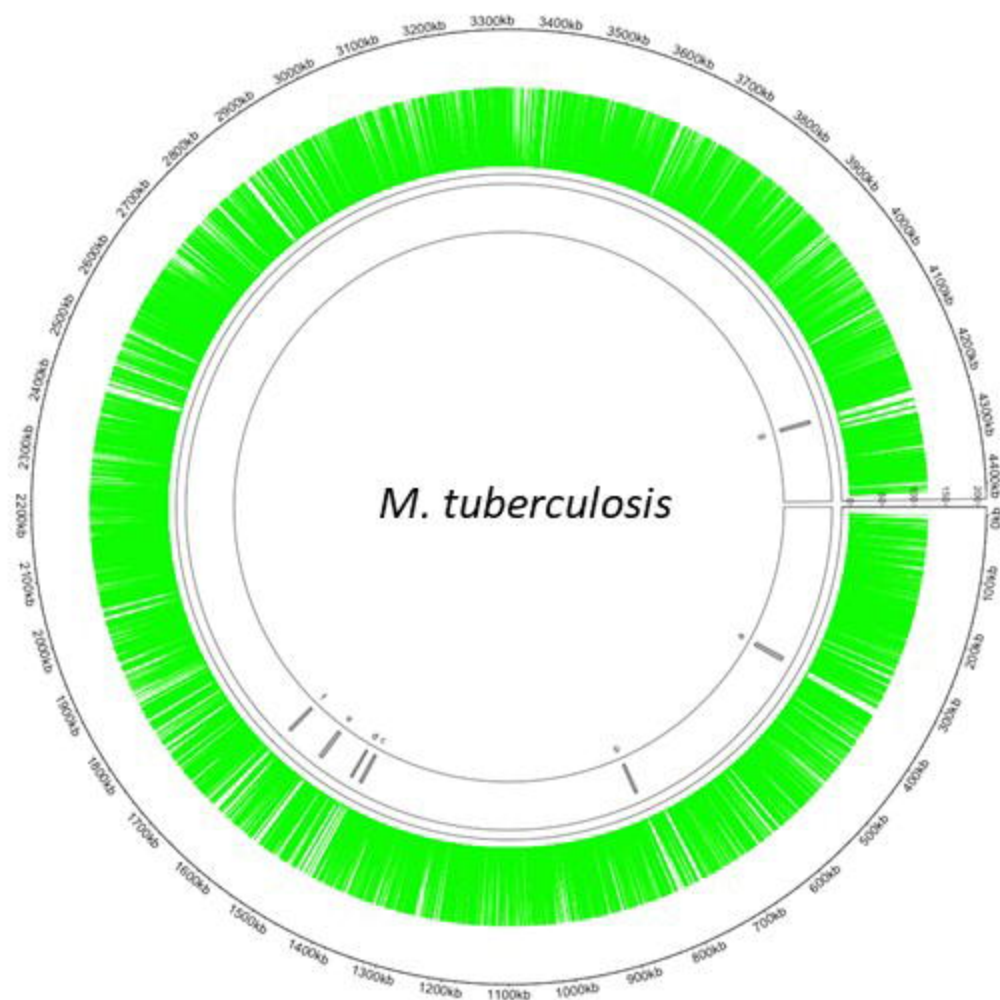843   doi:10.1093/BIOINFORMATICS/BTU393

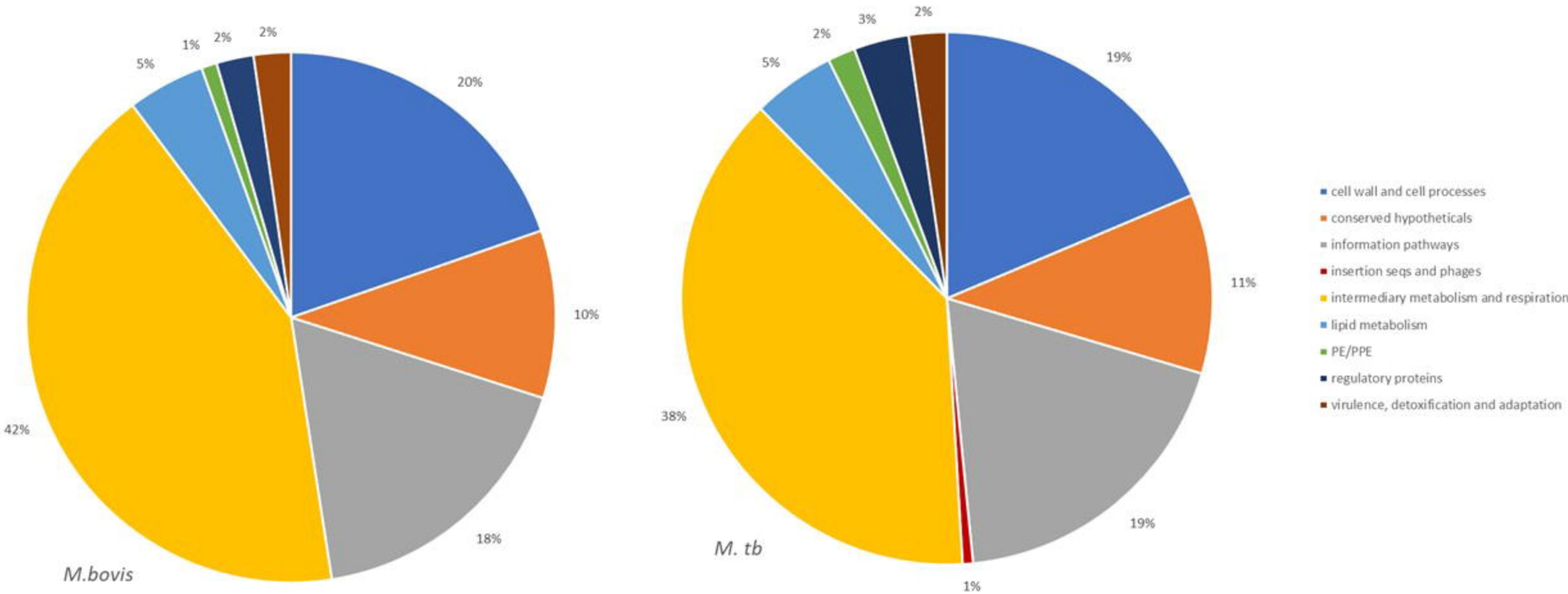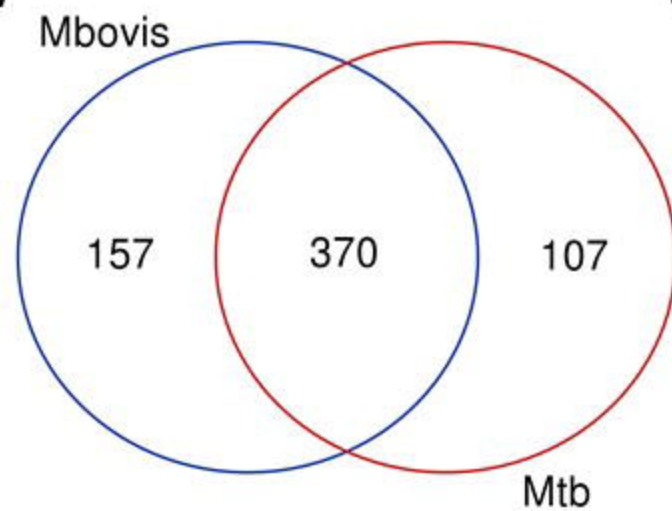844

Figure 1

Figure 2

Legend:
- 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

M.bovis: 20%, 10%, 18%, 42%, 5%, 1%, 2%, 2%

M. tb: 19%, 11%, 19%, 1%, 38%, 5%, 2%, 3%, 2%

Figure 3

(A)

Mbovis
157 370 107
Mtb

(B)

Mbovis
307 220 98
Mbovis (Butler)

(C)

Mtb DJ   Mtb G
1   179
Mtb   5   23   82   Mtb M
52   33   97   42
22   268   13
63   19
15

(D)

Mtb DJ   Mtb G
3   167
Mbovis   3   16   73   Mtb M
33   40   57   42
34   308   10
72   22
15

Figure 4

(A)

(B)

Figure 5

(A)

(B)

(C)