DrrS, a small non-coding *Mycobacterium tuberculosis* RNA, regulates the whole genome expression shifts consistent with adaptations for survival within host macrophages

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Abstract

Small non-coding RNAs play a significant role in regulation of bacterial transcription and translation. Their expression in response to external factors is important for the adaptation of bacteria to changing environmental conditions. We investigated the expression of DrrS, a small noncoding RNA of *Mycobacterium tuberculosis*, in the mouse model *in vivo*, in the *ex vivo* model based upon infected macrophages, and in bacterial cultures, and demonstrated its significant contribution to host-pathogen interactions. Activation of the host immune system triggers NO-inducible up-regulation of DrrS in macrophage-engulfed mycobacteria. Constitutive overexpression of DrrS in cultured mycobacteria launches a broad spectrum of shifts in the bacterial transcriptome profile very similar to those reported for *M. tuberculosis* adaptation to hostile intra-macrophage environment, and providing defense against oxidative and NO stresses. In addition, we observed dramatic up-regulation of genes for the PE/PPE proteins and proteins of the ESX-1 and ESX-5 secretion systems. Taken together, our results suggest a direct involvement on this small RNA in the interplay between mycobacteria and the host immune system during infectious process.

Author summary

Pathogenic mycobacteria, including *Mycobacterium tuberculosis*, are able to survive within host macrophages. In attempt to eliminate intracellular mycobacteria, innate and acquired immune responses of the host activate a number of effector reactions to achieve effective intracellular mycobacterial killing. Mycobacteria, in turn, evolved a plethora of molecular mechanism providing successful escape from host immunity, involving several metabolic pathways allowing transition to dormancy – the state of slow-to-no replicative activity and an increased resistance to external stresses. These mechanisms remain poorly characterized. Small non-coding bacterial RNAs are

40 expressed in response to external factors and play an important role in adaptation of bacteria to
41 changing environmental conditions and escape from host immune responses. We investigated DrrS,
42 a small non-coding RNA of *Mycobacterium tuberculosis*, in the mouse TB model *in vivo*, in infected
43 macrophages *ex vivo* and in bacterial culture, and demonstrated that DrrS up-regulation strictly
44 follows activation of the host immune defense. We established the strain of *M. tuberculosis*
45 overexpressing DrrS in culture and found that DrrS contributes to mycobacterial resistance to
46 reactive intermediates and activation of dormancy-associated genes, thus participating in bacterial
47 metabolic adaptations and interactions with the host immune system.

48 Introduction

49 Tuberculosis (TB), a chronic disease caused by *Mycobacterium tuberculosis*, takes nearly 2
50 million lives annually [1]. *M. tuberculosis* persistence in the infected host involve several stages and
51 may have different manifestations: initial infection followed by acute, semi-acute or chronic
52 diseases; latent infection characterized by the presence of viable bacteria with slow-to-no level of
53 replication and the lack of clinical manifestations; and transition from the latent state to reactivation
54 processes (reviewed in [2, 3]). The spectrum of the disease manifestations depends upon a dynamic
55 balance between protective host responses and defensive strategies of *M. tuberculosis*. Identification
56 of molecular mechanisms of *M. tuberculosis* adaptation to the host immune defense during its
57 persistence within macrophages and other phagocytes is an important scientific and medical
58 problem.

59 Long co-evolution of *M. tuberculosis* and its human host allowed the pathogen to develop
60 strategies that can effectively combat host defense systems. Regulatory proteins, non-coding RNAs
61 and their targets constitute complex adaptive metabolic networks that allow the pathogen to resist
62 host response at different stages of infection. Bacterial small RNAs participate in regulation of

63 transcription and translation by affecting the level of gene expression and mRNA stability. Mostly,
64 small RNAs are expressed in response to the external factors, helping bacteria to adaptively react to
65 the changing environmental conditions and regulate the key stages of pathogenesis (see recent
66 reviews [4-6]).

67 Application of the high throughput sequencing and computer algorithm approaches allowed
68 identification of dozens of small RNAs in mycobacterial species [7, 8]. Several *in vitro* studies have
69 elucidated the functioning of small RNAs in *M. tuberculosis* [9-12]. However, dissecting the role of
70 a particular small RNA in mycobacterial physiology appeared to be difficult, especially in *in vivo*
71 settings.

72 One of such RNAs, DrrS (DosR-associated sRNA, ncRv11733, MTS1338), is highly
73 expressed during the stationary phase of growth [13], and the dormancy state [14]. This small RNA
74 is present only in genomes of highly pathogenic mycobacteria and is highly conservative. *In vitro*
75 experiments demonstrated that its transcription is controlled by the transcriptional regulator DosR
76 and is activated under hypoxic and NO-induced stress conditions [11], suggesting that DrrS may
77 play a role during the stable phase of infection, when host responses confronts mycobacterial
78 multiplication more or less successfully. Indeed, we and others demonstrated a striking increase in
79 the DrrS transcription in animal models of chronic infection [9, 15]. Thus, it seems likely that DrrS
80 triggers adaptive biochemical cascades for intracellular bacterial persistence.

81 Here, we characterize dynamic changes in the DrrS expression in mycobacteria obtained
82 from the lungs of genetically susceptible and resistant TB-infected mice and provide a direct
83 evidence that the level of expression is regulated by the IFN-γ-dependent NO production. Using
84 high-throughput technologies, we describe the changes in the genome transcription profile that
85 accompany an increased DrrS transcription by mycobacteria. Overexpression of DrrS has led to the

86 transcriptional shifts similar to those previously observed during mycobacterial persistence in
87 macrophages: an enhanced expression of several transcription factors, an up-regulated expression of
88 the systems involved in superoxide- and NO-induced stress defense, a metabolic switch to anaerobic
89 respiration, as well as down-regulation of the cell division- and amino acid synthesis-linked systems.
90 Remarkably, we observed a dramatic up-regulation in the expression of genes involved in the
91 synthesis of PE/PPE proteins, whose functioning, remaining elusive, is very likely involved in the
92 interplay with the host immune system. Overall, we have identified the DrrS modulon that tunes the
93 network of interacting regulons, which, in turn, activates molecular mechanisms necessary for the
94 *M. tuberculosis* inter-macrophage survival.

95 Results and Discussion

96 DrrS expression in TB-infected mice

97 Earlier it was demonstrated that several *M. tuberculosis* non-coding RNAs, including DrrS,
98 are highly transcribed *in vivo* [15, 16]. We investigated the transcription profile of DrrS in
99 mycobacteria extracted from the mouse lungs in dynamics, from the initial to the terminal phases of
100 infection. Aerosol infection with low doses of *M. tuberculosis* leads to a chronic and temporary
101 effectively controlled infection in genetically resistant B6 mice, whilst in susceptible I/St mice fatal
102 pulmonary pathology develops relatively rapidly. Differences in mycobacterial lung CFU counts
103 between I/St and B6 mice reach about 1.5 logs during the first 2 months post challenge and remain
104 stable until I/St mice succumb to infection [17]. We profiled the DrrS expression along the
105 infectious course at 2 weeks (CFU numbers are similar in both strains, adaptive immune response
106 has not developed yet), 6 - 10 weeks (well-established adaptive immune response, significant inter-
107 strain differences in CFU numbers, bacterial transcriptome reflects adaptation), and 12 months (only
108 B6 mice survive, the late infection phase) post challenge (Fig 1A). For each time point, total RNA

109 was isolated from the lungs, and the level of DrrS expression was determined using quantitative
110 real-time PCR (Fig 1B). The highest level of expression was observed at week 10 post-challenge. In
111 B6 mice, it remained high throughout the experiment, although slowly decreased at the very late
112 phase of infection. At week 10 of infection, when I/St mice start to lose control of the disease
113 progression, the level of DrrS expression in their lung mycobacterial population was significantly
114 higher ($P < 0.01$) than that in more resistant B6 mice (Fig 1B). This may reflect an attempt of
115 mycobacteria residing in the I/St lungs to rapidly turn down metabolism, facing severe functional
116 failure in the surrounding tissue, providing aggressive, highly hypoxic and necrotic conditions to a
117 large proportion of mycobacterial population [18]. Overall, at the stage of flourishing infection, the
118 DrrS expression level in the lung-residing bacteria was more then 1000-fold higher compared to its
119 expression level during stationary phase of growth *in vitro* [15].

120 **The expression of DrrS is regulated by iNOS**

121 Our *in vivo* experiments demonstrated that the level of DrrS expression peaks at the stage of
122 fully developed adaptive immune response against mycobacteria. At this stage, B6 mice display
123 significantly higher levels of IFN-γ production compare to their I/St counterparts [18, 19]. Since
124 IFN-γ is the key cytokine activating macrophages for intracellular mycobacterial killing [20], we
125 compared DrrS expression levels in infected peritoneal B6 macrophages, either activated by the
126 external IFN-γ or not. At 2, 4, and 24 hours of macrophage infection, total RNA was isolated and
127 the level of DrrS expression was assessed in dynamics (Fig 2A). In IFN-γ-activated macrophages,
128 DrrS expression was significantly ($P < 0.001$, unpaired *t*-test) higher than in control macrophages at
129 every time point, and the difference reached more than 10-fold at 24 hours post infection. Thus, pre-
130 activation of macrophages with IFN-γ induced up-regulation of the DrrS expression in engulfed
131 mycobacteria. Given that the efficacy of mycobacterial killing by peritoneal macrophages

132 significantly increases in the presence of IFN-γ [21], this result suggests that the level of DrrS
133 expression correlates with the level of pressure emanating from infected macrophages.

134 Since the active nitrogen oxidative derivatives serve as the major trigger of DrrS
135 transcription activation in vitro [11], we decided to test whether this is true for the infected
136 macrophage system. As far as nitrogen oxidative derivatives production in macrophages depends
137 upon inducible NO-synthase (iNOS2), we compared mycobacteria-infected IFN-γ-activated and
138 control macrophages cultured in the presence or absence of L-NIL [N6-(1-iminoethyl)-L-lysine
139 hydrochloride] – a selective inhibitor of iNOS2. After 24-h incubation, total RNA was isolated and
140 analyzed (Fig 2B). Inhibition of NO production in IFN-γ-activated macrophages completely
141 abrogated elevation in the DrrS expression, reducing it to the control levels. L-NIL itself did not
142 affect DrrS expression in pure *M. tuberculosis* cultures. Thus, in macrophages, nitrogen oxidative
143 derivatives are an important trigger of DrrS expression, and this regulatory pathway seems to be
144 highly specific, since the expression of MTS0997, another small RNA abundantly present in
145 dormant mycobacteria [14], was not dependent upon the presence of L-NIL (Fig 2C).

146 In the *in vitro* system, DrrS expression was shown to be induced by the transcription
147 regulatory protein DosR [11], thus we assessed the dynamics of DosR transcription in our co-culture
148 system (Fig 2D). Remarkably, the level of DosR transcription in mycobacteria engulfed by activated
149 and control macrophages remained extremely low at the early stages of infection, indicating that the
150 difference in an early up-regulation of DrrS in mycobacteria residing in activated and control
151 macrophages was DosR-independent. In IFN-γ-activated macrophages, an elevation of the DosR
152 transcription was observed much later, whereas in control macrophages it remained at a very low
153 level even at 24 hours post infection (Fig 2D). Thus, regulation of DrrS transcription by *M.*
154 *tuberculosis* in culture and within the host cells is different.

155 **Transcriptome changes induced by the DrrS overexpression are consistent with mycobacterial**
156 **adaptation to stress conditions**

157 The involvement of DrrS in mycobacterial physiology suggested by the results obtained in
158 infected mice and IFN-γ-activated infected macrophages prompted us to create an experimental
159 system allowing the assessment of shifts in *M. tuberculosis* transcription profile resulting from
160 elevation of the DrrS expression level. To this end, we established a new *M. tuberculosis* strain
161 overexpressing DrrS (hereafter – OVER) and compared transcriptomes of this and of the control
162 strain (hereafter – pMV, transfected with the "empty" pMV261 vector) at the phase of exponential
163 growth in liquid culture using RNA-seq approaches. The DrrS expression level in the OVER strain
164 was more than 10-fold higher compared to the pMV strain, as confirmed by qRT-PCR (S1 Fig).

165 Mapping the processed reads against the reference *M. tuberculosis* genome (AL123456.3,
166 http://www.ncbi.nlm.nih.gov/), provided the following numbers of mapped reads: 22.6 x 10^6 for the
167 OVER strain (98% of all reads) and 11.8 x 10^6 for the pMV strain (98% of all reads). The
168 percentage of the protein-encoding part of the genome deduced from all reads mapped comprised
169 70% for pMV (8.2 x 10^6 reads) and 57% for OVER (12.8 x 10^6 reads). Statistical results were
170 visualized as transcription profiles using the Artemis genome browser [22].

171 Using the software package edgeR [23], we identified genes the expression of which
172 differed between the two strains. Overall, 235 genes were found to be differently expressed under
173 the DrrS overexpression condition (S1 Table), with 88 genes demonstrating a decreased and 147 an
174 increased expression. Further ascribing of genes to functional categories was performed using the
175 Mycobrowser database. The most prominent differentially expressed genes, with the expression
176 change module exceeding four, are displayed in Fig 3.

177 **Oxidative and NO-stress defense.** The most striking (>200-fold) difference between the expression
178 levels in the OVER and pMV strains was observed for the *glbN* gene encoding truncated
179 hemoglobin N. This protein protects *M. tuberculosis* from the oxidative host response in
180 macrophages by metabolizing NO into harmless nitrate [24, 25], preventing not only inhibition of
181 respiration, but also NO-mediated modification of thiol and metal centers of several enzymes and
182 regulatory proteins. The *glbN* gene is co-transcribed with the *lprI* gene (up-regulated 10-fold in
183 OVER strain) encoding the LprI lipoprotein which acts as a lysozyme inhibitor [26]. Binding of
184 LprI expressed on bacterial surface with lysozyme molecules inhibits activity of the latter and
185 supports the integrity of bacterial cells, allowing macrophage invasion. Invasion is facilitated by the
186 presence of glycosylated HbN, which protects bacilli from the macrophage-generated reactive
187 nitrogen derivatives. These two surface proteins work in concert and constitute a well-evolved
188 arsenal to disarm two important host defense mechanisms – NO and lysozyme production.

189 DrrS overexpression resulted in a significant elevation in the expression of four genes
190 involved in protection against oxidative stress. Firstly, the expression of genes for key components
191 of the peroxynitrite reductase/peroxidase antioxidant system, AhpC and AhpD, was ~10-fold higher
192 in the OVER compared to the pMV strain. AhpC is the NADH-dependent peroxidase whose thiol
193 groups in the active center are oxidized to form disulfide bridges whilst catalyzing H_2O_2 and organic
194 peroxides reduction to the water and alcohols, respectively [27]. In macrophages, AhpC catalyzes
195 the breakdown of the $ONOO^-$ group by forming an antioxidant complex bearing peroxidase and
196 peroxinitrite reductase activities, in conjunction with the dihydrolipoamide dehydrogenase (Lpd),
197 dihydrolipoamide succinyltransferase (SucB) and thioredoxin-like (AhpD) proteins [28]. Up-
198 regulation of the *ahpC* and *ahpD* transcription was previously reported for *M. tuberculosis*
199 experiencing the NO-mediated oxidative stress in macrophages [29]. Secondly, we observed up-

200 regulation of the genes for the peroxidase BpoA (~9-fold) and the ferric uptake regulation protein
201 FurA (4-fold). These genes are also involved in the oxidative stress response [30].

202 It was previously reported that in persisting *M. tuberculosis* the nuoA-N subunits of the
203 NADH-dehydrogenase complex are repressed [31]. Our results also demonstrate a 4-fold down-
204 regulation of transcription of the genes for all subunits in the OVER strain.

205 In line with the respiratory chain inhibition, overexpression of DrrS has led to a pronounced
206 up-regulation of genes associated with nitrogen metabolism. Under hypoxic conditions, nitrates
207 protect *M. tuberculosis* by replacing oxygen as a final electron acceptor in the respiratory chain [32,
208 33]. While assessing the expression of genes encoding four subunits of the NarGHJI nitrate
209 reductase, we observed the highest (4-fold) elevation in the expression of gene for NarG and 2-3-
210 fold increases in the expression of genes for other three. However, there is evidence that this nitrate
211 reductase is constitutively expressed at sufficiently high levels upon nitrate assimilation under both
212 anaerobic and aerobic conditions [34]. Another way of nitrogen assimilation by *M. tuberculosis* is
213 capturing organic amine-containing compounds of the host through a variety of transporters. The
214 gene for one of these transporters, the amino acid permease RocE [35], was 6-fold up-regulated in
215 the OVER strain. For further reduction of nitrites to ammonium, mycobacteria use the NADH-
216 dependent nitrite reductase NirBD [33], whose expression was dramatically (~40-fold) elevated in
217 the OVER strain. In addition, the expression of genes for NarU, NarK1 and NarK3, nitrite extrusion
218 transporters of unrecovered nitrites from bacterial cells, was also significantly increased (6, 15 and
219 11-fold, respectively).

220 **Amino acid biosynthesis.** During *M. tuberculosis* dormancy, protein transcription and synthesis is
221 profoundly inhibited due to a reduced expression of genes involved in biosynthesis of amino acids

222 and cofactors [36]. In line with this general tendency, we observed a ~4-fold reduction in the
223 expression of genes involved in synthesis of several amino acids (tryptophan, arginine, cysteine,
224 leucine, and alanine) and vitamin B6 in the OVER strain (Fig 3).

225 **Regulators.** Overexpression of DrrS significantly changed transcription of regulatory genes
226 increasing 10 and decreasing 7 of them (Fig 3). Among up-regulated, was the *Rv0079* (8-fold)
227 considered as a part of the DosR regulon [30]. In *E. coli* and *M. bovis* corresponding protein
228 significantly inhibits cell growth, apparently by the interaction with the 30S ribosome subunit, thus
229 inhibiting translation – the phenotype typical for transition to dormancy [37]. Another regulator of
230 transcription, whose expression was increased (8-fold), is the *Rv0081* gene – one of the two key
231 transcription factors mediating early response to hypoxia [38]. As an important "metabolic hub"
232 working in concert with other transcription regulators, *Rv0081* is associated with the processes of
233 lipid metabolism, protein degradation and cholesterol biosynthesis. In addition, our data confirmed
234 up-regulation of four of seven predicted and verified Rv0081 targets: *Rv2329c, Rv2699c, Rv1057c*
235 and *Rv3619c* [39]. Another up-regulated (~5-fold) transcription factor-encoding gene, *Rv2034*, is
236 known to be involved in the induction of transcription of the two major stress response genes, *dosR*
237 and *phoP* [40].

238 Evaluation of the expression of two transcription factors from the WhiB family provided a
239 22-fold increase in the *whiB3* expression, but a 4-fold decrease for *whiB2*. Up-regulation of the
240 transcriptional regulator WhiB3 in mycobacteria has been demonstrated during responses to a
241 number of stressful conditions, including low pH levels and drug pressure [41]. WhiB3 interacts
242 with the sigma factor RpoV, possibly playing a role in the disease progression and development of
243 tissue pathology [42]. In addition, WhiB3 is redox-dependent and undergoes activation in
244 mycobacteria residing in macrophages [43]. Under conditions of acidic stress, WhiB3 controls the

245 expression of multiple genes, including genes for complex lipids syntheses (*pks2*, *pks3-pks4*),
246 transcription factors (*whiB7*, *Rv0827c*, *Rv3183*), amino acid biosynthesis (*metH*, *metK*, *sahH*, and
247 *leuB*), ESX-1 secretion system (*Rv3614c*, *Rv3616c*) and nitrite transport (*narK1*). Remarkably,
248 unlike up-regulated WhiB3 involved in the stress/pathology metabolism, down-regulated WhiB2
249 controls the expression of several genes involved in cell division processes [44]. WhiB2 down-
250 regulation balanced by the WhiB3 up-regulation was observed in dividing intracellular bacteria [45,
251 46].

252 Another up-regulated (~5-fold) transcription factor, Rv2034, is involved in the transcription
253 induction of two major stress response genes, *dosR* and *phoP* [40]. Indeed, in our system *dosR*
254 transcription was increased about 3-fold in the OVER strain.

255 **PE/PPE system.** Among the genes encoding proteins belonging to the PE/PPE superfamily, the
256 transcription levels of 26 genes significantly (at least 4-fold) differed between the OVER and pMV
257 strains. Remarkably, an elevated transcription in the OVER strain was observed for 24 genes,
258 whereas only two genes (the tandem *Rv3477-3478* encoding the PE31 and PPE60 proteins) were
259 down-regulated.

260 PE and PPE proteins influence profoundly the survival of mycobacteria within host
261 macrophages by increasing bacterial resistance to oxidative, acidic and sodium dodecyl sulfate
262 stresses [47]. There is also an evidence that a group of these proteins expressed on bacterial surface
263 possesses antigenic properties, induces host immune responses and modifies immunologic and
264 inflammatory processes in many different ways [48-50]. Thus, the PPE25 protein (8-fold
265 transcriptional increase in the OVER strain) is believed to be involved in inhibition of phagosome
266 maturation [51]. PPE18 (8-fold up-regulation) was shown to bind TLR2 and trigger secretion of the

267 anti-inflammatory cytokine IL-10 in macrophages via activation of p38 MAPK [52], and PPE2 (4-
268 fold up-regulation) inhibits the reactive forms of nitrogen by inhibiting transcription of the gene for
269 iNOS [53].

270 At the genome level, some genes for PE/PPE proteins are clustered within the mycobacterial
271 ESX secretion system loci. In the experimental system based upon infection of human macrophages
272 with virulent *Mycobacterial marinum* it was shown that a few PE/PPE proteins are transported via
273 the ESX-5 secretion system. Moreover, loss-of-function mutations in the *ESX-5* locus resulted in the
274 inhibition of secretion of different cytokines dependent upon signaling of several TLR via Myd88
275 adaptive molecule in macrophages [54, 55]. In the OVER strain, many genes for the PE/PPE
276 components of ESX-5 (*PPE25, PE18, PPE26, PPE27, PE19*) were up-regulated compared to the
277 pMV strain, as were the *esxN* and *esxM* genes encoding putative ESAT-6-like proteins. Similar
278 expression shifts were found for the *PE13*, *PPE18*, *esxK* and *esxL* genes for the components of
279 another ESX secretion system, ESX-1. Recently, it was reported that the esxL protein down-
280 regulates the expression of CIITA/MHC-II by inducing hyper-methylation in histone H3 lysine9,
281 leading to inhibition of antigen presentation of mycobacterial antigens to the $CD4^+$ T lymphocytes
282 [56].

283 Taken together, the results obtained in the present study demonstrate tight connections
284 between the expression of small non-coding DrrS RNA and capacity of *M. tuberculosis* to respond
285 against stressful conditions *in vitro* and *in vivo*. Remarkably, overexpression of DrrS leads to the
286 transcriptional *in vitro* pattern similar to that reported for mycobacteria residing within host
287 macrophages. This particularly concerns mycobacterial responses to oxidative stresses. As the DrrS
288 expression displays a wide range of influence onto anti-stress transcriptional responses, we suggest
289 that this regulator works as a novel "DrrS modulon" in the mycobacterial genome.

290 **Conclusion**

291 In mycobacteria, small RNAs have been discovered much later then in many other bacterial species
292 [7], and their functions mostly remain unknown. However, the growing body of evidence obtained
293 *in vitro* and *in vivo* indicates that the DrrS small RNA may play a role in TB dormancy/latency [13].
294 In the present work, we demonstrate that up-regulation of DrrS *in vivo* is NO-inducible and parallels
295 iNOS activation in infected macrophages. *In vitro*, overexpression of DrrS in *M. tuberculosis*
296 triggers remarkable shifts in bacterial transcriptome, consistent with the activation of NO-induced
297 and other oxidative stress defense mechanisms, thus mimicking persistence within host
298 macrophages (Fig 4). Thus, elevated amounts of DrrS initiate changes in mycobacterial
299 transcriptional profile that are required to increase the resistance to stressful environmental
300 conditions. We suggest that a dramatic up-regulation of the genes for PE/PPE proteins and ESX-1
301 and ESX-5 secretion systems observed under DrrS overexpression conditions reflects the
302 importance of this small RNA for a broad range of stress adaptations *in vivo* and *in vitro*.

303 ## Materials and methods

304 **Ethics Statement**

305 Mice were maintained under conventional, non-SPF conditions at the Animal Facilities of
306 the Central Institute for Tuberculosis (CIT, Moscow, Russia) in accordance with the guidelines from
307 the Russian Ministry of Health # 755, and under the NIH Office of Laboratory Animal Welfare
308 (OLAW) Assurance #A5502-11. All experimental procedures were approved by the Bioethics
309 Committee of the Central Research Institute of Tuberculosis (IACUC), protocols # 2, 3, 7, 8, 11
310 approved on March 6, 2016.

311 **Bacterial strains, media and growth conditions**

312 For *in vitro* experiments, *M. tuberculosis* H37Rv, pMV and OVER *M. tuberculosis* strains
313 were initially grown from frozen stocks for 10 days in Sauton medium containing (per liter): 0.5 g
314 KH_2PO_4, 1.4 g $MgSO_4 \times 7H_2O$, 4 g L-asparagine, 60 ml glycerol, 0.05 g ferric ammonium citrate, 2 g
315 sodium citrate, 0.1 ml 1% $ZnSO_4$, pH 7.0 (adjusted with 1M NaOH), and supplemented with ADC
316 growth supplement [57], 0.05% Tween 80 and 50 µg/ml kanamycin (Sigma-Aldrich, St Louis, MO,
317 USA) at 37°C with agitation (200 rpm). The starter cultures were inoculated into fresh medium (the
318 same composition) and incubated for another 7-10 days until its optical density at 600 nm (OD_{600})
319 reached 1.2 (early log-phase).
320 For cloning procedures, *Escherichia coli* DH5α was grown in Luria Bertani (LB) broth and
321 LB-agar. When required, antibiotics were added at the following concentrations: kanamycin (Sigma-
322 Aldrich, St Louis, MO, USA), 50 µg/ml (*M. tuberculosis*); ampicillin (Invitrogen, Carlsbad, CA,
323 USA), 100 µg/ml (*E. coli*).

324 **M. tuberculosis OVER and pMV (control) strains establishment**

325 The DrrS gene containing vector was constructed on the basis of the pMV261 [58] as
326 described in Ref. [14]. The plasmid was transferred into mycobacteria by electroporation. DrrS
327 overexpression was confirmed by qPCR. The control strain was produced using an empty pMV261
328 vector.

329 **RNA extraction from cultured mycobacteria**

330 Bacterial cultures were grown up to the early log phase, rapidly cooled on ice, centrifuged,
331 and total RNA was isolated by phenol-chloroform extraction after cell disruption with BeadBeater
332 (BioSpec Products, Bartlesville, OK, USA) as previously described [59]. After isolation, RNA was
333 treated with Turbo DNase (Life Technologies, Carlsbad, CA, USA) to remove traces of genomic

334 DNA, and purified with the RNeasy mini kit (Qiagen, Venlo, Netherlands). Amounts and purity of
335 RNA were determined spectrophotometrically; integrity of RNA was assessed in 1% agarose gel.

336 **Libraries for RNA-seq and RNA-seq data analyses**

337 RNA samples were depleted of 16S and 23S rRNA using Ribo-Zero rRNA Removal Kit for
338 Gram-Positive Bacteria (Epicentre, Madison, WI, USA). Sequencing libraries were generated using
339 the resulting ribosomal transcript-depleted RNA and the TruSeq Stranded mRNA Library Prep Kit
340 (Illumina, San Diego, CA, USA) according to the manufacturers' protocol. Sequencing was
341 performed using the Illumina HiSeq 2500 as the double-ended 100 nt-long reads. Experiments were
342 performed in triplicates.

343 After quality control evaluation and trimming of bad qualitative reads the reads were mapped
344 on the reference *M. tuberculosis* genome (AL123456.3, http://www.ncbi.nlm.nih.gov/) by Bowtie2
345 [60]. The alignment was performed with the "local" option, which allows leaving 5' and 3' ends
346 uncharted. Calculation of the mapped reads for all genes was performed using functions of the
347 HTSeq-count package built into the author's script. The resulting statistics were visualized as
348 transcription profiles using the Artemis genome browser [22].

349 Differentially expressed genes were identified by the software package edgeR [23]. The
350 genes were considered to be differentially expressed, if the p-value was less than 0.05, the expected
351 measure of false deviations (FDR) was not higher than 0.1, and the expression change module (FC,
352 Fold change) was not less than 4. Further distribution of genes according functional categories was
353 performed using the Mycobrowser database (https://mycobrowser.epfl.ch/).

354 **Quantitative reverse transcription-PCR (qRT-PCR)**

355 One microgram of total RNA was used for cDNA synthesis with random hexanucleotides
356 and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Quantitative PCR

357 was performed using qPCRmix-HS SYBR (Evrogen, Russia) and the Light Cycler 480 real-time
358 PCR system (Roche, Switzerland); cycling conditions were as follows: 95C for20 s, 61C for 20 s,
359 72C for 30 s, repeat 40 times; primers are listed in S2 Table. In the end of amplification, a
360 dissociation curve was plotted to confirm specificity of the product. All real-time experiments were
361 repeated in triplicate. The results were normalized against the 16S rRNA gene.

362 **Infections in vivo and ex vivo**

363 **Mice.** C57BL/6Ycit (B6) and I/StSnEgYCit strain (I/St) mice were kept under standard conditions
364 in the Animal Facilities of the Central Research Institute of Tuberculosis. The mice had *ad libitum*
365 access to food and water. Female mice aged 2.5–3.0 months were used in experiments.

366 **Mycobacteria.** For infection of mice and macrophage cultures, *M. tuberculosis* H37Rv (substrain
367 Pasteur) from the collection of CIT were used. Mycobacteria were prepared to infect mice and
368 macrophages as described previously [61]. Briefly, to obtain log-phase bacteria for challenge, 50 µl
369 from a thawed aliquot was added to 30 ml of Dubos broth (BD Biosience, Sparks, MD, USA)
370 supplemented with 0.5% Fatty Acid-Poor BSA (Calbiochem-Behring Corp., La Jolla, CA, USA)
371 and oleic acid and incubated for 2 weeks at 37°C. The resulting suspension was washed two times at
372 3000 g, 20 min, 4°C with Ca^{2+}- and Mg^{2+}-free PBS containing 0,2 mM EDTA and 0,025% Tween
373 80. Cultures were filtered through a 45 µm-pore-size filter (Millipore, USA) to remove clumps. To
374 estimate the CFU content in the filtrate, 20 µl from each 5-fold serial dilution was plated onto Dubos
375 agar (BD), and the total number of micro-colonies in the spot was calculated under an inverted
376 microscope (200^x magnification) after being cultured for 3 days at 37°C. The bulk of the filtered
377 culture was stored at 4°C, and it was found that no change in the CFU content occurred during this
378 storage period.

379 **Infection of mice**. To infect mice, mycobacteria were resuspended in supplemented PBS. Mice
380 were infected via respiratory tract with ~100 viable CFU/mouse using an Inhalation Exposure
381 System (Glas-Col, Terre Haute, IN), as described in [18, 62]. Briefly, animals were exposed for 40
382 min to aerosol produced by nebulizing 8 ml of a bacterial suspension in PBS solution with 0,05%
383 Twin-80 at the concentration of 1.5 x 10^6 bacilli/ml. The size of challenging dose was confirmed in
384 preliminary experiments by plating serial 2-fold dilutions of 2-ml homogenates of the whole lungs
385 obtained from B6 and I/St females at 2 h post-exposure onto Dubos agar and counting colonies after
386 3-wk incubation at 37^0C. To assess CFU counts, lungs from individual mice were homogenized in
387 2.0 ml of sterile saline, and 10-fold serial dilutions were plated on Dubos agar and incubated at 37°C
388 for 20-22 days.
389 **Infection of macrophages, iNOS activation, RNA extraction.** To obtain peritoneal macrophages,
390 B6 mice were injected intra-peritoneally with 3% peptone (Sigma-Aldrich) in saline. Five days later,
391 peritoneal exudate cells (PEC) were eluted from the peritoneal cavities with Ca^{2+}- and Mg^{2+}-free
392 PBS supplemented with 2% FCS and 10 U/ml heparin, washed twice with PBS, and resuspended in
393 RPMI 1640 containing 5% FCS, 10 mM HEPES and 2 mM L-glutamine. The content of nonspecific
394 esterase-positive cells in PEC exceeded 85 %. PEC were plated onto 90 mm Petri dishes (Costar,
395 Corning Inc., Corning, NY, USA) at 10 x 10^6 cells/dish in 10 ml of RPMI-1640 containing 5% FCS,
396 10 mM HEPES and 2 mM L-glutamine to obtain macrophage monolayers. The cells were allowed
397 to adhere for 2 h at 37^oC, 5% CO_2 before mycobacteria were added in 10 ml of supplemented
398 RPMI-1640 at MOI = 30, 20, 15 and 5 for further culturing for periods indicated in Fig 2.
399 Macrophage-free mycobacterial cultures served as controls.

400 To activate macrophages, monolayers were treated with murine rIFN-γ (100 U/ml, Sigma)
401 for 14 h before adding mycobacteria. To block iNOS, 100 μM L-NIL (Sigma) was added 1 h before
402 rIFN-γ administration.

403 To extract RNA, dishes with cell monolayers were gently shaken, culture medium was
404 completely aspirated and macrophages were lysed with 5 ml/dish of Trisol (Invitrogen, Carlsbad,
405 CA,USA) as recommended by the manufacturer. Mycobacteria alone in control cultures were
406 suspended by pipetting and centrifuged at 3000 g, 20 min, 4°C. Pellets were suspended in 1 ml of
407 Trisol.

408 **Statistics**

409 Statistical analysis was performed using ANOVA test and unpaired t-test by GraphPad
410 Prism6.0 software (GraphPad Software, San Diego, CA, USA). *P*-value below 0.05 was assumed to
411 be statistically significant.

412

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577

578 ## Figure captions

579 **Fig 1. *M. tuberculosis* infection in resistant B6 and susceptible I/St mice.**

580 **(**A). Lung CFU counts along the disease progression (P<0.01 at 6 and 10 weeks post challenge,
581 ANOVA). (B). DrrS expression levels of at different time points. (**P < 0.01 and ***P < 0.001,
582 unpaired t-test). At indicated time points, samples of total RNA were analyzed by quantitative real-
583 time PCR, and the DrrS expression levels in the lung tissue were normalized to those of 16S rRNA.
584 Error bars represent the SD for three biological replicates.

585

586 **Fig 2. DrrS transcription is NO-dependent and correlates with activation of infected**
587 **macrophages.**

588 **(**A). The DrrS transcription dynamics in infected peritoneal macrophages of B6 mice. (B). The level
589 of DrrS transcription at 24 hrs post infection: control (mφ +MTb), IFN-γ-activated (mφ +MTb +
590 INF-γ), IFN-γ-activated and L-NIL treated (mφ +MTb + INF-γ + NIL). The levels of DrrS
591 transcription in pure *M. tuberculosis* cultures (MTb) and L-NIL-treated cultures (MTb + NIL) serve
592 as controls for the assessment of possible L-NIL influence onto cultured mycobacteria. (C). The
593 level of MTS0997 transcription at 24 hrs post infection: control (mφ +MTb), IFN-γ-activated (mφ
594 +MTb + INF-γ), IFN-γ-activated and L-NIL treated (mφ +MTb + INF-γ + NIL). (D). The dynamics
595 of DosR transcription in infected peritoneal macrophages.

596

597 **Fig 3. Differential gene expression under the DrrS overexpression conditions.**

598 (A). The numbers of differentially expressed genes by functional categories (Mycobrowser) are
599 given in circles. (B). The heat map of genes changing their transcription more then 4-fold under
600 DrrS overexpression. Gene expression ratios were determined using the edgeR package, log2

601 transformed, normalized to the average level across the replicates and displayed according to the
602 color code.
603
604 **Fig 4. Schematic representation of metabolic/transcriptomic cascades triggered by the elevated**
605 **transcriptional level of DrrS in the OVER mycobacterial strain.**
606 Up-regulated genes/processes are displayed in red, down-regulated – in green.
607

608 ## Supporting information

609 **S1 Table. Differential expression of genes in OVER strain vs pMV strain.**

610

611 **S2 Table. Primers used for qPCR mRNA transcript analysis**

612

613 **S1 Fig. DrrS transcription level in M. tuberculosis OVER strain comparable to H37Rv (wt)**

614 **and pMV strains.**

615 Transcription was measured by qRT-PCR in phase of exponential growth in liquid culture.

616

617

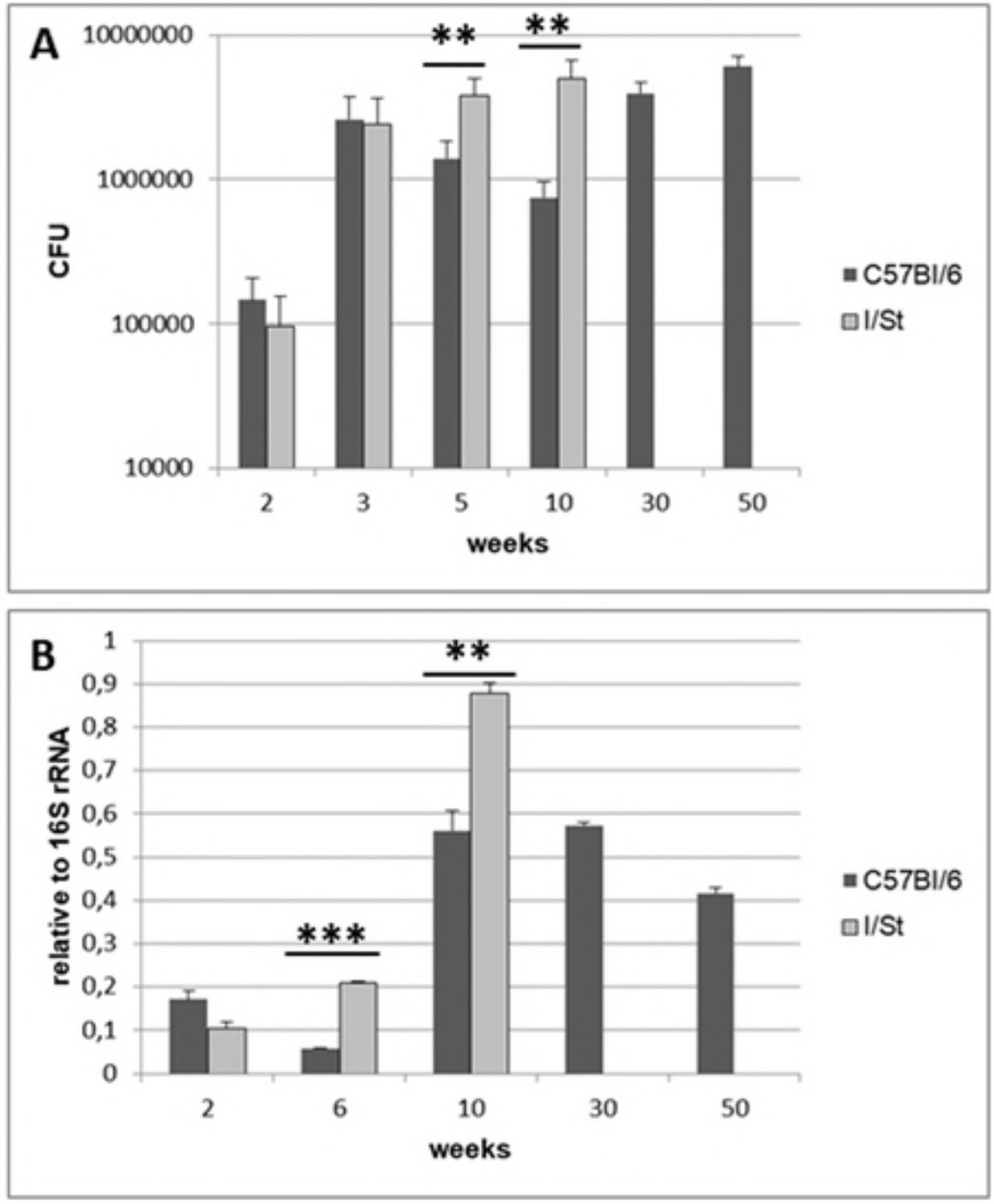
A
10000000
1000000
100000
10000
CFU
**
**
2
3
5
10
30
50
weeks
C57Bl/6
I/St
B
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
relative to 16S rRNA
***
**
2
6
10
30
50
weeks
C57Bl/6
I/St

Figure 1

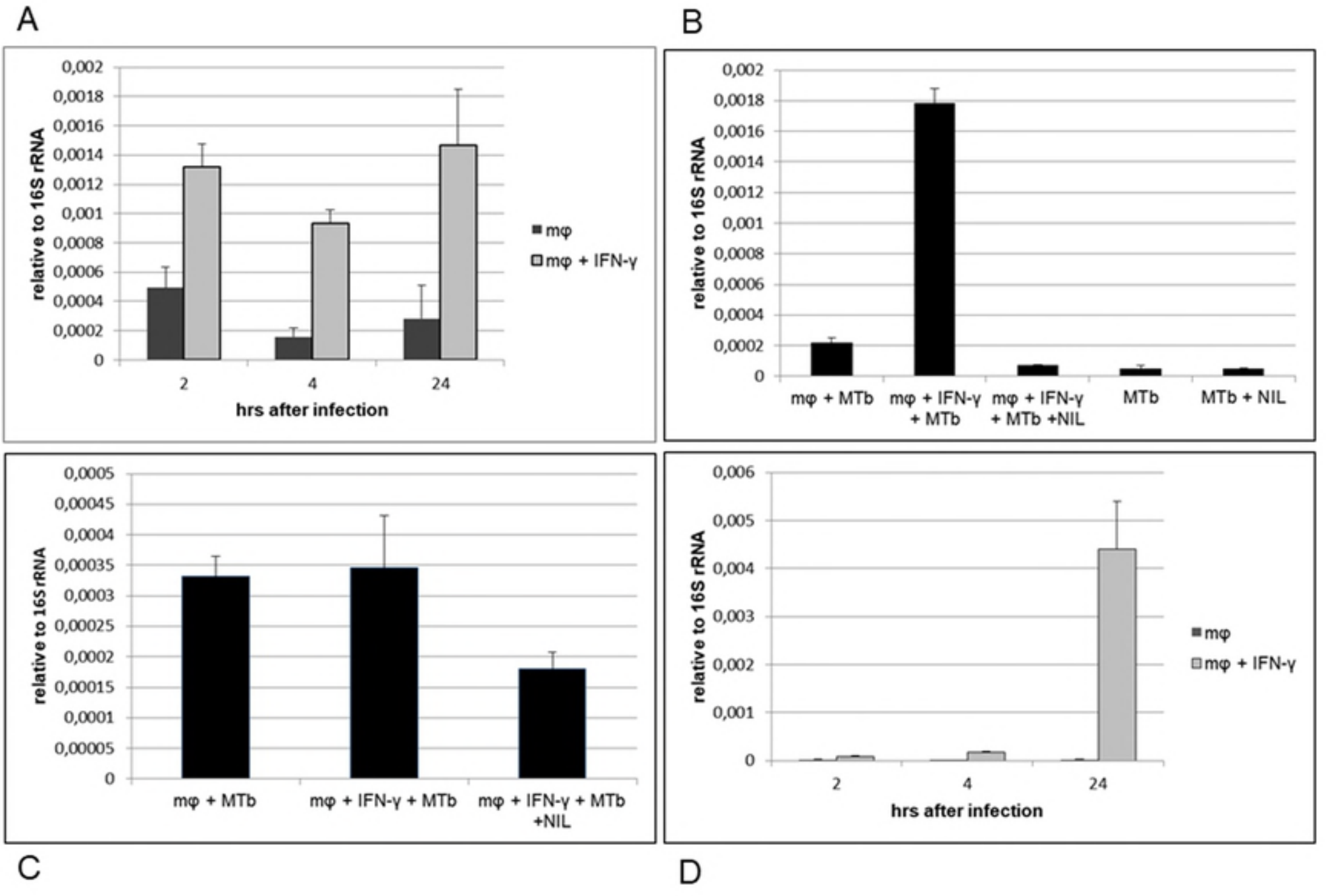
A
B
C
D
relative to 16S rRNA
hrs after infection
2
4
24
mφ
mφ + IFN-γ
mφ + MTb
mφ + IFN-γ + MTb
mφ + IFN-γ + MTb +NIL
MTb
MTb + NIL

Figure 2

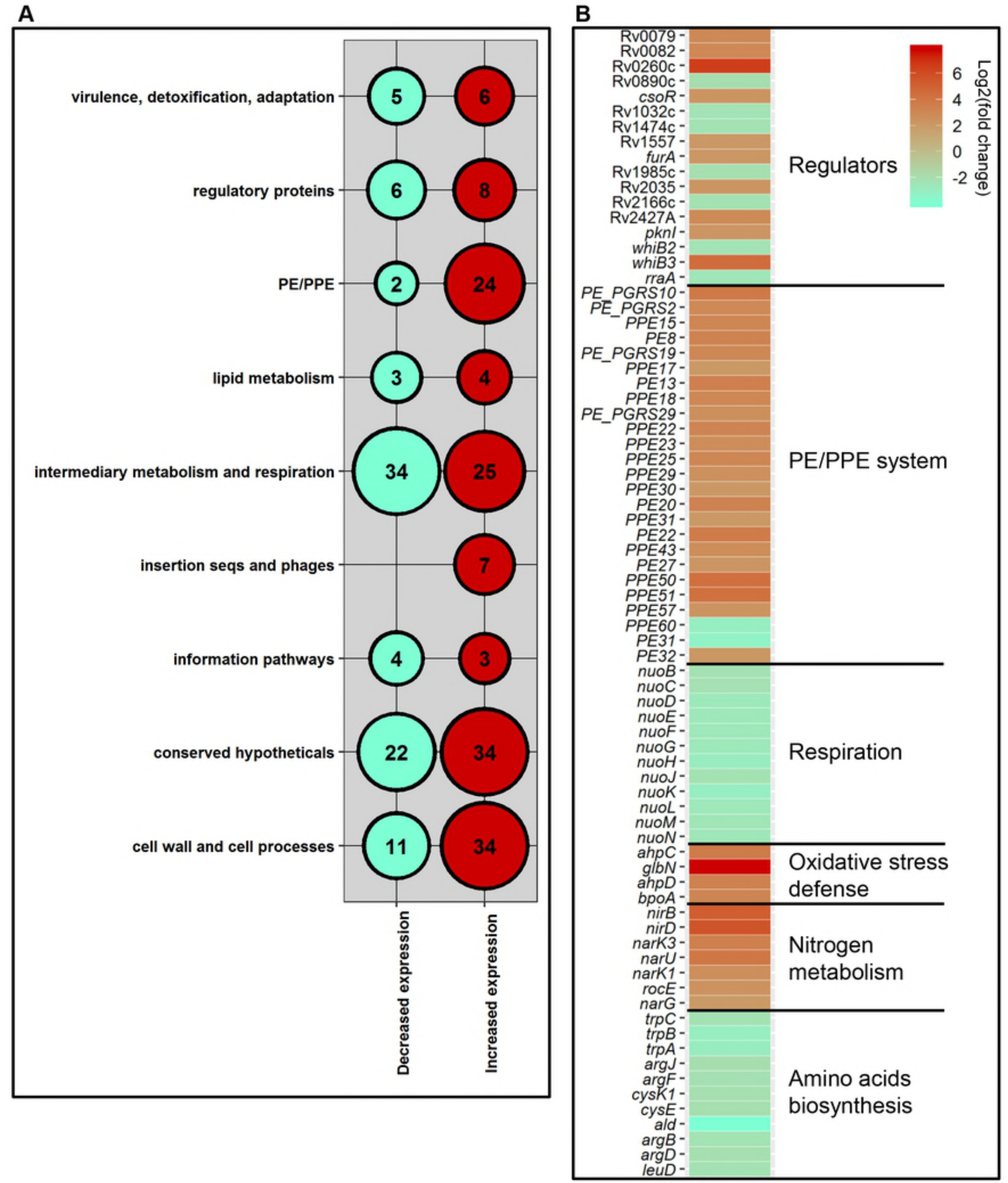
A
virulence, detoxification, adaptation
regulatory proteins
PE/PPE
lipid metabolism
intermediary metabolism and respiration
insertion seqs and phages
information pathways
conserved hypotheticals
cell wall and cell processes
5
6
6
8
2
24
3
4
34
25
7
4
3
22
34
11
34
Decreased expression
Increased expression
B
Rv0079
Rv0082
Rv0260c
Rv0890c
csoR
Rv1032c
Rv1474c
Rv1557
furA
Rv1985c
Rv2035
Rv2166c
Rv2427A
pknI
whiB2
whiB3
rraA
PE_PGRS10
PE_PGRS2
PPE15
PE8
PE_PGRS19
PPE17
PE13
PPE18
PE_PGRS29
PPE22
PPE23
PPE25
PPE29
PPE30
PE20
PPE31
PE22
PPE43
PE27
PPE50
PPE51
PPE57
PPE60
PE31
PE32
nuoB
nuoC
nuoD
nuoE
nuoF
nuoG
nuoH
nuoJ
nuoK
nuoL
nuoM
nuoN
ahpC
glbN
ahpD
bpoA
nirB
nirD
narK3
narU
narK1
rocE
narG
trpC
trpB
trpA
argJ
argF
cysK1
cysE
ald
argB
argD
leuD
Regulators
PE/PPE system
Respiration
Oxidative stress defense
Nitrogen metabolism
Amino acids biosynthesis
Log2(fold change)
6
4
2
0
-2

Figure 3

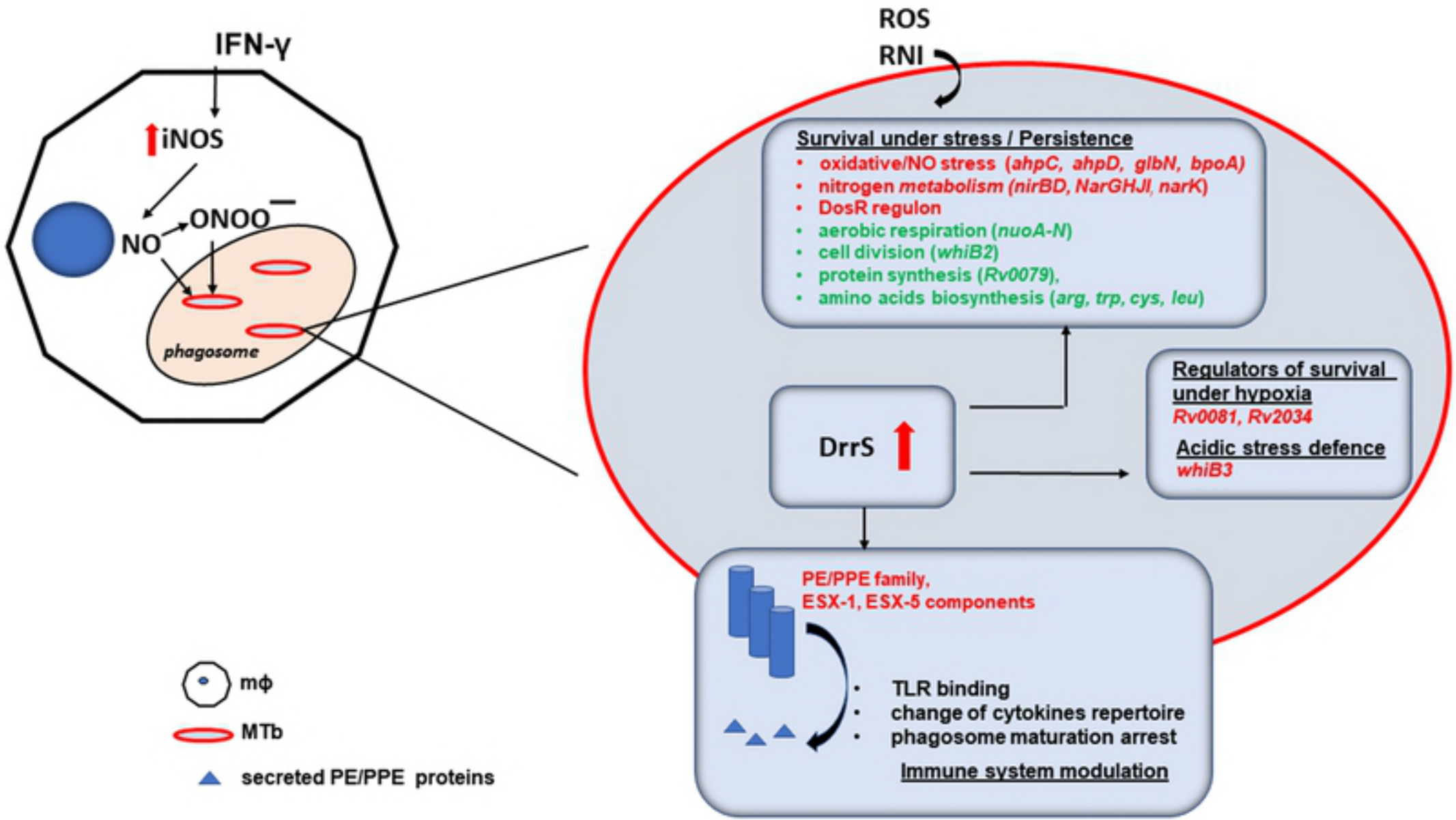
IFN-γ
iNOS
NO
ONOO⁻
phagosome
ROS
RNI
Survival under stress / Persistence
oxidative/NO stress (ahpC, ahpD, glbN, bpoA)
nitrogen metabolism (nirBD, NarGHJI, narK)
DosR regulon
aerobic respiration (nuoA-N)
cell division (whiB2)
protein synthesis (Rv0079),
amino acids biosynthesis (arg, trp, cys, leu)
Regulators of survival under hypoxia
Rv0081, Rv2034
Acidic stress defence
whiB3
DrrS
PE/PPE family,
ESX-1, ESX-5 components
TLR binding
change of cytokines repertoire
phagosome maturation arrest
Immune system modulation
mϕ
MTb
secreted PE/PPE proteins

Figure 4