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1 **Parallel experimental evolution in mice reveals that increased stress resistance**
2 **was a key event for the emergence of persistent tuberculosis bacilli**

3

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17 **Abstract**

18 Pathogenomic evidence suggests that *Mycobacterium tuberculosis* evolved from an environmental
19 ancestor similar to *Mycobacterium canettii*, a rare human pathogen. However, the adaptations
20 responsible for the transition from an environmental mycobacterium to an obligate human pathogen
21 are poorly characterised. The ability to persist in the human host appears to be one important trait.
22 Here we set out to identify the adaptations that contribute to the evolution of persistence in *M.*
23 *tuberculosis*. By using experimental evolution of 8 *M. canettii* populations in mice, we selected
24 mutants with enhanced persistence *in vivo* when compared to parental strains, which were thus
25 phenotypically closer to *M. tuberculosis*. Genome sequencing of 140 *M. canettii* mutants and
26 complementation analysis revealed that mutations in two loci were responsible for the enhanced
27 persistence phenotypes. Most of the tested mutants were more resistant than their parental strains
28 to nitric oxide, which is an important effector of immunity against *M. tuberculosis* infection. This
29 resistance was common to modern *M. tuberculosis* strains but not to *M. canettii* strains. Our findings
30 demonstrate phenotypic convergence during the experimental evolution of *M. canettii*, which mirrors
31 natural evolution of *M. tuberculosis*. Furthermore, they indicate that the ability to withstand host-
32 induced stresses, such as nitric oxide, was key for the emergence of persistent *M. tuberculosis*.

33 Introduction

34 The success of *Mycobacterium tuberculosis* (*MTB*), the primary aetiological agent of human
35 tuberculosis (TB), is largely attributed to its ability to evade host defences and thus persist for months
36 within the lungs. Indeed, it is estimated that one quarter of the world's population has been infected
37 with this pathogen and a substantial fraction still carries viable bacilli^{1,2}. The adaptations that allowed
38 the ancestor of *MTB* to colonise humans and evolve into the persistent pathogen that now causes an
39 estimated 1.5 million deaths each year remain mostly uncharacterised^{2,3}.

40 In contrast to *MTB*, *Mycobacterium canettii* are TB bacilli of only marginal epidemiological importance.
41 These rare human isolates can cause disease clinically indistinguishable from classical TB, however
42 epidemiological data indicate that they are not fully adapted for colonisation of the human host⁴⁻⁶.
43 Consistently, *M. canettii* strains are less able to persist and cause disease in animal models than *MTB*
44 strains⁷⁻⁹. During evolution, these strains diverged early from *MTB* and they are now believed to have
45 an environmental reservoir^{5,10}.

46 The current model is that *MTB* evolved by clonal expansion from an *M. canettii*-like progenitor through
47 adaptations that rendered them persistent in mammalian hosts^{9,11,12}. However, virtually all identified
48 *MTB* factors important for establishing chronic infection are also present in *M. canettii*⁹, and few genes
49 and loci are specific to *MTB*. It is therefore likely that the strict pathogenic phenotype differentiating
50 *MTB* from facultative human pathogens like *M. canettii* and other environmental mycobacteria is due
51 to modification of shared pathways. The *M. canettii* strains are therefore an outstanding resource for
52 investigating the molecular mechanisms driving enhanced persistence in tuberculosis bacilli, and for
53 gaining new insights into the evolutionary events involved in *MTB* emergence.

54 Following aerosol or intravenous (i.v.) infection of mice, *M. canettii* and *MTB* strains multiply in the
55 lungs similarly during the acute phase of infection (first 4 weeks). In the chronic phase of infection,
56 *MTB* persists and the bacterial load is comparable to that achieved during the acute phase. In sharp
57 contrast, the lung bacterial load for *M. canettii* strains declines substantially during the chronic

58 infection phase, and for STB-K, the most attenuated *M. canettii* isolate in this model, becomes
59 undetectable after 30 weeks of infection. This lower persistence is associated with less pathology⁹,
60 possibly explaining why there is no direct transmission in humans reported for *M. canettii*⁴⁻⁶.

61 Our objective for this study was to select *M. canettii* mutants with enhanced persistence *in vivo* in
62 order to identify what adaptations are associated with this phenotypic gain. We first implemented a
63 positive selection strategy based on unbiased experimental evolution of 8 *M. canettii* populations in
64 a mouse model. Then we used a combination of genome sequencing and phenotypic characterisation
65 of multiple evolved clones to identify and describe the genomic and functional adaptations associated
66 with enhanced persistence of *M. canettii*. Here we show that increased resistance to host-derived
67 stresses, and especially nitric oxide (NO), is a key adaptation required by *M. canettii* to survive for
68 several months *in vivo*.

69

70 **Results**

71 **Unbiased *in vivo* experimental evolution of *M. canettii* selects mutants with enhanced persistence**

72 It has previously been established that *M. canettii* are less persistent than *MTB* in different mouse
73 models^{8,9}. We therefore set up an experimental evolution procedure in mice to select for *M. canettii*
74 mutants with an improved ability to persist, thus resembling *MTB* strains. These mutants should
75 outcompete parental *M. canettii* strains in competitive experiments in mice.

76 Our experimental evolution strategy consisted of serial passages in BALB/c mice (**Fig. 1a**) starting from
77 two distinct *M. canettii* isolates; the STB-K and STB-D strains which are phylogenetically farthest from
78 and closest to *MTB* respectively⁹ (**Supplementary Fig. 1**). For both STB-K and STB-D, we generated four
79 evolution lineages (A, B, C and D) that evolved independently and were never mixed. We performed
80 15 cycles for STB-K and 6 cycles for STB-D. At each cycle, we evaluated the lung and spleen bacterial
81 loads at days 1, 28 and 56 post-infection, and we calculated a persistence index corresponding to the

82 log ratio of lung bacterial burden at day 56 vs day 28 (**Fig. 1b**). For both STB-K and STB-D, we found
83 that the persistence index increased throughout the cycles, suggesting that mutants with enhanced
84 abilities to persist emerged. To confirm this, we randomly collected clones from agar plates used to
85 evaluate the bacterial burden in the lungs during the experimental evolution cycles, and intranasally
86 (i.n.) infected BALB/c mice with 24 individual clones recovered after cycles 6, 9, 12 and 15 for strains
87 derived from STB-K, or cycle 6 for STB-D. We found that all the tested clones caused an increased
88 bacterial burden in the lungs and spleen relative to their parental strains (**Fig. 1c,d** and **Supplementary**
89 **Fig. 2**). This was most pronounced at later time points, indicating that mutants with enhanced
90 persistence were selected during experimental evolution of both *M. canettii* STB-K and STB-D.

91

92 **Small numbers of mutations were fixed in the populations during the experimental evolution**

93 To identify the mutations that arose in STB-K and STB-D during experimental evolution in mice, we
94 sequenced the whole genomes of 100 individual clones derived from STB-K and 40 clones derived
95 from STB-D, and searched for differences relative to their parental strains.

96 In the STB-K series, we found that mutations in two loci were fixed independently in the four evolution
97 lineages. An identical nucleotide substitution C>T at nucleotide position 1582889, corresponding to
98 the gene *BN42_21248*, was found in all clones recovered after cycle 6 (**Supplementary Table 1**). This
99 mutation changed a single amino acid P96L in the protein it encodes, which is the orthologue of
100 Rv1339, a *MTB* H37Rv protein, which was recently shown to encode a phosphodiesterase degrading
101 cyclic-AMP (cAMP)¹³ (**Fig. 2a**). In the second locus, three mutations became additionally fixed
102 independently in evolution lineages A, B and C: two single nucleotide insertion/deletions (indels) and
103 a 3073 bp deletion. The two small indels at positions 2112495 and 2113560 induce frameshifts in two
104 adjacent genes, *pe18* and *ppe26* respectively. The large deletion resulted in removal of the genes *pe18*
105 and *ppe26*, and fusion of the 5'-terminal part of *ppe25* with the 3'-terminal part of *ppe27*, probably
106 due to homologous recombination (**Fig. 2b**).

107 In the STB-D series, the analyses revealed a more complex situation. Indeed, we identified single
108 nucleotide polymorphisms (SNPs) present at relatively high frequency in the inoculum used for the
109 first cycle, suggesting three genetic profiles (**Supplementary Table 2**). Other SNPs were fixed
110 independently in these three different genetic backgrounds in orthologues of the genes *rv0200*,
111 *rv0246* and *rv2990c* in evolution lineages A, B and C respectively (**Supplementary Table 2**). No
112 mutation was clearly selected in the evolution lineage D.

113 These findings indicated parallel evolution in the different lineages derived from STB-K, whereas in
114 the STB-D background, such patterns were not visible in the framework of our experimental setting.
115 We decided to focus our studies on STB-K clones, given the limited number of mutations identified
116 and their apparent strong selective advantage. We first analysed the appearance and kinetics of
117 mutation selection in the *rv1339* orthologue and *pe18-ppe26* locus. Whole-genome sequencing (WGS)
118 of the bacterial populations from each evolution lineage and each cycle revealed that the *rv1339*
119 orthologue mutation was fixed very early, before cycle 4, in the four evolution lineages (**Fig. 2c**). In
120 contrast, mutations at the *pe18-ppe26* locus became fixed in the population later, between cycles 9
121 and 13 (**Fig. 2d**). These later mutations were only selected after fixation of the *rv1339* orthologue
122 mutation. Rapid emergence and parallel fixation of an identical SNP in the *rv1339* orthologue in the
123 four evolution lineages suggested that it may have pre-existed at low frequency in the initial STB-K
124 culture. However, deep sequencing did not detect the mutation above background variant frequency
125 (below 0.1%) in the starting STB-K inoculum (**Fig. 2c**). As our experimental evolution strategy included
126 a 14 day *in vitro* culture between each cycle, we hypothesised that the *rv1339* orthologue mutation
127 might have provided a selective advantage for growth *in vitro*. However, WGS of cultures subjected to
128 6 cycles of *in vitro* experimental evolution showed that this mutation was not enriched in the
129 population during these passages (**Fig. 2e**). Thus we concluded that the mutation in the *rv1339*
130 orthologue provides a strong *in vivo* selective advantage in the STB-K genetic background, whereas
131 mutations at the *pe18-ppe26* locus may provide an additional advantage only when *rv1339* is mutated.

132

133 **The persistence phenotypes of *rv1339* and *pe18-ppe26* STB-K mutants are not specific to the mouse**
134 **strain or route of infection**

135 To further investigate the effects of the selected mutations in the STB-K background, we chose two
136 STB-K-derived variants isolated after cycle 9: KC9A1 which only has the mutation in the *rv1339*
137 orthologue (hereafter referred to as the *rv1339* mutation), and KC9C1 which has both the *pe18-ppe26*
138 deletion and the *rv1339* mutation. To examine whether the persistence phenotypes of these strains
139 were specific to the BALB/c mouse genetic background, we infected C3HeB/FeJ mice, which are very
140 susceptible to TB infection^{14,15}, with either the parental STB-K strain, KC9A1, KC9C1, or *MTB* H37Rv.
141 The bacterial burden in the lungs and spleen of C3HeB/FeJ mice was consistent with an enhanced
142 persistence phenotype for KC9A1 and KC9C1 when compared to STB-K 77 days after i.n. infection (**Fig.**
143 **3a,b**), indicating that this phenotype was not restricted to a single mouse genetic background.
144 Furthermore, we found that this phenotype was also not specific to the route of infection: C3HeB/FeJ
145 mice infected i.v. with KC9A1 and KC9C1 also had substantially more bacteria in the lungs and spleen
146 at 76 days post-infection than had mice infected with STB-K (**Fig. 3c,d**), despite exhibiting similar
147 bacterial loads at 21 days post-infection (**Supplementary Fig. 3**). C3HeB/FeJ mice have been reported
148 to develop well-organised and necrotic granulomas in the lungs following aerosol infection with *MTB*
149 H37Rv^{16,17}. Therefore, we examined if there was a difference in granuloma formation 76 days after i.n.
150 infection with STB-K, KC9A1, KC9C1, and H37Rv. We found that H37Rv-infected mice had twice as
151 much lung inflammation as had mice infected with the *M. canettii* strains (**Fig. 3e**), and only H37Rv-
152 infected mice had mature granulomas in the lungs (**Supplementary Fig. 4**). There was, however, no
153 significant difference in lung inflammation between the three *M. canettii* strains (**Fig. 3e**). Analyses of
154 the cell types present in granulomas showed no difference between KC9A1 and STB-K, but KC9C1
155 exhibited a pattern more similar to H37Rv, i.e. higher percentages of CD3⁺ cells and neutrophils, and
156 lower percentages of B220⁺ cells than for STB-K (**Supplementary Fig. 5**). Similar concentrations of IFN-

157 γ , TNF and IL-10 were found in the organ homogenates from mice 21 and 76 days after i.v. infection
158 with the different strains (**Supplementary Fig. 6**). These results showed a convergence in the immune
159 response induced by the more persistent STB-K-derived mutant, KC9C1, and H37Rv. In contrast, the
160 persistence gain of KC9A1 was not associated with a change in inflammatory response.

161

162 **The *pe18-ppe26* and *rv1339* mutations confer enhanced persistence**

163 To demonstrate that the *pe18-ppe26* deletion is directly responsible for the difference in persistence
164 between KC9A1 and KC9C1, we complemented KC9C1 (which has both the *pe18-ppe26* deletion and
165 the *rv1339* mutation) with a plasmid containing the *ppe25-ppe27* region of STB-K (KC9C1::K), thus
166 rendering the strain genetically similar to KC9A1. We also constructed strains containing empty
167 vectors (EV), KC9A1::EV and KC9C1::EV, to control for potential effects of the plasmid on strain fitness.
168 We infected BALB/c mice i.n. with these strains and found that at 70 days post-infection, KC9C1::K
169 persisted in the lungs to the same extent as KC9A1 and KC9A1::EV (**Fig. 4a**). In contrast, mice infected
170 with KC9C1 or KC9C1::EV had approximately 10-fold more bacteria in the lungs (**Fig. 4a**). This trend
171 was also reflected in the spleen (**Fig. 4b**). These data indicate that the *pe18-ppe26* deletion is
172 responsible for the enhanced persistence phenotype of KC9C1 relative to KC9A1.

173 To extend these findings, we examined if the *pe18-ppe26* deletion selected after experimental
174 evolution of STB-K could also confer enhanced persistence to another *M. canettii* strain. To do so, we
175 deleted the *ppe25-ppe27* region in STB-D and complemented this strain with the *ppe25-ppe27* regions
176 from either STB-D (::D), STB-K (::K), or KC9C1 (::C1) (**Supplementary Fig. 7**). BALB/c mice infected i.n.
177 with these strains had very similar levels of bacteria in the lungs 70 days post-infection
178 (**Supplementary Fig. 8**), indicating that either the *pe18-ppe26* deletion confers a persistence
179 advantage in STB-K only, or that it requires an existing mutation in *rv1339* to have a significant impact.

180 To demonstrate that the *rv1339* mutation is directly responsible for the gain of persistence in KC9A1
181 and to examine if the gain associated with this mutation is specific to STB-K, we used the STB-D strain
182 in which the *rv1339* orthologue is 100% identical in sequence to that in STB-K. First we knocked out
183 the *rv1339* orthologue of STB-D (Δ rv1339), and then we complemented this strain by introducing a
184 plasmid containing either the wild-type *rv1339* orthologue (::rv1339K), or the mutated *rv1339*
185 identified in KC9A1 (::rv1339A1) (**Supplementary Fig. 9**). BALB/c mice infected i.n. with Δ rv1339 or
186 ::rv1339A1 had substantially more bacteria in their lungs 77 days post-infection than had mice
187 infected with STB-D or ::rv1339K (**Fig. 4c**). A similar phenomenon was also seen for ::rv1339A1 in the
188 spleen (**Fig. 4d**). These data confirm that the *rv1339* mutation is responsible for the enhanced
189 persistence phenotype of KC9A1 relative to STB-K, and suggest that it is a loss-of-function mutation.
190 They also demonstrate that although the *rv1339* mutation arose during experimental evolution of
191 STB-K, it can enhance persistence of the STB-D strain and is therefore possibly a general mechanism
192 for increasing the persistence of *M. canettii* strains.

193

194 **The *rv1339* mutation confers enhanced resistance to host-derived stresses**

195 As seen above, the enhanced persistence of KC9A1 relative to STB-K does not seem to relate to a
196 profound alteration of the immune response. Due to lymphocyte recruitment and phagocyte
197 activation with IFN- γ , bacilli must withstand various stresses¹⁸⁻²¹. These include low pH, in the range
198 of pH 4.5 – 5.5^{19,22,23}, nutrient restriction²⁴⁻²⁶, metal intoxication^{24,27,28}, or physical attacks by oxygen or
199 nitrogen radicals²⁹⁻³¹ and antimicrobial peptides³²⁻³⁵. Therefore, we examined if the selected mutants
200 have an enhanced capacity to survive these various bactericidal mechanisms. Growth of STB-K, KC9A1
201 and KC9C1 at various neutral or acidic pH and in phosphate-limited media was similar for the three
202 strains (**Supplementary Fig. 10**). H37Rv was more resistant to H₂O₂-induced stress than the three *M.*
203 *canettii* strains, which resembled each other in terms of resistance (**Supplementary Fig. 10**). In
204 contrast, both KC9A1 and KC9C1 were more tolerant than STB-K to high concentrations of copper and

205 to a lesser extent, zinc, two metals identified as important effectors of nutritional immunity against
206 *MTB*^{24,27,28} (Fig. 5a, b). Furthermore, both KC9A1 and KC9C1 were significantly more resistant to the
207 NO donor DETA/NO than was STB-K (Fig. 5c).

208 To link increased resistance to stress with enhanced persistence *in vivo*, we examined the persistence
209 of STB-K and KC9A1 in NOS2^{-/-} mice which are deficient for NO production, and in bone-marrow
210 derived macrophages (BMDMs) stimulated with IFN- γ and LPS and treated with L-NAME to inhibit NO
211 production (Supplementary Fig. 11). We found that, at 56 days post-infection, KC9A1 had higher
212 bacterial loads in the lungs and spleens of NOS2^{-/-} mice than in control mice. A similar trend was
213 observed for STB-K in the spleen. Consistently in BMDMs, treatment with L-NAME substantially
214 increased the survival of both STB-K and the two mutants KC9A1 and KC9C1. Together these findings
215 demonstrate that NO is an important factor reducing STB-K and KC9A1 persistence.

216 As KC9A1 and KC9C1 had similar levels of resistance to NO and tolerance to copper and zinc *in vitro*,
217 we hypothesised that these phenotypes were associated with their common mutation in the *rv1339*
218 orthologue. To test this, we treated STB-D, D Δ *rv1339*, ::*rv1339K*, and ::*rv1339A1* with DETA/NO for 48
219 hours. We found that D Δ *rv1339* and ::*rv1339A1* were significantly more resistant to killing by NO than
220 ::*rv1339K* and STB-D (Fig. 5d), demonstrating that the *rv1339* mutation is responsible for this
221 phenotype.

222 As the first barrier to exogenous toxic compounds is the bacterial cell envelope, we examined whether
223 the *rv1339* mutation impacts its permeability. To address this question, we used the non-pathogenic
224 *Mycobacterium smegmatis* model, which carries a *rv1339* orthologue (*MSMEG_4902*). First, we
225 disrupted this gene to generate *smeg* Δ *rv1339* (Supplementary Fig. 12). Then we complemented this
226 mutant strain by introducing a plasmid containing either the wild-type *rv1339* orthologue from STB-K
227 (*smeg*::*rv1339K*) or the mutated *rv1339* from KC9A1 (*smeg*::*rv1339A1*). Next, we performed a
228 permeability assay using ethidium bromide (EtBr) as a reporter molecule and found that both the

229 smegΔrv1339 mutant and the smeg::rv1339A1 complemented strain incorporated less EtBr in
230 comparison to the wild-type and smeg::rv1339K strains (**Fig. 5e**).

231 Together, these findings indicate that the rv1339 mutation decreases the cell envelope permeability
232 and confers increased resistance to several stresses encountered by the pathogen during infection.

233

234 ***MTB* exhibit higher NO resistance than *M. canettii* and this gain is important for persistence**

235 Persistent bacteria arising from experimental evolution of STB-K had mutations in rv1339, whereas
236 the persistent bacteria that evolved from the STB-D strain did not. As tuberculosis bacilli have acquired
237 multiple mechanisms to resist host-derived stress^{18-20,36-38}, we examined the sensitivities of several
238 persistent STB-D mutants selected after cycle 6 to NO as a representative stress. Two out of four
239 persistent mutants tested, DC6C1 and DC6D1, were substantially more resistant to DETA/NO
240 treatment than STB-D, and closely resembled *MTB* H37Rv (**Fig. 6a**). This indicated that phenotypic
241 convergence occurred during experimental evolution of the genomically distant STB-K and STB-D
242 strains, and highlighted that increased resistance to NO, and possibly to other stresses, are key
243 mechanisms driving persistence *in vivo*.

244 To assess the ability of phylogenetically diverse *M. canettii* strains (**Supplementary Fig. 1**) to resist NO,
245 we exposed five strains, STB-K, -D, -A, -J and -L, to DETA/NO for 48 hours. All the strains tested were
246 significantly more sensitive to killing by NO than *MTB* H37Rv (**Fig. 6b**). We also tested the
247 susceptibilities of three other *MTB* strains from various lineages to DETA/NO and found that there was
248 no significant difference in the resistance of *MTB* strains to NO with the exception of the lineage 1
249 strain IO367, which was slightly more susceptible to killing than H37Rv only (**Fig. 6c**). These results
250 thus demonstrate that *MTB* are more resistant than *M. canettii* to stresses, such as NO and indicate
251 that increased NO resistance is associated with enhanced persistence *in vivo*.

252

253 **Discussion**

254 Parallel molecular evolution and phenotypic convergence are canonical signatures of genes and
255 processes under strong adaptive selection, such as those related to pathogen adaptation to host
256 environments³⁹⁻⁴³. Here, the identification of parallel and convergent evolution during experimental
257 evolution of ancestor-like *MTB* strains in mice highlights that a key patho-adaptation differentiating
258 modern *MTB* from their environmental ancestor is enhanced resistance to stress, especially NO. NO
259 and other reactive nitrogen intermediates are produced by macrophages upon activation by T cell-
260 derived IFN- γ , and are major effectors in the immune response to TB^{29,31,44,45}. These radicals are
261 believed to protect TB-infected hosts through direct bacterial killing^{46,47}, and inhibition of granulocytic
262 inflammation and immunopathology^{48,49}. Enhancing resistance to these compounds, and possibly
263 other toxic molecules generated during the immune response such as metal ions, appears to be
264 required to increase persistence of ancestor-like *MTB* strains in the lungs, and was probably a major
265 step in the emergence of *MTB*.

266 In this study, we found that diverse mutations acquired by *M. canettii* STB-K and STB-D through
267 experimental evolution produce this phenotype. One of these mutations was in the *rv1339*
268 orthologue, and it conferred both STB-K and STB-D strains with enhanced resistance to NO. The gene
269 *rv1339* is highly conserved in TB bacilli (**Supplementary Fig. 13**) and other mycobacteria and a recent
270 study revealed that the Rv1339 protein degrades cAMP, an important signalling molecule controlling
271 peptidoglycan metabolism¹³. Inactivation of *rv1339* or expression of the mutated *rv1339* allele from
272 KC9A1 increased stress resistance in *M. canettii* STB-D and STB-K and reduced envelope permeability
273 in *M. smegmatis*. Consistently *MTB* strains containing point mutations or transposon insertions in
274 *rv1339* have increased resistance to antitubercular compounds^{50,51} and a reduced capacity to import
275 fatty acids during macrophage infection⁵². Therefore, the likely mechanism of enhanced persistence
276 associated with the *rv1339* mutation is a decrease in envelope permeability allowing bacilli to
277 withstand toxic stresses such as NO or metals. The other mutations selected in the STB-K-derived

278 persistent clones concerned *pe18* and *ppe26* genes located within the *esx-5* locus, which encodes one
279 of the five type VII protein secretion systems of *MTB*. Interestingly, clinical *MTB* isolates successfully
280 spreading in Tunisia harbour a single *pe-ppe* gene pair in the *esx-5* locus that likely arose by
281 homologous recombination between *ppe25-ppe27* genes⁵³ as in the mutant KC9C1. Furthermore,
282 *MTB* strains from the Beijing family exhibit *esx-5* locus mutations disrupting secretion of a large
283 number of PE/PPE proteins and enhancing their virulence⁵⁴. This convergence between experimental
284 evolution of ancestor-like *MTB* strains and the natural evolution of modern TB strains suggests that
285 the loss of certain PE/PPE proteins is another method exploited by TB bacilli to better survive and
286 enhance their virulence in mammalian hosts. The precise function of PE18-PPE26 is unknown but
287 PE/PPE proteins were recently found to mediate solute-specific import across the *MTB* outer
288 membrane^{52,55}. Therefore, *pe18-ppe26* mutations in STB-K might also limit the penetration of toxic
289 molecules allowing the *M. canettii* persistent mutants to better survive the immune response.
290 However, we found no evidence of enhanced resistance to stress associated with the *pe18-ppe26*
291 mutation. Therefore, an alternative explanation might relate to the difference in immune cell
292 recruitment observed in mice infected with the KC9C1 mutant that more closely resembles the
293 situation in H37Rv-infected mice.

294 In this study, we identified a major driving force for patho-evolution of TB bacilli and illustrated that
295 the required adaptations can be acquired by exploiting diverse bacterial pathways. This supports the
296 concept that phenotypic convergence can be achieved through diverse genetic modification due to
297 historical contingency. The associated loss-of-function mutational patterns apparent in STB-K further
298 highlight the key role of gene loss in driving evolutionary adaptation⁵⁶, in this case towards enhanced
299 persistence of a major pathogen. The rapid selection of *M. canettii* mutants with similar persistence
300 characteristics to *MTB* and diversity of mutations leading to a similar gain-of-function is surprising,
301 considering the accepted model that *MTB* emerged only once from an environmental ancestor⁹. Long-
302 term association with the human host requires both the ability to persist, and to transmit to
303 uninfected people. *M. canettii* strains have never been reported to be transmitted between humans⁴.

304 Therefore, another major adaptation may be the gain of function(s) favouring aerosol transmission,
305 which remains to be identified.

306 **Materials and methods**

307 **Mycobacterial strains and culture conditions**

308 The *M. canettii*, *MTB* and *M. smegmatis* strains used in this study are listed in **Supplementary Table**
309 **3**. All strains were cultured in Middlebrook 7H9 broth (BD Difco) supplemented with 0.2% glycerol
310 (w/v) and 10% Middlebrook albumin-dextrose-catalase enrichments (ADC; BD BBL), with 0.05% Tween
311 80 (Sigma) (7H9/ADC/Tween) unless otherwise described. To grow recombinant strains, the
312 antibiotics hygromycin (Euromedex) and kanamycin (Euromedex) were added as required at final
313 concentrations of 50 µg/ml and 40 µg/ml respectively. Cultures were agitated approximately twice
314 per week and maintained for 3-4 weeks at 37°C before use. During the experimental evolution cycles,
315 the antibiotic mixture PANTA (BD BBL) was added at a dilution of 25 µl for 10 ml 7H9/ADC/Tween for
316 *in vitro* cultures. For growth on solid medium, 7H11 agar base (BD BBL) was supplemented with 0.5%
317 glycerol and 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC; BD BBL). Counting of CFUs
318 on 7H11 agar plates was performed after 2, 3 and 4 weeks of incubation at 37°C.

319

320 **Experimental evolution strategy**

321 The experimental evolution procedure consisted of independent serial passages of strain STB-K
322 (phylogenetically farthest from *MTB*) and strain STB-D (phylogenetically closest to *MTB*) in BALB/c
323 mice. For both STB-K and STB-D, four independent cultures were passaged corresponding to evolution
324 lineages A, B, C and D, and they were never mixed. At cycle 1, 12 mice were infected i.n. with 10⁴ CFU
325 of STB-K or STB-D. STB-infected mice were then euthanised after 1 and 28 days of infection (4 mice
326 per time point) and the CFU in the lungs and spleens were enumerated to evaluate the initial bacterial
327 load and the multiplication during the acute phase of the infection. The 4 remaining STB-infected mice
328 were kept until 56 days post-infection and then sacrificed to determine CFU at the chronic infection
329 stage. The bacterial load in the organs was evaluated by plating part of the lung and spleen

330 homogenates on solid medium in order to get information on the entire population. Part of the lung
331 homogenates recovered from the 4 mice 56 days post-infection was also used to initiate four separate
332 cultures (evolution lineages A, B, C and D) which were allowed to grow for 14 days at 37°C before
333 being used as the new inocula for the next cycle. Each subsequent cycle was initiated by i.n. infecting
334 12 mice with approximately 10^4 CFU of the new STB-K or STB-D inocula prepared from cultures of
335 bacterial populations obtained 56 days post-infection in the previous cycle. This process of infection
336 and CFU monitoring was repeated for 15 cycles for STB-K and 6 cycles for STB-D. At cycles 3, 6, 9, 12
337 and 15 of STB-K, 20 individual colonies were picked up, briefly cultivated and stored at -80°C
338 (corresponding to 100 clones total). 20 of these individual clones (8 selected at cycle 6 and 4 selected
339 at cycles 9, 12 and 15) were characterised individually for their persistence in animal models. At cycles
340 3 and 6 of STB-D, 20 individual colonies were similarly picked up, briefly cultivated and stored at -80°C
341 (corresponding to 40 clones total). 4 of these individual clones (selected at cycle 6) were characterised
342 for their persistence in animal models.

343

344 **Sequencing and sequence analyses**

345 We performed Illumina-based WGS on 100 individual clones derived from STB-K (recovered at cycles
346 3, 6, 9, 12 and 15) and 40 clones derived from STB-D (recovered at cycles 3 and 6). Genomic DNA from
347 each clone was extracted from stationary phase culture using either the DNeasy Blood & Tissue kit
348 (Qiagen) or the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) according to the
349 manufacturer protocols.

350 For whole genome sequence analyses, CLC Genomics Workbench, version 20.0 (QIAGEN, Aarhus) was
351 utilised for sequence mapping, using the global alignment option and ignoring reads with more than
352 one match, and variant calling, using the basic variant calling mode with a minimum frequency filter
353 set to 85%, and other parameters set to default. Mapping was performed against previously
354 assembled reference genomes of STB-K and STB-D (accession number FO203509.1 and NC_019950.1

355 respectively), and a newly generated dataset obtained by PacBio for STB-K. WGS data generated from
356 STB-K and STB-D parental strain batches, used for the first cycle of experimental evolution, were used
357 to correct for SNPs that were pre-existing in the genome assemblies (i.e. before experimental
358 evolution). Manual curation was subsequently performed to discard probable false positive variants
359 typically identified in repetitive regions (e.g. PE/PPE protein-encoding genes), including variants found
360 within a 12 bp-distance range. The false positive variant calling rate with this analysing pipeline was
361 verified by mapping sequence reads from *MTB* H37Rv genomic DNA against a previously assembled
362 reference genome obtained after PacBio sequencing of the same genomic DNA preparation, resulting
363 in absence of any detectable SNP or indel.

364 For the large deletion identified in the *pe18-ppe26* gene region of STB-K evolution lineage C, a specific
365 read mapping analysis was performed against the parental reference sequence using bowtie2 version
366 2.3.5⁵⁷. Proportions of bacterial wild-type versus mutant (i.e. carrying this deletion) subpopulations
367 over the different cycles were determined after normalisation of the coverage depth detected around
368 the deleted region, by dividing this coverage depth by the mean genome wide coverage depth
369 obtained for the cycle considered. The proportion detected in the parental strain was also set at 100%,
370 to better visualise the progressive fixation of the deletion, starting from the initial population.

371 To search for *rv1339* variants among TB bacilli isolates, we analysed a collection of 6167 genome
372 assemblies available as of January 2020 from the NCBI by BLASTN search-based screening and we
373 looked at the distribution of mutations fixed in the *rv1339* gene in isolates belonging to lineages 1–8
374 or the animal lineages of the *MTB* complex.

375

376 **Strain and plasmid construction**

377 The plasmids and primers used in this study are listed in **Supplementary Tables 4** and **5**, respectively.

378 For the construction of the *BN44_11505* (*rv1339* orthologue) mutant in *M. canettii* STB-D, a 2kb DNA
379 fragment overlapping the *BN44_11505* gene was amplified by PCR using primers 39a and 39b and
380 cloned into the plasmid pJET (ThermoFisher Scientific) to give plasmid pWM355. A 0.8kb PCR fragment
381 containing the kanamycin (*km*) gene was amplified from plasmid pET26b (Addgene) using primers km1
382 and km2 and inserted into the EcoRV site of plasmid pWM355, which is located inside the *BN44_11505*
383 gene, to generate plasmid pWM359. The substrate for allelic exchange was produced by PCR
384 amplification of a 2.8kb fragment, carrying the *BN44_11505* gene disrupted by the *km* cassette, from
385 pWM359. This DNA fragment was electroporated in a recombinant *M. canettii* STB-D expressing the
386 recombinering system⁵⁸ from plasmid pJV53H and transformants were selected on km-containing
387 plates. Ten km-resistant clones were randomly picked and analysed by PCR using primers 39c / kmF,
388 39d / kmR and 39e / 39f. A clone giving the expected PCR profile (**Supplementary Fig. 9**) was selected
389 for further experiments and named PMM305 (or DΔrv1339).

390 For the construction of the DΔppe25-ppe27 mutant (also named PMM304), three PCR fragments were
391 amplified and fused. First, a 1kb fragment upstream of *ppe25* was generated from STB-D genomic DNA
392 by PCR amplification using primers esx1 and esx2 which carry DraIII restriction site at their 5'- end. A
393 second 1kb fragment downstream of *ppe27* was produced using primers esx5 and esx6 which also
394 carry DraIII restriction sites at their 5'- end. Finally, the third fragment corresponding to the *km* gene
395 was amplified using primers k1 and k2 which add DraIII sites on both side of the *km* gene. The three
396 PCR fragments were digested using the restriction enzyme DraIII for 1h at 37°C. The three fragments
397 were then ligated and the allelic exchange substrate corresponding to the ligated PCR fragments was
398 amplified by PCR as a 2.8kb product using primers esx1 and esx6. This DNA product was transferred
399 by electroporation into STB-D:pJV53H after induction of phage recombinase using acetamide as
400 previously described⁵⁸. Transformants were selected on km-containing plates. Ten colonies were
401 randomly picked and analysed by PCR using primers esx7 / kmR, esx8 / kmF and 26a / 26b. A clone
402 giving the expected PCR profile (**Supplementary Fig. 7**) was selected for further experimentation and
403 named PMM304 (or DΔppe25-ppe27).

404 For the construction of the *MSMEG_4902* (*rv1339* orthologue) mutant in *M. smegmatis* mc²155, two
405 1kb DNA fragments located upstream and downstream of *MSMEG_4902* gene were amplified by PCR
406 using either primers 4902A and 4902B, or primers 4902C and 4902D, and then purified. The two
407 fragments were ligated before amplification using primers 4902E and 4902F to generate a 2 kb DNA
408 fragment. This fragment was cloned into the plasmid pJET. A 0.8kb PCR fragment containing the *km*
409 gene was amplified from plasmid pET26b using primers km1 and km2 and inserted into the EcoRV site
410 located at the junction of the fused 1kb fragments to generate plasmid pAH06. The substrate for allelic
411 exchange was produced by PCR amplification of a 2.8kb fragment, carrying the *MSMEG_4902* gene
412 disrupted by the *km* cassette, from pAH06. This DNA fragment was ligated into the EcoRV site of
413 plasmid pJQ200⁵⁹ to generate plasmid pAH10. This plasmid was electroporated into *M. smegmatis*
414 mc²155 and transformants were selected on km-containing plates. One transformant was used to
415 grow a culture and then plated on solid medium containing km and sucrose 5%. Ten km-resistant
416 clones were analysed by PCR using primers 4902D / kmF, 4902A / kmR, and 4902A / 4902G. One clone
417 giving the expected PCR profile (**Supplementary Fig. 12**) was selected for further experiments and
418 named PMM280 (or smeg Δ rv1339).

419 The *BN42_21248* gene from *M. canettii* STB-K (orthologue of *rv1339* in *MTB* H37Rv) or the mutated
420 allele from KC9A1, were amplified from the corresponding strains using primers 1339G and 1339H.
421 They were then inserted between the Nde1 and Spe1 sites of a derivative of the integrative plasmid
422 pMV361⁶⁰ carrying a hygromycin resistance gene (*hyg*) and the mycobacterial promoter *pBlaF*^{*61}, to
423 give the complementation plasmids pAH01H (*BN42_21248* mutated) and pAH02H (*BN42_21248* wild-
424 type). In pAH01H and pAH02H, the wild-type *BN42_21248* or the mutated variant were expressed
425 under the control of the heterologous promoter *pBlaF*^{*}. These plasmids were transferred
426 independently into the strain PMM305 by electroporation to give PMM305::pAH01H (abbreviated
427 ::rv1339A1) and PMM305::pAH02H (abbreviated ::rv1339K). Plasmid pAH01 (carrying the gene
428 *BN42_21248* mutated from KC9A1) or pAH02 (carrying the wild-type gene *BN42_21248* mutated from
429 STB-K) were also transferred independently into the strain PMM280 by electroporation to give

430 PMM280::pAH01H (abbreviated smeg::rv1339A1) and PMM280::pAH02H (abbreviated
431 smeg::rv1339K).

432 Similarly, the *ppe25-ppe27* genes from *M. canettii* STB-K or the corresponding allele from KC9C1
433 (carrying the *pe18-ppe26* deletion) were produced by PCR amplification of the corresponding genomic
434 region using primers esxA and esxB. These regions were then inserted between the XbaI/HindIII
435 restriction sites from the pMV361 derivative carrying the *hyg* gene to give the complementation
436 plasmids pWM361 (*ppe25-ppe27* region from STB-K) and pWM386 (*ppe25-ppe27* region from KC9C1).
437 The corresponding genomic regions from STB-D were amplified using primers esxC and esxD and
438 cloned in the same vector to generate pWM385 (*ppe25-ppe27* region from STB-D). These three
439 complementation plasmids, or the corresponding empty vectors (EV), were transformed into strains
440 KC9A1, KC9C1, PMM304, or STB-D.

441

442 **Animal infection studies**

443 The persistence capacity of the *M. canettii* mutants and recombinant strains was assessed in four
444 different mouse models. 6-8 week old female BALB/c mice (Janvier; 3/4 mice per group per time point)
445 were infected i.n. with approximately 10^3 CFU per strain. 6-10 week old female C3HeB/FeJ mice (bred
446 in-house in the IPBS animal facility; 4/5 mice per group per time point) were infected either i.n. with
447 approximately 200 CFU per strain or i.v. with approximately 10^3 CFU per strain. All mice were
448 anaesthetised prior to infection: BALB/c mice were injected intraperitoneally (i.p.) with a combination
449 of valium, atropine and imalgene, and C3HeB/FeJ mice were injected i.p. with a ketamine-xylazine
450 mixture for i.n. infection, or were exposed to isoflurane for i.v. infection. For experiments with NOS2⁻
451 ^{-/-} mice, 7-15 week old NOS2^{-/-} mice (gift from E. Russo and P. Bousso, Institut Pasteur) or 6-8 week
452 old C57BL/6 control mice (Janvier) were infected via aerosol with approximately 100 CFU per strain.
453 Mice were housed under specific pathogen-free conditions. Mice displaying signs of health
454 deterioration were euthanised by cervical dislocation. At the indicated time points, mice were

455 sacrificed by cervical dislocation and the lungs and spleens were isolated and homogenised using a
456 GentleMACS Dissociator (Miltenyi Biotec) and gentlemacs M tubes. Bacterial load in the organs was
457 estimated by plating serial dilutions of the lung and spleen homogenates on solid medium. For
458 inoculum preparation, 3-4 week old bacterial cultures were re-cultured for 10 days in
459 7H9/ADC/Tween. 10 day old cultures were washed twice in PBS/0.05% Tween 80 and adjusted to the
460 final desired concentration in PBS/0.05% Tween 80 for infection. Inoculum accuracy was verified by
461 plating serial dilutions on 7H11 agar plates supplemented with 10% OADC.

462

463 **Histopathology**

464 For histopathological evaluation, C3HeB/FeJ mice were sacrificed by i.p. injection of Dolethal at 11
465 weeks post-infection, and lungs were isolated and fixed in 10% neutral buffered formalin for 48 hours.
466 Following fixation, lungs were transferred into 70% ethanol and then delivered to the Experimental
467 Histopathology service in Purpan Hospital, Toulouse (Anexplo). The lungs were embedded in paraffin
468 for 5 µm sectioning and sections were stained with hematoxylin and eosin. Stained samples (one
469 entire lung section for each mouse) were analysed using Panoramic Viewer software to quantify the
470 percentage lung inflammation per sample. For immunohistochemistry analyses, the epitopes were
471 unmasked by HIER treatment for 20 minutes at 95°C, either in citrate buffer pH6 for B cell and
472 neutrophil labelling or in citrate buffer pH9 for T-cell labelling. For macrophages, regeneration was
473 performed by PIER treatment for 10 minutes at room temperature. Endogenous peroxidase and
474 endogenous biotin were blocked by treatment with blocking solutions (S2023 Agilent for peroxidase
475 and NB306 Clinisciences for biotin) for 10 minutes and 20 minutes respectively. Then the tissue
476 sections were incubated with goat serum for 30 minutes. Specific labelling was performed by
477 incubation for 1 hour with primary antibodies for macrophages (F4/80, MCA497G, Serotec; 1/100
478 dilution), B lymphocytes (B220/CD45r, MCA1258G, Serotec; 1/400 dilution) and T lymphocytes (CD3,
479 ab16669, Abcam; 1/100 dilution) as well as overnight at 4°C for neutrophils (Neutrophil marker, sc-

480 71674, Santa Cruz biotechnology; 1/200 dilution). The lung sections were then incubated for 30
481 minutes with either biotinylated goat anti-rat secondary antibody for neutrophils, macrophages and
482 B cells or goat anti-rabbit biotinylated antibody for T cells and then incubated with horseradish
483 peroxidase (R.T.U., PK-7100, Vector Laboratories) for 30 minutes. The labelling was revealed in
484 Diaminobenzidine (DAB) with a counter-stain of modified hematoxylin. Quantification of positive cells
485 for F4/80, CD3, B220 and neutrophils markers per lesion was performed using the QuPath software⁶².
486 More precisely, regions of interest were drawn for each individual lung lesion using the wand
487 annotation tool. Next, cell detections were performed within each region of interest using
488 hematoxylin staining to detect the nucleus of individual cells combined with cell expansion option to
489 segment the whole cell. Finally, the mean intensity of DAB staining of cells from infected lung sections
490 stained with biotinylated secondary antibody only was used to define DAB intensity thresholds for
491 each marker and applied to quantify the percentage of positive cells within each lesion.

492

493 **Cytokine quantification in organ homogenates**

494 The lungs and spleens of infected mice were isolated and homogenised as described above. Samples
495 were centrifuged at 1800 rpm for 10 minutes at 4°C to pellet the remaining tissue, and supernatants
496 were filtered twice using 0.2 µm Millex-GP polyethersulfone filters (Merck Millipore). The cytokine
497 concentrations in the lung and spleen supernatants were quantified using standardised ELISA kits
498 according to the manufacturer's instructions; IL-10 (R&D), TNF (Invitrogen) and IFN-γ (BD).

499

500 **Bone-marrow-derived macrophage studies**

501 Bone-marrow derived macrophages were obtained from the femur, tibia, and hips from bones from
502 BALB/c mice. Bone marrow cells were cultured at 37°C and 5% CO₂ for 6 days in BMM medium to
503 obtain BMDMs. BMM medium consisted of high glucose DMEM medium with no glutamine or phenol

504 red (ThermoFisher) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 20% M-CSF
505 (Generous gift from Dr Etienne Meunier, Toulouse), 1% HEPES (Gibco), 200 mM Glutamine
506 (ThermoFisher), 100 mM Sodium pyruvate (Gibco) and 0.5% Penicillin/Streptomycin (Sigma), which
507 was filtered before use. After 6 or 7 days of culture, BMDMs were washed, counted and plated in flat-
508 bottomed 24 well cell culture plates at 2.5×10^5 cells per well in BMM containing no antibiotics.
509 BMDMs were then either left unstimulated, or activated by treatment for 16 hours with IFN- γ (100
510 U/ml, Biolegend) + LPS (100ng/ml, Sigma) to induce the production of NO, or activated for 16h with
511 IFN- γ (100 U/ml) + LPS (100ng/ml) and treated L-NAME (4mM, Sigma) to inhibit iNOS. In these
512 conditions, we detected no NO in the supernatant of unstimulated cells, between 10 and 20 μ M NO
513 in the supernatant of activated cells, and less than 5 μ M NO in activated cells treated with L-NAME,
514 using the Griess assay (Sigma). Following stimulation, BMDMs were washed and then incubated with
515 STB-K, KC9A1, KC9C1 or *MTB* H37Rv at MOI 0.5 bacteria per cell for 2 hours before washing again. To
516 evaluate bacterial counts just after infection on day 0 or 3 days after infection, BMDMs were lysed
517 using 0.1% Triton X-100 in H₂O and cell lysates were plated on 7H11 agar plates.

518

519 ***In vitro* sensitivity testing**

520 Before performing susceptibility testing to NO, 3-4 week old bacterial cultures were re-cultured for 10
521 days in 7H9/ADC/Tween to ensure bacteria were in the exponential phase. 10 day old cultures were
522 washed twice in PBS/0.05% Tween 80 and diluted into 7H9/ADC/Tween at a calculated final OD_{600nm}
523 of 0.0005, corresponding to 10^5 CFU/ml, and incubated in the presence of 5 mM DETA/NO (Sigma).
524 The number of viable bacteria in treated and non-treated control cultures was determined by CFU
525 plating at the times indicated in the figure legends.

526 To evaluate bacterial growth at neutral pH, 3-4 week old cultures were diluted 1 in 100 into medium
527 composed of 7H9/ADC/Tween at pH 6.8. For the acidic pH growth assay, 3-4 week old cultures were
528 diluted 1 in 100 into 7H9 supplemented with ADC and 0.05% Tyloxapol (Merck), which had been

529 adjusted to pH 5, 5.5, 6 or 6.6. 100 mM MOPS buffer (Sigma) was used to keep the pH stable in medium
530 adjusted to pH6.6, and 100 mM MES buffer (Sigma) was used for pH5, 5.5 and 6. Tyloxapol was used
531 as the dispersal agent for this experiment as Tween 80 was previously shown to be toxic to *MTB* at
532 acidic pH¹⁹. Growth was monitored by measuring OD_{600nm} at various intervals. Results are represented
533 as the log₁₀ OD_{600nm} and are representative of three independent experiments.

534 To evaluate bacterial growth in phosphate-limiting conditions, strains were grown for 3-4 weeks in
535 7H9/ADC/Tween, pelleted, washed three times in Sauton's medium minus phosphate and then diluted
536 to approximately OD_{600nm} 0.05 in Sauton's medium containing the standard concentration of
537 phosphate (2870 μM) or low-phosphate concentrations (0 μM, 50 μM). The composition of Sauton's
538 medium minus phosphate was 4g/L L-asparagine, 2g/L citric acid monohydrate, 0.05g/L ferric
539 ammonium citrate, 1% zinc sulfate, 0.5g/L magnesium sulfate heptahydrate and 60ml/L glycerol in
540 H₂O. Phosphate was added as required in the form of potassium phosphate dibasic trihydrate. Strain
541 growth was evaluated by monitoring the OD_{600nm}. Data are represented as the mean and standard
542 deviation from three independent experiments.

543 To evaluate bacterial tolerance to zinc and copper, 3-4 week old bacterial cultures were re-cultured
544 for 7 days in 7H9/ADC/Tween to ensure bacteria were in the exponential phase. 7 day old cultures
545 were diluted into 4 ml 7H9/ADC/Tween at a calculated final OD_{600nm} of 0.005 in McFarland tubes, and
546 incubated in the presence of various concentrations of zinc (ZnSO₄, Sigma), or copper (CuCl₂, Sigma).
547 At the indicated time points, each tube was vortexed before evaluation of turbidity using a McFarland
548 densitometer. Each measure was performed in triplicate and the experiment was performed four
549 times independently. Data are represented as the mean and standard deviation of the triplicates from
550 one experiment.

551

552 **Permeability assay**

553 To assess membrane permeability of the wild-type *M. smegmatis* (WT), PMM280 (*smeg* Δ rv1339), and
554 the complemented strains PMM280::pAH01H (*smeg*::rv1339A1) and PMM305::pAH02H
555 (*smeg*::rv1339K), an EtBr uptake assay on intact mycobacteria was performed, as previously
556 described⁶³. Briefly, logarithmic-phase strains grown in 7H9/ADC/Tween were centrifuged, normalised
557 to OD_{600nm} of 0.8 in PBS buffer containing glucose 0.4%. 100 μ l of bacterial suspension was added per
558 well into 96-well microplates. 10 μ l EtBr (25 μ M) was added to the bacterial suspension and the mix
559 was incubated at 37°C. Fluorescence, associated with accumulation of EtBr, was measured for 1 hour
560 at 3 minute intervals with an excitation wavelength of 525 nm and an emission wavelength of 615 nm.
561 The experiment was performed twice in quintuplicate for the WT and *smeg* Δ rv1339 strains and once
562 in quintuplicate for the *smeg*::rv1339A1 and *smeg*::rv1339K strains.

563

564 **Statistical analysis**

565 Statistical analysis was carried out using GraphPad Prism 5 and 7. One or two-way analysis of variance
566 (ANOVA) followed by the Bonferroni post hoc test was used to analyse the statistical significance
567 between three or more groups. P values less than 0.05 were considered to be statistically significant.
568 When bacteria were undetectable, the CFU value was set at the limit of detection (\log_{10} 1.7 for *in vivo*
569 studies and \log_{10} 1.3 for *in vitro* studies , with the exception of the study involving C57BL/6 and NOS2⁻
570 ⁻ mice where the limit was \log_{10} 2) for the purpose of statistical analysis. For comparison of cell types
571 in the lesions, the Brown-Forsythe and Welch ANOVA test with the Games-Howell post hoc test was
572 used.

573

574 **Ethics statement**

575 All studies with *M. canettii* and *M. tuberculosis* strains were performed in biosafety level 3 (BSL3)
576 conditions, strictly following all relevant biosafety guidelines for experimental work with wild-type and

577 genetically modified tuberculosis bacilli (Authorization N°6406 from the French Ministry for Higher
578 Education and Research).

579 Animal studies were performed in agreement with European and French guidelines (EC Directive
580 2010/63/UE and French Law 2013-118 issued on February 1, 2013) after approval by the relevant
581 Ethics Committee (Comité d'éthique en experimentation animale N°01 and N°89) and the French
582 Ministry for Higher Education and Research after ethical evaluation (number 201508271122464 v2
583 (APAFIS#1535) and number 2018060717283847 v1 (APAFIS#15409)).

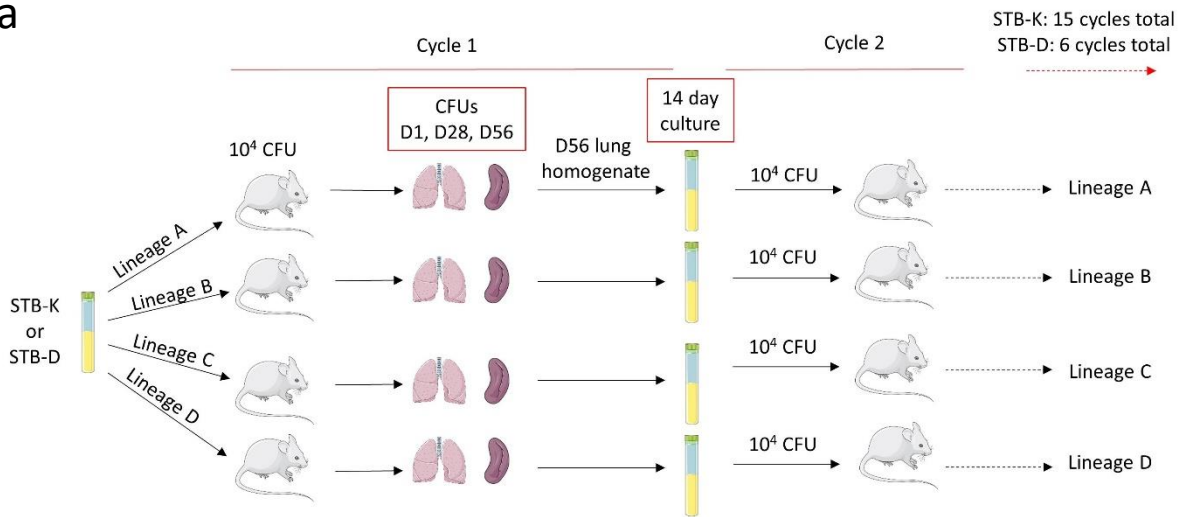
584

585 **Data availability**

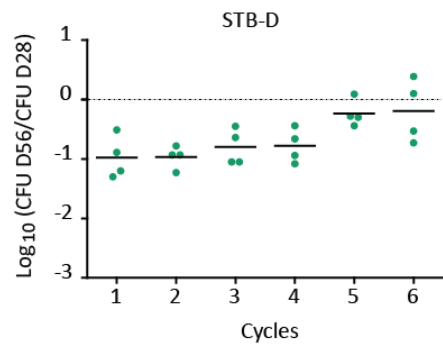
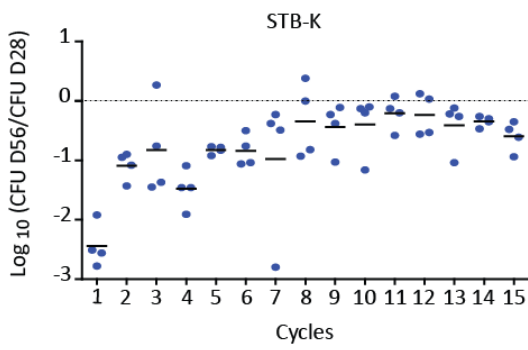
586 The sequence data were deposited in the NCBI repository under project PRJNA662472, PRJNA664307
587 and PRJNA665366 accession codes for Illumina-derived genome sequences of STB-K- and STB-D-
588 derived clones and populations and PacBio-derived genome sequence of STB-K respectively. All other
589 data that support the findings of this study are available from the corresponding author upon request.

590

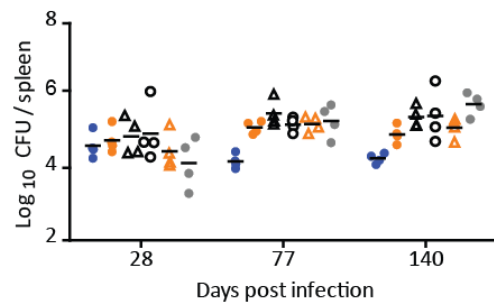
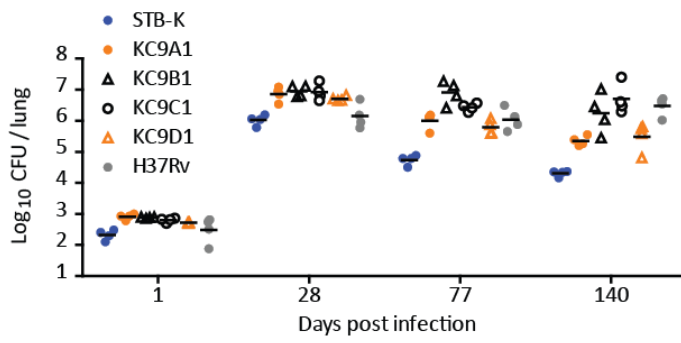
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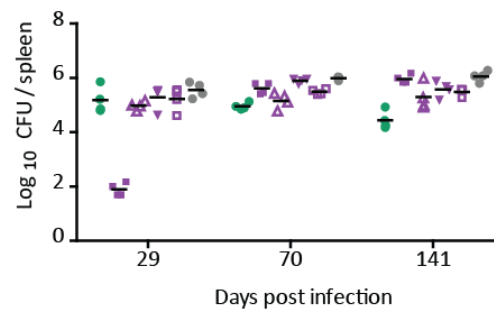
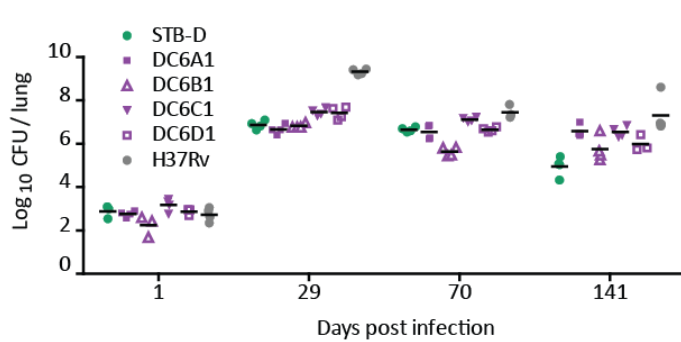
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d



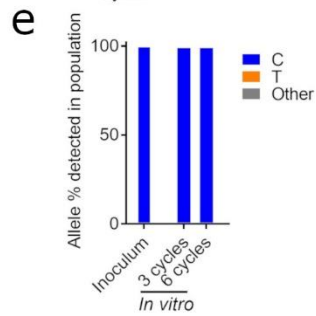
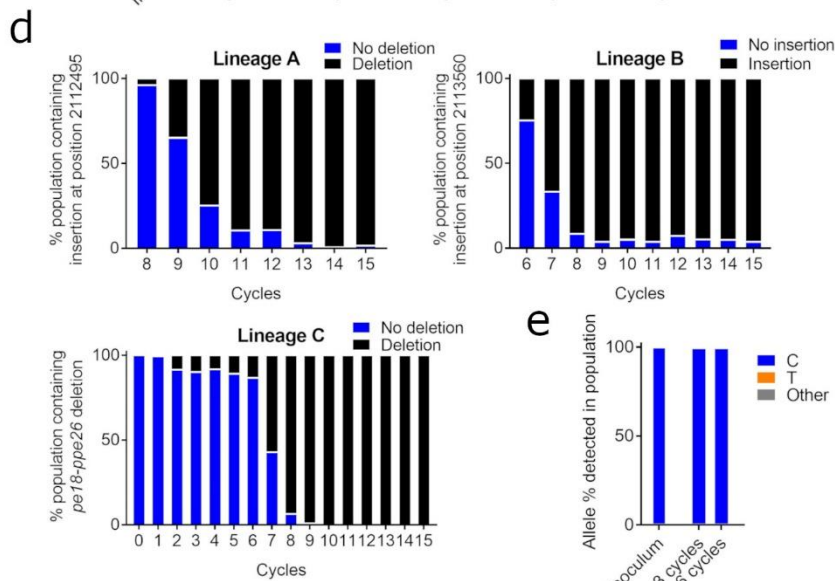
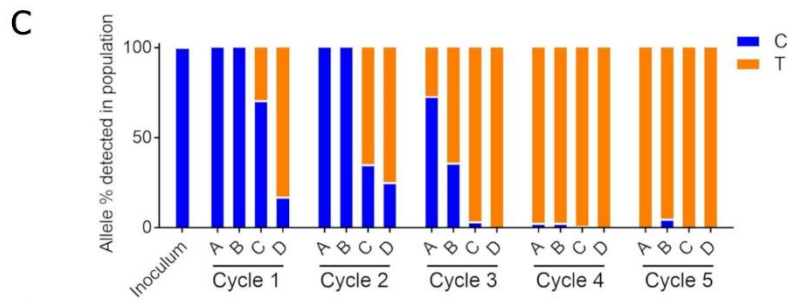
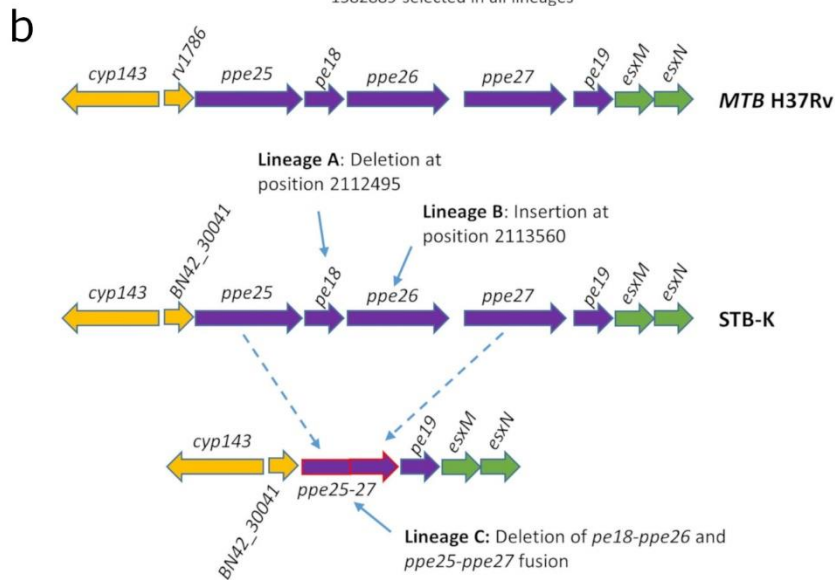
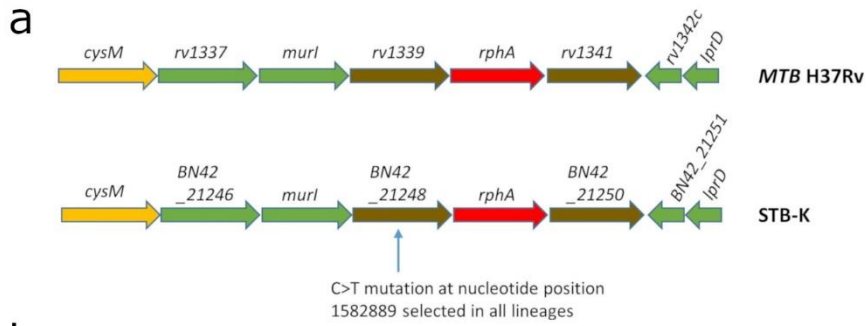
592 **Figure 1: Experimental evolution in mice selects *M. canettii* clones with enhanced persistence.**

593 a) Two experimental evolutions were performed in BALB/c mice using *M. canettii* STB-K and STB-D.
594 For both strains, 4 independent cultures were passaged corresponding to evolution lineages A, B, C
595 and D, and were never mixed. At the beginning of each cycle, 12 mice were infected i.n. with 10^4 CFU.
596 CFU numbers in the lungs and spleen were evaluated at 1, 28 and 56 days post-infection (4 mice per
597 time point) by plating. Part of the lung homogenates recovered from the mice sacrificed at 56 days
598 post-infection was also used to initiate 4 separate cultures. These cultures were grown for 14 days at
599 37°C and were then used as the new inocula for the next cycle. 15 cycles of experimental evolution
600 were performed for STB-K and 6 cycles were performed for STB-D. This diagram was created using
601 images from Servier Medical Art.

602 b) The persistence index was evaluated for each cycle in the experimental evolution by calculating the
603 log ratio of bacterial burden in the lung at day 56 vs day 28. Data are shown as \log_{10} CFU D56/D28 for
604 each lineage and the mean.

605 c) Persistence characteristics of 4 individual clones recovered after cycle 9 from STB-K evolution
606 lineages A, B, C and D. Mice were i.n. infected with 10^3 CFU of the parental STB-K strain, mutants
607 KC9A1, KC9B1, KC9C1, KC9D1, or *MTB* H37Rv and the bacterial burden in the lungs and spleen was
608 assessed at days 1, 28, 77 and 140 post-infection. Data are shown as \log_{10} CFU for individual mice and
609 the mean from an experiment performed with 3/4 mice per group.

610 d) Persistence phenotypes of 4 individual clones recovered after cycle 6 from STB-D evolution lineages
611 A, B, C and D. Mice were i.n. infected with 10^3 CFU of the parental STB-D strain, mutants DC6A1,
612 DC6B1, DC6C1, DC6D1 or *MTB* H37Rv and the CFU numbers in the lungs and spleen were assessed at
613 days 1, 29, 70 and 141 post-infection. Data are shown as \log_{10} CFU for individual mice and the mean
614 from an experiment performed with 3/4 mice per group. When bacteria were undetectable, the CFU
615 value was set at the limit of detection (\log_{10} 1.7).



617 **Figure 2: Mutations in the *rv1339* orthologue and the *ppe25-ppe27* region of STB-K mutants were**
618 **identified by whole-genome sequencing.**

619 a) Genetic region containing the *rv1339* gene in *MTB* H37Rv and the orthologue in *M. canettii* STB-K.
620 The blue arrow indicates the location of the point mutation identified in STB-K mutants following
621 experimental evolution.

622 b) The *ppe25-ppe27* locus of *MTB* H37Rv and STB-K. The blue arrows indicate the positions of the
623 mutations identified in the various STB-K evolution lineages between cycles 6 and 9 of the
624 experimental evolution.

625 c) Fixation of the C>T mutation in the *rv1339* orthologue occurred early during the experimental
626 evolution.

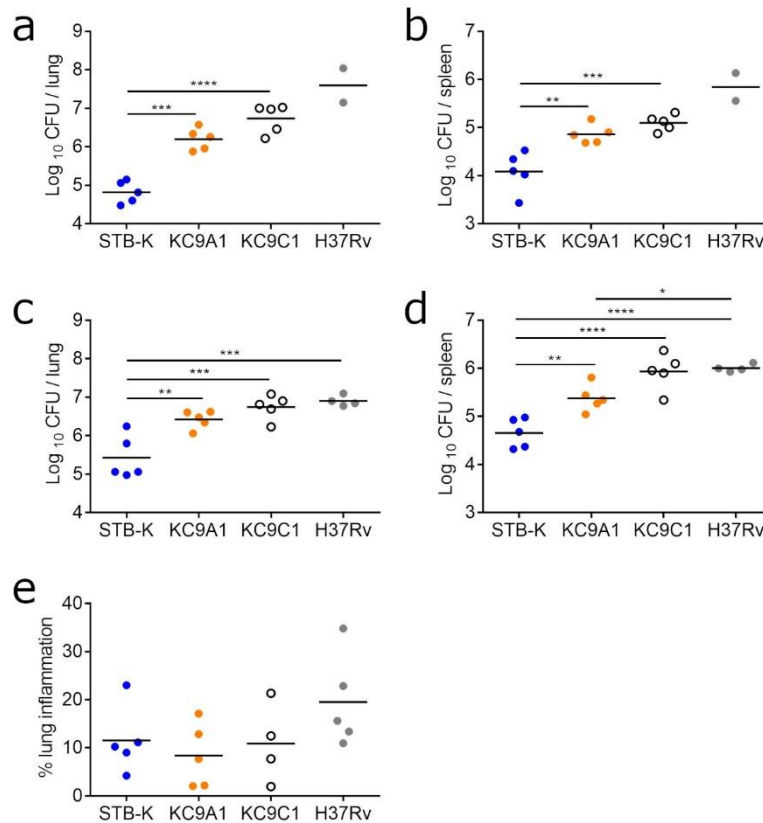
627 d) The mutations in the *ppe25-ppe27* region of the STB-K mutants became fixed in evolution lineages
628 A, B and C after 9-13 cycles of experimental evolution.

629 e) WGS demonstrating that the C>T mutation was not enriched following serial passaging of STB-K *in*
630 *vitro*.

631 For c), and d) whole -genome sequence data were generated from the bacterial populations
632 corresponding to the inoculum and to each evolution lineage recovered after cycles 1 to 15. Positions
633 corresponding to the mutation in the *rv1339* orthologue or the deletions in the *ppe25-ppe27* regions
634 were analysed to determine the distribution of wild-type and mutant alleles in the population.

635 For e), we performed 6 cycles of growth of STB-K in 7H9/ADC/Tween supplemented with panta (as for
636 the *in vitro* cultures between each mice infection cycle). Whole -genome sequence data were
637 generated from the bacterial populations corresponding to the inoculum and bacterial populations
638 recovered after cycles 3 and 6. For the position corresponding to the C>T in the *rv1339* orthologue,
639 the coverage depth was x98, x124 and x159 for the inoculum, cycle 3 and cycle 6 populations
640 respectively.

641 For **c**), **d**), and **e**), data are shown as the percentages of the wild-type and mutated alleles detected in
642 the STB-K-derived populations from each evolution lineage after each cycle by WGS.



643

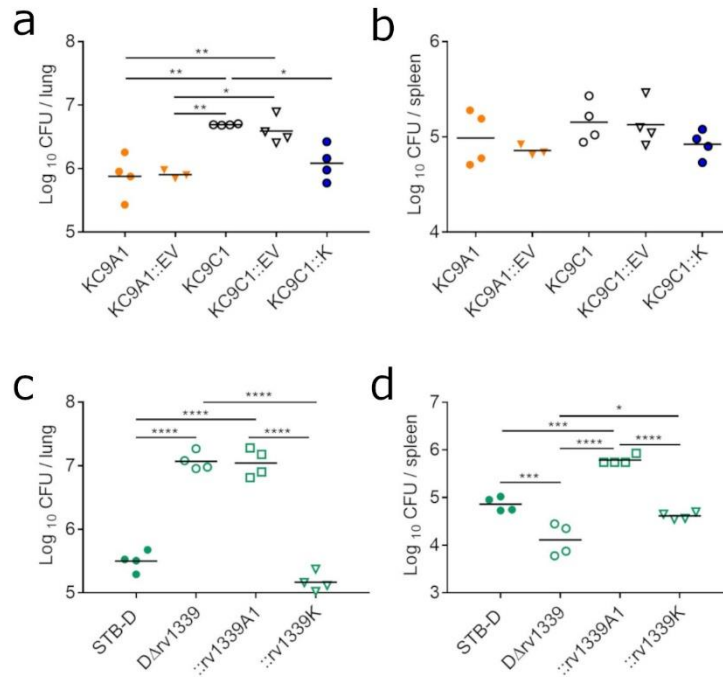
644 **Figure 3: The persistence phenotypes of *rv1339* and *pe18-ppe26* STB-K mutants are not specific to**
 645 **the mouse strain or route of infection.**

646 C3HeB/FeJ mice were i.n. infected with approximately 200 CFU of STB-K, KC9A1, KC9C1 or *MTB* H37Rv
 647 and bacterial burden in the **a)** lung and **b)** spleen was quantified at 77 days post-infection by CFU
 648 plating on solid medium. Results are shown as the log₁₀ CFU for individual mice and the mean (5 mice
 649 per group except for H37Rv because 3 mice had to be euthanised before the designated end point
 650 due to health deterioration), and are representative of two experiments. **P < 0.01, ***P < 0.001,
 651 ****P < 0.0001 by one-way ANOVA with the Bonferroni post hoc test.

652 C3HeB/FeJ mice were i.v. infected with approximately 10³ CFU of STB-K, KC9A1, KC9C1 or H37Rv and
 653 the CFU numbers in the **c)** lung and **d)** spleen were evaluated at 77 days post-infection. Results are
 654 shown as the log₁₀ CFU for individual mice (4/5 per group) and the mean, and are representative of

655 two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by one-way ANOVA
656 with the Bonferroni post hoc test.

657 e) C3HeB/FeJ mice were i.n. infected with approximately 200 CFU of STB-K, KC9A1, KC9C1 or *MTB*
658 H37Rv. After 76 days of infection, lungs were isolated for histopathology. Lung samples were fixed
659 and stained with hematoxylin and eosin. Percentage lung inflammation was quantified by dividing the
660 total inflamed area by the total lung area, and multiplying by 100. Data are represented as the
661 percentage lung inflammation for individual mice (one entire lung section per mouse) and the mean
662 from an experiment performed with 4/5 mice per group. Non-significant by one-way ANOVA with the
663 Bonferroni post hoc test.

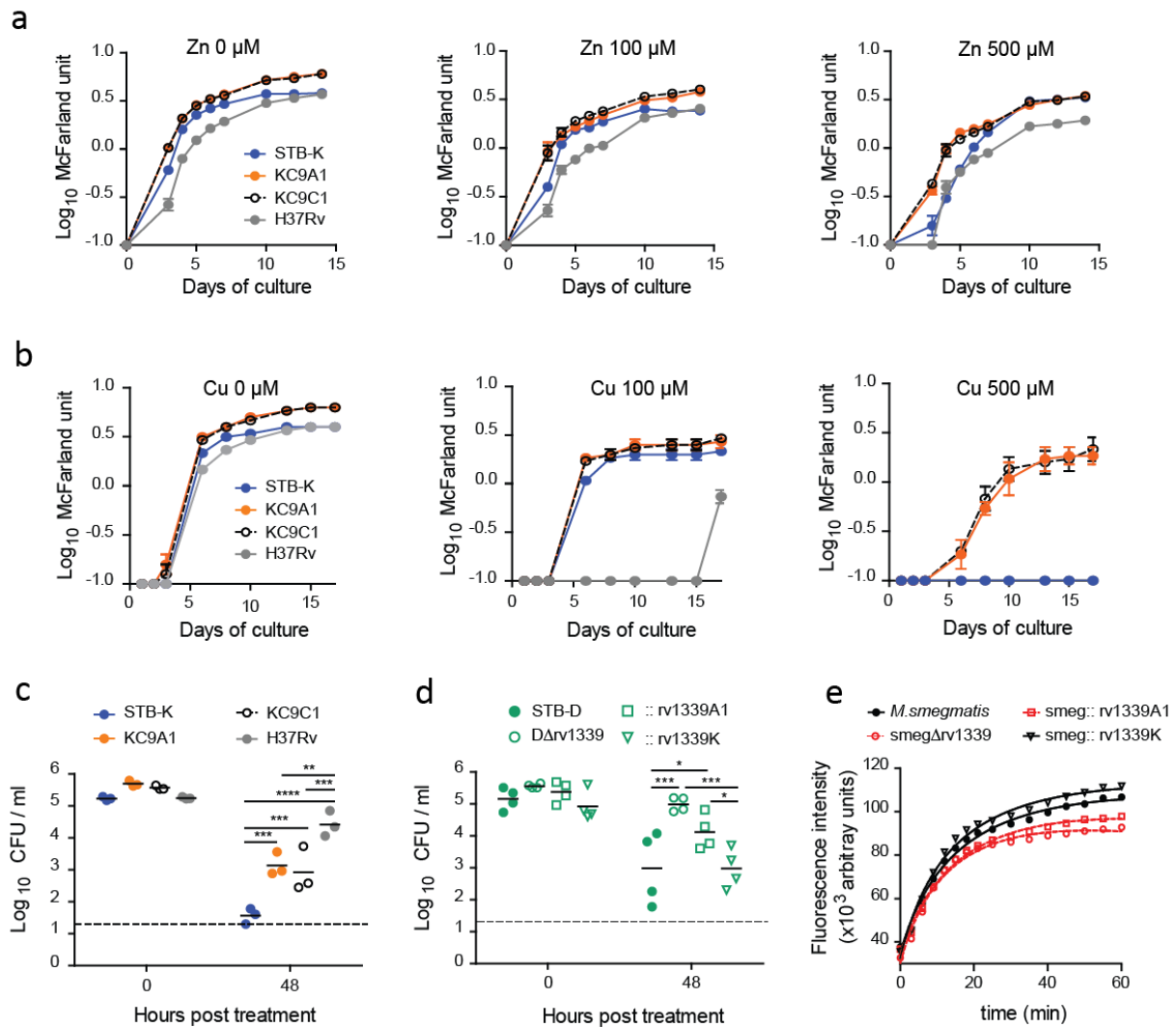


664

665 **Figure 4: The *pe18-ppe26* and *rv1339* mutations confer enhanced persistence.**

666 BALB/c mice were i.n. infected with approximately 10^3 CFU of *M. canettii* KC9A1, KC9A1::EV, KC9C1,
 667 KC9C1::EV, or KC9C1::K, and CFU counts in the **a)** lung and **b)** spleen were quantified at 70 days post-
 668 infection by plating serial dilutions of the organ homogenate on 7H11 agar plates. Results are shown
 669 as the log_{10} CFU for individual mice (3/4 per group) and the mean, and are representative of two
 670 independent experiments. * $P < 0.05$, ** $P < 0.01$, by one-way ANOVA with the Bonferroni post hoc
 671 test.

672 BALB/c mice were i.n. infected with approximately 10^3 CFU of STB-D, DΔrv1339, ::rv1339A1 or
 673 ::rv1339K. **c)** Lung and **d)** spleen bacterial burden was evaluated at 77 days post-infection by CFU
 674 plating on solid medium. Results are shown as the log_{10} CFU for individual mice (4 per group) and the
 675 mean, and are representative of two independent experiments. * $P < 0.05$, *** $P < 0.001$, **** $P <$
 676 0.0001 by one-way ANOVA with the Bonferroni post hoc test.



677

678 **Figure 5: The *rv1339* mutation contributes to enhanced resistance to host-derived stresses.**

679 Tubes of 7H9/ADC/Tween containing various concentration of **a)** ZnSO_4 , or **b)** CuCl_2 were inoculated
 680 with STB-K, KC9A1, KC9C1, and *MTB* H37Rv at a final $\text{OD}_{600\text{nm}}$ of approximately 0.005 and growth was
 681 evaluated by monitoring the density (evaluated as McFarland units). Data are represented as the
 682 mean and standard deviation from triplicates and are representative of four independent
 683 experiments.

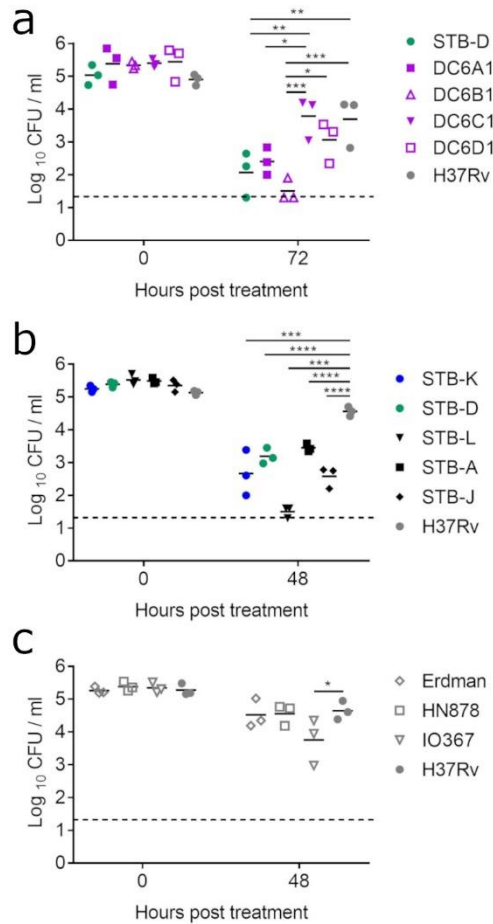
684 Flasks of 7H9/ADC/Tween +/- 5 mM DETA/NO were inoculated with 10^5 CFU of **c)** STB-K, KC9A1, KC9C1
 685 and H37Rv, or **d)** STB-D, Δrv1339 , $::\text{rv1339A1}$ and $::\text{rv1339K}$. Bacterial survival was monitored by
 686 evaluating CFU numbers at 0 and 48 hours post treatment with DETA/NO. Results are shown as \log_{10}
 687 CFU/ml for individual data points from **c)** three and **d)** four independent experiments and the mean.

688 When bacteria were undetectable, the CFU value was set at the limit of detection (\log_{10} 1.3) for the
689 purposes of statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by two-way ANOVA
690 with the Bonferroni post hoc test.

691 e) Exponentially growing *M. smegmatis*, smeg Δ rv1339, smeg::rv1339A1 and smeg::rv1339K were
692 incubated with EtBr and intracellular accumulation was followed over 60 minutes by measuring
693 fluorescence intensity. Data are represented as the mean and standard deviation from quintuplicates.
694 The experiment was performed twice in quintuplicate for the WT and smeg Δ rv1339 strains and once
695 in quintuplicate for the smeg::rv1339A1 and smeg::rv1339K strains.

696

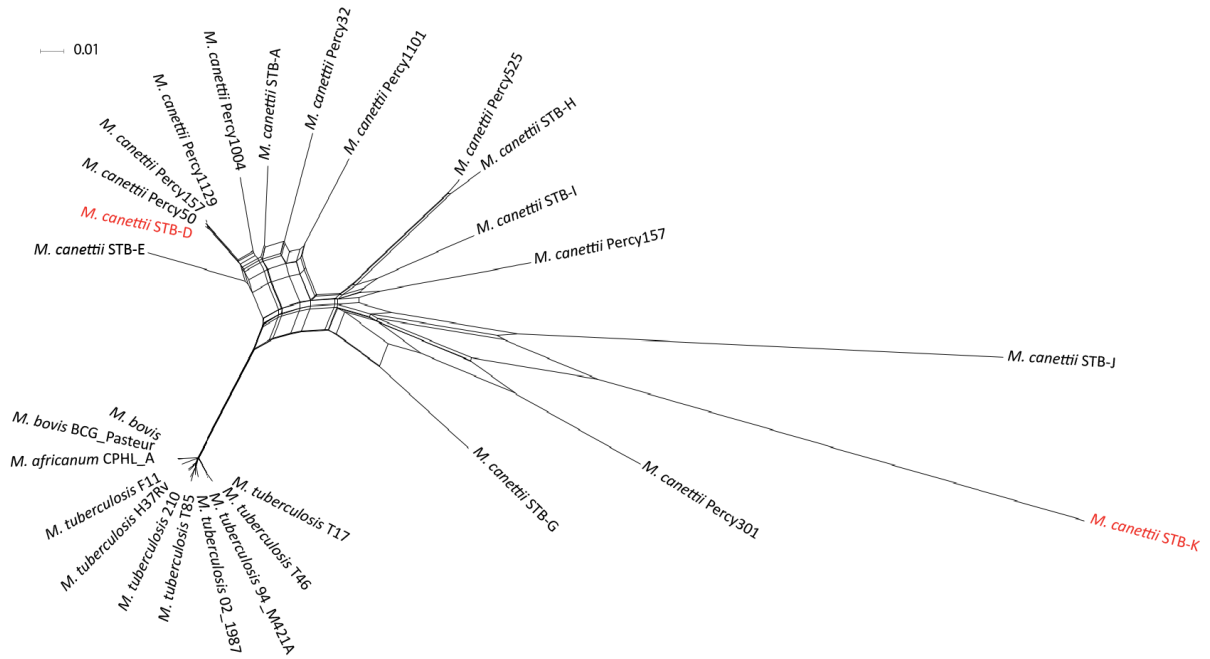
697



698

699 **Figure 6: *MTB* exhibit higher nitric oxide resistance than *M. canettii*.**

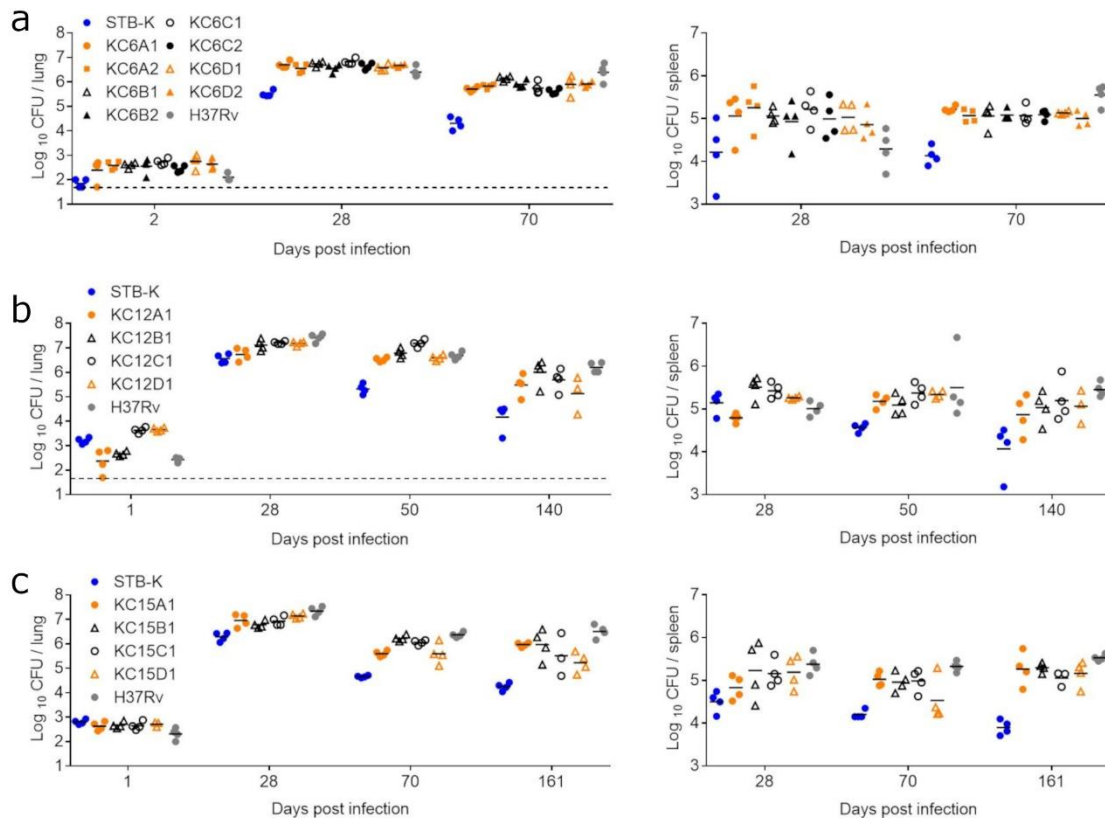
700 Flasks of 7H9/ADC/Tween +/- 5 mM DETA/NO were inoculated with 10^5 CFU of a) STB-D, DC6A1,
 701 DC6B1, DC6C1, DC6D1, and *MTB* H37Rv, b) *M. canettii* STB-K, -L, -A, -D, -J, and *MTB* H37Rv, or c) *MTB*
 702 Erdman, HN878, IO367, and H37Rv. Bacterial survival was monitored by evaluating CFU numbers at
 703 time 0 and 48 or 72 hours post treatment with DETA/NO. Results are shown as the \log_{10} CFU/ml for
 704 individual data points from three independent experiments and the mean. When bacteria were
 705 undetectable, the CFU value was set at the limit of detection (\log_{10} 1.3) for the purposes of statistical
 706 analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by two-way ANOVA with the Bonferroni
 707 post hoc test.



708

709 **Supplementary Figure 1: Phylogenetic tree showing the distribution of *M. canettii* and *MTB* strains.**

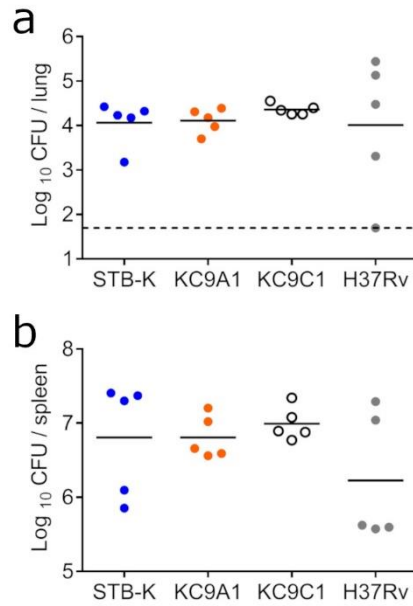
710 Network phylogeny inferred among 28 genomes by NeighborNet analysis, based on pairwise
 711 alignments of whole genome SNP data from a selection of strains (17 *M. canettii*, 8 *MTB* from various
 712 phylogenetic lineages and 3 strains from the *M. africanum* and *M. bovis* lineages), depicted by the
 713 SplitsTree software⁶⁴. The whole SNP dataset was originally generated for Supplementary Figure 1 of
 714 reference¹⁰, using genome data described by^{4,9,65}. Strains used in experimental evolution experiments
 715 are coloured in red.



716

717 **Supplementary Figure 2: Experimental evolution in mice selects *M. canettii* clones with enhanced**
 718 **persistence.**

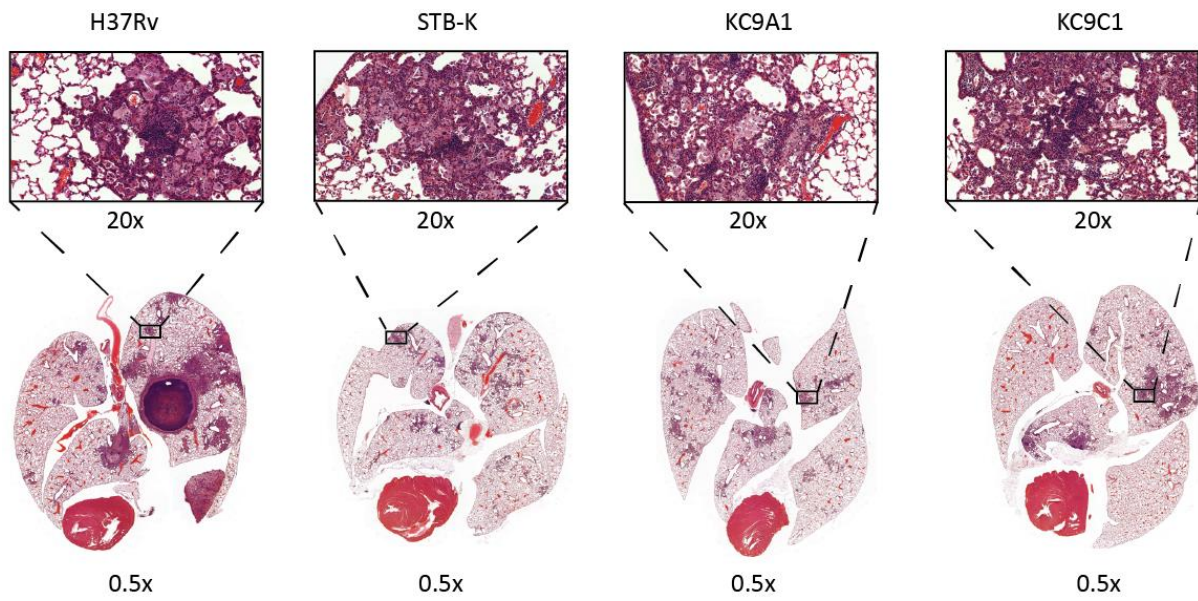
719 Persistence phenotypes of individual STB-K clones recovered from STB-K evolution lineages A-D after
 720 cycles **a)** 6, **b)** 12 and **c)** 15. Mice were i.n. infected with 10^3 CFU of **a)** KC6A1, KC6A2, KC6B1, KC6B2,
 721 KC6C1, KC6C2, KC6D1, and KC6D2, **b)** KC12A1, KC12B1, KC12C1, and KC12D1, or **c)** KC15A1, KC15B1,
 722 KC15C1, and KC15D1. Parental STB-K and control *MTB* H37Rv strains were included in all three
 723 experiments. The bacterial burden in the lungs and spleen was assessed at various intervals post-
 724 infection by plating serial dilutions of organ homogenates on 7H11 agar plates. Data are shown as
 725 \log_{10} CFU for individual mice and the mean, from an experiment performed with 3/4 mice per group.
 726 When bacteria were undetectable, the CFU value was set at the limit of detection (\log_{10} 1.7).



727

728 **Supplementary Figure 3: KC9A1 and KC9C1 display similar bacterial burden to STB-K during the acute**
 729 **phase of infection.**

730 C3HeB/FeJ mice were i.v. infected with approximately 10^3 CFU of STB-K, KC9A1, KC9C1, or *MTB* H37Rv,
 731 and CFU counts in the **a)** lung and **b)** spleen were quantified at 21 days post-infection by plating
 732 dilutions of the organ homogenate on 7H11 agar plates. Results are shown as the \log_{10} CFU for
 733 individual mice (5 per group) and the mean, and are representative of two independent experiments.
 734 Non-significant by one-way ANOVA with the Bonferroni post hoc test. When bacteria were
 735 undetectable, the CFU value was set at the limit of detection (\log_{10} 1.7).



736

0.5x

0.5x

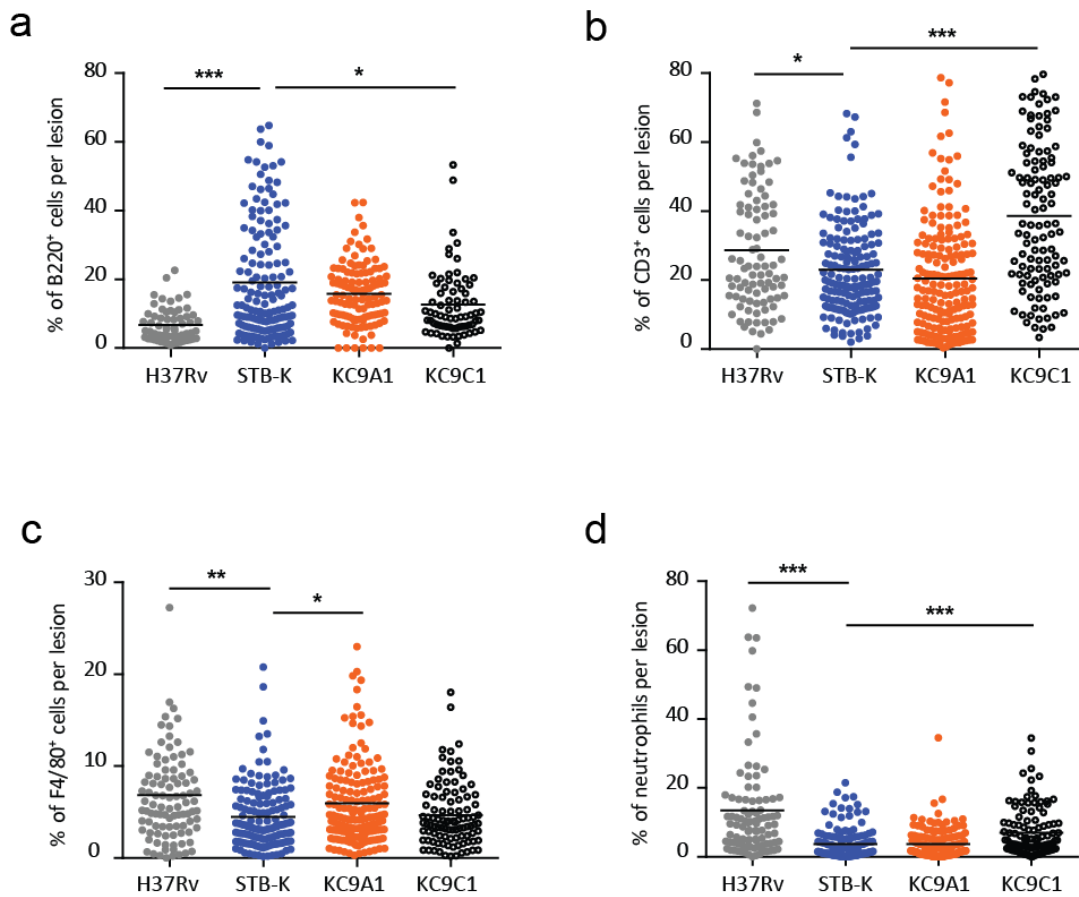
0.5x

0.5x

737 **Supplementary Figure 4: KC9A1 and KC9C1 do not induce a greater inflammatory response in the**
738 **lungs of mice than STB-K.**

739 Lungs were isolated from C3HeB/FeJ mice 76 days after i.n. infection with approximately 200 CFU of
740 STB-K, KC9A1, KC9C1, or H37Rv for histopathology. Lung samples were fixed and stained with
741 hematoxylin and eosin, and analysed using Panoramic Viewer. For each mouse (4/5 mice per group),
742 one entire lung section was analysed and the images shown are representative.

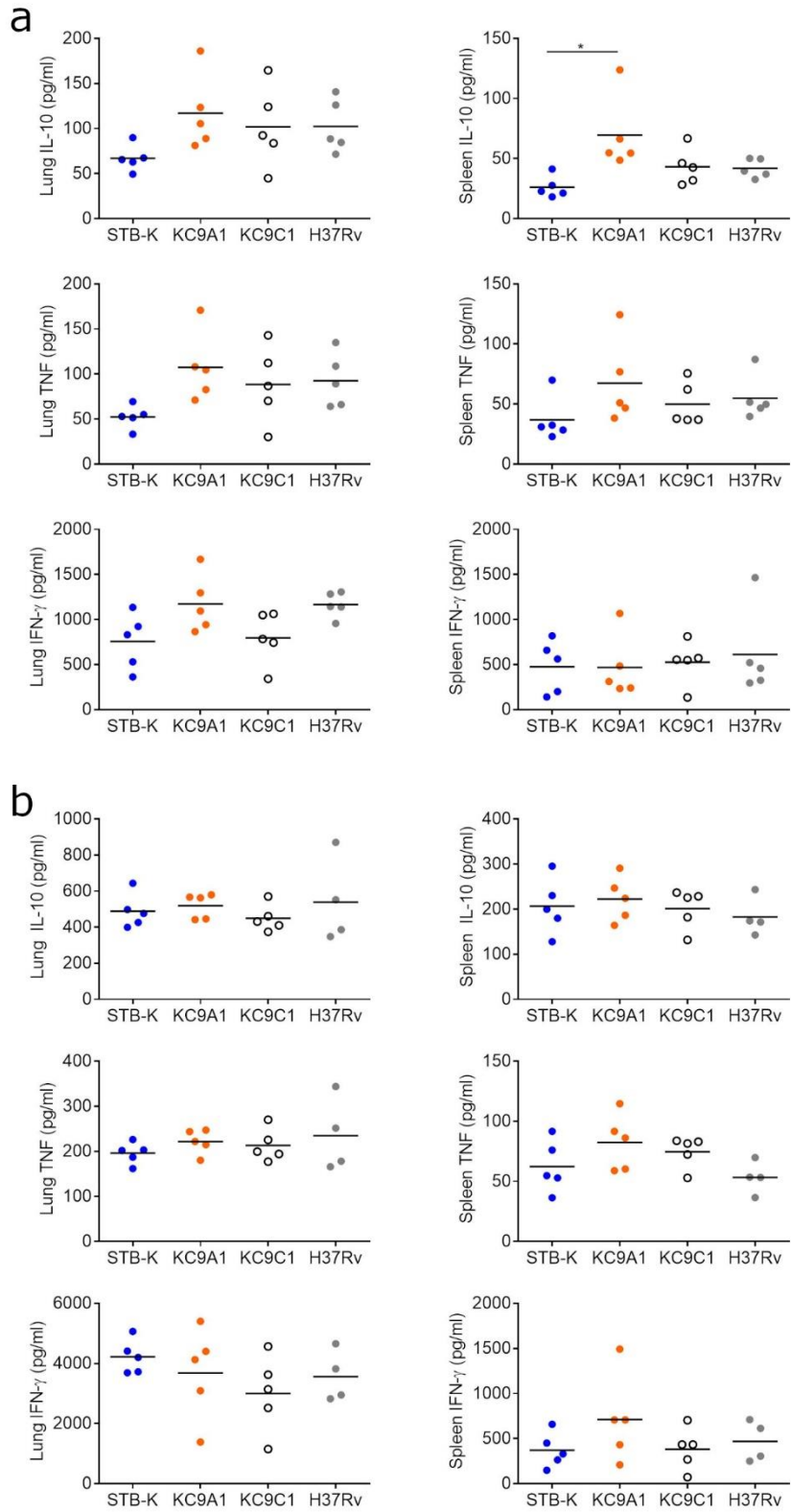
743



744

745 **Supplementary Figure 5: STB-K and KC9A1 induce a similar recruitment of immune cells to the lungs**
 746 **whereas the pattern for KC9C1 is closer to that of H37Rv.**

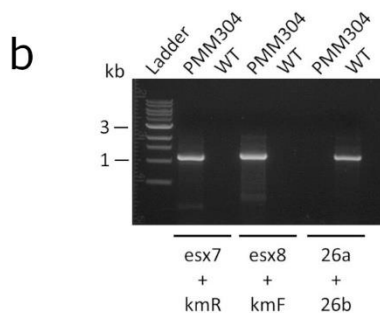
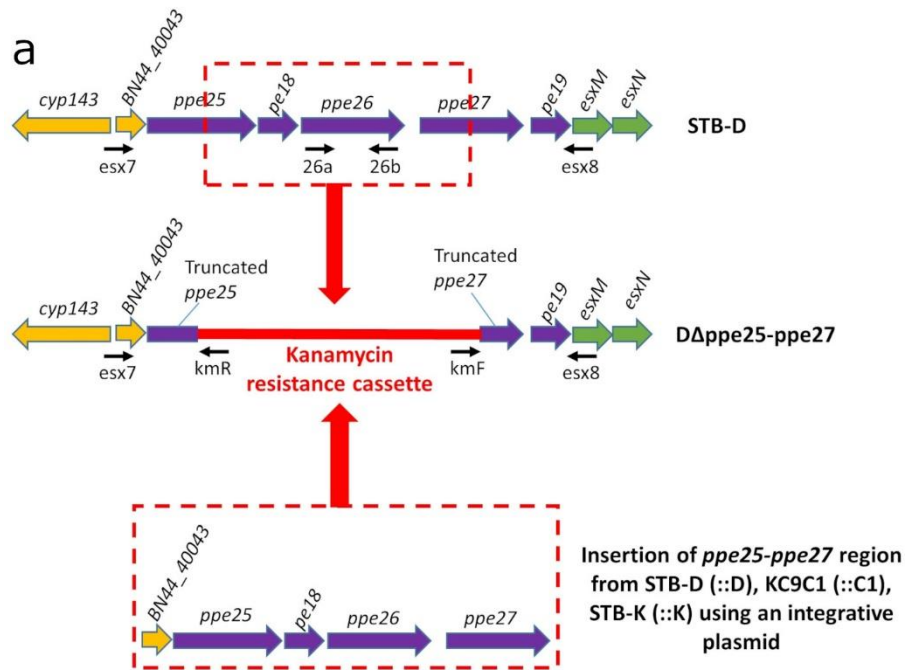
747 Lung samples from C3HeB/FeJ mice 76 days after i.n. infection with approximately 200 CFU of STB-K,
 748 KC9A1, KC9C1, or H37Rv were fixed, and analysed by immunohistochemistry. Serial lung sections, one
 749 per infected mouse and per primary antibody, were labelled with antibodies against **a**) B lymphocytes
 750 (B220), **b**) T lymphocytes (CD3), **c**) macrophages (F4/80), and **d**) neutrophils (neutrophil marker). At
 751 least four sections from 4 mice were analysed with each antibody. The percentage of each cell type in
 752 the various lesions was quantified. *P < 0.05, **P < 0.01, ***P < 0.001, by Brown-Forsythe and Welch
 753 ANOVA test with the Games-Howell post hoc test.



754

755 **Supplementary Figure 6: The persistent phenotypes of KC9A1 and KC9C1 are not associated with a**
 756 **change in cytokine production.**

757 C3HeB/FeJ mice were i.v. infected with approximately 10^3 CFU of STB-K, KC9A1, KC9C1, or *MTB* H37Rv,
758 and IL-10, IFN- γ and TNF concentrations in the lung and spleen homogenates were quantified at **a)** 21
759 days and **b)** 76 days post-infection by ELISA. Mean cytokine concentrations are shown, in addition to
760 the concentrations for individual mice (4/5 per group). *P < 0.05 by one-way ANOVA with the
761 Bonferroni post hoc test.

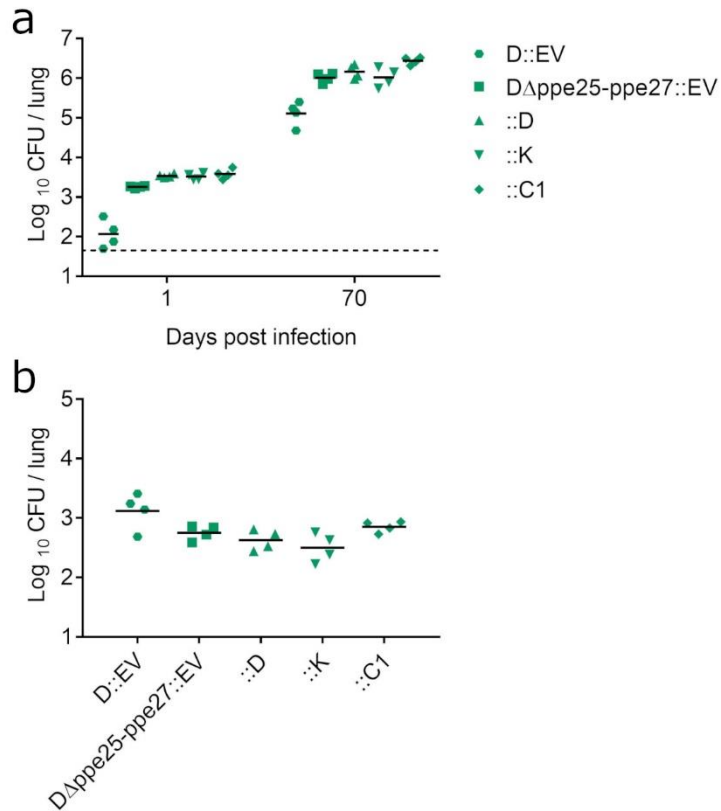


762

763 **Supplementary Figure 7: Insertion of the *ppe25-ppe27* regions from KC9C1 and STB-K into STB-D.**

764 **a)** The *ppe25-ppe27* region of STB-D was replaced with a *km* resistance cassette by allelic exchange to
 765 create D Δ ppe25-ppe27 (PMM304). This knockout strain was then complemented with an integrative
 766 plasmid containing the *ppe25-ppe27* regions from STB-D (::D), KC9C1 (::C1), or STB-K (::K). Only the
 767 region from STB-D is represented in this figure. The black arrows indicate the primers used to amplify
 768 the corresponding regions.

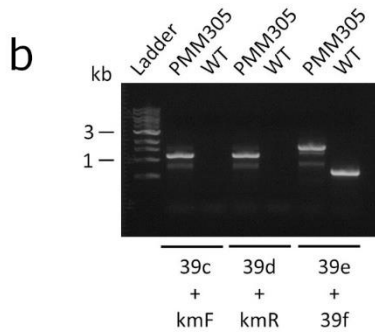
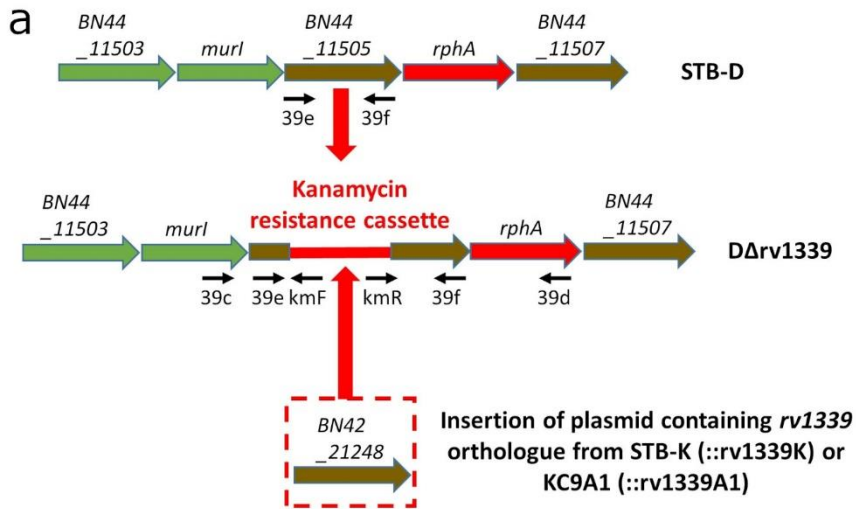
769 **b)** PCR profiles corresponding to a mutant deleted for *pe18-ppe26* (PMM304), or the wild-type STB-D
 770 strain (WT), when the indicated primer pairs were used for amplification.



771

772 **Supplementary Figure 8: The *pe18-ppe26* deletion does not confer enhanced persistence to STB-D.**

773 BALB/c mice were i.n. infected with approximately 10³ CFU of *M. canettii* D::EV, DΔppe25-ppe27::EV,
 774 ::D, ::K, or ::C1, and CFU counts in the lung were quantified at 1 and 70 days post-infection by plating
 775 dilutions of the organ homogenate on 7H11 agar plates. Results are shown as **a**) the log₁₀ CFU per lung
 776 for individual mice and the mean, and **b**) normalised values (log₁₀ CFU D77 – log₁₀ CFU D1), from an
 777 experiment performed with 4 mice per group. Differences between DΔppe25-ppe27::EV, ::D, ::K and
 778 ::C1 were deemed non-significant by **a**) two-way and **b**) one-way ANOVA with the Bonferroni post hoc
 779 test. The statistical difference between D::EV and the other groups is not shown. When bacteria were
 780 undetectable, the CFU value was set at the limit of detection (log₁₀ 1.7).

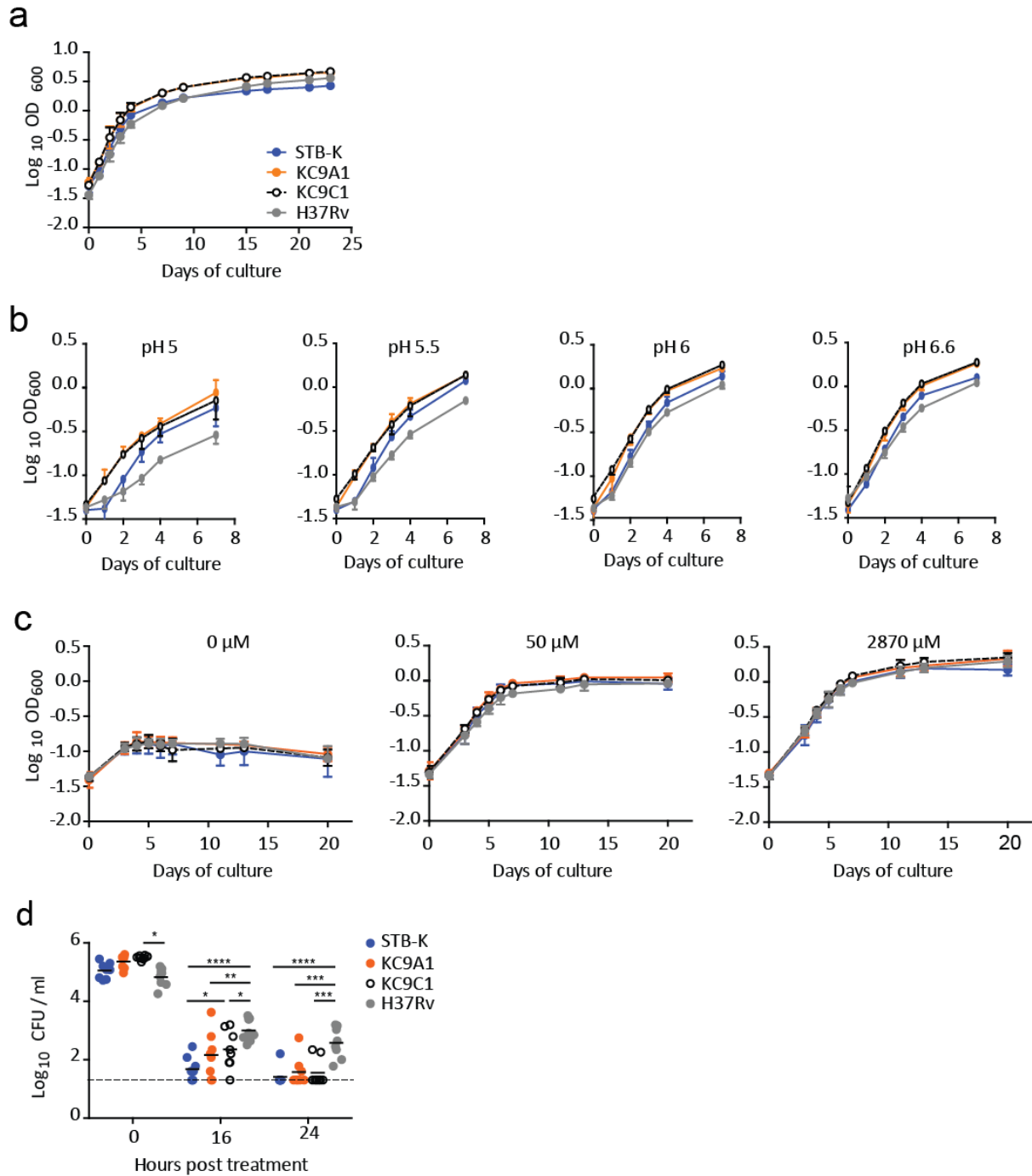


781

782 **Supplementary Figure 9: Replacement of the *rv1339* orthologue in STB-D with the *rv1339***
 783 **orthologue of STB-K or KC9A1.**

784 **a)** A *km* resistance cassette was inserted in the *rv1339* orthologue of STB-D (*BN44_11505*) by allelic
 785 exchange to create DΔ*rv1339* (PMM305). DΔ*rv1339* was then complemented with plasmids
 786 containing either the *rv1339* orthologue from STB-K (::*rv1339K*) or KC9A1 (::*rv1339A1*). Only the
 787 *rv1339* orthologue from STB-K is represented in this figure. The black arrows indicate the primers used
 788 to amplify the corresponding regions.

789 **b)** PCR profiles corresponding to a mutant interrupted for the *rv1339* orthologue (PMM305), or the
 790 wild-type STB-D strain (WT), when the indicated primer pairs were used for amplification.



791

792 **Supplementary Figure 10: STB-K and the two persistent clones KC9A1 and KC9C1 display similar**
 793 **resistance to H₂O₂ and grow similarly in phosphate-limited medium or at acidic pH.**

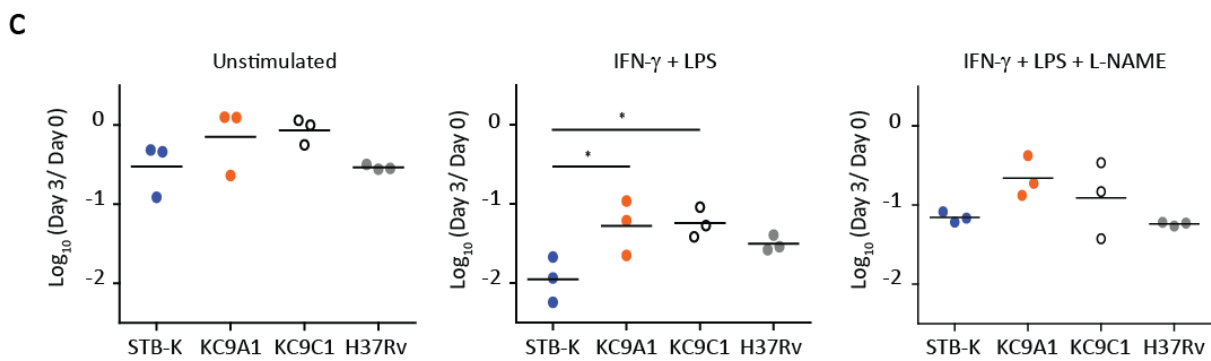
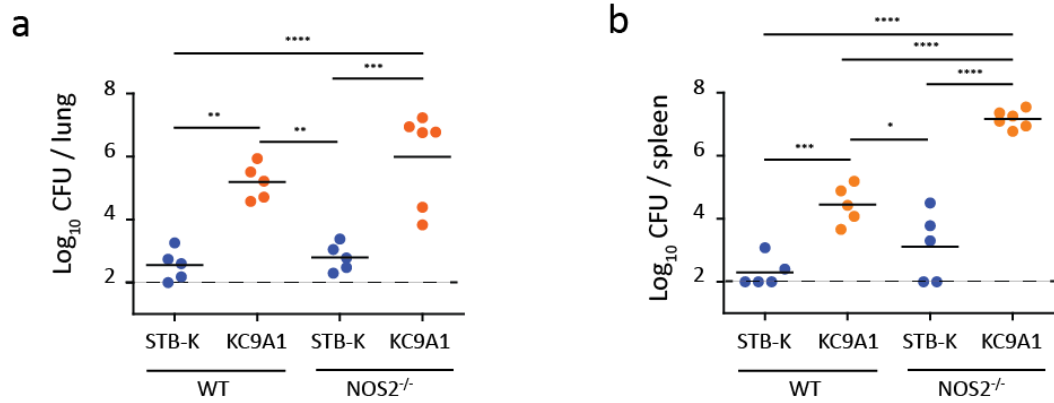
794 a) Flasks of 7H9/ADC/Tween at pH 6.8 were inoculated with STB-K, KC9A1, KC9C1, and *MTB* H37Rv at
 795 a final OD_{600nm} of approximately 0.05 and growth was evaluated by monitoring the OD600. Data are
 796 represented as the mean and standard deviation from three independent experiments.

797 **b)** STB-K, KC9A1, KC9C1, and H37Rv were inoculated to a final OD_{600nm} of approximately 0.05 into flasks
798 containing 7H9 supplemented with ADC and 0.05% Tyloxapol adjusted to pH 5, 5.5, 6 or 6.6. Strain
799 growth was evaluated by monitoring the OD₆₀₀. Data are represented as the mean and standard
800 deviation from three independent experiments.

801 **c)** STB-K, KC9A1, KC9C1, and H37Rv were inoculated to a final OD_{600nm} of approximately 0.05 into flasks
802 containing Sauton's medium where the final concentration of phosphate had been adjusted to either
803 0 μM, 50 μM or 2870 μM supplemented with 0.05% Tween 80. Strain growth was evaluated by
804 monitoring the OD₆₀₀. Data are represented as the mean and standard deviation from three
805 independent experiments.

806 **d)** Flasks of 7H9/ADC/Tween + 10 mM H₂O₂ were inoculated with 10⁵ CFU of STB-K, KC9A1, KC9C1 and
807 H37Rv. Bacterial survival was monitored by evaluating CFU numbers at 0, 16 and 24 hours post
808 treatment with H₂O₂. Results are shown as log₁₀ CFU/ml for individual data points from eight
809 independent experiments and the mean. When bacteria were undetectable, the CFU value was set at
810 the limit of detection (log₁₀ 1.3) for the purposes of statistical analysis. *P < 0.05, **P < 0.01, ***P <
811 0.001, ****P < 0.0001 by two-way ANOVA with the Bonferroni post hoc test.

812



813

814 **Supplementary Figure 11: The persistence phenotype of the *rv1339* STB-K mutant (KC9A1) is due in**
 815 **part to enhanced resistance to NO.**

816 NOS2^{-/-} mice or C57BL/6 wild-type controls (WT) were infected via aerosol with approximately 100
 817 CFU of STB-K or KC9A1 and bacterial burden in the **a)** lung and **b)** spleen was quantified at 56 days
 818 post-infection by CFU plating on solid medium. Results are shown as the log₁₀ CFU for individual mice
 819 and the mean from an experiment performed with 5-6 mice per group. *P < 0.05, **P < 0.01, ***P <
 820 0.001, ****P < 0.0001 by one-way ANOVA with the Bonferroni post hoc test.

821 Using this route of infection and the C57BL/6 mouse strain, the bacterial burden in the lungs and
 822 spleen at day 56 post-infection was consistent with the enhanced persistence phenotype of KC9A1
 823 when compared to STB-K. In NOS2^{-/-} mice, the persistence of STB-K in the spleen and KC9A1 in both
 824 organs were increased showing that NO is an important factor reducing STB-K and KC9A1 persistence.
 825 However, the difference between STB-K and KC9A1 was maintained in NOS2^{-/-} mice, and even

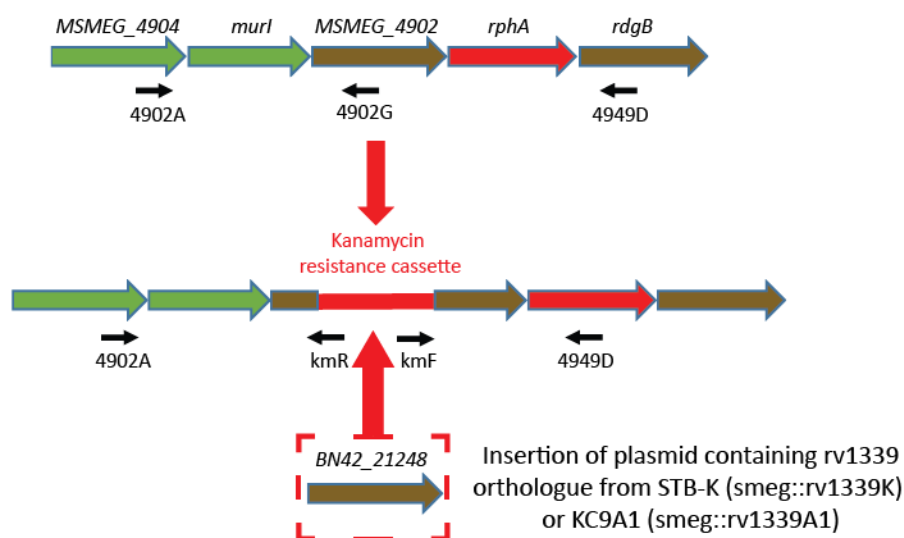
826 increased in comparison to C57BL/6 mice, indicating that the *rv1339* mutation also confers strong
827 resistance to NOS2^{-/-} independent mechanisms.

828 **c)** BMDMs from BALB/c mice were either unstimulated, or stimulated with IFN- γ (100 U/ml) + LPS (100
829 ng/ml), or stimulated with IFN- γ + LPS and treated with the inducible nitric oxide synthase (iNOS)
830 inhibitor L-NAME (4 mM). BMDMs (2.5 x 10⁵ cells/well) were infected at a multiplicity of infection
831 (MOI) of 0.5 bacteria/cell with STB-K, KC9A1, KC9C1 or *MTB* H37Rv and the number of surviving
832 bacteria were evaluated by plating on 7H11 solid medium. The indicated value are the Log₁₀ (ratio of
833 bacterial load at day 3 over bacterial load at day 0 in the same experiment). *P < 0.05 by one-way
834 repeated measure ANOVA with the Bonferroni post hoc test.

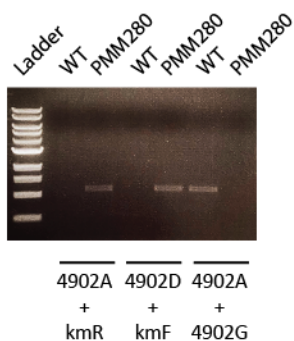
835 In these experiments, stimulation of BMDMs with IFN- γ and LPS reduced the survival of the 4 strains
836 and this effect is partially suppressed by inhibiting iNOS.

837

a



b



838

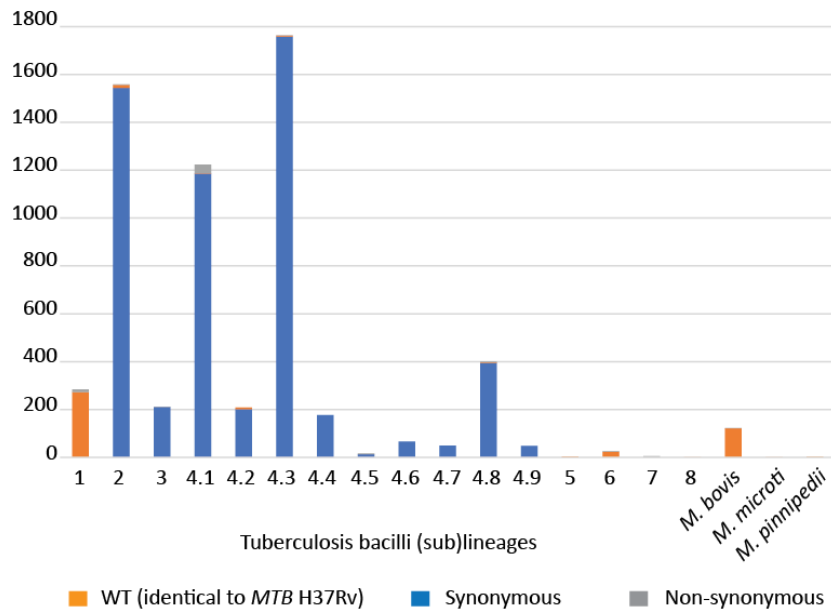
839 **Supplementary Figure 12: Replacement of the *rv1339* orthologue in *M. smegmatis* mc²155 with the**
 840 ***rv1339* orthologue of STB-K or KC9A1.**

841 a) A *km* resistance cassette was inserted in the *rv1339* orthologue of *M. smegmatis* mc²155
 842 (*MSMEG_4902*) by allelic exchange to create smegΔ*rv1339* (PMM280). smegΔ*rv1339* was then
 843 complemented with plasmids containing either the *rv1339* orthologue from STB-K (smeg::*rv1339K*) or
 844 KC9A1 (smeg::*rv1339A1*). The black arrows indicate the primers used to amplify the corresponding
 845 regions.

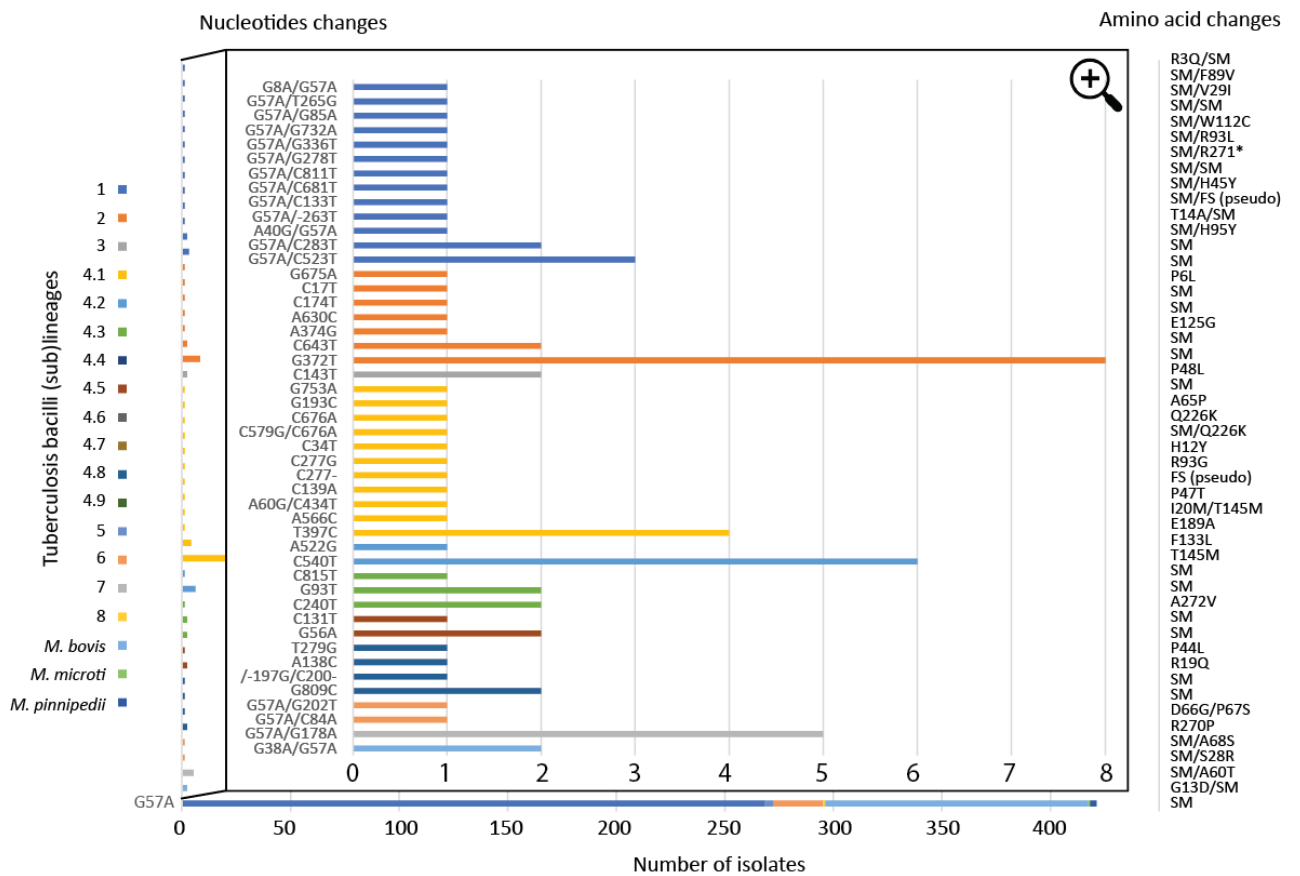
846 **b)** PCR profiles corresponding to a mutant interrupted for the *rv1339* orthologue (PMM280), or the
847 wild-type *M. smegmatis* mc²155 strain (WT), when the indicated primer pairs were used for
848 amplification.

849

a



b



850

851 **Supplementary Figure 13: Distribution of mutations within the *rv1339* orthologue among *MTB***

852 **complex lineages.**

853 **a)** Wild-type sequences (WT), as well as fixed synonymous and non-synonymous mutations were
854 identified in a collection of genome assemblies from 6167 isolates, which were classified in lineages
855 or main sub-lineages based on phylogenetic SNPs and the conventional nomenclature^{66,67}.

856 **b)** Nucleotide (left) and corresponding amino acid (right) changes or combined changes detected in
857 isolates from different (sub-) lineages are shown. The graph in the center is a zoom-in that represents
858 mutations detected in 8 isolates or less. The G57A mutation detected in >400 isolates (bottom) is a
859 synonymous SNP shared by basal lineages of the *MTB* complex (e.g. lineages 1, 5, 6 and animal
860 lineages). SM, synonymous; FS, frameshift.

861 These analyses identified about 30 different non-synonymous mutations in *rv1339* in this dataset,
862 which were phylogenetically linked in all cases, i.e. found in one (sub)lineage only. Of note, two
863 frameshift-causing indels and one non-sense mutation were detected, which were however
864 represented only in 2 isolates at most. These results indicate a strong conservation of the *rv1339* gene
865 among *MTB* isolates.

866 **Supplementary Table 1: Mutations identified in STB-K**

867 **Cycle 3**

Position	Ref	Alt	Mean depth	Ki00	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	D1	D2	D3	D4	D5	Gene (BN42_)	Gene (H37Rv)
1582889	C	T	61	0,6					97,8						95,7	93,6	94,9	95,6	94,5	100,0	100,0	100,0	97,4	95,9	21248	rv1339
4430235	G	-	88	0,4							95,45														90367	rv3839

869

870 **Cycle 6**

Position	Ref	Alt	Mean depth	Ki00	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	D1	D2	D3	D4	D5	Gene (BN42_)	Gene (H37Rv)
1582889	C	T	61	0,6					97,8						95,7	93,6	94,9	95,6	94,5	100,0	100,0	100,0	97,4	95,9	21248	rv1339
4430235	G	-	88	0,4							95,45														90367	rv3839

872

873 **Cycle 9**

Position	Ref	Alt	Mean depth	Ki00	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	D1	D2	D3	D4	D5	Gene (BN42_)	Gene (H37Rv)
355663	T	C	19	14,7	90																				20005	
934262	C	T	80	0,4															100,0						20588	rv0826
1582889	C	T	86	0,6	100,0	94,8	100,0	96,1	94,5	100,0	92,9	97,8	97,7	97,2	100,0	90,4	100,0	100,0	100,0	100,0	100,0	94,1	95,7	95,9	21248	rv1339
1602915	A	G	71	0,4							87,3														intergenic	
2111795	C	G	28	0,9											98,3		100,0	100,0	100,0						30042	rv1787
2111828	A	C	20	1,3											97,4		93,8	100,0	100,0						30042	rv1787
2113560	-	G	41	2,5						88,68		97,83	90,91	92,73											30044	rv1789
2451739	G	A	65	0,6									96,9												30386	rv2075c
2451798	T	C	10	2,9									90,0												40077	rv2162c
238782	G	C	20	3,8				90,0																	50005	rv0192
3952753	G	C	96	0,6																			96,9		50078	rv3419c
4202110	C	T	101	0,3							92,1														90152	rv3649

874

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876 **Cycle 12**

Position	Ref	Alt	Mean depth	Ki00	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	D1	D2	D3	D4	D5	Gene (BN42_)	Gene (H37Rv)
355663	T	C	19	14,7	90																				20005	
934262	C	T	80	0,4															100,0						20588	rv0826
1582889	C	T	86	0,6	100,0	94,8	100,0	96,1	94,5	100,0	92,9	97,8	97,7	97,2	100,0	90,4	100,0	100,0	100,0	100,0	100,0	94,1	95,7	95,9	21248	rv1339
1602915	A	G	71	0,4							87,3														intergenic	
2111795	C	G	28	0,9											98,3		100,0	100,0	100,0						30042	rv1787
2111828	A	C	20	1,3											97,4		93,8	100,0	100,0						30042	rv1787
2113560	-	G	41	2,5						88,68		97,83	90,91	92,73											30044	rv1789
2451739	G	A	65	0,6									96,9												30386	rv2075c
2451798	T	C	10	2,9									90,0												40077	rv2162c
238782	G	C	20	3,8				90,0																	50005	rv0192
3952753	G	C	96	0,6																			96,9		50078	rv3419c
4202110	C	T	101	0,3							92,1														90152	rv3649

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880 **Cycle 15**

Position	Ref	Alt	Mean depth	Ki00	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	D1	D2	D3	D4	D5	Gene (BN42_)	Gene (H37Rv)
240166	C	G	76	0,4																				100,0	10237	rv0193c
382112	C	T	18	0,7										100,0											20026	rv0297
386966	C	A	27	1,0			96,3																		20034	rv0305c
604649	-	T	113	0,0	92,92																				20231	rv0501
1216440	G	A	48	0,0	97,9																				20877	
1511865	G	C	24	1,2														91,7							21173	rv2885c
1582889	C	T	108	0,6	100,0	98,8	100,0	100,0	97,4	100,0	99,3	100,0	95,3	99,0	99,0	98,3	99,2	100,0	97,5	99,0	100,0	97,0	98,6	100,0	21248	rv1339
2111795	C	G	27	0,9											100,0	100,0	97,2	100,0	100,0						30042	rv1787
2111828	A	C	18	1,3											100,0	95,0	100,0	100,0	100,0						30042	rv1787
2112495	A	-	117	0,0	98,25	98,05	92,22	100	98,97																30043	rv1788
2113560	-	G	52	2,5						96		97,73	91,07	92,86											30044	rv1789
2246030	C	T	46	0,0					95,7																30180	
2405288	-	C	99	0,3				95,96																	30346	rv2042c
3440220	C	T	99	1,3			100,0																		40962	rv2962c
3440958	C	G	82	0,7			100,0																		40963	rv2963
3599034	T	G	20	1,8																		95,0			intergenic	
3964805	C	T	66	0,0						100,0	99,0	98,1													intergenic	
3628203	T	C	19	0,0				89,5																	90350	
4407518	T	C	10	0,8				100,0																	90350	

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882

883 **Notes: Position** indicates the mutated position in reference to the NCBI GenBank sequence
 884 FO203509.1, **Ref** and **Alt** refer to the wild-type and mutated alleles detected respectively, and **Ki00**
 885 refers to the frequency of the mutated allele detected in the inoculum. Table cells coloured in grey
 886 indicate the deleted region identified in evolution lineage C after cycle 6. The variants identified in this
 887 region are presumably false positives due to the recombination of *ppe25* and *ppe27*. Column four
 888 mentions mean depth calculated for the 20 sequenced clones from the corresponding cycle at the
 889 indicated position.

890

891 **Supplementary Table 2: Mutations identified in STB-D**

892 **Cycle 3**

Position	Ref	Alt	Mean depth	Di00	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	D1	D2	D3	D4	D5	Gene (BN44)	Gene (H37Rv)
77436	C	T	267	0,0												100,0			100,0						10083	rv0066c
156901	G	A	209	0,0											100,0										10152	rv0125
242924	G	A	213	0,0	99,35	100,0																			10234	rv0200
303690	G	T	222	0,0						100,0	100,0	100,0	100,0	100,0											10283	rv0246
399363	T	G	267	1,7												97,4									10367	rv0327c
443358	C	T	289	29,8	100,0	100,0	100,0	100,0		100,0	99,6	100,0	99,6	100,0	100,0		100,0	100,0		99,6	100,0	100,0	100,0	99,6	10398	rv0357c
1914166	G	A	231	0,0																			100,0		20251	rv1686c
2234377	C	T	163	0,0																100,0					40254	
2354094	C	T	204	0,0																			100,0		40367	rv2075c
2473696	C	T	236	0,0				99,5																	50119	rv2184c
2584587	G	A	228	0,0																100,0					50225	rv2281
2686044	C	T	218	1,3													99,1								intergenic	
2835137	C	A	112	0,8								92,1													50477	
2877937	C	A	177	0,5											99,5				100,0						50519	rv2524c
3746850	A	G	166	0,0																		98,5			70122	rv3109
4056021	G	A	130	0,0												99,5			100,0						110089	rv3594
4156062	A	T	281	2,6												99,6			100,0						120093	rv3696c
4156189	G	T	235	28,1	96,6	99,0	98,0	99,0		98,3	98,2	95,7	97,4	100,0	100,0		98,3	98,8		98,1	99,1	98,0	99,1	97,8	120093	rv3696c
4374850	C	T	171	3,3											100,0				100,0						120307	rv3876

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895 **Cycle 6**

Position	Ref	Alt	Mean depth	Di00	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	D1	D2	D3	D4	D5	Gene (BN44)	Gene (H37Rv)
77436	C	T	267	0,0															100,0			100,0	100,0		10083	rv0066c
242924	G	A	213	0,0	98,1	99,5	99,5	100,0	98,2																10234	rv0200
303690	G	T	222	0,0						100,0	100,0	100,0	100,0	100,0											10283	rv0246
399363	T	G	267	1,7															97,2			96,6	96,9		10367	rv0327c
443358	C	T	289	29,8	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0						100,0	100,0			100,0	10398	rv0357c
966533	C	T	208	0,0																100,0					10955	rv0873
1072346	G	C	209	0,0														88,2							11060	rv0959
1078392	C	T	154	0,7																98,5					11070	rv0969
1364332	C	G	230	0,0																	99,0				11369	rv1225c
1655576	G	A	214	0,0												99,5									20018	rv1464
2392213	G	A	162	0,0																			99,1		50026	rv2101
2877937	C	A	177	0,5															100,0			100,0	100,0		50519	rv2524c
3371167	A	T	187	0,0											100,0	100,0	100,0	100,0							60474	rv2990c
4100847	G	C	225	0,0																99,1					120034	rv3645
4156062	A	T	281	2,6															100,0			100,0	100,0		120093	rv3696c
4156189	G	T	235	28,1	98,9	98,2	99,5	100,0	100,0	100,0	98,4	97,9	99,4	98,2						99,6	98,9			99,4	120093	rv3696c
4338549	A	G	242	9,0											98,3	98,3	96,8	98,1							120265	
4374850	C	T	171	3,3															100,0			100,0	100,0		120307	rv3876

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897

898 **Notes: Position** indicates the mutated position in reference to the NCBI GenBank sequence

899 **NC_019950.1, Ref** and **Alt** refer to the wild-type and mutated alleles detected respectively, and **Di00**

900 refers to the frequency of the mutated allele detected in the inoculum. The light orange and green

901 boxes indicate SNPs segregated together in different evolution lineages. Most of these SNPs were

902 detected above background in the inoculum supporting the conclusion that the STB-D inoculum, used

903 to start the experimental evolution, contains at least three genetic profiles.

Supplementary Table 3: *M. canettii*, *MTB*, and *M. smegmatis* strains used in this study

Strain	Description
STB-K	<i>M. canettii</i> CIPT 140010070
STB-D	<i>M. canettii</i> CIPT 140060008
STB-A	<i>M. canettii</i> CIPT 140060001
STB-J	<i>M. canettii</i> CIPT 140070017
STB-L	<i>M. canettii</i> CIPT 140070008
KC6A1	Clone isolated from lineage A following 6 cycles of experimental evolution with STB-K
KC6A2	Clone isolated from lineage A following 6 cycles of experimental evolution with STB-K
KC6B1	Clone isolated from lineage B following 6 cycles of experimental evolution with STB-K
KC6B2	Clone isolated from lineage B following 6 cycles of experimental evolution with STB-K
KC6C1	Clone isolated from lineage C following 6 cycles of experimental evolution with STB-K
KC6C2	Clone isolated from lineage C following 6 cycles of experimental evolution with STB-K
KC6D1	Clone isolated from lineage D following 6 cycles of experimental evolution with STB-K
KC6D2	Clone isolated from lineage D following 6 cycles of experimental evolution with STB-K
KC9A1	Clone isolated from lineage A following 9 cycles of experimental evolution with STB-K
KC9B1	Clone isolated from lineage B following 9 cycles of experimental evolution with STB-K
KC9C1	Clone isolated from lineage C following 9 cycles of experimental evolution with STB-K
KC9D1	Clone isolated from lineage D following 9 cycles of experimental evolution with STB-K
KC12A1	Clone isolated from lineage A following 12 cycles of experimental evolution with STB-K
KC12B1	Clone isolated from lineage B following 12 cycles of experimental evolution with STB-K
KC12C1	Clone isolated from lineage C following 12 cycles of experimental evolution with STB-K
KC12D1	Clone isolated from lineage D following 12 cycles of experimental evolution with STB-K
KC15A1	Clone isolated from lineage A following 15 cycles of experimental evolution with STB-K
KC15B1	Clone isolated from lineage B following 15 cycles of experimental evolution with STB-K
KC15C1	Clone isolated from lineage C following 15 cycles of experimental evolution with STB-K
KC15D1	Clone isolated from lineage D following 15 cycles of experimental evolution with STB-K
DC6A1	Clone isolated from lineage A following 6 cycles of experimental evolution with STB-D
DC6B1	Clone isolated from lineage B following 6 cycles of experimental evolution with STB-D
DC6C1	Clone isolated from lineage C following 6 cycles of experimental evolution with STB-D
DC6D1	Clone isolated from lineage D following 6 cycles of experimental evolution with STB-D
KC9A1::EV	KC9A1 complemented with a derivative of the integrative plasmid pMV361
KC9C1::EV	KC9C1 complemented with a derivative of the integrative plasmid pMV361
KC9C1::K	KC9C1 complemented with integrative plasmid pWM361 containing <i>ppe25-ppe27</i> of STB-K
D::EV	STB-D complemented with a derivative of the integrative plasmid pMV361
DΔ <i>ppe25-ppe27</i> (PMM304)	STB-D deleted for the <i>ppe25-ppe27</i> region
DΔ <i>ppe25-ppe27</i> ::EV	PMM304 complemented with a derivative of the integrative plasmid pMV361
DΔ <i>ppe25-ppe27</i> ::D	PMM304 complemented with plasmid pWM385 containing <i>ppe25-ppe27</i> of STB-D
DΔ <i>ppe25-ppe27</i> ::K	PMM304 complemented with plasmid pWM361 containing <i>ppe25-ppe27</i> of STB-K
DΔ <i>ppe25-ppe27</i> ::C1	PMM304 complemented with plasmid pWM386 containing <i>ppe25-ppe27</i> of KC9C1
DΔ <i>rv1339</i> (PMM305)	STB-D interrupted for the gene <i>BN44_11505</i> (<i>rv1339</i> orthologue)
:: <i>rv1339A1</i> (PMM305::pAH01H)	PMM305 complemented with plasmid pAH01H containing <i>BN42_21248</i> of KC9A1 (mutated <i>rv1339</i>)
:: <i>rv1339K</i> (PMM305::pAH02H)	PMM305 complemented with plasmid pAH02H containing <i>BN42_21248</i> of STB-K (wild-type <i>rv1339</i>)
H37Rv	<i>MTB</i> Lineage 4
Erdman	<i>MTB</i> Lineage 4
IO367	<i>MTB</i> Lineage 1
HN878	<i>MTB</i> Lineage 2
mc ² 155	<i>M. smegmatis</i> mc ² 155
smegΔ <i>rv1339</i> (PMM280)	mc ² 155 interrupted for the gene <i>MSMEG_4902</i> (<i>rv1339</i> orthologue)
smeg:: <i>rv1339K</i>	PMM280 complemented with plasmid pAH02H containing <i>BN42_21248</i> of STB-K (wild-type <i>rv1339</i>)
smeg:: <i>rv1339A1</i>	PMM280 complemented with plasmid pAH01H containing <i>BN42_21248</i> of KC9A1 (mutated <i>rv1339</i>)

906 **Supplementary Table 4: Plasmids used in this study**

907

Name	Description
pWM355	<i>BN44_11505</i> gene from STB-D cloned into pJET
pWM359	<i>BN44_11505</i> gene disrupted by a <i>km</i> cassette carried by the pJET vector
pWM361	<i>ppe25-ppe27</i> fragment from STB-K cloned into a derivative of pMV361 containing a <i>hyg</i> resistance gene
pWM386	<i>ppe25-ppe27</i> fragment from KC9C1 cloned into a derivative of pMV361 containing a <i>hyg</i> resistance gene
pWM385	<i>ppe25-ppe27</i> fragment from STB-D cloned into a derivative of pMV361 containing a <i>hyg</i> resistance gene
pAH01H	<i>BN42_21248</i> gene from KC9A1 cloned into a derivative of pMV361 containing a <i>hyg</i> resistance gene
pAH02H	<i>BN42_21248</i> gene from STB-K cloned into a derivative of pMV361 containing a <i>hyg</i> resistance gene
pAH06	DNA fragments overlapping the N-terminal and C-terminal parts of <i>MSMEG_4902</i> cloned into pJET
pAH10	<i>MSMEG_4902</i> gene disrupted by a <i>km</i> cassette carried by the pJQ200 vector

908

909 **Supplementary Table 5: Primers used in this study**

Name	Sequence
39a	GCGTTGGTGATCGCCTGC
39b	CGGTGTCGGTAGCGACGA
39c	GCGACGATCTGGTCGGC
39d	CCCTGAATCTCTACCAGGG
39e	CCGTGCTCGGATGCTCC
39f	CGTCAGCAGCAGCTCACG
km1	GTCTGACGCTCAGTGGAAC
km2	TTCAGGTGGCACTTTTCGG
k1	TTTCACGAAGTGCATGAACAATAAACTGTCTGC
k2	TTTCACTCTGTGCATGAATTAATTCTTAGAAAACT
esx1	TTTCACAAAAGTGGCAAACGATCGTTTACGAG
esx2	TTTCACTTCGTGCCGAAGGTCAGCTGCTGT
esx5	TTTCACAGAGTGCTGCAGATAGCGATCGAC
esx6	TTTCACTTGTGCAGCATGTTACCGATG
esx7	ATCCTCGAGGAGGACGAC
esx8	CGTAGTTGTTGGCGTCGC
26a	ATCATATGGATTTTGGGGCGTTGC
26b	ATACTAGTCTATCCGGCAAGGGTGG
4902A	GGGTGTCGTGGCAGGGA
4902B	P-ATCGCACACTGCTAGCT
4902C	P-ATCGGCTGACCTGCGCTCG
4902D	GCCACGCACGCCAGCC
4902E	CCGAACGCAAGGCACCGG
4902F	GCGTTCTCCTCGAAAGTCG
4902G	CCGAAGTCGATGACCATCGG
kmF	GCCATCCTATGGAAGTCC
kmR	GCCTAGAGCAAGACGTTCC
1339G	ATCATATGCGTCGATGTATTCCGC
1339H	ATACTAGTCTAGCCGGCTCGC
esxA	ATTCTAGAGGGTTCTCATGCGCTCAAG
esxB	ATAAGCTTGGGACGATCAATTGGGCAT
esxC	ATTCTAGACGCTCAAGTCTTTCGTGTCT
esxD	ATAAGCTTCGGTCGATCGCTATCTGC

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911

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929

930 **Author contributions**

931 C.G., R.B. and P.S. planned the project. A.C.A., C.A.-D. and C.G. designed the experiments. A.C.A., W.
932 M., C.G., F.M., A.H., C.A.-D., A.P., R.A., A.P., W.F., C.B. and C.G. performed the experiments. A.C.A.,
933 W.M., C.G., A.V., A.H., C.A.-D., A.P., R.A., A.P., R.B., P.S. and C.G. analysed the data. A.C.A., C.A.-D.,
934 R.B., P.S. and C.G. contributed to the manuscript.

935

936 **Conflict of interest**

937 The authors declare no competing financial interests.

938

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