

1 **Defining the genes required for survival of *Mycobacterium bovis* in the bovine host offers**  
2 **novel insights into the genetic basis of survival of pathogenic mycobacteria**

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26 **Abstract**

27 Tuberculosis has severe impacts in both humans and animals. Understanding the genetic basis  
28 of survival of both *Mycobacterium tuberculosis*, the human adapted species, and  
29 *Mycobacterium bovis*, the animal adapted species is crucial to deciphering the biology of both  
30 pathogens. There are several studies that identify the genes required for survival of *M.*  
31 *tuberculosis* *in vivo* using mouse models, however, there are currently no studies probing the  
32 genetic basis of survival of *M. bovis* *in vivo*. In this study we utilise transposon insertion  
33 sequencing in *M. bovis* to determine the genes required for survival in cattle. We identify  
34 genes encoding established mycobacterial virulence functions such as the ESX-1 secretion  
35 system, PDIM synthesis, mycobactin synthesis and cholesterol catabolism that are required  
36 *in vivo*. We show that, as in *M. tuberculosis*, *phoPR* is required by *M. bovis* *in vivo* despite the  
37 known defect in signalling through this system. Comparison to studies performed in glycerol  
38 adapted species such as *M. bovis* BCG and *M. tuberculosis* suggests that there are differences  
39 in the requirement for genes involved in cholesterol import (*mce4* operon), oxidation (*hsd*)  
40 and detoxification (*cyp125*). We report good correlation with existing mycobacterial virulence  
41 functions, but also find several novel virulence factors, including genes involved in protein  
42 mannosylation, aspartate metabolism and glycerol-phosphate metabolism. These findings  
43 further extend our knowledge of the genetic basis of survival *in vivo* in bacteria that cause  
44 tuberculosis and provide insight for the development of novel diagnostics and therapeutics.

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49 **Importance**

50 This is the first report of the genetic requirements of an animal adapted member of the MTBC  
51 in a natural host. *M. bovis* has devastating impacts in cattle and bovine tuberculosis is a  
52 considerable economic, animal welfare and public health concern. The data highlight the  
53 importance of mycobacterial cholesterol catabolism and identifies several new virulence  
54 factors. Additionally, the work informs the development of novel differential diagnostics and  
55 therapeutics for TB in both human and animal populations.

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57 **Introduction**

58 Bacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) have devastating  
59 impacts in both animal and human populations. *Mycobacterium bovis*, an animal adapted  
60 member of the MTBC and one of the main causative agents of bovine tuberculosis (bTB),  
61 remains endemic in some high-income settings despite the implementation of a test and  
62 slaughter policy. In low- and middle-income settings, the presence of bTB in livestock  
63 combined with the absence of rigorous control measures contributes to the risk of zoonotic  
64 transmission (1, 2). Control measures based on cattle vaccination utilise the live attenuated  
65 vaccine *M. bovis* BCG but the efficacy of this vaccine still remains low in field situations (3, 4).  
66 In addition to vaccines, the development of diagnostic tools for the identification of infected  
67 individuals is crucial for the management of transmission. Currently, vaccination with *M. bovis*  
68 BCG sensitises animals to the diagnostic tuberculin skin test, therefore, sensitive and specific  
69 differentiating diagnostic strategies are a current imperative (5, 6).

70 The increased accessibility of whole genome fitness screens has allowed the assessment of  
71 the impacts of the loss of gene function on bacterial survival (7). Such screens have been

72 invaluable in identifying novel drug targets or candidates for the generation of new live  
73 attenuated vaccines in a number of bacterial pathogens, including *M. tuberculosis* (8–13).

74 Studies utilising whole genome transposon mutagenesis screens to examine gene fitness *in*  
75 *vivo* in *M. tuberculosis* have been limited to mouse models (8, 9, 13). These models do not  
76 faithfully replicate the granulomatous pathology associated with TB, nor do mice contain the  
77 same repertoire of CD1 molecules expressed by bovine T cells required to present  
78 mycobacterial lipid antigens (14). Whole genome transposon mutagenesis screens utilising  
79 non-human primates are limited because screening is restricted to smaller mutant pools (15).

80 To date, transposon insertion sequencing (Tn-seq) based studies in the context of bTB in cattle  
81 have only been performed using *M. bovis* BCG strains (16, 17).

82 In this study we use Tn-seq to determine the genes required for survival of *M. bovis* directly  
83 in cattle. We show that genes involved in the biosynthesis of phthiocerol dimycocerosates  
84 (PDIMs), the ESX-1 secretion system, cholesterol catabolism, and mycobactin biosynthesis are  
85 essential for survival in cattle, corroborating current knowledge of gene essentiality in  
86 members of the MTBC (8, 9, 13, 16, 17). We identify differences in the requirement for genes  
87 involved in cholesterol transport and oxidation in the fully virulent *M. bovis* strain. We also  
88 identify several novel genes required for survival *in vivo* that have not been previously  
89 described in members of the MTBC.

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94 **Results and Discussion**

95 **Generation and sequencing of the input library.**

96 We generated a transposon library in *M. bovis* AF2122/97 using the MycomarT7 phagemid  
97 system as previously described (18, 19). Sequencing of the input library showed that  
98 transposon insertions were evenly distributed around the genome and 27,751 of the possible  
99 73,536 thymine–adenine dinucleotide (TA) sites contained an insertion representing a  
100 saturation density of ~38% (Supplementary Figure S1 and Supplementary Table S1 -input  
101 library). The *M. bovis* AF2122/97 genome has 3,989 coding sequences and insertions were  
102 obtained in 3,319 of these, therefore the input library contained insertions in 83% of the total  
103 coding sequences.

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105 ***Mycobacterium bovis* specific immune responses were observed in cattle**

106 Twenty-four clinically healthy calves of approximately 6 months of age were inoculated with  
107 the library through the endobronchial route. Infection was monitored by IFN- $\gamma$  release assay  
108 (IGRA) at the time of inoculation and 2 weeks post infection. *M. bovis* specific immune  
109 responses were observed for all study animals at 2 weeks post infection (Figure 1A and B).  
110 Each animal presented very low background of circulating IFN- $\gamma$  together with a statistically  
111 significant increase in IFN- $\gamma$  release in response to PPD-B compared to PPD-A antigens (Figure  
112 1C; \*\*\* p ≤ 0.001). This indicates that infection with the library was successfully established  
113 in the cattle.

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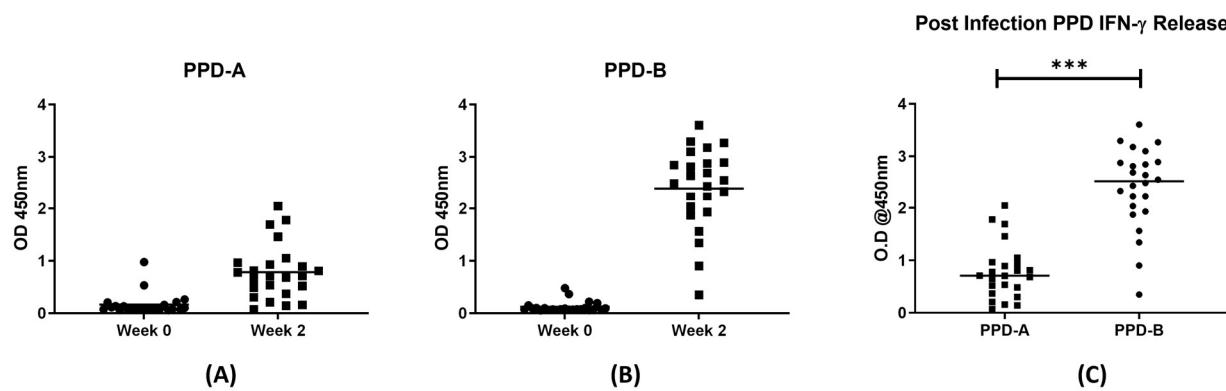
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121 **Figure 1. bTB specific IFN-gamma release in cattle infected with the *M. bovis* Tn-library.**

122 Blood was collected from all 24 animals on the day of infection and 2 weeks later. No response  
123 was detected to either PPD-A or PPD-B antigen stimulation prior to infection (Figure 1A and  
124 Figure 1B, week 0). All animals presented a significant and specific response to PPD-B  
125 compared to PPD-A as determined by a paired T-test using GraphPad Prism (Figure 1C). \*\*\*  
126  $p \leq 0.001$

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131 **Pathology associated with infection was greater in the lung and thoracic lymph nodes**

132 Animals were culled at 6 weeks post infection. Lung sections and upper (head and neck) and

133 lower (thoracic) respiratory tract associated lymph nodes were examined for gross lesions.

134 Lesions typical of *M. bovis* infection were observed in the tissues examined. Pathology scores

135 are shown in Figure 2A. Greater pathology was observed in lung and thoracic lymph nodes

136 compared to the head and neck lymph nodes.

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138 **Higher bacterial loads were associated with the lung and thoracic lymph nodes**

139 Bacterial counts were highest in lesions derived from the lung compared to those from the

140 thoracic lymph nodes and head and neck lymph nodes (Figure 2B). The lowest bacterial counts

141 were observed within the head and neck lymph nodes. However, this was not significant when

142 compared to thoracic lymph nodes. The volume of each macerate varied depending on lesion

143 size. Considering macerate volume, average bacterial loads of  $10^7$ ,  $10^6$  and  $10^5$  were

144 recovered from lesions from samples of the lungs, thoracic lymph nodes and head and neck

145 lymph nodes, respectively.

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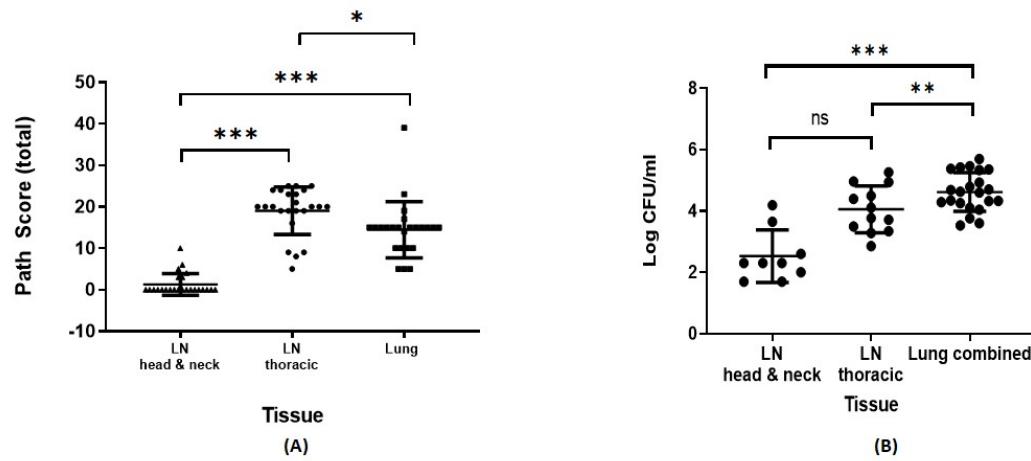
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157 **Figure 2. Tissue pathology and bacterial load in tissue sites. Six weeks after infection**  
158 **animals were subjected to post-mortem examination.** Gross pathology and evidence of TB-  
159 like granulomas lesions were scored. Data presented is the mean across animals of the total  
160 scores for each tissue group from 24 animals +/- the standard deviation. Lung and thoracic  
161 lymph nodes were observed to contain the highest pathology compared to head and neck  
162 lymph nodes (Figure 2A). For bacterial load estimation, aliquots of macerates were plated  
163 onto modified 7H11 agar containing kanamycin. Colonies were counted after 3-4 weeks  
164 growth. Data are presented as mean CFU/ml per collected tissue group +/- standard  
165 deviation. Lung tissue contained the highest bacterial burden compared to thoracic and head  
166 and neck lymph nodes as determined by one-way ANOVA analysis using GraphPad Prism  
167 (Figure 2b). \*\*\* p ≤ 0.001, \*\* p = 0.002, \*p=0.01

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171 **Recovery and sequencing of *in vivo* selected transposon libraries**

172 In order to recover the Tn library from harvested tissue  $\sim 10^5\text{-}10^6$  CFU from lungs and thoracic  
173 lymph nodes were plated onto several 140 mm modified 7H11 plates containing kanamycin  
174 to minimize competition between mutants. Samples from 4 cattle were lost due to fungal  
175 contamination, therefore the samples processed represent samples from 20 cattle. Lung  
176 samples were plated from all 20 animals and thoracic lymph nodes samples were plated from  
177 6 cattle. Bacteria were grown for 4-6 weeks before harvesting for genomic DNA extraction  
178 and subsequent sequencing (see Supplementary Table S1 for assignation of sequencing files  
179 to samples). The diversities of the output libraries were compared to the input library for each  
180 sample (Supplementary Figure S2 and Table S1). On average, libraries recovered from lung  
181 lesions from 20 different cattle contained 14,456 unique mutants and those recovered from  
182 the thoracic lymph nodes contained an average of 16,210 unique mutants. Given that the  
183 input library contained 27,751 unique mutants this represented a loss of diversity of  $\sim 40\text{-}50\%$ . Good coverage of coding sequences (CDSs) was maintained as the output libraries still  
184 contained insertions in (on average) 68-70% of the open reading frames.

186 Comparison of the read counts between the input and output libraries allowed a  
187 measurement of the impact of the insertion on the survival of mutants in cattle. The results  
188 are represented as a mean  $\log_2$  fold-change in the output compared with the input for each  
189 gene. The entire dataset is shown in supplementary Table S4 and a volcano plot from the  
190 lungs and thoracic lymph node of two representative animals is shown in Supplementary  
191 Figure S3. Comparison of the mean  $\log_2$  fold-change between lung and lymph node samples  
192 showed good correlation (Spearman's rho = 0.88, p-value <2.2e-16) (Supplementary Figure  
193 S4). TRANSIT resampling was performed to compare the composition of the mutant

194 population in the lungs and thoracic lymph nodes of paired cattle, it was also applied to  
195 compare all the thoracic lymph nodes with the lungs of all cattle samples. No statistically  
196 significant differences were observed indicating that there were no differences in mutant  
197 composition between the tissue sites.

198 No insertion mutants were significantly over-represented in the output library in any of the  
199 animals. Although interestingly, insertions in *MB0025*, a gene that is unique to *M. bovis*  
200 appeared to improve growth in cattle as mean  $\log_2$  fold-changes of +3.9 (lungs) and +4.2  
201 (lymph nodes) were observed; however, significance criteria were not met in any of the  
202 animals. In order to define a list of attenuating mutations, we used a similar approach to that  
203 used in a previous study with an *M. bovis* BCG library in cattle (16). Insertions in genes were  
204 defined as attenuating if they had  $\log_2$  fold-change of -1.5 or below and an adjusted p-value  
205 of <0.05 in at least half of the animals (Table S4, significant in 50% of cattle tab). When using  
206 these criteria, there were 141 genes where insertions caused significant attenuation in the  
207 lungs or the thoracic lymph nodes, 20 genes that reached significance only in the lungs (shown  
208 in red) and 16 genes that reached significance only in the thoracic lymph nodes (shown in  
209 green). Of the 141 genes, 109 had been previously described as being required *in vivo* in *M.*  
210 *tuberculosis* H37Rv in mouse models through the use of whole genome Tn screens  
211 representing ~77% overlap with the previous literature (8, 9, 13).

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217 **Comparison with mutations known to cause attenuation in the MTBC**

218 Insertions in the RD1 encoded ESX-1 type VII secretion system secreting virulence factors and  
219 immunodominant antigens *EsxA* (CFP-10) and *EsxB* (ESAT-6) are expected to cause  
220 attenuation (20). The impacts of insertions in this region are summarised in Figure 3 but are  
221 also available in Supplementary Table S4 (RD regions tab) and Supplementary Figure S5.  
222 Insertions in genes encoding the structural components of the apparatus (*eccB1*, *eccCa1*,  
223 *eccCb1*, *eccD1*) were severely attenuating ( $\log_2$  fold-change -6 to -9). Insertions in *eccA1*,  
224 which also codes for a structural component of the apparatus, were less impactful ( $\log_2$  fold-  
225 changes of -2 to -3) despite good insertion saturation in this gene. This is supported by the  
226 work of others who have shown that deletion of *eccA1* in *Mycobacterium marinum* leads to  
227 only a partial secretion defect (21). There were no impacts seen due to insertions in accessory  
228 genes *espJ*, *espK* and *espH*. The lack of attenuation seen in *espK* mutants is supported by other  
229 studies showing that this gene is dispensable for secretion through the apparatus and is not  
230 required for virulence of *M. bovis* in guinea pigs (22, 23). Insertions in *esxA* and *esxB* resulted  
231 in severe attenuation ( $\log_2$  fold-change of -6) but this did not reach significance cut-offs (adj.  
232  $p < 0.05$ ) in any of the cattle. This is likely to be due to the small number of TAs in these genes  
233 which makes it challenging to measure mutant frequency.

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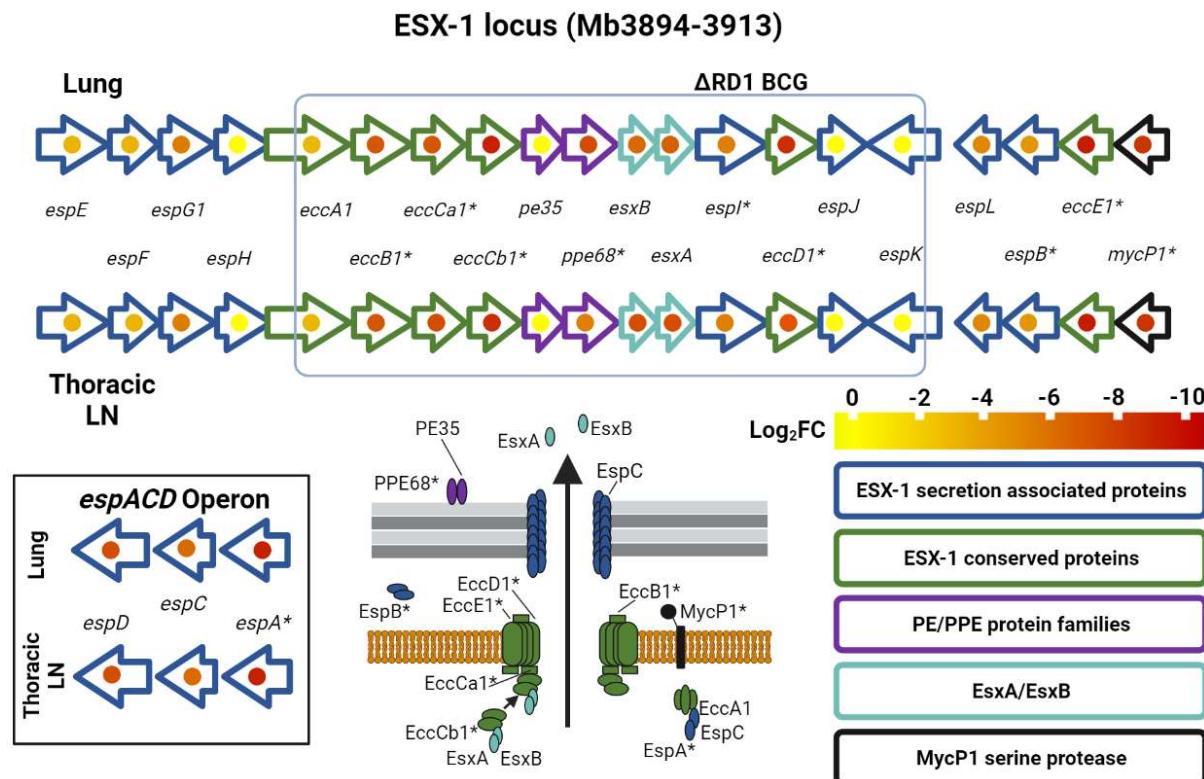
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244 **Figure 3. Fold-changes caused by transposon insertions in the ESX-1 secretion system in the**  
245 **lungs and lymph nodes of infected cattle.** Asterisks indicate that genes had an adjusted p-  
246 **value of <0.05 in at least half of the animals. The genes are grouped according to function as**  
247 **indicated by the colour scheme. The log<sub>2</sub> fold-change are indicated on a yellow to red scale**  
248 **and present as a dot in the centre of the gene.**

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253 The highest levels of attenuation seen were in genes involved in the synthesis of the cell wall  
254 virulence lipids PDIMs (*ppsABCDE* and *mas* with  $\log_2$  fold-changes of ~-10 commonly seen).  
255 PDIM synthesis is well known to be required for the survival of *M. tuberculosis* and *M. bovis*  
256 in mice and guinea pigs (24, 25). Insertions in genes involved in the synthesis of PDIMs were  
257 the most under-represented ( $\log_2$  fold-changes of -8 to -10) in the output library (Figure 4,  
258 Supplementary Table S4, mycolipids tab). MmpL7 is involved in PDIM transport and there is  
259 evidence that it is phosphorylated by the serine-threonine kinase PknD (26). PknD-MmpL7  
260 interactions are thought to be perturbed in *M. bovis* as *pknD* is split into two coding sequences  
261 in the bovine pathogen by a frameshift mutation (27). The data presented here suggest that  
262 MmpL7 still functions despite the frameshift mutation.

263 Iron restriction is thought to be a mechanism by which the host responds to mycobacterial  
264 infection, although different cellular compartments may be more restrictive than others (28).  
265 Insertion in many of the genes involved in mycobactin synthesis (*Mb2406-Mb2398*, *mbtJ*-  
266 *mbtH*) were attenuating in cattle (Figure 4, Supplementary Table S4, mycobactin synthesis  
267 tab). As mycobactin is required for the acquisition of iron, this confirms that, like other  
268 members of the MTBC, needs to scavenge iron from the host for survival (13, 16).

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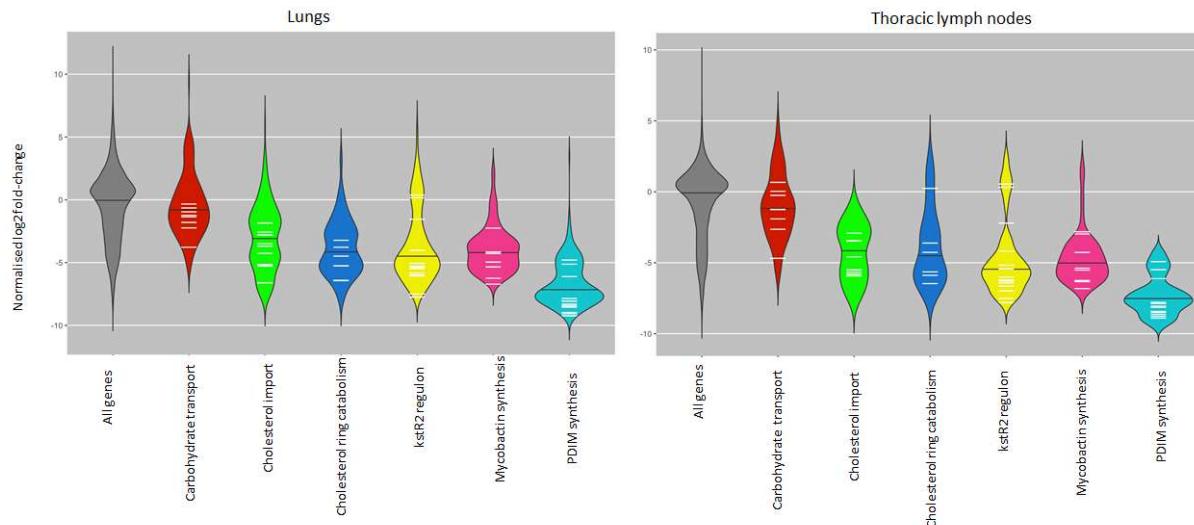
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281 **Figure 4. Violin plot of normalised  $\log_2$  fold changes in gene insertions recovered from**  
282 **bovine lung or thoracic lymph node tissue samples in selected gene groups.** Black bars  
283 indicate overall median of normalized  $\log_2$  fold-change among genes in grouping. White bars  
284 indicate mean  $\log_2$  fold-change for each gene in the group across all samples in either lung or  
285 lymph node tissue

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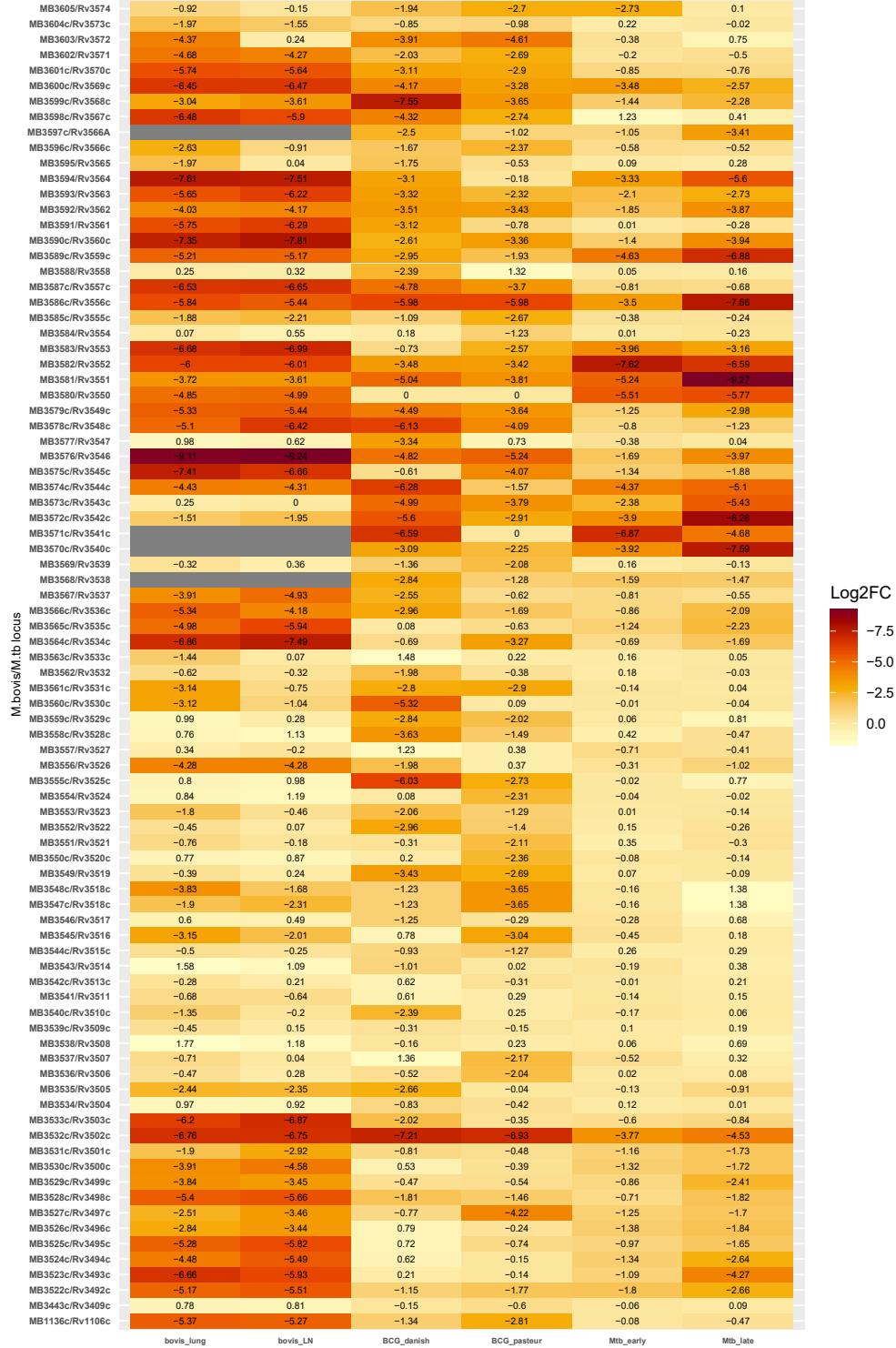
287 The role of the cholesterol catabolism in *M. tuberculosis* is well documented and it is required  
288 for both energy generation and manipulation of the immune response (29–31). Cholesterol  
289 uptake is mediated by the Mce4 transporter coded by the *mce4* operon *Rv3492c-Rv3501c*  
290 (*Mb3522c-MB3531c*) (32, 33). It has been suggested that an alternative cholesterol  
291 acquisition pathway operates in *M. bovis* BCG Danish as, unlike insertions in genes in the  
292 down-stream catabolic pathway, insertions in the *mce4* operon do not result in attenuation  
293 in this strain (16). In contrast, our study shows that cholesterol transport via the Mce4  
294 transporter is required in *M. bovis* (Figure 4, Supplementary Table S4 -cholesterol catabolism  
295 tab, Figure 5). This corroborates work performed in *M. tuberculosis*, where Mce4 has been  
296 shown to be required for growth in chronically infected mice (9, 32). Propionyl-coA generated  
297 from the catabolism of cholesterol is toxic and detoxification mechanisms include  
298 incorporation into PDIMs (34, 35). The observation that BCG Danish contains a lower amount  
299 of PDIMs compared to BCG Pasteur (16) suggests a correlation between Mce4 mediated  
300 cholesterol transport and PDIM synthesis and previous studies have demonstrated an  
301 increase in PDIM biosynthesis as a result of *mce4* over-expression (36). PDIMs biosynthesis  
302 genes are over-expressed in *M. bovis* compared to *M. tuberculosis* (27) and comparison of  
303 our dataset with Tn-seq studies performed in *M. tuberculosis* (9) indicates an over-reliance of  
304 *M. bovis* on cholesterol transport through the Mce4 transporter (Figure 5).

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310 **Figure 5. Comparison of reported  $\log_2$  fold-change in *M. bovis*, *M. bovis* BCG and Mtb**  
311 **transposon insertion sequencing experiments for orthologous genes in the cholesterol**  
312 **catabolic pathway.** Greatest attenuation (most negative  $\log_2$  fold-change) is coloured by  
313 darkest red. Studies used for comparison include Mendum et al., (24) and Bellarose et al., (9).  
314 Grey bars represent genes for which there is no information as they were either ES or GD in  
315 input library or had less than 5 insertions in any TA site in any sample (input and all output).

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317 Early stages of cholesterol catabolism involve the oxidation of cholesterol to cholestenone, a  
318 reaction catalysed by the  $3\beta$ -hydroxysteroid dehydrogenase (*hsd*) encoded by  
319 *Rv1106c/Mb1136c* (37). The cytochrome P450 Cyp125 (*Mb3575c/Rv3545c*) is required for the  
320 subsequent detoxification of cholestenone (38). Insertions in both *hsd* and *cyp125* in *M. bovis*  
321 were severely attenuating with  $\log_2$  fold-changes of ~-5 to -7 (Supplementary Table S4 -  
322 cholesterol catabolism tab, Figure 5). Previous studies have shown that these genes are not  
323 required for the survival of *M. tuberculosis* in macrophages or in guinea pigs and this is  
324 thought to be due to the availability of other carbon sources, including glycolytic substrates,  
325 *in vivo* (37, 39–43). *M. bovis* is more restricted in metabolic capabilities and is unable to  
326 generate energy from glycolytic intermediates, largely due to a disrupted pyruvate kinase  
327 encoded by *pykA* (44, 45). The essentiality of *hsd* and *cyp125* during infection for *M. bovis* but  
328 not *M. tuberculosis* supports the hypothesis of an over-reliance of *M. bovis* on cholesterol.  
329 Given the potential for the use of host cholesterol metabolites as diagnostic biomarkers, this  
330 observation might have applications in the development of differential diagnostics (46).

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332 **Genes that are differentially expressed between *Mycobacterium bovis* and *Mycobacterium***  
333 ***tuberculosis*.**

334 Several studies have identified key expression differences between *M. bovis* and *M.*  
335 *tuberculosis* (27, 47, 48). We examined the dataset for insights on the role of differentially  
336 expressed genes and transcriptional regulators during infection. One important regulatory  
337 system in *M. tuberculosis* is the two-component regulatory system PhoPR and deletions in  
338 the *phoPR* genes alongside *fadD26* are attenuating mutations in the live vaccine MTBVAC (49–  
339 51). Our data show that insertions in both *phoPR* and *fadD26* were severely attenuating with  
340  $\log_2$  fold-changes of -6 to -9 (Figure 6, Supplementary Table S4, *phoPR* regulon tab and

341 mycolipids tab). This reinforces the role of this system in virulence, despite the presence of a  
342 single nucleotide polymorphism (SNP) in the sensor kinase *phoR* that impacts signalling  
343 through the system in *M. bovis* (52). Signal potentiation via *phoR* is required for secretion of  
344 ESAT-6 through the ESX-1 secretory system and *M. bovis* is known to have compensatory  
345 mutations elsewhere in the genome, e.g. in the *espACD* operon, that restores ESAT-6  
346 secretion in the face of a deficient signalling system (49, 52, 53). Our data also show that Tn  
347 insertions in *espA* of the *espACD* operon (required for ESAT-6 secretion) and in *mprA*, a  
348 transcriptional regulator of that operon (54) were severely attenuating (log<sub>2</sub> fold-changes -7  
349 to -9), emphasising the relevance of ESAT-6 as a virulence factor.

350 Studies comparing differences in expression during *in vitro* growth between *M. bovis* and *M.*  
351 *tuberculosis* show that genes involved in sulfolipid (SL-1) biosynthesis are expressed at lower  
352 levels in *M. bovis* compared to *M. tuberculosis* (27, 47). Interestingly, insertions in genes  
353 involved in SL-1 biosynthesis (*Mb3850-Mb3856*) are not attenuating *in vivo* (Supplementary  
354 File S4, mycolipids tab), reinforcing the lack of importance of SL-1 for *M. bovis* *in vivo*, at least  
355 at the stages of infection studied here.

356 One of the most highly attenuating insertions occurred in *Mb0222/Rv0216* (log<sub>2</sub> fold change  
357 -8 to -9). This gene has been shown to be highly (> 10-fold) over-expressed in *M. bovis*  
358 compared with *M. tuberculosis* but the physiological function of this gene is not currently  
359 known. The secreted antigens MPB70 and MPB83, encoded by *Mb2900* and *Mb2898* are also  
360 over-expressed in *M. bovis* and play a role in host-specific immune responses, however,  
361 insertions in these genes did not cause attenuation *in vivo* in our dataset (55).

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379 **Figure 6. Fold-changes caused by transposon insertions in *phoP*, *phoR* and *fadD26* in the**

380 **lungs and lymph nodes of infected cattle.** Samples with adjusted p-values (BH-fdr corrected)

381  $< 0.05$  are indicated with purple points.

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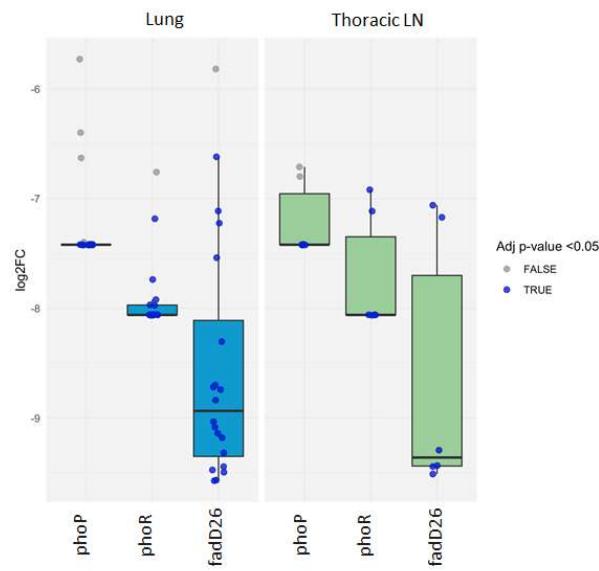
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390 **Novel attenuating mutations**

391 We identified 32 genes that were required for survival of *M. bovis* in cattle that had not been  
392 previously described as being essential *in vivo* through transposon mutagenesis screens of *M.*  
393 *tuberculosis* in mouse models (8, 9, 13) (see Supplementary Table 4, Significant in 50% of  
394 cattle tab). While writing this publication, a large scale Tn-seq study that utilised over 120 *M.*  
395 *tuberculosis* libraries and several diverse mouse genotypes (the collaborative cross mouse  
396 panel (56)) showed that the panel of genes required for the survival of *M. tuberculosis* *in vivo*  
397 is much larger than previously reported (57). A direct comparison of our dataset with the  
398 study by Smith et al., revealed that a further 13 genes were shown to be required in at least  
399 one mouse strain in that study. A summary set of the remaining 19 genes is given in  
400 Supplementary Table 4, Not in Mtb Tn-seqs tab. Some of these genes have been shown to be  
401 attenuated in the mouse model in *M. tuberculosis* through the use of single mutants (58–61).

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403 Included in this list are genes required for phenolic glycolipid synthesis (Figure 7). Insertions  
404 in *Mb2971c/Rv2947c* (*pks15/1*) and in *Mb2972c/Rv2948c* (*fadD22*) were attenuating in *M.*  
405 *bovis* but these genes are not required *in vivo* in *M. tuberculosis*, including in the extended  
406 panel of mouse genotypes (8, 9, 13, 57). Both *pks15/1* and *fadD22* are involved in the early  
407 stages of synthesis of phenolic glycolipids (PGLs) and are involved in virulence (62). The  
408 requirement for these genes in *M. bovis* but not in *M. tuberculosis* is consistent with the  
409 observation that the Tn-seq studies in *M. tuberculosis* are often carried out using lineage 4  
410 strains (H37Rv and CDC1551) that harbour a frameshift mutation in the *pks15/1* gene, which  
411 renders them unable to synthesise PGLs. This removes the requirement for these genes *in*

412 *vivo* in lineage 4 strains of *M. tuberculosis*. *pks15/1* has been previously reported to be  
413 required for survival of *M. bovis* in a guinea pig model of infection (63).

414

415 The list also includes genes that are involved in post-translational modifications such as  
416 glycosylation. *Rv1002c* is thought to add mannose groups to secreted proteins and over-  
417 expression of this protein in *M. smegmatis* was recently shown to enhance survival *in vivo*  
418 and inhibit pro-inflammatory cytokine production (64). The substrates of this protein  
419 mannosyltransferase are thought to be several secreted lipoproteins, including *LpqW* which  
420 is involved in the insertion of the virulence lipid LAM at the mycobacterial cell surface (64,  
421 65).

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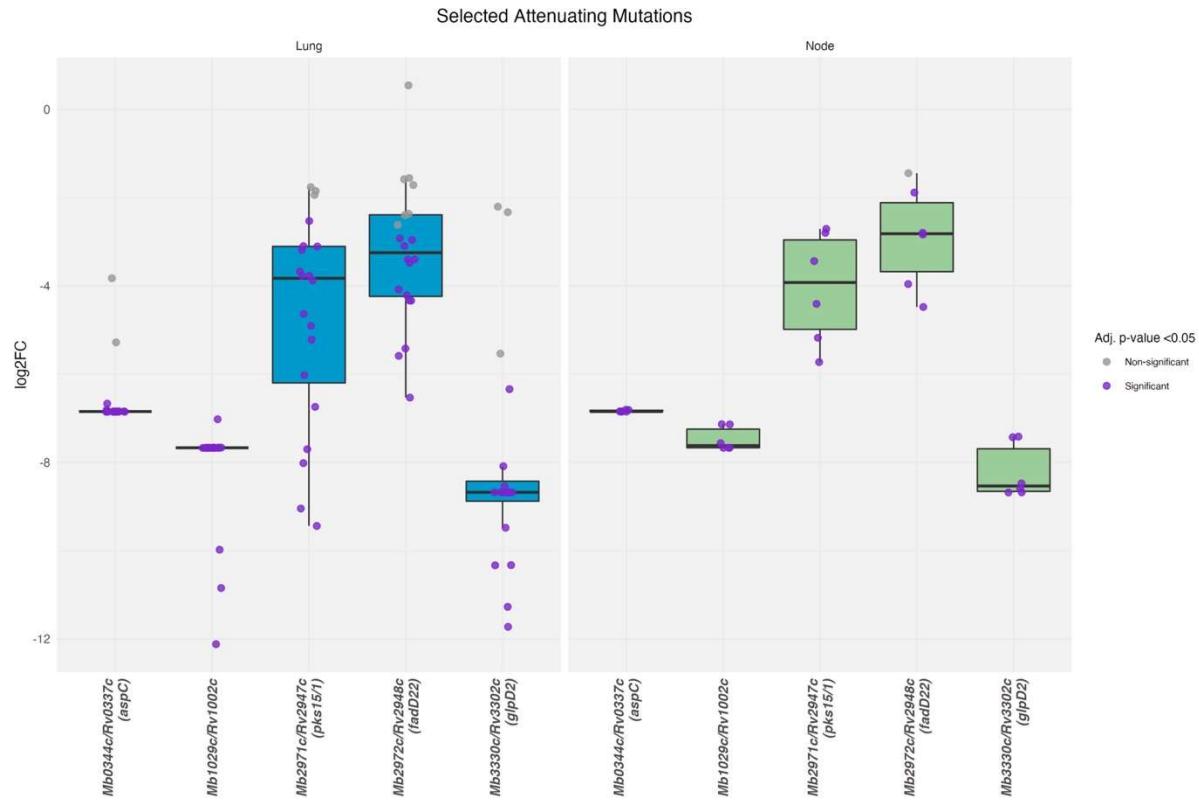
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437 **Figure 7. Fold-changes caused by transposon insertions in *pks15/1*, *fadD22*, *Rv1002c*, *aspc* and *glpD2* in the lungs and lymph nodes of infected cattle.** Samples with adjusted p-values (BH-fdr corrected) < 0.05 are indicated with purple points.

438

439

440 Finally, this list includes two genes (*aspC* and *glpD2*) that are essential *in vitro* in *M.*  
441 *tuberculosis* but not in *M. bovis* (10, 11, 18, 66). Information regarding *aspC* and *glpD2* from  
442 Tn-seq approaches is likely to be lacking in *M. tuberculosis* because Tn mutants will not be  
443 represented in the input pool. The absence of insertion mutants in these genes in the most  
444 recent large-scale *M. tuberculosis* Tn-seq study supports this (57). One of these genes  
445 *MB0344c/Rv0337c* (*aspC*) is an aspartate aminotransferase involved in the utilisation of  
446 amino acids (aspartate) as a nitrogen source (67). The other gene *Mb3303c/Rv3302c* (*glpD2*)  
447 is a membrane bound glycerol-phosphate dehydrogenase. In *Escherichia coli*, *glpD2* is an  
448 essential enzyme, functioning at the central junction of respiration, glycolysis, and  
449 phospholipid biosynthesis and catalyses the oxidation of dihydroxyacetone phosphate  
450 (DHAP) from glycerol-3-phosphate resulting in the donation of electrons to the electron  
451 transport chain (68). Its essentiality *in vitro* in *M. tuberculosis* might be explained by the usage  
452 of glycerol during *in vitro* growth in this species. The contribution of the membrane bound  
453 *glpD2* in donation of electrons to the electron transport chain, has been suggested but not  
454 yet explored in the MTBC (69). Given the interest in the electron transport chain as a  
455 chemotherapeutic target in *M. tuberculosis*, the data presented here suggests that inhibition  
456 of *glpD2* might be a fruitful approach in the development of new drugs for the treatment of  
457 TB in humans (70). The role of this gene in *M. bovis* *in vivo* is perhaps surprising, given the  
458 disruptions in glycerol phosphate uptake and pathways that phosphorylate glycerol in *M.*  
459 *bovis* AF2122/97 (71). However, *M. tuberculosis* is thought to engage in catabolism of  
460 membrane derived glycerophospholipids which may be a potential source of glycerol-3-  
461 phosphate in members of the complex (72).

462

463 **Materials and Methods**

464 **Bacterial strains and culture methods**

465 *M. bovis* strain AF2122/97 was maintained on modified Middlebrook 7H11 (BD Difco<sup>TM</sup>)  
466 medium (73). Liquid cultures of *M. bovis* were grown in Middlebrook 7H9 media (BD Difco<sup>TM</sup>)  
467 containing 75 mM sodium pyruvate, 0.05% v/v Tween<sup>®</sup>80 and 10% Middlebrook albumin-  
468 dextrose-catalase (ADC) (BBL BD Biosciences). Kanamycin at 25 µg/ml was used for selection  
469 where appropriate.

470

471 **Generation of input transposon mutant library and preparation of the inoculum**

472 Transposon libraries in *M. bovis* were generated as previously described using the  
473 MycomarT7 phagemid system as per Majumdar et al with modifications (19). Approximately  
474 66,000 kanamycin resistant transductants were scraped and homogenised in 7H9 medium  
475 and stored frozen at -80°C in 1 ml aliquots. CFU counting was performed on the homogenised  
476 culture to inform inoculum dosage.

477

478 **Cattle Infection**

479 Experiments were carried out according to the UK Animal (Scientific Procedures) Act 1986  
480 under project license PPL70/7737. Ethical permission was obtained from the APHA Animal  
481 Welfare Ethical Review Body (AWERB) (UK Home Office PCD number 70/6905). All animal  
482 infections were carried out within the APHA large animal biocontainment level 3 facility.  
483 Twenty-four Holstein-Friesian crosses of 6 months of age were sourced from an officially TB-  
484 free herd. An infectious dose of  $7 \times 10^4$  CFU was targeted for the “input” library, allowing

485 each mutant to be represented in the library ~ 2.5-fold. Retrospective counting of the  
486 inoculum revealed the actual inoculum for infection contained  $4 \times 10^4$  CFU. The inoculum was  
487 delivered endobronchially in 2 ml of 7H9 medium. In brief, animals were sedated with xylazine  
488 (Rompun® 2%, Bayer, France) according to the manufacturer's instructions (0.2 mL/100 kg, IV  
489 route) prior to the insertion of an endoscope through the nasal cavity into the trachea for  
490 delivery of the inoculum through a 1.8 mm internal diameter cannula (Veterinary Endoscopy  
491 Services, U.K.) above the bronchial opening to the cardiac lobe and the main bifurcation  
492 between left and right lobes.

493

494 **Infection Monitoring with the IFN- $\gamma$  release Assay (IGRA)**

495 Blood was collected by jugular venepuncture from animals on the day of the infectious  
496 challenge and two weeks after infection. Heparinized whole blood (250  $\mu$ l) was incubated  
497 with purified protein derivative (PPD) from *M. avium* (PPD-A) or PPD from *M. bovis* (PPD-B)  
498 (Prionics™) respectively at 25 IU and 30 IU final. Pokeweed mitogen was used as the positive  
499 control at 10  $\mu$ g/mL and a medium-only negative control. After 24 h incubation in 5% (v/v)  
500  $\text{CO}_2$ , 95% humidity, 37 °C atmosphere bloods were centrifuged (400  $\times g$  for 5 min); 120  $\mu$ l of  
501 supernatant was removed and stored at -80 °C for subsequent IFN- $\gamma$  quantification using the  
502 BOVIGAM® kit (Prionics™) in accordance with the manufacturer's instructions.

503

504 **Collection of tissues and gross pathology scores**

505 Six weeks after the initial infection animals were subjected to post-mortem examination.  
506 Initially the experiment was designed with two time points; an early time point (6 weeks) and

507 a later time point of 8 weeks. However, due to the unexpected high-levels of pathology seen  
508 at the earlier time-points all animals were culled at 6 weeks. Gross pathology and evidence of  
509 TB-like granulomas lesions was scored using a modified methodology to that previously  
510 described in (74). Tissue from head and neck lymph nodes (from the right and left sub-  
511 mandibular lymph nodes, the right and left medial retropharyngeal lymph nodes), thoracic  
512 lymph nodes (the right and left bronchial lymph nodes, the cranial tracheobronchial lymph  
513 nodes, the cranial and caudal mediastinal lymph nodes) and from lung lesions, was collected  
514 into sterile containers and frozen at -80 °C until further processing. Frozen tissues were  
515 thawed and homogenised in PBS using a Seward Stomacher Paddle Blender.

516

517 **Recovery of the output transposon mutant library from tissues**

518 Tissue macerates collected from study animals were thawed at room temperature, diluted in  
519 PBS and plated on modified 7H11 agar to determine bacterial loads. Colony counts were  
520 performed after 3-4 weeks growth. For recovery of the library from tissue macerates  $\sim 10^5$ -  
521  $10^6$  CFU were plated from lung lesions and thoracic lymph node lesions onto modified 7H11  
522 media containing 25 µg/ml kanamycin. The colonies were plated over several 140 mm petri  
523 dishes to minimise competition between mutants. The colonies were harvested after 4-6  
524 weeks growth and genomic DNA extracted.

525

526 **Genomic DNA extraction**

527 Genomic DNA from the input and recovered libraries was isolated by an extended bead  
528 beating procedure with detergent-based lysis, phenol-chloroform DNA extraction and

529 precipitation as previously described (18). DNA quality was assessed by nano-spectrometry  
530 (DeNovix) and gel electrophoresis and quantified by Qubit analysis using the Broad Range  
531 Assay Kit (ThermoScientific).

532

533 **Library preparation for transposon directed insertion sequencing**

534 DNA (2 µg) was resuspended in 50 µL distilled water and sheared to approximately 550 bp  
535 fragments using a S220 focussed-ultrasonicator (Covaris), according to the manufacturer's  
536 protocol. Fragmented DNA was repaired using NEBNext blunt-end repair kit (New England  
537 Biolabs) and purified using Monarch PCR clean-up kit (NEB). Blunted DNA was A-tailed using  
538 NEBNext dA-tailing kit (NEB) and column purified. Custom transposon sequencing adaptors  
539 (Supplementary Table S3) were generated by heating an equimolar mix of Com\_AdaptorPt1  
540 primer and Com\_AdaptorPt2 (P7+index) primers to 95°C for 5 min, followed by cooling by 1°C  
541 every 40 s to a final temperature of 4°C in a thermocycler. Adaptors were ligated to A-tailed  
542 library fragments using NEBNext quick ligase kit. Transposon-containing fragments were  
543 enriched by PCR using ComP7 primer (10 µM) and an equimolar mix of primers P5-IR2a-d  
544 primer (10 µM) in a reaction with 50 ng of adaptor ligated template and Phusion DNA  
545 polymerase (NEB) in a thermocycler with the following program 98°C 3 min; 4 cycles of 98°C  
546 20s, 70°C 20s, 72°C 1 min; 20 cycles of 98°C 20s, 67°C 20s, 72°C 1 min; 72°C 3 min. Transposon-  
547 enriched libraries were subsequently purified with AMPureXP beads (Beckman), pooled  
548 together and further purified using AMPure XP beads.

549

550

551 **Data analysis**

552 Indexed libraries were combined, spiked with 20% PhiX, and sequenced on the Illumina Hiseq  
553 3000 platform, using v2 chemistry, generating single-end reads of 250 bp. Raw .fastq  
554 sequencing files were analysed for quality and pre-processed using the TRANSIT TPP tool (75)  
555 set to default 'Sassetti' protocol, in order to remove transposon tags and adapter sequences,  
556 and to map reads using BWA-mem to TA sites to the *M. bovis* AF2122/97 genome  
557 (NC\_002945.3). The TRANSIT 'tnseq\_stats' tool was run on each sample to assess insertion  
558 density, skew, kurtosis and potential amplification bias.

559 The *M. bovis* AF2122/97 genome was scanned for the non-permissive Himar1 transposon  
560 insertion motif ('SGNTANCS', where S is either G or C and N is any base) as previously  
561 described [10]. 6605 sites were identified as non-permissive (approximately 9% of total TA  
562 sites) and excluded from resampling analysis. A custom annotation, '.prot-table' for TRANSIT,  
563 was created from the *M. bovis* AF2122/97 annotation file (NCBI Accession Number LT708304,  
564 version LT708304.1). TRANSIT HMM was run on the input library using the default  
565 normalisation (TTR) with LOESS correction for genomic position bias. Each TA site was  
566 assigned an essentiality state and genes were assigned an essentiality call based on the  
567 assigned state of the TA sites within annotated gene boundaries.

568 Resampling between the input library and each of the output sample libraries was performed  
569 independently using the TRANSIT resampling algorithm and the complete prot-table. TTR  
570 normalisation was used for 23 of the samples, and betageom normalisation for the three  
571 samples with skew of greater than 50. The initial resampling output files were evaluated to  
572 identify genes with very few, or no, reads at any TA site within the gene boundaries in both  
573 the input library and output sample libraries. Genes with no read counts greater than 4 at any

574 TA site, in any sample, and with a sum of all reads at any TA site across the 26 samples less  
575 than 55, were flagged. Essential and unchanged genes were removed from the prot-table  
576 prior to further evaluation. Resampling was further limited to protein-coding genes.  
577 Resampling was re-run for each sample using the attenuated prot-table and an edited  
578 TRANSIT resampling script to return the left-tail p-value, as the data were expected to reflect  
579 attenuation. All p-values were corrected for multiple testing with FDR adjustment.  
  
580 All analysis and plots were performed using R and R packages, tidyverse and circlize (76–78).  
581 Orthologous TB genes were obtained from supplementary data files published by Malone et  
582 al, 2018 (27). All scripts, prot-tables and insertion files are available at  
583 [https://github.com/jenjane118/Mbovis\\_in-vivo\\_Tnseq](https://github.com/jenjane118/Mbovis_in-vivo_Tnseq), DOI:10.5281/zenodo.6354151.  
584 Sequencing files (.fastq) are deposited in SRA (Bioproject ID: PRJNA816175, Submission ID:  
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586

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597 SK, DW, BW, BV-R and SB undertook funding acquisition and designed the study. AJG, VF, JM,  
598 SW, IP, MC carried out the experimental work. Data analysis was done by IN and JS. AJG, JS  
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600 revision, read, and approved the submitted version.

601

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886

887 **Figure Legends**

888 **Figure 1. bTB specific IFN-gamma release in cattle infected with the *M. bovis* Tn-library.**

889 Blood was collected from all 24 animals on the day of infection and 2 weeks later. No response  
890 was detected to either PPD-A or PPD-B antigen stimulation prior to infection (Figure 1A and  
891 Figure 1B, week 0). All animals presented a significant and specific response to PPD-B  
892 compared to PPD-A as determined by a paired T-test using GraphPad Prism (Figure 1C). \*\*\*

893  $p \leq 0.001$

894

895 **Figure 2. Tissue pathology and bacterial load in tissue sites. Six weeks after infection**

896 **animals were subjected to post-mortem examination.** Gross pathology and evidence of TB-  
897 like granulomas lesions were scored. Data presented is the mean across animals of the total  
898 scores for each tissue group from 24 animals +/- the standard deviation. Lung and thoracic  
899 lymph nodes were observed to contain the highest pathology compared to head and neck  
900 lymph nodes (Figure 2A). For bacterial load estimation, aliquots of macerates were plated  
901 onto modified 7H11 agar containing kanamycin. Colonies were counted after 3-4 weeks  
902 growth. Data are presented as mean CFU/ml per collected tissue group +/- standard  
903 deviation. Lung tissue contained the highest bacterial burden compared to thoracic and head  
904 and neck lymph nodes as determined by one-way ANOVA analysis using GraphPad Prism  
905 (Figure 2b). \*\*\*  $p \leq 0.001$ , \*\*  $p = 0.002$ , \* $p=0.01$

906

907 **Figure 3. Fold-changes caused by transposon insertions in the ESX-1 secretion system in the**

908 **lungs and lymph nodes of infected cattle.** Asterisks indicate that genes had an adjusted p-

909 value of <0.05 in at least half of the animals. The genes are grouped according to function as  
910 indicated by the colour scheme. The  $\log_2$  fold-change are indicated on a yellow to red scale  
911 and present as a dot in the centre of the gene.

912

913 **Figure 4. Violin plot of normalised  $\log_2$  fold changes in gene insertions recovered from**  
914 **bovine lung or thoracic lymph node tissue samples in selected gene groups.** Black bars  
915 indicate overall median of normalized  $\log_2$  fold-change among genes in grouping. White bars  
916 indicate mean  $\log_2$  fold-change for each gene in the group across all samples in either lung or  
917 lymph node tissue

918

919 **Figure 5. Comparison of reported  $\log_2$  fold-change in *M. bovis*, *M. bovis* BCG and *Mtb***  
920 **transposon insertion sequencing experiments for orthologous genes in the cholesterol**  
921 **catabolic pathway.** Greatest attenuation (most negative  $\log_2$  fold-change) is coloured by  
922 darkest red. Studies used for comparison include Mendum et al., (24) and Bellarose et al., (9).  
923 Grey bars represent genes for which there is no information as they were either ES or GD in  
924 input library or had less than 5 insertions in any TA site in any sample (input and all output).

925

926 **Figure 6. Fold-changes caused by transposon insertions in *phoP*, *phoR* and *fadD26* in the**  
927 **lungs and lymph nodes of infected cattle.** Samples with adjusted p-values (BH-fdr corrected)  
928 < 0.05 are indicated with purple points.

929